Cytidylyltransferase-binding Protein Is Identical to Transcytosis-associated Protein (TAP/p115) and Enhances the Lipid Activation of Cytidylyltransferase*

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We previously identified a protein from rat liver that binds CTP:phosphocholine cytidylyltransferase (CT). We have now purified this protein (cytidylyltransferase-binding protein (CTBP)) from rat liver. The purification involved precipitation at pH 5 and extraction of the precipitate with buffer, followed by sequential chromatography on DEAE-Sephrose and butyl-agarose. Final purification was accomplished by either preparative electrophoresis or hydroxylapatite chromatography. Amino acid sequences from six peptides derived from pure CTBP matched sequences in transcytosis-associated protein (TAP) with 98% identity. Thus, CTBP was positively identified to be TAP. Purified CTBP increased the activity of purified CT measured with phosphatidylcholine (PC)/oleic acid. In the absence of PC/oleic acid, CTBP did not stimulate CT activity. Dilution of CT to reduce the Triton X-100 concentration produced a loss of CT activity. The lost activity was recovered by the addition of CTBP plus PC/oleic acid to the assay, but not by the addition of either PC/oleic acid or CTBP alone. Removal of CTBP from purified preparations by immunoprecipitation with CTBP antibodies eliminated the activation of CT. Both CT and CTBP were shown to bind to PC/oleic acid liposomes. The formation of complexes between CT and CTBP in the absence of PC/oleic acid liposomes could not be demonstrated. These results suggest that CTBP functions to modify the interaction of CT with PC/oleic acid liposomes, resulting in an increase in the catalytic activity perhaps by the formation of a ternary complex between CT, CTBP, and lipid. Overall, these results suggest that CTBP (TAP) may function to coordinate the biosynthesis of phosphatidylcholine with vesicle transport.

CTP:phosphocholine cytidylyltransferase (CT)† is a major regulatory enzyme in the biosynthesis of phosphatidylcholine. CT exists intracellularly in inactive and active forms. Regulation of CT involves interconversion of these forms. There is abundant evidence indicating that the active form of CT is associated with membranes (reviewed in Refs. 1–3). A variety of mechanisms have been suggested to explain the activation of CT in response to a variety of stimulatory treatments. These include changes in the state of CT phosphorylation (4–8), changes in the content of phosphatidylcholine in membranes (9–11), alterations in the physical form of phospholipid at specific membrane domains (12), and the promotion of CT binding and activation by specific lipids (6, 13–16). The involvement of other cellular proteins in the overall regulation of CT is an additional possibility that has not been fully explored. The identification of a 110-kDa protein that binds CT (17) led us to consider in more detail the potential involvement of this protein in the regulation of CT. We subsequently found that complexes between CT and the cytidylyltransferase-binding protein (CTBP) were formed by incubation of liver cytosol with oleic acid (18). Further insight into the function of this protein required the isolation of purified protein.

We have now purified CTBP from rat liver cytosol. Amino acid sequences from six peptides derived from pure CTBP matched sequences in transcytosis-associated protein (TAP) from rat liver (19). Studies on the effects of purified CTBP on the activity of CT suggested that CTBP enhances the activation of CT by lipids.

EXPERIMENTAL PROCEDURES

Materials—We obtained [methyl-14C]phosphocholine from American Radiolabeled Corp. Protogel (30% acrylamide and 0.8% bisacrylamide) purchased from National Diagnostics, Inc. was used to prepare polyacrylamide gels. The ECL reagent was purchased from Amersham Corp. Polyvinylidene difluoride membrane and a minipreparative electrophoresis cell were purchased from Bio-Rad. Phosphorylase (Bio-Gel HTP) was from Bio-Rad. Pefabloc was from Boehringer Mannheim. Phosphatidylcholine, oleic acid, sodium oleate, CTP, phosphatidylcholine, protease inhibitors, and all other biochemicals were from Sigma.

Antiserum (N-1) raised against a synthetic peptide corresponding to the N-terminal sequence of CT was provided by Dr. Claudia Kent (University of Michigan) (20). The production and preparation of antisera (AS-II) that detects CTBP were described previously (17).

