Identification and Characterization of the Nuclear Isoform of *Drosophila melanogaster* CTP:Phosphocholine Cytidylyltransferase[†]

Dana M. Tilley,[‡] Chadrick R. Evans,[‡] Troy M. Larson,[§] Kevin A. Edwards,[§] and Jon A. Friesen*[,‡]

Department of Chemistry and Department of Biological Sciences, Illinois State University, Normal, Illinois 61790

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ABSTRACT: CTP:phosphocholine cytidylyltransferase (CCT) catalyzes the conversion of phosphocholine and cytidine 5′-triphosphate (CTP) to CDP-choline for the eventual synthesis of phosphatidylcholine (PC). The enzyme is regulated by reversible association with cellular membranes, with the rate of catalysis increasing following membrane association. Two isoforms of CCT appear to be present in higher eukaryotes, including *Drosophila melanogaster*, which contains the tandem genes *Cct1* and *Cct2*. Before this study, the CCT1 isoform had not been characterized and the cellular location of each enzyme was unknown. In this investigation, the cDNA encoding the CCT1 isoform from *D. melanogaster* has been cloned and the recombinant enzyme purified and characterized to determine catalytic properties and the effect of lipid vesicles on activity. CCT1 exhibited a V_{max} of 23904 nmol of CDP-choline min^{-1} mg^{-1} and apparent K_m values for phosphocholine and CTP of 2.29 and 1.21 mM, respectively, in the presence of 20 μM PC/oleate vesicles. Cytidylyltransferases require a divalent cation for catalysis, and the cation preference of CCT1 was found to be as follows: $Mg^{2+} > Mn^{2+} = Co^{2+} > Ca^{2+} = Ni^{2+} > Zn^{2+}$. The activity of the enzyme is stimulated by a variety of lipids, including phosphatidylcholine, phosphatidylinositol, phosphatidylglycerol, phosphatidylserine, diphosphatidylglycerol, and the fatty acid oleate. Phosphatidylethanolamine and phosphatidic acid, however, did not have a significant effect on CCT1 activity. The cellular location of both CCT1 and CCT2 isoforms was elucidated by expressing green fluorescent fusion proteins in cultured *D. melanogaster* Schneider 2 cells. CCT1 was identified as the nuclear isoform, while CCT2 is cytoplasmic.

CTP:phosphocholine cytidylyltransferase (CCT,[1] EC 2.7.7.15) is an enzyme critical for the biosynthesis of phosphatidylcholine (PC) and is part of the CDP-choline pathway found in many higher eukaryotes, including humans. PC is synthesized by the sequential action of three enzymes: choline kinase, CCT, and choline phosphotransferase (Figure 1). The plasma membrane of many plant and animal cells contains PC as the most abundant phospholipid. *Drosophila melanogaster*, however, is an exception as phosphatidylethanolamine (PE) constitutes approximately 50% of the membrane phospholipid, with PC being the second most abundant at ~25%. Other phospholipids present in significant amounts in *Drosophila* membranes are phosphatidylinositol (PI), phosphatidylserine (PS), and diphosphatidylglycerol (DPG or cardiolipin) with each contributing between 5 and 10% of the total membrane phospholipid (*1*).

In mammals, the first enzyme of the CDP-choline pathway, choline kinase, is soluble while the third enzyme, choline phosphotransferase, is an integral membrane protein. CCT catalyzes the rate-limiting step of the CDP-choline pathway and is regulated through reversible association with membranes. Central to this regulatory process is the property that mammalian CCT is present as both a soluble and membrane-associated form within the cell (*2*). Upon association with cellular membranes, enzyme activity is enhanced and the rate of PC synthesis increases, a phenomenon that can be mimicked in vitro by the addition of lipid vesicles. Human (*3, 4*) and rat (*5*) CCT isoforms have previously been characterized in addition to CCT from the nematode *Caenorhabditis elegans* (*6*), the yeast *Saccharomyces cerevisiae* (*7*), the protozoan *Plasmodium falciparum* (*8*), and the bacterium *Streptococcus pneumoniae* (*9, 10*). In *D. melanogaster*, humans, and several other higher eukaryotes, two isoforms of CCT appear to be present. In mammals, these isoforms are denoted as CCTα and CCTβ (*4*), while in *Drosophila*, they are CCT1 and CCT2 (*11, 12*). The specific role of each CCT isoform in cellular processes, especially the contribution of each isoform to cellular PC synthesis, remains under investigation. The activity of CCT and other enzymes of phospholipid biosynthesis undoubtedly is central to the maintenance of phospholipid composition in biological membranes.

In this report, the CCT1 isoform from *D. melanogaster* has been cloned, expressed, and purified, providing milligram amounts of the recombinant enzyme for biochemical characterization. The enzyme activity is enhanced in the presence

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* To whom correspondence should be addressed. Telephone: (309) 438-7850. Fax: (309) 438-5538. E-mail: jfriese@ilstu.edu.

[‡] Department of Chemistry.

[§] Department of Biological Sciences.

[1] Abbreviations: CCT, CTP:phosphocholine cytidylyltransferase; Sf9, *Spodoptera frugiperda* 9; GCT, glycerol-3-phosphate cytidylyltransferase; ECT, CTP:phosphoethanolamine cytidylyltransferase; CTP, cytidine 5′-triphosphate; PoC, phosphocholine; GFP, green fluorescent protein.

