Binding of CTP:Phosphocholine Cytidylyltransferase to Lipid Vesicles: Diacylglycerol and Enzyme Dephosphorylation Increase the Affinity for Negatively Charged Membranes†

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ABSTRACT: The regulation of membrane binding and activity of purified CDP:phosphocholine cytidylyltransferase (CT) by lipid activators and enzyme dephosphorylation was examined. The binding of CT to membranes was analyzed using sucrose-loaded vesicles (SLVs). Binding to phosphatidylcholine vesicles was not detected even at a lipid:protein ratio of ~2000 (1 mM PC). CT bound to vesicles containing anionic lipids with apparent molar partition coefficients between 2×10^5 and 2×10^6, depending on the vesicle charge. The vesicle binding and activation of CT showed very similar sigmoidal dependencies on the lipid negative charge. In addition, diacylglycerol interacted synergistically with anionic phospholipids to stimulate both binding and activation at lower mole percent anionic lipid. These results demonstrate parallel requirements for binding and activity. Dephosphorylation of CT without destabilization was accomplished using the catalytic subunit of protein phosphatase 1. Dephosphorylated CT required a lower mole percent anionic phospholipid than phosphorylated CT for binding to and activation by SLVs. The combination of 10 mol % diacylglycerol and enzyme dephosphorylation shifted the mole percent phosphatidic acid required for half-maximal activation from 25% to 12%. These results suggest a mechanism whereby large changes in CT activity can result from changes in the phosphorylation state combined with small alterations in the membrane content of diacylglycerol. We propose a mechanism whereby dephosphorylation on the domain adjacent to the membrane binding domain increases the affinity of the latter for a negatively charged membrane surface.

CTP:phosphocholine cytidylyltransferase (CT)[1] is a rate-limiting and regulatory enzyme in the synthesis of phosphatidylcholine (PC) in animal cells. The posttranslational regulation of CT involves its interconversion between a soluble, inactive form, and a membrane-bound, active form (Tronchère *et al*., 1994; Cornell, 1996). A primary determinant of membrane association is the lipid composition of the membrane. Anionic lipids such as oleic acid and diacylglycerol (DG) promote translocation to membranes *in vitro* (Choy & Vance, 1978; Feldman *et al*., 1981; Feldman & Weinhold, 1987; Kolesnick & Hemer, 1990; Cornell, 1991a,b; Arnold & Cornell, 1996) and *in vivo* (Pelech *et al*., 1983; Cornell & Vance, 1987; Slack *et al*., 1991; Utal *et al*., 1991; Wang *et al*., 1993; Tronchère *et al*., 1995). The activation by anionic lipid is clearly related to the head group charge (Cornell, 1991a; Arnold & Cornell, 1996), but hydrophobic interactions between enzyme and lipid constitute the dominating driving force for the binding and activation by anionic lipid vesicles (Arnold & Cornell, 1996). *In vitro* DG can synergize with anionic lipids such as phosphatidic acid (PA) to produce active transferase at very low, physiologically relevant mole fractions of the two lipid activators (Arnold & Cornell, 1996).

The phosphorylation state of CT also influences its membrane association.[2] The phosphatase inhibitor okadaic acid caused an increase in CT phosphorylation and an increase in the fraction of CT in the soluble form in hepatocytes and CHO cells (Hatch *et al*., 1992; Watkins & Kent, 1991). The effect of okadaic acid on CT distribution in hepatocytes was overcome by incubating cells with the lipid activator oleic acid (Hatch *et al*., 1992). On the other hand, the membrane translocation of CT induced by phospholipase C treatment of CHO cells was blocked by okadaic acid treatment (Watkins & Kent, 1991). Membrane translocation induced by phospholipase C or oleic acid treatment,

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[1] Abbreviations: CL, cardiolipin; PA, phosphatidic acid; PC, phosphatidylcholine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; DG, diacylglycerol; CT, CTP:phosphocholine cytidylyltransferase; [^{3}H]DPPC, [methyl-^{3}H]dipalmitoylphosphatidylcholine; SLVs, sucrose-loaded vesicles; SUV, small unilamellar vesicle; PMSF, phenylmethanesulfonyl fluoride; DTT, dithiothreitol; cdc2, cyclin-dependent kinase I; CS1, catalytic subunit of protein phosphatase 1.

[2] Although there is abundant evidence that the phosphorylation state modulates CT's distribution between soluble and particulate forms, a few systems have been described in which an increase in CT activity was associated with CT dephosphorylation, but no translocation between soluble and particulate forms was observed (Shiratori *et al*., 1994, 1995; MacDonald & Possmayer, 1995). Thus, the role of phosphorylation of CT may not be restricted to the regulation of its membrane association.

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or choline deprivation, was correlated with a decrease in the phosphorylation state (Watkins & Kent, 1991; Wang *et al*., 1993; Houweling *et al*., 1994; Weinhold *et al*., 1994). Kinetic analysis of the changes in CT in response to the oleic acid content of the membrane indicated that phosphorylation/dephosphorylation is subsequent to membrane dissociation/association (Wang *et al*., 1993; Houweling *et al*., 1994). These observations can be accommodated into a model in which CT equilibrates between membrane-bound and soluble forms. Phosphorylation stabilizes the soluble conformation, whereas dephosphorylation stabilizes the membrane-bound conformation. The relative significance of phosphorylation *vs* lipid signals in the control of the ratio of the two forms of CT was not clear from these studies.