Cytidylyltransferase Purification and Assay—Cultures of Sf9 insect cells infected with baculovirus cloning vector carrying the full-length coding sequence for CT (21) were provided by Dr. Claudia Kent and Joel Clement (University of Michigan). We purified cytidylyltransferase from baculovirus-infected Sf9 cells using the method previously reported for rat liver (21–23). Cytidylyltransferase activity was determined as described previously (24). Assays contained 1.6 mM [14C]phosphocholine (1000 dpm/nmol), 6 mM CTP (adjusted to pH 7.0), 24 mM magnesium acetate, 2 mM EDTA, and 50 mM imidazole, pH 7.0. All assays contained 100 μM phosphatidylcholine/oleic acid (1:1 molar ratio) unless otherwise indicated.

SDS Slab Gel Electrophoresis—SDS-polyacrylamide gel electrophoresis was performed on 10% polyacrylamide slab gels (12 cm × 16 cm × 1.5 mm) using a Bio-Rad Protein II system as described previously (17). The samples contained 2% SDS and 5% mercaptoethanol and were boiled for 5 min.

Protein Staining of Slab Gels—Silver stain analysis of SDS-poly-
acrylamide gels was performed using the method of DeMoreno et al. (25). Molecular mass standards (Sigma M-5505) were used to calibrate the gels. SDS-polyacrylamide slab gels were stained overnight using 0.1% Amido Black prepared in 30% methanol and 1.0% acetic acid. The gel was destained with 20% methanol and 80% acetic acid.

**RESULTS**—We detected CTBP immunologically on either Western blots or slot blots. Proteins separated by SDS gel electrophoresis were transblotted onto polyvinylidene difluoride membranes as described previously (17). For slot blots, 10–50-μl aliquots were diluted to 200 μl with TBS (25 mM Tris, pH 7.5, and 500 mM NaCl) and applied to nitrocellulose membranes using a microfiltration manifold (Bio-Rad). The detection of CTBP in gels and sucrose gradients was done by direct application of 5–10 μl to nitrocellulose membranes. The dried membrane was submerged briefly in 10% trichloroacetic acid, washed with TBS, and dried.

Membranes were blocked with 5% nonfat milk in TBS for 3 h at room temperature. The blot was probed overnight at 4 °C with N-1 or AS-II antiserum diluted in TBS containing 3% nonfat milk, 0.1% bovine serum albumin, and 0.1% Tween 20. For all subsequent procedures, we used glass roller bottles in a Technne hybridizer oven. The blot was washed four times with 100 μl of TBS containing 0.1% Tween 20. We probed the blot with goat anti-rabbit IgG conjugated to horseradish peroxidase (1:10,000 dilution) in TBS containing 3% nonfat milk, 0.1% bovine serum albumin, and 0.1% Tween 20 for 2 h at room temperature. We washed the blot three times with 100 μl of TBS containing 0.3% Tween 20 and three times with 100 μl of TBS containing 0.1% Tween 20. Immunoreactive bands were detected by chemiluminescence using the ECL reagent. We measured the relative optical densities and areas of the bands on a microcomputer imaging device (Imaging Research Inc., Ontario, Canada). We defined a scan density unit as the relative optical density multiplied by the area of the band.

**Purification of CTBP**—Male rats (200–250 g) were anesthetized with 1.0 M acetic acid. We diluted the cytosol to 8 mg/ml protein with Buffer A. The pH of the cytosol was reduced to 5.0 by the addition of 1.0 M acetic acid. We stirred the mixture on ice for 20 min and centrifuged the suspension at 12,500 × g for 20 min. We extracted the precipitate in a volume of Buffer A equal to the volume of the original cytosol. After stirring at 4 °C for 20 min, we centrifuged the mixture at 17,000 × g for 20 min to recover the soluble extract. Under these conditions, nearly all of the CTBP was solubilized along with ~35% of the CT activity.

A DEAE-Sepharose column (2.2 × 12 cm) was packed and equilibrated with Buffer A at a flow rate of 90 ml/h. The extract from the pH 5.0 fraction (350 μg of protein in 320 ml) was applied to the column at a flow rate of 45 ml/h. The column was washed with 250 ml of Buffer A. The column was eluted with a 500-ml gradient of NaCl (150–400 mM) in Buffer A containing 62 mM Tris, pH 6.8. The final concentrated pool contained 110 kDa protein, but did not contain the 95-kDa protein that was present in the native electrophoresis preparation. The CTBP preparation did not contain CT activity or CT detected by CTBP antiserum, suggesting that it was not a product of proteolytic digestion of CTBP.

