Comparison of the lipid regulation of yeast and rat CTP: phosphocholine cytidyltransferase expressed in COS cells

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The CTP:phosphocholine cytidyltransferase (CT) gene from yeast and cDNA from rat liver were over-expressed 20–30-fold in COS cells. Most of the CT activities were found in the cytosolic fraction. The regulation of the yeast CT activity (Y-CT) by lipids was characterized for the first time in comparison with the regulation of the well-studied rat CT (R-CT). Sonicated vesicles composed of egg phosphatidylcholine (PC) or 1-stearoyl-2-oleoyl PC had no effect on Y-CT and only slightly stimulated R-CT activity. Both CTs were activated 10–50-fold by the anionic lipids cardiolipin, phosphatidylglycerol, phosphatidylinositol and oleic acid. The effects of varying the vesicle concentration and the mol% of anionic lipid in PC vesicles were tested. The concentration optima for the activation of Y-CT by oleic acid or anionic phospholipids were 5–10-fold lower than those for R-CT. For example, the stimulation of Y-CT activity by phosphatidylglycerol vesicles was optimal between 5 and 15 μM and declined at higher concentrations, but R-CT activation by these vesicles saturated at $\sim 25 \mu\text{M}$. The positively charged aminolipid sphingosine antagonized the stimulation by oleic acid of both Y-CT and R-CT. Y-CT activity was insensitive to PC vesicles containing the neutral lipids diacylglycerol, monoacylglycerol or oleyl alcohol. However, R-CT was stimulated 10–20-fold by vesicles containing these neutral lipids. Translocation of the CTs to microsomal membranes enriched with anionic or neutral lipids was compared. Oleic acid enrichment promoted translocation of Y-CT and R-CT, whereas diacylglycerol promoted only R-CT translocation. These data show that the activity of Y-CT is lipid-sensitive. Y-CT is affected only by charged lipids, whereas R-CT responds to charged and neutral lipid activators. The data are consistent with different modes of interaction of the two CTs with lipids.

INTRODUCTION

In animal cells CTP:phosphocholine cytidyltransferase (CT) is a key regulatory enzyme in the synthesis of phosphatidylcholine (PC) via CDP-choline [1–3]. The activity of CT in liver and many animal cells in culture can be regulated by interconversion between an active membrane-bound form and an inactive cytosolic form [1,2]. Membrane binding (activation) can be modulated by the membrane lipid composition [4–9]. Purified rat liver CT is activated by anionic phospholipids and fatty acids [6–9], and antagonized by cationic lipids [10]. A second class of uncharged lipids exemplified by mono- and di-acylglycerol activate CT from rat liver or HeLa cells when these lipids are incorporated into a cellular or liposomal membrane [9,11,12].

In yeast the CDP-choline pathway serves as an auxiliary to the methylation pathway for synthesis of PC [13]. There is no evidence that CT activity regulates PC synthesis in yeast. Choline transport into the cell appears to be functionally co-ordinated with the CDP-choline pathway for PC synthesis [14]. This pathway may be regulated at the level of choline entry into the cell [15]. Most of the CT activity in homogenized yeast sediments with microsomes [15], but its mode of interaction with microsomes has not been studied. Yeast CT has never been purified, and its regulation by lipids has not been previously investigated.

Recently the yeast gene and rat liver cDNA for CT have been cloned and sequenced [16,17]. The gene and cDNA encode proteins of 49 and 42 kDa respectively. A large central domain of rat and yeast CTs share 75% identity, whereas the N- and C-

termini are non-conserved. Neither the rat nor the yeast sequences contain any hydrophobic segments indicative of a membrane-spanning helix, nor do they contain any signals for covalent lipid attachment. From the point of divergence at the C-terminal domain, the rat (but not the yeast) sequence predicts an extensive amphipathic α -helix containing three 11-mer repeats in tandem. We have postulated that the amphipathic helix serves as the membrane binding domain of the rat CT, by interacting with the membrane surface such that the hydrophobic face of the helix intercalates the lipid hydrophobic core [17]. This region in the yeast protein is not predicted to be helical.

As a first step in probing the nature of the membrane-interacting domain of the previously uncharacterized yeast CT (Y-CT), we monitored the effects of membranes of various lipid compositions on the activity of Y-CT in comparison to the well-characterized R-CT. For this purpose we expressed the yeast gene or the rat cDNA in COS cells so that the effects of the lipids could be investigated in a common cellular background. We found that Y-CT activity is lipid-sensitive, but that there are major differences between the responses of R-CT and Y-CT to membrane lipids.

