Phospholipid and cation activation of chimaeric choline/ethanolamine phosphotransferases

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The *Saccharomyces cerevisiae CPT*1 and *EPT1* genes encode for a cholinephosphotransferase (CPT) and choline/ethanolaminephosphotransferase, respectively. Both Cpt1p and Ept1p activities display an absolute requirement for cations and phospholipids. A mixed-micelle assay was employed to determine cation and lipid activators of parental and chimaeric Cpt1p/Ept1p enzymes to gain insight into their mechanism(s) of activation. Mg^{2+}, Mn^{2+} and Co^{2+} were the only cations capable of activating Cpt1p and Ept1p *in vitro*. Kinetic data revealed that only Mg^{2+} is present in appropriate amounts to activate CPT activity *in vivo*. The two enzymes displayed distinct activation profiles on the basis of their relative affinities for Mg^{2+}, Mn^{2+} and Co^{2+}. This allowed the use of chimaeric enzymes to determine the mechanism of cation activation. Cations do not activate Cpt1p or Ept1p by complexing with the substrate, CDP-choline, but instead bind to disparate regions within the enzymes themselves. Cpt1p and Ept1p also displayed distinct phospholipid activation profiles. Phospholipid activation required a phosphate and/or glycero-phosphoester linkage, with the phospho-head group moiety positioned at the surface of the micelle. Assays with parental and chimaeric Cpt1p/Ept1p constructs revealed that the phospholipid binding/activation domains are not located within linear segments of the protein, but instead are contained within distinct and separate regions of the proteins that require an intact tertiary structure for formation. Phosphatidylcholine (and its structural analogue sphingomyelin) were the best lipid activators of Cpt1p, the main biologically relevant CPT activity in *S. cerevisiae*. Hence CPT displays product activation. Because phosphatidylcholine is an efficient activator of CPT activity (and hence Cpt1p is not subject to feedback inhibition by its product), Cpt1p is incapable of functioning as a direct monitor of membrane phosphatidylcholine composition.

INTRODUCTION

By virtue of their physical properties, biological membranes physically define cellular and organellar boundaries. The lipid bilayer is a complex mesh of over 1000 distinct molecules [1] constantly undergoing synthesis and turnover to maintain appropriate physical properties [2], as well as serving as a reservoir for the release of many types of bioresponsive signalling molecules associated with efficient regulation of cell function and growth [3–10]. The dynamic nature of the lipid synthesizing, remodelling and turnover machinery must require the ability of the cell to monitor changes in lipid composition. Any changes detected would activate systems necessary to maintain cellular and organellar phospholipid composition. Many proteins embedded within the membrane also react to the lipid environment, including many that are responsible for phospholipid synthesis [11–23]. Hence not only would gross changes in membrane composition affect the physical properties of a membrane and its capability to serve as a source of lipid second messengers, but they would also grossly affect signalling processes and metabolic pathways dependent on enzymes responsive to specific lipids in their microenvironment. The mechanisms by which the cell monitors and maintains its required phospholipid composition are incompletely understood, as are the molecular mechanisms of phospholipid–protein interactions. Phosphatidylcholine (PC) is the major structural phospholipid present in eukaryotic membranes [24–26]. PC is a major source of the second messenger molecules diacylglycerol (DAG), phosphatidic acid, lysophosphatidic acid and arachidonic acid [6–8,10]. The main route for PC biosynthesis in mammalian cells is via the CDP-choline (Kennedy) pathway [27–29]. *Saccharomyces cerevisiae* also synthesizes a significant portion of PC via the CDP-choline pathway [30–32], whereby choline is phosphorylated to phosphocholine by choline kinase [33], phosphocholine is converted to CDP-choline by a phosphocholine cytidylyltransferase [34], and cholinephosphotransferases condense CDP-choline with DAG to form PC [35,36]. This laboratory has isolated the genes from *S. cerevisiae* for a cholinephosphotransferase, *CPT1* [37], and a choline/ethanolaminephosphotransferase, *EPT1* [38]. These are the only CPT genes isolated from any cell type. Together, Cpt1p and Ept1p comprise all of the CPT activity in *S. cerevisiae* [36]. *In vivo*, Cpt1p synthesizes 95 % of the PC made via the CDP-choline pathway in yeast, and Ept1p contributes the remaining 5 % [30,31]. In this study the phospholipid and cation dependences of Cpt1p, Ept1p and chimaeric Cpt1p/Ept1p constructs were examined to investigate the structural requirements for cation and phospholipid activation of these integral membrane-bound proteins. Additionally, the role of Cpt1p as a regulator of phospholipid composition, by acting as phospholipid sensor within the membrane, was assessed.

MATERIALS AND METHODS

Materials

[*methyl*-^{14}C]Cytidine diphosphocholine was purchased from American Radiolabeled Chemicals. Choline, phosphocholine,

Abbreviations used: CPT, cholinephosphotransferase; DAG, diacylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol.