CT consists of discrete catalytic, membrane-binding, and phosphorylation domains (domains C, M, and P, respectively). Domain C (residues 72−235) is homologous to other members of the CT superfamily (Bork *et al*., 1995). Domain M is composed of a long uninterrupted amphipathic helix (residues 242−298; Johnson & Cornell, 1994; Johnson *et al*., 1997; Dunne *et al*., 1996). Its role in membrane binding has been demonstrated using proteolysis (Craig *et al*., 1994), mutagenesis (Cornell *et al*., 1995; Wang & Kent, 1995b; Yang *et al*., 1995), direct lipid photolabeling (Johnson *et al*., 1997), and binding studies with synthetic peptides (Johnson & Cornell, 1994). The transferase as well as the domain M peptides intercalate into the lipid bilayer when they bind (Johnson & Cornell, 1994; Arnold & Cornell, 1996). Domain P (residues 312−367) resides at the C-terminal end of CT. This domain contains 16 serines which were between 50 and 90% phosphorylated as determined by sequencing of phosphopeptides generated from CT purified from Sf9 cells (MacDonald & Kent, 1994). Mutants lacking domain P were unphosphorylated when expressed in COS cells (Cornell *et al*., 1995), CHO cells (Wang & Kent, 1995b), and Sf9 cells (Yang & Jakowski, 1995).

Recently, mutations in rat liver CT lacking single, multiple, or all of the C-terminal phosphorylation sites have been characterized. None of the mutants have impaired catalytic properties. There were, however, indications of altered membrane lipid affinity. Alanine substitutions for 5, 7, or all 16 of the serines resulted in an increased ratio of membrane to soluble forms when expressed in a CT-defective CHO strain (Wang & Kent, 1995a). On the other hand, the distribution between membrane and soluble forms for a mutant lacking the entire domain P (312−367) was the same as wild-type CT when both were overexpressed in COS cells (Cornell *et al*., 1995). The same domain P mutation was expressed in insect cells (Sf9), and its activity was examined in delipidated cell lysates as a function of the molar concentration of lipid at fixed PC:lipid activator ratios (Yang & Jakowski, 1995). This study concluded that the K_m for lipid activator was decreased in the CT mutant lacking domain P.

The above mutants have at least two significant shortcomings as substitutes for dephosphorylated CT: (1) Deletion of the entire phosphorylation domain is not the same as whole dephosphorylated enzyme; (2) substitution of alanine for serine is not conservative in terms of the hydrophobicity change (Reithmeier & Deber, 1992). We have improved upon the experimental approach by generating dephosphorylated CT. Previous attempts at CT dephosphorylation using acid phosphatase produced dephosphoenzyme which was aggregated, inactive, and intractable to rephosphorylation by kinases *in vitro* (Cornell *et al*., 1995). In this study, the dephosphorylation of CT by the catalytic subunit of protein phosphatase 1 (CS1) was achieved without CT aggregation. We compare the intrinsic membrane affinity of purified phosphorylated and dephosphorylated CT. Our experiments explore the relationship of lipid activator and phosphorylation on the membrane affinity of CT.

MATERIALS AND METHODS

Materials

The lipids 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphate (PA), 1,2-dioleoyl-*sn*-glycero-3-[phospho-*rac*(1-glycerol)] (PG), heart cardiolipin (CL), egg yolk phosphatidylcholine (PC), soybean phosphatidylinositol (PI), and 1,2-dioleoyl-*sn*-glycero-3-[phospho-L-serine] (PS) were purchased from Avanti Polar Lipids. Diacylglycerol (DG) was prepared from egg PC by phospholipase C digestion as described by Myher and Kuksis (1979). Less than 1% was in the 1,3 isoform as determined by TLC. [methyl-^{3}H]Choline chloride and [^{3}H]-dipalmitoylphosphatidylcholine ([^{3}H]DPPC) were obtained from DuPont NEN.

[^{3}H]Phosphocholine was prepared from [^{3}H]choline as described by Cornell (1989). Cyclin-dependent kinase I (cdc2), MAP kinase, and casein kinase II were obtained from Upstate Biotechnology Incorporated. Leupeptin, chymostatin, antipain, pepstatin, *p*-aminobenzadine, benzamidine, phenylmethanesulfonyl fluoride (PMSF), cytidine triphosphate, dithiothreitol (DTT), Triton X-100, DEAE, and sucrose were supplied by Sigma. Microcystin was obtained from Calbiochem. Hydroxyapatite was purchased from Bio-Rad. All other chemicals were reagent grade. The α isoform of the catalytic subunit of protein phosphatase 1 (CS1) was expressed in *E. coli* and purified to homogeneity (Yang and DePaoli-Roach, unpublished results) by a modification of published procedures (Zhang *et al*., 1992). The specific activity toward glycogen phosphorylase was approximately 20 μmol min^{-1} mg^{-1}.

Methods

Purification of CT. CT was purified as described by MacDonald and Kent (1993), as modified by Cornell *et al.* (1995). Purified CT was stored at −80 °C in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 200 mM KH_2PO_4, 2 mM DTT, 0.8 mM Triton X-100, and protease inhibitors (2.5 μg/mL leupeptin, 2 μg/mL chymostatin, 1 μg/mL antipain, 2 μg/mL pepstatin, 10 μg/mL *p*-aminobenzadine, 10 μg/mL benzamidine, 2 mM PMSF).