MATERIALS AND METHODS

Materials

COS-1 cells [18] and the pAX111 expression vector [19] were gifts from Dr. Robert Kay, B.C. Cancer Research Center (Vancouver, B.C., Canada). All phospholipids were from Avanti

Abbreviations used: CT, CTP:phosphocholine cytidyltransferase; R-CT, rat CT; Y-CT, yeast CT; PC, phosphatidylcholine; SOPC, 1-stearoyl-2-oleoyl PC; PG, phosphatidylglycerol; PI, phosphatidylinositol.

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Polar Lipids (Alabaster, AL, U.S.A.). The lipids were > 99 % pure as estimated by t.l.c. Sphingosine, oleic acid, mono-olein and oleyl alcohol were from Sigma (St. Louis, MO, U.S.A.). *sn*-1,2-Diacylglycerol was generated by digestion of egg PC with phospholipase C as described in [20]. Restriction enzymes and other DNA-modifying enzymes were from Pharmacia (Uppsala, Sweden) or BRL (Burlington, Ont., Canada). Tissue-culture supplies were from Corning (Ithaca, NY, U.S.A.). Fetal-calf serum was from GIBCO/Life Technologies (Burlington, Ont., Canada). Other chemicals were from Sigma or BDH (Vancouver, B.C., Canada).

COS cell transfection of CT clones and preparation of cell extracts

COS cells were cultured and transfected as previously described [17] with pAX111 expression vector [19] containing no insert or the full-length R-CT [17]. For transfection with Y-CT, the pCC143 Y-CT clone [16] was digested in separate reactions with endonucleases *Rsa*I and *Eco*RI, and *Eco*RI and *Dra*I. The two separate fragments were gel-purified and ligated into pAX111 digested with *Sma*I in a three-way ligation. Cells from 15 cm-diam. dishes (one dish per sample) were harvested at 72 h after transfection by using phosphate-buffered saline with 2.5 mM-EDTA at 37 °C to release the cells from the dishes. The cells were pelleted at 1000 *g* for 5 min at room temperature. Subsequent steps were at 4 °C. The cell pellets were washed with homogenization buffer (10 mM-Tris/HCl, pH 7.4, 1 mM-EDTA, 0.2 mM-phenylmethanesulphonyl fluoride, 3 mM-MgCl₂, 2 mM-dithiothreitol). Cells were homogenized in 1 ml of homogenization buffer by 30 strokes of a Teflon/glass grinder. K₂HPO₄ (a stabilizer of CT activity) was added in four portions to the homogenate from a 2 M stock to a final concentration of 0.2 M. A sample was removed and the remainder was immediately centrifuged for 3 min at 3000 *g*. The supernatant was centrifuged at 100 000 *g* for 1 h. The pellet (microsomes) was resuspended in homogenization buffer with 0.2 M-phosphate by a 20–30 s sonication (Fisher Sonic Dismembrator, model 300). Fractions could be stored at –70 °C for several months and then thawed and refrozen three or four times without a decline in activity.

Digitonin permeabilization of transfected COS cells

The procedure used for digitonin permeabilization was based on the method described by Weinhold *et al.* [21]. COS cells transfected with Y-CT, R-CT or PAX vector were harvested 68 h after transfection. Cells were pelleted and resuspended in homogenization buffer containing 0.2 M-phosphate. Digitonin was added to a concentration of 0.8 mg/ml, and the cells were incubated at 2 °C for 3–10 min, followed by centrifugation for 5 min at 9700 *g*. The supernatant was removed and the pellet resuspended by sonication in homogenization buffer containing 0.2 M-phosphate. Lactate dehydrogenase activity was assayed [22] in all samples to quantify release of cytosolic proteins. In preliminary experiments, cholinephosphotransferase activity was also assayed [23] as a marker for the membrane fraction. More than 97 % of the cholinephosphotransferase activity was found in the digitonin-pellet fraction. Samples from the supernatant and pellet were assayed for CT activity in the presence of optimal egg PC/oleic acid vesicles: 0.5 mM for R-CT and 0.1 mM for Y-CT. CT from cells transfected with R-CT was also quantified by e.l.i.s.a. [8] after permeabilization.