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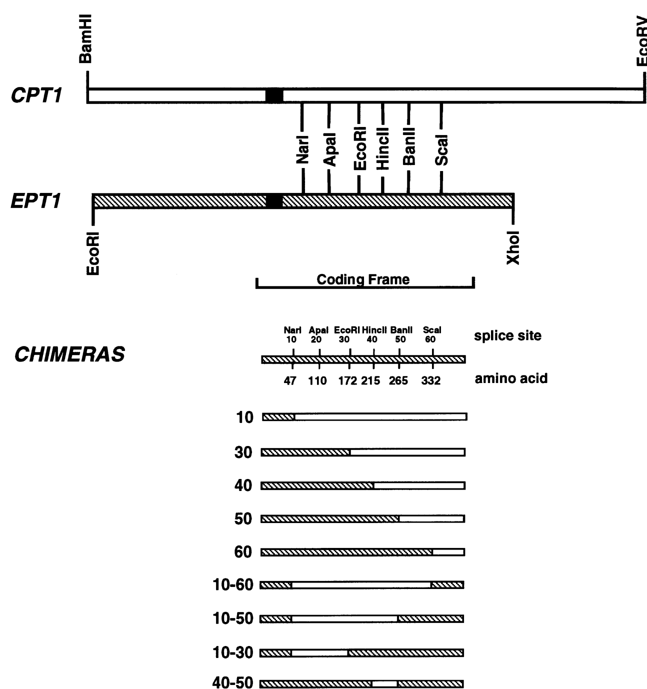

Figure 1 Construction and nomenclature of functional *CPT1*/*EPT1* chimaeras

Open areas indicate portions of the *CPT1* gene; hatched areas indicate regions of the *EPT1* gene [39]. The designations 10, 20, etc., are arbitrary and identify splice sites corresponding to the designated amino acids indicated in the Figure. The amino acids designated correspond to Ept1p junctions; corresponding Cpt1p junctions are 14 amino acids farther along owing to a predicted translation start site resulting in the addition of 14 amino acids to the N-terminus of Cpt1p not found in Ept1p.

CDP-choline, glycerophosphocholine and glycerophosphate were purchased from Sigma Chemical Company. Lipids were purchased from Avanti Polar Lipids. TLC plates (silica gel 60A) were obtained from Whatman Labsales. All other materials were of the highest grade available.

Chimaeric *CPT1*/*EPT1* gene construction

Functional chimaeric forms of *CPT1*/*EPT1* were constructed through mutual restriction sites within the coding region of each gene [39]. The sites used and the requisite nomenclature are indicated in Figure 1. Parental and chimaeric genes were subcloned into the multi-cloning site of YEp352 for high-copy-number expression of the gene products in the yeast strain HJ091 (α *leu2-3,112 his3-1 ura3-52 trp1-289 cpt::LEU2 ept1-1*). HJ091 is devoid of detectable CPT activity.

Preparation of cellular membranes

Yeast cells were grown in 250 ml synthetic dextrose minimal media (containing the appropriate supplements) to mid-exponential phase, then centrifuged at 1000 ***g*** for 10 min at 4 °C and washed with 25 ml ice-cold GTE buffer [20 % (v/v) glycerol, 50 mM Tris/HCl, pH 7.4, and 1 mM EDTA]. The pellet was resuspended in 0.7 ml ice-cold GTE buffer and transferred to a test tube containing 1 g of 0.5 mm glass beads. Cells were disrupted by vortexing at high speed for 5×30 s with 30 s of cooling on ice between bursts. The beads were washed twice with 0.5 ml of GTE buffer and the homogenate was centrifuged at 16000 ***g*** for 15 min at 4 °C. The supernatant was carefully removed and centrifuged at 100000 ***g*** for 15 min at 4 °C. The pelleted microsomal membranes were resuspended in 1 ml of GTE buffer using a glass–Teflon homogenizer.

CPT assays

CPT activity was determined in the microsomal membranes by using a Triton X-100 mixed micellar assay [40]. A 1,2-dioleoyl (di18:1) or 1,2-dipalmitoleoyl (di16:1) species of diacylglycerol was prepared from the appropriate PC by phospholipase C digestion. Normal assay conditions contained 50 mM Mops, pH 7.5, with 20 mM $MgCl_2$, 10 mol % di18:1 diacylglycerol, 10 mol % dioleoylPC and 0.5 mM [*methyl*-^{14}C]CDP-choline (2000 d.p.m. per nmol). Mol % assumes 130 molecules of Triton X-100 per micelle, e.g. 10 mol % lipid assumes 144 lipid or detergent molecules per micelle, of which 14 are lipid. A di16:1 species of diacylglycerol was used to determine the apparent K_a of Cpt1p for cations. Assays were initiated by the addition of 5–20 μg of membrane protein and incubated at 25 °C for 30 min. The reaction was stopped by the addition of 3 ml of $CHCl_3/CH_3OH$ (2:1, v/v), and 1.4 ml of 0.9 % (w/v) KCl was added to facilitate phase separation. The organic phase was washed twice with 1.5 ml of 40 % (v/v) CH_3OH and the label in the organic phase was determined.

