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Cloning, Genomic Organization, and Characterization of a Human Cholinephosphotransferase*

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A cholinephosphotransferase activity catalyzes the final step in the *de novo* synthesis of phosphatidylcholine *via* the transfer of a phosphocholine moiety from CDP choline to diacylglycerol. Ethanolaminephosphotransferase activity catalyzes a similar reaction substituting CDP ethanolamine as the phosphobase donor. We report the identification and cloning of a human cDNA (human cholinephosphotransferase (hCPT1)) that codes for a cholinephosphotransferase-specific enzyme. This was demonstrated using *in vitro* enzyme assays and *in vivo* measurement of the reconstitution of the phosphatidylcholine and phosphatidylethanolamine biosynthetic pathways in yeast cells devoid of their own endogenous cholinephosphotransferase and ethanolaminephosphotransferase activities. This contrasted with our previously cloned human choline/ethanolaminephosphotransferase cDNA that was demonstrated to code for a dual specificity choline/ethanolaminephosphotransferase. The hCPT1 and human choline/ethanolaminephosphotransferase (hCEPT1) predicted amino acid sequences possessed 60% overall identity and had only one variation in the amino acid residues within the CDP-alcohol phosphotransferase catalytic motif. *In vitro* assessment of hCPT1 and hCEPT1 derived cholinephosphotransferase activities also revealed differences in diradylglycerol specificities including their capacity to synthesize platelet-activating factor and platelet-activating factor precursor. Expression of the hCPT1 mRNA varied greater than 100-fold between tissues and was most abundant in testis followed by colon, small intestine, heart, prostate, and spleen. This was in marked contrast to the hCEPT1 mRNA, which has been found in similar abundance in all tissues tested to date. Both the hCPT1 and hCEPT1 enzymes were able to reconstitute the synthesis of PC in yeast to levels provided by the endogenous yeast cholinephosphotransferase; however, only hCEPT1-derived activity was able to complement the yeast *CPT1* gene in its interaction with *SEC14* and affect cell growth.

Phosphatidylcholine (PC)[1] and phosphatidylethanolamine (PE) are the two most abundant phospholipids found in eukaryotic cells, generally comprising 50 and 25% of cellular phospholipid mass, respectively (1). PC synthesis via the CDP choline pathway is responsible for essentially all *de novo* PC biosynthetic activity in all eukaryotic cell types thus far examined except (i) the liver where the methylation of phosphatidylethanolamine is predicted to contribute to 30% of net synthesis (2, 3) and (ii) yeast where the supply of exogenous choline dictates the relative contribution of the two PC biosynthetic pathways (4, 5). PE can also be synthesized by alternate routes with the CDP ethanolamine pathway supplemented by the decarboxylation of phosphatidylserine to PE, with the relative contribution of each route dependent on cell type (6–9).

The final step in the CDP alcohol pathways for the synthesis of PC and PE is catalyzed by cholinephosphotransferase and ethanolaminephosphotransferase enzyme activities, respectively. Cholinephosphotransferase catalyzes the transfer of phosphocholine from CDP choline to diacylglycerol (DAG) with the release of CMP and the formation of PC (10–13), whereas the ethanolaminephosphotransferase reaction catalyzes a similar transfer substituting CDP ethanolamine as the phosphobase donor resulting in the formation of PE (14–16). A cholinephosphotransferase has also been implicated in *de novo* synthesis of platelet-activating factor (PAF) (17, 18). This putative PAF-specific cholinephosphotransferase was identified based on DTT susceptibility of cholinephosphotransferase activities measured in microsomal membranes, with PAF-specific activity being DTT resistant and PC-specific activity being DTT sensitive (18).

The relative contribution of the CDP choline *versus* PE methylation pathways for the synthesis of PC have been implicated in regulating cell growth in the mammalian liver. PC synthesis through the CDP choline pathway favored proliferation, whereas PC synthesized by PE methylation inhibited cell growth and was negatively associated with the induction of liver tumor formation (19–21). PC molecules synthesized by these two pathways have different fatty acyl chain compositions and this has been postulated to be the major contributor to the observed effects on cell growth (3). The diacylglycerol specificity of cholinephosphotransferase regulates the fatty acyl composition of *de novo* PC synthesized through the CDP choline pathway and thus would be predicted to participate in the regulation of PC pathway-dependent liver tumor development. We recently cloned and characterized a human dual

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s)AF195623 and AF195624.

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[1] The abbreviations used are: PC, phosphatidylcholine; PE, phosphatidylethanolamine; DAG, diacylglycerol; PAF, platelet-activating factor; DTT, dithiothreitol; hCEPT1, human choline/ethanolaminephosphotransferase; hCPT1, human cholinephosphotransferase; RACE, rapid amplification of cDNA ends; kb, kilobase; PCR, polymerase chain reaction; *CPT1*, cholinephosphotransferase of *S. cerevisiae*; *EPT1*, choline/ethanolaminephosphotransferase of *S. cerevisiae*.

specificity choline/ethanolaminephosphotransferase (hCEPT1) capable of using both CDP choline and CDP ethanolamine as substrates *in vitro* (22). Metabolic labeling experiments demonstrated this enzyme was able to reconstitute the synthesis of both PC and PE when expressed in a yeast strain in which the endogenous cholinephosphotransferase and ethanolaminephosphotransferase genes were inactivated. In the work presented here we *de novo* cloned two cDNAs, each coding for a splice variant of a human cholinephosphotransferase (hCPT1). The cDNA product was expressed for characterization *in vitro* and *in vivo* and was found to code for a cholinephosphotransferase-specific enzyme.