Enrichment of microsomes with lipids and CT binding to microsomes

Sonicated dispersions of oleic acid, 1-stearoyl-2-oleoyl PC (SOPC), SOPC/diacylglycerol (2:1) and SOPC/sphingosine

(1:1) were prepared as described previously [9]. Rat liver microsomes (0.25 mg of protein, 130 nmol of phospholipid) prepared as described in [11] were mixed with 400 nmol of lipid dispersions. After incubation at 22 °C for 30 min, the samples were chilled to 4 °C and centrifuged for 60 min at 100 000 *g* in a Beckman TLA 45 rotor. In the presence of microsomes, all of the lipid dispersions sedimented, except for diacylglycerol, in which a minor fraction floated (see Fig. 7). The pellets were resuspended by sonication in homogenization buffer, and 10 units (0.15–0.25 mg) of COS-cell cytosolic extract containing R-CT or Y-CT were added. After 5 min at 37 °C the samples were chilled and re-centrifuged as above. Lipids were extracted [24] from supernatants and resuspended pellets for analysis by t.l.c. Both fractions were assayed for CT activity in the presence of an optimum concentration of PC/oleic acid (1:1) vesicles.

CT assay

The assay for CT activity [25] and the method for preparation of sonicated vesicles have been described previously [9]. Typically, 5 µg of R-CT or 10 µg of Y-CT transfected COS-cell cytosolic protein was assayed at 37 °C at pH 7.4, the optimum for R-CT. Similar results were obtained when assays were performed at 30 °C at pH 8.0, the optimum conditions for yeast CT [26]. Activities for each individual experiment were normalized to the activity obtained for that preparation of R-CT or Y-CT in the presence of egg PC/oleic acid (1:1) vesicles; 500 µM lipid was used for R-CT, and 100 µM for Y-CT. These controls were assayed along with each experiment. The endogenous CT activity in the COS cells transfected with vector alone ranged from < 1 (no lipid) to 5 nmol/min per mg (in the presence of anionic lipids). These values were subtracted from the activity values plotted in Figs. 1–6. One unit of activity is defined as 1 nmol of CDP-choline formed/min. CT activity was normalized to cytosolic protein.

Miscellaneous

Protein was assayed by the method of Bradford [27]. Rat liver CT was purified by the method of Weinhold *et al.* [6], with minor modifications [25]. For t.l.c. we utilized heat-activated (30 min, 100 °C) Merck silica-gel 60 plates.

RESULTS

Expression of Y-CT and R-CT in COS cells

CT activity in cytosolic extracts from COS cells transfected with the PAX vector alone was 2–3 nmol/min per mg when assayed in the presence of a saturating concentration of PC/oleic acid (1:1) vesicles (Table 1). Transfection with the Y-CT or R-CT clones resulted in an elevation to ~ 60 nmol/min per mg in COS-cell homogenates prepared 72 h after transfection (Table 1). These expression levels are higher than those previously obtained in COS cells [17]. Table 1 also shows that the activities of transfected Y-CT and R-CT were subject to regulation by lipids. The activities in the presence or absence of PC/oleic acid vesicles differed by factors of 8 (Y-CT) or 25 (R-CT). The intracellular distribution of CT activity in the cells transfected with Y-CT, R-CT or the vector alone was similar; 80–90 % was found in the cytosolic fraction obtained by high-speed centrifugation. This distribution is similar to that of rat liver. The cellular distribution was also analysed by digitonin permeabilization. Table 1 shows that ~ 70 % of both the Y-CT and R-CT activities was released from permeabilized cells, and the remaining ~ 30 % was cell-membrane-associated. The distribution of R-CT was also determined via an e.l.i.s.a. using a rat CT-specific anti-peptide antibody (gift from H. Jamil and D. E. Vance, Edmonton, Canada). The results were similar to the values

Table 1. CT specific activity and percentage distribution in transfected COS-cell cytosols

Cytosols were prepared from transfected COS cells as described in the Materials and methods section. CT activity was assayed in the presence of optimal concentration of egg PC/oleic acid (1:1) vesicles. Data are presented as activity units/mg of cytosolic protein (means $\pm$ S.D. of 3–6 separate transfections). To measure CT in cytosol, COS cells were permeabilized with digitonin, and permeabilized (supernatant) and cell-associated (pellet) fractions were assayed for CT activity, as described in the Materials and methods section. The percentage of CT activity in the cytosol was determined by dividing the supernatant activity units by the sum of the units obtained in the supernatant + pellet fractions. Data are means $\pm$ S.D. of 4–10 determinations, normalized to an LDH release of 100%. The mean LDH release was $95 \pm 2\%$.