Standard procedures

Protein was estimated by the procedure of Lowry et al. [41]. Lipid phosphorus was determined by the method of Ames and Dubin [42]. Diacylglycerol concentration was assessed according to the method of Stern and Shapiro [43].

RESULTS

Rationale

Cpt1p (a CPT) and Ept1p (a dual-specificity choline/ethanolamine phosphotransferase) are integral membrane-bound proteins and their activities possess an absolute requirement for both cations and phospholipids [12]. In the present study, a mixed-micelle assay was employed to determine the cation and phospholipid activation mechanisms of Cpt1p and Ept1p. Mixed micelles allow for the solubilization of integral membrane-bound proteins and facilitate the homogeneous delivery of the DAG lipid substrate and phospholipid activators while simultaneously diluting endogenous lipid activators present in the membrane preparations [44]. The soluble mixed micelle is then suitable for delivery of the soluble substrate, CDP-choline, as well as the soluble cation activators. Radiolabelled CDP-choline was employed as the substrate in all assays for several reasons. Most importantly, chimaeric Cpt1p/Ept1p enzymes are all capable of utilizing CDP-choline [39]. Because the chimaeric enzymes were to be employed to dissect the mechanisms by which Cpt1p and Ept1p are activated by cations and phospholipids, a common substrate was employed to facilitate the study of all functional chimaeric enzymes. The yeast strain HJ091 (*cpt1::LEU2 ept1-1*) was used as a null background with which to express the parental and chimaeric CPTs.

Cation activation of Cpt1p, Ept1p and chimaeric Cpt1p/Ept1p enzymes

Several cations were employed to assess their abilities to activate Cpt1p- and Ept1p-derived CPT activity (Table 1). Only Mg^{2+},

Table 1 Activation of Cpt1p and Ept1p by divalent cations

Mixed-micelle enzyme assays were performed with CDP-choline and di18:1 diacylglycerol as substrates as described in the Materials and methods section. Cations were added at 1, 4 or 16 mM. Results are expressed, in nmol per min per mg protein, as the means ± S.D. for three separate experiments. n.d., not detectable.

[Cation] (mM)	Mg	Mn	Co	Ni	Zn	Cu	Cd	Ca	Sr
Cpt1p-derived CPT activity:									
1	3.5 ± 0.4	16.2 ± 2.6	17.1 ± 2.2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
4	9.1 ± 1.4	11.8 ± 1.1	18.5 ± 2.0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
16	19.8 ± 2.6	7.9 ± 1.1	16.2 ± 2.3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Ept1p-derived CPT activity:									
1	6.9 ± 0.7	5.0 ± 0.7	3.9 ± 0.4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
4	12.2 ± 1.6	5.0 ± 0.6	3.5 ± 0.3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
16	17.1 ± 2.4	5.2 ± 0.5	3.0 ± 0.4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

