Biochemical and Biophysical Research Communications 449 (2014) 26–31

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Human CTP:phosphoethanolamine cytidylyltransferase: Enzymatic properties and unequal catalytic roles of CTP-binding motifs in two cytidylyltransferase domains

Siqi Tian [a], Jun Ohtsuka [b], Shipeng Wang [b], Koji Nagata [b], Masaru Tanokura [b], Akinori Ohta [c], Hiroyuki Horiuchi [a], Ryouichi Fukuda [a,*]

[a] Department of Biotechnology, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan
[b] Department of Applied Biological Chemistry, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan
[c] Department of Biological Chemistry, College of Bioscience and Biotechnology, Chubu University, 1200 Matsumoto-cho, Kasugai, Aichi 487-8501, Japan

ARTICLE INFO

Article history:
Received 7 April 2014
Available online 4 May 2014

Keywords:
Phosphatidylethanolamine
CTP:phosphoethanolamine cytidylyltransferase
Kennedy pathway
Human

ABSTRACT

CTP:phosphoethanolamine cytidylyltransferase (ECT) is a key enzyme in the CDP-ethanolamine branch of the Kennedy pathway, which is the primary pathway of phosphatidylethanolamine (PE) synthesis in mammalian cells. Here, the enzymatic properties of recombinant human ECT (hECT) were characterized. The catalytic reaction of hECT obeyed Michaelis–Menten kinetics with respect to both CTP and phosphoethanolamine. hECT is composed of two tandem cytidylyltransferase (CT) domains as ECTs of other organisms. The histidines, especially the first histidine, in the CTP-binding motif HxGH in the N-terminal CT domain were critical for its catalytic activity *in vitro*, while those in the C-terminal CT domain were not. Overexpression of the wild-type hECT and hECT mutants containing amino acid substitutions in the HxGH motif in the C-terminal CT domain suppressed the growth defect of the *Saccharomyces cerevisiae* mutant of *ECT1* encoding ECT in the absence of a PE supply via the decarboxylation of phosphatidylserine, but overexpression of hECT mutants of the N-terminal CT domain did not. These results suggest that the N-terminal CT domain of hECT contributes to its catalytic reaction, but C-terminal CT domain does not.

1. Introduction

Phosphatidylethanolamine (PE) is one of the major phospholipids that constitute eukaryotic membranes and comprises ~25% of mammalian phospholipids. PE has unique functions in various cellular processes in eukaryotic cells [1]. It has a tendency to form hexagonal phase II structure in physiological conditions, and this property is believed to contribute to the dynamics of eukaryotic membranes, including fission and fusion, by modulating the formation of membrane curvature. PE is involved in autophagy through lipidation of Atg8 in *Saccharomyces cerevisiae* and LC3, the mammalian ortholog of Atg8 [2]. It also functions as a donor of phosphoethanolamine moieties that anchor glycosylphosphatidylinositol (GPI) to C-termini of proteins [3]. In mammalian cells, PE is a precursor of anandamide, the ligand of cannabinoid receptors [1]. In addition, PE functions as a lipid chaperone for a subset of integral membrane proteins in *Escherichia coli* [4].

In mammalian cells, PE is synthesized mainly through two pathways, the CDP-ethanolamine branch of the Kennedy pathway (CDP-ethanolamine pathway), and by the decarboxylation of phosphatidylserine (PS) [1,5]. PE is also synthesized through an acylation of lyso-phosphatidylethanolamine and through a base-exchange reaction between PS and ethanolamine, although the contributions of these pathways are minor [1]. In the Kennedy pathway, PE is synthesized from diacylglycerol and ethanolamine through three reactions. First, ethanolamine kinase catalyzes the phosphorylation of free ethanolamine. In the next step, CTP:phosphoethanolamine cytidylyltransferase (ECT) catalyzes the conversion of phosphoethanolamine (P-Etn) and CTP to CDP-ethanolamine (CDP-Etn) and pyrophosphate. Finally, *sn*-1,2-diacylglycerol ethanolaminephosphotransferase catalyzes the synthesis of PE from CDP-Etn and diacylglycerol. The second reaction cata-

Abbreviations: CCT, CTP:phosphocholine cytidylyltransferase; CDP-Etn, CDP-ethanolamine; CT, cytidylyltransferase; DTT, dithiothreitol; ECT, CTP:phosphoethanolamine cytidylyltransferase; GCT, CTP:glycerol-3-phosphate cytidylyltransferase; hECT, human ECT; PE, phosphatidylethanolamine; P-Etn, phosphoethanolamine; PS, phosphatidylserine; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

* Corresponding author. Fax: +81 3 5841 8015.
E-mail address: afukuda@mail.ecc.u-tokyo.ac.jp (R. Fukuda).

http://dx.doi.org/10.1016/j.bbrc.2014.04.131
0006-291X/

lyzed by ECT appears to be the rate-limiting step under most metabolic conditions [6,7].