Preparation of SLVs. Lipids were stored as stock solutions in chloroform. The concentrations of the stock solutions were determined by phosphorus assay (Bartlett, 1959). SLVs were prepared by drying chloroform solutions of lipid mixtures and trace amounts of [^{3}H]DPPC under vacuum. The lipids were rehydrated in 20 mM Tris, pH 7.4, and 170 mM sucrose, vortexed vigorously, subjected to 5 freeze−thaw cycles, and stored at −20 °C. Upon thawing, the lipid suspensions were extruded with a Liposofast extruder (Avestin, Inc., Ottawa, ON) through a 0.1 μm polycarbonate filter. The extruded vesicles were diluted 4-fold in 20 mM Tris, pH 7.4, 133 mM NaCl, and then centrifuged at 100000*g*, 20 °C, for 30 min. Three-fourths of the supernatant was

removed, and the pellet was resuspended by vortexing. The concentration of the lipid was calculated from the dpm of tracer [^{3}H]DPPC. SLVs were used immediately in the assays. The sizes of the SLVs were determined using a NICOMP Submicron Particle Sizer Model 370. The diameter of the SLVs containing 100 mol % PC was 200 ± 3 nm, similar to the SLVs containing 50 mol % PA, 190 ± 1 nm.

Dephosphorylation of CT. To remove phosphate, purified CT was dialyzed at 20 °C for seven 1 h intervals against 100× volumes of 50 mM Tris, pH 7.4, 0.7 mM Triton X-100, 150 mM NaCl, 1 mM EDTA, 2 mM DTT, and 1 mM PMSF, followed by dialysis overnight at 4 °C. After dialysis, CT was stored at −80 °C. When partially purified ^{32}P-labeled CT was used (octyl glucoside extract; Cornell *et al*., 1995), dialysis was unnecessary. CT was dephosphorylated in the presence of 40 mM imidazole, pH 7.1, 2 mM DTT, 0.2 mM $MnCl_2$, 0.5% glycerol, 110 μM Triton X-100, 20 mM NaCl, 7 mM Tris, and 0.1 mM EDTA and CS1α. A weight ratio of 40:1 CT to phosphatase was present in the final reaction mixture. The reaction was stopped after 1.5−2 h at 30 °C by the addition of microcystin to a final concentration of 15 μM. Controls were included, omitting the addition of the phosphatase. These samples were used immediately in binding or activity assays.

Binding Assays. Freshly prepared SLVs were incubated with 3−5 μg of CT in a total volume of 100−260 μL. The final concentrations of species in the solution were 20 mM Tris, pH 7.4, 10 mM DTT, 110 mM NaCl, 5 mM sucrose, 30−65 μM Triton X-100, 0−9 mM KH_2PO_4, and 50−1000 μM lipid. After incubation for 10−30 min at 20 °C, the samples were centrifuged at 100000*g* for 30 min at 20 °C. The Triton X-100 present did not interfere with the sedimentation of the lipid. The pellets contained greater than 90% of the lipid as determined by liquid scintillation counting of [^{3}H]DPPC. In experiments where activity and binding were compared, lipid, CT, $MgCl_2$, CTP, and DTT were combined at 3× the concentration in the final assay mixture and samples removed and diluted 3-fold for binding and activity assays. Final concentrations were the same as above except that 3 mM CTP and 12 mM $MgCl_2$ were also present. Eighty percent of the supernatant was removed, and the quantity of CT in the different fractions was analyzed by SDS−PAGE (Laemmli, 1970) followed by densitometry of Coomassie-stained gels (Microtek Scanmaker Densitometer). Electrophoresis sample buffer contained 1 μg of BSA/30 μL for quantitation of CT bands. Lipid concentrations were determined from [^{3}H]DPPC dpm in aliquots of mixture before pelleting and aliquots of the supernatant after pelleting. The % CT in the pellet was calculated as follows:

$$\% \text{ CT in pellet} = [(\text{pellet})/(\text{pellet} + \text{sup})] \times 100$$

where sup = density units of CT relative to density units of BSA in supernatant/0.8 and pellet = density units of CT relative to density units of BSA in pellet − (sup × 0.2).

These calculations take into account the 20% supernatant remaining in the pellet fraction. The apparent molar partition coefficient was calculated as K_x = (nmol of CT bound/nmol of CT free) × (nmol of H_2O/nmol of accessible lipid). Accessible lipid was 0.5 × total lipid, i.e., the outer monolayer only: $K_x = K_p \times 55.5$ M.

Activity Assays. A solution of CT, DTT, NaCl, Tris (pH 7.4), CTP, and $MgCl_2$ was added to SLVs, and the reaction was initiated with [^{3}H]phosphocholine (≈10 Ci/mol) in a final volume of 50 μL. The final concentrations of components in the reaction mixture were 0.2−1.5 μg of CT, 10 mM DTT, 110 mM NaCl, 20 mM Tris, pH 7.4, 10 mM CTP, 15 mM $MgCl_2$, 7 mM sucrose, 1.7 mM phosphocholine, and 0−700 μM lipid. When dephosphorylated CT was assayed, 4.6 mM imidazole, 1.7 μM microcystin, and 5 ng of CS1α were also present. The reaction was allowed to proceed for 10 min at 37 °C unless otherwise indicated, and was stopped by the addition of 30 μL of methanol/ammonia (9:1). The rate of the reaction was linear with time. The formation of [^{3}H]CDP-choline was analyzed as described previously (Sohal & Cornell, 1990).

