CTP:phosphocholine cytidylyltransferase is a major regulator of phosphatidylcholine biosynthesis. A single isoform, CCTα, has been studied extensively and a second isoform, CCTβ, was recently identified. We identify and characterize a third cDNA, CCTβ2, that differs from CCTβ1 at the carboxyl-terminal end and is predicted to arise as a splice variant of the CCTβ gene. Like CCTα, CCTβ2 is heavily phosphorylated in vivo, in contrast to CCTβ1. CCTβ1 and CCTβ2 mRNAs were differentially expressed by the human tissues examined, whereas CCTα was more uniformly represented. Using isoform-specific antibodies, both CCTβ1 and CCTβ2 localized to the endoplasmic reticulum of cells, in contrast to CCTα which resided in the nucleus in addition to associating with the endoplasmic reticulum. CCTβ2 protein has enzymatic activity in vitro and was able to complement the temperature-sensitive cytidylyltransferase defect in CHO-58 cells, just as CCTα and CCTβ1 supporting proliferation at the nonpermissive conditions. Overexpression experiments did not reveal discrete physiological functions for the three isoforms that catalyze the same biochemical reaction; however, the differential cellular localization and tissue-specific distribution suggest that CCTβ1 and CCTβ2 may play a role that is distinct from ubiquitously expressed CCTα.

PtdCho is the major membrane phospholipid in higher eukaryotes and is also secreted by particular tissues for important extracellular tasks. For example, it is a significant component of lung surfactant, serum lipoproteins, and bile. CCT is a key regulator of PtdCho biosynthesis (1) and membrane-protein interaction is one important mechanism that governs cellular CCT activity (1, 2). Recently a second isoform, CCTβ, was discovered which is encoded by a second gene (3). CCTα and CCTβ have nearly identical amino acid sequences in the catalytic domain which extends approximately from residues 72 to 233 in both proteins, and also near identity in the membrane-interaction domain which extends approximately from residues 256 to 288. Both isoforms are dependent on interaction with phospholipids for catalytic activity (3–9), as would be predicted from the high degree of identity in the membrane-interaction domains. These domains are characterized by three 11-residue amphipathic repeats that form α-helices upon association with phospholipid regulators (10–13).

The amino terminus of CCTβ bears no resemblance to the amino terminus of CCTα and does not include a nuclear localization sequence as was identified in the CCTα protein (14, 15). CCTα has been localized predominantly in the nucleus but the physiological significance of the nuclear localization of CCTα remains unclear. CCTβ protein was localized outside the cell nucleus by indirect immunofluorescent microscopy (3). CCTβ consists of 330 amino acids, in contrast with the 367 residues of CCTα, and lacks most of the carboxyl-terminal phosphorylation domain that is found in the CCTα protein (9, 16). Phosphorylation of CCTα interferes with the lipid stimulation of enzyme activity in vitro (17) and correlates with a reduction of PtdCho biosynthesis in vivo (18–24). Despite the differences at the amino and carboxyl termini of the proteins, both CCTα and CCTβ exhibit high activity when overexpressed in COS-7 cells (3, 9, 25, 26) resulting in accumulation of cellular CDP-choline and increased radiolabeling of PtdCho (3, 27).

In this work we identify a third isoform of CCT, called CCTβ2, which is a splice variant of CCTβ. CCTβ2 encodes a 369-amino acid protein which is identical to the CCTβ1 isoform described previously from amino acids 1 to 320. However, CCTβ2 also has a carboxyl-terminal sequence that resembles the phosphorylation domain of CCTα. The existence of two distinct CCT genes and two CCTβ splice variants raises the possibility of regulation of CCT activity at the level of gene expression as well as subcellular localization and phosphorylation (3). Thus, we investigated the expression of the CCT isoforms in human tissues, determine whether CCTβ2 has a phosphorylated carboxyl-terminal domain, and whether these structural differences alter the cellular localization of or the ability of CCT isoforms to complement defective CCT activity in vivo (28).

