Enzymatic and Cellular Characterization of a Catalytic Fragment of CTP:Phosphocho line Cytidylyltransferase α*

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To probe the mechanism of lipid activation of CTP:phosphocho line cytidylyltransferase (CCTα), we have characterized a catalytic fragment of the enzyme that lacks the membrane-binding segment. The kinetic properties of the purified fragment, CCTα236, were characterized, as well as the effects of expressing the fragment in cultured cells. CCTα236 was truncated after residue 236, which corresponds to the end of the highly conserved catalytic domain. The activity of purified CCTα236 was independent of lipids and about 50-fold higher than the activity of wild-type CCTα assayed in the absence of lipids, supporting a model in which the membrane-binding segment functions as an inhibitor of the catalytic domain. The kcat/Km values for CCTα236 were only slightly lower than those for lipid-activated CCTα. The importance of the membrane-binding segment in vivo was tested by expression of CCTα236 in CHO58 cells, a cell line that is temperature-sensitive for growth and CCTα activity. Expression of wild-type CCTα in these cells complemented the defective growth phenotype when the cells were cultured in complete or delipidated fetal bovine serum. Expression of CCTα236, however, did not complement the growth phenotype in the absence of serum lipids. These cells were capable of making phosphatidylcholine in the delipidated medium, so the inability of the cells to grow was not due to a defective phosphatidylcholine synthesis. Supplementation of the delipidated medium with an unsaturated fatty acid allowed growth of CHO58 cells expressing CCTα236. These results indicate that the membrane-binding segment of CCTα has an important role in cellular lipid metabolism.

CTP:phosphocholine cytidylyltransferase (CCT)1 is a critical participant in the CDP-choline pathway, catalyzing the synthesis of CDP-choline for the biosynthesis of phosphatidylcholine (PC), a major component of eukaryotic cell membranes (1, 2). CCT is rate-limiting for the CDP-choline pathway and is expressed (2). The recently discovered CCTα (3) exhibits tissue-specific expression (3). Mammalian CCTα contains several functional regions: an N-terminal nuclear localization signal, a central catalytic domain, a membrane/lipid (M/L) activation segment, and a C-terminal phosphorylation region (see Fig. 1). There is a high degree of sequence similarity within the catalytic domain of all known forms of CCT, with the yeast catalytic domain being 56% identical to that of mammalian CCTα, and the catalytic domains of CCTα and CCTβ being 90% identical. By contrast, there is much less conservation of primary structure in the N-terminal sequence or the C-terminal M/L and phosphorylation regions. Truncated forms of rat CCTα have been constructed to determine the regions responsible for lipid activation. Both full-length CCTα and truncated forms lacking the phosphorylation segment require lipids for full activity (4, 5). Truncations lacking the M/L region, however, are not lipid-activated (4–6) and do not translate to membranes in the cell (5). It is notable that CHO58 cells that are temperature-sensitive for growth and CCTα activity can be complemented by exogenous expression of the truncated CCTα lacking the M/L region, which suggested that this segment is not important for growth under normal cell culture conditions (5).

These studies have contributed to the general understanding that the M/L region is truly responsible for membrane binding in the cell and lipid activation in vitro. On the other hand, two important questions regarding the M/L segment remain. First, what is the mechanism by which the M/L segment controls the activation of the enzyme by lipids? Second, what is the importance of the M/L segment in vivo? The answer to the first question depends on an understanding of the level of activity remaining in the truncated enzyme lacking the M/L region, which has been a matter of some debate. The truncation expressed by Wang and Kent (5), CCTα236 (Fig. 1), is constitutively active as assessed by measuring enzyme activity in crude extracts and enzyme levels by quantitative immunoblotting. Slightly different forms expressed by Yang et al. (6) or Cornell et al. (4) are less than 10% as active as full-length, lipid-activated CCTα (7). Thus the precise role of the M/L segment in enzyme activation is not clear. Wang and Kent (5) proposed that the M/L segment interacts with the catalytic domain to cause inhibition and that removal of the M/L segment by lipid binding results in activation of catalysis. Jackowski and co-workers (6), however, proposed that lipids and the M/L domain are co-activators of the catalytic domain.