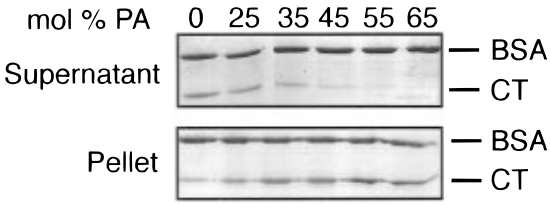

FIGURE 1: Binding of CT to SLVs with increasing mole percent PA. Three micrograms of CT was incubated with 1 mM PC SLVs containing the indicated mole percent PA; the vesicle-bound (pellet) and free CT (supernatant) were separated as described under *Methods*. Each fraction was analyzed by SDS−PAGE (12%) and stained with Coomassie Blue. The pellet fraction contained 20% of the total supernatant. This amount was taken into account to calculate the percent CT in the pellet as in Figures 2−4, 6, and 7.

RESULTS

To carry out this work, it was necessary to establish a lipid vesicle-binding assay. At the onset of the project, there had been no published studies of the direct binding of purified CT to lipid vesicles. Binding had merely been inferred from enzyme activity increases. We adapted a sucrose-loaded vesicle (SLV) binding assay, which enables facile separation of free and vesicle-bound protein by sedimentation (Rebecchi *et al*., 1992). This method has been used to study the membrane interactions of phospholipase C (Rebecchi *et al*., 1992) and protein kinase C (Mosior & Epand, 1993, 1994; Mosior & Newton, 1996). Using SLVs, we compared the requirements for CT activation and membrane binding with respect to the negative charge of acidic lipids, synergism between acidic phospholipids and DG, and the phosphorylation state of the enzyme.

Negative Surface Charge Regulates CT Binding to SLVs. The binding of purified CT to pure phospholipid membranes was investigated using SLVs. Figure 1 shows the result of a typical binding assay. As the mole fraction of PA increased from 0 to 65 mol %, the amount CT in the supernatant decreased, and the amount in the pellet increased. Greater than 90% of the CT was recovered in the binding experiments.

Figure 2A shows the binding as a function of increasing concentration of SLVs composed of 100% PC or 40 mol % PA/60 mol % PC. The lowest concentration of lipid that could be tested was 15 μM, due to the presence of at least 30 μM Triton X-100 accompanying the CT. Below a Triton: lipid molar ratio of 2, the vesicular nature of the lipids was preserved, since >90% of the lipid sedimented at 100000*g* × 30 min. CT bound poorly to SLVs composed of egg PC between 30 and 450 μM. Binding to 40 mol % PA vesicles plateaued at approximately 80% bound and was ∼75%

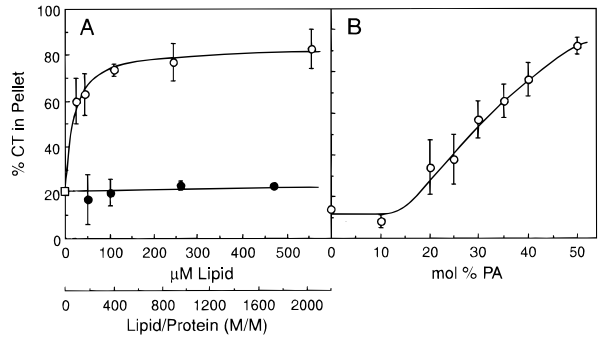

FIGURE 2: CT binding to SLVs: dependence on total lipid concentration (A) and mole percent lipid activator (B). (A) CT binding to SLVs was measured at 20 °C in the presence of increasing concentrations of PC alone (●) or 40 mol % PA/60 mol % PC (○) as described under *Methods*. In the absence of lipid (□), 20% of the CT was in the pellet. (B) Binding of CT to SLVs composed of egg PC and 0−50 mol % PA was measured at 25 °C. The total lipid concentration was 1 mM. Error bars represent the standard error of the mean of two experiments (except for 470 μM egg PC, which is one data point) (A) and three experiments (B).

Table 1: Binding of CT to SLVs Is Related to Lipid Charge[a]

lipid	total lipid concn (μM)	% CT in pellet	N	K_x ($\times 10^{-5}$)	N
none		19 ± 3	7		7
PC	100 ± 3	16 ± 2	6		6
25 mol % CL[b]	110 ± 3	75 ± 6	7	26 ± 3.2	7
25 mol % PA	110 ± 4	59 ± 3	6	11 ± 1.4	6
40 mol % PA	110 ± 3	75 ± 2	3	25 ± 3.5	3
40 mol % PG	100 ± 6	36 ± 7	4	2.1 ± 0.4	4
40 mol % PS	110 ± 5	29 ± 3	8	2.1 ± 0.3	6
40 mol % PI	100 ± 2	22 ± 3	9	1.6 ± 0.5	7

[a] Binding of CT was measured at 25 °C as described under *Methods*. The bulk lipid present in the vesicles was egg PC. Error represents the standard error of the mean of *N* sets of data. K_x values were calculated as described under *Methods*. [b] 100% of the CT was found in the pellet with SLVs containing 40 mol % CL.

complete at 30 μM lipid. Binding curves using other lipid compositions indicated that binding was saturated or nearly saturated at 30 μM lipid (data not shown), corresponding to a total lipid to protein molar ratio (L/P) of 110:1. The influence of the mole fraction of lipid activator is demonstrated in Figure 2B. The total lipid concentration was 1 mM (L/P = 1300). Binding to the SLVs was observed only above 10 mol % PA, or 100 μM PA. By comparison, 80% binding was observed in the presence of 40 μM PA when the total lipid concentration was 100 μM (Figure 2A). These data demonstrate that the relative rather than the absolute concentration of lipid activator determines the partitioning of CT into the membrane. Figure 2B also shows the sigmoidal dependence of CT binding on the mole percent PA. In addition, a sigmoidal dependence was observed with CL (Figure 4), PG (Figure 7A), and PS (data not shown).