**EXPERIMENTAL PROCEDURES**

**Materials**—Sources of supplies were: Accurate Chemical & Scientific Corp., anti-mouse protein disulfide isomerase antibody; American Radiolabel Co., Inc., phospho-l-methyl-14Ccholesterol (specific activity, 55 mCi/mmol); Amersham Pharmacia Biotech, [35S]methionine (specific activity, >1000 Ci/mmol); Life Technologies, Inc., LipofectAMINE reagent; Molecular Probes, Oregon Green™ 488; Texas Red™, Hoechst and FluoReporter™ labeling kits, Oregon Green™, and Texas Red™, concanavalin A, and wheat germ agglutinin conjugates, Prolong™ antifade kit with mounting medium; Nalge Nunc International, LabTek™. This paper is available on line at http://www.jbc.org
II Chamber Slides. Promega, restriction endonucleases and other molecular biology reagents; Invitrogen, pDNA3 vector plasmid, cDNA cycle kit, human poly(A)− RNAs; Genome Systems, Inc., cDNA clone A6852866; Research Genetics, Inc., cDNA clone A041180; Sigma, anti-FLAG M2 monoclonal antibody, CTP, phosphoethanol and buffers; Ana- litec, and multiple 3-D DNA sequencing plates. All other supplies were reagent grade or better.

**Antibodies**—Anti-CCTb rabbit polyclonal antiserum was raised against a synthetic peptide (MDAQSSAKYNRKRKE) corresponding to the first 17 amino acids of CCTb. Anti-CCTb antibody (B1 epitope) was a rabbit polyclonal antiserum raised against a peptide (MER- EHESSHSCPPL) corresponding to amino acids 27–35 of CCTb and CCTb2. The anti-CCTb antibody (B2 epitope) was a rabbit polyclonal antiserum raised against a synthetic peptide (TPDASETGTIPKSL- SNEP) corresponding to amino acids 5–22 of CCTb and CCTb2. Anti- CCTb2 antibody (B3 epitope) was a rabbit polyclonal antiserum raised against a synthetic peptide (PFPSKKAARSISISSEG) corresponding to amino acids 347–366 of CCTb. Resequencing of the CCTb2 clone identified that the correct residue at position 10 of the B3 peptide is an alanine instead of an arginine. The B1 and B2 epitope antibodies recognized both CCTb1 and CCTb2 whereas the B3 epitope antibody recognized only CCTb2. Peptides and peptide antigens were prepared by the Molecular Resource Center of St. Jude Children’s Research Hospital. The B1 and B2 antigens were prepared by coupling each peptide to agarose and addition of DNA insert; the carboxy terminus of the peptide whereas the B3 antigen was coupled at the amino terminus. Immunization of rabbits and collection of antisera was performed by Rockland, Inc., according to their standard schedule. Antiseras were purified by affinity chromatography on Affi-Gel 10 cross-linked to the peptide as described previously (3).

**Isolation of the CCTb2 cDNA and Construction of Expression Plasmids**—The EST data base was searched using the published CCTb sequence (GenBankTM/EHI Data Bank accession number AF052510). A clone from human brain was identified (GenBankTM accession number AA683266) and purchased from Genome Systems. The cDNA sequence was determined 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. A second EST clone from human testis was identified (GenBankTM accession number AI041180) and purified as described previously (3).

**RNA Analysis**—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.

**Immunoblots and Immunoprecipitation**—Cell lysates (50 μg of protein) were separated by SDS-gel electrophoresis in 12% polyacrylamide gels and transferred by electroblotting onto nitrocellulose membranes. Immunoblotting was performed by incubation of the membranes with purified anti-CCTb (1:2000 dilution), purified anti-CCTb1 (B2 epitope) (1:2000 dilution), or purified anti-CCTb2 (B3 epitope) (1:2000 dilution) as primary antibody. The Amersham Pharmacia Biotech ECL Western blotting reagents and protocol were used to identify the immunoreactive proteins. For immunoprecipitations, cells were washed twice with PBS and lysed in the culture dish with lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Triton X-100, 2% aprotinin, 5 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 50 μM sodium fluoride, 100 μM Na3VO4) for 30 min in 4 °C with gentle agitation. Cell lysates and debris were scraped from the dish and centrifuged for 10 min at 10,000 × g at 4 °C. Lysate supernatants were incubated for 1 h with 8 μg of anti-CCTb (B2 epitope) purified antibody at 4 °C and then with the protein A-Sepha-
Distribution of CCT Isoforms

rose beads pre-equilibrated in lysis buffer for 1 h at 4°C. The beads were collected and washed thoroughly. Complexes were disrupted by addition of Laemmli buffer and heated in boiling water for 3 min. Proteins were separated by SDS-gel electrophoresis and proteins were detected by autoradiography. 

