Functions of the C-terminal domain of CTP:phosphocholine cytidylyltransferase

Effects of C-terminal deletions on enzyme activity, intracellular localization and phosphorylation potential

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The role of the C-terminal domain of CTP: phosphocholine cytidylyltransferase (CT) was explored by the creation of a series of deletion mutations in rat liver cDNA, which were expressed in COS cells as a major protein component. Deletion of up to 55 amino acids from the C-terminus had no effect on the activity of the enzyme, its stimulation by lipid vesicles or on its intracellular distribution between soluble and membrane-bound forms. However, deletion of the C-terminal 139 amino acids resulted in a 90% decrease in activity, loss of response to lipid vesicles and a significant decrease in the fraction of membrane-bound enzyme. Identification of the domain that is phosphorylated *in vivo* was determined by analysis of ^{32}P-labelled CT mutants and by chymotrypsin proteolysis of purified CT that was ^{32}P-labelled *in vivo*. Phosphorylation was restricted to the C-terminal 52 amino acids (domain P) and occurred on multiple sites. CT phosphorylation *in vitro* was catalysed by casein kinase II, cell division control 2 kinase (cdc2 kinase), protein kinases Cα and βII, and glycogen synthase kinase-3 (GSK-3), but not by mitogen-activated kinase (MAP kinase). Casein kinase II phosphorylation was directed exclusively to Ser-362. The sites phosphorylated by cdc2 kinase and GSK-3 were restricted to several serines within three proline-rich motifs of domain P. Sites phosphorylated *in vitro* by protein kinase C, on the other hand, were distributed over the N-terminal catalytic as well as the C-terminal regulatory domain. The stoichiometry of phosphorylation catalysed by any of these kinases was less than 0.2 mol P/mol CT, and no effects on enzyme activity were detected. This study supports a tripartite structure for CT with an N-terminal catalytic domain and a C-terminal regulatory domain comprised of a membrane-binding domain (domain M) and a phosphorylation domain (domain P). It also identifies three kinases as potential regulators *in vivo* of CT, casein kinase II, cyclin-dependent kinase and GSK-3.

INTRODUCTION

CTP:phosphocholine cytidylyltransferase (CT) is an important regulatory enzyme of the phosphatidylcholine metabolic cycle [1], which, under most conditions, controls the rate of phosphatidylcholine (PC) synthesis by interconversion between an inactive soluble form and an active membrane-bound form [2,3]. The soluble form appears to be stabilized by phosphorylation at specific sites, whereas the active form is stabilized by interaction with lipids [4–6]. Translocation from a soluble form to membranes is associated with dephosphorylation, while the reverse process is accompanied by phosphorylation [6–10]. During the cell cycle of a macrophage cell line, Bac-1, the degree of phosphorylation of CT varied in concert with changes in enzyme activity [11]. The lipid interaction depends on the presence within the membrane of certain lipid activator molecules, such as anionic phospholipids, fatty acids or diacylglycerol [12].

The primary structures of several mammalian CTs [13–18] and CT from *Saccharomyces cerevisiae* [19] have been determined. The mammalian CTs are highly conserved with ⩾ 96% identity at the amino acid level. Secondary structure predictions suggest a tripartite organization: (1) a globular N-terminal domain (residues 1–235); (2) a pair of amphipathic α-helices (residues 236–315); and (3) a C-terminus dominated by turns (residues 316–367). This basic structure is supported by limited proteolysis, which shows that the N-terminal two-thirds is more resistant to digestion than the C-terminal one-third [20].

The structure and function of the N-terminal 75 residues is relatively unexplored. CT appears, by immunofluorescence, to be a nuclear enzyme [21]. Deletion of a polybasic site (residues 12–16) alters the nuclear localization of the enzyme [22]. The domain between amino acids 75 and 235 is highly conserved between the yeast and mammalian CTs and is believed to house the active site [13,23,24]. There is now strong evidence that amino acids 239–298 function in membrane binding. This region constitutes a discrete exon in the mouse CT gene [16]. Synthetic peptides from this region were transformed from random coil to α-helix in the presence of anionic lipid vesicles and intercalated into the hydrophobic core of these vesicles [25]. Furthermore, loss of membrane binding directly correlated with loss of the amphipathic helix when residues from the C-terminus were progressively removed using limited proteolysis [20].

C-Terminal to the lipid-binding amphipathic helix there is a short domain (17 residues) of unknown function, which is predicted to be helical. The C-terminal 55 amino acids contain 16 serines which fall within substrate consensus sequences for several protein kinases. Phosphopeptide analysis of ^{32}P-labelled CT immunoprecipitated from several sources [6,8,10,26] has shown that mammalian CT is phosphorylated on multiple sites. CT from cell extracts is often visualized on SDS gels as a set of two

Abbreviations used: CT, CTP:phosphocholine cytidylyltransferase; PKC, protein kinase C; MAP kinase, mitogen-activated kinase; GSK-3, glycogen synthase kinase-3; cdc2 kinase, cell division control 2 kinase; PC, phosphatidylcholine; DAG, diacylglycerol; PG, phosphatidylglycerol; CSF-1, colony-stimulating factor-1; DTT, dithiothreitol; EGF, epidermal growth factor; TBS, Tris-buffered saline; PAP, potato acid phosphatase.

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or three bands between 42 and 44 kDa, which is also indicative of multiple phosphorylated forms [6,11]. Recently MacDonald and Kent [26] demonstrated that the C-terminal 55 residues constitute the sole site of phosphorylation *in vivo*, and that all 16 serines in this domain are phosphorylated to some extent in rat liver CT, which was overexpressed using baculovirus and purified from insect cells.

The phosphorylation domain is very rich in prolines. At least seven serines are candidates for phosphorylation by mitogen-activated kinases (MAP kinases) [27] or cyclin-dependent kinases [28]. Ser-362 resides within an acidic context (DISEDEED) that resembles the target site for casein kinase II [28]. There is also the potential for phosphorylation by glycogen synthase kinase-3 (GSK-3) on several sites in the proline-rich region, in response to phosphorylation on downstream sites by other kinases [29,30]. Up to ten potential protein kinase C (PKC) sites are dispersed throughout the N-and C-terminal domains of CT. Treatment with phorbol esters, however, did not result in detectable changes in the phosphorylation state of CT [31,32]. Recently Wieprecht et al. [33] reported that CT was a substrate for phosphorylation *in vitro*, catalysed by $p44^{mpk}$.

To probe the role of the C-terminal lipid-binding and phosphorylation domains we have constructed mutations in the rat liver CT cDNA, deleting up to 140 amino acids from the C-terminus. In addition, Ser-362 was mutated to alanine. These mutants were expressed in COS cells. We also analysed chymotryptic fragments of wild-type CT, which lacked portions of the C-terminus. Our results show that the C-terminal domain P (Figure 1) is not necessary for enzyme activity, but is required for phosphorylation *in vivo* and for phosphorylation *in vitro* by casein kinase II, cell division control 2 kinase (cdc2 kinase) and GSK-3.

