Lipid Activation of CTP:Phosphocholine Cytidylyltransferase α: Characterization and Identification of a Second Activation Domain†

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ABSTRACT: The CTP:phosphocholine cytidylyltransferase (CCT) governs the rate of phosphatidylcholine (PtdCho) biosynthesis, and its activity is governed by interaction with membrane lipids. The carboxy-terminus was dissected to delineate the minimum sequences required for lipid responsiveness. The helical domain is recognized as a site of lipid interaction, and all three tandem α-helical repeats from residues 257 through 290 were found to be required for regulation of enzymatic activity by this domain. Truncation of the carboxy-terminus to remove one or more of the α-helical repeats yielded catalytically compromised proteins that were not responsive to lipids but retained sufficient activity to accelerate PtdCho biosynthesis when overexpressed in vivo. The role of the helical region in lipid-activation was tested further by excising residues 257 through 309 to yield a protein that retained a 57-residue carboxy terminal domain fused to the catalytic core. This construct tested the hypothesis that the helical region inhibits activity in the absence of lipid rather than activates the enzyme in the presence of lipid. This hypothesis predicts constitutive activity for CCTα[Δ257−309]; however, this protein was tightly regulated by lipid with activities comparable to the full-length CCTα, in both the absence and presence of lipid. Activation of CCTα[Δ257−309] was dependent exclusively on anionic lipids, whereas full-length CCTα responded to either anionic or neutral lipids. Phosphatidic acid delivered in Triton X-100 micelles was the preferred activator of the second lipid-activation domain. These data demonstrate that CCTα can be regulated by lipids by two independent domains: (i) the three amphipathic α-helical repeats that interact with both neutral and anionic lipid mixtures and (ii) the last 57 residues that interact with anionic lipids. The results show that both domains are inhibitory in the absence of lipid and activating in the presence of lipid. Removal of both domains results in a nonresponsive, dysregulated enzyme with reduced activity. The data also demonstrate for the first time that the 57-residue carboxy-terminal domain in CCTα participates in lipid-mediated regulation and is sufficient for maximum activation of enzyme activity.

The dominant pathway for phosphatidylcholine (PtdCho)[1] synthesis in all mammalian tissues occurs via a CDP-choline intermediate produced by the CTP:phosphocholine cytidylyltransferase (CCT). An abundance of data demonstrates that CCT activity and PtdCho biosynthesis are regulated primarily by the membrane lipid environment. The rate of PtdCho biosynthesis is stimulated by hydrophobic perturbants such as fatty acids (*1*−*5*) or treatment with phospholipases (*6*−*11*). CCT activity and PtdCho formation are reduced by lysoPtdCho (*12*, *13*), sphingosine (*14*), and antineoplastic ether lipids (*5*, *12*, *15*−*18*). Reduction of the membrane PtdCho content, often coupled with elevation of the PtdEtn ratio, also stimulates PtdCho biosynthesis (*19*−*23*). CCT activity and PtdCho biosynthesis correlate with the cellular DG levels (*24*−*27*), consistent with the activation of the enzyme by PtdCho/DG mixtures in vitro (*24*, *28*, *29*). For example, colony-stimulating factor 1 addition to BAC1.2F5 macrophage cells results in biphasic elevation of the DG level (*30*), which can contribute to the stimulation of CCT activity and PtdCho biosynthesis following growth factor addition (*31*−*33*).

The CCTα (*34*), CCTβ1 (*35*), and CCTβ2 (*36*) isoforms have nearly identical membrane-binding domains characterized by three amphipathic α-helical repeats, and all three isoforms associate with the endoplasmic reticulum (*36*). CCTα and CCTβ2 have carboxy-terminal domains in which the last 55 residues include multiple phosphorylation sites (*36*, *37*), whereas CCTβ1 is smaller, lacking the additional phosphorylation domain (*35*, *36*). In addition to the endoplasmic reticulum, the majority of CCTα is distributed throughout the interphase nucleus (*36*, *38*−*40*) and associates with the nuclear membrane following exposure to oleic acid (*1*) or DG generated by phospholipase C treatment (*41*). A reduction in the diffuse nuclear CCTα staining correlates with stimulation of cellular PtdCho synthesis (*1*, *33*, *41*) and

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[1] Abbreviations: CCT, CTP:phosphocholine cytidylyltransferase; PtdOH, phosphatidic acid; PtdIns, phosphatidylinositol; PtdEtn, phosphatidylethanolamine; DG, diacylglycerol; and PtdCho, phosphatidylcholine.

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is associated either with punctate staining at the nuclear membrane in response to oleic acid (*1*) or phospholipase C (*41*) or with increased staining outside the nucleus in response to serum (*33*). Soluble CCT released from permeabilized cells may represent nucleoplasmic CCTα (*36*, *39*) together with CCT that is loosely bound to intracellular membranes (*42*) and exhibits a basal activity that can be stimulated upon addition of mixed PtdCho vesicles (*11*, *43*−*45*). Thus, the CCTα that is not associated with the membrane fraction has been postulated to be essentially inactive (*43*−*48*). However, the basal activity of soluble CCT is eliminated by extraction of the protein with nonionic detergents (*12*, *15*, *29*, *32*, *49*−*51*) or organic solvent (*13*), and subsequently enzymatic activity is restored upon readdition of lipid activators (*12*, *13*, *15*, *29*, *32*, *43*, *49*−*51*). These data suggest the presence of endogenous lipid tightly bound to soluble CCT and support the strict dependence of enzyme activity on lipid interaction with the protein.

The CCTα amphipathic helical domain interacts with PtdCho vesicles and membranes (*52*−*60*) and a transition from random coil to a helix conformation occurs upon the addition of lipid (*52*, *55*, *57*). The analyses to date of CCTα carboxy-terminal truncation mutants are consistent with the idea that the helical domain is both necessary and sufficient for responsiveness to lipid vesicles (*50*, *51*, *54*, *61*), and residues 267−277, encompassing the central 11mer repeat, are reported to be required for the interaction of CCTα with vesicles (*60*). Interaction between lipid and the helical domain has been proposed to remove an inhibitory constraint on activity (*51*, *61*, *63*), and the CCTα catalytic fragment has activity greater than the full-length protein in the absence of lipid (*63*). The nature of the lipid interaction with the helical region has been studied extensively, and electrostatic interaction between lipid and protein (*64*−*66*), peptide hydrophobicity (*42*, *64*), and lipid packing (*29*, *67*, *68*) are all contributing factors. Interestingly, the yeast CCT is also activated by lipid but lacks an identifiable amphipathic α-helical domain (*69*). In contrast to the mammalian CCTα, yeast CCTα is responsive to anionic lipids but not neutral lipids (*70*), suggesting the presence of a distinct lipid regulatory domain.