The gene encoding ECT was first isolated from the yeast *S. cerevisiae*, and was named *ECT1* [8]. *ECT1* is not essential for growth, but deletion mutants of *ECT1* require PE supply through the decarboxylation of PS for growth, since PE is one of the essential phospholipids in yeast [9,10]. ECT-encoding genes have been cloned and characterized from various eukaryotes, including human [11–15]. In mice, homozygous disruption of *Pcyt2* that encodes ECT results in embryonic lethality [16], indicating the importance in mouse development of ECT and of PE synthesized through the Kennedy pathway. In addition, heterozygous disruption of *Pcyt2* causes increased triacylglycerol and diacylglycerol accumulation in the livers and the development of features of metabolic diseases in mice [17], and hepatocyte-specific homozygous disruption of *Pcyt2* in mice leads to liver steatosis [18], suggesting an important role of ECT in the lipid homeostasis in the liver.

One of the most prominent structural features of ECT is that it consists of two tandem cytidylyltransferase (CT) domains within the one molecule, in marked contrast to bacterial CTP:glycerol-3-phosphate cytidylyltransferase (GCT) or eukaryotic CTP:phosphocholine cytidylyltransferase (CCT), both of which only have a single CT domain. The roles of the two CT domains in ECT have been characterized in the human malaria parasite *Plasmodium falciparum*, and it was shown that only the N-terminal CT domain is catalytically active [14]. However, the roles of two CT domains in other ECTs remain to be determined. The crystal structure of the recombinant human ECT (hECT) protein, in which both CT domains are included but N- and C-terminal ends are removed, has been solved by the Structural Genomics Consortium (PDB code 3ELB). Interestingly, CMP was found in the pocket of the C-terminal CT domain of hECT, raising the possibility that the C-terminal CT domain is involved in the regulation of ECT activity by binding to CMP or structurally-related compounds, or involved more directly in the catalytic reaction. In this study, we characterized the enzymatic properties of the recombinant hECT and analyzed the contributions of the two CT domains to catalytic activity.

2. Materials and methods

2.1. Strains, media, and growth conditions

Escherichia coli strain BL21(DE3) (Novagen) was used to produce the wild-type and mutant hECT proteins, and a strain DH5α was used for plasmid construction. *S. cerevisiae* strain TKY12Ga (*MAT*a *ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 Δpsd1*::*kanMX4 Δpsd2*::*LEU2 ect1*::*HIS3-GAL1p-ECT1*) [19] was used as a host to evaluate activities of the wild-type and mutant hECT proteins *in vivo*.

E. coli was cultured in LB medium or 2xYT medium at 25 °C. The minimal medium for yeast contained 0.17% yeast nitrogen base without amino acids and ammonium sulfate (Difco), 0.5% ammonium sulfate, and 2% glucose (SD medium) or 2% galactose (SG medium). Ethanolamine and choline were added at a final concentration of 1 mM. Yeasts were cultured at 30 °C.

2.2. Construction of plasmids

Primers used for plasmid constructions were listed in Table S1.

hECT cDNA (DDBJ accession No. D84307) was amplified from cDNA of Saos-2 cells (gift from Dr. Michihiko Fujii) by PCR using primers hECT-U-Nde and hECT-L-Bam. Obtained fragment was digested with NdeI and BamHI, and cloned into pET15b digested with the same restriction enzymes, resulting in pET15b-hECT.

Point mutations were generated by PCR using KOD-Plus-Neo (TOYOBO) with pET15b-hECT as a template. Used primers were hECT-H35A-U and hECT-H35A-L for H35A, hECT-H38A-U and hECT-H38A-L for H38A, hECT-H226A-U and hECT-H226A-L for H226A, and hECT-H229A-U and hECT-H229A-L for H229A. Obtained fragments were digested by DpnI and introduced into the *E. coli* DH5α strain. Plasmids were digested with NdeI and BamHI, and DNA fragments carrying hECT mutants were ligated with pET15b digested with the same restriction enzymes, resulting in pET15b-hECTH35A, pET15b-hECTH38A, pET15b-hECTH226A, and pET15b-hECTH229A. Mutations were confirmed by DNA sequencing.

Plasmids to express the wild-type and mutant hECT proteins in yeast were constructed as follows: the terminator region of *TDH3* was excised from pYPR2831 [20] with SalI and HindIII and cloned into SalI and HindIII sites of YCplac22 [21] to obtain YCplac22-TGAP. The promoter region of *PGK1* was amplified by PCR with primers pPGK1-EcoRI-fw and pPGK1-SmaI-rv using the total DNA of *S. cerevisiae* strain W3031A, and digested with EcoRI and SmaI. The coding region of hECT with one copy of HA epitope tag was amplified by PCR with primers HA-Nde-hECT and hECT-L-Bam using pET15b-hECT as a template. Using obtained fragment as a template, the coding region of hECT with three copies of HA epitopes tag was amplified by PCR with primers SmaI-ATG-3xHA-NdeI and hECT-L-Bam, and digested with SmaI and BamHI. These fragments were ligated into EcoRI/BamHI digested YCplac22-TGAP, resulting in YCplac22-hECT. The coding regions of mutant hECT proteins were excised with NdeI and BamHI from pET15b-hECTH35A, pET15b-hECTH38A, pET15b-hECTH226A, and pET15b-hECTH229A, and ligated with vector fragments obtained by digestion of YCplac22-hECT with the same restriction enzymes, resulting in YCplac22-hECTH35A, YCplac22-hECTH38A, YCplac22-hECTH226A, and YCplac22-hECTH229A, respectively.