EXPERIMENTAL PROCEDURES

Materials—[α-^{32}P]dATP (3000 Ci/mmol) and [γ-^{32}P]ATP (3000 Ci/mmol) were purchased from NEN Life Science Products. [Methyl-^{14}C]CDP choline, [methyl-^{14}C]choline, and [1,2-^{14}C]ethanolamine were purchased from American Radiolabeled Chemicals. [Ethanolamine-1,2-^{14}C]CDP ethanolamine was a product of ICN. λGT11 forward sequencing primer, λGT11 human Quick-Clone cDNA libraries, and advantage cDNA polymerase were purchased from CLONTECH. The pCMV-Sport human brain cDNA library, custom oligonucleotides, and T4 DNA ligase were products of Life Technologies, Inc. Manual dideoxy sequencing was performed utilizing the ^{T7}sequencing kit (Amersham Pharmacia Biotech). Lipids were purchased from Avanti Polar Lipids. All other reagents were of the highest quality commercially available.

Isolation of a Full-length hCPT1 cDNA—An analysis of the expressed sequence tags data bases (23) for clones with significant similarity to the active site of *Saccharomyces cerevisiae* cholinephosphotransferase (Cpt1p) resulted in the identification of two groups of mammalian cDNAs. Each group likely represented an isoform of mammalian cholinephosphotransferase. Alignment of the sequences within these groups revealed there were no full-length cDNAs represented for one of these groups. To obtain a full-length cDNA the longest expressed sequence tag clone (expressed sequence tag clone 67440) was obtained from the IMAGE consortium and sequenced in its entirety on both strands utilizing a combination of manual dideoxy (24) and automated techniques (Li-cor apparatus, National Research Council of Canada/Dalhousie University Joint Laboratory). The 5′-end of the cDNA was successfully extended through two successive rapid amplification of cDNA ends (RACE) protocols (25) from human λGT11 cDNA libraries. However, the 5′-end of the cDNA did not code for an obvious initiator Met residue. A full-length cDNA was isolated using the Genetrapper method (Life Technologies, Inc.) (26) by biotinylating an oligonucleotide corresponding to the most 5′-RACE extended sequence, 5′-TATCGGCGGGTGGAGCACCGCTACAGC-3′, using terminal deoxynucleotide transferase and hybridizing the oligonuceltoide in solution to a single-stranded human brain cDNA library that had been generated by digestion of the library with Gene II protein and exonuclease III. Avidin bound to magnetic beads was used to selectively precipitate the biotinylated probe/hybridized cDNA complex to enrich the library for cDNAs complementary to the hCPT1-specific oligonucleotide. The single-stranded cDNAs were extended using Klenow, *Escherichia coli* were transformed, and colonies were screened for positive clones by hybridization *versus* an end-labeled hCPT1-specific antisense oligo 5′-CCAGTCAGGATAAGTTCCTAAGCGA-3′. Separately, cDNA libraries for adult human eye tissues were constructed in the pCMVSPORT6 vector (Life Technologies, Inc.). Clones were picked for sequencing at the National Institutes of Health Intramural Sequencing Center (NISC). Novel, potential full-length clones were identified from 5′ sequencing analyzed using BLAST at NCBI.

Northern Blot Analysis—Random primed ^{32}P-labeled probes were synthesized *versus* either the entire 1.2-kb coding region of hCPT1 or a 2.0-kb region of human β-actin cDNA. Multiple human tissue Northern blots (CLONTECH) were hybridized at 68 °C in ExpressHyb solution (27) for 1 h and washed as per the manufacturer's instructions. Blots were exposed to x-ray film for 1–3 days.

Protein Expression—The coding sequence for hCPT1 was amplified by PCR and subcloned into pET23a (Novagen) resulting in the addition of the coding sequence for a T7 epitope tag to the 5′-end of the open reading frame of hCPT1. The PCR-amplified fragment was sequenced in its entirety to ensure polymerase fidelity. The T7-tagged hCPT1 insert was then subcloned into p416GPD (28) for constitutive expression of hCPT1-encoded protein in *S. cerevisiae*. The HJ091 strain of *S. cerevisiae* is devoid of endogenous cholinephosphotransferase and ethanolaminephosphotransferase activities because of inactivated alleles at the loci coding for these activities (*cpt1::LEU2 ept1*$^-$) (29, 30).

Western Blot Analysis—Membranes were prepared from HJ091 *S. cerevisiae* cells (*cpt1::LEU2 ept1*$^-$) grown to mid-log phase in appropriate medium to ensure plasmid maintenance (30, 31). Proteins were incubated with SDS-polyacrylamide gel electrophoresis sample buffer at 37 °C for 30 min, separated on a 12% SDS-polyacrylamide gel electrophoresis gel, and transferred to polyvinylidene difluoride membranes (32). Blots were probed with a T7 epitope tag-specific monoclonal antibody (1:5000, Novagen) coupled to horseradish peroxidase for subsequent detection using the ECL (Amersham Pharmacia Biotech) system.