Binding of CT to SLVs was related to the charge on the lipids present. Table 1 shows the percent CT in the pellet and the apparent molar partition coefficients for SLVs composed of various mole percentages of anionic phospholipids. CT bound SLVs containing CL, net negative charge of 2, with the highest affinity followed by PA with a net negative charge between 1 and 2. CT bound with weaker but similar affinity to PG and PS, each having a net negative charge of 1. CT bound with weakest affinity to PI, net negative charge of 1. This binding preference resembles the activation preference (Arnold & Cornell, 1996). Apparent

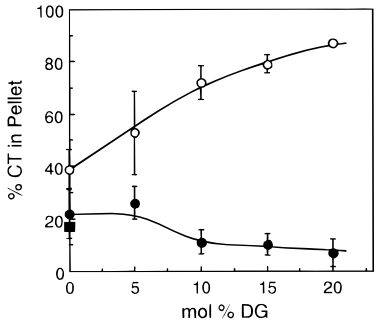

FIGURE 3: DG synergism with PA: effects on binding. CT binding to SLVs was measured at 20 °C as described under *Methods*. The SLVs were composed of egg PC and 0−20 mol % DG (●) or 50−70 mol % egg PC, 0−20 mol % DG, and 30 mol % PA (○). The total lipid concentration was 1 mM. The amount of CT in the pellet in the absence of lipid is also shown (■). Error bars represent the standard error of the mean of three experiments.

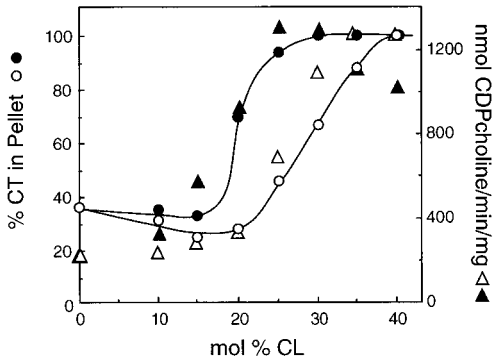

FIGURE 4: DG lowers the mole percent CL required for CT binding and activation. CT binding to SLVs was measured at 25 °C. The SLVs were composed of egg PC and 0−40 mol % CL (○) or egg PC, 0−40 mol % CL, and 10 mol % DG (●). The total lipid concentration was 0.7 mM. 12 mM $MgCl_2$ and 3 mM CTP were included in the binding assay which was performed at 25 °C. Activity was measured at 37 °C for 30 min and is shown with (▲) and without (△) 10 mol % DG. The data represent one experiment which was repeated with similar results using PG.

K_x values were calculated from the binding data (Tamm, 1991). The same effect of lipid charge on the K_x values was observed at 50 and 100 μM lipid. To determine the effect of anionic lipid on the K_x value, the amount of CT that pelleted in the presence of PC alone was subtracted from the amount of CT in the pellet of SLVs composed of the various anionic phospholipids. The K_x values ranged from 2×10^5 to 2×10^6. The lower limit of K_x that could be measured in our binding assay was approximately 1×10^5, due to the substantial sedimenting of CT in the absence of lipid.

Synergism between Anionic Phospholipid and DG. DG and anionic lipids synergize to activate CT (Arnold & Cornell, 1996). Synergistic effects of these lipids on the binding of CT to SLVs were investigated, and the results are shown in Figures 3 and 4. Figure 3 demonstrates that 0−20 mol % DG had no significant effect on the amount of CT in the pellet. Higher concentrations of DG could not be tested because the vesicles were unstable. The amount of CT in the pellet nearly doubled when vesicles containing PC alone were compared to vesicles containing 30 mol % PA. CT binding was further enhanced by vesicles containing 30 mol % PA plus 5−20 mol % DG. Whereas only ~10% of the CT sedimented with vesicles containing 20 mol % DG, nearly 90% sedimented with vesicles containing 20 mol

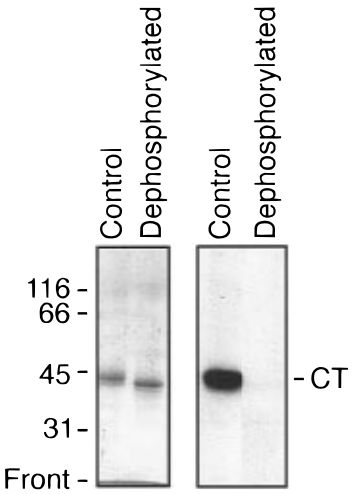

FIGURE 5: Dephosphorylation of CT. (A, left panel) Coomassie-stained gel (9%). CT purified from the baculovirus expression system was incubated at 30 °C for 1.5 h in the absence (control) or presence (dephosphorylated) of CS1. An aliquot (3 μg) was removed for electrophoresis. (B, right panel) Autoradiograph of SDS gel. ^{32}P-Labeled CT, partially purified from a COS cell expression system, was treated for 1.25 h at 30 °C in the absence (control) or presence (dephosphorylated) of CS1.

% DG plus 30 mol % PA. Because DG alone did not promote binding, and 30 mol % PA merely doubled the binding, this increase must be attributed to synergism between PA and DG.