A plasmid that produces *EKI1*-encoding ethanolamine kinase of *S. cerevisiae* in *E. coli* was constructed as follows: the coding region of *EKI1* was amplified by PCR using primers EKI1-f and EKI1-r from the total DNA of *S. cerevisiae* strain MHY501 [22] as a template. Obtained fragment was digested with EcoRI, blunt-ended, and ligated with NdeI-digested and blunt-ended pET15b, resulting in pETEKI1.

2.3. Expression and purification of the wild-type and mutant hECT proteins

The wild-type and mutant hECT proteins were produced in *E. coli* and purified as described for yeast Ect1p [23] with modifications. Cells were cultured to OD_{600} = 0.4–0.5 in LB medium or to 0.6 in 2xYT medium at 25 °C. Following 4 h of incubation with 0.1 mM isopropyl β-D-1-thiogalactopyranoside, cells were harvested and lysed by French pressure cell press (SLM Instruments) in breaking buffer [25 mM HEPES-KOH (pH 7.4), 300 mM KCl, 10% (w/v) glycerol, 2 mM 2-mercaptoethanol] containing 0.5% (v/v) protease inhibitor cocktail for use in purification of histidine-tagged proteins (Sigma). Cell lysate was centrifuged at 18,000×*g* at 4 °C for 30 min. The supernatant was incubated overnight at 4 °C with Profinity IMAC Ni-charged resin (Bio-Rad) equilibrated with breaking buffer. The resin was extensively washed first with breaking buffer and then with breaking buffer containing 20 mM imidazole. Protein was eluted from the resin with breaking buffer containing 500 mM imidazole. The buffer was exchanged using NAP-5 column (GE Healthcare) to buffer A [25 mM HEPES-KOH (pH 7.4), 300 mM KCl, 10% (w/v) glycerol, 1 mM dithiothreitol (DTT)].

2.4. Preparation of phospho[1,2-^{14}C]-ethanolamine

[1,2-^{14}C]Ethanolamine hydrochloride was purchased from Moravek Biochemical Inc. (Brea, CA). Phospho[1,2-^{14}C]-ethanolamine was synthesized enzymatically and purified according to the pub-

lished protocol [24], except that purified recombinant Eki1p of *S. cerevisiae* was used as ethanolamine kinase. *E. coli* BL21(DE3) cells harboring pETEKI1 were cultured and His_6-tagged Eki1p was purified similarly to hECT described above. The purity of phospho[1,2-^{14}C]-ethanolamine was checked by thin layer chromatography on Silica Gel 60 (Merck No. 5721) with 99.5% ethanol – 2% ammonia (1:2, v/v) as a developing solvent.

2.5. Enzyme activity assay

The reaction mixture contained following components in 25 μl: 20 mM Tris–HCl (pH 7.7), 10 mM $MgCl_2$, 2 mM or 10 mM CTP, 1 mM or 10 mM phospho[1,2-^{14}C]-ethanolamine (5 μCi/ml or 12.5 μCi/ml, respectively), 1 mM DTT, and enzyme, unless otherwise indicated. The reaction was carried out at 37 °C, unless otherwise specified, for 15 min, and stopped by incubation at 100 °C for 3 min. A 10 μl aliquot of the reaction was separated by thin-layer chromatography on Silica Gel 60 (Merck, 5721). After development with 99.5% ethanol – 2% ammonia (4:3 v/v), the spots corresponding to P-Etn and CDP-Etn, which were visualized with ninhydrin spray (Kanto Chemical), were scraped off, and the radioactivity was measured by a scintillation counter (Aloka). Kinetic parameters were calculated from the obtained curve using GraphPad Prism.

2.6. Preparation of whole cell extract and immunoblotting

Yeast cells cultured in SG medium containing 1 mM ethanolamine and 1 mM choline to the logarithmic phase at 30 °C were collected and washed with ice-cold PBS. Cells were suspended in the breaking buffer 2 [25 mM HEPES-KOH (pH 7.4), 300 mM KCl, 10% (w/v) glycerol, 1 mM DTT] containing 1% (v/v) protease inhibitor cocktail (Sigma), and disrupted with glass beads using Multi-Beads Shocker (YASUI-KIKAI). After centrifugation at 1000×*g* for 10 min at 4 °C, the supernatant was used as the whole cell extract. The whole cell extract was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), followed by immunoblotting analysis. The anti-HA monoclonal antibody (clone 16B12) was used at 1:1000 dilution, and the HRP-linked anti-mouse IgG Antibody (Cell Signaling Technology) was used at 1:4000 dilution as a secondary antibody.