The CCTα carboxy-terminal domain is phosphorylated at multiple sites (*37*) in a reversible manner (*1*, *32*, *71*, *72*). Dephosphorylation of the carboxy-terminal domain correlates with the regulation of CCTα membrane association in response to exposure to exogenous fatty acids (*1*) or treatment with phospholipase C (*71*). However, CCTα also translocates prior to dephosphorylation (*72*) or becomes dephosphorylated without an associated translocation event (*73*). Truncation of the carboxy-terminal domain (*54*) or substitution of all of the serine phosphorylation sites with either alanine or glutamate does not quantitatively alter the cellular distribution of the protein (*74*), suggesting that CCTα phosphorylation and membrane translocation are independent events. However, the phosphorylated carboxy-terminal domain alters the kinetic properties of lipid activation by exerting a negative cooperative effect on the affinity of the enzyme for lipid (*50*), illustrating a role for the carboxy-terminal domain in the lipid regulation of enzyme activity.

In this study, the CCTα helical region was truncated successively to determine the number of helical repeats that were sufficient for lipid-responsive regulation of enzyme activity. The helical region was also selectively deleted to test its inhibitory role and to determine whether activation by lipids was abrogated. Surprisingly, CCTα protein that lacked the helical domain but retained the carboxy terminal domain exhibited the same activities as full-length CCTα in the absence and presence of lipid, suggesting that the last 57 residues of the carboxy-terminus also regulated lipid responsiveness. This region of the CCTα protein mediated interaction with anionic lipids and not neutral lipids, similar to the yeast CCT. These data delineate the lipid-mediated regulatory functions for the CCTα carboxy terminus and suggest that a domain within the last 57 residues of the carboxy-terminus can influence the catalytic domain either alone or together with the helical domain.

EXPERIMENTAL PROCEDURES

Materials. Sources of supplies were Amersham Pharmacia Biotech, phospho[*methyl*-^{14}C]choline (specific activity 58 mCi/mmol); American Radiolabeled Chemicals, Inc., [*methyl*-^{3}H]choline (specific activity 80 Ci/mmol); Life Technologies, Inc., LipofectAMINE reagent; Promega, restriction endonucleases and other molecular biology reagents; Invitrogen, pcDNA3 and pcDNA3.1 vectors; Sigma, CTP, rabbit IgGs, phosphocholine, oleic acid, Triton X-100, DEAE-Sepharose, and buffers; Analtech, thin-layer chromatography plates; Avanti Polar Lipids, dioleoyl-PtdCho and dioleoyl-PtdOH; and Serdary, diolein. Anti-CCTα rabbit polyclonal antiserum was raised against a synthetic peptide (MDAQSSAKVNSRKRRKE) corresponding to the first 17 amino acids of CCTα. Antiserum was purified by affinity chromatography on Affi-Gel-10 cross-linked to the peptide as described previously (*75*). All other chemicals were reagent-grade or better.

Construction of CCTα Mutants. Construction of CCTα-[Δ312−367], CCTα[Δ257−367], and CCTα[Δ229−367] has been described previously (*51*). CCTα[Δ236−367] was constructed as described (*63*). The deletion mutants CCTα-[Δ299−367], CCTα[Δ287−367], CCTα[Δ276−367], and CCTα[Δ265−367] were constructed using a common forward primer 5′-AGAAGATGGATGCACAGAGTTC and specific reverse primers that had a stop codon and a *Hind*III site: 5′-TCAGCATAAGCTTCTACGCTCCT for CCTα-[Δ299−367]; 5′-CCAGGAAGCTTCAAATGAACT for CCTα[Δ287−367]; 5′-TCCTCAAGCTTCTAGATGAGGTCG for CCTα[Δ276−367]; 5′-CTCCTCAAGCTTCTACACAAATTC for CCTα[Δ265−367]. CCTα[Δ257−309] was constructed using the forward primer 5′-AGGGAAAAGATCTGATGCTGC that inserts a *Bgl*II site before residue 309 and the reverse primer 5′-TTCCTCCAGATCTTTCACTT, which inserts a *Bgl*II site after residue 256 and converts valine at position 256 to leucine. The sequences of the above mutants were confirmed, and expression vectors were constructed in pcDNA3.1. CCTα[Δ257−309] was amplified by PCR and subcloned into the pET-15b bacterial expression vector using the *Nde*I and *Bam*HI sites. The primers used for the amplification were forward primer 5′-CATATGGATGCACAGAGTTCAGC, which creates an *Nde*I site in front of the starting methionine and the reverse primer 5′-GGATCCTTAGTCCTCTTCATCCTCGC, which creates a *Bam*HI after the stop codon. The DNA sequence was confirmed.

Preparation of Lipid Mixtures. Lipid activators were prepared either as PtdCho vesicles or as Triton X-100 mixed micelles. The vesicles were prepared by combining the appropriate amounts of chloroform solutions, evaporating the solvent under nitrogen and resuspending the mixture in 1.5 M bis-Tris-HCl, pH 6.5. The vials that contained PtdCho were sonicated 3 × 30 s. The micelles were prepared by resuspending the appropriate amount of lipid in 1.5 M bis-Tris-HCl, pH 6.5, that contained the appropriate concentration of Triton X-100. The vials were vortexed and rotated at 4 °C overnight.

CCT Assay. CCT activity was determined essentially as described previously (*50*). The standard assay contained 150 mM bis-Tris-HCl, pH 6.5, 10 mM $MgCl_2$, 1.04 mM phospho-[^{14}C]choline (specific activity 2.4 mCi/mmol) and the concentration of CTP and lipid activator indicated in the figure legends. The final volume of the reactions was 50 μL. The reaction mixtures were incubated at 37 °C for 10 min. The reaction was stopped by the addition of 5 μL of 0.5 M EDTA, and the tubes were vortexed and placed on ice. Next, 35 μL of the reaction mixtures were spotted on preadsorbent Silica Gel G thin-layer chromatography plates, which were developed in 2% ammonium hydroxide and 95% ethanol (1:1, v/v). CDP-[^{14}C]choline was identified by comigration with a standard, scraped from the plate, and quantified by liquid scintillation counting. Protein was determined according to the Bradford method (*76*).

Removal of Endogenous Lipid. COS-7 cell pellets were lysed by incubation in lysis buffer (10 mM HEPES, pH 7.4, 10 mM NaCl, 2 mM dithiothreitol, 1 mM EDTA, 1 mM phenylmethanesulfonyl fluoride, 50 mM NaF, and 100 mM Na_3VO_4) for 1 h on ice. The cells were disrupted by sonication, and the particulate matter was removed by centrifugation. The supernatant was loaded onto a 0.5-mL DEAE-Sepharose column, and the column was washed with 1.5 mL of each of the following in succession: lysis buffer, lysis buffer plus 1% Nonidet P-40, lysis buffer, lysis buffer plus 0.25 M NaCl, and lysis buffer plus 0.5 M NaCl. CCT activity was eluted in the 0.25 M NaCl fraction.