The mechanism of the effect of DG was clarified by examining the binding of CT to SLVs containing increasing mole percent CL in the absence or presence of 10 mol % DG (Figure 4); 10 mol % DG significantly shifted the anionic lipid dependence to the left. The concentration of CL for maximal CT binding was 40 mol % CL in the absence of DG and 25 mol % CL in the presence of 10 mol % DG. SLVs containing 10 mol % DG alone had no effect on the amount of CT in the pellet. The same mixtures were analyzed for effects on CT activity. Synergism between CL and DG was also observed in the activity assays. Activity and binding were nearly coincident (Figure 4). Coincidence between binding and activity curves was also observed for SLVs containing variable PG content (data not shown). The dissimilarity in the values in Table 1 and Figure 4 for the percent CT in the pellet at 25 mol % CL is a result of $MgCl_2$ present in the experiment shown in Figure 4. CT binding in the presence of Mg^{2+} required a higher mole percent CL or PA, thus shifting the binding curves to the right.

Effect of CT Phosphorylation State on Binding, Activity, and Stability. We explored the effect of CT phosphorylation on its affinity for SLVs. CT purified from the baculovirus expression system has been shown to be phosphorylated on up to 16 serine residues on the C-terminus (MacDonald & Kent, 1994). Upon treatment of this CT with the catalytic subunit of phosphatase 1 (CS1α), we saw a characteristic gel shift (SDS−PAGE) to lower apparent molecular mass (2.0 ± 0.6 kDa shift; $N = 4$), indicative of a reduced phosphorylation state (Figure 5A, left panel). Similar phosphatase treatment of partially purified ^{32}P-labeled CT from a COS cell expression system revealed a similar (1.9 ± 0.5 kDa; $N = 3$) gel shift and a reduction of greater than 90% of the ^{32}P-label as determined by densitometry of the autoradiogram (Figure 5B, right panel). The solubility of CT was not reduced by the dephosphorylation. The amount

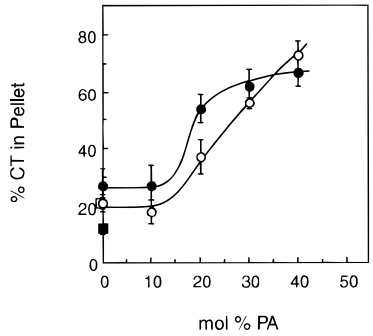

FIGURE 6: Effect of dephosphorylation on the binding to SLVs containing PA. Binding was measured at 20 °C to SLVs containing 0−50 mol % PA (circles) with control (○, □) or dephosphorylated (●, ■) CT. The total lipid concentration in the assay was 0.5 mM. The amount of CT in the pellet in the absence of lipid is also shown (squares). Error represents standard error of the mean of four sets of data.

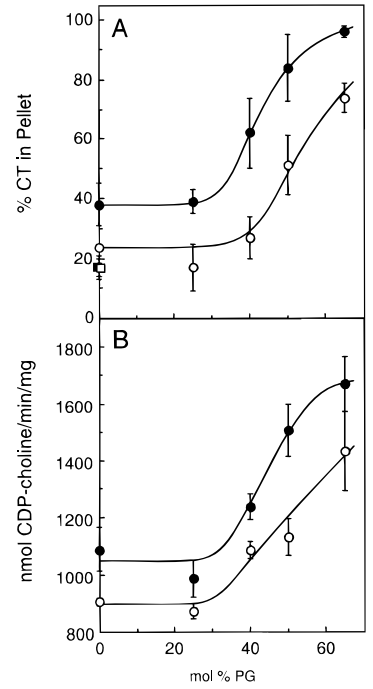

FIGURE 7: Effect of dephosphorylation on binding to/activation by SLVs containing PG. Binding (A) and activity (B) were measured at 20 °C in the presence of SLVs containing 0−65 mol % PG (circles) with control (○, □) or dephosphorylated (●, ■) CT. The total lipid concentration was 0.5 mM for the binding assay and 140 μM for the activity assay. Activity measurements were for 20 min. The amount of CT in the pellet in the absence of lipid is also shown (squares). Binding is the average of two experiments. The error in the activity measurements represents the standard error of the mean of three data sets.

of CT sedimenting in the absence of lipid remained at ~20% (Figures 6 and 7A).

We compared the binding of phosphorylated and dephosphorylated CT to SLVs containing varying mole percentages PA or PG. The binding curves for the dephosphorylated enzyme were shifted to the left of control enzyme (Figures 6 and 7A). This indicates that less anionic lipid was needed to stimulate binding. Dephosphorylated enzyme had a higher binding affinity for PC alone in comparison to phosphorylated CT. Different degrees of enzyme dephosphorylation may account for the differences in the effects on CT binding in the PA *vs* PG curves (Figures 6 and 7A). Partition coefficients (K_x) for SLVs containing 20 mol % PA increased

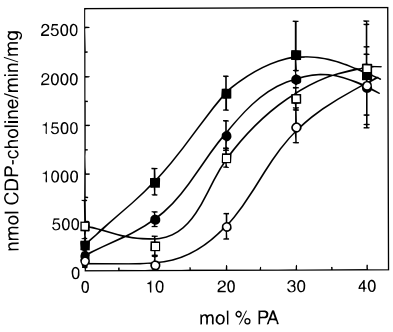

FIGURE 8: Effect of DG and CT dephosphorylation on the activation by PA. Activity was measured at 37 °C in the presence of SLVs (200 μM lipid) containing 0−40 mol % PA, with control (○, ●) or dephosphorylated (□, ■) CT in the absence (open symbols) or presence (closed symbols) of 10 mol % DG. Error represents the standard error of the mean of four sets of data.