MATERIALS AND METHODS

Materials

Purified PKCα and PKCβII were generous gifts from Dr. A. Newton (University of California, San Diego), and purified GSK-3β was kindly provided by Dr. J. Woodgett (Ontario Cancer Institute, Toronto, Ontario, Canada). All three kinases were recombinant, rat brain- and baculovirus-expressed. Casein kinase II, cdc2 kinase and MAP kinase were purified from sea star as described [34,35]. Potato acid phosphatase (PAP) was from Boehringer. Heparin, heparin–Sepharose, digitonin, cyanogen bromide-activated Sepharose, peroxidase-antibody conjugate, ATP, CTP, β-glycerophosphate, dithiothreitol (DTT) and oleic acid were from Sigma. Dioleoyl phosphatidylserine, dioleoyl phosphatidylglycerol (PG) and egg PC were from Avanti. 1,2-Di-oleoylglycerol was generated from egg PC [36]. Restriction enzymes were from BRL/Life Technologies. Sequenase was from US Biochemicals and Vent polymerase was from New England Biolabs. [γ-^{32}P]ATP (3000 Ci/mmol) and sodium [^{32}P]orthophosphate were from Amersham. Antiserum against the C-terminal 12 amino acids of rat liver CT was a gift from Dr. C. Kent (University of Michigan). Rabbit antiserum against the N-terminal 15 amino acids of rat liver CT conjugated to keyhole-limpet haemocyanin was generated by monthly subcutaneous neck injections of 50 μg of protein in Freund's adjuvant, bleeding 10 days post-injection.

Methods

Construction of mutants

The construction of CT-2 (wild-type rat liver CT) from CT-1 has been described by Walkey et al. [37]. CT-1 is the original rat liver clone [13] containing two nucleotide mutations that encode for the replacement of Gly-91 with serine and of Ser-114 with cysteine. All mutations in this paper contained the wild-type sequence at amino acids 91 and 114. The start codon begins at nucleotide 25 in all CT-2 constructs.

To generate mutants Δ228 and Δ312, the CT-2 cDNA in pAX142, a derivative of pAX114 [38] which utilizes an elongation factor-1α promoter [39], was digested with *Sst*I at nucleotide 708 and *Hae*III at nucleotide 961 respectively. The Δ336 mutant was generated using the exonuclease *Bal*31 according to the manufacturer's instructions (Gibco/BRL), which digested from the 3′-end to nucleotide 1033. The cDNAs were ligated into a pUC-derived vector, pEND, which has three stop codons in each of three frames within several nucleotides downstream of the cDNA insertion site. There were up to four additional amino acids appended to the C-termini of the truncated CT sequences (see Figure 1). The mutant CT cDNAs were transferred back to pAX142 for sequencing to confirm the mutation and for expression in COS cells.

To generate the Δ349 mutant, we amplified the rat CT-2 cDNA by PCR in a CTV3 retroviral vector [40], using a vector specific 5′ primer and the 3′ CT-specific mutagenic primer: 5′-GCGTCGACTCAA**GC**TGCTGGGGAGGAAGATGG-GGA-3′. The PCR product was digested with *Sst*I and *Sal*I and the 368 bp fragment was ligated with the 810 bp *Mlu*I-*Sst*I fragment of pAX142-CT-2 and *Mlu*I-*Sal*I-digested pAX142. This mutant terminates at Ala-349 and contains one additional alanine before the stop codon.

The S362ΔA site-specific mutation was generated by PCR amplification of pAX142-CT-2 using the mutating primer 5′-GGGGATCCTTAGTCCTCTTCATCCTCG**GC**GATG-3′, and a 5′ primer RCT-1 [13]. The PCR product was cleaved at the *Sst*I site and ligated into *Sst*I-*Bam*HI-digested pAX142-CT-2. All mutant constructs were confirmed by DNA sequencing.

Transfection and extraction of CT from COS cells

COS cells were transfected as previously described [20,37,41], except that the cells were harvested 48 h after transfection and were not subcultured after transfection. Homogenates, cytosol and microsomes were prepared as described [20,37]. Potassium phosphate was added to 0.2 M after homogenization. CT activity was determined as described [42].

Digitonin permeabilization

Transfected COS cells were permeabilized at 0–2 °C as follows. The cells were washed three times with PBS and incubated for 12 min with permeabilization buffer: 0.5 mg/ml digitonin in 20 mM Tris/HCl, pH 7.4, 0.13 M KCl, 3 mM $MgCl_2$, 1 mM PMSF, 2 mM DTT and 60 mM β-glycerol phosphate. The digitonin lysate was transferred to a cold tube. The cell ghosts were collected by scraping into permeabilization buffer without digitonin. Enzyme activity controls for permeabilization were the cytosolic enzyme lactate dehydrogenase [43], 90% of which was in the lysate after 12 min, and the microsomal enzyme choline-phosphotransferase [44], which was not released after 12 min.

Kinase assays *in vitro*

For the phosphorylation of CT, purified CT from the baculovirus expression system, transfected COS cell homogenates, cytosol or heparin–Sepharose-treated cytosol were utilized. Phosphorylation assays catalysed by casein kinase II, cdc2 or MAP kinase

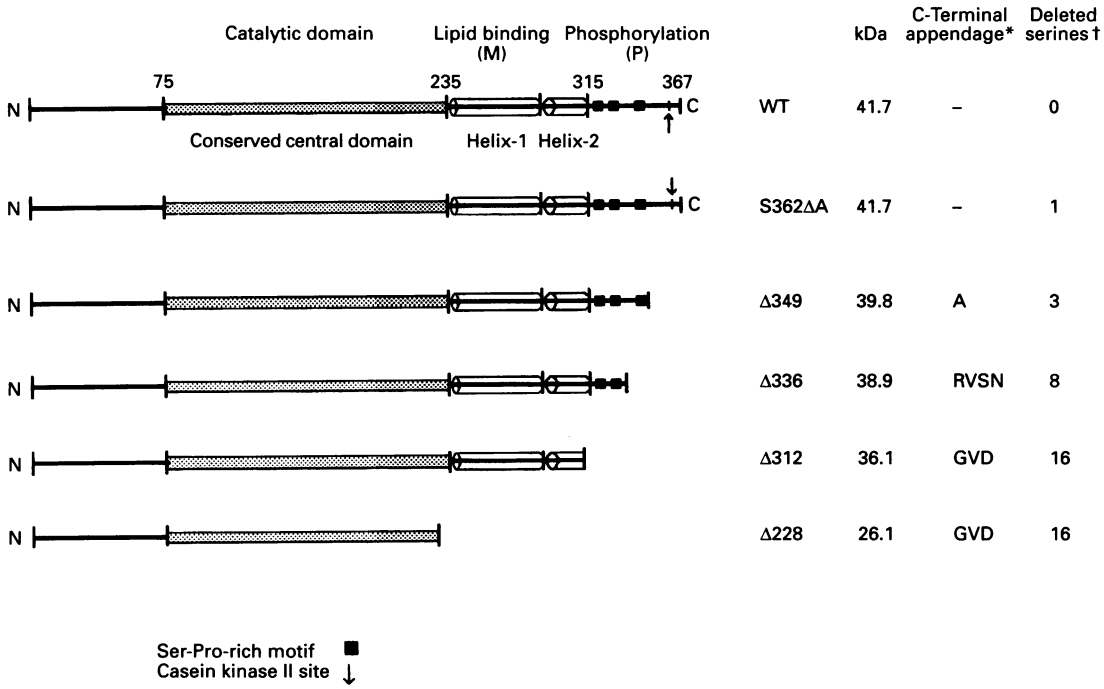

Figure 1 Domain structure of wild-type and mutant CTs

* Amino acids appended to C-terminus (not encoded by CT cDNA); † number of serines deleted within the region 315–367. WT, wild type.