**Purity of CTBP**—The purity of the final preparation was examined by SDS-polyacrylamide gel electrophoresis (Fig. 1). The major protein component migrated to a position just beyond the 110-kDa protein standard, corresponding to a molecular mass of 110 kDa. This protein band was detected by CTBP antiserum (AS-II) on a Western blot. There was a small amount of a 95-kDa protein contaminant. The 95-kDa protein was not detected by CTBP antiserum, suggesting that it was not a product of proteolytic digestion of CTBP...

**Measurement of CTBP**—...
slab gel. After electrophoresis, we detected the band of CTBP by staining the gel with Amido Black. We then excised the band with a scalpel. We submitted the gel segment to the Protein Sequencing Facility at the University of Michigan. Peptide fragments were prepared by cyanogen bromide cleavage. Amino acid sequence analysis was performed on six different peptides.

As shown in Fig. 4, the sequences from each peptide (18–31 amino acids) generated from CTBP matched regions in the sequence of TAP from rat liver (19). The six peptide sequences of CTBP contained a total of 164 amino acids, and 161 amino acids were identical to the corresponding region in TAP. The calculated molecular mass of TAP was 107,161 Da (19). This value closely matches the molecular mass of CTBP (110 kDa) obtained by SDS gel electrophoresis. Barroso et al. (19) found that TAP was 92% identical to a vesicular transport protein (p115) from bovine brain (28).

**Effects of CTBP on CT Activity**—In these experiments, we used purified CT. We store purified CT at −40 °C in buffer containing 50 mM Tris, 200 mM ammonium phosphate, 150 mM NaCl, 2 mM EDTA, 2 mM DTT, 0.025% sodium azide, and 0.03–0.06% Triton X-100, pH 7.5. Under these conditions, CT is stable, and the activity remains constant for many months. Since purified CT contained high activity, it was necessary to dilute the preparation for routine experimental use. We diluted purified CT 1:40 either in Buffer C (50 mM Tris, 200 mM ammonium phosphate, 150 mM NaCl, 2 mM EDTA, and 0.025% sodium azide, pH 7.5) or in Buffer C containing 0.06% Triton X-100 resulted in a 70–80% decrease in CT activity, measured with optimal concentrations of PC/oleic acid. The addition of purified CTBP to the assay produced an increase in CT activity (Fig. 5A). Thus, the addition of CTBP resulted in the recovery of the CT activity that was lost by the dilution. Dilution of CT with Buffer C containing 0.06% Triton X-100 did not produce a decrease in CT activity (Fig. 5B). Under these conditions, CTBP increased CT activity to values that were nearly 70% higher than in the original undiluted CT preparation. Half-maximal activation was obtained with 10 ng of CTBP. Maximal stimulation of CT activity, in the presence of 100 μM PC/oleic acid, was obtained at an ~1:1 molar ratio of CTBP to CT, assuming that both CT and CTBP were present as homodimers. CT activity after dilution in the presence or absence of Triton X-100 was not increased by ovalbumin, thyroglobulin, or bovine serum albumin (Fig. 5, A and B). Thus, the effect was specific for CTBP. CTBP produced a similar increase in CT activity in the original undiluted preparation (Fig. 5C). These assays contained 16 times more CT than the dilution experiments. Maximal activation was obtained with ~16 times more CTBP. Thus, the molar ratios of CT to CTBP required for optimal activation were similar to those for diluted CT.

