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The Association of Lipid Activators with the Amphipathic Helical Domain of CTP:Phosphocholine Cytidylyltransferase Accelerates Catalysis by Increasing the Affinity of the Enzyme for CTP*

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The biochemical mechanism for the regulation of enzyme activity by lipid modulators and the role of the amphipathic α-helical domain of CTP:phosphocholine cytidylyltransferase (CT) was investigated by analyzing the kinetic properties of the wild-type protein and two truncation mutants isolated from a baculovirus expression system. The CT[Δ312–367] mutant protein lacked the carboxyl-terminal phosphorylation domain and retained high catalytic activity along with both positive and negative regulation by lipid modulators. The CT[Δ257–367] deletion removed in addition the region containing three consecutive amphipathic α-helical repeats. The CT[Δ257–367] mutant protein exhibited a significantly lower specific activity compared to CT or CT[Δ312–367] when expressed in either insect or mammalian cells; however, CT[Δ257–367] activity was refractory to either stimulation or inhibition by lipid regulators. Lipid activators accelerated CT activity by decreasing the K_m for CTP from 24.7 mM in their absence to 0.7 mM in their presence. The K_m for phosphocholine was not affected by lipid activators. The activity of CT[Δ257–367] was comparable to the activity of wild-type CT in the absence of lipid activators and the CTP K_m for CT[Δ257–367] was 13.9 mM. The enzymatic properties of the CT[Δ231–367] mutant were comparable to those exhibited by the CT[257–367] mutant indicating that removal of residues 231 through 257 did not have any additional influence on the lipid regulation of the enzyme. Thus, the region between residues 257 and 312 was required to confer lipid regulation on CT, and the association of activating lipids with this region of the protein stimulated catalysis by increasing the affinity of the enzyme for CTP.

CT[1] is considered a key rate-controlling step in the biosynthesis of PtdCho, a major phospholipid component of mammalian membranes, and modulation of CT function by lipid regulators is an important element in the control of enzyme activity (for reviews, see Refs. 1–3). Purified CT has essentially no activity in the absence of lipids (4, 5), and its activity is revealed by the addition of anionic lipids (such as oleic acid or phosphatidylglycerol) or neutral activators (such as diacylglycerol) presented in PtdCho vesicles (4–7). The significance of lipid regulation of CT to the control of PtdCho biosynthesis is supported by the stimulation of the pathway following the treatment of cells with oleic acid (8, 9) or the generation of diacylglycerol by the addition of exogenous PtdCho-specific phospholipase C (10–12). The stimulation of PtdCho synthesis by these treatments correlates with the translocation of CT to cellular membranes and the dephosphorylation of the enzyme (9–14). The correlation between cellular diacylglycerol (15–17) or PtdCho (18–20) content with the rate of PtdCho synthesis and CT membrane translocation provides compelling support for the physiological importance of CT-lipid interactions. CT is also negatively regulated by associations with lipids. CT activity *in vitro* is inhibited by sphingosine (21), lysoPtdCho (22), and the antineoplastic lysoPtdCho analog, ET-18-OCH_3 (22). Treatment of cells with either lysoPtdCho or ET-18-OCH_3 inhibits PtdCho synthesis and triggers the accumulation of phosphocholine indicating that CT is a target for these compounds *in vivo* (22). The observation that the inhibition of CT activity by these three lipids is competitive with respect to the PtdCho/oleic acid activator suggests that both positive and negative lipid regulators bind to the same site on the enzyme (21, 22).

CT can be divided into several discrete functional domains (Fig. 1). The focus of this work is the region between residues 228 and 315 which is predicted to be primarily α-helical with subdomains that exhibit significant amphipathic character (23). This helical domain is highly conserved in all sequenced mammalian CT proteins (23–26), although the yeast CT sequence does not contain a homologous domain (27). The helical region contains a positively charged cluster that is immediately followed by a series of three, 11-residue repeats that are strongly predicted to form an amphipathic α-helix (23). The predicted α-helix is broken at residues 294–297, but this interruption is followed by another predicted α-helix through residue 315 that also has amphipathic character (23). Protease protection experiments (28) implicate the α-helical region between residues 236 and 293 as responsible for the interaction of CT with phospholipid bilayers. Also, antibodies directed against residues 247 to 257 interfere with CT membrane association (29) supporting the idea that this region of CT is involved in lipid-protein interactions.

There are two possible mechanisms that could account for the regulation of CT activity by lipids. First, the amphipathic α-helical domain may inhibit CT activity, and this inhibition

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[1] The abbreviations used are: CT, CTP:phosphocholine cytidylyltransferase; PCR, polymerase chain reaction; PtdCho, phosphatidylcholine; lysoPtdCho, lysophosphatidylcholine; ET-18-OCH_3, 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine; bp, base pair(s); DMEM, Dulbecco's modified Eagle's medium; bis-Tris, 2-[bis(2-hydroxyethyl)-amino]-2-(hydroxymethyl)propane-1,3-diol.

might be relieved by the binding of the amphipathic α-helix to phospholipid vesicles. Second, the binding of lipid regulators to the amphipathic α-helix may trigger a conformational change that activates the enzyme. The goal of the present work is to verify that the amphipathic helical domain is required to confer lipid regulation to CT and to determine if the interaction between this domain and lipid regulators is responsible for increasing the catalytic activity of the protein or relieving the inhibition of the enzyme.