Enzyme Assays—Several assays for the measurement of cholinephosphotransferase and ethanolaminephosphotransferase activities were used in this study. Method one was routinely used unless otherwise indicated. This assay delivers lipids as an emulsion, whereby diradylglycerols or ceramides were dried under nitrogen gas and resuspended in 0.015% (w/v) Tween 20 by sonication. Unless otherwise indicated, assay buffer 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. Components were incubated at room temperature for 5 min to allow for the lipids to be incorporated into the microsomal membrane and this was followed by the addition of CDP choline or CDP ethanolamine (0.4 mM, 2000 dpm/nmol). Assays were incubated at 37 °C for 15 min (33). The second method used also delivered lipids in a lipid emulsion of either 0.1% Tween 20 or 0.1% deoxycholate (w/v) as the final detergent concentration (modified from Sleight and Kent (34)), but otherwise assay conditions were identical to method one. Method three used a PC liposome to deliver diacylglycerol to the membranes and was performed as described (35). The final two methods used excess detergent to form mixed micelles using either Triton X-100 or sodium cholate. The Triton X-100 assay was performed as described (36). The sodium cholate assay was modifed from the assay described by Bru *et al.* (37) and was performed in 50 mM Tris-HCl (pH 8.0), 20 mM $MgCl_2$, 20% glycerol, and 10 mol % DAG and 10 mol % PC in 1% sodium cholate using 10–25 μg of membrane protein as the enzyme source. The assay mixture was incubated at 25 °C for 5 min to allow for mixed micelle formation and then initiated by the addition of CDP choline or CDP ethanolamine (0.4 mM, 2000 dpm/nmol) and incubated at 25 °C for 20 min.

The PC liposome assay was terminated as described (35). Each of the other assays was terminated by the addition of 3 ml of $CHCl_3/CH_3OH$ (2/1, v/v) and 1.5 ml of 0.9% (w/v) KCl to each 0.1-ml assay (38). Tubes were vortexed, and phase separation was facilitated by centrifugation at 2000 $\times$ *g* for 10 min. The aqueous phase was aspirated and the organic phase was washed twice with 1.5 ml of 40% CH_3OH/H_2O (v/v). An aliquot of the organic phase was dried in a scintillation vial, and radioactivity was determined. Samples were routinely analyzed by thin layer chromatography on silica gel plates in a solvent system of $CHCl_3/CH_3OH/NH_4OH/H_2O$ (70/30/4/2, v/v) to confirm products. Some assays resulted in low levels of activity in the absence of added exogenous diradylglycerol because of utilization of endogenous diacylglycerols present in the membrane preparations. These values were subtracted from standard enzyme assays to obtain specific enzyme activity values for each diradylglycerol species.

Metabolic Labeling—S. cerevisiae HJ091 cells (*cpt1::LEU2 ept1*$^-$) transformed with either hCPT1 or hCEPT1 in the constitutive expression vector p416GPD, or empty p416GPD vector, were grown to mid-log phase in synthetic dextrose medium containing appropriate nutritional supplements to ensure plasmid maintenance (31). [^{14}C]Choline (10 μM, 1×10^5 dpm/nmol) was added to the cultures for 1 h (30). For [^{14}C]ethanolamine experiments, the cells were washed twice in synthetic dextrose medium plus required nutritional supplements but without ammonium sulfate and resuspended minus ammonium sulfate medium before the addition of [^{14}C]ethanolamine (6.7 μM, 2.2×10^5 dpm/nmol) for 1 h (22). The reduced nitrogen-containing medium was required for the efficient uptake of ethanolamine. Subsequent to incubation with radiolabel, cells were concentrated by centrifugation, washed twice with water, and resuspended in 1 ml of $CHCl_3/CH_3OH$ (1/1, v/v). Cells were disrupted for 1 min at 4 °C using a BioSpec Multi Bead Beater containing 0.5 g of 0.5-mm acid-washed glass beads. The beads were washed with 1.5 ml of $CHCl_3/CH_3OH$ (2/1, v/v). To facilitate phase separation 1.5 ml of water and 0.5 ml of $CHCl_3$ were added. Phospholipids in the organic phase were routinely analyzed by thin layer chromatography on Whatman silica gel 60A plates using the solvent system $CHCl_3/CH_3OH/H_20/CH_3COOH$ (70/30/4/2, v/v) for subsequent radioactivity determination by scintillation counting.

Yeast Phenotype Complementation—S. cerevisiae strain CTY434 (**a**

ura3–52 ade2–101 leu2–3, 112 his4–519 s14^{ts} *cpt1::LEU2*, a kind gift from Dr.Vytas Bankaitis) transformed with expression plasmids containing hCPT1, hCEPT1, yeast *CPT1*, or vector controls were grown overnight in synthetic dextrose medium under selection for plasmid maintenance at 25 °C (31). Cell density was estimated by monitoring A_{600} of a 1:10 dilution into water. Identical cell numbers were serial-diluted 1:10 into water, and 1 μl of each dilution was spotted onto synthetic minimal medium agar plates supplemented as required for cell growth and plasmid maintenance. Plates were incubated for 4 days at 25 or 37 °C.

Protein and Lipid Determinations—Protein was determined by the method of Lowry *et al.* (39) using bovine serum albumin as the standard. Diradylglycerols not available commercially were prepared from PC by digestion with *Bacillus cereus* phospholipase C, and the yield was estimated using the method of Stern and Shapiro (40). Phospholipid phosphorus was determined by the method of Ames and Dubin (41).

