Cloning and Characterization of a Second Human CTP:Phosphocholine Cytidyltransferase*

(Received for publication, March 2, 1998)

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CTP:phosphocholine cytidyltransferase (CCT) is a key regulator of phosphatidylcholine biosynthesis, and only a single isoform of this enzyme, CCTα, is known. We identified and sequenced a human cDNA that encoded a distinct CCT isoform, called CCTβ, that is derived from a gene different from that encoding CCTα. CCTβ transcripts were detected in human adult and fetal tissues, and very high transcript levels were found in placenta and testis. CCTβ and CCTα proteins share highly related, but not identical, catalytic domains followed by three amphipathic helical repeats. Like CCTα, CCTβ required the presence of lipid regulators for maximum catalytic activity. The amino terminus of CCTβ bears no resemblance to the amino terminus of CCTα, and CCTβ protein was localized to the cytoplasm as detected by indirect immunofluorescent microscopy. Whereas CCTα activity is regulated by reversible phosphorylation, CCTβ lacks most of the corresponding carboxyl-terminal domain and contained only 3 potential phosphorylation sites of the 16 identified in CCTα. Transfection of COS-7 cells with a CCTβ expression construct led to the overexpression of CCT activity, the accumulation of cellular CDP-choline, and enhanced radiolabeling of phosphatidylcholine. CCTβ protein was posttranslationally modified in COS-7 cells, resulting in slower migration during polyacrylamide gel electrophoresis. Expression of CCTβ/CCTα chimeric proteins showed that the amino-terminal portion of CCTβ was required for posttranslational modification. These data demonstrate that a second, distinct CCT enzyme is expressed in human tissues and provides another mechanism by which cells regulate phosphatidylcholine production.

CCTα proteins have been identified and sequenced in rat (3), hamster (4), mouse (5), and human (6), and there are only minor differences among these mammalian cDNAs (see Ref. 6 for a comparison). Their catalytic properties are thought to be essentially identical, and CCTα can be divided into four distinct functional domains (see Fig. 1). The amino-terminal domain between residues 1 and 71 contains a sequence that specifies the nuclear localization of the protein between residues 2 and 28 (7, 8). The catalytic core extends from residues 72 to 233. This region of the protein is conserved from yeast to mammals and is responsible for substrate binding and catalysis. In particular, the conserved HXGH motif is essential for cytidylyltransferase activity (9, 10). The third domain, located between residues 256 and 288, contains three 11-residue amphipathic repeats that form α-helices following association with lipid regulators and contribute to the reversible membrane association of the enzyme (11–15). The binding of stimulatory lipids to this region greatly enhances catalytic activity by lowering the Km of the enzyme for CTP into the range corresponding to cellular concentrations of the nucleotide (16). CCTα is also negatively regulated by lipids and is potently inhibited by sphingosine (17), lysophosphatidylcholine (18), and antineoplastic phospholipids (18, 19). The fourth domain of CCTα is the carboxyl-terminal phosphorylation domain between residues 315 and 367. CCTα membrane association and activity are modulated by reversible phosphorylation (20, 21), and all of the phosphorylation sites are located in the carboxy-terminal region (22). Phosphorylation attenuates CCTα biochemical activity by interfering with lipid stimulation (23), and unphosphorylated CCTα exhibits a greater degree of membrane association in cells (20).

CCTα has been localized using cellular in situ methods to the nucleus in Chinese hamster ovary cells (7, 8), but in rat hepatocytes, the protein has been detected in both the nuclear and cytoplasmic compartments (24). CCTα has also been identified in association with Golgi membranes (25, 26), endoplasmic reticulum, and transport vesicles (27) using biochemical methods. There is only one isoform of CCT expressed in yeast (28), and only one isoform of CCT (CCTα) has been identified, purified, or cloned from mammalian sources (2). The existence of a conditionally lethal Chinese hamster ovary cell mutant with a temperature-sensitive defect in CCTα activity (29) also suggested that there was only a single CCT isoform in mammalian cells. A single genetic locus for CCTα was identified on mouse chromosome 16 (5), and the murine CCTα gene has been cloned (30). In this work, we identify a unique, second human CCT isoform, called CCTβ. CCTβ catalyzes the same enzymatic reaction as CCTα and requires the presence of lipids for full activity. However, CCTβ lacks the nuclear targeting sequence and the phosphorylation domain of CCTα, suggesting that CCTβ is distinct from CCTα with regard to its subcellular localization and regulation.