EXPERIMENTAL PROCEDURES

Materials—The rat CT cDNA was provided by Dr. R. B. Cornell (23), the murine CT cDNA was isolated in our laboratory (24), and the CT recombinant baculovirus vectors were constructed and expressed in Sf9 cells as described previously (30). Baculovirus DNA was purchased from Clontech. Baculovirus vectors and pcDNA3 were from Invitrogen. Flag-tag M2 monoclonal antibody was from Kodak. Phospho[*methyl*-^{14}C]choline (specific activity 55 mCi/mmol) was from DuPont NEN. Lipids were from Avanti Polar Lipids, Inc. Cell culture media were obtained from Life Technologies, Inc., thin layer plates were from Analtech, and molecular biology reagents were obtained from Promega. All other materials were reagent grade or better.

Construction of the Deletion Mutants CT[Δ312–367], CT[Δ257–367], and CT[Δ231–367]—The original rat CT cDNA has a cloning artifact containing 2 incorrect bases generating a G91S and S114C mutant protein (25). These mutations were removed from all our constructs by replacing the *Eco*RI/*Sty*I fragment (bp 189–783) of the rat CT cDNA clone with the corresponding fragment of the murine CT cDNA clone.

The carboxyl-terminal deletion mutant pCTD312 was constructed starting with a CT mutant containing an *Msc*I site at bp 1040. This new restriction site was generated by adding an additional A at the 3′ end by *Taq* DNA polymerase, which changed A^{1037} to T^{1037}, when PCR was performed using primer 5′-GGCCATCgcTCCCAAGCAGAG-3′ to generate the CT[S315A] mutant constructed in a previous study (31). The sequence was confirmed by DNA sequencing. The region between bp 1040 and bp1342 was deleted by digestion of the resulting plasmid with *Msc*I/*Not*I. The *Not*I site was filled with Klenow fragment, and the plasmid religated. In this construct, the codons for Gln-312 through Asp-367 were deleted, and the codons for five additional amino acid residues (Leu-Gly-Pro-His-Ala) were added after the codon for Leu-311.

Plasmid pCTD231 was constructed from the *Nco*I/*Sac*I fragment of CT cDNA subcloned into pTrc99A (Pharmacia). The pTrc99ACTN/S containing the *Nco*I/*Sac*I fragment was digested with *Sac*I and *Bam*HI, and the plasmid fragment was isolated by electrophoresis. The *Bam*HI overhang was filled with Klenow fragment, and the plasmid religated. The insert contained amino acid residues 1–230 plus Ile-Leu and was subcloned into *Nco*I/*Hin*dIII sites of pBlueBacIII. The resulting construct was called pCTD231. The pCTD257 was constructed by PCR site-directed mutagenesis. The *Sac*I/*Bam*HI fragment of rat CT (0.57 kilobase) was subcloned into pBS and used as the template for the deletion primer which was designed based on the nucleotide sequence from bp 861 to bp 882. The primer, 5′-GAAAGATGTGtAGTAAAAGTCG-3′, substituted the original GAG codon for Glu-257 with TAG, thus changing Glu-257 to a stop codon. Mutagenesis was performed using the deletion primer and the M13 reverse primer to generate the first round PCR product and then using the first round PCR product and the M13 forward primer to generate the second round PCR product (32). The second round PCR product was digested with *Sac*I/*Bam*HI, ligated into the pBS plasmid, and the DNA sequence was confirmed. The mutated fragment was isolated by digesting the mutated pBS construct with *Sac*I/*Hin*dIII and co-ligated with the CT *Nco*I/*Sac*I fragment into pBlueBacIII yielding pCTD257.

The flagCT construct was made by co-ligation of the CT *Nco*I/*Bam*HI fragment with the flag-tag oligonucleotide, 5′-AGCTTATGGACTACAAGGACGACGATGACAAGGC-3′ ((+)-strand) and 5′-CATGGCCTTGTCATCGTCGTCCTTGTAGTCCATA-3′ ((−)-strand), into the *Hin*dIII/*Bam*HI sites of plasmid pcDNA3. The flagCT[Δ312–367] construct was made by replacing the *Sse*8387I/*Xho*I fragment in flagCT with the corresponding fragment from pBSIIKSCTD312. The pBSIIKSCTD312 was obtained by subcloning the insert of pCTD312 into the *Bam*HI/*Hin*dIII sites of pBluescript II KS (Stratagene). The flagCT[Δ257–367] construct was made by replacing the *Sse*8387/*Bam*HI fragment with the corresponding fragment from pCTD257.

In all cases, the identities of the mutations were confirmed by DNA sequencing.

Expression of CT and the Mutants in Sf9 Cells—The expression of CT and CT[Δ312–367] in Sf9 cells and the transfection of baculovirus and the recombinant constructs of CT[Δ257–367] and CT[Δ231–367] were performed as described previously (30).