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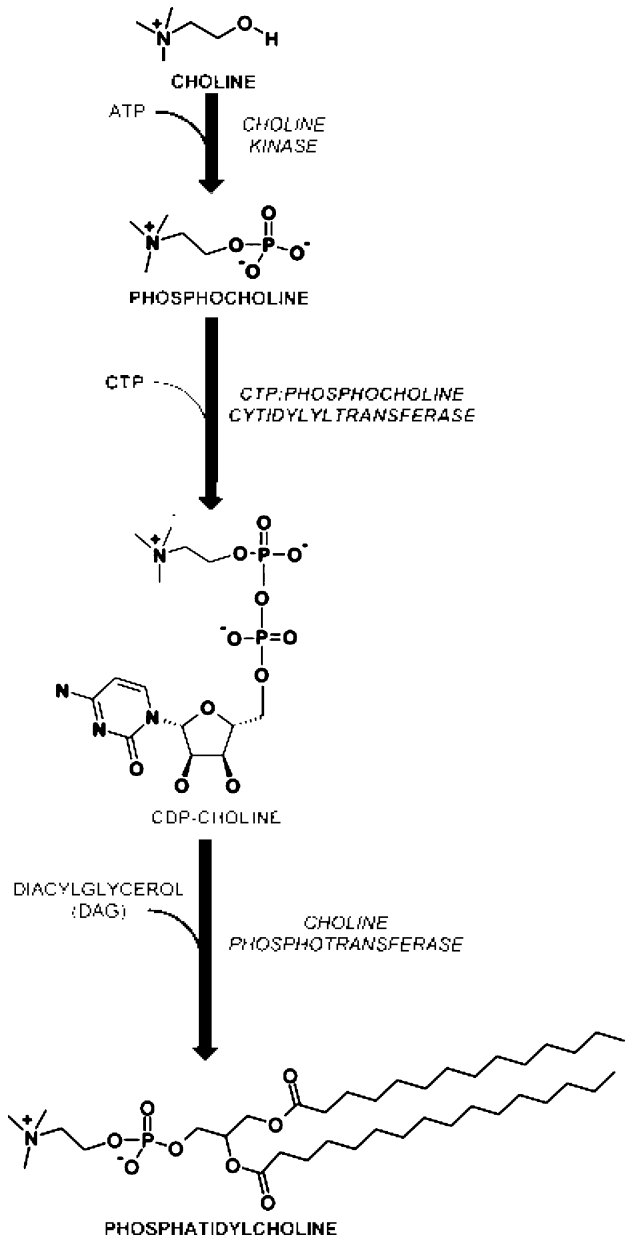

FIGURE 1: CDP-choline pathway. CTP:phosphocholine cytidylyltransferase is the second of three enzymes in this pathway.

of a variety of lipids, including PC, PI, PG (phosphatidylglycerol), PS, DPG, and the fatty acid oleate. In addition, the cellular location of both CCT1 and CCT2 isoforms has been determined in *D. melanogaster* Schneider 2 (S2) cells, with CCT1 present in the nucleus and CCT2 in the cytoplasm.

EXPERIMENTAL PROCEDURES

Expression of CCT1 in Sf9 Insect Cells. The full-length cDNA encoding CCT1 was amplified from a *D. melanogaster* pOT2 cDNA library (*12*) using PCR and sequence-specific oligonucleotides. The forward primer had the sequence 5′-CGCCGCGGATCCCATatgGCCACCTC-3′ and contained a *Bam*HI site (underlined) prior to the ATG start codon (lowercase). The 5′ primer had the sequence 5′-CGGCGGGGTACCtcaATTGCTTCGACGCTCGTACTC-CGC-3′ and resulted in a *Kpn*I site (underlined) being incorporated after the stop codon (lowercase). Thermocycler conditions were as follows: 94 °C for 15 s, 45 °C for 30 s, and 68 °C for 1 min for 30 cycles using Pfx DNA polymerase (Invitrogen). Expression in Sf9 insect cells utilized the Bac-to-Bac Baculovirus Expression System (Invitrogen). The 1.6 kb PCR product was cloned into pFASTBACHTb using *Bam*HI and *Kpn*I, which appends a nucleotide sequence encoding 29 additional amino acids (MSYYHHHHHHDY-DIPTTENLYFNGAMGSH) at the amino terminus that includes a six-histidine tag. The resultant recombinant plasmid, pFASTBACHTb-CCT1, was used as a donor plasmid for baculovirus expression following procedures outlined in the Bac-to-Bac Baculovirus Expression System manual. Expression of CCT1 in Sf9 cells was accomplished by the addition of 5 mL of high-titer virus to a 400 mL culture of Sf9 cells at a density of 1×10^6 cells/mL incubated at 27 °C with shaking at 125 rpm. After 2 days, cells were harvested by centrifugation at 3000*g* for 5 min.

Purification of CCT1. Sf9 cells expressing CCT1 were resuspended in 20 mL of lysis buffer [20 mM Tris and 100 mM NaCl (pH 7.5)] and lysed using a Dounce homogenizer to yield the cell lysate. After centrifugation at 27000*g* for 30 min at 4 °C, the pellet was discarded and the supernatant, containing soluble cellular components, was passed over a 3 mL TALON metal affinity column (BD Biosciences). The proteins that did not bind to the column were collected as the flow-through fraction. The column was washed successively with 5 column volumes of lysis buffer, lysis buffer containing 500 mM NaCl, 1 mM imidazole, 1% NP-40, and 1% glycerol, and lysis buffer containing 10 mM imidazole. Elution of CCT1 from the metal affinity column was accomplished using lysis buffer containing 200 mM imidazole. Protein was quantified by the method of Bradford (*13*) using a Bio-Rad protein assay kit and bovine serum albumin as the standard.

Enzyme Assays and Kinetic Characterization. CCT1 was assayed essentially as described previously (*14*). A standard reaction mixture contained 50 mM imidazole, 20 mM magnesium acetate, 20 μM phosphatidylcholine and oleate (1:1 molar ratio), 4 mM [^{14}C]phosphocholine (specific activity of 55 μCi/mmol), 10 mM CTP, and enzyme in a final volume of 100 μL at pH 7.0. Reactions were conducted at 37 °C for 15 min and terminated by the addition of 100 μL of 150 mM phosphocholine in 10% trichloroacetic acid; 500 μL of 10 mg/mL activated charcoal was added, and the reaction mixture was incubated on ice for 1 h. The charcoal was pelleted and washed twice with water, and the radioactivity of [^{14}C]CDP-choline was quantitated by liquid scintillation counting. The specific activity was calculated in units of nanomoles of CDP-choline produced per minute per milligram of enzyme. Phospholipid vesicles containing the indicated lipids were prepared by sonication as described previously (*15*). The kinetic parameters V_{max} and K_m with respect to phosphocholine and CTP were determined using primary plots of initial velocity versus substrate concentration [S]. Data were analyzed by nonlinear regression using SigmaPlot (Systat Software, Inc.). Initial velocity versus [S] data were fit to the Michaelis−Menten equation: $v = (V_{max}[S])/(K_m + [S])$, where v is the rate of the reaction and K_m is the Michaelis constant. For studies investigating cation usage by CCT1, each divalent cation at 25 mM was utilized.