*This work was supported by National Institutes of Health Grant GM 45737, Cancer Center (CORE) Support Grant CA 21765, and the American and Lebanese Syrian Associated Charities. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

†The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF052510.

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§§The abbreviations used are: CCT, CTP:phosphocholine cytidyltransferase; PtdCho, phosphatidylcholine; α-isoform, the previously discovered CCT; β-isoform, the new CCT described in this paper; kb, kilobase(s); bp, base pair(s).
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EXPERIMENTAL PROCEDURES

Materials—Sources of supplies were as follows: American Radiolabel Company, Inc., phospho[methyl-14C]choline (specific activity, 55 mCi/ mmol) and [methyl-3H]choline (specific activity, 85 mCi/mmol); Amer- sham Pharmacia Biotech [3H]methionine (specific activity, >1000 Ci/ mmol); CLONTECH, human multiple tissue Northern blots; Life Technologies, Inc., LipofectAMINE reagent; Promega, restriction en- zymes and other molecular biology reagents; Invitrogen, pcDNA3 plasmid; FMC Corp., Sea-Kem, molecular biology grade agarose; American Type Culture Collection, cDNA clone AA382871; Sigma, CTP and buffers; Avanti Polar Lipids, PtdCho and fatty acids; Analtech, thin- layer chromatography plates. All other supplies were reagent grade or better.

Antisera—Antibodies raised against a synthetic peptide (MDAQSSAVSNSSRKRE) corresponding to the first 17 amino acids of CCTα. AntiCCTβ rabbit polyclonal antiserum was raised against a synthetic peptide (MEEIEHCTCPQPRL) corresponding to the first 17 amino acids of CCTβ. Peptides and peptide antigens were prepared by the Molecular Resource Center of St. Jude Children's Research Hospital. To prepare antigen, each peptide was coupled to keyhole limpet hemocyanin via an additional cysteine at the carboxyl terminus of the peptide. Immunization of rabbits and collection of antisera was performed by Rockland, Inc., according to their standard schedule. Antisera were purified by affinity chromatography on Affi-Gel 10 cross-linked to the peptide.

Isolation of the CCTβ cDNA—Human Genome Systems identified and provided a clone that exhibited significant sequence similarity to CCTα. The protein expressed from this cDNA, however, did not exhibit significant amino acid identity. Sequence information from this clone was used to search the public expressed sequence tagged data base. We identified a clone (GenBankTM accession no. AA382871) that contained 40 bp of related sequence at the 5’ end. We purchased this clone from American Type Culture Collection and sequenced the cDNA on both strands using primers that flanked the multiple cloning sites and internal primers that were synthesized to ensure a complete read on both strands. The sequence contained a single open reading frame. The sequence was sequenced in both directions using primers flanking the reading frame. The sequence was submitted to GenBankTM.

Plasmid pPJ34 was digested with NcoI and the resulting 2185-bp fragment was ligated into pcDNA3 that had been previously digested with NcoI, and the resulting 2185-bp fragment was ligated into pcDNA3 that had been previously digested with NcoI and XhoI. The resulting plasmid pPJ34, which expressed CCTβ from the constitutive cytomegalovirus promoter, was sequenced. Plasmid DNA was isolated, transcribed, translated, and labeled with [35S]methionine using the Promega T7-coupled transcription/translation kit according to the manufacturer's instructions. The labeled proteins were analyzed by SDS-gel electrophoresis and visualized by autoradiography.

Construction of Plasmids for Expression of CCT Chimeras and CCTβ Amino-terminal Truncation—Rodent CCTα (pWYCT) and human CCTβ (pPJ34) cDNAs cloned into pcDNA3 were digested with SspI. The pcDNA3 vector has an SspI site distanced approximately 1 kb from the 5’-end of the T7 promoter. The CCTα cDNA has an SspI site at nucleotide 1980, 110 bp from the 5’-end of the T7 promoter. The cDNA has an SspI site at nucleotide 2014. The first and last 2 kb of the 2185-bp fragment that contained either CCTα or CCTβ sequence plus vector sequence were purified. The 1.3-kb fragment generated from CCTβ cDNA that encoded the amino terminus was ligated to the 5-kb fragment of pWYCT to generate pCCTα/CCTβα, and the 1.3-kb fragment from the CCTα cDNA encoding the amino terminus was ligated to the 5-kb fragment of pPJ34 to generate pCCTβ/CCTββ. The resulting plasmids were checked for correct orientation with the polymerase chain reaction using the T7 and SP6 primers of pcDNA3. Plasmid pPJ34 was digested with NcoI, and the resulting 2185-bp fragment was purified. The DNA overhanging sequences were filled in with Klenow fragment and purified. The DNA was subsequently gel purified. The DNA was digested with XhoI to yield a 1180-bp fragment, and after purification, this fragment was ligated into pcDNA3 that had been previously digested with EcoRV and XhoI, resulting in plasmid pPJ35. Inserts in pcDNA3 were screened by polymerase chain reaction using T7 and SP6 primers. DNA sequencing confirmed the truncation of the first 26 amino acids of CCTβ.