3−4-fold upon dephosphorylation. Activity curves showed similar effects of CT dephosphorylation (Figures 7B and 8).

Conversely, purified CT was phosphorylated by the combination of cdc2 kinase, MAP kinase, and casein kinase II using conditions previously described (Cornell *et al*., 1995). The lipid activation curves for control and kinase-treated CT were not significantly different. The low stoichiometry of kinase-catalyzed phosphorylation (0.003 mol of phosphate/mol of CT) explains the lack of an effect. Dephosphorylation of CT with CS1 did not improve the stoichiometry of phosphorylation by these kinases (data not shown). This suggests that the purified CT preparation provides a very poor substrate for these kinases *in vitro*.

The above work demonstrated that both dephosphorylation and DG increased the sensitivity of CT to the anionic lipid surface concentration. Subsequently, we determined the effect of the combination of enzyme dephosphorylation and DG on the activation by anionic lipids. Control and phosphatase-treated CT were assayed for activity in the presence of SLVs containing 0−40 mol % PA, with or without 10 mol % DG (Figure 8). The combination of CT dephosphorylation and DG resulted in large changes in CT activity in response to anionic lipid. Thus, vesicles containing 20 mol % PA provided minimal stimulation of phosphorylated CT, whereas vesicles containing 20 mol % PA plus 10 mol % DG produced nearly full activation of dephosphorylated CT (compare open circles and closed squares in Figure 8).

A recent report by Wieprecht *et al*. (1996) demonstrated that treatment of cells with olomoucine, an inhibitor of proline-directed kinases, reduced the phosphorylation of CT in comparison to untreated cells. The olomoucine-treated CT appeared to be more sensitive to proteolysis by endogenous proteases, suggesting that phosphorylation of the enzyme resulted in a more stable enzyme. We explored this possibility further by examining the proteolytic sensitivity of dephosphorylated CT by chymotrypsin. The fragmentation pattern of CT resulting from chymotrypsin proteolysis has previously been characterized (Craig *et al*., 1994; Cornell *et al*., 1995). Control and dephosphorylated CT showed no detectable differences in their pattern of proteolysis as a function of chymotrypsin concentration (results not shown). Therefore, it does not appear that dephosphorylation of the purified CT sensitized the enzyme to proteolysis by chymotrypsin *in vitro*. In agreement with our findings, Wang and Kent (1995a) found that 40−60% of a fully dephosphorylated CT mutant was stable in a soluble form.

DISCUSSION

Parallel Requirements for Activation and Binding. Investigations of the lipid−protein interactions of CT have mainly relied on an assumed equivalence between enzyme binding to lipid vesicles and enzyme activation by those vesicles, the enzyme activity being much simpler to monitor than binding. However, we have recently shown that CT can bind electrostatically to gel phase lipids without activation, indicating that binding is not always synonymous with activation. In the present work, we have adapted a vesicle binding assay for CT. We show parallel requirements for enzyme activation and vesicle binding with respect to the negative surface charge density of lipid vesicles and the synergism between two classes of lipid activators, anionic phospholipids and DG. The parallel effects were especially evident in the data of Figure 4, where the binding and activity curves were virtually coincident.

The binding of amphiphilic peptides to lipids is often described as a partitioning between two phases which is determined by the intrinsic solubility difference of the peptide for the two phases and the relative concentrations of the two phases. For charged peptides, the partitioning is also dependent on the electrostatic potential at the surface (Tamm, 1991, 1994). Using vesicles containing varying mole percent PA, we observed that CT partitioning was limited strictly by the mole fraction of negatively charged lipid. Thus, the enzyme does not respond to an absolute molar concentration of the negatively charged lipid, but responds to the surface concentration of acidic lipid relative to the bulk inert lipid. This phenomenon emphasizes the important role of electrostatics in the binding of CT to acidic vesicles. The surface potential attracts CT to the interface, and could increase its effective concentration in the zone 50 Å from the phospholipid surface by up to 6×10^4, depending on the strength of the surface potential.[3] The interfacial zone of the domain M amphipathic helix is dominated by lysines. We propose that these basic side chains respond to the negative surface potential. The sigmoidal nature of the binding curves can be attributed to a requirement for a threshold surface potential, and the reduction in dimensionality (Mosior & McLaughlin, 1992) resulting from the electrostatic attraction. The sigmoidal behavior was not a result of curvature promotion. The diameters of vesicles with 0 or 50 mol % PA were very similar (200 nm).

Complete activation of CT by small unilamellar vesicles (SUVs) occurred in the presence of 10 mol % PA (Arnold & Cornell, 1996). However, the complete binding and activation of CT by SLVs required 50 mol % PA. Activation by SLVs required a higher concentration of all lipid activators than did SUVs. The lack of correlation between SUVs and SLVs may be a result of curvature differences. Our SUVs were 25−50 nm in diameter (Arnold & Cornell, 1996), while

[3] The calculation of the ratio of total aqueous volume to the total surface phase volume of a 0.1 mM suspension of SLVs made the following assumptions: vesicles were treated as spheres; volume per lipid=1600 Å^3; total surface phase volume = (surface phase volume per vesicle) × (total number of vesicles); surface phase volume per vesicle = (volume of sphere, r = 1050 Å) − (volume of sphere, r = 1000 Å). Number of vesicles was calculated based on 2.6×10^6 lipids per SLV.

our SLVs were approximately 200 nm in diameter. In addition, SLVs can take on discoid shapes (Mui *et al*., 1993). Therefore, in SLV assays, CT was exposed to lipids in a more planar arrangement. The promotion of positive curvature, which is a factor in the potency of anionic lipids presented as SUVs, is not a factor when the same lipids are presented as SLVs.