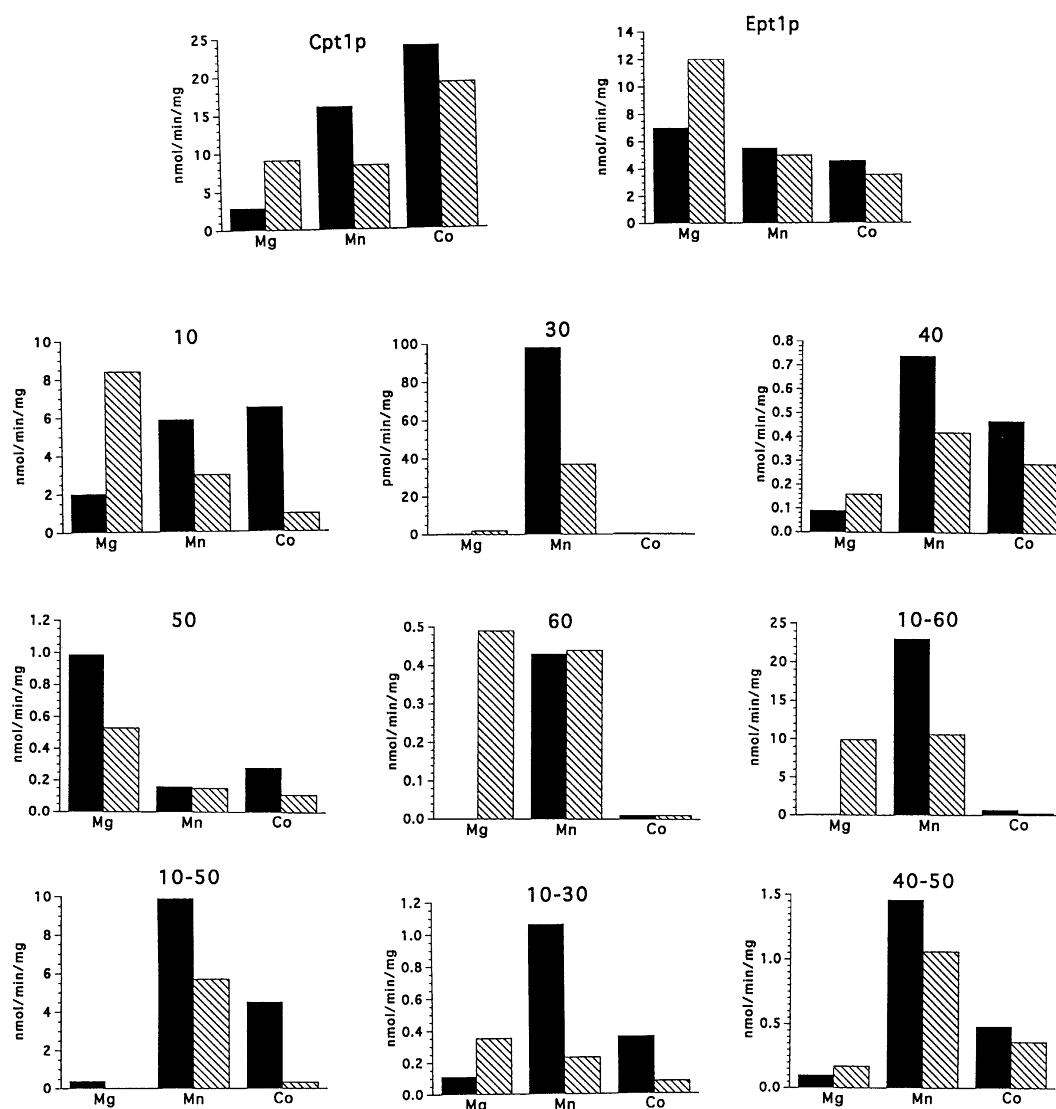

Figure 2 Activation of Cpt1p/Ept1p chimaeric enzymes by cations

Mixed-micelle enzyme assays were performed as described in the Materials and methods section. Cation concentrations: solid columns, 0.5 mM; hatched columns, 4 mM. Results are the means of two separate experiments performed in duplicate. Variation was less than 15% per value between experiments. Chart headings correspond to chimaeric enzyme splice sites (see Figure 1).

Mn^{2+} and Co^{2+} were capable of activating Cpt1p and Ept1p. Neither enzyme was responsive to Zn^{2+}, nor were the enzymes activated by Ca^{2+}, implying that changes in intracellular calcium concentration do not directly affect Cpt1p or Ept1p enzyme activities. Cpt1p contains the major biologically relevant CPT activity, responsible for synthesizing 95 % of PC made via the CDP-choline pathway [30,31]. Although the activation of Cpt1p by three different cations could constitute a complex regulatory system *in vivo*, it is unlikely that competition for binding to Cpt1p by Mg^{2+}, Mn^{2+} and Co^{2+} exists for this CPT, which resides in the endoplasmic reticulum [24,45], with the active site facing the cytoplasm [46]. Although the apparent K_a values for the cations capable of activating Cpt1p were 4.1 mM for Mg^{2+}, 1.0 mM for Mn^{2+}, and 450 μM for Co^{2+} (kinetic results not shown), only Mg^{2+} is found in concentrations in the cytoplasm at high enough levels to activate Cpt1p efficiently [47]. Cellular Mn^{2+}, and enzymes activated by this cation, tend to be concentrated in organelles, most notably in the vacuole and mitochondria [48]. Indeed, cytoplasmic Mn^{2+} levels are estimated to be less than 1 μM [47]. Co^{2+} is also an unlikely biologically relevant activator of Cpt1p owing to its being very rare [47]; indeed, minimal medium for the growth of *S. cerevisiae* is devoid of Co^{2+} [49].

To determine the exact mechanism of cation activation as well as the segment(s) within Cpt1p and Ept1p that are responsible for binding cations, chimaeric enzymes were used. Because Cpt1p-derived CPT activity was activated by cations at both 0.5 and 4 mM (Figure 2) in the order $Co^{2+} > Mn^{2+} > Mg^{2+}$, whereas Ept1p was activated in the converse order, $Mg^{2+} > Mn^{2+} > Co^{2+}$, this difference in the activation profiles of the parental enzymes allowed the chimaeric enzymes to be used to determine the mechanism of cation activation of Cpt1p and Ept1p. Three results would be predicted from the analysis of cation activation of chimaeric Cpt1p/Ept1p enzymes. First, cation activation could be the result of a required complex formation between CDP-choline and cation activators (reminiscent of Mg^{2+}/ATP complexes in kinases); in this event, the cation activation domain would map to the CDP-choline-binding domain of Cpt1p/Ept1p (region 10–30; residues 61–186 of Cpt1p, 47–172 of Ept1p). Secondly, the cation could have a specific binding site within Cpt1p/Ept1p in a specific linear domain of Cpt1p/Ept1p, and the chimaeric enzymes would allow the assignment of this domain. Thirdly, the cation-binding site could consist of specific discontinuous domains within the proteins, resulting in chimaeric cation activation profiles dissimilar to the parental enzymes.