CCT Assay—CCT activity was determined essentially as described previously (31). The standard assay contained 64 μM lipid activator (methyl-[3H]choline, 4 mM CTP, 10 mM MgCl2, 150 mM NaCl, pH 8.5, 1 mM phospho[14C]choline (specific activity, 4.5 mCi/mmol), in a final volume of 50 μl. The reaction mixtures were incubated at 37 °C for 10 min. The reaction was stopped by the addition of 5 μl of 0.5 M Na3EDTA, and the tubes were vortexed and placed on ice. Next, 40 μl of each sample was spotted on preadsorbent Silica Gel G thin layer plates, which were developed in 2% ammonium hydroxide/95% ethanol (1:1, v/v). CDP-[14C]choline was identified by co-migration with a standard, scraped from the plate, and quantitated by liquid scintillation counting. Protein was determined according to the Bradford method (32).

Isolation of CCTβ from Endogenous Lipids—CCTβ was isolated from COS-7 cells 48 h after transfection with plasmid pPJ34. Cells were prepared by the Molecular Resource Center of St. Jude Children's Research Hospital. To prepare antigen, each peptide was coupled to keyhole limpet hemocyanin via an additional cysteine at the carboxyl terminus of the peptide. Immunization of rabbits and collection of antisera was performed by Rockland, Inc., according to their standard schedule. Antisera were purified by affinity chromatography on Affi-Gel 10 cross-linked to the peptide.

Isolation of the CCTβ cDNA—Human Genome Systems identified and provided a clone that contained 40 bp of related sequence at the 5’ end. We purchased this clone from American Type Culture Collection and sequenced the cDNA on both strands using primers that flanked the multiple cloning sites and internal primers that were synthesized to ensure a complete read on both strands. The sequence contained a single open reading frame we called CCTβ. A 1.3-kb BamHI-XhoI fragment was excised and subcloned into the mammalian expression vector, pcDNA3, to generate plasmid pPJ34, which expressed CCTβ from the constitutive cytomegalovirus promoter.

Plasmid DNA was isolated, transcribed, translated, and labeled with [35S]methionine using the Promega T7-coupled transcription/translation kit according to the manufacturer’s instructions. The labeled proteins were analyzed by SDS-gel electrophoresis and visualized by autoradiography.

Northern Blots—Three multiple human tissue Northern blots were purchased from CLONTECH and were hybridized and washed according to the manufacturer’s instructions. The blots were first hybridized with 32P-labeled probe prepared from a 1.3-kb BamHI-XhoI fragment that contained the entire cDNA. The blots were then stripped and hybridized with a 32P-labeled probe prepared from a 200-bp SacI fragment of the human phosphatidylinositol synthase (pis1) cDNA (33). The blots were stripped again and hybridized with a 32P-labeled probe prepared from a 320-bp PstI-Apal fragment representing the 3’ region of the CCTβ cDNA. This area of the CCTβ cDNA did not show any sequences in common with CCTα.

Immunoblots—Cell lysates (50 μg of protein) were separated by SDS-gel electrophoresis on 12% polyacrylamide gels and transferred by electroblotting onto nitrocellulose membranes. Immunoblotting was performed by incubation of the membranes with either purified anti-CCTα (1:200 dilution) or purified anti-CCTβ (1:200 dilution) as the primary antibody. The Amersham Pharmacia Biotech ECL Western blotting reagents and protocol were used to identify the immunoreac tant proteins.

