

The selective utilization of substrates in vivo by the phosphatidylethanolamine and phosphatidylcholine biosynthetic enzymes Ept1p and Cpt1p in yeast

Henry A. Boumann^{a,*}, Ben de Kruijff^a, Albert J.R. Heck^b, Anton I.P.M. de Kroon^a

^aDepartment Biochemistry of Membranes, Centre for Biomembranes and Lipid Enzymology, Institute of Biomembranes, Padualaan 8, 3584 CH Utrecht, The Netherlands

^bDepartment of Biomolecular Mass Spectrometry, Bijvoet Center for Biomolecular Research and Utrecht Institute for Pharmaceutical Sciences, Sorbonnelaan 16, 3584 CA Utrecht, The Netherlands

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Abstract In yeast, the aminoalcohol phosphotransferases Ept1p and Cpt1p catalyze the final steps in the CDP-ethanolamine and CDP-choline routes leading to phosphatidylethanolamine (PE) and phosphatidylcholine (PC), respectively. To determine how these enzymes contribute to the molecular species profiles of PE and PC in vivo, wild-type, *cpt1Δ*, and *ept1Δ* cells were pulse labeled with deuterated ethanolamine and choline. Analysis of newly synthesized PE and PC using electrospray ionization tandem mass spectrometry revealed that PE and PC produced by Ept1p and Cpt1p have different species compositions, demonstrating that the enzymes consume distinct sets of diacylglycerol species in vivo. Using the characteristic phospholipid species profiles produced by Ept1p and Cpt1p as molecular fingerprints, it was also shown that in vivo CDP-monomethylethanolamine is preferentially used as substrate by Ept1p, whereas CDP-dimethylethanolamine and CDP-propanolamine are converted by Cpt1p.

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1. Introduction

Phosphatidylethanolamine (PE) and phosphatidylcholine (PC) are two of the major membrane lipid constituents in eukaryotic cells. In the model eukaryote *Saccharomyces cerevisiae*, these phospholipid classes together account for 60–75% of the total phospholipid content (see e.g. [1]), and they share a highly interconnected metabolic relationship [2]. In yeast, the biosynthesis of PE proceeds mainly via decarboxylation of phosphatidylserine (PS) [3,4], and the subsequent triple methylation of PE leads to the production of PC [5]. Apart

from these routes, yeast has preserved the Kennedy pathways, i.e., the CDP-ethanolamine and the CDP-choline routes yielding PE and PC, respectively [6]. In the Kennedy pathways an aminoalcohol, choline or ethanolamine, is phosphorylated by a kinase, and then converted to a CDP-aminoalcohol by a CTP-phosphoaminoalcohol cytidylyltransferase. In the third and final step mediated by the aminoalcohol phosphotransferases Cpt1p and Ept1p, the phosphoaminoalcohol moiety is transferred to diacylglycerol (DAG) (for review see [2]). Cpt1p and Ept1p exhibit 54% sequence homology [7], and the encoding genes most likely arose from a common ancestor by whole genome duplication [8].

In yeast strains with defects in PS decarboxylation or in PE methylation, the CDP-ethanolamine and CDP-choline pathways fulfill the requirements for PE and PC, respectively, provided that ethanolamine and choline are supplied in the medium [3,9]. The notion that the CDP-choline route serves primarily as an auxiliary pathway in the synthesis of PC in wild-type yeast was proven incorrect, as this biosynthetic pathway also contributes to PC formation in the absence of exogenous choline [10]. By recycling degradation products of PC, the CDP-choline route may contribute to maintaining the proper PC species composition, i.e., the profile of acyl chains in PC [11]. Very little is known about the physiological role of the CDP-ethanolamine route. It may recycle ethanolamine and ethanolaminephosphate derived from PE and sphingoid base phosphates, respectively, into PE [12]. Both Kennedy pathways, in particular the membrane-associated enzymes Ept1p and Cpt1p consume DAG, which has been implicated in the yeast secretory pathway (reviewed in [2,13,14]). Whereas inactivation of genes in the CDP-choline pathway bypasses the essential function of Sec14p, inactivation of genes in the CDP-ethanolamine pathway does not [15,16].