Delipidation of Sf9 Cell CT Lysate—All procedures were performed at 4 °C. A baculovirus-infected Sf9 cell pellet was resuspended in 200 μl of Sf9 lysis buffer (10 mM HEPES, pH 7.4, 10 mM NaCl, 1 mM EDTA, 2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 2% aprotinin, 1 μg/ml leupeptin, 50 mM NaF, 100 μM Na_3VO_4, 100 nM okadaic acid) and sonicated 3 × 30 s. The lysate was centrifuged at 10,000 × *g* for 20 min. The supernatant (200 μl) was loaded onto a DEAE-Sepharose CL-6B column (0.7 × 1.0 cm) which was equilibrated with 2 ml of buffer I (10 mM HEPES, pH 7.4, 10 mM NaCl, 1 mM EDTA, 2 mM dithiothreitol) plus 1% Nonidet P-40. The column was sequentially eluted by 1.5 ml of buffer I plus 1% Nonidet P-40 (to remove endogenous lipids), 1.5 ml of buffer I, 1.7 ml of buffer I plus 0.25 M NaCl, and 1.7 ml of buffer I plus 0.5 M NaCl. The 0.5-ml fraction between 200 and 700 μl of buffer I plus 0.25 M NaCl contained the CT activity.

Expression of flagCT and flagCT Mutants in COS7 Cells—COS7 cells were seeded at a density of 3 × 10^6 in a 100-m dish (about 30% confluence) with DMEM complete medium (DMEM + 10% fetal calf serum + 1% glutamine) and cultured overnight at 37 °C. The medium was removed, and the cells were gently washed with 3 ml of DMEM. Then the cells were cultured with 2.5 ml of transfection medium (DMEM + 5 μg/ml plasmid DNA + 200 μg/ml DEAE-dextran) for 4 h at 37 °C. The transfection medium was removed and the cells were incubated with 2 ml of HBS buffer (20 mM Hepes, pH 7.5, 135 mM NaCl, 10% dimethyl sulfoxide) for 2 min at 25 °C and washed twice with DMEM. The cells were then cultured in DMEM complete medium for 72 h at 37 °C. The cells were harvested and resuspended in 100 μl of lysis buffer (0.25 M sucrose, 1 mM EDTA, 10 mM Hepes, pH 7.3, 1 μg/ml leupeptin). The cells were sonicated 3 × 30 s at an output of 10 at 80% duty cycle. The protein of the cell lysate was measured (33), and CT activity of the lysate was determined.

CT Assay—The standard CT activity assay contained the delipidated CT or mutant CT fraction in a final volume of 40 μl containing 120 mM bis-Tris-HCl, pH 6.5, 1 mM phospho[*methyl*-^{14}C]choline (0.5 μCi), 2 mM CTP, 80 μM or the indicated amount of PtdCho/18:1 (1:1). The reaction was for 10 min at 37 °C and stopped by the addition of 5 μl of 0.5 M EDTA and incubated on ice. CDP-[^{14}C]choline formation was determined by thin layer chromatography (30). CT assays were linear with time and protein. Protein was determined according to the method of Bradford (33) using γ-globulin as a standard. The CT protein was also estimated by comparing the density of the CT bands to a standard curve of carbonic anhydrase following SDS gel electrophoresis and staining with Coomassie Blue.

Kinetic Analysis—The Hill constant for CTP binding to CT was determined by analyzing the data according to a transformation of the Hill equation: $\log [(v/(V_{max} - v)] = n_H \log[A] - \log K$, where v is the CT catalytic rate, V_{max} is the CT maximum catalytic rate, n_H is the Hill coefficient, A is the concentration of CTP, and K is a constant (34). The Hill number, n_H, represents the power to which the CTP concentration (A) must be raised such that the plots of initial rate *versus* activator concentration fit a rectangular hyperbola. Under these conditions, the constant (K) approximates the K_m value predicted by the Michaelis-Menten equation. Linear correlation coefficients for the data used to determine the slope (n_H) in the Hill plots exceeded 0.98 in all cases. V_{max} values used in the Hill plots were determined independently by double reciprocal plots using the data obtained either in the presence or absence of 80 μM PtdCho/oleate (1:1).

RESULTS

Rationale—As outlined in the introduction, the α-helical domain between residues 228 and 312 has been proposed to be the region of the protein involved in protein-lipid interactions. A series of CT deletion mutants were constructed to test this idea (Fig. 1). The CT[Δ312–367] mutant removed the carboxyl-terminal phosphorylation domain, but left the entire α-helical region intact. The CT[Δ257–367] mutant removed the three consecutive 11-residue repeats between residues 256 and 288 that were strongly predicted to form an amphipathic α-helix (23). This mutant also lacked the predicted α-helix between residues 294 and 312 that was predicted to have a significant hydrophobic moment. We expressed these deletion mutants in insect and mammalian cells and analyzed the biochemical properties of the enzymes to determine if the residues between