In this paper we report the kinetic properties of purified

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1 The abbreviations used are: CCT, CTP:phosphocholine cytidylyltransferase; PC, phosphatidylcholine; FBS, fetal bovine serum; M/L, membrane/lipid; GCT, CTP:glycerol-3-phosphate cytidylyltransferase.
Catalytic Domain of CCTα

Fig. 1. Functional regions of CCTα and CCTα236. Full-length wild-type rat CCTα contains a nuclear localization sequence (NLS), catalytic domain, membrane and lipid binding segment (M/L), and phosphorylation region (P). CCTα236 is truncated after residue 236, so it lacks the M/L segment and phosphorylation region.

CCTα236, showing that it is nearly as active as full-length CCTα and is indeed lipid-independent, supporting the model that the M/L segment is inhibitory. In addition, we report conditions under which the presence of the M/L segment in CCTα is necessary for cell growth.

EXPERIMENTAL PROCEDURES

Materials

CTP, phosphocholine, protease inhibitors, lipids, CM-Sepharose, DEAE-Sepharose, and Sephacryl S-200-HR were obtained from Sigma. The cross-linkers, BS3 and Sulfo-MBS, were from Pierce. [3H]Choline and [14C]phosphocholine were from Amersham Pharmacia Biotech. The Bac-to-Bac baculovirus expression system, SF9 cells adapted for serum-free growth, Sf900II serum-free medium, and Ham’s F-10 and F-12 media were from Life Technologies, Inc. Restriction endonucleases were from New England Biolabs. Fetal bovine serum was from BioWhittaker. Blue-Sepharose was from Amersham Pharmacia Biotech.

Baculovirus Expression of CCTα and CCTα236

A cDNA encoding rat liver CCTα (8) was placed in the baculovirus donor plasmid pFASTBAC1 using the BamHI and SpH1 sites of the multiple cloning site. To generate a cDNA encoding truncation mutant CCTα236 with appropriate restriction sites for the vector, oligonucleotide-directed polymerase chain reaction mutagenesis was used to introduce a stop codon following amino acid 236. The following oligonucleotides were utilized: 5’-oligo, 5’-CGCGGATCCAGATCATTGATGACGAGTCTCA-3’ and 3’-oligo, 5’-ACATGAGCTCGGTACCTTTGATAGTG-3’.

Stocks of virus encoding wild-type CCTα and CCTα236 were prepared as described in the Bac-to-Bac Baculovirus expression system instruction manual. For protein production, 1-liter cultures of SF9 cells at 1 × 10⁶ cells/ml were infected at a multiplicity of infection of 2. Cells were harvested at 48 h post-infection for protein purification.

Protein Purification

Wild-type CCTα—SF9 cells expressing wild-type CCTα were collected, lysed, and centrifuged as described (8). The 100,000 × g SF9 cell supernatant was loaded directly onto a DEAE-Sepharose column equilibrated with buffer A (10 mM Tris-Cl, pH 7.5, 2 mM diithiothreitol, 1 mM EDTA) containing 150 mM NaCl. The column was washed with 10 column volumes of buffer A containing 150 mM NaCl and 1% Nonidet P-40 followed by 10 column volumes of buffer A containing 150 mM NaCl. The enzyme was eluted from the column with a 150–400 mM NaCl gradient in buffer A. The fractions constituting the peak of CCTα activity were pooled, diluted 5-fold in buffer A, and loaded onto a CM-Sepharose column equilibrated with buffer A containing 30 mM NaCl. The enzyme was eluted from the column with a gradient of NaCl from 30 to 150 mM, and the fractions containing the peak of the CCTα activity were pooled.

CCTα236—the high speed SF9 cell supernatant was loaded onto a CM-Sepharose column equilibrated with buffer A. The column was washed sequentially with 10 column volumes each of buffer A and buffer A containing 0.1 mM NaCl. CCTα236 was eluted from the CM-Sepharose with buffer A containing 0.2 mM NaCl. The CM-Sepharose elution fraction was loaded onto a Blue-Sepharose 6 fast flow column. The column was washed with 10 column volumes of buffer A containing 0.2 mM NaCl. CCTα236 was eluted from Blue-Sepharose with buffer A containing 0.5 mM NaCl.