Immunofluorescence Microscopy—HeLa cells grown on coverslips were fixed with 3% formaldehyde, permeabilized with cold acetone, and processed as described (34). Affinity-purified anti-CCTβ primary antibody was diluted in 0.15 M NaCl, 10 mM Tris-HCl, pH 8.0. The cells were incubated with anti-CCTβ antibodies at increasing dilutions followed by fluorescein-conjugated secondary antibodies. The coverslips
were mounted with p-phenyldiamine, the cells viewed in a Zeiss IM-35 microscope equipped with fluorescence optics, and photographs were made on Kodak Tri-X pan film. Controls from which the primary antibody was excluded showed no significant fluorescence. Preincubation of the primary antibody with the peptide did not yield significant fluorescence. Two other controls assured selective labeling of the nuclear and cytoplasmic compartments. Both a nuclear marker (anti-p120 antibodies, Becton Dickinson) and a cytoplasmic (cytoskeletal) marker (anti-vimentin antibodies, Boehringer Mannheim) were used to label the cells to confirm appropriate staining of cellular compartments.

RESULTS

Identification of the CCTβ Clone—A BLAST search of the proprietary human expressed sequence tagged data base of Human Genome Sciences revealed the existence of a cDNA with considerable similarity to mammalian CCTs. However, the protein expressed by this cDNA was not catalytically active, indicating that it did not contain a complete CCT coding sequence. Sequence information from this clone was used to search the public expressed sequence tagged data base. We identified a second clone (GenBank™ accession no. AA382871) isolated from a human testis library that contained a 140-bp related sequence at the 5’ end. This clone, called CCTβ, was purchased from American Type Culture Collection. Both cDNA strands were sequenced, and the clone contained the entire CCTβ coding sequence. The cDNA sequence of human CCTβ was compared with the cDNA sequence of human CCTα (see Fig. 2). The analysis of the sequence (see below) indicated that the CCTβ cDNA encoded a new CCT isoform.

Similarities and Differences between the Predicted Protein Sequences and the cDNAs of CCTα and CCTβ—The predicted amino acid sequences of human CCTα and CCTβ are compared in Fig. 1. The catalytic core of human CCTα and CCTβ are nearly identical and extend from amino acids 72 through 233. The catalytic core in CCTβ has 64% identity with the yeast CCT domain that is located between amino acids 99 and 260 of the yeast protein (28). Three of the amino acids in CCTβ that are different from CCTα (N120K, V136L, and R162K) are identical to the yeast CCT sequence. Three other amino acids that are different in CCTβ compared with CCTα (E126D, D134E, and E160K) are identical to the residues found in the catalytic core of the yeast MUQ1 sequence, which has been identified as phosphoethanolamine cytidylyltransferase (36). Also, the catalytic domains of human phosphoethanolamine cytidylyltransferase are highly related to the analogous domains in CCTα and CCTβ (37). These sequence similarities strongly suggested that the CCTβ cDNA encoded a protein with cytidylyltransferase activity.

The amino-terminal domain of CCTβ is distinct from the amino-terminal region of human CCTα (Fig. 1). CCTβ lacks the human CCTα sequence KVaRRKKeaPGPNGAED28, which is postulated to mediate transit of the protein to the nuclear compartment. Amino acids 11 and 18 are designated in lowercase because they differ from the rodent CCTα sequence that is known to be the minimal protein sequence that is both necessary and sufficient for localization of rodent CCTα to the nucleus (8). The significant difference in the amino-terminal sequences of CCTα and CCTβ suggests that the lack of a discernible nuclear localization motif in CCTβ strongly suggested that the CCTβ cDNA encoded a protein with cytidylyltransferase activity.

The predicted CCTβ protein sequence exhibits some significant similarities and distinct differences from the CCTα sequence in the amphipathic helical and phosphorylation regulatory domains (Fig. 1). The helical domain of human CCTβ is highly related to the analogous domain in CCTα, with 90% amino acid identity between residues 256 and 288 and with conservative substitutions at K259R, Q265N, and K266R. These sequence similarities indicate that the amino terminus of CCTβ is more related to the yeast than mammalian CCTα; however, the significance of this correlation is not obvious because a specific function for the amino terminus of yeast CCT has not been described.