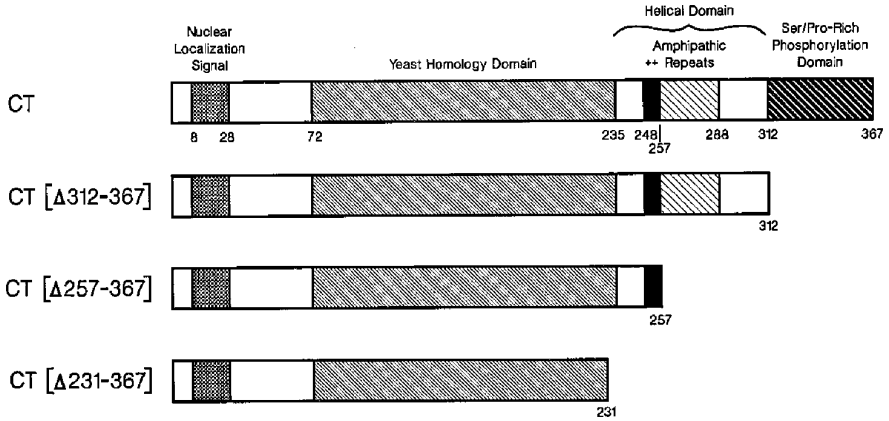

FIG. 1. **Domain structure of CT.** CT contains four distinct domains. There is an amino-terminal nuclear localization signal located between residues 8 and 28, a central yeast homology domain that is thought to be the catalytic center of CT between residues 72 and 235, an α-helical domain between residues 228 to 312 that we have conceptually subdivided into a cluster of positively charged residues (++) followed by three consecutive 11-residue repeats that are strongly predicted to form an amphipathic α-helix between residues 257 and 288, and a carboxyl-terminal Ser/Pro-rich domain extending from residue 312 to 367 that contains all of the CT phosphorylation sites. The helical region between 298 and 315 is also predicted to have a significant hydrophobic moment. The CT[Δ312–367] truncation removes the carboxyl-terminal phosphorylation domain, the CT[Δ257–367] deletion removes the three 11-residue amphipathic α-helical repeats as well as the region between residues 294 and 312. The CT[Δ231–367] mutant removes the entire α-helical domain and 4 residues that extend into the yeast homology domain.

257 and 312 were required for lipid regulation and to determine the biochemical mechanism that accounts for lipid activation.

Expression and Activity of CT Mutants—CT and the two truncation mutants were expressed in Sf9 insect cells using recombinant baculoviruses, the proteins were partially purified and endogenous lipids were removed by DEAE-column chromatography, and their specific activities were determined (Fig. 2). The highest specific activity was exhibited by the CT[Δ312–367] which was 1.6-fold higher than CT. A higher specific activity for CT[Δ312–367] was expected since the phosphorylated carboxyl-terminal domain inhibits lipid activation (31). Although CT[Δ257–367] activity was detected easily, the specific activity of this mutant was considerably lower than both CT (23-fold) and CT[Δ312–367] (38-fold). The expression level of all three CT constructs in Sf9 cells were essentially the same; however, the cytidylyltransferase activity in lysates containing CT[Δ257–367] was only 4% of the activity in lysates from cells expressing wild-type CT. Furthermore, like CT and CT[Δ312–367], CT [Δ257–367] retained >80% of its initial activity following 3 days of storage at 4 °C. These data indicated that the lower specific activity of CT[Δ257–367] was not due to an inherent instability of the protein or the loss of activity during the DEAE-Sepharose chromatography step. The delipidated protein preparations contained essentially the same amount of CT (Fig. 2*A*), and the protein concentrations were verified by measuring the total protein content and the relative CT protein levels using densitometric analysis of Coomassie-stained gels as described under "Experimental Procedures." The wild-type CT band was consistently more diffuse than the truncation mutants because wild-type CT is extensively phosphorylated in Sf9 cells (35) and CT phosphorylation results in the retardation of its electrophoretic mobility (36). These data show that the removal of the amphipathic α-helical domain between residues 257 and 312 results in a marked reduction in the catalytic activity of CT in the presence of PtdCho/oleic acid vesicles.

Lipid Activation of CT Deletion Mutants—The sensitivity of CT and its deletion mutants to lipid activators was examined by determining the ability of PtdCho/oleic acid vesicles, a potent lipid activator of the enzyme, to stimulate activity (Fig. 3). Both CT and CT[Δ312–367] possessed extremely low activity in the absence of PtdCho/oleic acid and were stimulated 700- to 1,400-fold by the addition of the lipid activator. In contrast, the activity of CT[Δ257–367] was not altered by the addition of

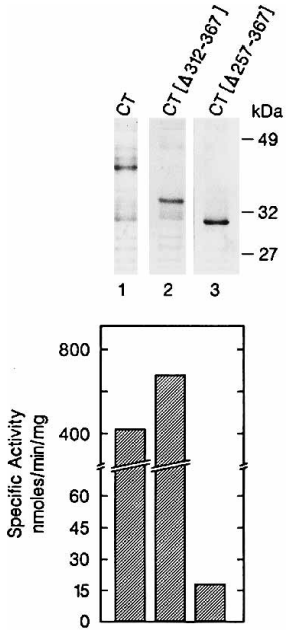

FIG. 2. **Expression and activity of CT and the three deletion mutants.** CT and the truncation mutants were expressed in Sf9 cells by infection with recombinant baculoviruses. The proteins were delipidated and partially purified by DEAE-Sepharose chromatography. *A*, SDS-gel electrophoresis analysis of the protein preparations showing the purity and the apparent molecular weights of CT and the mutant proteins. *B*, specific activities of CT and the two mutant proteins were compared by assaying CDP-choline formation in the presence of 80 μM PtdCho/oleic acid as described under "Experimental Procedures." Assays were linear with time and protein.