Kinetic Characterization

Activity of purified enzymes was determined with a charcoal binding assay as described previously (9). The kinetic parameters kcat and Km with respect to substrates CTP and phosphocholine were determined from secondary plots. Kinetic parameters were determined in the presence and absence of PC:oleate vesicles (10); the final concentration of each lipid was 100 μM.

Cell Growth and Maintenance

CHO58 cells expressing full-length CCTα or CCTα236 were obtained previously (5). Cell stocks were maintained at 34 °C in 5% CO2 in Ham’s F-12 medium supplemented with 25 mM HEPES and 10% fetal bovine serum (FBS). The stably transfected cell lines were maintained in the presence of 0.8 mg/ml G418 during one-third of the passages. For growth curves, cells were plated at 34 °C in Ham’s F-12, 10% FBS at 50,000 cells/35-mm dish or 50,000 cells/well of a 6-well plate. After 24 h the medium was removed, cells were washed twice in Ca2+- and Mg2+-free phosphate-buffered saline, and then F-10 medium supplemented with 25 mM HEPES, 50 μg/ml gentamycin, and either 10% FBS or 10% delipidated serum was added. The cells were then maintained at 37 °C, and viable cell density was determined after trypsinization by counting in trypan blue with a hemocytometer. Lipids were diluted into the medium from a 1000× stock in ethanol. For the choline uptake experiment, cells were plated at 250,000 cells/60-mm dish in Ham’s F-10, 10% FBS, and the medium changed as for growth curves.

Delipidation of Serum

Fetal bovine serum was delipidated by extraction with isopropyl ether and n-butanol as described (11) and then sterilized by filtration.

Incorporation of [3H]Choline

The protocol used is a modification of that described previously (5). 24 h after plating, cells were washed twice with Ca2+- and Mg2+-free phosphate-buffered saline and shifted into Ham’s F-10, 25 mM HEPES, 10% delipidated serum, and 50 μg/ml gentamycin. Cells were then grown for another 24 h at 37 °C. Cells were labeled by incubating at 37 °C in 1.5 ml of medium supplemented with 2 μCi/ml [3H]choline for the indicated times. Cells were harvested by washing three times in Ca2+- and Mg2+-free phosphate-buffered saline and scraping into 1.0 ml of water at 0 °C. Total lipids were extracted from 0.8 ml of the extract as described previously (12).

Protein Assay

Protein concentration was determined by the assay of Bradford (13) or Lowry et al. (14).

RESULTS

Protein Purification—Because we were interested in lipid activation of CCTα, we devised a new purification procedure for CCTα that does not include treatment with lipids, as did the classical procedure (7). The new procedure involves ion exchange chromatography on both DEAE-Sepharose and CM-Sepharose at pH 7.5. Apparently the distribution of charged residues in the enzyme is suitable to allow it to bind to both resins at pH 7.5, unlike most other proteins. The baculovirus-expressed enzyme that was purified by this procedure appeared homogeneous by Coomassie Blue staining of SDS gels (Fig. 2A). The specific activity of the purified enzyme was similar to the activity of the enzyme purified by the classical procedure (7, 8) (Table I).