Transfection and Metabolic Labeling Experiments. COS-7 cells were grown in 100-mm dishes to 80% confluency in Dulbecco's modified eagle's medium supplemented with 10% fetal bovine serum and 1% glutamine at 37 °C. CHO58 cells were grown in 100-mm dishes at 33 °C in Ham's F-12 medium supplemented as above. COS-7 or CHO58 cells were transfected using the LipofectAMINE reagent according to the manufacturer's instructions, and 48 h post transfection CHO58 cells were transferred to 40 °C. After incubation for an additional 72 h at 40 °C, CHO58 cells were washed with 10 mL of phosphate-buffered saline and incubated in trypsin. Five minutes later, the cells were collected, centrifuged, resuspended in 5 mL of medium, and counted. The metabolic labeling experiments were performed 48 h post transfection by washing the cells, incubating at 33 °C or 40 °C for 12 h, and then exchanging the cells into medium containing [^{3}H]-choline (5 μCi/mL). Cells were incubated for an additional 6 h either at the permissive or the restrictive temperature. At the end of the 6 h, cells were washed with 10 mL phosphate-buffered saline and collected, and the cell pellets were extracted using the Bligh−Dyer method (*77*).

Bacterial Expression and CCTα[Δ257−309] Purification. BL21-CodonPlus(DE3)-RIL *Escherichia coli* strain was transformed with the pET15b expression vector containing CCTα[Δ257−309]. Expression of the mutated CCT was induced by addition of 1 mM isopropyl-β-D-thiogalactopyranoside in the culture medium for 3 h. Cells were collected and lysed, and the His-tagged CCTα[Δ257−309] was purified using a Ni^{2+}-NTA agarose column according to the manufacturer's instructions.

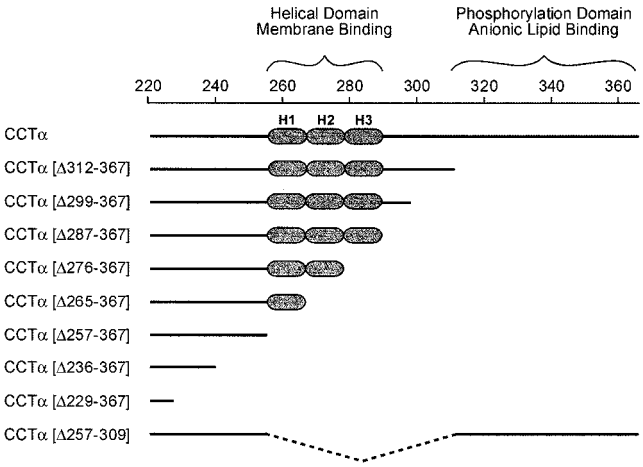

FIGURE 1: Structure of the CCTα deletion mutants. Diagrammatic representation of the structure of CCTα and CCTα deletion mutants. The entire structure of CCTα is not shown and the diagram begins at residue 220 located just after the predicted catalytic core of the protein. CCTα[Δ312−367] lacks the carboxyl-terminal phosphorylation/anionic lipid regulatory domain. The CCTα[Δ299−367] and CCTα[Δ287−367] retain the three 11-residue repeated amphipathic α-helices. The CCTα[Δ276−367] lacks one helical repeat, CCTα[Δ265−367] lacks two repeats, and CCTα[Δ257−367] lacks three repeats. CCTα[Δ236−367] lacks the helical domain but retains the entire predicted catalytic domain, while CCTα[Δ229−367] lacks seven residues of the catalytic region. CCTα[Δ257−309] lacks the membrane interaction domain defined by three consecutive amphipathic α-helical repeats (H1, H2, and H3), and fusing this domain to residue 257 retains the carboxy-terminus of the protein.

Immunoblots and Protein Determination. Cell lysates (50 μg of protein) were separated by SDS−gel electrophoresis on 12% polyacrylamide gels and transferred by electroblotting onto nitrocellulose membranes. Immunoblotting was performed by incubation of the membranes with purified anti-CCTα (1:2000 dilution) antibody. ^{125}I-protein A was used to identify the immunoreactive proteins. Specified amounts of IgGs were separated by SDS−gel electrophoresis on 12% polyacrylamide gels and transferred by electroblotting onto nitrocellulose membranes. ^{125}I-protein A was coupled to IgGs, and the signal was quantitated using a phosphoimager (Molecular Dynamics). A calibration curve was created by plotting the signal versus the amount of IgGs (Figure 2). On the basis of this curve and the quantitation of the signal on CCT immunoblots, we calculated the amount of CCT protein in each sample. Our calculations were based on an IgG molecular mass of 55 kDa, on the fact that two molecules of IgG react with one epitope and took into consideration the molecular mass of each mutant.

RESULTS

CCTα Truncation Mutants. A series of deletion mutants in CCTα were constructed to examine the role of the carboxy-terminal domains in lipid regulation (Figure 1). The CCTα[Δ312−367] mutant lacked the carboxy-terminal

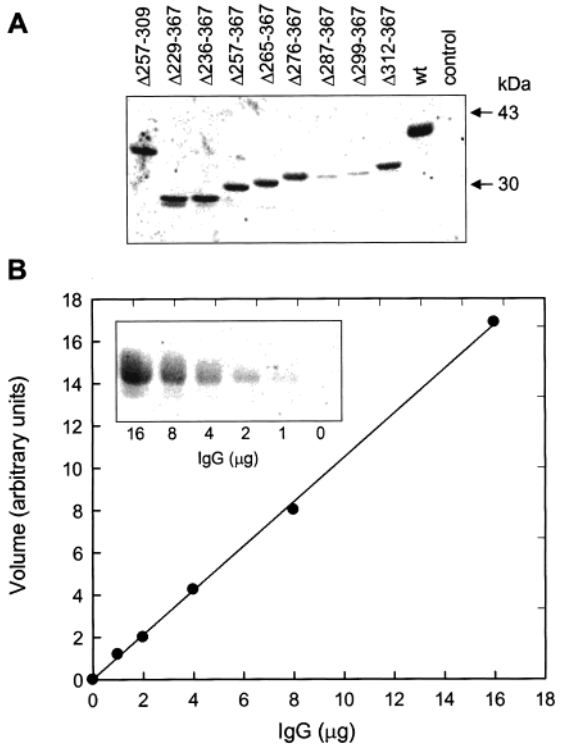

FIGURE 2: Quantitation of protein expression of CCTα deletion mutants. Panel A, COS-7 cells were transfected with vector alone or vectors carrying cDNAs encoding the specified CCTα mutants. Cell lysates were analyzed by immunoblotting 48 h later. Samples of the total cell lysates were probed with anti-CCTα antibody and detected with ^{125}I-labeled protein A. The signal was quantitated and the amount of each protein was calculated as described under Experimental Procedures. Panel B, calibration curve for the quantitation of CCTα deletion mutants. The indicated amounts of IgGs were probed with ^{125}I-labeled protein A and the signal was quantitated as described under Experimental Procedures. The calibration curve was created by plotting the signal versus the amount of IgGs. The experiment was representative of the results from three independent transfection experiments.