contained 25 mM Mops, pH 7.4, 6 mM $MgCl_2$, 25 mM β-glycerol phosphate, 25 μM [γ-^{32}P]ATP (2000–8000 c.p.m./pmol), 2 mM DTT and ~ 50 ng of purified kinase from sea star [34,35]. Reactions were for 1 h at 30 °C. PKC was assayed in 25 mM Tris/HCl, pH 7.4, 12.5 mM $MgCl_2$, 50 μM [γ-^{32}P]ATP, 1 mM DTT, 1 mM $CaCl_2$, mixed micelles composed of 1.6 mM Triton X-100, 0.28 mM phosphatidylserine and 0.14 mM diacylglycerol (DAG) and ~ 200 ng of purified PKCα or PKCβII. Reaction was for 1 h at 37 °C. GSK-3 was assayed in 25 mM Tris/HCl, pH 7.0, 5 mM $MgCl_2$, 50 μM [γ-^{32}P]ATP, 0.2 mM EGTA and 2 mM DTT, for 1.5 h at 30 °C. All kinase reactions were in 50 μl volumes and were stopped with 20 μl of Laemmli sample buffer [45]. The orthophosphate concentration (due to 0.1–0.2 M phosphate in the CT samples) was < 50 mM in all kinase assays. The kinases were added in two or three batches at zero time and at 30 min intervals. Reactions were saturating with respect to kinase, ATP concentrations and time of incubation.

In some experiments using pure CT, chymotrypsin (protease: CT, 1:20 w/w) was added during the last 5 min of the incubation with kinases. The digestion was quenched with 2 mM PMSF followed within 1 min by Laemmli sample buffer and boiling.

Labelling with ^{32}P *in vivo*

At 42–44 h after transfection, COS cells were washed three times with phosphate-free DMEM and labelled for 4 h with the same medium containing 0.1–0.2 mCi/ml [^{32}P]orthophosphate. The cells were harvested as described [41] and were homogenized in buffer A [16] containing 50 mM β-glycerolphosphate to inhibit phosphatases. In some experiments CT was purified up to the octylglucoside solubilization step [16]. The purity of the CT was 80–90 %, based on densitometry of Coomassie-Blue-stained gels.

Baculovirus-mediated expression and purification of CT

CT was purified from the baculovirus expression system [16] with some modifications: *T. ni* cells (Invitrogen) were initially cultured in T-75 flasks in Grace's medium supplemented with 10 % fetal bovine serum. Before infection with recombinant baculovirus containing rat liver CT cDNA (a gift from Dr. C. Kent), cells were transferred to three 100-ml spinner bottles. After reaching a density of 1.5×10^6 cells/ml, cells were resuspended in SF-900 medium and inoculated with virus at 30×10^8plaque-forming units/100 ml (multiplicity of infection = 20) and were harvested 65–70 h later. The subsequent protocol followed that of MacDonald and Kent [16] with the following minor modifications: the eluant from the DEAE–Sepharose column was diluted 2.5-fold before loading onto the hydroxylapatite column; the Triton X-100 concentration in the final eluant from this column was 0.05 %.

Electrophoresis, autoradiography, quantification of radioactivity of bands on gels and Western blotting

Proteins were separated using 11, 12 or 13 % acrylamide/SDS gels [45]. Autoradiography of ^{32}P-labelled proteins on dried gels utilized reflectance film (Dupont/NEN) with or without screen, depending on the degree of labelling. Radioactivity in bands was quantified by liquid-scintillation counting of the excised bands, or with the use of a BioRad GS-250 phosphorimager. Protein mass associated with CT bands was quantified using a Microtek Scanmaker densitometer and NIH image software. For some experiments using purified wild-type CT, the density of the Coomassie Blue-stained CT bands was normalized to 1 μg of BSA co-electrophoresed in each lane. For experiments using COS cell extracts, the CT bands were normalized to a 45 kDa COS cell protein.

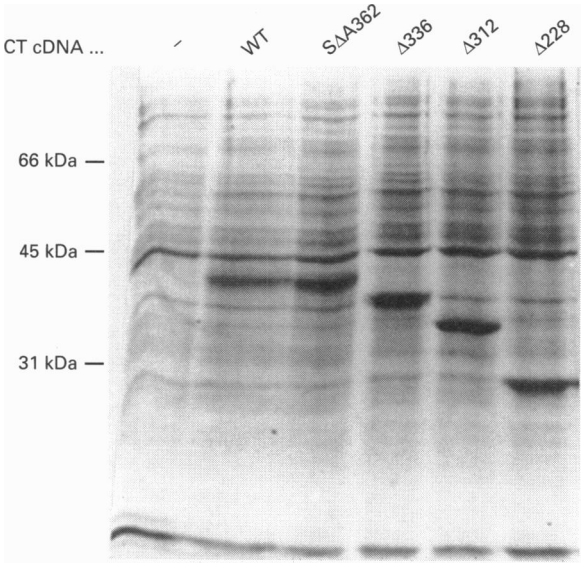

Figure 2 Expression of wild-type and mutant CTs in COS cells

Homogenates from COS cells transfected with vector alone, or vector containing wild-type (WT) or mutant CT cDNAs were prepared. Samples containing 25 μg of homogenate protein were electrophoresed on an 11% gel. The intensities of the Coomassie Blue-stained bands at 42, 39, 35 and 30 kDa relative to the prominant band at ~ 45 kDa were determined by densitometry. The expression of each CT was equivalent ±10%.

Proteins were transferred from Laemmli gels to polyvinylidene difluoride membranes (BioRad) at 2 mA/cm² for ~ 100 min using an LKB 2117 Multiphor Trans-blot apparatus. Blots were blocked with 5% milk powder in Tris-buffered saline (TBS) [20 mM Tris/HCl (pH 7.4)/0.15 M NaCl) for 2 h at room temperature, and incubated in TBS/ 0.05% Tween-20 solution containing the anti-CT peptide antisera (1:500) for 1 h at room temperature, followed by overnight incubation at 4 °C. After washing twice with TBS/Tween-20, blots were incubated with goat anti-rabbit–(horseradish peroxidase) conjugate (1:1000) in TBS/Tween-20 for 2 h at room temperature, washed twice with TBS/Tween-20, twice with TBS and developed with diaminobenzidine [20].