Purification of the truncation mutant CCTα236 to homogeneity involved cation exchange chromatography on CM-Sepharose as well as Blue-Sepharose chromatography (Table I). The enzyme was homogeneous as assessed by SDS gels (Fig. 2B). CCTα236 did not bind to DEAE-Sepharose at pH 7.5, suggesting that the C-terminal regulatory segments in CCTα are negatively charged at that pH and mediate interaction with the anion exchanger support. CCTα236 Is Constitutively Active—The ability of the purified forms of CCTα to be activated by lipids was determined by assaying in the presence and absence of vesicles of PC:oleate, 1:1 (10). The activity of wild-type CCTα, purified in the absence of lipids, is very responsive to the addition of lipid (Fig. 3). Over the range of 0–20 μM lipid, CCTα is activated approximately 50-fold. In contrast, the activity of CCTα236 was not affected by the addition of lipid; the specific activity of CCTα236 with and without lipid was comparable with lipid-activated wild-
type CCTα (Fig. 3). The kinetic parameters of purified wild-type CCTα and CCTα236 were measured in the presence and absence of 100 μM PC:oleate (1:1), a concentration at which CCTα is maximally active. The $k_{\text{cat}}$ value for wild-type CCTα in the absence of lipid was 86-fold less than that for wild-type CCTα with 100 μM lipid (Table II). The $k_{\text{cat}}$ values for CCTα236 were independent of the presence of lipid and were only about 30% lower than that for wild-type CCTα in the presence of lipid. The $K_m$ values for phosphocholine were similar for both enzyme forms in the presence and absence of lipids. The $K_m$ values for CTP were somewhat higher for CCTα236 than for wild-type CCTα. The catalytic efficiency ($k_{\text{cat}}/K_m$) values, therefore, for CCTα236 in the absence of lipid were 30–50% as high as those for wild-type CCTα in the presence of lipids. In contrast, the $k_{\text{cat}}/K_m$ values for CCTα236 were 30–70-fold higher than those for wild-type CCTα in the absence of lipids (Table II). The fact that the activity of CCTα236 is so much greater than the activity of wild-type CCTα in the absence of lipids supports the model in which the M/L region is inhibitory to catalysis.

**CCTα236 Is a Homodimer**—Wild-type CCTα is known to be a homodimer (15, 16), but the residues interacting at the dimer interface have not been determined. If the M/L region were important in determining the oligomerization state of CCTα, then CCTα236 might be expected to be monomeric. The quaternary structure of CCTα236 was therefore assessed by intermolecular cross-linking using the bifunctional cross-linkers BS3 and sulfo-MBS. BS3 is specific for cross-linking two amine-containing functional groups, whereas sulfo-MBS targets an amine and sulfhydryl. Both wild-type CCTα and CCTα236 were cross-linked by BS3 and sulfo-MBS (Fig. 4). The major cross-linked form for both species appeared to be dimeric. Wild-type CCTα also appeared to form high molecular weight species, whereas CCTα236 formed only the cross-linked dimer.

The quaternary structure of CCTα236 was also investigated by gel filtration on Sephacryl S-200-HR. The truncated enzyme eluted between the protein standards bovine serum albumin and ovalbumin. The calculated molecular weight was 50,300, and the expected molecular weight of a dimer of CCTα236 is 53,500. Thus, both cross-linking and gel filtration indicated that CCTα236 is a homodimer and support the concept that the dimerization interface of CCTα involves residues in the catalytic domain and not the C-terminal regulatory domains.

**Expression of CCTα236 in CHO58 Cells in Delipidated Medium**—Although it is clear that the M/L region of CCTα has a profound effect on enzyme activity, the function of this domain in the cell remains open to question. Expression of CCTα236 in the CHO58 cell line, which is temperature-sensitive for growth and CCTα activity (17), fully complements the temperature-
sensitive growth defect (5). This suggests that the M/L region is not required for routine lipid metabolism and cell cycling. However, we have more recently discovered conditions under which expression of CCT\textsubscript{a}236 is not sufficient to complement the CHO58 defect. When CHO58 cells stably transfected with CCT\textsubscript{a}236 were transferred to medium containing delipidated serum, the cells could not continue to grow at 40 °C (data not shown). Furthermore, these cells could not grow in delipidated serum at 37 °C, a temperature that is permissive for the growth of untransfected CHO58 in either complete serum or delipidated serum (Fig. 5). Thus the inability of cells expressing CCT\textsubscript{a}236 to grow in delipidated medium is a dominant negative effect. CHO58 cells expressing wild-type CCT\textsubscript{a} were capable of growth at 37 °C in either complete or delipidated serum (Fig. 5).