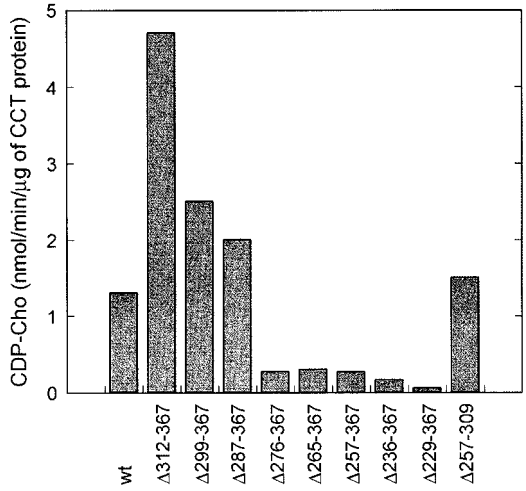

FIGURE 3: Specific activity of CCTα deletion mutants. COS-7 cells were transfected with vector alone or vectors carrying cDNAs encoding the specified CCTα mutants. The cells were harvested 48 h after transfection, and extracts were prepared and assayed for CCT activity in the presence of 10 mM CTP as described under Experimental Procedures. The mass of each CCTα mutant was determined based on the calibration curve shown in Figure 2 and taking into account the molecular mass of each mutant. The assays were carried out using delipidated COS-7 cell lysates in the presence of 160 μM PtdCho/oleic acid to maximally activate the enzymes, and the plotted values are the average of duplicate determinations of enzyme specific activity and are representative of two independent trials.

residues known as the phosphorylation domain and retained the entire helical domain (*50*, *51*). The CCTα[Δ299−367] and CCTα[Δ287−367] were shorter truncation mutants that still retained all three 11-residue repeated amphipathic α-helices. The CCTα[Δ276−367] lacked one repeat, CCTα-[Δ265−367] lacked two repeats, and CCTα[Δ257−367] lacked three helical repeats. CCTα[Δ236−367] retained the predicted catalytic domain, while CCTα[Δ229−367] lacked seven residues of the predicted catalytic region. CCTα-[Δ257−309] was an internal deletion mutant constructed by removing the α-helices and fusing the remaining carboxy-terminus to the catalytic domain. The mutated cDNAs were subcloned into pcDNA3 (or pcDNA3.1) vector and transfected into COS-7 cells. After 48 h, COS-7 cells were lysed and protein levels were quantitated by immunoblotting with a CCTα-specific rabbit antibody raised against the amino-terminal epitope followed by incubation with ^{125}I-protein A. The phosphoimager exposures for each CCTα protein were compared to a calibration curve of rabbit IgG protein blotted with ^{125}I-protein A in the same manner (Figure 2). This approach was necessary because the expression levels of CCTα[Δ299−367] and CCTα[Δ287−367] were consistently lower than the other proteins.

Enzymatic Activity of the Truncation Mutants. CCT activity was assayed in cell lysates in the presence of 160 μM PtdCho/oleic acid vesicles (1:1), a potent lipid mixture for activation. The CTP concentration was increased to 10 mM to permit evaluation of the low range of activities encountered with the truncated proteins. Comparison of the specific activities of the fully activated proteins (Figure 3) demonstrated that removal of the carboxy terminus in CCTα-[Δ312−367] resulted in a 3-fold increase and suggested that the carboxy-terminus attenuated the maximum response to PtdCho mixed vesicles. These data were consistent with the idea that the carboxy-terminal domain exerted a negative effect on the regulation of activity when the helices were fully engaged with lipid (*50*).

Further truncations of the protein resulted in progressively less total activity in the presence of PtdCho/oleic acid as compared to the CCTα[Δ312−367]. CCTα[Δ287−367] retained the three amphipathic helical repeats and had slightly higher activity as compared to the full-length protein, whereas a significant decrease in the activity of the mutants was observed in the truncations beginning with CCTα-[Δ276−367], indicating that lipid-induced conformational change was lost in all these proteins. Truncation of the remainder of the helical domain in constructs CCTα[Δ236−367] and CCTα[Δ229−367] resulted in further loss of activity. Selective excision of the helical region in CCTα-[Δ257−309] resulted in activity equivalent to the full-length protein (Figure 3).

The CCT activity in COS cells without expression of any construct was 2.8 nmol min^{-1} (mg of lysate protein)$^{-1}$, as compared to overexpression of the full-length wild-type CCTα, which was 150-fold higher at 440 nmol min^{-1} (mg of lysate protein)$^{-1}$ or the CCTα[Δ236−367], which was 4.5-fold higher at 12.6 nmol min^{-1} (mg of lysate protein)$^{-1}$.

Lipid Regulation in CCTα Deletion Mutants. The prevailing model for lipid regulation of CCTα predicted that the

Table 1: Activities of CCTα Mutants in the Presence and Absence of Lipid Activator[a]

enzyme	nmole min^{-1} mg^{-1}	
	no lipid	PtdCho/oleic acid
CCTα	9.2 ± 2.1	435.2 ± 20.3
CCTα[Δ312−367]	8.3 ± 1.6	488.6 ± 32.4
CCTα[Δ257−309]	7.9 ± 1.8	422.4 ± 18.5
CCTα[Δ299−367]	2.4 ± 0.5	32.9 ± 4.3[b]
CCTα[Δ287−367]	7.2 ± 1.1	48.8 ± 5.6[b]
CCTα[Δ276−367]	20.7 ± 1.6	26.2 ± 3.8
CCTα[Δ265−367]	24.7 ± 0.8	21.9 ± 2.2
CCTα[Δ257−367]	17.1 ± 2.2	19.1 ± 1.5
CCTα[Δ236−367]	12.9 ± 0.5	13.6 ± 1.4