The predicted CCTβ protein sequence exhibits some significant similarities and distinct differences from the CCTα sequence in the amphipathic helical and phosphorylation regulatory domains (Fig. 1). The helical domain of human CCTβ is highly related to the analogous domain in CCTα, with 88% amino acid identity between residues 256 and 288 and with conservative substitutions at K259R, Q265N, and K266R. This domain in rodent CCTα is required for the lipid-dependent decrease in the CTP $K_m$ associated with stimulation of CCT activity (16) and mediates the reversible binding of CCTα to phospholipid bilayers (11–15). A similar amphipathic helical domain is absent in the yeast CCT (28), although this enzyme is activated by lipids (38). The similarity in the helical regions
of CCTα and CCTβ suggested that CCTβ activity would be stimulated by the interaction with lipids in a manner similar to mammalian CCTα and that CCTβ would undergo reversible association with cellular membranes. Lipid stimulation of CCTα activity is regulated by phosphorylation of the carboxy-terminal domain (23). Sixteen serine residues are located in the CCTα domain, which extends from amino acid 315 through 367, and all of these serines are phosphorylated to some extent (22). In contrast, the shorter carboxy-terminal domain of CCTβ contains only three possible serine phosphorylation sites (Ser315, Ser319, and Ser323). Ser315 and Ser319 are potential proline-directed phosphorylation sites analogous to Ser315 and Ser319 in CCTα. Thus, the opportunities for the regulation of CCTβ activity by reversible phosphorylation are more restricted in CCTβ than in CCTα.

The cDNA sequences of CCTα and CCTβ are distinct but there are regions that have significant similarity (Fig. 2). The similarities are most pronounced in the catalytic core and amphipathic helix domains that are the most conserved peptide regions between CCTα and CCTβ. However, there are many differences in the cDNA sequence in these regions, illustrating that CCTα and CCTβ arise from the transcription of different genes and not by the alternative splicing of a single gene. The 5’ and 3’ regions of the cDNAs reflect the lack of similarity between the two proteins in the amino and carboxy-terminal regions.

Pattern of CCTβ mRNA Expression—The relative abundance of CCTβ mRNA expression in a wide variety of human tissues was addressed by Northern blot analysis (Fig. 3). The blots were probed with the human phosphatidylinositol synthase (pis1) cDNA as a loading control. This enzyme in phosphatidylinositol biosynthesis is a “housekeeping” protein that is expressed at a relatively uniform level in human tissues. The blots were then probed with both the entire CCTβ cDNA (Fig. 3) and with a 32P-labeled fragment from the 3’ untranslated region of the CCTβ cDNA. The pattern of expression was the same with both probes (not shown). Two sizes of CCTβ transcripts were detected. The largest CCTβ mRNA (~6.5 kb) was most abundant in brain, ovary, testis, and all fetal tissues examined. The second class of CCTβ mRNAs were found between 1.1 and 1.9 kb. The 1.1-kb mRNA was detected in placenta, which was the most abundant source for CCTβ mRNA in our survey. Testis also was an abundant source for CCTβ transcripts, and two mRNAs of 1.6 and 1.9 kb were detected in this tissue. Although it is difficult to see in Fig. 3 due to the very high expression of CCTβ in placenta and testis, CCTβ mRNA species of either 1.1 or 1.9 kb were faintly detected in all tissues examined. Thus, CCTβ mRNA is widely distributed in human tissues and expressed at very high levels in testis and placenta.

Substrate Specificity of CCTβ—The similarity of the catalytic core domains of CCTβ and CCTα suggested that CCTβ was a phosphocholine cytidylyltransferase. This prediction was confirmed by transfecting COS-7 cells with a CCTβ expression construct (pPJ34) and measuring the CCT enzymatic activity in cell lysates (Fig. 4A). The introduction of the CCTβ expression vector into the COS-7 cells led to a significant (8-fold) increase in the CCT specific activity from 4.8 to 38.4 nmol/min/mg of protein in cell extracts (Fig. 4A). These data establish that the CCTβ cDNA encodes an active CTP:phosphocholine cytidylyltransferase. Alternative substrates for CCTβ (phosphoethanolamine, glycerol 3-phosphate, phosphatidic acid, and lysophosphatidic acid) were also screened. Substitution of these compounds for phosphocholine in the biochemical assay did not yield significant activity. Substitution of deoxyCTP for the CTP in the assay at concentrations up to 10-fold higher also did not yield significant activity.