To determine whether a single specific lipid would restore the ability of CHO58 cells expressing CCT\textsubscript{a}236 to grow, a number of different lipids were added to the delipidated medium. The unsaturated fatty acid oleate was capable of supporting growth at the same rate as in complete serum (Fig. 6). Optimal growth was seen at oleate concentrations from 40 to 100 \textmu{}M oleate. Other unsaturated fatty acids, linoleate and palmitoleate, could also fully support growth at 40 \textmu{}M (not shown). The saturated fatty acids myristate, palmitate, and stearate at 40 \textmu{}M could not support growth. The combination of 20 \textmu{}M cholesterol plus 200 \textmu{}M mevalonate could not support growth, and the addition of cholesterol and mevalonate to the oleate-containing medium did not enhance growth further than oleate alone. These results indicate that expression of CCT\textsubscript{a}236 had transformed CHO58 cells into unsaturated fatty acid auxotrophs.

**Synthesis of Phosphatidylcholine in CCT\textsubscript{a}236/58 Cells**—We had observed previously that CHO58 cells expressing CCT\textsubscript{a}236 in complete serum were considerably more active at making PC than cells expressing wild-type CCT\textsubscript{a} (5), which is consistent

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![FIG. 4. Cross-linking of CCT\textsubscript{a}236. Cross-linking reactions were with the indicated concentration of BS3 or sulfo-MBS at 20 °C for 30 min. The enzyme concentration was 5 \textmu{}M.](image-url)

![FIG. 5. Growth curves in complete and delipidated serum. Cells were plated in medium containing complete fetal bovine serum at time 0, then changed to medium with either complete serum (FBS) or delipidated (DLS) at 24 h (indicated by the arrow). The number of viable cells was determined as indicated under "Experimental Procedures." □, CHO58 cells; ○, CHO58 cells expressing CCT\textsubscript{a}; △, CHO58 cells expressing CCT\textsubscript{a}236.](image-url)

![FIG. 6. Growth of cells in delipidated serum supplemented with oleate. Cells were plated in medium containing complete fetal bovine serum at time 0 and then changed at 24 h to medium with complete serum (○), delipidated serum (□), or delipidated serum containing 40 \textmu{}M oleate (△).](image-url)

![FIG. 7. Phosphatidylcholine biosynthesis in delipidated medium. Cells were grown in medium containing delipidated serum, and incorporation of \(^{3}H\)choline was determined as described under "Experimental Procedures." ●, untransfected CHO58 cells; ■, CHO58 expressing wild-type CCT\textsubscript{a}; ▲, CHO58 expressing CCT\textsubscript{a}236. Data points represent the average ± S.D. from three determinations in one experiment. The experiment was performed a total of five times, with different clonal isolates, with similar results.](image-url)
with the CCTα236 being constitutively active. To discover whether the ability of these cells to make PC was defective in delipidated serum, rates of choline incorporation into PC were measured 24 h after transfer to delipidated medium, a time at which the cells were still viable as determined by trypan blue exclusion. As indicated in Fig. 7, CCTα236/58 cells were fully capable of making PC in delipidated serum. The rate of PC synthesis was higher by a factor of 1.7 ± 0.6 (n = 5) in cells expressing CCTα236 than in cells expressing wild-type CCTα, as had been observed when these cells were grown in complete serum. The higher rate of PC synthesis in cells expressing CCTα236 is consistent with the M/L domain being inhibitory in the cell.

**DISCUSSION**

**Role of M/L Region**—Two distinct models can be envisioned for the role of lipid binding in regulating catalysis by CCTα (Fig. 8). In model A, the M/L region interacts with the catalytic domain in the absence of lipids, and this interaction inhibits enzyme activity. Binding of lipids to the M/L segment causes it to dissociate from the catalytic domain, and the inhibition is relieved. In model B, the catalytic domain in the inactive enzyme does not associate with the M/L region. When the M/L region becomes complexed with lipids it then associates with the catalytic domain to activate catalysis. Both models predict that CCTα236 would have lipid-independent activity. Model A, however, predicts that CCTα236 would be as active as the wild-type enzyme in the presence of lipids, whereas model B predicts that CCTα236 would be as inactive as the wild-type enzyme in the absence of lipids.

The results in this manuscript show that the activity of CCTα236 is indeed independent of lipids. Moreover, CCTα236 is nearly as active as full-length CCTα in the presence of lipids and much more active than full-length CCTα in the absence of lipids. The presence of the regulatory regions in the absence of lipid lowers the maximal velocity attainable by CCTα about 50-fold. These data clearly support model A, in which the M/L region acts as an inhibitor in the absence of lipids.