[a] COS-7 cells were transfected with vectors expressing CCTα deletion mutants, harvested and lysed 48 h post transfection, and the cell lysates were prepared. Endogenous lipids were removed by ion-exchange chromatography and enzyme activities were measured as described under Experimental Procedures. The enzyme specific activities were determined in the absence and presence of 160 μM PtdCho/oleic acid (1:1) using 10 mM CTP, and the error bars represent the range of data points. [b] The maximum activities in these two samples in the presence of lipid were less than observed with CCTα, CCTα[Δ312−367], and CCTα[Δ257−309] due to the lower level of protein expression (see Figure 2, panel A).

amphipathic helical repeats between residues 257 and 310 were responsible for lipid interaction. Clearly, the removal of the helical domain by carboxy-terminal truncations beginning at residue 257 yielded proteins that exhibited lipid dysregulation characterized by either increased activity in the absence of lipid or an inability to be stimulated by activating lipid mixtures (*51*, *61*, *63*). We analyzed the activities of the mutant enzymes and characterized their response to lipid regulators in vitro to delineate in more detail the protein domains involved in regulation. The lipid responsiveness of the mutants was examined after removal of the endogenous lipids as described under Experimental Procedures. We determined the activities of the delipidated proteins in the absence or presence of 160 μM PtdCho/oleic acid (Table 1). Two major observations were made in Table 1. First, CCTα[Δ257−309] activity was very low in the absence of lipid and was stimulated about 50-fold upon addition of lipids, similar to the full-length CCTα and CCTα-[Δ312−367]. This was surprising since the amphipathic helices were absent in the CCTα[Δ257−309] mutant (Figure 1), and it was anticipated that the protein would be nonresponsive to lipids. Second, removal of one of the identical helical repeats (between residues 276−287) resulted in higher basal activity in the absence of lipid, and lipid activation was also lost. These data suggested that both the latter 57-residue portion of the carboxy-terminal domain and the full-length amphipathic helical domain conferred the capacity for regulation of CCTα by lipid. These domains suppressed the basal activity of the enzyme in the absence of lipid and also mediated the dramatic stimulation of activity in association with lipid.

To further characterize the lipid response of the CCTα-[Δ257−309] mutant, we determined the abilities of three lipid mixtures to reactivate the full-length CCTα, CCTα-[Δ312−367] lacking the terminal domain, or CCTα[Δ257−309] lacking the helical region (Figure 4). Removal of the endogenous lipids diminished the activity of all three enzymes to barely detectable levels (Table 1, Figure 4), which were restored to the same extent by the addition of

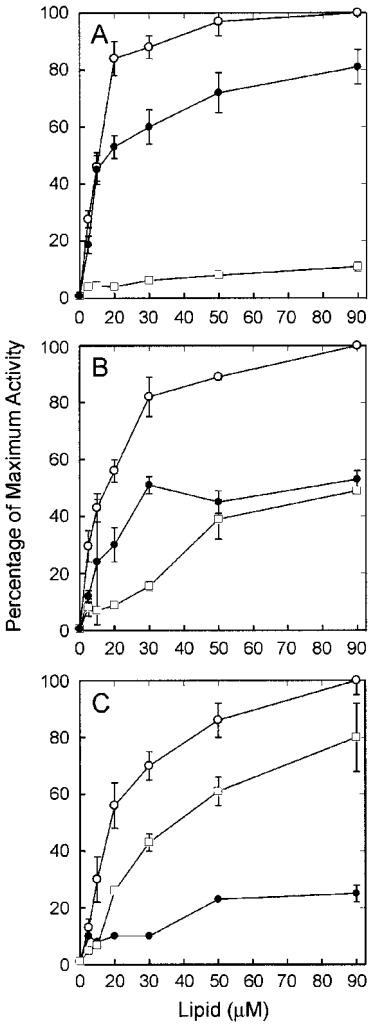

FIGURE 4: CCTα[Δ257−309] was activated by anionic lipids. COS-7 cells were transfected with CCTα, CCTα[Δ312−367], or CCTα[Δ257−309] expression constructs, and 48 h later, the cells were harvested and lysed. Endogenous lipid activators were removed by anion exchange chromatography, and the ability of PtdCho/oleic acid (1:1) (○), Triton/oleic acid (1:1) (●), or PtdCho/diolein (1:1) (□) to restore CCT activity was determined in the presence of 2 mM CTP as described under Experimental Procedures. Panel A, CCTα[Δ257−309] specific activities ranged from 6.5 to 395 nmol min^{-1} mg^{-1} and was activated by PtdCho/oleic acid (○) and Triton/oleic acid (●) but not significantly by PtdCho/diolein (□). Panel B, CCTα specific activities ranged from 8.8 to 440 nmol min^{-1} mg^{-1} and was activated by all three lipid mixtures. Panel C, CCTα[Δ312−367] specific activities ranged from 11.7 to 470 nmol min^{-1} mg^{-1} and was activated by PtdCho/oleic acid (○) and PtdCho/diolein (□) and to a lower extent by Triton/oleic acid (●). The experiments were repeated twice in duplicate, with essentially identical results. The error bars represent the range of duplicate data points in one experiment.

PtdCho/oleic acid (1:1) vesicles to each of the protein samples (Figure 4 and Table 1). The data in Table 1 point out that the activities of these three constructs in the absence of lipid were about 50% as compared to truncated CCTα proteins that lacked both the extreme carboxy-terminal domain and the three helices. Mixtures of Triton/oleic acid (1:1) were also able to stimulate the CCTα enzyme to about 50% of maximum activity and the CCTα[Δ257−309] to about 80%. This lipid composition did not activate the CCTα[Δ312−367] efficiently, however, to only about 25% of the maximum, suggesting that the molecular context

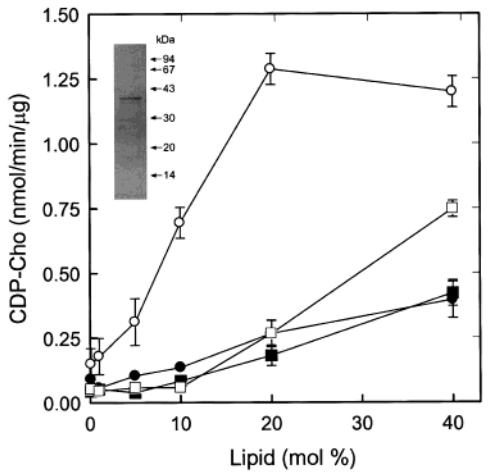

FIGURE 5: Triton/PtdOH selectively activated CCTα[Δ257−309]. CCTα[Δ257−309] specific activity was determined in the presence of different concentrations of either PtdOH, presented in either Triton X-100 (○) or in PtdCho (●), and oleic acid, presented in either Triton X-100 (□) or PtdCho (■). The final concentrations of lipids in the reaction mixtures were 160 μM. CCTα[Δ257−309] was expressed in *E. coli* and purified as described under Experimental Procedures. The inset shows the protein following SDS−gel electrophoresis through a 12% polyacrylamide gel and stained with Coomassie Blue. The error bars represent the range of duplicate data points in one of two experiments, which yielded similar results.