The results shown in Fig. 5 were obtained with CTBP purified by hydroxylapatite chromatography. Similar results were obtained using CTBP purified by native polyacrylamide electrophoresis. Each preparation contained >80% CTBP. How-
ever, the population of contaminating proteins was different in the two preparations. The similarity in the results obtained with the two preparations suggested that the contaminating protein(s) were not responsible for the stimulatory activity. To obtain direct evidence that CTBP was responsible for the activation of CT, we specifically removed CTBP from the preparation with CTBP antibodies. Two separate approaches were used. In the first, we used protein A-Sepharose to isolate IgG fractions from CTBP antiserum and preimmune serum. IgG recovered from each was coupled to Affi-Gel 10 (Bio-Rad). Treatment of CTBP preparations with Affi-Gel containing CTBP antibodies removed CTBP (determined by dot-blot assay). The immunodepleted preparation produced an 8% increase in CT activity. Treatment of the CTBP preparation with Affi-Gel containing preimmune IgG did not remove CTBP. The treated CTBP preparation retained the ability to activate CT (45% increase in CT activity). In the second set of experiments, CTBP (150 ng) was incubated with protein A-Sepharose containing IgG from either CTBP antiserum or preimmune antiserum. A third incubation contained buffer in place of the protein A-Sepharose. After removal of the Sepharose by centrifugation, an aliquot of the supernatant was used to activate diluted CT. CT activity was increased by 30.5% with the untreated CTBP preparation. The preparation treated with protein A-Sepharose containing preimmune IgG produced a 29.9 ± 2.2% increase in CT activity compared with an 8.8 ± 3.4% increase with the preparation treated with protein A-Sepharose containing CTBP IgG. Western blot analysis of the supernatants indicated that protein A-Sepharose containing CTBP IgG removed CTBP. Protein A-Sepharose containing preimmune IgG did not remove CTBP. Thus, CTBP IgG specifically adsorbs the activating protein in preparations of purified CTBP. Taken together, these results indicated that CTBP/TAP specifically activates CT.

The activation of CT by CTBP was dependent upon the presence of PC/oleic acid in the assay (Fig. 6A). CTBP did not increase CT activity in the absence of PC/oleic acid. In the presence of PC/oleic acid, CT activity was activated by CTBP to nearly 80% of the activity in the undiluted preparation (Fig. 6A). This was nearly a 4-fold increase in activity over the activity obtained with PC/oleic acid alone. When we diluted CT in the presence of 0.06% Triton X-100, ~80% of the activity was recovered with assays containing PC/oleic acid (Fig. 6B). The addition of CTBP to the assays increased CT activity to values nearly 50% higher than in the original undiluted preparation. This is consistent with the results shown in Fig. 5B. CTBP alone did not increase CT activity in the presence of Triton X-100. The PC/oleic acid dependence for CT activity and for CTBP stimulation was similar for undiluted CT and for CT diluted with Triton X-100 in the dilution buffer (Fig. 6, compare C with B).

**CT and CTBP Bind to Liposomes**—Since CTBP activated CT only in the presence of PC/oleic acid, it seemed likely that CTBP may enhance or stabilize the binding of CT to PC/oleic acid liposomes. To assess the effect of CTBP on the binding of CT to PC/oleic acid liposomes, we incubated CT, CTBP, or CT plus CTBP with PC/oleic acid. We then separated proteins bound to PC/oleic acid from the unbound proteins by flotation of the PC/oleic acid liposomes to the top of a sucrose gradient (29).

The results from these experiments indicated that both CT and CTBP bind to PC/oleic acid liposomes (Fig. 7A). All of the CTBP was bound to PC/oleic acid, but a portion of the CT (~25%) did not bind to PC/oleic acid and remained at the bottom of the gradient. CTBP appeared to bind to CT at the bottom of the gradient (Fig. 7A, compare A and C). The amount of CT bound to PC/oleic acid was not increased by CTBP (Fig. 7A, compare and ). The activity of CT bound to PC/oleic acid in the absence of CTBP was measured with and without the addition of CTBP to the assay. The addition of CTBP stimulated CT activity (Fig. 7B, compare and ). When CT was incubated with CTBP and PC/oleic acid, the activity of CT bound to PC/oleic acid was increased (Fig. 7, compare B, and C, ). The increase in CT activity appeared to be due to activation by CTBP because the mass of CT bound to PC/oleic acid was not increased (Fig. 7A). Furthermore, CT activity was not increased by the addition of more CTBP to the assay (Fig. 7C, compare and ). These results suggested that CT, CTBP, and PC/oleic acid formed a relatively stable complex in which CT was optimally active.