RESULTS

Expression and activity of C-terminal mutants in COS cells

A diagram of the mutants constructed from the wild-type rat liver CT cDNA is shown in Figure 1. The putative phosphorylation domain C-terminal to the amphipathic helices contains a total of 16 serines within 53 amino acids. The S362ΔA mutant lacks a potential phosphorylation site by casein kinase II, Δ349 lacks an additional two serines (S352 and S350), Δ336 lacks an additional five serines from the proline-rich domain, while Δ312 and Δ228 lack all the proline-rich motifs. Δ228 is also missing the amphipathic helix domain. Transfection of COS cells with the CT cDNAs resulted in approximately equal levels of expression of wild-type and mutant proteins and generated a major protein component in the COS cells (Figure 2). Cells transfected with vector alone (Figure 2, lane 1) contained no major bands corresponding to the molecular masses of wild-type CT (42 kDa), or any of the mutants (42, 40, 39, 36 and 30 kDa). (The mobility of the ΔK228 mutant on SDS gels gives an apparent molecular mass of 30 kDa. However, its true mass, based on its sequence, is 26.1 kDa. The reason for its anomalous mobility is unknown. The mobility of the other mutants agrees well with their molecular mass obtained from sequence analysis.) These same bands were the principal bands in homogenates detected with anti-CT antibodies in a Western blot (results not shown). Densitometry of the CT bands relative to a 45 kDa COS cell protein verified equivalent expression (±10%).

Table 1 Activities and intracellular distribution of CT C-terminal mutants

Homogenates, digitonin lysates and cell ghosts were assayed in the presence of 0.5 mM PC–oleic acid (1:1) sonicated vesicles. Cytosols were assayed in the absence or presence of 0.5 mM PC–oleic acid vesicles. CT activities in extracts from COS cells transfected with vector alone (< 1 nmol/min per mg) were subtracted to obtain the values in this Table. Data are averages of 2–5 independent transfections. * [Activity in lysate ÷ (activity in lysate + ghosts)] × 100%; † $P < 0.005$, significance of the difference compared with wild type (WT).

Transfected cDNA	Homogenate	Cytosol		Digitonin lysate (%)*
		−Lipid	+Lipid	
WT	87±15	40±3	101±1	66±8
S362ΔA	90±13	42±7	119±3	66±5
Δ349	95±3	28±3	99±3	69±4
Δ336	98±8	25±3	96±6	59±10
Δ312	97±5	38±9	101±6	71±3
Δ228	5.6±1.1	8.2±0.2	8.2±0.7	86±6†

The activities of the C-terminal mutants are shown in Table 1. Neither substitution of Ser-362 with alanine nor deletion of the final 18, 31 or 55 residues had an effect on the activity of CT in the presence or absence of activating vesicles (PC:oleic acid, 1:1) using a standard assay [45]. These mutants all contain the complete amphipathic helix domain of CT (see Figure 1) and were stimulated 3–4-fold by anionic lipid vesicles (Table 1). On the other hand, the mutant missing the last 140 amino acids, including the amphipathic helix and eight residues of the conserved domain, had less than 10% of the catalytic activity of the wild type in the presence of PC–oleic acid vesicles. The activity of this mutant (Δ228) was not influenced by lipid vesicles composed of PC–oleic acid (1:1) (Table 1), PC–diacylglycerol (3:1) or PG (results not shown).

Effects of the mutations on intracellular distribution

The subcellular distribution of the Δ228 mutant was altered. Whereas the other mutants were distributed, according to activity measurements, in approximately the same manner as the wild-type CT (63±3%, cytosol; 15±3%, microsomes; 22±7%, low speed pellet), less than 3% of the Δ228 mutant activity was in the microsomal fraction. This result was confirmed when we used digitonin permeabilization to assess the intracellular distribution. In cells transfected with wild-type CT or any of the mutants other than Δ228, 60–70% of the CT activity was found in the cell lysate (soluble or cytosolic fraction). On the other hand 86% of the Δ228 activity was associated with the lysate (Table 1). Analysis of the distribution, based on protein-stained gels, confirmed the result that much less of the Δ228 mutant was in the cell ghosts (results not shown). The changes in lipid responsiveness and membrane localization of the Δ228 mutant are in keeping with other evidence suggesting that the amphipathic helix is the lipid-responsive, membrane-binding domain [20,25,46].

Mapping the *in vivo* phosphorylation domain

To determine the site of phosphorylation, cells transfected with

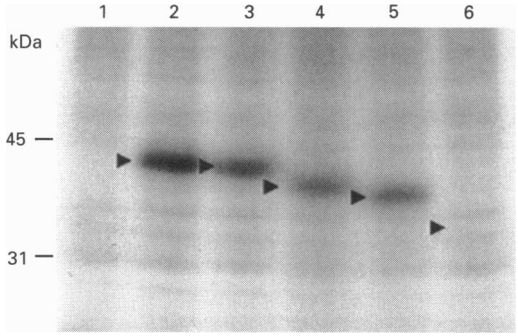

Figure 3 Phosphorylation *in vivo* of wild-type and mutant CTs

COS cells transfected with vector alone (lane 1), wild-type CT (lane 2), S362ΔA (lane 3), Δ349 (lane 4), Δ336 (lane 5) or Δ312 (lane 6) were labelled *in vivo* with ^{32}P-phosphate and cytosols were prepared. Samples containing 25 μg of protein were electrophoresed on 11% gels and the gel was exposed to film. The arrows indicate the positions corresponding to the Coomassie Blue-stained CT bands.

each of the mutants were labelled with [^{32}P]orthophosphate and the degree of labelling was measured after gel electrophoresis of homogenates or cytosol. CT was the major phosphoprotein visible in the COS cell extracts (Figure 3). The amount of ^{32}P associated with the CT band decreased with progressive deletion of the C-terminus (Figure 3). The radioactivity associated with each CT band was examined using four separate gels from two independent labelling experiments. Substitution of Ser-362 with alanine resulted in a $21 \pm 6\%$ reduction in label; deletion of the C-terminal 18 or 31 amino acids yielded a $26 \pm 14\%$ or $41 \pm 6\%$ reduction in ^{32}P label respectively, and deletion of the C-terminal 55 amino acids eliminated all ^{32}P label. This result suggests that the C-terminal 55 amino acids constitute the sole phosphorylation domain in CT and that there are multiple sites of phosphorylation. These findings are in agreement with previous analysis of the *in vivo* phosphorylation sites of rat liver CT expressed in insect cells [26].