2.7. Measurement of yeast growth

Cells from 2 ml culture in SG medium containing 1 mM ethanolamine and 1 mM choline were suspended in 2 ml SD medium containing 1 mM ethanolamine and 1 mM choline at a starting OD_{600} = 0.005. After 24 h preculture at 30 °C to reduce endogenous Ect1p, ethanolamine, and/or PE [25], cells were suspended in 2 ml SD medium containing 1 mM ethanolamine and 1 mM choline at a starting OD_{600} = 0.005. Cells were incubated at 30 °C for 48 h and OD_{600} was measured.

3. Results and discussion

3.1. Characterization of recombinant hECT

hECT derived from the smaller splice variant of human *PCYT2* transcripts [11,15] was produced in *E. coli* as a fusion protein with a 6 × His tag at its N-terminus, and purified. The activity of hECT was measured with phospho[1,2-^{14}C]-ethanolamine, which was prepared by the phosphorylation of [1,2-^{14}C]-ethanolamine using purified recombinant ethanolamine kinase Eki1p of *S. cerevisiae*. The optimum pH range of hECT was 7.2–9.0, and the optimum temperature was 37 °C (Fig. S1). hECT required Mg^{2+} for its optimal activity, and it exhibited partial activity in the presence of Mn^{2+} (Fig. S1), similarly to rat ECT [7].

The reaction of hECT exhibited Michaelis–Menten kinetics with a K_m for CTP of 48 ± 9 μM and a K_m for P-Etn of 179 ± 27 μM (Fig. 1 and Table 1). The K_m values of hECT are similar to those of rat and mouse ECTs [7,26]. The maximum velocity (V_{max}) of hECT was 0.33 ± 0.01 μmol/min/mg protein for CTP, corresponding to a turnover rate (k_{cat}) of 0.25 ± 0.01 s^{-1}. The V_{max} of hECT for P-Etn was 0.33 ± 0.02 μmol/min/mg protein, corresponding to a k_{cat} of 0.25 ± 0.02 s^{-1}. These values are lower than those of ECTs from rat and *P. falciparum* [7,14], but higher than those of mouse ECTs [26].

3.2. Contribution of the two CT domains to the enzymatic function

The roles of the two CT domains have been analyzed in the ECT of *P. falciparum*, and it was shown that only the N-terminal CT domain is catalytically active [14]. hECT also possesses two CT domains and the CTP-binding motifs HxGH sequences are well conserved in both CT domains of hECT (Fig. 2). Several studies have demonstrated the critical role of histidine residues of HxGH motifs in the activity of various CTs, including those of GCT from *Bacillus subtilis*, rat CCT, and the N-terminal CT domain of ECT from *P. falciparum* [14,27–29]. To clarify the role of the two CT domains of hECT, H35A, H38A, H226A, and H229A mutants, in which histidine residues in the HxGH motifs of the two CT domains were replaced by alanines, were produced in *E. coli* and purified, and their activities were measured *in vitro* (Table 1, Fig. S2). The activity of the H35A mutant was low, and its K_m and k_{cat} values could not be determined. The K_m values of the H38A mutant for CTP and P-Etn were increased, and the k_{cat} values were decreased to less than 10% of the wild-type hECT. In contrast, although the K_m values of the H226A mutant were increased, significant effects were not observed on the k_{cat} values of the H226A and H229A mutants. These results suggest that the N-terminal CT domain is critical for the activity of hECT.

We also analyzed the roles of the two CT domains of hECT in *S. cerevisiae* using a mutant defective in *ECT1* [8]. In *S. cerevisiae*, PE is synthesized through two pathways, the CDP-diacylglycerol pathway, in which PS is decarboxylated to PE by Psd1p or by Psd2p, and the Kennedy pathway [30]. PE is essential for the growth of *S. cerevisiae*, and deletion of both *PSD1* and *PSD2* is lethal without PE synthesis through the Kennedy pathway [9,10]. The yeast strain TKY12Ga, in which *PSD1* and *PSD2* are deleted and the promoter of *ECT1* is replaced with the *GAL1* promoter [19], can grow in media containing galactose with ethanolamine, since the expression of *ECT1* is induced by galactose and PE is synthesized through the Kennedy pathway. In contrast, the TKY12Ga strain cannot grow in media containing glucose due to the transcriptional repression of *ECT1*. Nikawa and colleagues reported that the defect of *ect1* in the *S. cerevisiae* mutant was suppressed by the overexpression of hECT cDNA [11]. In agreement with this, the overexpression of a gene encoding a fusion protein of the wild-type hECT with a 3 × HA epitope tag at its N-terminus using the constitutive strong promoter of *TDH3*, which encodes glyceraldehyde-3-phosphate dehydrogenase, suppressed the growth defect of the TKY12Ga strain in media containing glucose (Fig. 3B). Using this system, we examined the activities of hECT mutants in the yeast cells. The hECT mutants were produced at similar levels to the wild-type hECT in the TKY12Ga strain cultured in SG medium containing ethanolamine, in which all strains were viable (Fig. 3A). Significant growth was not observed when the TKY12Ga strain producing the H35A or H38A mutant was cultivated in the medium containing glucose (Fig. 3B). In contrast, substantial growth was observed when the TKY12Ga strain producing the H226A mutant was cultured in the glucose-containing medium, and the TKY12Ga strain producing the H229A mutant weakly but reproducibly grew in this medium.