RESULTS

Isolation of a Full-length Human Cholinephosphotransferase 1 cDNA—Previous structure/function analysis of the integral membrane-bound yeast cholinephosphotransferase (*CPT1*) and choline/ethanolaminephosphotransferase (*EPT1*) enzymes resulted in several predictions (11, 29). The first three membrane-spanning helices are the maximum region required for DAG binding, and the first cytoplasmic loop is required for CDP alcohol binding. 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 (Asp-Gly-X_2-Ala-Arg-X_8-Gly-X_3-Asp-X_3-Asp). Site-directed mutagenesis analysis studies predict this motif performs catalysis by employing the final two Asp residues in a general base reaction that does not proceed through an enzyme-bound intermediate (30). A search of the expressed sequence tag data base (23) 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 previously expressed and found to be capable of (i) synthesizing PC and PE *in vitro* and (ii) metabolically reconstituting both the CDP choline and CDP ethanolamine pathways *in vivo* in a yeast strain where the endogenous cholinephosphotransferase and ethanolaminephosphotransferase genes had been inactivated (22). 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. In the present study, we extended the longest of these partial cDNAs by an additional 322 base pairs by two successive 5′-RACE protocols, and a subsequent screen of a human brain cDNA library with an oligonucleotide derived from the most 5′-end of the furthest RACE extension resulted in our 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 (plus poly(A) tails) indicating the cDNAs extended through to the 3′-end of the mRNA transcript. Three of the cDNAs coded for a protein of 407 amino acids (hCPT1, GenBank^{TM} accession number AF195623). cDNA clones for hCPT1 were also isolated as part of NEIBANK and expressed sequence tag survey of human eye tissues. Of ~2000 expressed sequence tags from the adult human iris, two cDNAs for hCPT1 were obtained including one full-length clone designated bx02f09. One additional clone was identified of a total of over 8000 clones from adult human retinal pigment epithelium/choroid. The full-length hCPT1 amino acid sequence is 60% identical to hCEPT1 (Fig. 1*A*), and hydropathy plots predict proteins with very similar secondary structures (Fig. 1*B*). An inspection of the hCPT1 amino acid sequence revealed the presence of the CDP-alcohol phosphotransferase motif at amino acids 114–136 and an estimated seven membrane-spanning helices.

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 resulted in a termination codon coded for at amino acid 219 (hCPT1β, GenBank^{TM} accession number AF195624). This would produce a hCPT1β protein consisting of the CDP alcohol binding motif and the first two predicted membrane-spanning helices. Based on previous analysis of the highly similar yeast *CPT1* gene product (11, 29, 30) the hCPT1β protein would consist of only the catalytic portion of the enzyme.

Genomic Organization of Human Cholinephosphotransferase 1—Similarity searches of public data bases were used for determination of the genomic locations of hCEPT1 and hCPT1. The hCEPT1 gene has been mapped to chromosome 1 between microsatellite markers D1S2865 and D1S418 by several groups but its precise DNA sequence is not known (42). The hCPT1 gene was positioned to chromosome 12q between microsatellite markers D12S346 and D12S78, and a BAC clone from the human genome sequencing project contained the entire hCPT1 gene sequence and flanking DNA (GenBank^{TM} accession number AC010205). The hCPT1 gene is 32 kb in length and is comprised of 9 exons (Fig. 2). An analysis of the hCPT1 gene indicated that the extra DNA sequence found in the hCPT1β cDNAs was 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 (Fig. 2). Each of the libraries possessed cDNAs coding for hCPT1 and the hCPT1β splice variant, except for those derived from the iris and retinal pigment epithelium, which contained only the hCPT1 cDNA. We have been successful in expressing hCPT1-derived protein for subsequent enzymatic and metabolic assessments and are currently working to establish if hCPT1β codes for an active enzyme.

hCPT1 mRNA Expression—The relative distribution of hCPT1 mRNA in human tissues was assessed by Northern blot (Fig. 3). Because of 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 of hCEPT1, which was found in similar amounts in each of the above tissues (22).

Expression and Enzymatic Characterization of hCPT1 and hCEPT1—To enzymatically assess hCPT1 substrate specificities, the hCPT1 coding region was inserted in frame with a T7 epitope tag for expression in the *S. cerevisiae* strain HJ091 (*cpt1::LEU2 ept1*$^{-}$). This yeast is devoid of its endogenous cholinephosphotransferase and ethanolaminephosphotransferase activities because of inactivation of the genomic loci coding for these activities (29, 30). This ensured any enzyme activity observed was derived from the transformed hCPT1 or hCEPT1 coding sequences. The hCPT1 protein was successfully expressed in *S. cerevisiae* as a full-length protein as determined by Western blot analysis, albeit at levels significantly

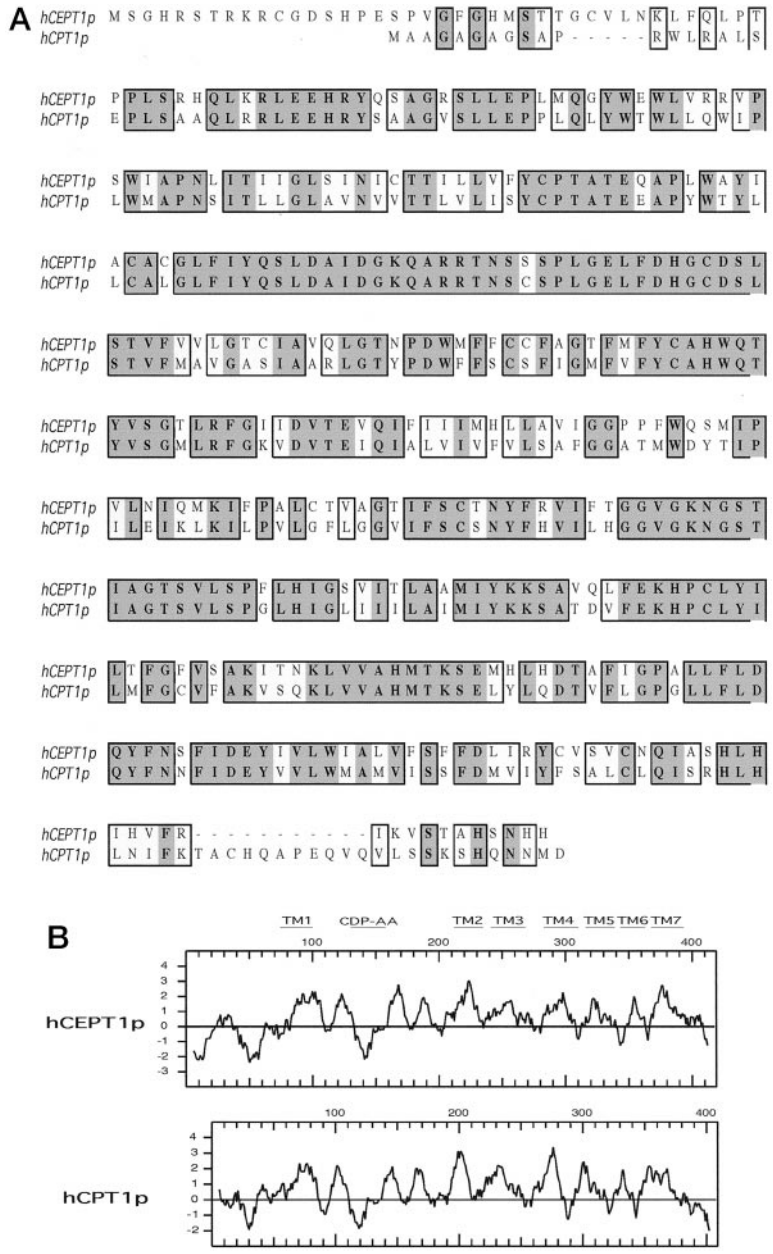
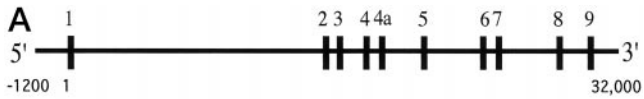
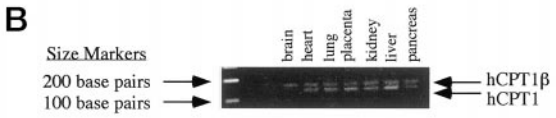