Transfection	Cytosolic CT specific activity		CT in cytosol (%)
	No lipid	+PC/oleic acid	
Vector alone	0.4 ± 0.3	2.4 ± 0.7	67 ± 13
R-CT	2.4 ± 1.1	61 ± 14	73 ± 10
Y-CT	8 ± 4	64 ± 13	66 ± 16

Fig. 1. Dependence of CT activity on the concentration of egg PC/oleic acid or SOPC vesicles

Cytosolic extracts from COS cells expressing R-CT (●) or Y-CT (○) were assayed in the presence of the indicated concentration of egg PC/oleic acid (1:1, mol/mol; continuous line) or SOPC vesicles (broken line). Data are duplicate determinations of single experiments that were repeated three or four times with similar results.

obtained by activity assays: $84 \pm 7\%$ of the CT was released by digitonin permeabilization ($n = 2$).

Effect of oleic acid and sphingosine

Sonicated unilamellar vesicles composed of egg PC and oleic acid (1:1, mol/mol) have been used as a standard for comparing the activation of purified rat liver CT by vesicles of different lipid compositions. Fig. 1 shows that oleic acid stimulates both Y-CT and R-CT activity. PC/oleic acid vesicles stimulated Y-CT and R-CT activity up to 30-fold, whereas vesicles of egg PC had little effect. The activation of Y-CT, but not of R-CT, was decreased at lipid concentrations above $100 \mu\text{M}$. In Fig. 1 the oleic acid content of the vesicles used was 50 mol%. The mol% of oleic acid in SOPC vesicles was varied between 0 and 50% (Fig. 2). Since Y-CT was not stimulated by PC alone, the effect of varying the mol% of oleic acid in PC vesicles on Y-CT activity was more dramatic than that on R-CT. Fig. 3 shows that the activation of

Fig. 2. Dependence of CT activity on the mol% of oleic acid in SOPC vesicles

Cytosolic extracts from COS cells expressing R-CT (●) or Y-CT (○) were assayed in the presence of $100 \mu\text{M}$ -SOPC vesicles containing the indicated mol% of oleic acid. Data are duplicate determinations of a single experiment that was repeated three times with similar results.

Fig. 3. Antagonism between oleic acid and sphingosine

Cytosolic extracts from COS cells expressing R-CT (●) or Y-CT (○) were assayed in the presence of $100 \mu\text{M}$ vesicles containing 50 mol% SOPC and the indicated mol% of oleic acid (OA) and sphingosine (Sph). Data are duplicate determinations of a single experiment that was repeated twice with similar results.

both CTs by oleic acid can be completely blocked by incorporation of equimolar sphingosine into the vesicles (sphingosine equimolar with oleic acid). This amount of sphingosine should neutralize the negative charge of the fatty acid. This antagonism between oleic acid and sphingosine has been previously documented for purified rat liver CT [10], and indicates the role of the surface charge in the activation by oleic acid.

Effects of anionic phospholipids

Vesicles composed of cardiolipin, phosphatidylglycerol (PG) or phosphatidylinositol (PI) were tested as a function of vesicle concentration (Fig. 4) or mol% anionic phospholipids in vesicles composed of variable ratios of anionic phospholipid and SOPC (Fig. 5). The concentration-dependence of R-CT activity became saturated at $10 \mu\text{M}$ -cardiolipin, $\sim 50 \mu\text{M}$ -PG and $\sim 100 \mu\text{M}$ -PI.

Fig. 4. Dependence of R-CT (a) or Y-CT (b) activity on the concentration of anionic phospholipids

Cytosolic extracts from COS cells were assayed in the presence of vesicles containing cardiolipin (CL; ○), PI (■) or PG (□). Data are averages of two to four separate experiments.