of the lipid activator was important for interaction with the helical domain. In contrast, PtdCho/diolein (1:1) mixtures activated CCTα to about 50% of the activity obtained with PtdCho/oleic acid and activated CCTα[Δ312−367] to about 80% but failed to significantly stimulate CCTα[Δ257−309] (only 10% activation) above basal activity (6.5 nmol min^{-1} mg^{-1}) in the absence of lipids. Under the same conditions, vesicles made up of PtdCho alone increased activity by only 5% (data not shown). These data suggested that removal of the carboxy-terminal domain increased the protein's responsiveness to neutral lipid. We also assayed the three enzymes in the presence of PtdCho/monoacylglycerol (1:1) vesicles. The activation of both CCTα and CCTα[Δ312−367] by these lipid vesicles was identical to the activation of these proteins by PtdCho/diolein shown in Figure 4, panels B and C. Likewise, the response of CCTα[Δ257−309] to PtdCho/monoacylglycerol was the same as to PtdCho/diolein shown in Figure 4, panel C, indicating that the carboxy-terminal domain was nonresponsive to neutral lipids. On the other hand, responsiveness to neutral lipids was a distinguishing characteristic of the helical domain. The helical domain also interacted with anionic lipids and mediated enzyme activation together with the carboxy-terminus in response to anionic lipids.

Lipid Activation Specificity of the Carboxy Terminal Domain. We investigated the ability of other anionic phospholipids to activate CCTα[Δ257−309] to determine if there was any selectivity for this mode of CCTα lipid regulation. These experiments were performed with His-tagged CCTα-[Δ257−309] that was expressed in *E. coli* and purified by affinity chromatography to corroborate the results in the COS-7 cell system (see insert, Figure 5). This enzyme preparation was activated by oleic acid presented either as PtdCho vesicles or in Triton X-100 (Table 2). In addition to oleic acid, PtdOH, LPA, and PtdIns stimulated the activity

Table 2: Activation of CCTα[Δ257−309] by Anionic Phospholipids[a]

lipid activator		
vehicle	anionic lipid, 20 mol %	nmol min^{-1} μg^{-1}
Triton X-100	PtdOH	1.287
PtdCho	PtdOH	0.266
Triton X-100	oleic acid	0.286
PtdCho	oleic acid	0.182
Triton X-100	LysoPtdOH	0.077
Triton X-100	PtdIns	0.257
Triton X-100	PtdInsP	0.042
Triton X-100	$PtdInsP_2$	0.035
none	PtdOH (100%)	0.039
none	none	0.032

[a] CCTα[Δ257−309] was expressed in *E. coli* and purified as described under Experimental Procedures. The specific activity (2 mM CTP) was determined in the absence of lipids or in the presence of 20 mol % anionic lipids in PtdCho or Triton X-100 (final concentration of lipid activator 160 μM) as described under Experimental Procedures. Data are the average of duplicate determinations.

of the CCTα[Δ257−309], whereas PtdInsP or $PtdInsP_2$ did not restore substantial enzyme activity when presented with Triton X-100 (Table 2). This survey showed that PtdOH was the most potent of the anionic lipids tested. Regulation by PtdOH was further investigated by examining CCTα[Δ257−309] activation as a function of the mole fraction of anionic lipid in Triton X-100 (Figure 5). The enzyme was selectively activated by PtdOH in Triton X-100 between 5 and 20 mol % (Figure 5) supporting the conclusion that the carboxy-terminal domain has a preference for this anionic lipid. Increasing the PtdOH concentration to 40 mol % did not stimulate the enzyme further (1.195 nmol min^{-1} μg^{-1}). However, lipid mixtures of PtdCho/PtdOH, Triton X-100/oleic acid, and PtdCho/oleic acid containing 40 mol % anionic lipid elevated enzyme activity over the values obtained at 20 mol % (0.920, 0.845, and 0.733 nmol min^{-1} μg^{-1}, respectively) illustrating that higher concentrations of anionic lipids were able to substitute for PtdOH.

All Three Helical Repeats are Required for Activation Mediated by the Helical Domain. The data in Table 1 suggested that deletion of the residues 276−287 resulted in a protein that was no longer activated by lipids. To further characterize the minimal length of the helical domain required for lipid activation, we delipidated CCTα[Δ287−367], CCTα[Δ276−367], and CCTα[Δ265−367] and monitored the ability of PtdCho/oleic acid vesicles to stimulate CCT activity. As shown in Figure 6 only the CCTα[Δ287−367] mutant that retains all three helical repeats is activated by lipids. On the contrary, both CCTα[Δ276−367] and CCTα[Δ265−367] (missing one and two helical repeats, respectively) are not responsive to lipid. Taking into consideration that the helical repeats are almost identical, the above data indicated that the presence of two of the three helices was not sufficient for significant conformational activation of the protein following lipid-induced helix formation.

Biological Activity of the Mutants. The in vivo activities of the CCTα mutant proteins were evaluated by determining if they were capable of complementing the temperature-sensitive growth phenotype of the CHO58 cell line, which has a defect in CCT activity. The CHO58 cell line is conditionally defective for CCT activity (*78*, *79*) and cannot

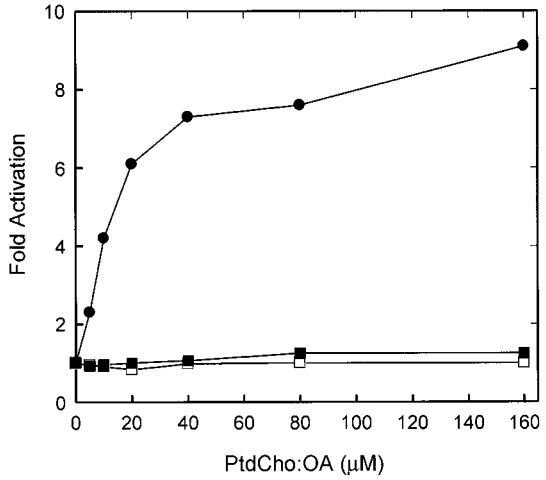

FIGURE 6: All three helices are required for lipid activation by the α-helical domain. COS-7 cells were transfected with vector alone or vectors carrying cDNAs encoding mutants CCTα[Δ287−367] (●), CCTα[Δ276−367] (■), and CCTα[Δ265−367] (□). The cells were harvested 48 h after transfection, cell lysates were delipidated as described under Experimental Procedures, and the ability of PtdCho/oleic acid (1:1) vesicles to activate catalytic activity was tested. CCT assays were performed in the presence of 10 mM CTP. The experiment was performed twice with similar results. The data points are the average of duplicate determinations from one representative experiment.

synthesize sufficient PtdCho to support growth at 40 °C. CHO58 cells were transfected with cDNAs encoding the full-length CCTα, CCTα[Δ312−367], CCTα[Δ257−309], CCTα-[Δ299−367], CCTα[Δ287−367], CCTα[Δ276−367], CCTα-[Δ265−367], CCTα[Δ257−367], CCTα[Δ236−367], CCTα-[Δ229−367], and an empty vector control. The plates were transferred to the restrictive temperature 48 h after transfection, and following incubation for 72 h at 40 °C, the cell number in each dish was determined. All of the CCT constructs supported the growth of CHO58 cells at the restrictive temperature, and there was no significant difference among the cell numbers of mutants and full-length CCTα, which were all significantly different from the control vector dishes (Figure 7, panel A).