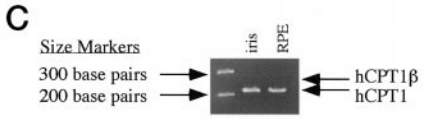
FIG. 1. **Comparison of hCPT1 and hCEPT1 predicted amino acid sequences.** *A,* the predicted amino acid sequence coded by hCEPT1 and hCPT1 were aligned using the CLUSTAL method. *B,* hydropathy plots for hCEPT1 and hCPT1. *TM*, transmembrane.

lower than that for hCEPT1 expressed from the identical vector (Fig. 4). There are several commonly used assays for measurement of mammalian cholinephosphotransferase and ethanolaminephosphotransferase activities (33–37). The assays are very similar except for (i) the nature in which the DAG substrate is solubilized for subsequent delivery in the assay mixture and (ii) the use of Mg^{2+} or Mn^{2+} as the essential cation. We tested the delivery of DAG in Tween 20 emulsions (33), a deoxycholate emulsion (34), complexed with PC in liposomes (35), in Triton X-100-mixed micelles (36) or in sodium cholate-mixed micelles (37), using either Mg^{2+} or Mn^{2+} as an essential cation donor.

We first determined the best method for delivery of DAG to hCPT1 (Table I). 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 ethanolaminephosphotransferase activity, with any of the diradylglycerol species tested, under each of the assay conditions employed. We chose to characterize hCPT1 further using the Tween 20 assay conditions as (i) this was the assay used in our initial characterization of hCEPT1 activity (22) and (ii) this assay was also used to demonstrate the existence of a putative microsomal PAF-specific cholinephosphotransferase activity (17, 18). We also determined the Mg^{2+} and Mn^{2+} requirement for hCPT1-derived cholinephosphotransferase activity and an essential requirement for either cation was observed (Fig. 5); however, Mg^{2+} activated enzyme activity to levels 10-fold higher than Mn^{2+}. The specificity of the DAG donor was also determined in the presence of either Mg^{2+} or Mn^{2+} (Table II). In general, Mg^{2+}-derived activities were higher than those obtained using Mn^{2+} as the essential cation for most substrates. 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 cholinephosphotransferase 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. Most notably there was no activity when diradylglycerols capable of synthesizing PAF (16:0(O)/2:0) or PAF precursor 16:0(O)/20:4) were used as substrates for hCPT1. We also tested hCEPT1-derived activity for the ability to synthesize







D

	Gene (base pair)	hCPT1 amino acid
Exon 1	1-443	1-91
Exon 2	16604-16751	92-141
Exon 3	17018-17159	141-188
Exon 4	19243-19327	188-216
Exon 5	22639-22771	217-260
Exon 6	25686-25844	261-313
Exon 7	26365-26490	314-355
Exon 8	28938-29048	366-392
Exon 9	31547-31719	393-407

FIG. 2. **Structure of the hCPT1 gene and analysis of hCPT1 splice variants.** *A,* the predicted intron/exon splice junctions of the hCPT1 gene. *B,* PCR analysis of cDNA libraries generated from the indicated human tissues using primers that span the predicted hCPT1β exon. The hCPT1-specific primers used were 5′-GAACAGGAAAATATTACTCCACC-3′ and 5′-GCTTTAGTGATTGTCTTTGTGTTG-3′. 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,* PCR analysis of cDNA libraries generated from human iris and retinal pigment epithelium using primers that span the predicted hCPT1β exon. The hCPT1-specific primers used were 5′-GTTGAGATTTGGAAAAGTGGATGTAAC-3′ and 5′-GGATCCATTCTTGCCAACACCACCATG-3′. Amplification of the hCPT1 cDNA would result in a DNA fragment of 222 base pairs, whereas the hCPT1β cDNA would produce a 249-base pair fragment. *D,* the hCPT1 amino acids encoded by each hCPT1 exon.