On the other hand, the dependence of Y-CT activity on anionic-phospholipid concentration was biphasic, where the activation curve reached a plateau at 2–5 μM, and declined at higher concentrations (Figs. 4a and 4b). The biphasic behaviour was the most pronounced for cardiolipin, and the least pronounced for PI. When assayed at the vesicle concentration optimum, determined from the data of Fig. 4 (100 μM for R-CT), the activity dependence of R-CT on the mol% of anionic phospholipids was saturable at ~50 mol% for cardiolipin, PG and PI (Fig. 5a). Cardiolipin produced the strongest stimulation. For Y-CT, assayed at the optimal lipid concentration (5 μM), the mol% curves saturated at 25% cardiolipin and 50% PG or PI (Fig. 5b). At higher than optimal lipid concentrations (e.g. 25 and 100 μM) the mol% of anionic lipid required for maximal stimulation of Y-CT shifted to lower values and the stimulation declined at high mol% of PI, cardiolipin or PG (results not shown). The loss of stimulatory effects of anionic phospholipids at high concentrations is not due to metabolism of the added lipid to an inhibitory species. Extraction and t.l.c. analysis of the lipids after incubation of cardiolipin, PI, PG or PC/oleic acid vesicles with COS-cell cytosol under conditions identical with the enzyme assay revealed no new species (results not shown).

Effects of neutral lipid activators

It has been shown that CT from mammalian sources binds to and is activated by cellular or liposomal membranes enriched with diacylglycerol or other neutral lipids with small uncharged polar head groups [9,11,12]. Anionic phospholipids are not needed in conjunction with the neutral lipid activator. Fig. 6

Fig. 5. Dependence of R-CT (a) or Y-CT (b) activity on the mol% of anionic phospholipid

Cytosolic extracts from COS cells were assayed in the presence of SOPC vesicles containing the indicated mol% of anionic lipid: cardiolipin (CL; ○), PI (■) or PG (□). The total lipid concentration was 100 μM in (a) and 5 μM in (b). Data are duplicate determinations of single experiments that were repeated twice with similar results.

Fig. 6. Dependence of CT activity on the mol% of neutral lipid in SOPC vesicles

Cytosolic extracts from COS cells expressing R-CT (continuous lines) or Y-CT (broken lines) were assayed in the presence of 100 μM-SOPC vesicles containing the indicated mol% of egg diacylglycerol (DAG, ●), mono-oleoylglycerol (MG, ○), or oleyl alcohol (OH, □). Data are duplicate determinations of single experiments that were repeated three times with similar results.

compares the effects of three neutral lipids on the activities of Y-CT and R-CT: diacylglycerol (derived from egg PC), mono-oleoylglycerol and oleyl alcohol. The mol% of the neutral lipids

Fig. 7. T.l.c. of lipid extracts

Rat liver microsomes (0.4 mg) were incubated with 0.4 μ mol of the indicated lipid, then centrifuged at 100 000 g for 1 h. Lipids were extracted from the supernatant (S) and pellet (P) fractions. Solvent: (a) chloroform/methanol/water/ NH_3 (65:35:5:1, by vol.); (b) hexane/diethyl ether/acetic acid (60:40:1, by vol.). Abbreviations: Sph, sphingosine; DAG, diacylglycerol; OA, oleic acid.

Table 2. Y-CT and R-CT translocation to lipid-enriched microsomes

R-CT or Y-CT (10 units; 0.15–0.25 mg) from COS-cell cytosolic extracts were incubated with 0.25 mg of untreated microsomes or microsomes enriched in the indicated lipid, as described in the Materials and methods section. The samples were centrifuged at 100 000 g for 1 h, and CT activity was determined in the supernatant and pellet fractions under optimal conditions for enzyme activity. For comparison, the numbers presented (units of CT activity) have been normalized to 10 units in the supernatant + pellet fractions. Recovery of added CT activity ranged from 60 to 90%, except for Y-CT treated with oleic acid, in which the recovery was 40–50%. The lower recovery is due to the inhibitory effect of high concentrations of oleic acid in the membrane; see, e.g., Fig. 1.