Each chimaeric enzyme displayed a novel cation activation profile (Figure 2). With a few exceptions, the general trend was $Mn^{2+} > Mg^{2+} = Co^{2+}$. Several conclusions can be drawn from these results. Activation of Cpt1p and Ept1p by cations is not via complex formation of cations with the CDP-aminoalcohol substrate, because the cation activation domain did not map to the CDP-aminoalcohol-binding region of the chimaeric enzymes (region 10–30) [31,39]. Instead, the data are consistent with the cation binding/activation domain of Cpt1p and Ept1p requiring disparate segments of Cpt1p/Ept1p that do not form an intact cation-binding site until the protein has assumed its proper tertiary structure within the membrane. Additionally, because an intact tertiary structure is required for cation activation, and the chimaeric Cpt1p/Ept1p proteins were functional, the two proteins must form similar tertiary structures within the membrane.

Table 2 Phospholipid activation of Cpt1p and Ept1p

Mixed-micelle enzyme assays with CDP-choline and di18:1 diacylglycerol as substrates were performed as described in the Materials and methods section. Activation is expressed as a ratio compared with activation of the enzymes by PC. Phospholipids were present at a concentration of 10 mol%. For reference, specific activities for over-expressed CPT1p were 19.2 nmol per min per mg protein; those for Ept1p were 18.6 nmol per min per mg. Results are expressed as the means ± S.D. for three separate experiments performed in duplicate. *n.d., not detectable. Abbreviations: CER, ceramide; CL, cardiolipin; CDP-DG, CDP-diacylglycerol; Cho, choline; GP, glycerophosphate; GPC, glycerophosphocholine; LPE, lysophosphatidylethanolamine; LPC, lysophosphatidylcholine; PA, phosphatidic acid; Pcho, phosphocholine; PG, phosphatidylglycerol; PS, phosphatidylserine; SM, sphingomyelin; SPH, sphingosine.

	CPT activity (nmol per min per mg)	
Lipid activator	Cpt1p-derived	Ept1p-derived
PC	1.00 ± 0.08	1.00 ± 0.06
PE	0.42 ± 0.05	0.85 ± 0.09
PS	0.37 ± 0.04	0.58 ± 0.06
PI	0.70 ± 0.10	0.42 ± 0.05
PG	0.55 ± 0.07	1.24 ± 0.15
CL	0.38 ± 0.06	0.72 ± 0.08
CDP-DG	0.39 ± 0.05	0.08 ± 0.02
PA	0.32 ± 0.05	0.92 ± 0.09
LPC	0.89 ± 0.09	1.44 ± 0.13
LPE	0.40 ± 0.06	1.08 ± 0.12
SM	1.42 ± 0.18	0.98 ± 0.10
CER	0.10 ± 0.02	0.25 ± 0.04
SPH	n.d.*	n.d.
DAG (20 mol%)	n.d.	n.d.
GPC (1 mM)	n.d.	n.d.
GP (1 mM)	n.d.	n.d.
Pcho (1 mM)	n.d.	n.d.
Cho (1 mM)	n.d.	n.d.
None	n.d.	n.d.

Activation of Cpt1p and Ept1p by phospholipids

Both Cpt1p and Ept1p absolutely require exogenous phospholipid activators for their enzyme activities. This is evidenced by the inability of microsomal preparations alone to activate either Cpt1p or Ept1p (Table 2). Lipid activation of Cpt1p in descending order was sphingomyelin > phosphatidylcholine > lysophosphatidylcholine > phosphatidylinositol > phosphatidylglycerol > phosphatidylethanolamine = lysophosphatidylethanolamine = phosphatidylserine = cardiolipin = phosphatidic acid = CDP-diacylglycerol > ceramide; of the lipids that activated Ept1p, lysophosphatidylcholine > phosphatidylglycerol > lysophosphatidylethanolamine = phosphatidylcholine = phosphatidylethanolamine = phosphatidic acid = sphingomyelin > cardiolipin > phosphatidylserine > phosphatidylinositol > ceramide > CDP-diacylglycerol (Table 2). These results proved effective in dissecting structural requirements within the phospholipid molecule for activation of Cpt1p and Ept1p. First, DAG added up to 20 mol % was unable to activate either Cpt1p or Ept1p. Hence DAG is used only as a substrate and does not act as both a substrate and an activator, a finding in contrast with the *Escherichia coli* DAG kinase [13]. Secondly, the inability of DAG to activate both Cpt1p and Ept1p, whereas PA activates both Cpt1p and to a greater extent Ept1p, indicates that the *sn*-glycerol 3-phosphate and/or phosphoester bond is necessary for activation. Hence it is the addition of different head groups to the phosphate that results in the differential activation of the enzymes by different phospholipids. Sphingomyelin activates both Cpt1p and Ept1p, whereas ceramide is a very poor activator and sphingosine is completely ineffective, consistent with the requirement for a phosphate and a head group (in this case phosphocholine) for efficient lipid activation of Cpt1p (and Ept1p). Additionally, the soluble head group and glycerol backbone molecules glycerophosphocholine, glycerophosphate, phosphocholine and choline were all unable to activate either