In another approach, the *in vivo* phosphorylation domain was identified using chymotrypsin proteolysis of wild-type CT [20]. After ^{32}P-labelling *in vivo*, CT was partially purified from COS cells up to the octylglucoside solubilization step [16]. Chymotrypsin digestion generated fragments of 40, 39, 35 and 28 kDa and a faint set of fragments of between 16 and 19 kDa (Figure 4, lane 2). All the fragments greater than 20 kDa reacted with an antibody against an N-terminal peptide, but not with an anti-C-terminal antibody (Figure 4, lanes 2, 5 and 7). The antibody against the C-terminal peptide reacted only with the 19 kDa fragment, in addition to the undigested 42 kDa species (Figure 4, lane 7). The autoradiogram indicated ^{32}P-labelling of the 42, 40, 39 and 16–19 kDa bands, but the 35 and 28 kDa bands had no label above the background (Figure 4, lanes 8 and 9). The strongly ^{32}P-labelled 16–19 kDa fragments were also labelled by a photo-activatable lipid probe and by an antibody directed against the amphipathic helix domain of CT, suggesting they all contain the amphipathic helix domain. The N-terminal sequences of the 19, 17 and 16 kDa fragments are identical, beginning at Thr-226 (J. E. Johnson, R. Aebersold and R. B. Cornell, unpublished work). These results indicate that the *in vivo* phosphorylation domain is restricted to the C-terminal ~ 7 kDa.

Phosphorylation *in vitro* of wild-type and mutant CT forms in cell homogenates

Homogenates were prepared from COS cells transfected with vector alone, with wild-type or with mutant CTs. Protein phosphorylation was assayed *in vitro*, catalysed by cdc2 kinase, casein kinase II, MAP kinase, GSK-3, PKCα and PKCβII, or kinases endogenous to the COS cells (Figure 5). CT was a major phosphorylated protein in these assays. When no exogenous kinase was added, we observed phosphorylation of, predominantly, the wild-type CT (Figure 5a). Label associated with mutant

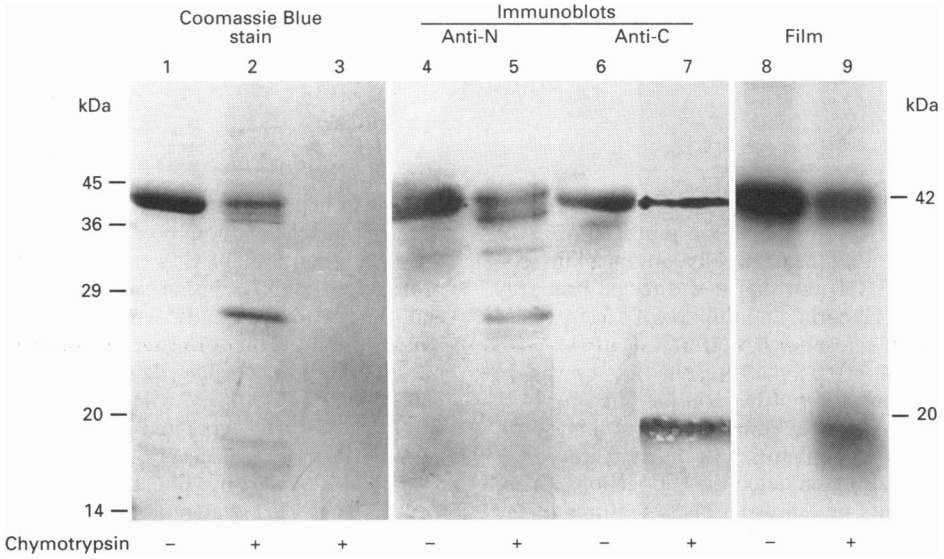

Figure 4 Determination by proteolysis of the domain phosphorylated *in vivo*

COS cells were transfected with wild-type CT and labelled with ^{32}P-phosphate. The CT was partially purified. Untreated sample (2 μg) and a sample digested with chymotrypsin (protease:CT, 1:1 w/w) were electrophoresed and stained with Coomassie Blue (lanes 1–3), or transferred for immunoreaction with antipeptide antibody against the CT N-terminus (lanes 4 and 5), or C-terminus (lanes 6 and 7), or exposed to film (lanes 8 and 9). Lane 3 contained the same amount of chymotrypsin as in lanes 2, 5, 7 and 9, but lacked CT.