The substrate specificities of Ept1p and Cpt1p toward a limited set of DAG species and different CDP-aminoalcohols have been characterized in vitro using microsomes containing either one of the two enzymes in a mixed micellar assay [17,18]. In the presence of detergent both enzymes were found to exhibit broad but distinct specificities for their DAG substrates [17,18]. Here, the predictive value of the in vitro data for the in vivo condition was tested, by comparing the utilization of substrates by Ept1p and Cpt1p, in intact yeast cells, in order to analyze how the Kennedy pathways contribute to the PE and

* Corresponding author. Fax: +31-30-2533969.

E-mail address: h.a.boumann@chem.uu.nl (H.A. Boumann).

Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; (P)MME, (phosphatidyl)monomethylethanolamine; (P)DME, (phosphatidyl)dimethylethanolamine; PPrN, phosphatidylpropanolamine; DAG, diacylglycerol; ESI-MS/MS, electrospray ionization tandem mass spectrometry

PC species profiles. Using electrospray ionization tandem mass spectrometry (ESI-MS/MS) in phospholipid analysis [19], in combination with stable isotope labeling [11,20], it is now possible to examine the substrate use of these enzymes in vivo. Pulse labeling yeast cells with deuterated ethanolamine and choline revealed that Ept1p and Cpt1p utilize different sets of DAG species. In addition, the aminoalcohol specificities of Ept1p and Cpt1p in vivo have been addressed. The results are discussed in the light of the substrate selectivity of the enzymes in vitro, and of the physiological functions of both phospholipid biosynthetic pathways.

2. Materials and methods

2.1. Materials

Yeast extract was supplied by Sigma (St. Louis, MO). Yeast Nitrogen Base (YNB) without amino acids was obtained from Difco (Detroit, MI). Deuterium labeled (D_4)-ethanolamine and (D_{13})-choline were purchased from Cambridge Isotope Laboratories (Andover, MA). 2-(Methylamino)-ethanolamine was supplied by Fluka (Buch, Switzerland), *N,N*-dimethylethanolamine and 3-amino-1-propanol were from Aldrich (Milwaukee, WI). All other chemicals were of analytical grade.

2.2. Yeast strains

The parental strain *S. cerevisiae* BY4742 (*MAT α his3 Δ 1 leu2 Δ 0 lys2 Δ 0 ura3 Δ 0*) and the congenic *cho2 Δ* strain (*cho2::KanMX*) were obtained from Research Genetics (Paisley, UK). The strains *ept1 Δ* (*ept1::KanMX*) and *cpt1 Δ* (*cpt1::KanMX*) were from Euroscarf (Frankfurt, Germany). The *LEU2* marker [21] was introduced into the *cho2 Δ* strain to substitute for the *OPI3* gene (nucleotides –11 to 621), thereby generating the *cho2 Δ opi3 Δ* strain (*cho2::KanMX opi3::LEU2*). PCR was carried out to verify the correct integration of the *LEU2* marker into the yeast genome.

2.3. In vivo deuterium pulse labeling of phospholipids

Yeast strains were cultured aerobically at 30 °C in 0.8 L of semi-synthetic lactate (SSL) medium [22] supplemented with 20 mg/L histidine, 60 mg/L leucine, 230 mg/L lysine and 40 mg/L uracil. Lactate was used as carbon source because of our interest in phospholipid metabolism under conditions of optimal mitochondrial development. The medium of the *cho2 Δ opi3 Δ* strain was additionally supplemented with 1.0 mM choline. Cells in the midlog phase of growth were harvested, washed with, and resuspended in 0.8 L of synthetic lactate (SL) medium [0.67% YNB without amino acids, 2% lactate, 0.1% glucose, pH 5.5] with the supplements mentioned above except choline. In order to obtain sufficient incorporation of label to enable accurate mass spectrometry analysis, cells were pulsed with 2.0 mM (D_4)-ethanolamine for 30 min, whereas 10 min labeling with 0.2 mM (D_{13})-choline was sufficient to detect labeled PC (not shown). Pulsing the cells with (D_{13})-choline for 30 min instead of 10 min, did not significantly affect the species profile of labeled PC. The *cho2 Δ opi3 Δ* strain was also pulsed with 4.0 mM monomethylethanolamine (MME) or 4.0 mM dimethylethanolamine (DME) for 30 min, or with 20 mM propanolamine for 2 h. After the pulse, cellular processes were arrested by adding an ice-cold mixture of KCN, NaF, and NaN₃ to final concentrations of 15 mM each. Cell pellets corresponding to ~100 OD₆₀₀ units were homogenized using glass beads and phospholipids were extracted as described [23].