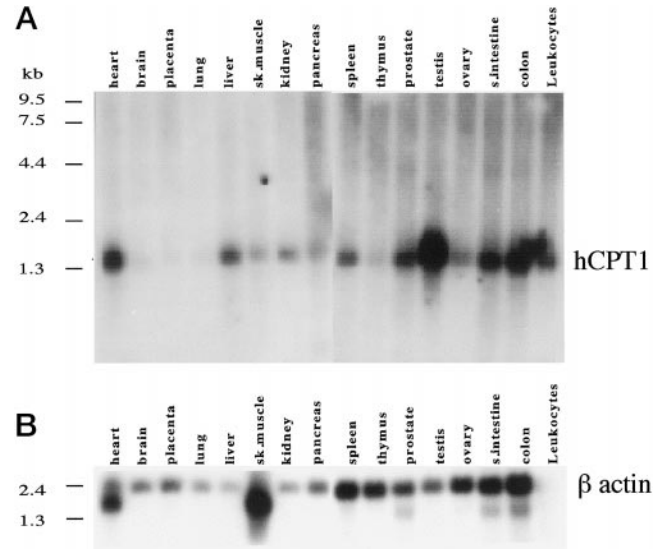


FIG. 3. **Abundance and pattern of hCPT1 mRNA.** Human multiple tissue mRNA Northern blots were hybridized against probes generated using the random priming method from the entire hCPT1 cDNA or 2.0 kb of a human actin cDNA.

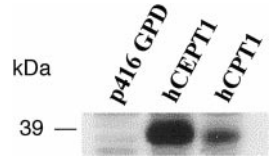


FIG. 4. **Expression of hCPT1 and hCEPT1 in *S. cerevisiae*.** The hCPT1 and hCEPT1 coding sequences were spliced in frame with a T7 epitope tag at their N termini and expressed from the constitutive *GPD* promoter in HJ091 (*cpt1::LEU2 ept1*$^-$) yeast. Protein was detected by Western blot with a T7 epitope tag-specific monoclonal antibody.

TABLE I
Cholinephosphotransferase activity of hCPT1

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

[a] ND, not detectable.

PAF *in vitro* and 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 PAF precursor were observed.

A PAF-specific cholinephosphotransferase activity detected in crude subcellular extracts was previously differentiated from PC-specific activity based on DTT sensitivity (17, 18). The PAF-specific activity was slightly stimulated by DTT, whereas the PC-specific activity was greatly inhibited by DTT. We repeated these experiments using the cloned hCPT1 and hCEPT1 as specific enzyme sources. Low concentrations of DTT moderately increased the activity of hCPT1 for the synthesis of PC and surprisingly allowed hCPT1 to now synthesize PAF (Fig. 6*A*). Hence, hCPT1 does have the capacity to synthesize PAF *in vitro*, and this ability is dependent upon the inclusion of DTT in the assay mixture. Activities for both substrates peaked at 0.1–1.0 mM DTT with a slight decrease in activity at 5 mM DTT compared with peak values. The hCEPT1-derived cholinephosphotransferase activity was also capable of synthesizing both PAF and PC; however, DTT did not alter the activity of hCEPT1 toward either substrate (Fig. 6*B*).

In Vivo Assessment of hCPT1 and hCEPT1 CDP Alcohol Specificity—Although we 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 a yeast strain devoid of its endogenous cholinephosphotransferase and ethanolaminephosphotransferase activities because of genomic inactivation of the alleles coding for the yeast enzymes (HJ091, *cpt1::LEU2 ept1*). We then tested 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, by the addition of radiolabeled choline or ethanolamine, respectively. Expression of hCPT1 resulted in the ability to incorporate radiolabeled choline into PC, but we were unable to detect any radiolabel in the lipid fraction of the ethanolamine-labeled cells (Fig. 7). Expression of hCEPT1 resulted in the reconstitution of both the PC and PE biosynthetic pathways. Hence, the *in vivo* results for hCPT1

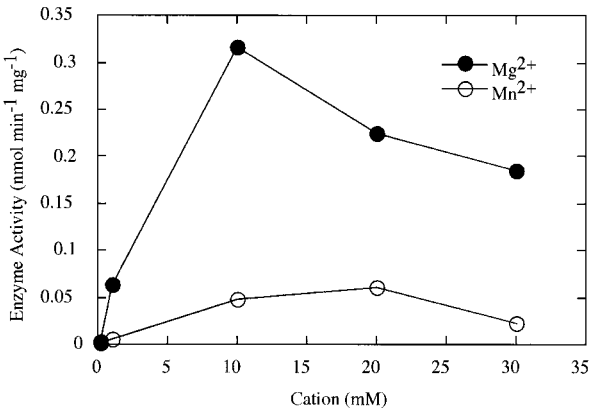


FIG. 5. **Cation requirement of hCPT1.** Enzyme activities were determined using the Tween 20 (0.004%) assay with di18:1 diacylglycerol as described under "Experimental Procedures" from microsomal membranes of yeast devoid of their endogenous cholinephosphotransferase activity (*cpt1::LEU2 ept1*$^-$) but constitutively expressing hCPT1.

TABLE II
Substrate specificity of human cholinephosphotransferase 1

Diradylglycerol substrate	Cholinephosphotransferase activity	
	Mg^{2+}	Mn^{2+}
	nmol min^{-1} *mg*$^{-1}$	
di8:0	ND[a]	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	
16:0(*O*)/20:4	ND	ND
18:1/2:0	ND	0.243

[a] ND, not detectable.

and hCEPT1 correlated with our *in vitro* assessment of their substrate specificities. It should be pointed out that although the expression of hCEPT1 was ~10-fold higher than hCPT1 in yeast as determined by Western blot and 20-fold higher as assessed by *in vitro* enzyme activities, both hCPT1 and hCEPT1 reconstituted the PC biosynthetic pathway to similar levels. This rate of PC synthesis was analogous to that observed for the endogenous yeast cholinephosphotransferase enzyme (11, 30). The lack of correlation of expression levels with the rate of PC synthesis is consistent with previous observations that the cholinephosphotransferase 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 (11).