**CT Does Not Bind to CTBP in the Absence of Lipid**—To assess the ability of CT to form complexes with CTBP in the absence of lipid, we incubated purified CT with purified CTBP. A mixture of CT and CTBP and samples of CT and CTBP alone were then subjected to glycerol density centrifugation. CT sedimented in the glycerol gradient to a position corresponding to a molecular mass of 90–100 kDa (Fig. 8, A (activity) and C (immunoassay)). There was no evidence for the formation of a stable complex between CT and CTBP because incubation of CT with CTBP did not change the position of CT in the gradient (Fig. 8B). Interestingly, CT that was sedimented alone was nearly inactive (Fig. 8A). The addition of CTBP to the assay produced a 4–5-fold increase in activity. When CT was sedimented with CTBP, the recovery of CT activity was increased.

**Fig. 3. SDS gel analysis of CTBP purified by preparative SDS gel electrophoresis**. The protein composition and immunostain pattern of CTBP were assessed by SDS electrophoresis on a 10% polyacrylamide slab gel. An aliquot of the CTBP preparation containing 4.5 μg of protein was applied to both lanes A and B. Lane A was detected by silver staining. Lane B was detected by Western blot immunostaining with CTBP antiserum.
Furthermore, CT activity was not increased appreciably when CTBP was added to the assay. This was likely due to the fact that CTBP sedimented to approximately the same position as CT in the gradient (Fig. 8B). Therefore, CTBP would have been present in the assay along with CT.

**DISCUSSION**

In previous studies, we identified a protein (CTBP) with a subunit molecular mass of 110–112 kDa that binds CT, forming heterogeneous complexes (17, 18). In the present study, we purified CTBP from rat liver. Amino acid sequences from six separate peptides derived from purified CTBP exactly matched sequences in TAP that had previously been isolated from rat liver and sequenced (19).

CTBP Is Identical to TAP/p115

The dilution experiments were repeated for CTBP, ovalbumin, thyroglobulin, and bovine serum albumin. The average activity of purified CT, determined in 13 separate experiments, was 1.06 ± 0.09 nmol/min/ml. This activity was used to calculate the percent of the total.
CTBP Is Identical to TAP/p115

Purified CTBP stimulated CT activity under a variety of conditions. The increase in CT activity occurred after dilution in the presence of Triton X-100 or without Triton X-100. Dilution of CT without maintaining the Triton X-100 concentration resulted in a loss of CT activity, measured under optimal conditions for a function of TAP/p115 in the docking of vesicles to target membranes (32).

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Fig. 6. CTBP stimulation of CT activity requires PC/oleic acid. Purified CT (12 μl) was diluted to 480 μl in Buffer C (A) or in Buffer C containing 0.06% Triton X-100 (B). The diluted mixtures were kept on ice for 30 min. CT activity was measured in 5-μl aliquots of the diluted mixtures. CT activity was also measured in 2-μl aliquots of undiluted CT (C). CT activity was measured in the presence of varied amounts of PC/oleic acid with (●) or without (○) CTBP. In A and B, 94 ng of CTBP were added to the assay. CTBP was added to assay mixtures immediately before the addition of CT. The results are calculated as the percent recovery of the activity in undiluted CT measured with 100 μM PC/oleic acid.