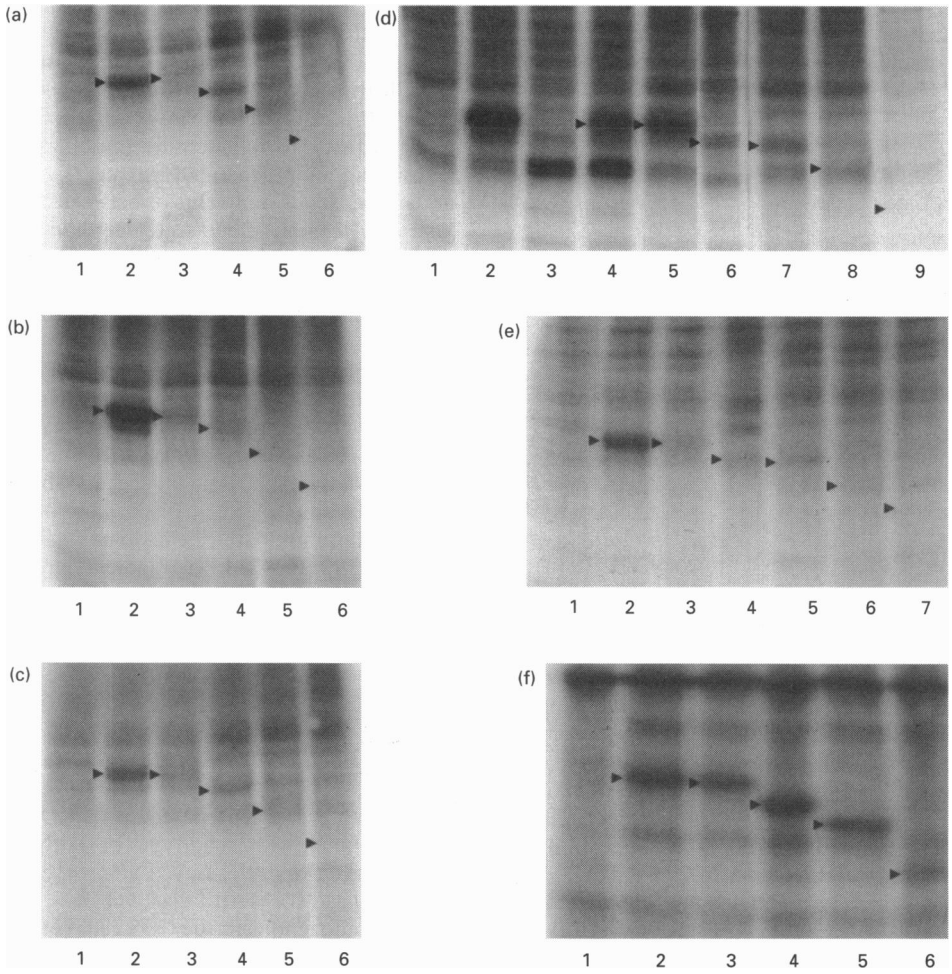

Figure 5 Phosphorylation *in vitro* of wild-type and mutant CT

Homogenates were prepared from COS cells transfected with vector alone, wild-type or mutant CTs. Phosphorylation reactions using 25 μg of homogenate protein were catalysed by: (**a**) endogenous kinase; (**b**) casein kinase II; (**c**) MAP kinase; (**d**) cdc2 kinase; (**e**) GSK-3; and (**f**) PKCβII. Panels are autoradiograms after electrophoresis of samples on 11% gels. For (**a**) (**b**) (**c**) and (**f**): lane 1, vector alone; lane 2, wild-type CT; lane 3, S362ΔA; lane 4, Δ336; lane 5, Δ312; and lane 6, Δ228. For (**d**): lanes 1 and 3, vector; lanes 2 and 4, wild-type CT; lane 5, S362ΔA; lane 6, Δ349; lane 7, Δ336; lane 8, Δ312; and lane 9, Δ228. Lanes 3 and 4 (**d**), reactions in the presence of 50 μg/ml heparin. Other lanes, reactions contained no heparin. For (**e**): lane 1, vector; lane 2, wild-type; lane 3, S362ΔA; lane 4, Δ349; lane 5, Δ336; lane 6, Δ312; and lane 7, Δ228. The phosphorylated band at the top of the gel in (**f**) is autophosphorylated PKCβII. Arrows indicate the positions of CT bands on the gels.

S362ΔA or Δ336 was < 20% of wild type, and no phosphorylation above background was observed for mutants Δ312 or Δ228. This result suggested that the COS cells contain a kinase(s) that is very active towards CT and that it is directed primarily towards one site, Ser-362. Heparin, an inhibitor of casein kinase II, inhibited by 70% the phosphorylation of CT in the COS extracts at a concentration of 50 μg/ml (results not shown). In the same assay, this concentration of heparin inhibited by 94% the phosphorylation of casein by purified casein kinase II. Chromatography on heparin–Sepharose effectively removed activity towards casein and also eliminated the endogenous kinase activity towards CT (results not shown). These features of the endogenous kinase suggest it is casein kinase II.

When casein kinase II was added to the extracts, we observed phosphorylation above the endogenous levels of only the wild-type CT (Figure 5b). This suggests Ser-362 as the sole casein kinase II site. In the presence of MAP kinase there was no significant phosphorylation above the endogenous levels (Figure 5c). cdc2 kinase was added to the extracts in the presence or absence of heparin (to inhibit endogenous kinase). Heparin only affected phosphorylation by endogenous kinase of wild-type, not the C-terminal mutant CTs (results not shown). The phosphoprotein at ~ 34 kDa in lanes 3 and 4 of Figure 5d is a species endogenous to COS cells, which was phosphorylated by cdc2 kinase only in the presence of heparin. We observed the phosphorylation of wild-type CT and three of the mutants: the S362ΔA mutant (90% of wild type in the presence of heparin), Δ349 (58% of wild type) and Δ336 (43% of wild type), but the mutants lacking 55 (Δ312) or 139 residues of the C-terminal domain (Δ228) were not phosphorylated (Figure 5d, lanes 8 and 9). This suggested that multiple sites were phosphorylated by cdc2 kinase between residues 312 and the C-terminus.

When GSK-3 was added, no phosphorylation above endogenous levels was observed even for wild-type CT (Figure 5e). As described below, this absence of phosphorylation of CT by GSK-3 was due to inhibitory factors in the COS cell extract rather than the lack of phosphorylation sites for this kinase. When PKCβII was added, we observed phosphorylation not

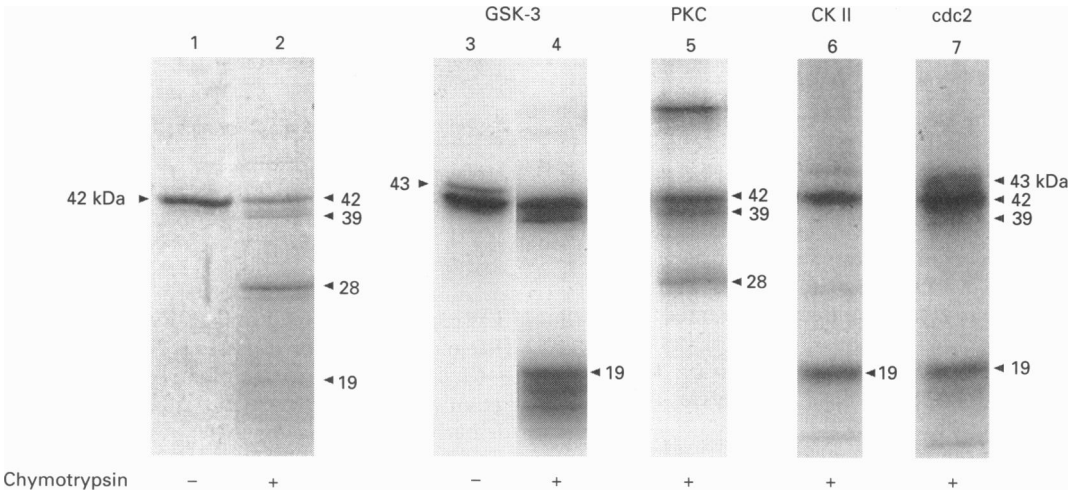

Figure 6 Determination of the domain of purified CT phosphorylated *in vitro* by chymotrypsin proteolysis

Wild-type CT purified from the baculovirus expression system (6 μg) was phosphorylated using the indicated kinase (CK II, casein kinase II), and a portion of the sample was digested with chymotrypsin (protease:CT, 1:20 w/w). Samples were electrophoresed on 13% gels. Lanes 1 and 2, representative Coomassie Blue-stained gels showing undigested and digested CT; lanes 3–7, autoradiograms of phosphorylated CT treated as indicated. Arrows indicate the primary proteolytic fragments (values are in kDa); in lanes 3 and 7 the ~ 43 kDa radiolabelled band is probably hyperphosphorylated, undigested CT.

only of wild-type S362ΔA and Δ336, but also of the Δ312 and Δ228 mutants (Figure 5f). Quantification of the ^{32}P-labelled CT bands suggested that ~ 50% of the PKC-catalysed phosphorylation was directed towards the N-terminal domain (Δ228 mutant; Figure 5f, lane 6). Similar results were obtained using PKCα.

Phosphorylation *in vitro* of purified wild-type CT

CT was purified to homogeneity from insect cells overexpressing CT via the baculovirus system. Phosphorylation of purified CT was achieved using casein kinase II, cdc2 kinase, PKC or GSK-3, but not MAP kinase.

GSK-3-catalysed phosphorylation resulted in a gel shift. Approximately 20% of the radioactivity was associated with a 43 kDa band (Figure 6, lane 3), whereas by Coomassie Blue staining, only the 42 kDa band was readily visible (lane 1). Thus GSK-3 catalyses the hyperphosphorylation of a small fraction of the CT population. cdc2 kinase-catalysed phosphorylation also produced diffuse labelling in the 42–44 kDa region (lane 7). No ~ 43 kDa phosphoprotein was observed when the reactions contained the kinase but lacked CT (not shown), indicating that this species does not represent phosphorylation of the cyclin B subunit of cdc2 kinase.