S2 Cell Culture. Serum-free S2 cells were grown in suspension culture in Drosophila-SFM growth medium (Invitrogen Corp.) at 28 °C and 125 rpm in a temperature-

controlled shaking incubator. Cell densities were maintained between 3×10^5 and 3×10^7 cells/mL of medium. The doubling time for S2 cells was observed to be approximately 23 h.

Preparation of CCT−GFP Fusion Constructs. The forward primers used for amplifying CCT1 and CCT2 cDNAs for CCT−GFP vector construction had the sequences 5′-ACATTCACTAGTAGATCTatgGCCACCTCATCGATACTCGC-3′ and 5′-ACATTCACTAGTAGATCTatgGACAGAATGCCGAAAGAGG-3′ containing *Spe*I and *Bgl*II sites (underlined) prior to each ATG start codon (lowercase). The reverse primers for amplification of CCT1 and CCT2 cDNAs had the sequences 5′-AATGTCGGATCCGCATTGCTTCGACGCTCGTACTCC-3′ and 5′-AATGTCGGATCCGCGTCGCTGCGTCGCTCGTCTACG-3′, respectively, and contained a *Bam*HI site (underlined). *Spe*I (ACTAGT) on the forward primer and *Bam*HI (GGATCC) on the reverse primer were used to ligate the CCT1 and CCT2 cDNAs to the GFP cDNA, while the *Bgl*II site (AGATCT) on the forward primer and a *Xba*I site downstream of the GFP cDNA were used to clone the CCT−GFP fusion constructs into vector *pUAST* (*16*). The stop codon in both CCT1 and CCT2 cDNAs was removed for fusion of the C-terminal GFP tag. DNA sequences were confirmed by automated DNA sequencing using a BigDye cycle sequencing kit (Applied Biosystems) and an Applied Biosystems 3130 genetic analyzer.

Cellular Localization of CCT Isoforms. Transcription of *Cct1* and *Cct2*, which were cloned into *pUAST*, can be activated by yeast GAL4, which binds to the *UAS* sequences in the *pUAST* vector. GAL4, in turn, is provided by a helper plasmid ("driver") with a GAL4 coding region expressed via a constitutive promoter. *Actin 5C-GAL4* is often used as a driver in S2 cells, but this leads to very high levels of *UAS* activation and protein overexpression. For more moderate expression of *Cct1* and *Cct2*, we instead cotransfected with the *sqh-GAL4* driver (gift from R. Wharton, Duke University, Durham, NC). In the *sqh-GAL4* driver, *sqh* encodes the regulatory light chain of non-muscle myosin II. *D. melanogaster* S2 cells, 1.6×10^6 cells/3.5 cm well, were transfected at 28 °C with 200 ng of the desired pUAST construct and 200 ng of *sqh-GAL4* construct using Effectene reagent (Qiagen). After the addition of fresh Drosophila-SFM medium (Invitrogen), the transfected cells were incubated at 28 °C for 48 h. Cells were resuspended in the medium present in the well and pelleted at 4000*g* for 4 min. The S2 cell pellet was resuspended in approximately 100 μL of the remaining medium, while the rest of the medium was discarded. Approximately 40 μL of the transfected cells was placed on a microscope slide. Analysis of transfected cells was accomplished using a Leica TCS SP2 confocal microscope (Leica Microsystems) utilizing a HCX PL APO 63× oil immersion objective. Excitation of GFP was accomplished with a 488 nm laser, while FM 4-64 membrane stain (Invitrogen) and TO-PRO-3 DNA stain (Invitrogen) were excited at 633 nm.

RESULTS AND DISCUSSION

Gene Structure and Cloning. The *D. melanogaster* genome sequencing project (*17*) and Flybase, a repository of *Drosophila* genetic and biochemical resources available at www.flybase.org, provide an extensive knowledge database for those investigating the function of genes in the fruit fly. The *Cct1* gene is found on chromosome 3 of *D. melanogaster*. The cytological map location is 62A3-5, and the sequence location is 3 L:1,546,081..1,553,235. *Cct1* contains three coding exons, with the two introns being 189 and 63 nucleotides in length. Exon 1 is 495 nucleotides in length and encodes amino acids 1−165; exon 2 is 324 nucleotides in length and encodes amino acids 166−273, and exon 3 is 762 nucleotides in length and encodes amino acids 274−526. Amplification of the cDNA encoding CCT1 resulted in isolation of a 1581-nucleotide sequence in which four nucleotide changes were observed when compared to the database sequence (AE014296), but all were silent and did not change the amino acid sequence. Discrepancies were thymines at positions 348, 390, and 1555 and an adenine at position 480.

Previous analysis of the *D. melanogaster* genome revealed two putative *Cct* genes predicted to encode two enzyme isoforms, denoted here as CCT1 (NM_139364) and CCT2 (NM_139365) (*11*). *Cct1* and *Cct2* are tandem genes, with *Cct2* present at sequence location 3 L:1,553,493..1,555,325, less than 300 bp downstream from *Cct1*. The exon−intron structure of *Cct2* is similar to that of *Cct1*, also containing three exons and two introns. The two *Cct2* introns are 67 and 59 nucleotides in length, and exons 1−3 of *Cct2* encode amino acids 1−38, 39−146, and 147−381, respectively (*12*). The *Drosophila Cct* gene structures are simpler than that of *C. elegans* gene F08C6, which contains five exons (*6*), and the mouse *Ctpct* gene, containing eight translated exons (*18*). An alignment of the *Cct1* and *Cct2* coding regions reveals an overall level of nucleotide identity of 68%. The central regions of the nucleotide sequences that encode the highly conserved catalytic domain are greater than 80% identical, whereas the 3′ regions of approximately 200 bp encoding the extreme C-terminal region of each CCT isoform exhibit a level of identity of approximately 25%. The *Cct1* gene is significantly longer at the 5′ end, containing more than 300 nucleotides at the beginning of exon 1 that do not have a sequence match in *Cct2*.