2.4. ESI-MS/MS

Lipid extracts were dissolved at 0.5 mM total phospholipid-phosphorus in CHCl₃/CH₃OH/H₂O (2:15:3, v/v/v) containing 1.0% (v/v) formic acid. Samples were analyzed on a Quattro Ultima triple quadrupole MS instrument (Micromass, Manchester, UK) equipped with a nano-electrospray ion source, and introduced into the instrument at a flow rate of ~50 nL/min. The capillary and cone voltages were set at 1.5 kV and 30 V, respectively, and all measurements were performed in the positive ion mode. Collision-activated dissociation was applied using argon and collision energies of 30 eV. The [M + H]⁺ and [M + Na]⁺ adducts of unlabeled PE, (D_4)-labeled PE, phosphati-

dylmonomethylethanolamine (PMME), phosphatidylmethylethanolamine (PDME), and phosphatidylpropanolamine (PPrN) were monitored by neutral loss scanning for *m/z* 141, 145, 155, 169, and 155, respectively. The [M + H]⁺ ions of unlabeled and D_{13} -labeled PC were detected by parent ion scanning at *m/z* 184 and 197, respectively. Data were collected by averaging 40–100 repetitive scans with a scan time of 10 s, obtained from at least two independent experiments. For quantification, ESI-MS/MS data were processed using Masslynx NT software (Micromass), thereby taking into account the three most prominent isotopic signals of each molecular species. Correction for the inverse relationship between mass and signal response of the MS instrument was performed as described previously [11,19]. This correction was not applied to spectra of the labeled phospholipids, since these lipids were introduced into the instrument at low concentrations (<0.005 mM). In that concentration range the instrument response was found to be virtually independent of the acyl chain length in the relevant *m/z* range (cf. [24]).

3. Results

3.1. Comparison of the species compositions of newly synthesized PE and PC via the Kennedy pathways

In order to monitor the molecular species composition of newly synthesized PE and PC, wild-type yeast cells were pulse labeled with (D_4)-ethanolamine and (D_{13})-choline, respectively. The ESI-MS/MS spectrum of (D_4)-PE obtained after 30 min labeling with (D_4)-ethanolamine revealed the presence of 34:2, 34:1, 32:2, and 32:1 species (Fig. 1A), that presented paired combinations of C16:0, C16:1, C18:0, and C18:1, the predominant acyl chains in yeast [25]. Although the PE synthesized via the CDP-ethanolamine pathway can be used for the formation of PC via methylation [16], parent ion scanning at *m/z* 188 did not reveal significant conversion of (D_4)-PE to (D_4)-PC upon 30 min labeling with (D_4)-ethanolamine (data not shown). This indicates that there is no appreciable methylation of the CDP-ethanolamine route derived PE under the conditions used. The (D_4)-PE species composition was quantified and compared to that of (D_{13})-PC newly synthesized via the CDP-choline route [11], revealing that the PE species profile is different from that of PC (Fig. 1B). Whereas 34:2 dominated the species profile of (D_4)-PE, 32:2 was the major species of newly synthesized (D_{13})-PC (Fig. 1B). These findings demonstrate that Ept1p and Cpt1p consume distinct sets of DAG species to produce PE and PC in vivo, respectively. Both profiles of newly synthesized (D_4)-PE and D_9 -PC differed from the corresponding steady-state species profiles (cf. [11]), indicating that PS decarboxylation and PE methylation are major contributors to the steady-state molecular compositions of PE and PC, respectively. In addition, remodeling by acyl chain exchange could contribute to the steady state species profiles as was recently demonstrated for PC [11].