The phosphorylated CT was digested briefly with chymotrypsin to determine the domain labelled *in vitro*. When CT phosphorylation was catalysed by GSK-3, the undigested 42 kDa band, the 40 and 39 kDa fragments and three species of ~ 16, 17.5 and 19 kDa were labelled with approximately equal intensity, but the 28 kDa band was not labelled (Figure 6, lane 4). On the other hand, PKCβII-catalysed phosphorylation resulted in strong labelling of the 28 kDa band, along with the 42, 40, 39 and 35 kDa fragments, and no labelling of the bands of < 20 kDa (Figure 6, lane 5). Digestion of CT after phosphorylation using PKCα resulted in labelling of all the C-terminal and N-terminal fragments (results not shown). When CT phosphorylation was catalysed by casein kinase II only the undigested 42 kDa band and a fragment of ~ 19 kDa were labelled (Figure 6, lane 6). cdc2 kinase-catalysed phosphorylation strongly labelled the 42 kDa parent species and the ~ 19 kDa fragment and faintly labelled the fragments at 40, 39 and ~ 17.5 kDa (Figure 6, lane 7). These results suggest that phosphorylation catalysed by casein kinase II, cdc2 kinase and GSK-3 is directed exclusively to the C-terminal domain, while that of PKC-βII is directed primarily against sites in the N-terminal 28 kDa segment.

To determine why GSK-3 was active towards pure CT, but not towards the CT in COS cell homogenates, we added COS cell homogenate to purified CT and phosphorylated in the presence of GSK-3. The addition of 25 μg of homogenate completely inhibited GSK-3 activity towards 2 μg of pure CT substrate (results not shown). This suggests an inhibitory component in the crude cell homogenate. On the other hand, CT phosphorylation by MAP kinase was negligible using pure CT (not shown) or COS extracts (Figure 5c). Inclusion of COS cell homogenate or purified CT reduced the activity of MAP kinase towards myelin basic protein by only ~ 50%.

The stoichiometries of phosphorylations catalysed by PKCα, cdc2 kinase, casein kinase II and GSK-3 were in the range 0.1–0.2 mol phosphate/mol CT monomer. Samples were removed from these same phosphorylation reactions to assay CT enzyme activity in the presence or absence of saturating levels of lipid-activating vesicles (0.5 mM egg PC:oleic acid, 1:1). Phosphorylated CT was compared with controls not containing kinase. The small increase in the kinase-catalysed phosphorylation state of CT produced no significant changes in CT activity, assayed in the presence or absence of lipid (results not shown). Apparently the purified CT is in a relatively highly phosphorylated form when extracted from cells, even though the purification was carried out in the absence of phosphatase inhibitors.

Treatment of pure CT with PAP linked to Sepharose was effective at removing covalently bound phosphate (70 to > 90% reduction in c.p.m. of ^{32}P). However, the dephosphorylated CT self-aggregated and/or aggregated with the PAP–Sepharose such that it sedimented at very low *g* force. The activity of the aggregated, dephosphorylated CT was reduced ~ 70% and was recalcitrant to rephosphorylation by cdc2 kinase and casein kinase II (results not shown).

DISCUSSION

Domain M, but not domain P, is required for enzyme activity, lipid responsiveness and intracellular localization

Domain M refers to the 62-residue amphipathic helix between Lys-238 and Leu-299, and domain P refers to the C-terminal 53 amino acids of CT. That domain P is not required for enzyme activity is supported by the lack of effect on catalytic activity of deletion of up to 55 residues. Since the expression levels of wild-type and C-terminal-deletion mutants were equivalent, specific-activity measurements in COS cell extracts from wild-type and mutant CT transfections could be directly compared. None of the deletion mutants lacking portions or all of domain P were affected with respect to maximum catalytic activity. They were also activated 3- to 4-fold by PC–oleic acid vesicles and other lipids that stimulate the wild-type CT.

On the other hand, the mutant missing the C-terminal 139 amino acids (domains M + P) was characterized by large reductions in activity, responsiveness to lipid activators and localization in the membranes. In addition to domains M and P, this mutant lacks eight residues of the conserved domain, which is postulated to contain the catalytic site. This latter factor may be the reason for the low activity. The loss of lipid responsiveness and membrane localization of the Δ228 mutant is compatible with the idea that the amphipathic helix is responsible for membrane binding and is the principal determinant of the distribution between soluble and membrane-bound forms.

Much data suggests that the phosphorylation state of CT influences membrane association. For example, okadaic acid treatment elevates the degree of phosphorylation and lowers the proportion of membrane-bound CT [6,10]. Fatty acid or phospholipase C treatment of cells [6–8] or choline deprivation [9] decreases the phosphorylation state and increases the proportion of membrane-bound CT. On the other hand, kinetic analysis of translocation in reponse to oleic acid or phospholipase C revealed that CT binds to cell membranes in its phosphorylated form and is only subsequently dephosphorylated [8]. Moreover, cholecystokinin stimulates CT dephosphorylation but does not alter its intracellular distribution [47]. We observed that deletion of the entire phosphorylation domain did not change the equilibrium between soluble and membrane-bound forms. Absence of the entire phosphorylation domain is quite different from retention in a dephosphorylated form. When domain P is missing, the membrane affinity may be dictated solely by domain M. When the protein contains a complete M + P regulatory domain, membrane affinity may be dictated by domain M as well as the degree of phosphorylation of domain P: the fewer sites phosphorylated, the more hydrophobic the protein. Our preliminary experiments showing that dephosphorylation of CT decreases its solubility support this hypothesis. To understand how domain P might fine-tune membrane affinity, characterization of its interactions with domain M and the catalytic domain will be needed.

Phosphorylation *in vivo* is restricted to the C-terminus

Our ^{32}P-labelling, *in vivo*, of a mammalian cell expressing CT, confirms the findings using an insect cell expression system [26] that all the phosphorylation sites are restricted to the C-terminal 53 amino acids. The progressive decrease in ^{32}P associated with successive C-terminal deletions suggests multiple phosphorylation sites, including Ser-362, Ser-352 and/or Ser-350 and at least several of the serines in the proline-rich motifs. Several of these sites (Ser-345, Ser-346 and Ser 350) are not conserved between the rat liver CT and a CT clone from human K562 cells [14] and thus may be of lesser regulatory importance.

^{315}**S**$^{*+}$ P K Q **S*** P S S S* P T H E R **S*** P S* P S^{+} F R W P F

^{339}**S**$^{+}$ G K T **S*** P S S S* P A S^{+} L S R C K A V T C D I S$^{\#}$ E D E E D^{367}

Figure 7 Sequence of the C-terminus of rat liver

Potential phosphorylation sites are indicated as follows: *, cdc2 or MAP kinase [SP]; +, protein kinase C [$SX_{1,2}K(R)$]; #, casein kinase II (C-terminal acidic); **S**, GSK-3 [$SXXXS(P_i)$].