Fluorescein Labeling of Antibodies—The antibodies were labeled according to the instructions provided with Molecular Probes’ FluoroReporter™ labeling kits. Briefly, 200 μl of the 1–2 mg/ml antibody in PBS was combined with 20 ml of 1 m sodium bicarbonate, pH 8.0. An appropriate amount of 5 mg/ml reactive dye solution in Me2SO was added to the mixture. The amount of dye was calculated according to the following formula: μl of dye stock solution = (mg/ml protein × 0.2 ml × MWdye/2 × 200 × MR/MWdye) Where 200 is a unit conversion factor, and MR is the molar ratio of dye to protein in the reaction mixture. The reaction was stirred in the dark for 1 h and stopped by the addition of 5.5 μl of hydroxylamine provided with the kit and additional stirring for 15 min. Labeled antibodies were purified using spin columns provided with the kit. The degree of labeling was determined by measuring protein and dye concentrations (extinction coefficients provided by Molecular Probes) in a spectrophotometer and calculating protein/dye ratio. Typical labeling reaction resulted in 5–10 molecules of dye per one bivalent antibody molecule.

Direct Immunofluorescence Experiments—BAC1.2F5 cells (32) and HeLa cells (33) were cultured as described previously. Cells were grown in 4% FCS II Chamber Slides28. The cells were fixed twice with PBS, fixed, and permeabilized. Six different fixation and permeabilization procedures were investigated to evaluate the reproducibility of staining patterns with Oregon Green™-labeled anti-CCT antibodies. The procedure of choice entailed fixation in 3.7% formaldehyde for 20 min at 25°C followed by washing with PBS and permeabilization with 0.2% Triton X-100 for 30 min at 25°C followed by the addition of 5.5 μl of hydroxylamine provided with the kit and additional stirring for 15 min. Labeled antibodies were purified using spin columns provided with the kit. The degree of labeling was determined by measuring protein and dye concentrations (extinction coefficients provided by Molecular Probes) in a spectrophotometer and calculating protein/dye ratio. Typical labeling reaction resulted in 5–10 molecules of dye per one bivalent antibody molecule.

CCT Isoform Protein Sequence Comparison—The predicted amino acid sequence of CCTβ2 was aligned with the sequences of CCTα and CCTβ1 (Fig. 2). The predicted CCTβ2 protein had 369 amino acids and was identical to CCTβ1 from amino acids 1 to 320. After residue 320 there were 39 additional amino acids, including two groups of 5 and 4 amino acids (SSPTR, residues 321–325, and RSPS, residues 328–331), respectively, which were identical to sequences in CCTα and missing from CCTβ1. The carboxyl terminus of CCTβ2 had 21 potential phosphorylation sites after position 310, including 19 serines and 2 threonine residues. As shown in Fig. 2, only 9 serines and 1 threonine of CCTβ2 align with the corresponding residues of CCTα.

Phosphorylation of CCTβ Isomorphs—The existence of 21 potential serine and threonine phosphorylation sites in the predicted carboxyl-terminal domain of CCTβ2 suggested that this enzyme was phosphorylated similarly to the modification of CCTα protein. This point was tested by transfecting COS-7 cells with CCTβ1, CCTβ2, or a vector control and followed labeling 48 h later with [32P]orthophosphate (160 μCi/ml) for 60 min. Both CCTβ isomorphs were immunoprecipitated with the amino-terminal anti-CCTβ antibody (B2 epitope), fractionated by SDS-PAGE, and the radiolabeled proteins were visualized by autoradiography. CCTβ2 was highly phosphorylated (Fig. 3) confirming the prediction made from the analysis of the primary structure of its carboxyl terminus. CCTβ1 was also phosphorylated, although to a significantly lesser extent as was predicted from the fact that CCTβ1 had only 3 potential phosphorylation sites after amino acid 310. These data are consistent with the idea that the carboxyl-terminal domains of CCTβ1 and CCTβ2 were the exclusive sites of phosphorylation, as was shown with CCTα (9, 16).