Fig. 7. CT and CTBP binding to PC/oleic acid liposomes. Purified CT (19 nmol/min), purified CTBP (3.9 μg), or CT and CTBP were incubated with PC/oleic acid (2 mg of PC and 0.75 mg of oleic acid, 1:1 molar ratio) containing trace amounts of sn-1,2-dipalmitoylphosphatidylcholine (100 ng) in a final volume of 60 μl of buffer containing 50 mM imidazole, 2 mM EDTA, and 2 mM DTT, pH 7.0. After 5 min at 37 °C, 60 μl of 70% sucrose were added. The mixture (100 μl) was layered under a 15–30% sucrose gradient (1.0-ml total volume). The gradient was centrifuged in an SW 50.1 rotor for 18 h at 40,000 rpm. Fractions (200 μl) were collected from the top. CT and CTBP masses were measured by immunoassays. The amount of radioactive PC determined in each fraction was used to measure the distribution of PC/oleic acid (expressed as percent distribution of radioactive PC; ○). CT activity was determined with and without the addition of 65 ng of CTBP to the assay. A, mass distribution of CT and CTBP. ●, CT mass after incubation with PC/oleic acid; ▲, CTBP mass after incubation with PC/oleic acid; ○, CT mass after incubation with CT and PC/oleic acid; ●, CTBP mass after incubation with CT and PC/oleic acid. B, distribution of CT activity after incubation with PC/oleic acid. The activity was measured with and without CTBP added to the assay. ●, without added CTBP; ■, with 65 ng of CTBP. C, distribution of CT activity after incubation with CTBP and PC/oleic acid. CT activity was measured with and without CTBP added to the assay. ●, without added CTBP; ■, with 65 ng of CTBP.
centrations of PC/oleic acid. However, the addition of CTBP to the assay increased CT activity to the original activity measured before dilution. With both undiluted CT and CT diluted with Triton X-100, the activity of CT was increased by CTBP to levels 50–60% higher than those obtained with PC/oleic acid alone. Under all conditions, stimulation of CT activity by CTBP occurred only when PC/oleic acid was present. CTBP did not stimulate CT activity in the absence of PC/oleic acid. Both CT and CTBP bound to PC/oleic acid liposomes. The isolated complexes contained active CT, but the activity was increased when CTBP was present. Complexes between CT and CTBP were not formed in the absence of lipid, in agreement with the observation that CTBP alone did not stimulate CT activity. These results are in general agreement with our previous observations that oleic acid or PC/oleic acid promoted the formation of complexes between CT and CTBP in liver cytosol (17, 18). Overall, the results indicated that the coincident binding of CT and CTBP to PC/oleic acid formed a ternary complex in which CT activity was increased above the activity achievable with PC/oleic acid. At present, the mechanism for this cooperative effect is unknown.

The finding that CTBP is identical to TAP/p115, together with the results suggesting a role of CTBP in the regulation of CT activity leads to the interesting possibility that this protein enables communication between phosphorylcholine synthesis and vesicular transport. Conceptually, this dual function would allow the rate of vesicular transport to be coordinated with the biosynthesis of phosphatidylcholine needed for the production of membrane vesicles. There is some relevant information in the literature to support a connection between vesicular transport and phosphatidylcholine synthesis. Cytidylyltransferase has been reported to be present in Golgi membranes (33, 34). Studies by Slomiany et al. (35) on the biogenesis of endoplasmic reticulum transport vesicles in gastric mucosal tissue suggested that CT was associated with the transport vesicle. Indirect evidence led the authors to speculate that the formation of vesicles from the endoplasmic reticulum may be regulated by the activity of cytidylyltransferase. The results suggesting that CT is located in the nucleus (6, 20, 36) are difficult to reconcile with a function of CT in vesicular transport. However, the subcellular localization of CT is not completely established. For example, recent studies by Houweling et al. (37), using immunofluorescence, immunogold electron microscopy, and biochemical techniques, indicate that CT is not exclusively located in the nucleus in all cells. For example, CT was found to be distributed throughout the cytoplasm in isolated rat hepatocytes.

A series of genetic studies with Saccharomyces cerevisiae provided independent evidence for a regulatory connection between cytidylyltransferase and Golgi function. Sec14p, a phosphatidylinositol/phosphatidylcholine transfer protein, is bound peripherally to Golgi membranes and is required for transport from Golgi compartments (38–40). Skinner et al. (41) provided both in vitro and in vivo evidence that Sec14p decreases the
CDP-choline pathway for phosphatidylcholine synthesis by inhibiting cytidylyltransferase. Furthermore, the data suggest that Sec14p is inhibitory when in the phosphatidylcholine-bound form. This provides a potential feedback process to regulate the amount of phosphatidylcholine in Golgi membranes. Thus, it is possible that a Sec14p-type action and the effects of CTBP/TAP are both examples of a complex mechanism to coordinate the biosynthesis of phosphatidylcholine with membrane formation required for vesicular transport. Full recognition of the importance of CTBP/TAP in regulating CT activity and in vivo demonstration of the effects of CTBP on CT activity and in vivo demonstration of the cooperative effect of CTBP on CT activity.

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