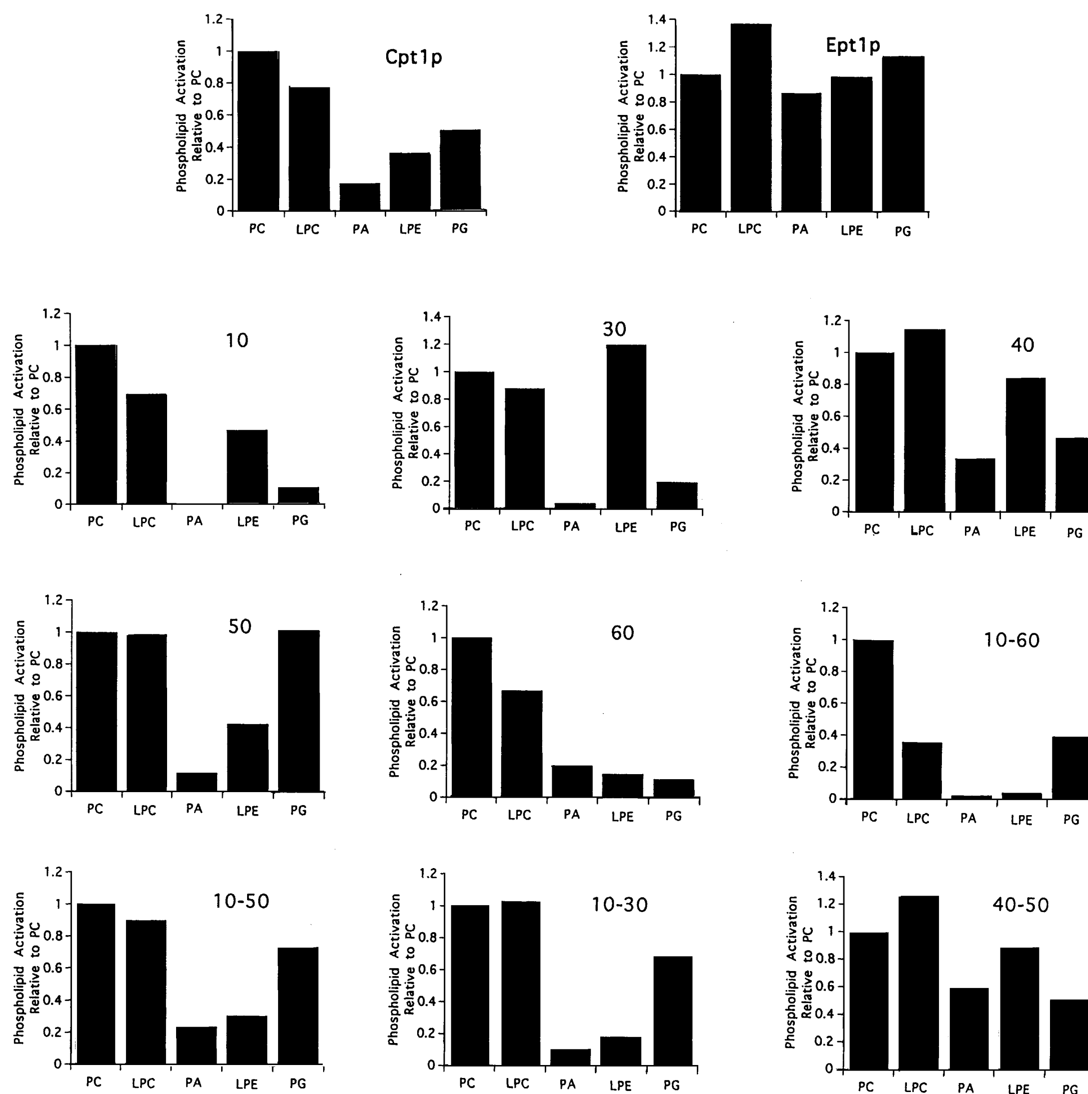

Figure 3 Activation of Cpt1p/Ept1p chimaeric enzymes by phospholipids

Mixed-micelle enzyme assays were performed as described in the Materials and methods section. Activation is expressed as a ratio compared with the activation of the enzymes by PC. Phospholipids were present at a concentration of 10 mol%. The results are expressed as the means of two separate experiments performed in triplicate. Variations obtained were less than 15% per value between experiments. Abbreviations: LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; PA, phosphatidic acid; PG, phosphatidylglycerol. Chart headings correspond to chimaeric enzyme splice sites (see Figure 1).

enzyme, indicating that appropriate attachment of the head group to the lipid backbone and association with the surface membrane are essential for activation of both Cpt1p and Ept1p.