Role of hCPT1 and hCEPT1 in Vesicle Trafficking—In yeast, the regulation of PC synthesis has been specifically implicated in *SEC14*-dependent vesicle trafficking from the Golgi apparatus (43–46). *SEC14* codes for a PC/phosphatidylinositol transfer protein, which *in vitro* catalyzes transport of phospholipid monomers from one membrane bilayer to another (47) and *in vivo* is believed to act as a sensor of PC levels that in turn regulates lipid homeostasis for subsequent downstream regulation of Golgi-derived vesicle trafficking (48). Inactivation of *SEC14* function is lethal; however, a temperature-sensitive conditional allele of *SEC14* (*sec14*^{ts}) was used in a screen to isolate bypass mutations of the normally essential *SEC14* function. This screen demonstrated that inactivating mutations in several structural genes relieved the cell of its essential requirement for *SEC14* (44). Three of these genes coded for each of the enzymes of the CDP choline pathway for PC biosynthesis. Inactivating mutations in genes of the CDP ethanolamine pathway for PE synthesis did not bypass the requirement for *SEC14*. Hence, the synthesis of PC by the CDP choline pathway specifically regulated *SEC14*-mediated Golgi-derived vesicle trafficking events. To preliminarily test if hCPT1 or hCEPT1 might be involved in the regulation of vesicle trafficking, these enzymes were expressed in a yeast strain containing

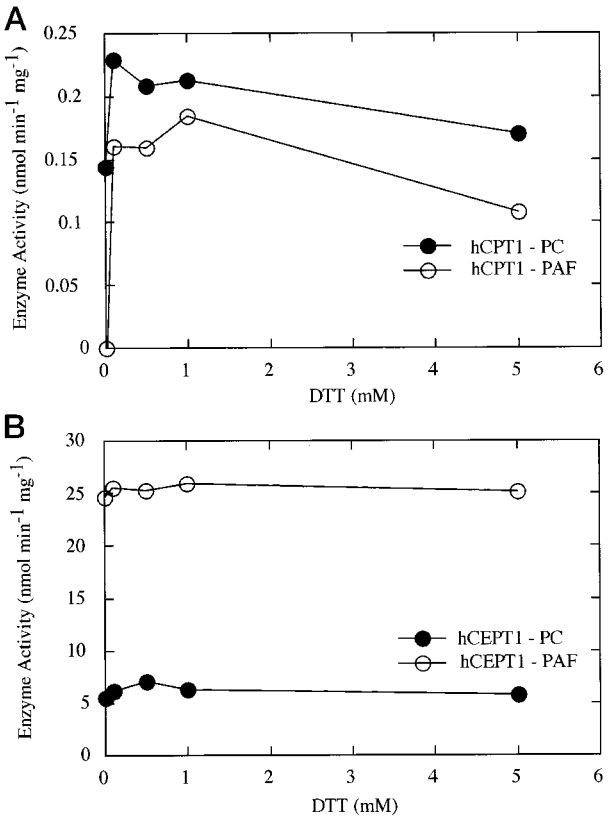


FIG. 6. **DTT sensitivity of hCPT1 and hCEPT1.** Enzyme activities were determined from microsomal membranes of yeast devoid of their endogenous cholinephosphotransferase activity (*cpt1::LEU2 ept1*$^-$) expressing either hCPT or hCEPT1 using the Tween 20 (0.004%) assay as described under "Experimental Procedures." The product formed is indicated by each symbol. *A,* hCPT1 activity. *B,* hCEPT1 activity.

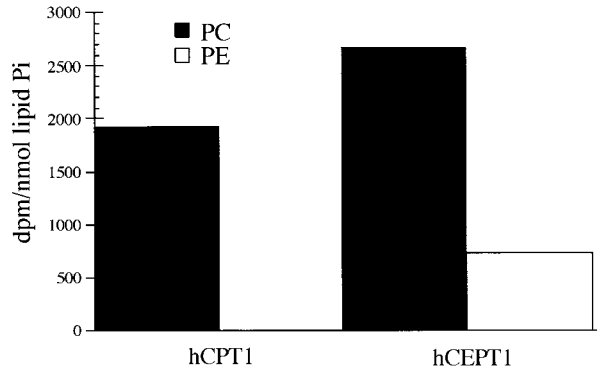


FIG. 7. **Reconstitution of phosphatidylcholine and phosphatidylethanolamine metabolic pathways *in vivo* by hCPT1 and hCEPT1.** The hCPT or hCEPT1 proteins were expressed in yeast devoid of their endogenous cholinephosphotransferase and ethanolaminephosphotransferase activities (*cpt1::LEU2 ept1*$^-$), and radiolabeled choline or ethanolamine was added to exponential phase cells for 1 h as described under "Experimental Procedures." Lipids were extracted from the cells, and the amount of radiolabel incorporated into lipid was determined by scintillation counting.