Based on above results, it is suggested that the N-terminal CT domain of hECT plays an important role *in vivo*.

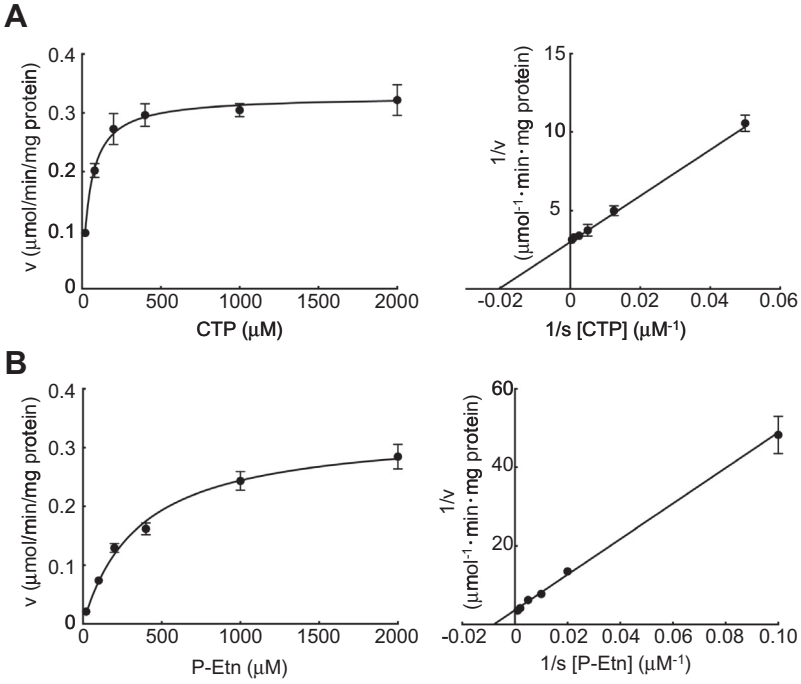

Fig. 1. Kinetic analysis of hECT. hECT activity was measured with increasing amount of CTP (A) and P-Etn (B). The double-reciprocal plots of the kinetic data were also shown. Experiments were performed in triplicate. Results are expressed as means ± SE.

Table 1
Kinetic parameters of the wild-type and mutant hECTs.

	K_m CTP (μM)	k_{cat} CTP (s^{-1})	K_m P-Etn (μM)	k_{cat} P-Etn (s^{-1})
Wild-type	48 ± 9	0.25 ± 0.01	179 ± 27	0.25 ± 0.02
H35A	N.D. [a]	N.D. [a]	N.D. [a]	N.D. [a]
H38A	74 ± 10	0.015 ± 0.001	880 ± 115	0.016 ± 0.001
H226A	111 ± 16	0.19 ± 0.01	275 ± 44	0.23 ± 0.02
H229A	42 ± 9	0.20 ± 0.01	301 ± 59	0.26 ± 0.02

Kinetic analyses were performed in triplicate. The parameters are the mean ± SE.
[a] Not determinable due to a weak activity.

3.3. Role of C-terminal CT domain of hECT

In the crystal structure of hECT, CMP was found in the pocket of the C-terminal CT domain, but not in that of the N-terminal domain (PDB code 3ELB). This result raises the intriguing possibility that CMP regulates the activity of hECT through an interaction with the C-terminal CT domain. We tested this possibility by adding CMP to the reaction of hECT (Fig. 4). However, the addition of CMP at concentrations of up to 2 mM did not significantly activate or inhibit the activity of hECT *in vitro*. Therefore, the significance of the interaction of CMP with the pocket of the C-terminal CT domain is currently unclear.

```
H. sapiens      -------RRAVRVWCDGCYDMVHYGHSNQLRQARAMG----DYLIVGVHTDEEIAKHKGP  68
M. musculus     -------QRIVRVWCDGCYDMVHYGHSNQLRQARAMG----DYLIVGVHTDEEIAKHKGP  68
R. norvegicus   -------QRTVRVWCDGCYDMVHYGHSNQLRQARAMG----DYLIVGVHTDEEIAKHKGP  68
S. cerevisiae   ------------KVWIDGCFDFTHHGHAGAILQARRTVSKENGKLFCGVHTDEDIQHNKGT  57
S. pombe        --------IKHRLWLDGCMDFFHYGHSNAILQAKQLG----ETLVIGIHSDEEITLNKGP  54
A. nidulans     -------IAEGRVWVDGCFDFSHHGHAGAMLQARRLG----DELLVGVHSDEAILENKGP  73
A. thaliana     -WRILRKRKPVRVYMDGCFDMMHYGHCNALRQARALG----DQLVVGVVSDEEIIANKGP 100
T. brucei       -------PGTIRVWVDGCFDMLHFGHANALRQARSMG----DELFVGCHTDEEIIRHKGP  80
P. falciparum   STSTQEKTKETRIYVDGIFDLSHSGHFNAMRQAKKLG----DIVVVGINSDEDALNSKGV 176
                             ::: **  *: * ** . : **:         :. *  :**     **

H. sapiens      IQFASGK---EPQPGETVIYVAGAFDLFHIGHVDFLEKVHRLAER--------------- 242
M. musculus     IQFASGK---EPQPGETVIYVAGAFDLFHIGHVDFLQEVHKLAKR--------------- 242
R. norvegicus   IQFASGK---EPQPGETVIYVAGAFDLFHIGHVDFLQEVHKLAKR--------------- 242
S. cerevisiae   ----VLVNGGYKFDAEDCVYVDGDFDLFHMGDIDQLRKLKMDLHPDKK------------ 229
S. pombe        ETLISGTTLLRLNPEKNIIYIDGDWDLFTEKHISAL-ELCTRMFPGIP------------ 233
A. nidulans     GSFDKLVSGKPPRPGQRIVYVDGGFDLFSSGHIEFLRQVLAIEESDGRQRGWYDQEQREQ 291
A. thaliana     VQFSNGK---GPGPDARIIYIDGAFDLFHAGHVEILRRARELGD---------------- 282
T. brucei       AQFSNKL---APPVGATVVYVDGAFDLFHAGHIRFLQKARALGD---------------- 242
P. falciparum   YQFIDNNELIKKKKNKKVVYVDGSFDIFHIGHLRILENAKKLGD---------------- 434
                                :*: * :*:*    .:   * .
```