We also found that DG, by itself, did not stimulate CT binding or activity when it was presented in egg PC SLVs. DG did not activate CT when added as a component of a Triton X-100 micelle (Cornell, 1991a). The stimulation of CT−vesicle binding by DG was only evident in SUVs (Arnold & Cornell, 1996). Thus, the lipid packing arrangement influences the efficacy of DG as a CT activator. The manner of presentation of DG to CT is of critical importance when using model membrane systems.

DG synergized with anionic lipids in SLVs to bind and activate CT. This effect is in agreement with results observed using SUVs (Arnold & Cornell, 1996). The effect of DG was to lower the mole percent anionic lipid required for membrane binding and activation of CT. One possible mechanism for this phenomenon is the promotion of the formation of lateral domains of anionic lipid by DG. This mechanism has been proposed for the effect of saturated DG on the activation of phospholipase A_2 by lysoPC, using membranes near the gel to liquid-crystalline phase transition, T_m (Sheffield *et al*., 1995; Bell *et al*., 1996). Our lipid mixtures, however, were unsaturated and well above T_m; thus, lateral domain formation of the acidic lipid is unlikely. Changes in the mole fraction of membrane DG in response to agonists could increase CT's membrane affinity and activity without alteration in the total acidic lipid content [see, e.g., Tronchere *et al*. (1995)].

Modulation of Membrane Affinity by Phosphorylation. One view of the relationship between lipid and phosphorylation regulators of CT is that the primary regulator of membrane association and activation is the surface concentration of lipid activator, while the phosphorylation state serves a secondary regulatory role. This view is suggested by several observations: (1) dephosphorylation/rephosphorylation of CT was subsequent to membrane association/dissociation in cells enriched or depleted in the lipid activator oleic acid (Houweling *et al*., 1994; Wang *et al*., 1993); (2) the lipid activator oleic acid promoted membrane binding of phosphorylated CT in cells treated with okadaic acid (Hatch *et al*., 1992); (3) mutants lacking some or all of the phosphorylation sites had altered distribution between membrane and soluble cell fractions, but could be induced to translocate to membranes in the presence of excess lipid activator (Wang & Kent, 1995a). In these experiments, the membrane concentrations of lipid activator which reversed the effects of phosphorylation were undefined, but were likely higher than physiological concentrations. Our data (Figures 6−8) explain clearly why the effects of increased phosphorylation could be overcome by excess lipid activator.

An alternative view is that phosphorylation/dephosphorylation serves to fine-tune CT's membrane affinity at the levels of acidic lipid and DG normally encountered in cell membranes (Yang & Jackowski, 1995). Our results concur with this notion.

We have examined the relationship between lipid activators and the phosphorylation state of CT by analyzing the effects of dephosphorylation of pure enzyme on its membrane affinity as a function of the surface concentration of lipid activator. We have observed that dephosphorylation reduced the mole percent anionic lipid required for activation. Dephosphorylated CT also had a higher affinity for PC than did phosphorylated CT. This suggests that in some instances a change in CT's membrane affinity and hence activation could be induced solely by changes in the phosphorylation state, i.e., without a change in the membrane content of lipid activators. Our studies suggest how different modes of regulation could influence CT under different cellular conditions. CT's activation requires its association with membranes, and a negatively charged membrane surface greatly promotes that association. Thus, an increase in CT activity will result when the membane content of acidic lipids is elevated, *or* when the acidic lipid content is static but additional factors decrease the negative surface charge requirement; factors such as DG and/or CT dephosphorylation.

How could changes in the phosphorylation state of CT modulate its affinity for anionic membrane surfaces? We propose the following hypothesis: Domain P is adjacent to domain M in the linear sequence. Domain M is an amphipathic helix containing many interfacial lysine side chains, which are believed to interact electrostatically with the negatively charged lipid polar head groups in CT's membrane-bound form (Dunne *et al*., 1996). The interactions of these lysine side chains in the soluble form of the enzyme are unknown. It is conceivable that they ion pair with the negatively charged phosphates of domain P. The greater the degree of phosphorylation, the greater the electrostatic attraction for the positively charged groups of domain M. The phosphates on domain P would essentially compete for the phosphates on the membrane polar head groups. Dephosphorylation of domain P would eliminate this competition, thereby promoting the CT−membrane interation. The idea that each phosphate of domain P contributes to the stabilization of the soluble form is intriguing (Yang & Jackowski, 1995). There are 16 phosphoserines in domain P; there are 16 basic residues in domain M.

This mechanism for regulating the membrane association of CT resembles the hydrophobic−electrostatic switch proposal for the regulation of myristoylated amphitropic proteins such as the Src family of kinases and MARCKS (McLaughlin & Aderem, 1995). These proteins require, in addition to a hydrophobic myristoyl anchor, a polybasic region which interacts electrostatically with acidic phospholipids. The electrostatic attraction is weakened by phosphorylation of serines in the polybasic region, leading to membrane dissociation. The hydrophobic and electrostatic interaction surfaces of CT are created in the formation of an amphipathic α-helix in domain M. The effect of phosphorylation of sites in domain P on the stability of this α-helix is an important question for future work.

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