Expression and Purification of CCT1. The 1581-nucleotide *Cct1* cDNA encodes a 526-amino acid protein with a calculated molecular mass of 58.2 kDa. In this study, using baculovirus-mediated expression, approximately 5 mg of purified six-His-tagged CCT1 was isolated from a 400 mL culture of Sf9 cells upon purification using metal affinity chromatography. The six-His tag appended to the enzyme from pFASTBACHTb added an additional mass of 3.5 kDa. After purification, SDS−PAGE analysis revealed a single band that migrated at a molecular mass of approximately 70 kDa, slightly larger than the calculated mass of 61.7 kDa for His-tagged CCT1. *Drosophila* CCT1 is the largest CCT characterized to date and is 142 amino acids longer than CCT2, which has 384 amino acids and a mass of 43.8 kDa (*12*). The majority of the additional sequence is at the amino-terminal end of CCT1 with 127 amino-terminal amino acids lacking a sequence match in CCT2 (Figure 2). Inspection of the sequence reveals 23 serines and 16 threonines within this 127-amino acid stretch, suggesting this region may be phosphorylated, a phenomenon that could affect enzyme activity or interaction with other proteins. Since CCT1 is shown in this study to be the nuclear isoform, it is also

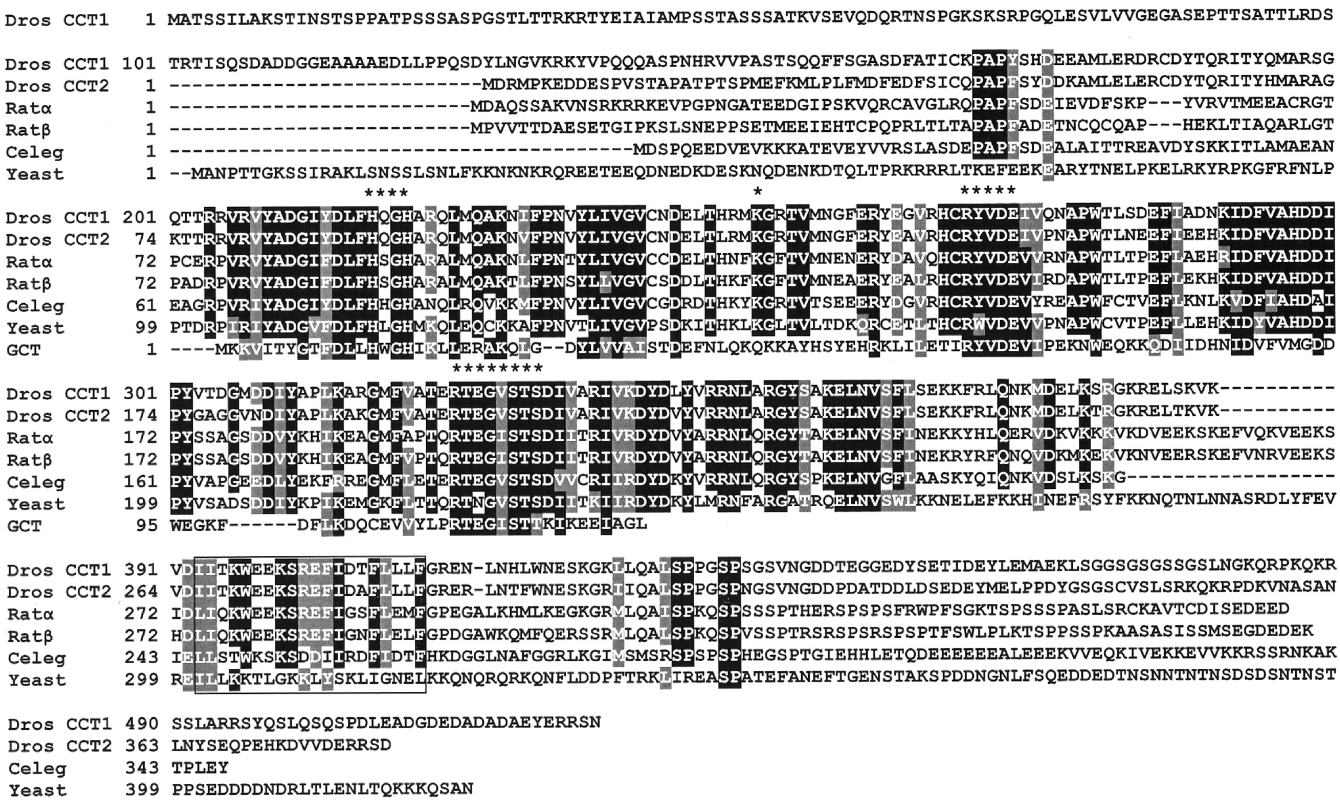

FIGURE 2: Amino acid sequence alignment. Identical amino acids are boxed in black and similar amino acids boxed in gray. Conserved amino acids implicated in catalysis or structural stability are denoted with an asterisk above the amino acid. A rectangle around amino acids 393−412 of CCT1 indicates the putative lipid binding region.

possible that the additional amino-terminal sequence plays a role in cellular localization.

In Figure 2, an amino acid sequence alignment is shown that compares CCT1 to CCT2 and other CCT isoforms. Included in this alignment is glycerol-3-phosphate cytidylyltransferase (GCT) from *Bacillus subtilis*, a prototypical member of the cytidylyltransferase family with a known three-dimensional structure, which facilitates prediction of functional roles for conserved regions of primary sequence in CCT (*19*−*21*). Conserved in CCT1 are several cytidylyltransferase signature sequences, including the 218-HxGH, 269-RYVDE, and 325-RTEGxST motifs and Lys251 shown to be critical for catalysis in other CCTs or GCT. The first histidine of the HxGH motif is postulated to play a role in transition state stabilization, while the second histidine is thought to be involved in CTP binding (*22*). Lysine 122 of rat CCTα, sequence aligned with Lys251 in CCT1, has been identified as a critical catalytic residue since mutation results in a large decrease in V_{max} and an increase in the K_m for phosphocholine (*23*). Arg325 of CCT1 is aligned with Arg196 of the RTEGxST motif in rat CCTα, which is thought to interact with CTP in the active site (*23*). The amino acids of the RYVDE motif in the related enzyme glycerol-3-phosphate cytidylyltransferase are implicated in the maintenance of structural stability (*24*).