Expression and Amino-terminal Modification of CCTβ Isoforms—In our previous report (3) describing CCTβ1 we suggested that the CCTβ1 protein was modified when expressed in COS-7 cells. Two immunoreactive proteins with apparent molecular masses of approximately 40 or 35 kDa were identified using anti-CCTβ (epitope B1) following transfection of COS-7 cells with CCTβ1 cDNA. The faster migrating protein species co-migrated with the major [35S]methionine-labeled product of an in vitro transcription/translation reaction using the CCTβ1 cDNA as template. We proposed that the slower migrating CCTβ1 form identified in COS-7 cells may result from post-translational modification. We therefore attempted to obtain evidence of possible glycosylation, acylation or ubiquitination of the CCTβ1 protein, however, our efforts to identify the biochemical nature of the putative modification were unsuccessful. Experiments with a different lot of the commercial preparation of reticulocyte lysate also yielded two CCTβ1 translation products in vitro (Fig. 4), rather than the single product that
was originally described (3), and addition of microsomes to the lysate did not alter the relative amounts of the two radiolabeled protein products. Therefore, we tested the hypothesis that the faster migrating protein produced in the in vitro transcription at translation originated from an alternative translational initiation at methionine 27, since this second predicted methionine in the CCT\textsubscript{b} open reading frame was within a favorable Kozak consensus context (34). Methionine 27 was changed to alanine by mutagenesis of the CCT\textsubscript{b} cDNA and the derived expression construct, plasmid pPJ82, was used as a template for in vitro expression. CCT\textsubscript{b}(M27A) protein migrated at the same position as the "modified" CCT\textsubscript{b} expressed in cells (Fig. 4). These data indicated that the initiation site in vivo was the first methionine in the open reading frame and also identified the correct mobility in SDS-PAGE for the full-length protein. On the other hand, expression of CCT\textsubscript{b}[\text{D}1–26] in which the first 26 amino acids were deleted from the amino terminus yielded a protein that co-migrated with the "unmodified" or faster-migrating CCT\textsubscript{b} expressed in cells (Fig. 4), and the major band produced by the in vitro transcription/translation system. These data showed the correct mobility for a protein that was 26 amino acids smaller corresponding to a CCT\textsubscript{b} protein initiating at methionine 27 in the open reading frame. Thus, CCT\textsubscript{b} was not post-translationally modified at the amino terminus when expressed in a cellular context and the differences in the in vivo and in vitro results was due to the artificial initiation at an alternative methionine in the in vitro experiments.

**Tissue-specific Expression of CCT Isoforms**—The tissue distribution and indication of the relative abundance of the CCT mRNAs was addressed by RT-PCR in a series of human tissues. The forward primer for the detection of both CCT\textsubscript{b} isoforms was complementary to sequence within the 5' coding region of CCT\textsubscript{b} and a sequence-specific reverse primer corresponded to the 3' ends of each of the two coding sequences for CCT\textsubscript{b}1 and CCT\textsubscript{b}2. The anticipated size of the CCT\textsubscript{b}1 product was 256 bp whereas the CCT\textsubscript{b}2 product was predicted to be 586 bp. The CCT\textsubscript{a} primers were predicted to yield a PCR product 345 bp long. We incubated the CCT\textsubscript{b} primers with the CCT\textsubscript{a} purified cDNA as template, and conversely, the CCT\textsubscript{a} primers were incubated with the CCT\textsubscript{b}2 purified cDNA as template to verify the specificity of the primers under the thermocycling conditions. In both cases no DNA products were detectable (data not shown).

The data indicated that CCT\textsubscript{a} was expressed in all tissues approximately at the same levels (Fig. 5). In contrast, the expression of the CCT\textsubscript{b} isoforms differed among the tissues tested. Both isoforms of CCT\textsubscript{b} were expressed in brain, with CCT\textsubscript{b}2 being predominant. Liver also expressed both isoforms, with CCT\textsubscript{b}2 purified cDNA as template and, conversely, the CCT\textsubscript{a} primers were incubated with the CCT\textsubscript{b}2 purified cDNA as template to verify the specificity of the primers under the thermocycling conditions. In both cases no DNA products were detectable (data not shown).