PtdCho/oleic acid. These data indicated that the lower specific activity of the CT[Δ257–367] mutant was due to the inability of the lipid activators to stimulate the deletion mutant. Thus, a region of CT required for lipid activation lies between residues 257 and 312.

Inhibition of Activity by an Antineoplastic LysoPtdCho Analog—CT activity was potently inhibited by the ether-linked lysoPtdCho analog, ET-18-OCH_3 (22). Kinetic analysis of the inhibition of CT by ET-18-OCH_3 illustrated that the inhibition was competitive with respect to PtdCho/oleic acid vesicles (22).

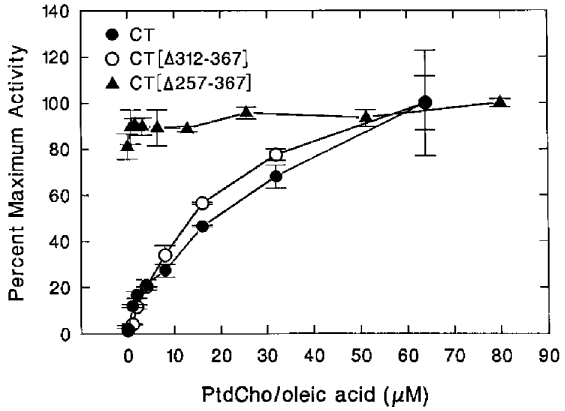

FIG. 3. **The activity of CT[Δ257–367] was not stimulated by PtdCho/oleic acid vesicles.** The ability of the potent lipid activator mixture (PtdCho/oleic acid) to stimulate the activity of CT, CT[Δ312–367], and CT[Δ257–367] was determined as described under "Experimental Procedures." The maximum activities for the protein preparations used in this experiment were: CT, 305 nmol/min/mg; CT[Δ312–367], 628 nmol/min/mg; and CT[Δ257–367], 22.7 nmol/min/mg.

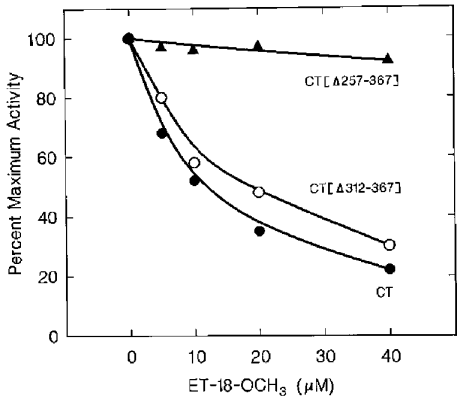

FIG. 4. **Effect of ET-18-OCH_3 on the activities of CT, CT[Δ312–367], and CT[Δ257–367].** The three proteins were assayed in the presence of 80 μM PtdCho/oleic acid and the indicated concentration of ET-18-OCH_3 as described under "Experimental Procedures." The maximum activities for the protein preparations used in this experiment were: CT, 420 nmol/min/mg; CT[Δ312–367], 680 nmol/min/mg; and CT[Δ257–367], 17.8 nmol/min/mg.

These data led to the hypothesis that the same region of the protein responsible for the association with lipid activators was also the site for regulation by inhibitors suggesting that CT[Δ257–367] would be refractory to ET-18-OCH_3 inhibition. Indeed, CT[Δ257–367] activity was not significantly diminished by ET-18-OCH_3, whereas CT and CT[Δ312–367] were both inhibited by ET-18-OCH_3 (Fig. 4). CT and CT[Δ312–367] were inhibited 50% by 10–14 μM ET-18-OCH_3, whereas CT[Δ257–367] activity was only decreased by 4–6% at these ET-18-OCH_3 concentrations. Thus, the region between residues 257 and 312 was required for regulation of enzyme activity by both positive and negative lipid modulators.

Expression of flagCT, flagCT[Δ312–367], and flagCT[Δ257–367] in COS7 Cells—To determine if CT[Δ257–367] also possessed lower specific activity when expressed in mammalian cells, COS7 cells were transfected with epitope-tagged CT expression constructs, and, after 48 h, the transfected cells were harvested and the extracts were analyzed for protein expression and CT activity (Fig. 5). CT protein expression was analyzed by immunoblotting with the M2 monoclonal antibody that detects the flag-tag epitope. Control cells did not express epitope-tagged proteins, whereas cells transfected with flagCT, flagCT[Δ312–367], and flagCT[Δ257–367] all expressed significant and comparable amounts of protein (Fig. 5*A*). Extracts of the transfected cell populations were assayed for CT activity (Fig. 5*B*). Lysates prepared from cells transfected with flagCT and flagCT[Δ312–367] expression vectors possessed significantly higher specific activities for CT compared to the lysates prepared from cells transfected with the vector control. In contrast, CT specific activity in lysates from cells transfected with the flagCT[Δ257–367] construct was similar to that found in the vector control lysates. Since the level of flagCT[Δ257–367] protein expression was essentially the same as flagCT[Δ312–367] protein expression as judged by immunoblotting, we conclude that CT[Δ257–367] was also catalytically impaired when expressed in mammalian cells.