Fig. 2. Alignment of amino acid sequences of ECTs. Amino acid sequences of HxGH motifs and the surrounding regions in the N- and C-terminal CT domains of ECTs were aligned with ClustalW. The UniProtKB/Swiss-Prot or UniProtKB/TrEMBL accession numbers of the sequences are as follows: *Homo sapiens* (isoform 1, Q99447); *Mus musculus* (A2ABY3); *Rattus norvegicus* (isoform 2, O88637); *Saccharomyces cerevisiae* (P33412); *Schizosaccharomyces pombe* (Q9UTI6); *Aspergillus nidulans* (C8V991); *Arabidopsis thaliana* (Q9ZVI9); *Trypanosoma brucei* (B9WNA0); and *Plasmodium falciparum* (Q8IDM2).

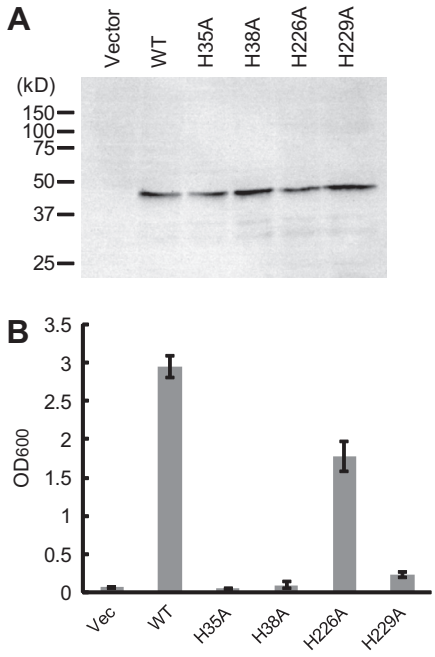

Fig. 3. Expression and function of the wild-type and mutant hECTs in the strain TKY12Ga. (A) Expression of the wild-type and mutant hECTs. The TKY12Ga strains containing YCplac22, YCplac22-hECT, YCplac22-hECTH35A, YCplac22-hECTH38A, YCplac22-hECTH226A, and YCplac22-hECTH229A were cultured to the logarithmic phase at 30 °C in SG medium containing 1 mM ethanolamine and 1 mM choline. Whole cell extracts were separated by SDS–PAGE, and analyzed by immunoblotting using anti-HA monoclonal antibody. (B) Growth of the TKY12Ga strains expressing the wild-type and mutant hECTs. Growth of the TKY12Ga strains containing YCplac22, YCplac22-hECT, YCplac22-hECTH35A, YCplac22-hECTH38A, YCplac22-hECTH226A, and YCplac22-hECTH229A was analyzed as described in Section 2. Results are expressed as means ± SE from three independent experiments.

The tandem repeat structures of the two CT domains are well conserved in ECTs from yeasts to mammals. It has been shown that only the N-terminal CT domain is active in the ECT of *P. falciparum* [14]. Our results suggest that the contributions of the N-terminal CT domains to their catalytic activities might be common features of ECTs. Indeed, the RTXGVSTT motif, which has been proposed to be involved in the interaction with CTP [27], is less conserved in the C-terminal CT domains of ECTs [14,15]. In addition, the HxGH motif is not perfectly conserved in the C-terminal CT domains of the fungal ECTs, *S. cerevisiae* Ect1, in which the corresponding sequence is $H^{210}MGD^{213}$ [8], *Schizosaccharomyces pombe* Ect1, in which the corresponding sequence is $T^{215}EKH^{218}$, and the *Aspergillus nidulans* ortholog, in which the corresponding sequence is $S^{260}SGH^{218}$ (Fig. 2).