The data indicated that CCT\textsubscript{a} was expressed in all tissues approximately at the same levels (Fig. 5). In contrast, the expression of the CCT\textsubscript{b} isoforms differed among the tissues tested. Both isoforms of CCT\textsubscript{b} were expressed in brain, with CCT\textsubscript{b}2 being predominant. Liver also expressed both isoforms, with CCT\textsubscript{b}2 giving a stronger signal. Placental tissue contained CCT\textsubscript{b}1 transcripts with no detectable signal for CCT\textsubscript{b}2. On the other hand, CCT\textsubscript{b}2 was the predominant isoform expressed in HeLa cells whereas lower amounts of CCT\textsubscript{b}1 were detected. An interesting variation in the development of lung tissue was suggested in that CCT\textsubscript{a} and both CCT\textsubscript{b} isoforms were expressed in fetal lung whereas mRNA from adult lung

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**Fig. 1. Comparison of the cDNA sequences of CCT\textsubscript{b}2, CCT\textsubscript{b}1, and CCT\textsubscript{a}.** The human CCT\textsubscript{b}2 cDNA sequence determined in this paper (GenBank\textsuperscript{TM} accession number AF148464) was compared with the published cDNA sequences of human CCT\textsubscript{b}1 (GenBank\textsuperscript{TM} accession number AF052510) or human CCT\textsubscript{a} (GenBank\textsuperscript{TM} accession number L28957). Identical bases are boxed.
did not yield a signal for CCTβ and only CCTα was expressed. These data are consistent with the results from Post's lab (35) where cDNAs encoding only CCTα were cloned from an adult lung library.

Subcellular Localization of CCTβ Isoforms—
We have developed two new antibodies, anti-CCTβ (B2 epitope) and anti-

CCTα overexpressed in CHO58 cells was found to be localized mainly in the cell nucleus using a specific peptide antibody directed against the amino terminus of the protein (15, 36). On the other hand, CCTα was also found to be both a nuclear and cytoplasmic protein in primary hepatocytes using an antibody
that recognized the membrane interaction domain of CCTα and that could potentially cross-react with CCTβ (37). In our previous report (3) we showed that CCTβ1 was an extranuclear protein using a specific anti-CCTβ amino-terminal antibody (B1 epitope). The above studies utilized conventional immunofluorescent microscopy to visualize the CCT proteins. In the present report, we used confocal microscopy to investigate the cellular localization of the β isoforms in more detail. Confocal microscopy was advantageous because it detected proteins at the same focal plane, thoroughly increasing the resolution of cellular structures compared with previously used techniques. We also coupled the fluorescent dyes directly to the affinity-purified primary antibodies at a high molar ratio (5–10 mol of dye/mol of bivalent antibody), thus increasing the sensitivity of detection of endogenously expressed protein. We compared the distribution of CCTβ2 in cells with the distribution of the two other CCT isoforms, CCTα and CCTβ1, using direct immunofluorescence microscopy with confocal imaging and affinity-purified isoform-specific antibodies.

All of the CCT antipeptide antibodies used were raised in rabbits and direct coupling of different dyes to the antibodies also allowed the co-visualization of the CCT isoforms in the same in situ context. Fixation and permeabilization conditions were optimized as described under “Experimental Procedures” and the conditions (3.7% formaldehyde, 0.2% Triton) were chosen on the basis of reproducibility, consistency with the other methods, and preservation of morphology. An antibody dilution series was performed following each coupling reaction and cellular fluorescence patterns were recorded using antibody preparations at as high a dilution as possible to minimize possible nonspecific detection of unrelated proteins. The specificity of the fluorescent signal in cells was confirmed by preincubation of the antibodies with the corresponding peptide epitopes (Fig. 7). Multiple CCTβ antibodies (B1, B2, and B3 epitopes) were used to confirm the results for CCTβ. The CCTα proteins from both human and rodent species are known to be identical at the amino terminus (38–41) and the mouse and human CCTβ proteins are also identical at the amino terminus.2