Research efforts of the early 1990s concluded mammalian CCTα contains several functional regions (*2*). The extreme N-terminus harbors a nuclear localization signal; the central portion constitutes a highly conserved catalytic domain, and the C-terminus contains both a lipid binding and phosphorylation region. The catalytic domain is conserved among the members of the cytidylyltransferase family, including CCT, CTP:phosphoethanolamine cytidylyltransferase (ECT) (*25*), and GCT (*20*). ECT, critical for cellular PE synthesis, appears to possess two catalytic repeats but lack a lipid binding region. GCT, an enzyme of bacterial teichoic acid production, is a significantly smaller enzyme, with the entire amino acid sequence corresponding to the catalytic domain of CCT. Among CCTs from yeast, plants, and animals, the catalytic domain is highly conserved at the level of primary sequence. However, the lipid binding region, postulated to form an amphipathic α-helix that facilitates interaction with biological membranes (*26*), is not highly conserved throughout evolution. Analysis of the sequence alignment suggests the central catalytic domain of CCT1 is between amino acids 204 and 364 (Figure 2).

Biochemical Characterization of CCT1. The kinetic parameters V_{max} and K_m were determined for recombinant CCT1 by varying the concentration of substrates CTP and phosphocholine (Figure 3). Assays were conducted using 20 μM PC/oleate vesicles (1:1 molar ratio). The maximal experimental rate that can be attained by CCT1 is dependent on the composition of the lipid vesicles included in the enzyme assay. Since a higher rate of product formation is theoretically possible with vesicles containing a different lipid composition, the kinetic parameters reported here are considered to be apparent K_m and V_{max} values. Other isoforms of CCT have been kinetically characterized in the presence of PC/oleate vesicles; therefore, PC/oleate vesicles were also used in this study to allow comparison to kinetic parameters reported for other CCT isoforms. CCT1 exhibited a V_{max} of 23904 enzyme units (eu) per milligram of enzyme, with an enzyme unit defined as nanomoles of CDP-choline produced per minute, and apparent K_m values for phosphocholine and

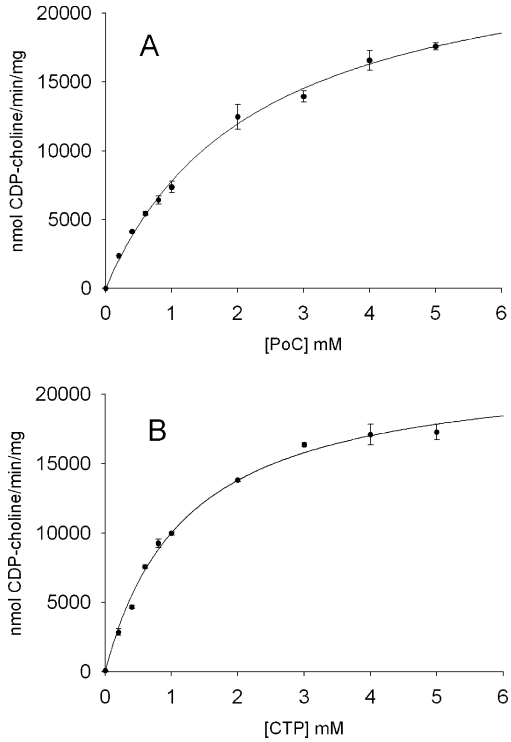

FIGURE 3: Substrate dependence of CCT1. Assays were conducted in the presence of 20 μM PC/oleate vesicles. The concentration of (A) phosphocholine (PoC) or (B) cytidine 5′-triphosphate (CTP) was varied. Data were fit to the Michaelis−Menten equation as described in Experimental Procedures to determine kinetic parameters.

Table 1: Kinetic Parameters of Purified *Drosophila* CCT1 and Comparison to Those of Other CCTs[a]

		K_m (mM)		V_{max}/K_m	
	V_{max} (eu[b]/mg)	PoC	CTP[c]	PoC	CTP
CCT1	23904	2.29	1.21	10438	19674
CCT2	2254	0.63	0.81	3600	2800
rat CCTα	25480	0.65	1.29	39200	19752
S. cerevisiae	35550	0.80	1.42	44375	25035
C. elegans	30166	1.6	5.2	18854	5801

[a] Kinetic data for CCT1 were obtained and analyzed as described in Experimental Procedures. [b] One enzyme unit (eu) is the number of nanomoles of CDP-choline produced per minute. [c] Phosphocholine is abbreviated PoC. Data for CCT2 are derived from ref *12*, for rat CCTα from ref *27*, for *S. cerevisiae* from ref *7*, and for *C. elegans* from ref *6*. For CCT2, rat CCTα, and *C. elegans* CCT, the CTP K_m is a K' value due to observed cooperativity.

CTP of 2.29 and 1.21 mM, respectively. The kinetic parameters for CCT1 are presented in Table 1 and compared to kinetic parameters for forms of CCT from other organisms. CCT1 has a V_{max} comparable to those of rat CCTα, yeast CCT, and *C. elegans* CCT, which exhibit V_{max} values between 25000 and 36000 eu/mg. It is interesting to note that CCT1 has a V_{max} approximately 10-fold greater than the V_{max} exhibited by the CCT2 isoform (*12*). Whether the difference in maximal activity is indicative of each isoform's cellular role is not known. The CCT1 apparent K_m value of 2.29 mM for phosphocholine is 1.5−3.5-fold higher than those of the other isoforms listed in Table 1, while the CTP apparent K_m value of 1.21 mM is similar to those of CCT2 (*12*) and CCTs from rat (*27*) and yeast (*7*).

Cytidylyltransferases require a divalent cation, such as Mg^{2+}, for activity. A possible role for the divalent cation

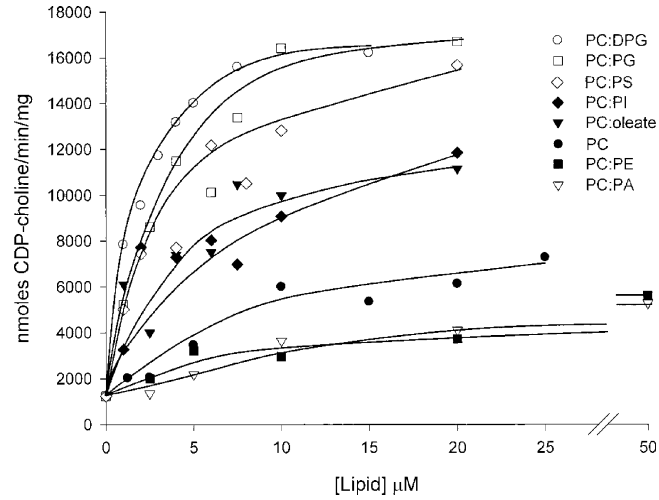

FIGURE 4: Increase in CCT1 activity in the presence of lipid vesicles containing PC or 50 mol % PC and a second lipid (at 50 mol %), either DPG, oleate, PA, PE, PG, PI, or PS as indicated.