Kinetic Mechanism for Lipid Activation—We examined the effect of PtdCho/oleic acid vesicles on the K_m for the substrates, phosphocholine and CTP, to determine the kinetic mechanism responsible for CT activation by lipids. We first examined the effect of lipid activators on the kinetic constants for phosphocholine (Fig. 6). The K_m values for phosphocholine were the same for CT assayed either in the presence or absence of PtdCho/oleic acid activator (Fig. 6). Thus, alterations in the kinetics of phosphocholine did not contribute to the regulation of CT activity by lipids.

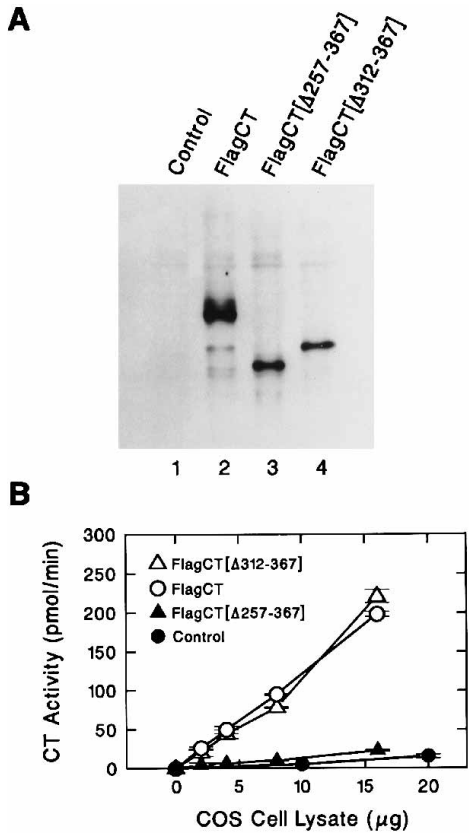

FIG. 5. **Expression of flagCT, flagCT[Δ312–367], and flagCT[Δ257–367] in COS7 cells.** COS7 cells were transfected with plasmids directed to express epitope-tagged proteins from the cytomegalovirus promoter as described under "Experimental Procedures." At 48 h after transfection, the cells were harvested. *A*, a sample of the cell lysate (100 μg of protein) was separated by SDS-gel electrophoresis, the proteins were transferred to nitrocellulose membrane, and the levels of flagCT, flagCT[Δ312–367], and flagCT[Δ257–367] were determined by immunoblotting with the M2 monoclonal antibody to the flag-tag epitope. *B*, cell lysates were assayed for CT activity to determine the specific activity of CT in the transfected cell populations as described under "Experimental Procedures." Assays were linear with time and protein.

A different result was obtained when the CTP K_m was de-

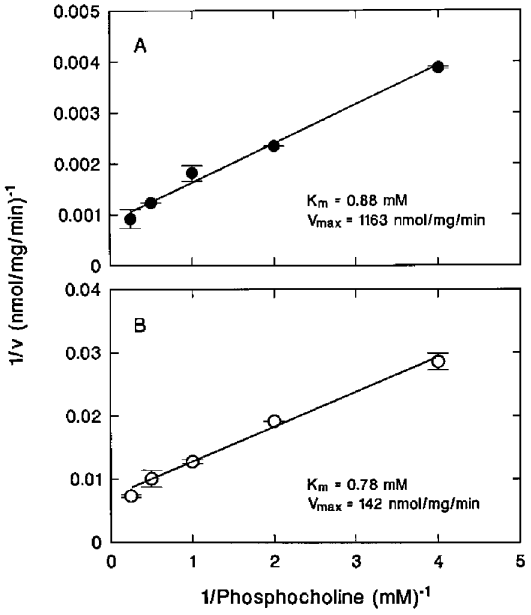

FIG. 6. **Phosphocholine kinetic constants for CT in the presence and absence of lipid activators.** The K_m for phosphocholine was determined for CT in the presence of PtdCho/oleic acid vesicles and 2 mM CTP (*A*) or in the absence of lipid activators in the presence of 32 mM CTP (*B*). Enzyme assays were performed as described under "Experimental Procedures."