The CCTα and -β isoforms were visualized in several different cell types, including BAC1.2F5 murine macrophage cells, HeLa human carcinoma cells, and the CHO58 hamster ovary cells (Fig. 8A). In all three cell lines, CCTα protein was largely found in the nucleus but a significant signal was also detected outside of the nucleus. The extranuclear CCTα co-localized with concanavalin A (Fig. 8B), an agglutinin with a high affinity for mannose residues and a marker for the ER (42), as determined by computer-mediated overlay of the two distinct fluorescent images. Since there was only one antibody specific for CCTα, a cDNA encoding an FLAG epitope-tagged CCTα was also transfected into CHO58 cells and localized with anti-FLAG antibody (M2 antibody) to confirm results obtained with the anti-CCTα antibody and ensure that an unrelated protein did not possess the same peptide epitope (Fig. 8C). CCTβ2 protein was also found in the three cell lines and was situated outside of the nucleus (Fig. 8A). Antibodies that recognized both CCTβ isoforms (B1 and B2 epitopes) and those that were specific for CCTβ2 co-localized not only with concanavalin A but also with anti-protein disulfide isomerase (Fig. 9), another marker protein for the ER (43). Antibodies for CCTβ and CCTβ2 also co-localized with each other (Fig. 10). Neither the CCTα nor CCTβ antibodies associated to a high degree with the Golgi bodies as determined by co-staining with fluorescently tagged wheat germ agglutinin (data not shown), a marker for the Golgi (44). These data support the conclusion that CCTα was found both in the nucleus and associated with the ER and that the CCTβ isoforms were associated with the ER. Direct evidence of CCTβ2 expression and ER association was obtained with this approach but the specific occurrence of CCTβ1 in CHO58 cells could not be determined with these immunological reagents.

Overexpression of CCTβ2 in COS-7 Cells—The similarities among amino acid sequences of CCTβ2, CCTβ1, and CCTα suggested that CCTβ2 would also exhibit CCT enzyme activity. Transfection of COS-7 cells with plasmid pAL2 containing the CCTβ2 cDNA resulted in significantly increased CCT enzyme specific activity (Fig. 10), from 2.5 to 27 nmol/min/mg, in the crude cell lysates. Overexpression of CCTβ2 activity also resulted in an increased incorporation of [methyl-3H]choline into cellular CDP-choline, PtdCho, and glycerophosphocholine (data not shown) comparable to the levels of radioactive metabolites following overexpression of CCTβ1 in COS-7 cells (3).

CCTβ Rescued CHO58 Cells—The CHO58 cell line is conditionally defective for CCT activity (28) and cannot synthesize sufficient PtdCho to support growth at 40 °C. Transfection of CHO58 cells with a CCTα cDNA complements the defective CCT activity and the overexpression of the α isoform supported the proliferation of CHO58 cell colonies after shifting the cultures to the restrictive temperature (45). The differences in primary structure and subcellular localization between CCTα and CCTβ2 raised a question as to whether CCTβ2 had a cellular function similar to that of CCTα. To address this issue, CHO58 cells were transiently transfected with cDNAs encoding CCTβ1, CCTβ2, and CCTα as a positive control, or vector alone as a negative control (Fig. 11). After 72 h at 40 °C, 7 colonies remained, in the control dishes transfected with vector alone, indicating that reversion of the background genetic phenotype did not occur under these experimental conditions. In contrast, the dishes transfected with CCTα, CCTβ1, or CCTβ2 cDNAs, hundreds of colonies were evident, indicating that overexpression of any of the CCT isoforms could complement the CCT defect in the CHO58 cells. These data suggest that CCTα, CCTβ1, and CCTβ2 perform equivalent biochemical functions.

\[\text{M. Kavim and S. Jackowski, unpublished data.}\]
DISCUSSION

A major finding of this study is the identification of the CCTα cDNA and the characterization of the protein. CCTα, CCTβ1, and CCTβ2 have very similar catalytic and amphipathic helical domains consistent with their stimulation by lipid regulators (1–3). Also, both the α (6) and β isoforms are inhibited by antineoplastic phospholipids as would be predicted from the similarity of their primary sequences and the metabolic redistribution of the PtdCho precursors in drug-treated cells that express all isoforms (33). CCTβ1 and CCTβ2 likely arise from alternate splicing of the CCTβ mRNA which results in the production of two mRNAs that encode proteins that differ only at their carboxyl terminus. CCTβ1 is a protein of 330 amino acids whereas CCTβ2 has 369 amino acids. The additional 39 carboxyl-terminal residues in CCTβ2 closely resembles the carboxyl-terminal phosphorylation domain of CCTα. Within this domain, CCTβ2 has 22 potential phosphorylation sites (19 serines and 2 threonines) compared with the 13 serine residues known to be phosphorylated in the carboxyl-terminal domain of CCTα (16). Accordingly, CCTβ2, like CCTα, is extensively phosphorylated in vivo. CCTβ1 lacks the numerous phosphorylation sites present in CCTα and CCTβ2 and is phosphorylated to a minor extent in vivo indicating that CCTβ1 may not be subject to regulation by protein kinases. After the splice junction at amino acid 323, CCTβ2 has two regions of five (SSPTR) and four (RSPS) residues identical to sequences known to be phosphorylated in CCTα (16). Also, CCTβ2 contains a unique sequence, SSPTRSRSPSRSP, containing the RSPXR motif similar to the one found in neurofilament H (KSPXK) that specifies phosphorylation by cyclin-dependent kinase 5 (46). Phosphorylation attenuates CCTα biochemical activity by interfering with lipid stimulation (17) and unphosphorylated CCTα exhibits a higher degree of membrane association in cells (24). Phosphorylation is predicted to exert the same regulatory influence on CCTβ2 as on CCTα.