Effect of CCTβ Expression on PtdCho Metabolism—COS-7 cells were transfected with the CCTβ expression plasmid and were labeled with [3H]choline for 24 h to determine whether CCTβ functions as a CCT in vivo and whether the overexpression of this isoenzyme effects the PtdCho biosynthetic pathway (Fig. 4B). Although the cellular content of [3H]-labeled choline and phosphocholine were the same in control and CCTβ-transfected cells, there was a 3.4-fold increase in the CDP-choline pool from 1963 ± 13 to 6706 ± 847 cpm/mg. This finding was

**Fig. 2. A comparison of the cDNA sequences of CCTα and CCTβ.** The human CCTβ cDNA sequence determined in this paper (GenBank™ accession no. AF052510) was compared with the published cDNA sequence of human CCTα (GenBank™ accession no. L28957). Identical bases are boxed.
consistent with the identification of CCTβ as a phosphocholine cytidylyltransferase and illustrated that overexpression of the enzyme leads to increased accumulation of its product, CDP-choline in vivo. There was also a 50% increase in the incorporation of [3H]choline into PtdCho from 715,228 ± 28,317 to 1,061,310 ± 15,959 cpm/mg in CCTβ-transfected cells indicating that CCTβ overexpression accelerated PtdCho synthesis.

The 5-fold increase in the amount of glycerophosphocholine, a breakdown product of PtdCho, from 7027 ± 251 to 35,835 ± 7083 cpm/mg in the CCTβ-expressing cells indicated an acceleration of PtdCho turnover similar to that previously observed with CCTα overexpression (39).

Lipid Stimulation of CCTβ Activity—The similarities in the amphipathic helical domains between CCTα and CCTβ suggested that lipids would stimulate CCTβ activity in a similar manner to CCTα. The addition of the PtdCho:oleic acid lipid activator mixture to crude cell lysates did not enhance CCTβ activity (not shown). However, the lack of lipid regulation in cell lysates could be attributed to the presence of endogenous lipid activators. This point was tested by removing the endogenous lipid activators from the transfected COS-7 cell lysates by ion-exchange chromatography (16, 23) and determining the ability of PtdCho:oleic acid vesicles to activate the enzyme (Fig. 4C). CCTβ activity was not detected following the removal of endogenous lipids, and it was potently stimulated by the addition of PtdCho:oleic acid vesicles to the sample. There remain many kinetic details related to the specificity of the lipid regulation of CCTβ to be investigated with purified CCTβ, and there may be subtle differences between the two proteins because the amphipathic helical domains are not identical. Nonetheless, our experiments establish that CCTβ, like CCTα, is critically dependent on the presence of stimulatory lipids for activity.

Expression and Modification of CCTβ Protein—The predicted molecular size of the CCTβ protein was confirmed by transcription and translation of the CCTβ cDNA in vitro using a reticulocyte lysate (Fig. 5). The expressed proteins were ra-
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Fig. 5. Antibody specificity and in vivo modification of CCTβ. Left three lanes, transcription and translation in vitro of CCTα or CCTβ cDNAs using a reticulocyte lysate system (Promega) containing 40 μCi/50 μL [35S]methionine (1000 Ci/nmol). The products were separated by SDS gel electrophoresis on 12% gels, and the bands were visualized by autoradiography. Middle and right lanes, cellular expression of CCTα or CCTβ protein species. COS-7 cells were transfected with pcDNA3 plasmids carrying CCTα or CCTβ cDNAs, and cell lysates were analyzed by immunoblotting 48 h later. Samples (50 μg protein) of the total cell lysates were probed with either anti-CCTα or anti-CCTβ antibodies as described under “Experimental Procedures.” Control samples were obtained using the pcDNA3 vector without a cDNA insert for expression plasmid was transfected into COS-7 cells, was expressed in the transcription/translation system, was tranlated by in vitro transcription/translation using CCTα or CCTβ protein of an apparent size of 35 kDa was identified in reactions using CCTβ cDNA as template and was consistent with the predicted size of 36.3 kDa for CCTβ protein. As a control, CCTα was expressed in the transcription/translation system, and the expected 42-kDa protein was detected (Fig. 5). The CCTβ expression plasmid was transfected into COS-7 cells, and cell lysates were analyzed for expression of CCTβ protein by immunoblotting with an antibody against amino acids 27–39 of the CCTβ polypeptide sequence (Fig. 5). Two forms of CCTβ were detected following expression in COS-7 cells. The less abundant species migrated at the same apparent size as the protein made in vitro (CCTβ), and there was a second, slower migrating form (CCTβ_M). The location of both CCTβ_M and CCTα at approximately the same position on the gel was not due to cross-reactivity of the two affinity-purified antibodies. The specificities of the anti-CCTα and anti-CCTβ amino-terminal antibodies were clearly demonstrated in the same experiment (Fig. 5). The larger apparent size of CCTβ_M suggests that a significant portion of the expressed protein is modified posttranslationally.