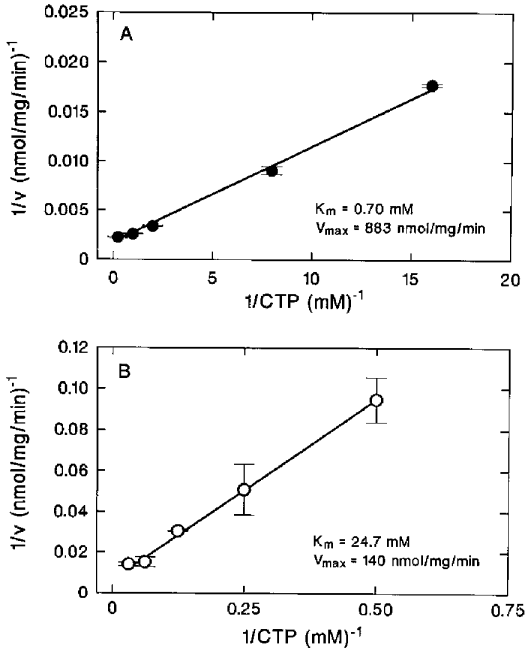

FIG. 7. **Kinetic constants for CTP in the presence and absence of lipid activators.** The K_m for CTP was determined for CT in the presence of PtdCho/oleic acid and 1 mM phosphocholine (*A*) or in the absence of lipid activator and 1 mM phosphocholine (*B*). Assays were performed using the indicated concentrations of CTP as described under "Experimental Procedures."

termined in the presence and absence of PtdCho/oleic acid activator (Fig. 7). The presence of activating lipids significantly increased the affinity of CT for CTP (Fig. 7, *A* and *B*). In the presence of PtdCho/oleic acid, the CTP K_m was 0.7 mM which increased to 24.7 mM (35-fold) in the absence of lipids. The V_{max} in the presence of lipids was 6.3-fold higher than in the absence of lipids (Fig. 7, *A* and *B*), indicating that the 700-1400-fold increase in CT specific activity by PtdCho/oleic acid when measured at 2 mM CTP was due almost entirely to the ability of lipid activators to increase the affinity of CT for CTP. The CTP K_m values for CT and CT[Δ312–367] in the presence of lipid activators were similar to those reported for purified CT by ourselves and other investigators (31, 37), and, in both cases, the CTP kinetics were a close match to the Michaelis-Menten equation showing little cooperativity, with Hill coefficients (n_H) close to 1.0 (Table I). The CTP K_m calculated for CT[Δ257–367] was 13.2 mM (Table I). This value was considerably higher than the values for the CT and CT[Δ312–367] in the presence of lipid activators, but was close to the K_m determined for CT and CT[Δ312–367] in the absence of lipid activators. These data indicated that the PtdCho/oleic acid stimulates CT activity by lowering the CTP K_m by 20–30 fold and that the CT[Δ257–367] truncation mutant cannot undergo this allosteric transition because it lacks the lipid interaction domain.

These data predicted that there would be little difference between the activity of CT and CT[Δ257–367] when assayed in the absence of lipid activators. Therefore, we compared the activities of these two proteins in the absence of lipids as a function of CTP concentration and found that indeed the two proteins possessed almost identical activities under these assay conditions (Fig. 8). In both cases, the enzymes required high concentrations of CTP for maximal activity. Thus, the presence of the amphipathic α-helical domain did not inhibit CT activity in the absence of lipids, but rather was required along with lipid activators to lower the K_m for CTP.

Analysis of the CT[Δ231–367] Mutant—The CT[Δ231–367] mutant lacked the entire α-helical domain and differed from the CT[Δ257–367] in that it lacked a cluster of positively charged resides between positions 248 and 256, the α-helical region between residues 232 and 256, and the deletion extended four residues into the yeast-homology domain of the protein. Like the CT[Δ257–367] mutant, the CT[Δ231–367] mutant protein possessed lower catalytic activity compared to wild-type CT, was not activated by the PtdCho/oleic acid vesicles, and exhibited a high K_m for CTP (Table I). These data showed that the enzymatic properties of the CT[Δ231–367] mutant were essentially the same as the CT[Δ257–367] mutant and indicated that the region between residues 231 and 257 was not sufficient to confer either positive or negative regulation either in the presence or absence of lipid modulators.

DISCUSSION

Our data reveal that the association of wild-type CT with activating lipids accelerates catalysis by increasing the affinity of the enzyme for CTP. This model asserts that the binding of activating lipids to the amphipathic α-helical domain induces a conformational change that lowers the CTP K_m. The increase in CT specific activity triggered by activating lipids when assayed at CTP concentrations around 1–2 mM is so large (≈1,000-fold) that most investigators report that CT has essentially no activity when stripped of lipid activators (4, 5, 22, 31). However, when delipidated CT is assayed at high CTP concentrations (approximately 30 mM), the difference in the specific activity in the presence and absence of lipid activators is reduced to approximately 6-fold (Table I, Figs. 3 and 7). Thus, the ability of lipid mixtures to induce a conformational change that leads to higher affinity CTP binding to the enzyme is the primary mechanism that accounts for the ability of activating lipids to accelerate CT catalysis. Indeed, the activities of CT and the CT[Δ257–367] mutant are nearly identical as a function of the CTP concentration in the absence of lipid activators (Fig. 8) clearly showing that the binding of lipids to the amphipathic α-helical domain is responsible for activating CT as opposed to relieving the inhibition of the enzyme.