CCTβ1 was proposed to be post-translationally modified following overexpression in COS-7 cells, resulting in slower migration during SDS-PAGE (3). Truncation of the amino-terminal 26 amino acids resulted in a protein that co-migrated slightly faster than the full-length product of in vitro transcription/translation of the CCTβ1 cDNA (3). Examination of new data that was obtained during comparison with the most recently discovered isoform, CCTβ2, revealed that the faster migrating product of the in vitro transcription/translation was an artifact where translation was initiating at Met-27. CCTβ1 and CCTβ2 proteins initiated at Met-1 when expressed in vivo as demonstrated by interaction with the antibody specific for residues 5 through 22 (B2 epitope) and confirmed by co-migration with the M27A mutant (Fig. 4).

CCTβ1 and CCTβ2 are distinguished from CCTα by their selective localization to the ER suggesting that the β isoform plays a special role in PtdCho metabolism in the ER compartment. Our use of laser-scanning confocal microscopy and direct labeling of the primary antibodies with fluorescent tags resulted in improved resolution of cellular structures and a more sensitive detection of endogenous CCT isoforms than in previous studies. Computer overlays of the immunofluorescent images confirm that both CCTβ isoforms colocalize with each other and with ER-specific markers. Although, significant amounts of CCTα are found distributed throughout the interphase nucleus, except the nucleolus, CCTα also colocalizes with ER-specific markers. These results are in general agreement with the available information from other laboratories. An investigation of the cellular distribution of CCTα in hepatocytes using immunoelectron microscopy and an antibody that was potentially cross-reactive with CCTβ localized CCTα to both the nuclear and extranuclear compartments (37). CCTα
FIG. 8. **CCTα and CCTβ isoforms in three cell types.** A, murine macrophage (BAC1.2F5) cells, human carcinoma (HeLa) cells, or Chinese hamster ovary fibroblast (CHO) cells were stained with Oregon Green™-labeled anti-CCTα (three upper panels) or Texas Red™-labeled anti-CCTβ2 (B3 epitope) antibodies (three lower panels). Images were acquired with the ×63 objective and digitally zoomed. B, BAC1.2F5 cells were co-stained with Oregon Green™-labeled anti-CCTα antibodies and Texas Red™-labeled concanavalin A. The images were acquired with the ×63 objective and digitally zoomed to bring the single cell into the field of view. Two image files were obtained with different filter sets and the files were overlaid by computer (third panel). The orange color indicates co-localization. C, CHO cells were transfected with cDNA encoding FLAG-CCTα and co-stained with Texas Red™-labeled anti-FLAG antibody and Oregon Green™-labeled anti-CCTα antibody. Images were acquired with the ×63 objective and digitally zoomed. The overlay of the two image files obtained with different filter sets was computer-mediated (third panel). The orange color indicates region of co-localization.
was assigned to both the cytoplasm and the ER, however, membranous structures were not distinct in the images used as evidence for cytoplasmic localization, probably due to the limitations of the fixation procedure. It is clear from our images (Fig. 8) that there is very little extranuclear CCTa that is not associated with the ER. Indirect immunofluorescence employed by the Kent group (15, 21, 22, 36, 47) showed CCTa to predominantly reside in the nucleus. Although there is an indication of

Fig. 9. CCTβ2 associates with the ER. BAC1.2F5 cells were co-stained with Texas Red™-labeled anti-CCTβ (B2 epitope), and Oregon Green™-labeled concanavalin A (first row); co-stained with Texas Red™-labeled anti-CCTβ (B2 epitope) antibodies and Oregon Green™-labeled anti-mouse protein disulfide isomerase antibodies (second row); or Texas Red™-labeled anti-CCTβ2 (B3 epitope) antibodies and Oregon Green™-labeled anti-CCTβ (B2 epitope) antibodies (third row). The images were acquired with the ×63 objective and digitally zoomed to bring the single cell into the field of view. The computer overlays of the image files are shown in the far left column of each row.