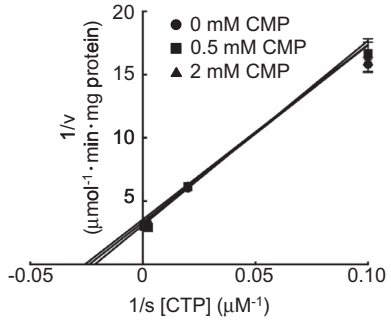

Fig. 4. Effect of CMP on hECT activity. hECT activity was measured with increasing amount of CTP in the presence of 0 mM (closed circles), 0.5 mM (closed squares), and 2 mM CMP (closed triangles). Experiments were performed in triplicate. Results are expressed as means ± SE.

The roles of the C-terminal CT domains of ECT remain to be determined. The C-terminal CT domains might contribute to the structural stability of ECTs. Analyses of crystal structures have shown that *B. subtilis* GCT and rat CCT, which are structurally related cytidylyltransferases but only possess one CT domain each, form homodimers [28,31]. Thus, cytidylyltransferases might be stabilized by dimeric or dimer-like structures, and, therefore, ECT is composed of two tandem CT domains. Alternatively, the C-terminal CT domains might be involved in the regulation of the enzymatic activities of ECT by binding ligands. Although the addition of CMP did not have any effect on the activity of hECT, an unidentified ligand that could be structurally similar to CMP may regulate ECT activity *in vivo*. Lower level of growth defect suppression of TKY12Ga by overexpression of the H229A mutant, which has substantial catalytic activity *in vitro*, may be explained by its regulatory role. In addition to measuring the ECT activities directly *in vitro*, it is very useful to express them in the yeast strain defective in *ECT1* in order to identify and characterize important residues for its *in vivo* function and for the elucidation of the regulation of hECT activity.

Acknowledgments

This work was partly supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan and the Platform for Drug Discovery, Informatics, and Structural Life Sciences of the Ministry of Education, Culture, Sports, Science and Technology of Japan. This work was performed using the facilities of the Biotechnology Research Center of the University of Tokyo.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.04.131.

References

[1] J.E. Vance, G. Tasseva, Formation and function of phosphatidylserine and phosphatidylethanolamine in mammalian cells, Biochim. Biophys. Acta 1831 (2013) 543–554.
[2] Y. Ichimura, T. Kirisako, T. Takao, Y. Satomi, Y. Shimonishi, N. Ishihara, N. Mizushima, I. Tanida, E. Kominami, M. Ohsumi, T. Noda, Y. Ohsumi, A ubiquitin-like system mediates protein lipidation, Nature 408 (2000) 488–492.
[3] A.K. Menon, V.L. Stevens, Phosphatidylethanolamine is the donor of the ethanolamine residue linking a glycosylphosphatidylinositol anchor to protein, J. Biol. Chem. 267 (1992) 15277–15280.
[4] W. Dowhan, M. Bogdanov, Lipid-dependent membrane protein topogenesis, Annu. Rev. Biochem. 78 (2009) 515–540.
[5] Z. Pavlovic, M. Bakovic, Regulation of phosphatidylethanolamine homeostasis—the critical role of CTP:phosphoethanolamine cytidylyltransferase (Pcyt2), Int. J. Mol. Sci. 14 (2013) 2529–2550.
[6] R. Sundler, B. Åkesson, Biosynthesis of phosphatidylethanolamines and phosphatidylcholines from ethanolamine and choline in rat liver, Biochem. J. 146 (1975) 309–315.
[7] R. Sundler, Ethanolaminephosphate cytidylyltransferase. Purification and characterization of the enzyme from rat liver, J. Biol. Chem. 250 (1975) 8585–8590.
[8] R. Min-Seok, Y. Kawamata, H. Nakamura, A. Ohta, M. Takagi, Isolation and characterization of *ECT1* gene encoding CTP: phosphoethanolamine cytidylyltransferase of *Saccharomyces cerevisiae*, J. Biochem. 120 (1996) 1040–1047.
[9] R. Birner, M. Bürgermeister, R. Schneiter, G. Daum, Roles of phosphatidylethanolamine and of its several biosynthetic pathways in *Saccharomyces cerevisiae*, Mol. Biol. Cell 12 (2001) 997–1007.
[10] M.K. Storey, K.L. Clay, T. Kutateladze, R.C. Murphy, M. Overduin, D.R. Voelker, Phosphatidylethanolamine has an essential role in *Saccharomyces cerevisiae* that is independent of its ability to form hexagonal phase structures, J. Biol. Chem. 276 (2001) 48539–48548.
[11] A. Nakashima, K. Hosaka, J. Nikawa, Cloning of a human cDNA for CTP-phosphoethanolamine cytidylyltransferase by complementation *in vivo* of a yeast mutant, J. Biol. Chem. 272 (1997) 9567–9572.
[12] B.A. Bladergroen, M. Houweling, M.J. Geelen, L.M. van Golde, Cloning and expression of CTP:phosphoethanolamine cytidylyltransferase cDNA from rat liver, Biochem. J. 343 (1999) 107–114.