Other truncated forms of rat CCTα have been expressed with considerably lower activity than CCTα236. Two of these, CCTα228 (4) and CCTα230 (6), were missing several residues that are highly conserved in all CCTs from yeasts to mammals (2). It is possible that these residues are important for catalysis or for enzyme structure. In addition both these truncations contained additional amino acids at their C termini, which may have adversely affected activity. CCTα256 also had low activity (6), which presents the interesting possibility that residues 237–256 contain part of the inhibitory region of the M/L region. We originally chose to end our truncation at 236 based on sequence conservation (5), but later analysis of the mouse CCTα gene indicated that a splice junction terminates the last exon of the catalytic domain at this site (18). This suggests that the catalytic domain does truly end at residue 236.
The role of lipid in the activation of CCTα has been reported to be due to both a lowering of the $K_m$ for CTP as well as an increase in $V_{\text{max}}$ (6). Our analysis of kinetic parameters of wild-type CCTα revealed only an increase in $k_{\text{cat}}$ in the presence of lipids, with no substantial decrease in $K_m$ for CTP (Table II). It is not yet clear why there is a discrepancy in these $K_m$ determinations. It should be remembered that the kinetic constant $K_m$ is a complex term of rate constants that is rarely a simple reflection of substrate binding affinity (19). It has been argued that the effect of an activator or inhibitor on $K_m$ is not as useful as a determination as the effect on $V_{\text{max}}/K_m$ or $k_{\text{cat}}/K_m$ (20). It is interesting that the $V_{\text{max}}/K_m$ values for CCTα ± lipids vary by a factor of 220 in the studies of Yang et al. (6), and the $k_{\text{cat}}/K_m$ values for CCTα ± lipids in the present study differed by a factor of 114. That these values are quite similar supports the argument that the ratio of catalytic constant to $K_m$ rather than $K_m$ itself is the appropriate constant for comparing the effects of lipids on activity.

Quaternary Structure of CCTα236—Results obtained by cross-linking (Fig. 4) and gel filtration indicate that CCTα236 is a dimer. This would indicate that the residues responsible for dimer formation in wild-type CCTα are largely within the catalytic domain or the N terminus. CTP:glycerol-3-phosphate cytidylyltransferase (GCT), a small enzyme similar in primary sequence to the catalytic domain of CCT, is also a homodimer (21), supporting the concept that residues in the catalytic domain are sufficient for dimer formation. We have recently completed the crystal structure of GCT. The majority of residues at the dimer interface of GCT are similar or identical to the corresponding residues in CCTα, suggesting that the interface within the catalytic domain is a conserved feature of this cytidylyltransferase family. A role for the M/L region in lipid-free medium has afforded an experimental system in which that role can be elucidated.

Cellular Expression of CCTα236—Determining the role of the M/L region in vitro should aid in our understanding of the function of this enzyme in the cell. It was disappointing, therefore, that previous expression results had indicated that the regulatory regions of CCTα are not needed by cells, at least under the usual cell culture conditions (5). The present results indicate, however, that the presence of the M/L region can be critical under certain conditions. Expression of CCTα236 in CHO58 cells maintained in delipidated serum was toxic unless the cells were supplied with an unsaturated fatty acid. The toxicity was a dominant effect in that it occurred even at a temperature permissive for growth of untransfected CHO58 cells. The toxicity was not due to an inability of the cells to make PC under these conditions (Fig. 7). Although we do not yet know the reason for the requirement for an unsaturated fatty acid, two possibilities come to mind. One is simply that the cells are making so much more PC that the desaturase responsible for making unsaturated fatty acids cannot keep up with the demand, with the result that too much saturated phospholipid is made and put into membranes. The other possibility is that CCTα is somehow involved in the induction of the desaturase in lipid-free medium, and the M/L region plays a role in that induction. The latter possibility would mean that CCTα has a role in coordinating lipid biosynthesis, which might explain its puzzling nuclear location. The demonstration of a role for the M/L region in lipid-free medium has afforded an experimental system in which that role can be elucidated.

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