The effect of deleting portions of the CCTα protein on PtdCho biosynthesis was compared to the full-length protein by metabolic labeling of transfected CHO58 cells with [^{3}H]-choline. CHO58 cells were transiently transfected with the CCTα[Δ257−309], CCTα[Δ236−367], and CCTα[Δ229−367] deletion constructs, full-length CCTα, and an empty control vector and transferred to 40 °C 48 h later. After 12 h at the restrictive temperature, the cells were labeled for 6 h with [^{3}H]choline. [^{3}H]Choline incorporation into PtdCho was the same with all the constructs tested as compared to the empty vector control, with the exception of CCTα-[Δ229−367], which had reduced PtdCho biosynthesis (Figure 7, panel B). CCTα[229−367] had the lowest in vitro activity as compared to the other constructs (Figure 7, panel B), and yet overexpression still increased PtdCho biosynthesis sufficiently above what was required for cell survival at 40 °C (Figure 7, panel B). These data show that dysregulated or catalytically defective CCTα proteins functioned in vivo. However, the overexpression experiments were a blunt tool for the assessment of CCTα functionality since endogenous levels of CCT activity were low and the highly expressed enzymes (Figure 3) only needed to retain

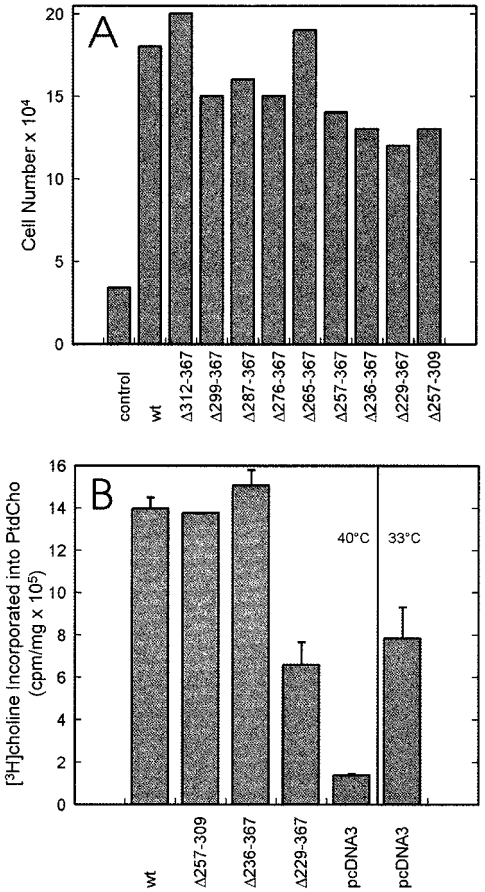

FIGURE 7: Biological activity of CCTα deletion mutants. Panel A, CHO58 cells were transfected with a vector control and vectors carrying cDNAs encoding the full-length CCTα and the deletion mutants. Forty-eight hours after transfection, the cells were transferred to 40 °C and incubated for an additional 72 h following determination of the cell number in each dish. The data represent the average of duplicate cell number determinations in duplicate dishes. Panel B, CHO58 cells were transfected with vector alone or vectors expressing CCTα, CCTα[Δ257−309], CCTα[Δ236−367], and CCTα[Δ229−367]. Forty-eight hours post-transfection, the dishes were transferred to 40 °C and incubated for 12 h. Cells were subsequently labeled with [^{3}H]choline for 6 h at 40 °C. The cells were harvested, and the incorporation of label into PtdCho was determined. Dishes transfected with vector alone were labeled as above but remained at 33 °C. The error bars represent the range of duplicate data points in the experiment, and the results were confirmed by a second, independent set of transfection experiments.

marginal catalytic activity to exceed this level (Figure 3) and support the operation of the CDP-choline pathway.

DISCUSSION

The discovery of a second lipid regulation domain on CCTα helps to explain the observed complexity of the lipid regulation of CCTα and ascribes a regulatory function to the extreme carboxy-terminal domain for the first time. Two domains, a helical region spanning residues 257 through 287 and a domain within the last residues 314 through 367, mediate lipid activation of CCTα. Lipid interactions with either domain relieve the suppression of basal activity and promote the protein transition to a highly active conformation. Our data, together with previous investigations by others, suggest that the two domains can mediate enzyme activation of the full-length protein either independently or cooperatively. Full-length CCTα is activated by PtdCho/

diolein that does not interact with the carboxy-terminus alone (Figure 4). Full-length CCTα also interacts with Triton/PtdOH, which can fully activate the enzyme in the absence of the helical region (Figure 5). This model is consistent with previous observations. First, proteolysis protection experiments indicated that the helical region was embedded in PtdCho/diolein, whereas both the helical and the carboxy-terminal domains were protected by PtdCho/anionic lipid vesicles (*53*). Second, CCTα is activated in a synergistic manner using low concentrations of PtdOH and diolein in mixed PtdCho vesicles (*64*, *66*), consistent with cooperative binding of lipid to two sites on the protein. Finally, the biphasic binding of CCTα to PtdCho/PtdOH vesicles (*66*) also supports a model in which the lipid interaction is mediated by two distinct mechanisms that we propose represent the two lipid interaction domains on the protein.

The second lipid activation domain has a dual role in the regulation of CCTα. The extreme carboxy-terminus is a site for interaction with anionic lipid and also suppresses the maximum activity in the presence of PtdCho/oleic acid vesicles (Figure 3). This conclusion is consistent with an earlier investigation of the kinetics of lipid activation suggesting that the last 57 residues interfered with lipid activation of CCTα (*51*). It is possible that this terminal peptide could bind to the helical domain when the latter is intercalated with lipid or, alternatively, compete with the helical domain for the available anionic lipid. In the absence of lipid, the second lipid activation domain alone can suppress the basal enzyme activity, similar to the helical domain in the absence of lipid (Table 1). These data suggest that the latter portion of the carboxy-terminus undergoes a conformation change in response to anionic lipids, by analogy with the lipid-induced helix formation of the peptide corresponding to the CCTα helical region (*57*). The interaction of low concentrations of PtdOH in Triton with full-length CCTα is sufficient to support full enzyme activation (data not shown) (*64*). Although it is difficult to ascertain whether a lipid interaction with either the helical or extreme carboxy terminal domain is preferred, these data raise the possibility that CCT regulation and activation in vivo are not strictly dependent on association with membrane bilayers.