[13] A. Poloumienko, A. Coté, A.T. Quee, L. Zhu, M. Bakovic, Genomic organization and differential splicing of the mouse and human Pcyt2 genes, Gene 325 (2004) 145–155.
[14] S. Maheshwari, M. Lavigne, A. Contet, B. Alberge, E. Pihan, C. Kocken, K. Wengelnik, D. Douguet, H. Vial, R. Cerdan, Biochemical characterization of *Plasmodium falciparum* CTP:phosphoethanolamine cytidylyltransferase shows that only one of the two cytidylyltransferase domains is active, Biochem. J. 450 (2013) 159–167.
[15] M. Bakovic, M.D. Fullerton, V. Michel, Metabolic and molecular aspects of ethanolamine phospholipid biosynthesis: the role of CTP:phosphoethanolamine cytidylyltransferase (Pcyt2), Biochem. Cell Biol. 85 (2007) 283–300.
[16] M.D. Fullerton, F. Hakimuddin, M. Bakovic, Developmental and metabolic effects of disruption of the mouse CTP:phosphoethanolamine cytidylyltransferase gene (Pcyt2), Mol. Cell. Biol. 27 (2007) 3327–3336.
[17] M.D. Fullerton, F. Hakimuddin, A. Bonen, M. Bakovic, The development of a metabolic disease phenotype in CTP:phosphoethanolamine cytidylyltransferase-deficient mice, J. Biol. Chem. 284 (2009) 25704–25713.
[18] R. Leonardi, M.W. Frank, P.D. Jackson, C.O. Rock, S. Jackowski, Elimination of the CDP-ethanolamine pathway disrupts hepatic lipid homeostasis, J. Biol. Chem. 284 (2009) 27077–27089.
[19] L. Deng, T. Kakihara, R. Fukuda, A. Ohta, Construction of a yeast strain with regulatable phospholipid synthesis for analysis of the uptake and metabolism of phosphatidylethanolamine with short acyl chains, Biosci. Biotechnol. Biochem. 71 (2007) 2313–2315.
[20] R. Fukuda, H. Horiuchi, A. Ohta, M. Takagi, The prosequence of *Rhizopus niveus* aspartic proteinase-I supports correct folding and secretion of its mature part in *Saccharomyces cerevisiae*, J. Biol. Chem. 269 (1994) 9556–9561.
[21] R.D. Gietz, A. Sugino, New yeast-*Escherichia coli* shuttle vectors constructed with *in vitro* mutagenized yeast genes lacking six-base pair restriction sites, Gene 74 (1988) 527–534.
[22] P. Chen, P. Johnson, T. Sommer, S. Jentsch, M. Hochstrasser, Multiple ubiquitin-conjugating enzymes participate in the *in vivo* degradation of the yeast MAT alpha 2 repressor, Cell 74 (1993) 357–369.
[23] J. Ohtsuka, K. Nagata, W.C. Lee, Y. Ono, R. Fukuda, A. Ohta, M. Tanokura, Crystallization and preliminary X-ray analysis of CTP:phosphoethanolamine cytidylyltransferase (ECT) from *Saccharomyces cerevisiae*, Acta Crystallogr., Sect. F: Struct. Biol. Cryst. Commun. 62 (2006) 1003–1005.
[24] L.B. Tijburg, P.S. Vermeulen, L.M. van Golde, Ethanolamine-phosphate cytidylyltransferase, Methods Enzymol. 209 (1992) 258–263.
[25] L. Deng, R. Fukuda, T. Kakihara, K. Narita, A. Ohta, Incorporation and remodeling of phosphatidylethanolamine containing short acyl residues in yeast, Biochim. Biophys. Acta 1801 (2010) 635–645.
[26] A. Tie, M. Bakovic, Alternative splicing of CTP:phosphoethanolamine cytidylyltransferase produces two isoforms that differ in catalytic properties, J. Lipid Res. 48 (2007) 2172–21781.
[27] Y.S. Park, P. Gee, S. Sanker, E.J. Schurter, E.R. Zuiderweg, C. Kent, Identification of functional conserved residues of CTP:glycerol-3-phosphate cytidylyltransferase. Role of histidines in the conserved HXGH in catalysis, J. Biol. Chem. 272 (1997) 15161–15166.
[28] C.H. Weber, Y.S. Park, S. Sanker, C. Kent, M.L. Ludwig, A prototypical cytidylyltransferase: CTP:glycerol-3-phosphate cytidylyltransferase from *Bacillus subtilis*, Structure 7 (1999) 1113–1124.
[29] D.P. Veitch, D. Gilham, R.B. Cornell, The role of histidine residues in the HXGH site of CTP:phosphocholine cytidylyltransferase in CTP binding and catalysis, Eur. J. Biochem. 255 (1998) 227–234.
[30] S.A. Henry, S.D. Kohlwein, G.M. Carman, Metabolism and regulation of glycerolipids in the yeast *Saccharomyces cerevisiae*, Genetics 190 (2012) 317–349.
[31] J. Lee, J. Johnson, Z. Ding, M. Paetzel, R.B. Cornell, Crystal structure of a mammalian CTP: phosphocholine cytidylyltransferase catalytic domain reveals novel active site residues within a highly conserved nucleotidyltransferase fold, J. Biol. Chem. 284 (2009) 33535–33548.