Our results show that the region between residues 257 and

TABLE I
Kinetic constants for CT and the three truncation mutants

Protein	V_{max}		CTP K_m		Hill coefficient (n_H)	
	+Lipid[a]	−Lipid	+Lipid[a]	−Lipid	+Lipid[a]	−Lipid
	nmol/min/mg		*mM*			
CT	883	140	0.7	24.7	0.90	0.98
CT[Δ312–367]	892	29	0.5	13.9[a]	1.46	1.39
CT[Δ257–367][b]	86		13.2[c]		1.46	
CT[Δ231–367][b]	32		19.2[c]		1.90	

[a] Lipid refers to 80 μM PtdCho/oleic acid (1:1) vesicles.
[b] These truncation mutants were not activated by lipids.
[c] Due to the high degree of positive cooperativity exhibited by these truncation mutants, the CTP K_m was estimated from the Hill equation.

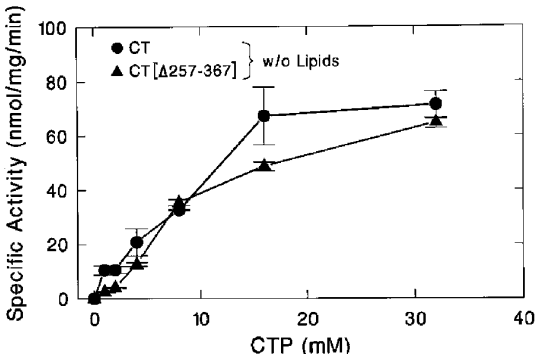

FIG. 8. **A comparison of the specific activity of CT and CT[Δ257–3367] as a function of CTP concentration and in the absence of lipid activators.** The activities of CT and CT[Δ257–367] as a function of CTP concentration were compared in the absence of lipid activators and in the presence of 1 mM phosphocholine using the CT assay described under "Experimental Procedures."

312 is necessary for the regulation of CT activity by lipids. Although removal of this region in CT[Δ257–367] resulted in a 30-fold reduction in specific activity of the expressed protein, significant residual catalytic activity remained that was refractory to activation by lipid regulators (Fig. 3). Our data also indicate that the region between residues 257 and 312 of CT is involved in negative regulation of CT activity by lipids. Craig *et al.* (28) report that all of the chymotrypsin fragments bind to PtdCho/sphingosine vesicles and suggest that CT interaction with this negative lipid modulator is not mediated by the amphipathic α-helix, but rather by the amino-terminal domain. However, our experiments with ET-18-OCH_3 (Fig. 4) are consistent with the hypothesis that negative lipid modulators associate with the same domain of the protein as positive regulators. The idea that both positive and negative lipid modulators bind to the same domain on the protein is supported by the finding that inhibition by lipid effectors (sphingosine, ET-18-OCH_3, and lysoPtdCho) is competitive with respect to lipid activator concentration (21, 22). Thus, the cumulative data point to the three 11-residue repeats that are strongly predicted to form an amphipathic α-helix as a region of CT that is essential for all lipid-protein interactions. However, although our data show that the region from residues 257 through 312, inclusive, is necessary for lipid-protein interaction, the data do not prove that it is sufficient and other regions of the protein may also participate in the regulation of CT by lipids. The role of the region between residues 231 and 312 needs to be tested further by the construction and analysis of additional carboxyl-terminal truncation mutants and internal deletion mutants that specifically remove one or more of the α-helical repeat motifs.

Modulation of the CTP K_m by lipid regulators is an effective mechanism for controlling CT activity *in vivo*. The average intracellular concentration of CTP in a variety of cell types is 278 ± 242 μM (29 ± 19 μM for dCTP) (38), which is close to the CTP K_m found by us and others *in vitro* (Table I) (31, 37). These data indicate that if CT is not associated with lipid activators *in vivo*, the enzyme would be essentially inactive due to the high CTP K_m (≈25 mM). The fact that the *in vivo* CTP concentration is close to the CTP K_m for CT suggests that alterations in the intracellular CTP levels could affect the rate of PtdCho synthesis. Indeed, the elevation of the intracellular CTP concentration accelerates PtdCho synthesis in neuronal cells (39, 40) supporting the idea that modulation of intracellular CTP levels could participate in the regulation of enzyme activity *in vivo*. Cytosolic CT preparations have a CTP K_m of 2 mM which is lowered to 0.2 mM by the addition of lipid activators (41) indicating that "soluble" CT is lipid-associated. Increased phosphorylation of the CT carboxyl-terminal domain leads to reduced enzyme activity by interfering with the association of activating lipids with the enzyme (31). Thus, CT phosphorylation fits into the regulatory scheme by interfering with the ability of activating lipids to lower the CTP K_m. In summary, the large change in the CTP K_m following lipid binding to the CT amphipathic α-helical domain between residues 257 and 312 represents an extremely effective and physiologically relevant on-off switch that governs CT catalysis and hence the rate of PtdCho production.

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Note Added in Proof—Our finding of a requirement for the residues between 257 and 312 for lipid regulation is consistent with the recent results of Wang and Kent (42); however, our conclusion that lipid binding triggers a conformational change that activates CT by increasing its affinity for CTP does not agree with their conclusion that lipid binding or carboxyl-terminal truncation activates CT by removing an inhibitory domain.

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