Enriched lipid	R-CT		Y-CT	
	Supernatant	Pellet	Supernatant	Pellet
None ($n = 3$)	8.3 $\pm$ 0.3	1.7 $\pm$ 0.3	8.3 $\pm$ 0.7	1.7 $\pm$ 0.7
PC ($n = 4$)	9.1 $\pm$ 0.2	0.9 $\pm$ 0.2	8.7 $\pm$ 0.9	1.3 $\pm$ 0.9
Oleic acid ($n = 2$)	1.2 $\pm$ 0.1	8.8 $\pm$ 0.1	2.1 $\pm$ 1	7.9 $\pm$ 1
PC/sphingosine ($n = 2$)	8.5 $\pm$ 0.4	1.5 $\pm$ 0.4	9.5 $\pm$ 0.1	0.5 $\pm$ 0.1
PC/diacylglycerol ($n = 3$)	6.7 $\pm$ 0.5	3.3 $\pm$ 0.4	9.0 $\pm$ 0.3	1.0 $\pm$ 0.3

was varied in SOPC vesicles up to 25%. All three lipids were activators of R-CT activity ($\sim$ 10-fold stimulation). Diacylglycerol and oleyl alcohol did not stimulate R-CT above 25 mol%. Diacylglycerol is known to induce non-bilayer phases above 25 mol% [28], which could be related to the lack of stimulation. Y-CT activity was not affected by any of these lipids. The mol% curves were examined for three different concentrations (5, 25 and 100 μ M) without effect on Y-CT activity. Diacylglycerol, monoacylglycerol and oleyl alcohol were not metabolized to other lipid species, as evidenced by extraction and t.l.c. analysis of the lipids after incubation with COS-cell cytosols under conditions identical with the enzyme assay (results not shown).

Translocation to microsomes

The preceding studies showed that Y-CT and R-CT activities have different sensitivities to lipid vesicles, Y-CT being activated only by class I (anionic) lipids. We investigated whether Y-CT and R-CT can be distinguished by differences in enzyme binding to membranes enriched with lipids of class I or class II (neutral lipids). Microsomal membranes were used for this analysis, owing to the ease of separation of microsomal-bound CT from

unbound CT. Microsomes were enriched with oleic acid, SOPC, diacylglycerol or sphingosine by incubating the membrane suspensions with sonicated dispersions of oleic acid or sonicated vesicles of SOPC, SOPC/diacylglycerol (2:1) or SOPC/sphingosine (1:1). The extent of incorporation of oleic acid and sphingosine from the vesicles to the microsomes was virtually complete, as assessed by t.l.c. of lipids extracted from the sedimented microsomes or supernatants (Fig. 7). In some experiments a small fraction of the PC/diacylglycerol vesicles was not incorporated into the microsomes (see Fig. 7). Y-CT or R-CT binding to the enriched microsomes was assessed by sedimentation and assay of activity in the supernatant and pellet fractions at an optimum concentration of PC/oleic acid vesicles. In preliminary experiments, rat liver and COS-cell microsomes showed equally effective capacity to bind CT after oleic acid enrichment. Rat liver microsomes were used, owing to a more abundant supply.

The results are shown in Table 2. The CT activity endogenous to rat liver microsomes was subtracted from the total pellet-associated CT activity. The percentage distributions show that oleic acid promoted both R-CT and Y-CT binding. Diacylglycerol enrichment resulted in a moderate increase in R-CT binding, but had negligible effect on Y-CT binding. Assuming complete incorporation of the added PC/DAG into microsomes, the DAG content was $\sim$ 25 mol% of total phospholipid, which was quantified by lipid phosphorus determination [29]. The stimulation of R-CT activity by 25 mol% DAG in SOPC vesicles was about half as strong as the maximum stimulation achieved by oleic acid/SOPC vesicles (see Figs. 1 and 6). Thus both the microsome translocation experiments and the lipid-vesicle activation experiments suggest that diacylglycerol is less potent an effector than oleic acid for R-CT. That the sedimenting CT activity represents microsome-bound CT and not dense CT aggregates promoted by contact with lipids was demonstrated by controls in which PC/oleic acid vesicles were incubated with cytosolic R-CT or Y-CT in the absence of microsomes. Minimal CT activity ($< 10\%$) appeared in the pellet after the standard centrifugation (results not shown).

DISCUSSION

This paper represents the first characterization of the modulation of yeast CT activity by lipids. In light of the differences in metabolic control of PC synthesis in yeast and animal cells [30,31], and the divergence in the sequences of cloned Y-CT and R-CT [16,17], we were surprised to find that the yeast CT activity is lipid-dependent. However, two striking differences between the response of R-CT and Y-CT were discovered: (1) Y-CT appears to be modulated only in response to negatively charged lipids (class I), whereas R-CT activity is modulated both by charged and by neutral lipids such as diacylglycerol (class II); (2) the activation of Y-CT by charged lipids is only seen over a small concentration range, and the maximum stimulation is at 5–10-fold lower concentration than that for R-CT.