Table 2: Increases in CCT1 Activity in the Presence of Lipid Vesicles Containing 50 mol % PC and a Second Lipid at 50 mol %[a]

	$K_{1/2}$ (μM)[b]	eu/mg[c]	*x*-fold increase	increase/$K_{1/2}$
no lipid	−	1236 (0)	−	−
PC	5	7278 (25)	5.9	1.2
PC/PA	6	5274 (20)	4.3	0.7
PC/PE	6	5602 (15)	4.5	0.7
PC/oleate	2.5	11154 (50)	9.0	3.6
PC/PI	2.5	11850 (50)	9.6	3.8
PC/PS	2.5	15681 (20)	12.7	5.1
PC/PG	2.5	16705 (20)	13.5	5.4
PC/DPG	1	16206 (20)	13.1	13.1

[a] Kinetic data were obtained as described in Experimental Procedures. [b] $K_{1/2}$ is the estimated lipid concentration necessary to achieve half of the maximal observed activity listed in the table. [c] eu/mg represents the maximal specific activity achieved with a particular lipid preparation. The number in parentheses indicates the micromolar concentration of lipid at which the specific activity was observed.

may be to coordinate the negatively charged phosphates of CTP. To probe the cation specificity of CCT1, enzyme assays were conducted in the presence of various divalent cations. Mg^{2+} was the preferred cation for catalysis, but Mn^{2+} and Co^{2+} also resulted in significant activity at a concentration of 25 mM, with 51 and 41% of the activity seen with 25 mM Mg^{2+}, respectively. Ca^{2+} and Ni^{2+} were not as well utilized, both yielding approximately 9% of the activity seen with Mg^{2+}. Zn^{2+} was the least preferred divalent cation studied, resulting in a catalytic activity of only 1% when compared to the activity in the presence of Mg^{2+}.

Effect of Lipid Vesicles on CCT1 Activity. CCT has been shown to be the rate-limiting enzyme of the CDP-choline pathway and is believed to be extensively regulated intracellularly via translocation to the membrane surface. Enzyme assays for studying the effect of lipid on catalysis by CCT1 were conducted using either PC vesicles or PC vesicles containing 50% (molar ratio) oleate, diphosphatidylglycerol (DPG), phosphatidylserine (PS), phosphatidylglycerol (PG), phosphatidylinositol (PI), phosphatidylethanolamine (PE), or phosphatidic acid (PA). As illustrated in Figure 4 and summarized in Table 2, PC vesicles enhance CCT1 activity 6-fold at a concentration of 25 μM. PE and PA, however, do not appear to have a significant effect on CCT1 activity, even at lipid concentrations of up to 50 μM, since adding either to PC vesicles did not result in an activity greater than

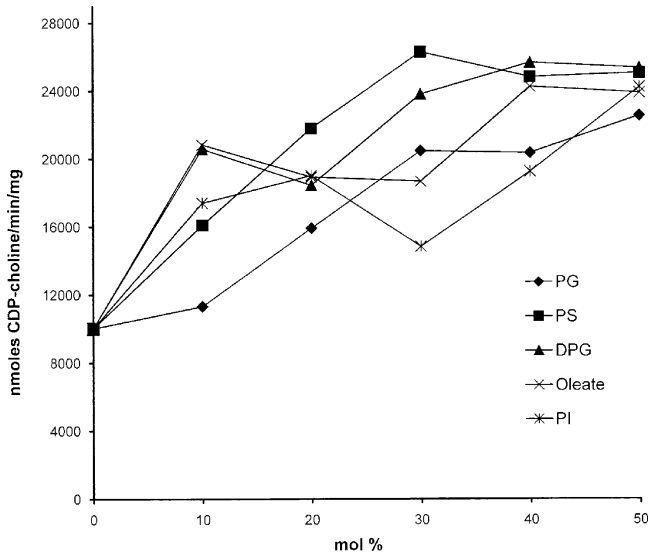

FIGURE 5: Increases in CCT1 activity in the presence of PC lipid vesicles containing the indicated mole percent of a second lipid, either PS, oleate, DPG, PG, or PI as indicated. Each data point is the average of three determinations.

that seen with PC alone. A rate increase of slightly less than 10-fold was seen in the presence of 20 μM PC vesicles containing 50% PI or oleate, while a 13-fold rate increase was observed when the vesicles contained 50% PS, PG, or DPG. The concentration of lipid required for half of the maximal observed activity, defined as $K_{1/2}$ in Table 2, was estimated from each plot in Figure 4. $K_{1/2}$ values of approximately 2.5 μM were observed for PC/oleate, PC/PI, PC/PS, and PC/PG vesicles, while enhancement of catalysis required a lower concentration of PC/DPG vesicles with a $K_{1/2}$ of approximately 1 μM. PC, PC/PA, or PC/PE vesicles had higher $K_{1/2}$ values of 5−6 μM. The activity of CCT1 was also greatest in the presence of PC/oleate, PC/PI, PC/PS, or PC/PG vesicles, each resulting in an increase in specific activity of approximately 13-fold compared to the activity in the absence of lipid. The ratio of rate increase to $K_{1/2}$ provides a measure of the efficiency of each lipid with respect to enhancing the catalytic ability of CCT1. PC/DPG vesicles exhibited the greatest efficiency with a ratio of approximately 13 (Table 2). These results parallel those seen in the characterization of CCT2, which also exhibits a significant increase in activity in the presence of vesicles containing DPG (*12*).

Additional assays were conducted by varying the mole percent of lipids PI, PS, PG, DPG, and oleate within the PC vesicle (Figure 5). At a total lipid concentration of 20 μM, PC vesicles resulted in an activity increase of 5-fold when compared to CCT1 vesicles without added lipid. The optimal mole percent within a PC vesicle for PS was 30%, for DPG and oleate 40%, and for PI and PG 50%. For PS, DPG, and oleate, addition of each lipid to PC vesicles at the optimal molar ratio increased the activity of CCT1 approximately 12-fold when compared to the activity without lipid.