A second approach was used to verify that a posttranslational modification of the CCTβ sequence was responsible for the retarded electrophoretic mobility of CCTβ_M. The cDNA sequences corresponding to the dissimilar amino-terminal regions of CCTα and CCTβ were exchanged. Proteins were expressed from the chimeric cDNAs that represented the first 84 amino acids of CCTβ followed by the remaining 283 amino acids of CCTα (CCTα/CCTβ, Fig. 6) or the first 84 amino acids of CCTα followed by the latter 246 amino acids of CCTβ (CCTα/CCTβ, Fig. 6). The anti-CCTβ antibody recognized both the authentic CCTβ and CCTβM proteins (Fig. 6, lane 8) expressed in COS-7 cells as well as the CCTβ/CCTα and CCTβM/CCTα chimeras (lane 6). The CCTβ/CCTα chimera exhibited a larger apparent molecular size than CCTβ as anticipated due to the longer carboxyl terminus of CCTα (lane 6) and also displayed the previously reported gel shifts due to multiple phosphorylation of the CCTα carboxyl-terminal domain (40). In contrast, the anti-CCTα antibody only detected the gel shifts due to phosphorylation of the authentic CCTα protein (lane 4) and did not reveal any modification of the smaller CCTα/CCTβ chimeric protein (lane 7). The dissimilarities of the CCTα and CCTβ NH2 termini, the demonstrated specificities of the antibodies, and the reproduction of the gel shift with in vivo expression of a CCTβ/CCTα fusion protein strongly support the idea that CCTβ is biochemically modified. This modification results in a protein that migrates with approximately the same apparent molecular weight as CCTα following denaturing gel electrophoresis.

To further localize the sequences in CCTβ required for the posttranslational modification, the first 26 amino acids were deleted from the NH2 terminus. The truncated protein, called CCTβ[Δ1–26], was expressed in COS-7 cells and detected by immunoblotting with the anti-CCTβ antibody (Fig. 6). The apparent molecular size of CCTβ[Δ1–26] was smaller than CCTβ, as expected; however, a CCTβM[Δ1–26] species was not observed, indicating that posttranslational modification of the truncated protein did not occur (Fig. 6, lane 11). These data suggested that the first 26 amino acids of CCTβ were necessary for cellular processing of the protein.

Regulation of CCTβ Activity—CCTβ activity was examined in vitro to determine whether the NH2-terminal sequence and modification of the protein influenced expression and/or catalytic activity. COS-7 cells were transfected with the plasmids encoding CCTβ, CCTα, the chimeric CCT proteins (CCTβ/
CCTα and CCTα/CCTβ, and the NH₂-terminal truncated CCTβ[Δ1–26]. The CCT protein species were expressed at approximately equivalent levels based on the immunoblots (Fig. 6). The CCT protein species in the cell lysates were assayed in the presence of excess stimulatory lipids to evaluate their relative activities. Although cells overexpressing (CCTβ plus CCTβ[Δ1]) had higher activity (76 nmol/min/mg) than the endogenous control activity (8 mmol/min/mg), (CCTβ plus CCTβ[Δ1]) was less active than CCTα and its multiply phosphorylated species (1105 nmol/min/mg) (Fig. 7). Substitution of the NH₂-terminal domain of CCTα onto CCTβ enhanced biochemical activity (215 nmol/min/mg), whereas the amino-terminal domain of CCTβ dramatically reduced the activity of CCTα (91 nmol/min/mg). These data suggested that the protein modification directed by the amino terminus of CCTβ attenuates biochemical activity. In support of this hypothesis, truncation of the first 26 amino acids of CCTβ elevated activity almost 10-fold (686 nmol/min/mg), supporting the idea that the NH₂ terminus plays a role in the cellular regulation of CCTβ activity.