Fig. 10. CCTβ2 expression in COS-7 cells. CCT specific activity was determined in COS-7 cells transfected with plasmids expressing CCTβ2 (pAL2) (●) or an empty vector control (pcDNA3) (○). The cells were harvested, extracts were prepared and assayed for CCT activity 48 h after transfection as described under “Experimental Procedures.” The results are representative of duplicate experiments.

Fig. 11. CCTβ1 and CCTβ2 can rescue CHO58 cells. Cells grown at 33 °C were transfected with cDNAs encoding CCTa, CCTβ1, CCTβ2, or pcDNA3 vector alone. Cells were transferred to 40 °C 24 h after transfection and cultured at 40 °C for an additional 72 h. Dishes were then washed twice with phosphate-buffered saline and adherent colonies were stained with Coomassie Blue R-250 as described under “Experimental Procedures.”
cytoplasmic fluorescence in some of their images, the bright nuclei coupled with the inability of this technique to examine thin sections through the cells may have obscured extranuclear CCT. This may be particularly relevant in their experiments using CHO58 cells to localize overexpressed CCTα (15, 36, 40) since it is possible that the number of CCT sites on the ER is limiting and that supraphysiological concentrations of CCTα accumulate in the nucleus. Nuclear CCTα is the likely source of the soluble CCT pool defined by subcellular fractionation and digitonin permeabilization experiments (1) since the remaining CCT staining in cells is associated with membrane systems. CCT is primarily regulated by the membrane lipid environment (1, 2) and localization of CCT to the ER places the enzyme in a prime position to respond to changes in the bulk membrane environment to maintain homeostasis.

All CCT isoforms have the same biochemical function and accelerate PtdCho synthesis when overexpressed in cells (Fig. 10) (3, 26). Also, CCTα, CCTβ1, or CCTβ2 can supply the enzymatic activity necessary to support the growth of a cell line conditionally defective in CCT activity. We detected both CCTα and CCTβ proteins in the mutant CHO58 cell line (Fig. 8A) indicating that both proteins may be conditionally defective to obtain the temperature-sensitive defect in PtdCho biosynthesis (28). The ability of either CCTα or CCTβ to complement the temperature-sensitive phenotype suggests that reversion at either the CCTα or CCTβ genetic locus could give rise to a temperature-resistant derivative cell line (48). However, any conclusions reached on the basis of expression studies in CHO58 cells must be tempered by the understanding that overexpression studies are a very blunt experimental tool to address the functionality of isoforms or mutants. Catalytically compromised or mislocalized proteins can complement mutant phenotypes if the defective proteins are expressed at a high enough level. Supraphysiological concentrations of CCT swamp the cells with CDP-Chol and trigger a compensatory response to the overproduction of PtdCho by enhancing degradation (3, 26, 27). Thus, enforced CCTβ may complement a CCTα function(s) by swamping the cells with CDP-Chol. Also, CCTα proteins defective in catalytic activity, regulatory function, or cellular localization may complement the CHO58 cells when overexpressed, whereas they may not be able to sustain growth if present as a single copy. The definition of the specific functions of the CCT isoforms and the importance of nuclear versus ER localization await more detailed genetic experiments.

A specific cellular function for CCTβ remains speculative, although analysis of the tissue-specific distribution of CCTβ isoforms does suggest some hypotheses. CCTβ2 could play an important role in neuronal development and function since the brain has the highest levels of CCTβ2 expression (Fig. 5) and PtdCho biosynthesis is critical to axons (49). The finding of CCTβ associated with the ER suggests that this isoform may be involved in tissues that secrete PtdCho. For example, CCTβ1 is highly expressed in placenta (Fig. 5) and may play a role in PtdCho biosynthesis in this lipidic tissue which secretes and supplies phospholipid to a developing embryo (50). Liver and fetal lung also express both CCTβ isoforms, but CCTβ1 is predominant (Fig. 5). The absence of CCTβ expression in adult lung (Fig. 5) does not fit with this hypothesis since PtdCho biosynthesis plays an important role in surfactant secretion and pulmonary function. A precise determination of a function for CCTβ will require the analysis of genetically engineered animals that do not express this isoform.

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