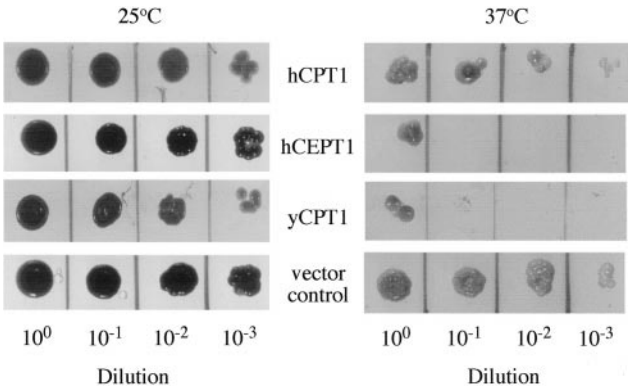


FIG. 8. **Ability of hCPT1 and hCEPT1 to affect *SEC14*-dependent cell growth.** *S. cerevisiae* cells containing a temperature-sensitive *SEC14* allele (*sec14*^{ts}) and an inactivated *CPT1* gene (*cpt1::LEU2*) were transformed with plasmids for the constitutive expression of hCPT1, hCEPT1, yeast *CPT1*, and an empty vector control. Cells were grown overnight in liquid broth at 25 °C, and identical numbers of cells were serial diluted 1:10 and spotted onto minimal medium plates containing the required nutrients for plasmid maintenance. Plates were incubated at 25 or 37 °C for 4 days.

both a *sec14*^{ts} temperature-sensitive allele and an inactivated yeast cholinephosphotransferase gene (*cpt1::LEU2*). The *sec14*^{ts} *cpt1::LEU2* yeast can grow at the nonpermissive temperature for *sec14*^{ts} function because of the inactivated *CPT1* allele (*cpt1::LEU2*). Because both hCPT1 and hCEPT1 could reconstitute PC synthesis in yeast lacking endogenous cholinephosphotransferase activity, we tested whether hCPT1 or hCEPT1 could also complement yeast *CPT1* function with respect to *SEC14*-mediated Golgi vesicle transport. Either hCPT1, hCEPT1, or yeast *CPT1* were expressed in the *sec14*^{ts} *cpt1::LEU2* yeast strain, and the ability of the cells to grow at both the permissive (25 °C) and nonpermissive (37 °C) *sec14*^{ts} temperature was monitored. As expected, expression of the yeast *CPT1* enzyme resulted in cells that were unable to grow at 37 °C because of restoration of the *sec14*^{ts} phenotype (Fig. 8). Expression of the hCEPT1 protein in *sec14*^{ts} *cpt1::LEU2* 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 vesicle transport events. Surprisingly, expression of hCPT1 in the same yeast did not affect growth at the nonpermissive temperature of 37 °C. Hence, although expression of both hCEPT1 and hCPT1 both reconstituted PC synthesis to levels similar to those provided by the endogenous yeast *CPT1* gene, hCEPT1, but not hCPT1, was able to affect *SEC14*-mediated cell growth.

DISCUSSION

The final steps in the Kennedy pathways for the synthesis of PC and PE are catalyzed by cholinephosphotransferase and ethanolaminephosphotransferase activities, respectively (10–16). The hCPT1 cDNA described in this study codes for an enzyme that demonstrated cholinephosphotransferase activity in *in vitro* enzyme assays, and in yeast devoid of their endogenous cholinephosphotransferase activity, hCPT1 was able to reconstitute the PC biosynthetic pathway *in vivo*. The hCPT1 enzyme was unable to use CDP ethanolamine as a substrate *in vitro* and was unable to reconstitute the PE biosynthetic pathway *in vivo*. This is in contrast to our previously isolated human hCEPT1 cDNA, which encoded an activity capable of using both CDP choline and CDP ethanolamine as substrates *in vitro* and *in vivo* (22). It has been presumed that Kennedy pathways for the synthesis of PC and PE are completely separate in mammalian cells (49, 50); however, the redundancy of cholinephosphotransferase activity in both the hCPT1 and hCEPT1 enzymes and the dual specificity of hCEPT for the synthesis of PC and PE imply that these two pathways may not be metabolically distinct. Indeed, the hCEPT1 mRNA distribution was similar in all tissues tested, whereas the hCPT1 mRNA varied greater than 100-fold between the same tissues, implying tissue-specific expression of hCPT1. Consistent with different functions for hCPT1 and hCEPT1 was the ability of hCEPT1, and not hCPT1, to complement the yeast *CPT1* protein in mediating *SEC14*-dependent cell growth, even though both hCEPT1 and hCPT1 both reconstituted *in vivo* PC synthesis to levels similar to those provided by the yeast *CPT1* gene product. In plants there is evidence that PC and PE are synthesized by a dual specificity choline/ethanolaminephosphotransferase (51, 52), and it will be interesting to determine if hCEPT1 can perform both functions in mammalian cells. A Chinese hamster ovary cell line had been produced by random mutagenesis with a specific defect in ethanolaminephosphotransferase activity (53), and it would have been illuminating to express the hCPT1 and hCEPT1 products in this cell line; unfortunately, this cell line is no longer available.

Consistent with our hypothesis that hCPT1 and hCEPT1 functions may not be metabolically distinct was an examination of the expressed sequence tag data bases, 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 cholinephosphotransferase- and ethanolaminephosphotransferase-encoded activities. This would be similar to the demonstrated gene complement in *S. cerevisiae* (*CPT1* and *EPT1*) (29), and an examination of the completed *C. elegans* data base also revealed only two genes coding for enzymes with similarity to hCPT1 and hCEPT1 (54). 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 ethanolaminephosphotransferase-specific enzyme purified to near homogeneity from bovine liver (15). It will be interesting to determine if the purified ethanolaminephosphotransferase is indeed coded for by the bovine CEPT1 gene, but confirmation awaits peptide sequencing of the purified protein.

A PAF-specific cholinephosphotransferase activity had been previously distinguished from the PC-specific cholinephosphotransferase enzyme based on DTT sensitivity (17, 18). Microsomal membranes contained a PAF-specific cholinephosphotransferase activity that was moderately stimulated by DTT and a PC-specific cholinephosphotransferase 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 and dependent on DTT, whereas 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 cholinephosphotransferase activities reflected 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, we predict 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; these are currently under development.

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