All three of the amphipathic helical repeats spanning residues 257 through 287 are important for regulation of CCTα activity by this domain (Figure 6). A smaller fourth helix spanning residues 299 through 312 is not critical for lipid regulation as detected by our assays, although its removal results in decreased expression as compared to the CCTα[Δ312−367] (Figure 3). Truncation of one out of the three, 11-residue repeats results in a dysregulated protein whereby the basal activity is increased in the absence of lipid and the proteins cannot be activated (Table 1; Figure 6). A recent report concluded that residues 267 through 277 were necessary and sufficient for binding of CCTα fusion peptides to lipid vesicles (*60*). Since the 11mer repeats are nearly identical and a short central helical segment is all that is required for membrane association (*62*), it is not clear why this central portion of the helix would be unique. Our data suggest that vesicle binding mediated by a short helix is not sufficient for enzyme activation.

These data address the tenets of two proposed models for the regulation of CCT by lipids. One model states that the lipid-binding region of CCT inhibits CCT activity and that this inhibition is relieved upon binding of lipids (*61*, *63*). The second model states that binding of lipids triggers a conformational change that activates the enzyme (*51*). Delipidation and the use of high CTP concentrations allow the influence of the helical region on the basal catalytic activities in the absence of lipid to be assessed, and the data in this report are in general agreement with both hypotheses. We find that the removal of the lipid binding domains leads to a 2−3-fold increase in basal activities when different CCT mutant proteins are compared under the same conditions, whereas Kent and co-workers reported a 50-fold increase based on different kinetic parameters with different assay conditions for the CCT[Δ237−367] and the full-length protein (63). Nevertheless, the data from both labs are consistent with a role for the lipid regulatory domains in suppressing the activity of the enzyme in the absence of lipid. On the other hand, enzymes that contain intact lipid regulatory domains are activated at least 50-fold by the presence of lipid (Table 1), illustrating that the activation is the predominate mode of lipid regulation when all constructs are assayed under the same conditions. Kent and co-workers also report a 50-fold activation of the full-length enzyme by lipid (63), and thus, both mechanisms for the control of CCT activity are not mutually exclusive. The removal of the lipid regulatory domains results in a dysregulated protein that lacks both positive and negative control over CCT catalytic activity.

The extensive analysis of the response of CCT to lipids has been generally interpreted in light of a single domain involving the three α-helical repeats between residues 257 and 287. The amphipathic nature of the helical domain (*34*), the random coil to α-helix conformational change that occurs upon binding to PtdCho vesicles containing activating lipids (*55*, *57*), and the fact that truncation of this domain eliminates lipid responsiveness (*50*, *51*, *61*, *63*) strongly support a role in CCT regulation. Vesicle structure (*29*, *67*, *68*), hydrophobic forces (*42*, *64*), the net charge of the lipid within a PtdCho vesicle (*64*−*66*), and reduction of the CCT net charge (*64*, *66*) have all been suggested as mechanisms involved in CCT lipid regulation. A preference for the packing of the choline phospholipid headgroups in vesicles is not consistent with inhibition by lysoPtdCho, lysoPtdCho analogues, or sphingosine, and electrostatic interactions alone cannot account for the stimulation of CCT activity by uncharged lipids, such as DG and PtdEtn. However, these nonionic lipids share the ability to induce the polar/apolar interface of the bilayer to bend toward the polar environment with anionic fatty acids and PtdOH and are collectively known as type II amphiphiles (*80*−*82*). In contrast, lipid inhibitors of CCT, such as the antineoplastic lysophospholipid analogues, are characterized by their ability to induce the opposite bilayer conformation in which the polar/apolar interface curves away from the polar region and are known as type I amphiphiles (*80*−*82*). Both positive and negative CCT regulation by lipid vesicle mixtures (*29*, *67*, *67*, *68*) correlates with the biophysical characteristic termed stored curvature elastic stress (*83*). According to this hypothesis, vesicle curvature tendency determines the degree of CCT helix intercalation into the phospholipid matrix, which, in turn, determines activity (*67*). This hypothesis explains both positive and negative regulation and is consistent with the conclusion that the amphipathic helical domain adjusts CCT

activity by sensing the composition of the phospholipid bilayer that has evolved from a great deal of research in the area (*43*, *58*). However, our unanticipated discovery of a second anionic lipid-specific regulatory domain within the CCT carboxy terminal domain complicates the interpretation of CCT lipid regulation and opens the door to a new line of inquiry into the structure and function of this protein. We are currently investigating the influence of phosphorylation on the nature of lipid activation by the carboxy-terminal 57 residues.

There are interesting parallels between the regulation of yeast CCT and the mammalian CCT[Δ257−309] mutant that may suggest a role for this mode of regulation in cell physiology. Yeast CCT was not anticipated to be activated by lipids since it lacks an amphipathic α-helical domain (*84*); however, anionic lipids potently activate yeast CCT (*70*). Like CCTα[Δ257−309], yeast CCT is stimulated by PtdCho: anionic lipid but is not regulated by PtdCho/DG. Unlike mammalian CCT, yeast CCT is not required for the formation of the major phospholipid classes, but rather represents a salvage pathway (*85*) that is implicated in the regulation of vesicular trafficking (*86*, *87*, *87*−*89*). PtdOH and DG both play a role in vesicular fission and fusion (*87*, *90*, *91*), and the specific regulation of yeast and mammalian CCT by PtdOH may reflect the role of the CDP-choline pathway in controlling the levels of these lipid mediators in intracellular membranes. The association of CCTβ2 and significant amounts of CCTα with the intracellular membrane systems suggests a function distinct from the role of nucleoplasmic CCTα, or CCTβ1, which lacks the carboxy-terminal regulatory domain.

Note added in proof: A peptide spanning residues 236 through 268 and including a highly-charged, lysine-rich region together with the first 11mer repeat of the helical region has been shown to bind to acidic lipid vesicles (*57*), and one would expect the CCTα[Δ257-367] truncation mutant to respond to lipids if, in fact, this lysine-rich region was sufficient to promote CCTα enzyme activation. However, the data in Figure 3 and Table 1 show that the CCTα[Δ257-367] is not responsive to lipids and argue against interaction between lipids and the lysine-rich region in the intact protein.

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