Whereas the final step in the CDP-ethanolamine route proceeds exclusively via Ept1p, both Ept1p and Cpt1p have been reported to be capable of transferring phosphocholine to DAG both in vitro [26,27], and in vivo, with the relative contribution of Ept1p to PC synthesis in vivo depending on the strain background [10,28]. To evaluate the contribution of both aminoalcohol phosphotransferases to PC production in BY4742, and to assess the role of the CDP-aminoalcohol substrate in the specificity of Ept1p for DAG species, *ept1 Δ* and *cpt1 Δ* cells were examined in pulse labeling experiments. ESI-MS/MS analysis showed that the species composition of (D_{13})-PC produced in *ept1 Δ* cells via Cpt1p was similar to that

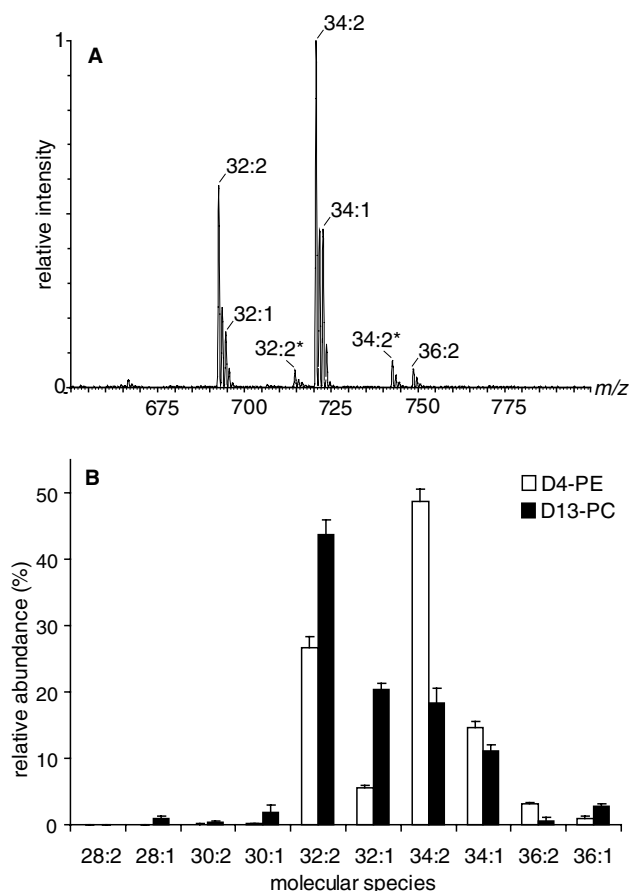


Fig. 1. The CDP-ethanolamine and the CDP-choline routes use distinct sets of DAG species in wild-type yeast in vivo. (A) ESI-MS/MS spectrum of newly synthesized (D₄)-PE in wild-type yeast labeled with (D₄)-ethanolamine for 30 min. (D₄)-PE species were detected by neutral loss scanning for m/z 145. Both $[M+H]^+$ and asterisk-marked $[M+Na]^+$ PE species are shown. The unmarked peaks represent isotopic signals originating from the natural abundance of ¹³C. (B) Quantification of the species composition of (D₄)-PE (white, means \pm S.D., $n = 4$) in wild-type yeast, compared to that of newly synthesized (D₁₃)-PC (black, means \pm S.D., $n = 3$) obtained by pulsing cells for 10 min with (D₁₃)-choline (taken from [11]).

of D₁₃-labeled PC in wild-type cells (compare Figs. 1B and 2, black bars). The species profiles of (D₁₃)-PC and (D₄)-PE observed in *cpt1Δ* were similar (compare gray to white bars in Fig. 2), and resembled that of (D₄)-PE rather than that of (D₁₃)-PC in wild-type cells, except for significant increases in the relative amounts of the mono-unsaturated species at the expense of the di-unsaturated species (cf. Fig. 1B, white bars). The combined results indicate (i) that the nature of the CDP-aminoalcohol substrate of Ept1p does not significantly influence its specificity for the DAG species used as lipid substrate; (ii) that Cpt1p activity affects the substrate use by Ept1p; (iii) that Ept1p plays only a minor role in PC biosynthesis in wild-type yeast in agreement with previous studies [10,29].

3.2. CDP-aminoalcohol specificity of Ept1p and Cpt1p in vivo

Apart from ethanolamine and choline, the Kennedy pathways can also use MME and DME as substrates to yield PMME and PDME, respectively [17]. By adding MME or DME to the culture medium, PC synthesis and growth of *cho2Δ* yeast strains with an impaired PE methylation pathway