CPT catalyses the ultimate step in PC synthesis. Subcellular fractionation studies have determined that the bulk of this enzyme activity resides in the endoplasmic reticulum [24,45,46]. The phospholipid composition of *S. cerevisiae* microsomes, which consist mainly of endoplasmic reticulum membrane, is similar to that of other eukaryotic cell types. PC comprises 50 % of the total phospholipid, followed by phosphatidylethanolamine (PE) at 35 % and phosphatidylserine and phosphatidylinositol (PI) at 7 % each [24,26]. Other phospholipid species comprise only minor components of these membranes. We have demonstrated *in vitro* that CPT activity is efficiently activated by its product, PC (Table 2). Because PC is also the main phospholipid component of the membrane in which CPT resides, this lipid most probably also plays the role of activator *in vivo*. Indeed, the local concentration of PC at the site of CPT is most probably even higher than that of the organelle in which Cpt1p resides.

Activation of chimaeric Cpt1p/Ept1p enzymes by phospholipids

The fact that low mixed-micelle concentrations (3–7 mol %) of phospholipid [12] were sufficient to fully activate Cpt1p and Ept1p implied that a small number of molecules are sufficient for activation. Hence regions within the primary structure of the enzyme must exist that comprise the physical site of phospholipid cofactor–enzyme interaction. Previous work from this laboratory that used the differential activation of PE and PC in a competitive manner reached a similar conclusion: these two particular lipids were predicted to share specific binding sites within the enzyme(s) [12]. To explore the regions within Cpt1p and Ept1p responsible for phospholipid activation, chimaeric Cpt1p/Ept1p enzymes were used. To this end, phospholipids that were found to activate

the two parental enzymes differentially were used as activators of the chimaeric enzymes. Based on earlier studies of DAG substrate specificity, the chimaeras were expected to display either parental patterns of phospholipid activation or novel patterns of activation by phospholipids. Although chimaeric enzymes displayed both parental and novel phospholipid activation profiles, a definitive pattern of activation could not be delineated from the studies of chimaeric enzymes (Figure 3). The inability to correlate specific linear segments within the chimaeric enzymes with phospholipid activation implies that phospholipid activation domains for Cpt1p and Ept1p are formed from discontinuous, non-linear regions. This predicted multiplicity in phospholipid activation is a logical extension of kinetic observations drawn from the mixed-micelle analysis of the parental Cpt1p and Ept1p proteins, where 5–10 phospholipid molecules were predicted to be required for maximal activation of the enzymes [12]. Hence functional phospholipid activation domains exist only once the protein has been folded into its correct tertiary structure within the membrane.

DISCUSSION

The present work defines the mechanisms for the required cation and phospholipid activation of the cholinephosphotransferases Cpt1p and Ept1p. These enzymes condense DAG with CDP-choline, catalysing the ultimate step in PC biosynthesis via the CDP-choline pathway [31–33]. They also act as potential sinks for attenuating DAG signalling pools in some cell types [50]. Together, Cpt1p and Ept1p comprise the total CPT activity present in *S. cerevisiae* [32,36] and are the only CPT genes so far isolated. Although Cpt1p and Ept1p account for half of the measurable CPT activity *in vitro* [35], Cpt1p is responsible *in vivo* for the synthesis of 95 % of PC, whereas Ept1p synthesizes the remaining 5 % [31].

The CPT activities of Cpt1p and Ept1p possess an absolute requirement for cations. Of the cations tested, only Mg^{2+}, Mn^{2+} and Co^{2+} activated Cpt1p and Ept1p. Most of the cellular CPT activity resides in the endoplasmic reticulum [45,46], with the active site facing the cytoplasm [46]. Of the three cations capable of activating the CPTs *in vitro*, only Mg^{2+} is present in the cytoplasm in sufficient levels to be used *in vivo* [47]. Cation activation of CPT activity was predicted to occur by one of three mechanisms: complex formation between the CDP moiety of the CDP-choline substrate and the cation activator, interaction of cations with a specific linear domain of the protein, or a requirement for distinct disparate regions within the proteins. Cation activation was found to occur via direct binding of the cation to the enzymes at discontinuous sites that require an intact tertiary structure for formation.