**Cellular Localization of CCTβ**—The amino terminus of CCTβ bears little resemblance to the amino terminus of CCTα, which harbors a nuclear localization sequence. CCTα is reported to be predominately an intranuclear protein based on indirect immunofluorescence using an antibody raised against an amino-terminal peptide of CCTα (7, 8). The lack of a nuclear localization motif in CCTβ suggested that it would not be found in the cell nucleus. This hypothesis was tested by evaluating the distribution of CCTβ in human HeLa cells using the anti-CCTβ peptide antibody and indirect immunofluorescence microscopy (Fig. 8). The affinity-purified peptide antibody detected CCTβ protein in the cytoplasm and did not detect the protein in the nucleus, even at the lowest antibody dilutions. The cytoplasmic staining appeared diffuse but was higher than the apparent background staining of the nucleus. The background signal associated with the cell nucleus/nucleolus was due to reaction with the fluorescein-conjugated secondary antibodies used in the assay (Fig. 8B). Monoclonal anti-p120 antibody positively identified the nucleolus in these cells (Fig. 8C), and anti-vimentin monoclonal antibody, which signaled the cytoskeleton, was used as a cytoplasmic marker (Fig. 8D). These data confirm that CCTβ is localized primarily outside of the nucleus.

**DISCUSSION**

The existence of a second isoform of CCT opens the door to a series of experiments to determine the physiological function of CCTβ. Our data indicate that the biochemical properties of the two CCT isoforms are similar and the overexpression of either isoform is capable of perturbing PtdCho biosynthesis and metabolism. However, the distinct differences between the amino and carboxyl-terminal domains of CCTα and CCTβ indicate that the two isoforms likely have unique regulatory properties. The two isoforms are clearly the products of different genes and are differentially expressed in tissues and perhaps also during development. Because CCTα is the only isoform detected in the large volume of work in this area (1, 2), it is possible that CCTβ is expressed at lower levels or in a specific developmental setting compared with the more widely distributed CCTα.

Previous data may have to be reinterpreted in light of the existence of CCTβ. For example, data on whole tissue CCT activity and distribution will need to be reevaluated. The Northern blots cannot be used to predict the relative levels of CCTα and CCTβ proteins in particular tissues. Total CCT specific activity is relatively low in most tissues, illustrating that neither isoform is expressed at high levels. The properties of CCTβ when expressed in vivo and assayed in vitro suggest that there are no significant differences in biochemical characteristics that could distinguish between the two isoforms in crude extracts. The general impression is that CCTβ may be more restrictive in its expression compared with CCTα because CCTα has been the only mammalian isoform cloned to date. Nonetheless, we think that these data will be important to gather because some of the controversial issues in the CCT field may be explained by the presence of two isoforms.

The CCTα anti-peptide antibody used previously to identify...
its nuclear localization (7, 8) does not cross-react with CCTβ (Figs. 5 and 6). Houweling et al. (24) report that CCT is both a nuclear and cytoplasmic protein in primary hepatocytes using a peptide antibody raised against amino acids 164–176 of CCTa. This sequence in the 164–176 region (DFVAHD-DIPYSSA) is identical in human CCTa and CCTβ; therefore, antisera raised against this peptide would be predicted to react with both CCT isoforms. We detect both CCTα and CCTβ transcripts in rodent liver,2 and it will be very interesting to determine whether the cytoplasmic CCT detected in primary rodent hepatocytes can be attributed to the presence of CCTβ in this tissue.

The co-migration of CCTα and CCTβM on denaturing gels and their similar reliance on lipid activators for biochemical activity makes it difficult to determine whether CCTβ is a component of purified CCT preparations from mammalian sources. The regulation of CCTα is governed by phosphorylation of its carboxyl terminus at multiple sites, resulting in at least two species that migrate more slowly on denaturing gels (Figs. 5 and 6 and Ref. 40). Phosphorylation interferes with the stimulatory action of lipids on CCT activity (23), and there is some correlation with membrane dissociation of CCTα in vivo (20), but the regulation by this mechanism is not an absolute on/off switch (23, 35). On the other hand, the activity of CCTβM, a protein with approximately the same molecular weight as CCTα, is lower (Fig. 7) due to posttranslational modification that is dependent on the amino terminus. The nature of the modification of CCTβ protein and its role in the regulation of PtdCho biosynthesis is currently under investigation.

Acknowledgments—We thank Pam Jackson for her expert technical assistance, Wannian Yang for the construction of pWYCT, and Chuck Rock for his comments on the research. We thank Human Genome Sciences for providing us with information on the CCT-like cDNA contained in their library.

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