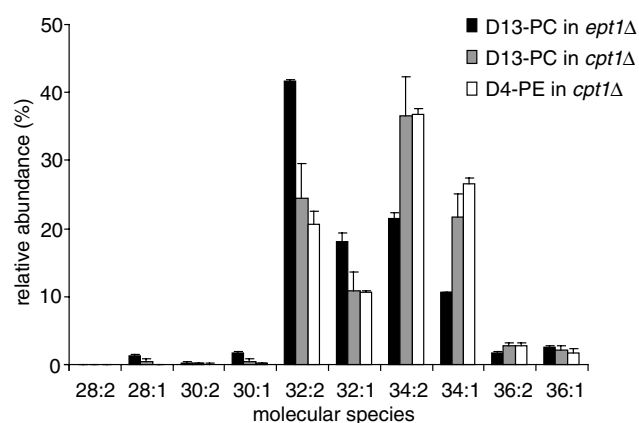


Fig. 2. The species profiles of (D₁₃)-PC (black, means \pm S.D., $n = 3$) synthesized by Cpt1p in *ept1Δ*, and of (D₁₃)-PC (gray bars, means \pm S.D., $n = 3$) and (D₄)-PE (white, means \pm S.D., $n = 3$) synthesized by Ept1p in *cpt1Δ* yeast cells. Cells were pulsed with (D₁₃)-choline for 10 min and with (D₄)-ethanolamine for 30 min, and total lipid extracts were subjected to ESI-MS/MS in the parent ion scan mode (m/z 197), and neutral loss mode (m/z 144), respectively.

is restored to wild-type levels [9,30]. To investigate which of the two aminoalcohol phosphotransferases is used in vivo for the incorporation of the aminoalcohols MME and DME into phospholipids, a *cho2Δopi3Δ* strain was used. This strain lacks the phospholipid methyltransferases, thereby permitting the detection of newly formed PMME and PDME, which are short-lived intermediates in wild-type cells [31]. First, we analyzed the steady-state species compositions of PE and PC and observed that both profiles were very similar to those in the parental wild-type strain (Fig. 3A) (cf. [11]). Subsequently, *cho2Δopi3Δ* cells were pulse labeled with (D₄)-ethanolamine and (D₁₃)-choline. As the compositions of new Kennedy pathway derived PE and PC were comparable to those observed in the wild-type strain (Figs. 1 and 3B), we concluded that the *cho2Δopi3Δ* strain is a valid in vivo model to examine the aminoalcohol preferences of Ept1p and Cpt1p.

When *cho2Δopi3Δ* cells were pulsed with MME, ESI-MS/MS analysis revealed that the species profile of PMME strongly resembled that of newly formed PE (Fig. 3B). In contrast, pulse labeling with DME resulted in a PDME species composition matching that of newly synthesized PC. These findings demonstrate that CDP-MME is preferentially used as substrate by Ept1p, whereas CDP-DME is preferentially used by Cpt1p in vivo.

3.3. Incorporation of propanolamine into phospholipids

Recently, it was shown that the artificial phospholipid PPrN comprised up to 40% of total phospholipids in a *psd1Δpsd2Δ* mutant supplemented with propanolamine, and strongly reduced the requirement for PE in yeast [32]. Here, we assessed the metabolic origin of PPrN, using the characteristic species profiles of the Kennedy pathway derived PE and PC as molecular fingerprints. *Cho2Δopi3Δ* cells were labeled with propanolamine and PPrN was monitored by neutral loss scanning for the headgroup m/z 155 [32]. The composition of the PPrN species strongly resembled that of newly formed PC (compare Fig. 4 inset with Fig. 3B, black bars), demonstrating that Cpt1p rather than Ept1p transfers the propanolaminephosphate moiety to DAG.