In previous analysis of other CCT isoforms, it has been shown that association with lipid vesicles in vitro results in an increase in the catalytic activity of rat (*27*), *C. elegans* (*6*), and yeast (*7*) CCT of 84-, 37-, and 21-fold, respectively. CCT2 also exhibits a substantial rate enhancement upon association with lipid vesicles, with an approximately 100-fold stimulation in the presence of 20 μM PC/oleate vesicles (*12*). The catalytic rate of CCT1 does not increase as substantially in the presence of lipids as the rates of these other CCT isoforms; however, the enzyme retains a relatively high activity in vitro in the absence of added lipid. Though the lipid-dependent rate increase exhibited by CCT1 is not as great as that of rat CCTα, it is interesting to note that CCT1 requires approximately 10-fold fewer PC/oleate vesicles for maximal activity than the mammalian CCTα isoform (*27*). In general, the activity of CCT1 appears to be enhanced in vitro by lipid vesicles, although to a lesser extent than those of other CCT isoforms, and is relatively nonspecific since the rate increase is similar in the presence of a variety of lipids.

Association of CCT with membranes is thought to proceed via two steps. Initially, an electrostatic interaction with a charged membrane surface occurs, promoting an α-helical conformation for the lipid binding region of CCT, a phenomenon that has been confirmed by NMR and circular dichroism studies (*28, 29*). The hydrophobic face of the amphipathic α-helix next inserts into the nonpolar membrane bilayer (*26, 30*). The lipid binding region has been proposed to be inhibitory in the absence of lipid, and the adoption of an α-helical conformation in the lipid binding region results in an improvement in the catalytic ability of the catalytic domain by relieving inhibition (*26*). It is difficult to compare the primary sequence of the lipid binding regions from various CCT isoforms because of the low level of sequence conservation; however, a putative amphipathic α-helix located between amino acids 393 and 412 in CCT1 likely contains the lipid binding region (Figure 2). This region is virtually identical to the corresponding amino acid sequence in CCT2.

Cellular Localization of CCT1 and CCT2. Recent studies in the field of phosphatidylcholine biosynthesis have revealed the presence of two isoforms of CCT in mammals, denoted CCTα and CCTβ. CCTα was shown to be located in the nucleus (*31*), while CCTβ was determined to be a cytosolic isoform (*4*). The role(s) of each isoform within a cell, including the contribution of each enzyme to cellular phosphatidylcholine synthesis, continues to be studied. Sequencing of the *D. melanogaster* genome (*17*) revealed that *Drosophila* also contains two genes, *Cct1* and *Cct2*, that appear to encode two CCT isoforms, CCT1 and CCT2, respectively. An alignment of CCT1 and CCT2 amino acid sequences reveals an overall level of identity of 53% at the level of primary structure with a level of identity of 90% within the catalytic domain. Alignment with human CCTα and CCTβ reveals CCT1 is 35% identical to CCTα and 34% identical to CCTβ while CCT2 is 46% identical to CCTα and 44% identical to CCTβ. Unfortunately, this comparison does not yield any clues about whether CCT1 or CCT2 is likely to correspond to either CCTα or CCTβ from mammals or which *Drosophila* CCT isoform is likely to be nuclear or cytoplasmic.

In this investigation, efforts to elucidate the cellular location of CCT1 and CCT2 utilized the *D. melanogaster* cell line Schneider 2, or S2, established from an embryo in 1972 (*32*). Intracellular visualization of each CCT isoform was accomplished using carboxy-terminal green fluorescent fusion proteins. Two cDNA constructs were created, one encoding a CCT1−GFP fusion protein and a second encod-

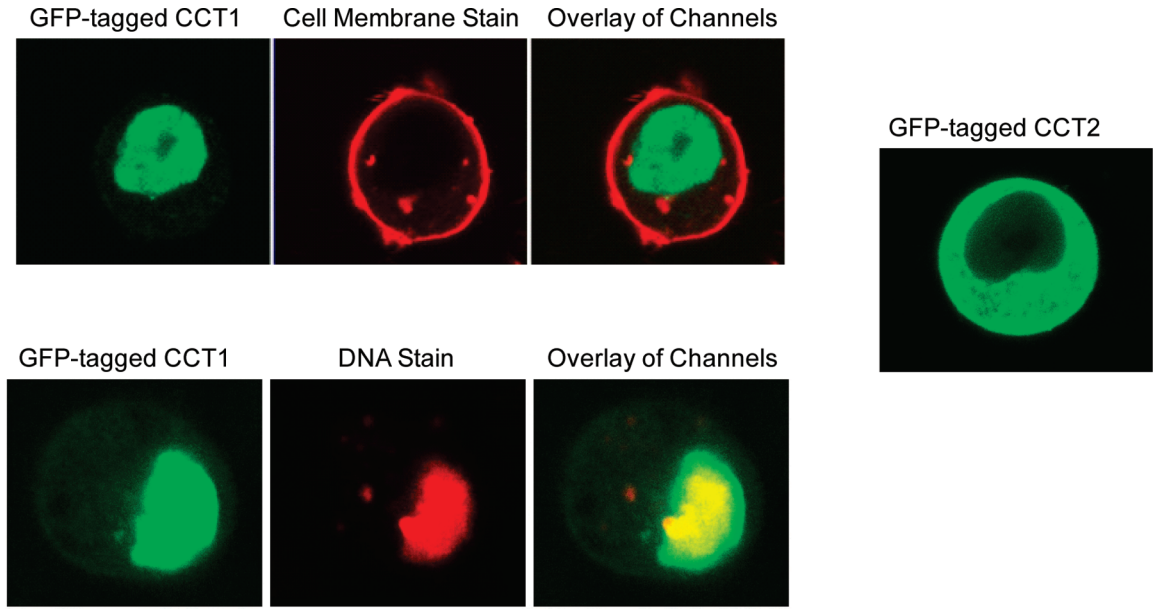

FIGURE 6: Cellular localization of *Drosophila* CCT isoforms. Green fluorescent protein was fused to the C-terminus of CCT1 (six panels on the left) or CCT2 (panel on the right). Shown are the green channel (GFP), the red channel (cell membrane or DNA stain), and an overlay of the green and red channels from confocal microscopy. The red FM 4-64 stain was used in conjunction with CCT1 in the top three images to stain the plasma membrane. The red TO-PRO-3 stain was used to stain DNA and verify localization of CCT1 within the nucleus.