Chymotrypsin digestion of *in vivo* ^{32}P-labelled CT generated a set of ^{32}P-labelled fragments of < 20 kDa, which have been definitively identified by N-terminal sequencing and reactivity with antibodies. All fragments begin with Thr-226 and reacted with antibody specific for the three 11-mer repeats of domain M (J. E. Johnson, R. Aebersold and R. B. Cornell, unpublished work), but only the fragment running at $\sim$ 19 kDa reacted with an anti-C-terminal antibody. Thus the 19 kDa species is the complete regulatory domain, composed of domains M + P. The discrepancy between calculated mass of the regulatory domain (16.1 kDa) and apparent mass of this fragment (19 kDa) could be due to hyperphosphorylation, which would cause aberrantly slow mobility.

Identification of putative kinases directed towards CT

The C-terminus of CT contains potential sites for phosphorylation by casein kinase II, MAP kinase, cdc2 kinase, GSK-3 and PKC (Figure 7). All of these kinases catalyse the phosphorylation of CT *in vitro*, except MAP kinase.

Casein kinase II

Casein kinase II is a ubiquitous kinase that is translocated to the nucleus in response to mitogens [48,49] and has many nuclear targets such as Max, Myc, Myb, RNA polymerase I and II, nucleolar protein B23 and DNA topoisomerase I and II. Regulation of CT by casein kinase II is a reasonable prospect in light of the evidence that CT is a nuclear enzyme [21,22] and its phosphorylation state changes in response to colony-stimulating factor-1 (CSF-1) [11], insulin, or epidermal growth factor [33]. There are potential casein kinase II sites at Thr-25, Ser-47 and Ser-178. However, only the C-terminal site, Ser-362, was targeted, as evidenced by the complete loss of casein kinase II-catalysed phosphorylation upon substitution of Ser-362 with alanine and also by the lack of phosphorylation of any of the chymotryptic fragments other than that containing the C-terminus. The fidelity of the phosphorylation *in vitro* to the one acidic site that is phosphorylated *in vivo* offers some reason to believe that casein kinase II phosphorylation of CT is functionally relevant. Casein kinase II appears to be the dominant kinase activity in COS cell extracts that is directed against CT. This conclusion is based on the response of the endogenous COS cell kinase activity to heparin and on the nearly complete loss of phosphorylation of CT by the endogenous kinase upon substitution of Ser-362.

Removal of the single serine at the 362 site results in a 20% decrease in the phospho-labelling of CT *in vivo*. Since all 16 serines C-terminal of Ile-314 are likely to be phosphorylated to some extent [26], this result suggests that, (1) the casein kinase II site is a predominant site, or (2) phosphorylation of Ser-362 is required for phosphorylation of some other upstream sites.

Phosphorylation at the casein kinase II site had no effect on enzyme activity. Sweitzer and Kent [17] found that a S362ΔA mutation had no effect on enzyme activity and that this mutant could restore the CDP-choline pathway when transfected into a CHO mutant defective in CT.

PKC

Four potential sites for PKC-catalysed phosphorylation are found in domain P and up to six are found in the N-terminal domain. Analysis of PKC-catalysed phosphorylation of deletion mutants in COS cell extracts suggested that, *in vitro*, PKCα and PKCβII phosphorylated both N-terminal and C-terminal sites. Analysis of chymotrypsin fragments from wild-type CT phosphorylated in the presence of PKCα or PKCβII suggested targeting of N-terminal sites, primarily. The lack of restriction of PKC action *in vitro* to domain P suggests a lack of relevance to phosphorylation of CT *in vivo*. At best 0.2 mol of phosphate was incorporated per mol of CT monomer and this level of phosphorylation did not alter CT activity.

MAP kinase

We were unable to achieve MAP-kinase-catalysed phosphorylation using COS cell extracts or purified CT. The MAP kinase was from sea star, but the consensus phosphorylation sequence is highly conserved and the specific activity towards myelin basic protein was 0.3 μmol/min per mg and was only partially inhibited by components in the CT preparations. Either CT is not a substrate for MAP kinase or all of the potential sites were completely occupied in the CT extracted from COS or *T. ni* insect cells. Wieprecht et al. [33] recently reported that phosphorylation of purified CT could be catalysed by MAP kinase, but only after treatment with a crude alkaline phosphatase–agarose from Sigma. The stoichiometry obtained was 0.15 mol P/mol CT. We were unable to dephosphorylate CT using purified alkaline phosphatase from Boehringer. After complete dephosphorylation using acid phosphatase, aggregation of CT prevented rephosphorylation catalysed by MAP kinase or any other kinase.

cdc2 kinase

The phosphorylation state of CT varies during the cell cycle [11]. In CSF-1-dependent Bac-1 cells the enzyme is dephosphorylated in early G_1 phase. Phosphorylation steadily increases during late G_1 and continues through the cell cycle, dropping dramatically after mitosis. The peak of the enzyme activity corresponds to the dephosphorylated state in G_1. Regulation by cyclin-dependent kinases was proposed to account for these cell-cycle-dependent variations in CT phosphorylation [11]. We found that CT functioned as a substrate for cdc2 kinase *in vitro* and could be hyperphosphorylated, resulting in a retarded mobility on gels. CT participates in the control of the PC metabolic cycle in which degradation of PC by phospholipases is closely coupled to PC synthesis [1,3,37]. Activation of CT could result in increased utilization of DAG or other lipid signalling molecules released via the action of signal-regulated phospholipases. Inhibition of CT activity by cyclin-dependent kinases in late G_1 could be a mechanism to augment the levels of DAG or other lipid second messengers which participate in the relaying of the mitogenic signal to the nucleus.

GSK-3

GSK-3 is constitutively active in non-proliferating cells and has diverse substrates, including glycogen synthase, protein phosphatase 1, ATP citrate lyase, the R subunit of protein kinase A, *c-Jun*, *c-Myc*, *c-Myb* and CREB. GSK-3 inactivation by EGF or insulin treatment is mediated via the MAP kinase/p90 ribosomal S6 kinase pathway [50–53], and this inactivation of GSK-3 may be important in mitogenic signalling. Phosphorylation by GSK-3 is usually associated with inactivation of its substrates [54]. The function of CT phosphorylation by GSK-3 might be to inactivate CT during growth arrest. We found that CT was a substrate for GSK-3β *in vitro* and mapped the site using chymotrypsin proteolysis to at least three C-terminal fragments differing in mass by 1–2 kDa. All three of these bands begin at Thr-226 and differ at their C-termini. Since the shortest fragment extending from Thr-226 to, most likely, Phe-334 (apparent mass, 16 kDa; actual mass, 13 kDa) was as intensely labelled as the largest (apparent mass, 19 kDa; actual mass, 16 kDa), this suggests that the sites targeted by GSK-3 lie in domain P between residues 314 and 334 (see Figure 7).

Identification of the kinases that regulate CT activity will require an '*in vivo*' approach in addition to the '*in vitro*' approach taken here. This is an important task, as it will help to define the role of CT in signal transduction pathways involving the PC metabolic cycle.

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