The differences between Y-CT and R-CT responses to class II lipids were observed both in activity assays and in membrane translocation assays. The results of the membrane translocation assays support the model that the process of activation of CT is linked to physical binding of enzyme to membranes. The translocation data also provide an explanation for the inhibitory (antagonizing) action of sphingosine. We have postulated that sphingosine antagonizes the activation by anionic lipids by neutralizing the negative surface charge of the membrane, thereby attenuating the signal for binding [10]. This proposal is supported by our finding that neither Y-CT nor R-CT bound to microsomes enriched with sphingosine.

After disruption and homogenization of yeast cells, most of the CT activity appeared in the microsomal fraction ([16]; P. S. Sohal, unpublished work). In COS cells the over-expressed Y-CT accumulates mainly in the cytosol. There may be a mechanism present in yeast cells for membrane association of CT during or after synthesis which is missing from the COS cells. It should be noted, however, that the sedimentation behaviour in this case did not distinguish microsomal binding from aggregation of the yeast CT.

Our working model for the interaction of mammalian (i.e. rat liver) CT with membranes involves two components: an electrostatic attraction between a positively charged domain on the protein and a negatively charged lipid surface, and a hydrophobic interaction which probably involves intercalation of protein into the bilayer core. The role of the electrostatic component has been demonstrated by the dependence of the activity of purified rat liver CT on the membrane surface charge density and on the ionic strength of the medium [8]. Evidence for an intercalating hydrophobic component comes from studies showing that dissociation of membrane-bound CT requires detergent [12], and studies demonstrating that activation depends on the acyl-chain length of the phospholipids and vesicle curvature, and is enhanced at the lipid phase transition [9]. The discovery of a class of uncharged activating lipids suggested that the electrostatic interaction was not absolutely required. These properties supported a role for the C-terminal amphipathic α -helix in membrane binding. Further support for this domain in membrane binding has emerged recently. An R-CT fragment produced by chymotrypsin treatment which includes the N-terminal two-thirds, but lacks the entire amphipathic helix domain, does not bind to vesicles containing either oleic acid or diacylglycerol (L. Craig, J. E. Johnson & R. B. Cornell, unpublished work). This implicates the amphipathic helix domain in the interaction of R-CT with both classes of lipid activators.

There are no studies on the mechanism of Y-CT-membrane interaction to guide our explanations of the observed differences in lipid activation. The finding that Y-CT activity is modulated only by the anionic class of lipids suggests that the electrostatic interaction dominates in the activation. Since the curves for activation by anionic lipids were different for Y-CT and R-CT, it is possible that the anionic lipid-binding sites in Y-CT and R-CT are completely different. Y-CT, which has no amphipathic helix, has no class II lipid-binding site. This supports a role for the amphipathic helix of R-CT in the interaction with class II lipid activators.

We found that Y-CT activation by anionic lipids depends on the concentration of vesicles added, where only a narrow concentration range supports activity. The concentration-dependence observed could be the composite of two separate effects of the lipid vesicles, one inhibitory and one stimulatory. The inhibitory effect on Y-CT activity at high anionic-lipid vesicle concentration was not due to metabolism of the added lipid to some inhibitory species catalysed by enzymes in the COS-cell extract. T.l.c. analysis of lipids incubated with COS-cell extract showed no evidence for metabolism of added lipids. These distinct effects could originate from two distinct anionic-lipid-binding sites on Y-CT. Site A, the stimulatory site, would have higher affinity for anionic lipids; thus at low lipid concentration more A sites than B sites would be filled, resulting in enzyme activation. Site B, the inhibitory site, would have lower affinity for anionic lipids, so that B sites would become occupied only at higher lipid concentration. This would result in a diminution of

the activation at higher lipid concentration. This two-site model could also explain why in the translocation experiments (Table 2) not all of the Y-CT activity lost from the cytosolic fraction was recovered in the microsomal fraction.

This study has been the first step in the elucidation of the CT domains involved in the interaction with lipids. The data support a role for the amphipathic helix of the rat CT in lipid interactions, in that the yeast CT, which lacks this domain, has different lipid specificity and appears to have a different mode of interaction with membrane lipids. Mutagenesis of R-CT and Y-CT clones and photochemical labelling of R-CT and Y-CT with lipid derivatives could be used to identify the lipid-interactive sites of both CTs.

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