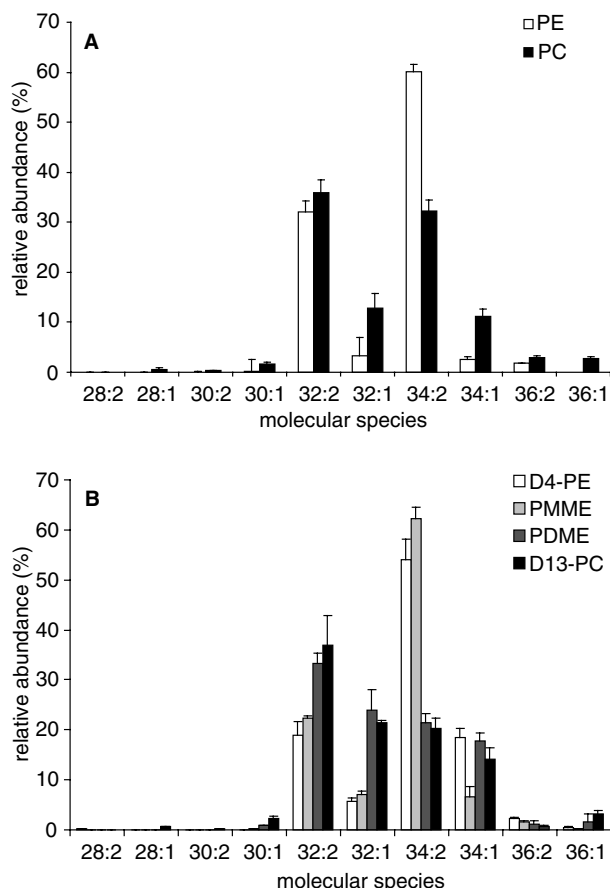


Fig. 3. The specificity of Cpt1p and Ept1p for CDP-aminoalcohols in vivo, assessed in a *cho2Δopi3Δ* strain. (A) Steady-state species compositions of PE and PC in *cho2Δopi3Δ* cells (\pm S.D., $n \geq 3$). (B) Species profiles of newly synthesized (D₄)-PE, PMME, PDME, and (D₁₃)-PC in *cho2Δopi3Δ* cells pulse-labeled with (D₄)-ethanolamine (white), MME (light gray), DME (dark gray), and (D₁₃)-choline (black), respectively (mean \pm variation, $n = 2$). For experimental details, see Section 2.

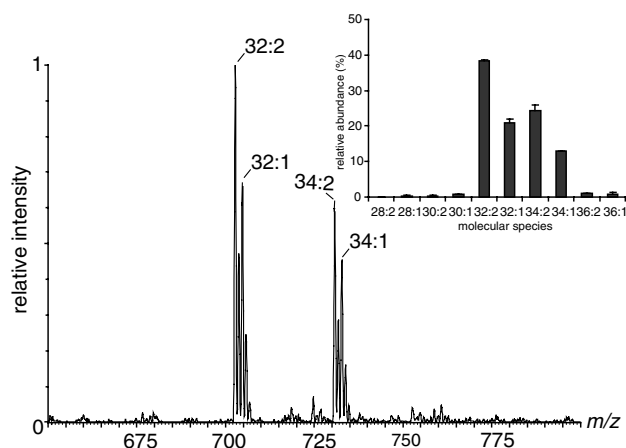


Fig. 4. The species profile of the artificial phospholipid PPRn reflects synthesis by Cpt1p. ESI-MS/MS spectrum (neutral loss scanning for m/z 155) of PPRn in a total lipid extract of *cho2Δopi3Δ* cells grown in the presence of 20 mM propanolamine for 2 h. The inset depicts the quantified species distribution (means \pm variation, $n = 2$).

4. Discussion

The substrate specificities of the aminoalcohol phosphotransferases Ept1p and Cpt1p were investigated in vivo, by pulse labeling yeast cells with deuterated lipid precursors and analyzing the phospholipids by ESI-MS/MS. Ept1p and Cpt1p were found to convert the endogenous DAG species to different extents, resulting in characteristic profiles of PE and PC species newly synthesized by Ept1p on the one hand, and of PC species produced by Cpt1p on the other.

The selectivity in the consumption of DAG species is most likely the result of the specific catalytic properties of the enzymes and/or the availability of DAG substrates at the sites of the enzymes. The DAG substrate specificities of Ept1p and Cpt1p were studied previously in vitro in a mixed micellar assay [18], in which the conversion of several synthetic DAG species was tested. Ept1p showed the highest activity toward di-unsaturated DAG species ($36:2 > 32:2 > 34:1$), consistent with the present in vivo results ($34:2 > 32:2 \sim 34:1$, Figs. 1–3). Cpt1p was found to preferentially use 32:2 in vitro ($32:2 \gg 34:1 > 36:2$) [18], again in agreement with the in vivo data ($32:2 > 32:1 \sim 34:2 > 34:1$, Figs. 1–3).