ing a CCT2−GFP fusion protein. For expression in cultured S2 cells, the vector *pUAST* was employed, which drives protein expression in S2 cells (*16*). After transfection of *pUAST* constructs into S2 cells, protein expression was visualized using confocal microscopy. To visualize the plasma membrane, the water soluble red fluorescent lipophilic styryl stain FM 4-64 was used, which inserts into the outer leaflet of the plasma membrane and is commonly used to visualize the plasma membrane in cultured eukaryotic cells. The nucleus was stained using the cyanine stain TO-PRO-3, which detects double-stranded nucleic acids. Upon transfection of S2 cells with the expression vector *pUAST-CCT1-GFP* and excitation with a 488 nm laser, GFP fluorescence was evident in the nucleus of the cell (Figure 6). Nuclear localization of CCT1 was verified by colocalization with TO-PRO-3, excited with a 633 nm laser. In contrast, when S2 cells were transfected with *pUAST-CCT2-GFP* and excited at 488 nm, the fluorescence was cytoplasmic. Therefore, we concluded that CCT1 is the nuclear isoform of CCT in *D. melanogaster* while CCT2 is cytoplasmic.

Possible Cellular Role of CCT1. Genetic studies of *D. melanogaster* have provided insight into metabolic and developmental roles of CCT1. Most of the *Cct1* transcription unit is occupied by a series of four alternate promoters dispersed across approximately 4 kb. Gupta and Schüpbach (*11*) found that mutations which disrupt the first promoter are viable, while mutations that disrupt the entire *Cct1* gene are lethal; this indicates that the downstream promoters are essential and suggests unexpected complexity in the regulation of CCT expression. *Cct2* transcription starts less than 300 bp downstream of the 3′ end of *Cct1*. Thus, *Cct2* has far less room than *Cct1* for 5′ regulatory elements, suggesting that its expression pattern may be simpler. However, *Cct2* expression might also be influenced by regulatory elements from *Cct1*.

Gupta and Schüpbach further took advantage of the viability of promoter 1 mutants to explore adult functions for CCT, specifically in oogenesis. Remarkably, reduced CCT leads to several specific developmental defects, most of which are tied to known signaling pathways. Early development of the ovary is defective in *Cct1* mutant pupae, in a manner akin to *Wnt4* mutant animals. Later, in adulthood, *Cct1* ovaries have several defects: failure to maintain germline stem cells, faulty packaging of germ cell cysts into egg chambers, and improper positioning of the oocyte within the growing egg chamber. *Cct1* shows a dynamic expression pattern, with an increased level of expression in somatic cells that will make specialized structures at the anterior of the eggshell. *Cct1* expression responds to both *dpp* and EGFR signaling in these cells, and *Cct1* mutant eggs have defects in anterior structures. Surprisingly, clonal analyses showed that *Cct1* does not act cell autonomously, either in ovarian morphogenesis or in oocyte positioning. This suggests that CCT or PC may be required to synthesize, process, or secrete an extracellular signaling molecule involved in these processes.

Weber et al. (*33*) also characterized mutations affecting *Cct1*'s first promoter and likewise found linkages to specific signaling pathways. *Cct1* acts positively in both the EGFR and Notch pathways on the basis of genetic interactions. To investigate the mechanism, Weber et al. analyzed membrane phospholipid concentration. PC was reduced in *Cct1* transposon insertion mutants, indicating that *Cct1* is limiting for PC production and cannot be substituted with *Cct2* (assuming the insertions do not also compromise *Cct2* expression). The total amount of PI increased substantially, perhaps as a compensation mechanism for the loss of PC. Given the importance of phospholipids in membrane trafficking, they next asked whether CCT was required for proper endocytosis. Cells from *Cct1* null animals were found to have increased

abundance of endocytic vesicles (based on dextran uptake) and more clathrin-coated pits and vesicles (by TEM). Mutant tissue also displayed increased numbers of Notch- and EGFR-bearing vesicles. In sum, the data suggest that with less PC (and more PI), the level of endocytosis increases, receptors are prematurely cleared from the plasma membrane, and signaling through these receptors is impeded. It remains unclear whether CCT acts solely by controlling PC/PI ratios or whether it plays other roles in signaling or trafficking, as suggested by its lack of cell autonomy (*11*).

Another study by Landis et al. (*34*) used a transposable element to induce overexpression of genes in *Drosophila* and determine mutations that increase life span. *Cct1* was one of six genes identified as contributing to life span; a 1.7-fold overexpression resulted in an increase in life span of 7%. The underlying mechanism that results in the effect on life span remains to be determined.

CONCLUSIONS

The CDP-choline pathway for PC synthesis was discovered 50 years ago by E. Kennedy (*35*), who soon thereafter showed CCT associated with biological membranes (*36*). Despite research on CCT for the next 40 years, the subcellular localization of CCT remained a matter of vigorous debate. It was only 10 years ago that the first report of a second isoform of CCT was published (*4*), providing an explanation for why a consensus about whether the enzyme was nuclear or cytoplasmic could not be reached. This relatively recent discovery of both a nuclear and a cytoplasmic isoform in humans has resulted in the realization that additional research is now needed to elucidate the specific role each isoform plays in phospholipid synthesis and cellular development in eukaryotes.

This study is part of a greater long-term goal of determining the role of each CCT isoform in eukaryotes. To achieve this goal, it is imperative that the subcellular location of each enzyme isoform be determined as well as the catalytic properties and factors that affect enzyme activity. This characterization of CCT1 from *Drosophila* complements the in vitro characterization of CCT2 reported previously (*12*). In addition to investigating in vitro catalysis by CCT1 and determining the effect of lipids on activity, we report the subcellular localization of both CCT1 and CCT2. This study is significant in that it provides fundamental knowledge that can be applied to cellular and genetic studies aimed at elucidating individual roles of each CCT isoform. With the sequencing of the *D. melanogaster* genome and the genetic investigations already conducted on the CCT1 isoform, the prospect of exploiting fruit flies as a model genetic organism for the examination of CCT function and phospholipid biosynthesis is appealing. This study of CCT isoforms from *D. melanogaster* will allow for greater utilization of this powerful genetic system in future studies striving to determine the role of CCT isoforms in the growth and development of higher eukaryotes.

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