Cpt1p and Ept1p CPT activities also possess an absolute requirement for phospholipid activators [12,40]. The abilities of a defined set of phospholipids to activate Cpt1p and Ept1p enzyme activities allowed the assignment of structural requirements within phospholipids for activation of Cpt1p and Ept1p. These include a phosphate moiety and/or glycero-phosphoester linkage from which head group additions to the phosphate resulted in the differential ability of each lipid type to activate Cpt1p and Ept1p. Additionally, the phospho-head group must be properly positioned at the membrane surface, because soluble head group precursors were unable to activate the enzymes. Most of the CPT activity resides in the endoplasmic reticulum, and Cpt1p is responsible for the bulk of this activity *in vivo* [31]. PC, the product of the CPT reaction, is the main phospholipid component of the endoplasmic reticulum in eukaryotic cells, including *S. cerevisiae*. The best phospholipid activators for Cpt1p-derived CPT activity are PC and its structural analogue, sphingomyelin. This is biochemically interesting in that the product of the reaction is a very efficient activator. CPT lies downstream of the rate-limiting step in phosphatidylcholine biosynthesis, phosphocholine cytidylyltransferase. Intracellularly, CPT resides mainly in the endoplasmic reticulum; phosphatidylcholine is the major phospholipid present in this organelle. The efficient activation of CPT activity by its product, PC, would ensure that the regulation imposed on the rate of PC synthesis by changes in cytidylyltransferase activity would not be impeded. Indeed, the concentration of PC within the micro-environment of increased CPT activity would be predicted to be even higher than that of the surrounding organellar PC concentration. The property of CPT to act efficiently *in vivo* is well established and the surprising biochemical observation that CPT is efficiently activated by its product, PC, unites this known physiological property of CPT with a biochemical phenomenon.

The mechanisms by which cells regulate their phospholipid composition, and respond and regulate the enzymes involved in phospholipid metabolism to maintain the required phospholipid composition, is unknown. The CDP-choline pathway is the main route for PC synthesis in most eukaryotic cell types [28,50] and also synthesizes PC in *S. cerevisiae* [30–32]. The pathway comprises three steps: choline is phosphorylated by a cytosolic choline kinase to form phosphocholine; phosphocholine is converted to CDP-choline by a cytidylyltransferase residing in both soluble and membrane-bound forms in yeast and mammalian cells, with the membrane-bound form being the active form *in vivo* [28,50,51], and CDP-choline is condensed with DAG to form PC by integral membrane-bound CPTs. Is the CDP-choline pathway itself capable of sensing PC levels and modulating PC synthesis accordingly? Both the cytidylyltransferase and the CPT associate with the membrane, the former transiently and the latter intimately. However, cytidylyltransferase (both mammalian and yeast) activity is completely unaffected by PC levels [52], making it an ineffective candidate for a direct sensor/responder to PC levels. The data presented here reveal that CPT (Cpt1p) activity is modulated by PC. Hence Cpt1p is the only enzyme within the CDP-choline pathway with the capability of directly sensing/responding to changes in PC membrane composition. However, it would be predicted that if Cpt1p were to play a role in monitoring and responding to phospholipid composition, a simple feedback inhibition mechanism would exist whereby increased PC synthesis would down-regulate enzyme activity and, conversely, decreased PC levels would up-regulate enzyme activity. An examination of the data reveal that the converse is true. PC (and its structural analogue sphingomyelin) are the best activators of Cpt1p. Because Cpt1p is the CPT responsible for the synthesis of 95 % of the PC via the CDP-choline pathway, the efficient activation of Cpt1p by PC is in direct contrast with the predicted response of Cpt1p if Cpt1p were to act as a direct sensor of PC synthesis/composition within the cell. Indeed, because Cpt1p is downstream of cytidylyltransferase, the rate-limiting step in PC biosynthesis, the efficient activation of Cpt1p by PC demonstrated in this study would ensure that the pathway is at its most active when cytidylyltransferase is driving the synthesis of CDP-choline through to the cholinephosphotransferases for its subsequent conversion to PC. Pulse-labelling studies have established that CPT activities, *in vivo*, are incredibly adept at converting any available CDP-choline to PC and are not normally rate-limiting in this process [28–31,53].

An indirect mechanism for sensing and responding to PC levels in the cell has recently been described in *S. cerevisiae*. Cytidylyltransferase, the rate-limiting step in PC biosynthesis, is

regulated by a phospholipid-sensing protein Sec14p [54,55]. Sec14p specifically binds PI and PC [55,56]. When Sec14p is bound to PC, it inhibits cytidylyltransferase and results in decreased PC synthesis via the CDP-choline pathway *in vivo*. In its PI-bound form, Sec14p is incapable of inhibiting cytidylyltransferase activity. Hence Sec14p acts as an efficient feedback inhibitor: an increase in PC content will result in an increased proportion of Sec14p binding PC and an inhibition of PC synthesis to maintain an optimal level of PC within the cell membrane. The results presented here, showing that CPT is an Mg^{2+}-dependent enzyme that displays the unusual property of product (PC) activation, are consistent with the above mechanism.

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