However, when comparing the preferential use of DAG species by Ept1p and Cpt1p in vivo versus in vitro, it should be realized that two endogenous species, 32:1 and 34:2, were not tested in vitro, and that the species composition of DAG available to Ept1p and Cpt1p in vivo is not known and not necessarily the same. The subcellular localizations of Ept1p and Cpt1p have yet to be unambiguously resolved, in contrast to those of their mammalian counterparts that have been localized to ER and Golgi, respectively [33]. Subcellular fractionation studies in yeast showed an enrichment of Cpt1p activity in microsomal fractions and Golgi [34–36]. More recently, green fluorescent protein-tagged versions of both Ept1p and Cpt1p revealed punctate composite localizations, with Ept1p co-localizing with a Golgi-marker [37]. If the subcellular localizations of Ept1p and Cpt1p differ like in higher eukaryotes, the enzymes may have access to spatially separate pools of DAG with different species compositions, as the spontaneous intermembrane exchange of DAG is extremely slow [38]. Irrespective of the localization, our finding that the profile of DAG species converted by Ept1p was affected by deleting the *CPT1* gene (Fig. 2) indicates that the putative separate DAG pools do communicate.

The bypass of the essential function of Sec14p by genetic inactivation of the CDP-choline pathway but not the CDP-ethanolamine route has been attributed to an increased content of DAG in the Golgi membranes, based on the assumption that Cpt1p is localized in the Golgi membranes while Ept1p is not [33]. Alternatively, based on the present results one could postulate that the differential consumption of molecular species of DAG by the two routes accounts for their functional difference with respect to Sec14p defects, that is, if indeed DAG is instrumental in bypassing defects in Sec14p function (for a discussion see [2,13,39]).

The present data show that the profile of PE species newly synthesized by the CDP-ethanolamine route in wild-type cells does not match the PE steady-state species distribution (cf. [11]). It is very well possible that this pathway plays a physiological role in maintaining the proper PE species distribution via recycling of ethanolamine upon turnover of PE. Establishing such a role for the CDP-ethanolamine route awaits the

analysis of the PE species synthesized by decarboxylation of PS in wild-type yeast and appropriate mutants. With respect to the CDP-choline route we have previously shown that this pathway may contribute significantly to the newly synthesized mono-unsaturated PC species in wild-type yeast [11].

The characteristic species profiles of the products of Ept1p and Cpt1p were used as molecular fingerprints for establishing the specificities of Ept1p and Cpt1p toward the CDP-aminoalcohol derivatives of MME and DME in vivo in a *cho2Δopi3Δ* strain. A remarkable dichotomy was observed with PMME carrying the species signature of Ept1p and PDME that of Cpt1p. These results show that the nature of the CDP-aminoalcohol substrate does not influence the enzymes' specificity for the DAG species. Whereas the selective incorporation of CDP-MME by Ept1p in intact yeast cells is in agreement with previous in vitro results [17], the selective use of CDP-DME by Cpt1p in vivo is unexpected in view of the in vitro data showing that the K_m and V_{max} values of Ept1p for CDP-DME, CDP-MME, and CDP-ethanolamine were similar and compared favorably to those of Cpt1p for CDP-DME [17]. Conversion of CDP-DME by Cpt1p apparently outweighs that by Ept1p in vivo, possibly due to differences in enzyme abundance. Alternatively, metabolic channeling between Ept1p and Cpt1p, and the CTP-phosphoaminoalcohol cytidylyltransferases Ect1p and Pct1p, catalyzing the previous step in the Kennedy pathways, might be involved (cf. [40]). In this respect it is noteworthy that the partitioning of the CDP-aminoalcohols over Ept1p and Cpt1p in vivo, follows that of the corresponding aminoalcoholphosphates utilized by Ect1p and Pct1p, respectively [41].

Contrary to expectation, CDP-propanolamine, the precursor of the artificial phospholipid PPrN [32], was preferentially used as substrate by Cpt1p rather than by Ept1p. Since propanolamine differs from ethanolamine by only one methylene group and contains a primary amine it was expected to serve as substrate for Ept1p and not for Cpt1p. This result implies that the extra methylene group impairs a productive interaction of CDP-propanolamine with Ept1p leaving it as a substrate for Cpt1p, unless metabolic channeling is involved.

ESI-MS/MS combined with stable isotope labeling has proven a powerful method for resolving the substrate preferences of phospholipid biosynthetic enzymes in vivo. This approach adds a new dimension to research on the many functions of lipids in cell biology by its ability to visualize changes in molecular species in vivo.

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