Identification of an 11-residue portion of CTP–phosphocholine cytidylyltransferase that is required for enzyme–membrane interactions

Jilin YANG, Jinxia WANG, Irene TSEU, Maciej KULISZEWSKI, Wensu LEE and Martin POST*

The Medical Research Council Group in Lung Development and the Neonatal Research Division, Hospital for Sick Children, Department of Pediatrics, University of Toronto, Toronto, Ontario M5G 1X8, Canada

CTP–phosphocholine cytidylyltransferase (CT) is a key regulatory enzyme in the biosynthesis of phosphatidylcholine (PC) in many cells. Enzyme–membrane interactions appear to play an important role in CT activation. A putative membrane-binding domain appears to be located between residues 236 and 293 from the N-terminus. To map the membrane-binding domain more precisely, glutathione S-transferase fusion proteins were prepared that contained deletions of various domains in this putative lipid-binding region. The fusion proteins were assessed for their binding of [^{3}H]PC/oleic acid vesicles. Fusion proteins encompassing residues 267–277 bound to PC/oleic acid vesicles, whereas fragments lacking this region exhibited no specific binding to the lipid vesicles. The membrane-binding characteristics of the CT fusion proteins were also examined using intact lung microsomes. Only fragments encompassing residues 267–277 competed with full-length ^{125}I-labelled CT, expressed in recombinant *Sf*9 insect cells, for microsomal membrane binding. To investigate the role of this region in PC biosynthesis, A549 and L2 cells were transfected with cDNA for CT mutants under the control of a glucocorticoid-inducible long terminal repeat (LTR) promoter. Induction of CT mutants containing residues 267–277 in transfectants resulted in reduced PC synthesis. The decrease in PC synthesis was accompanied by a shift in endogenous CT activity from the particulate to the soluble fraction. Expression of CT mutants lacking this region in A549 and L2 cells did not affect PC formation and subcellular distribution of CT activity. These results suggest that the CT region located between residues 267 and 277 from the N-terminus is required for the interaction of CT with membranes.

INTRODUCTION

Phosphatidylcholine (PC) is the major phospholipid component of mammalian membranes. The major pathway for PC biosynthesis in animal cells is the CDP-choline pathway. CTP–phosphocholine cytidylyltransferase (CT) (EC 2.7.7.15) catalyses a rate-limiting step in the CDP-choline pathway in many cells [1,2]. Overwhelming evidence suggests that CT is regulated by enzyme–membrane interactions [1,3,4]. The enzyme exists in an inactive soluble form and an active membrane-bound form [1,5]. In many cells the rate of PC synthesis appears to correlate directly with the amount of membrane-bound CT [1,3,6]. The intracellular localization of CT is somewhat controversial. In Chinese hamster ovary (CHO) cells CT displays a largely nuclear localization [7–9], whereas in hepatocytes it is predominantly a cytoplasmic enzyme [7]. In CHO cells transfected with a CT mutant in which the nuclear targeting sequence is deleted, modified CT is mainly found outside the nucleus, but CT function is not affected [10]. The mechanism for membrane binding and subsequent activation of the enzyme remains to be elucidated. The soluble form of CT is highly phosphorylated at the C-terminus, and translocation of CT to membranes is accompanied by extensive dephosphorylation [8,11]. Several proline-directed protein kinases, such as casein kinase II, glycogen synthetase kinase-3, cyclin-dependent kinase 2 and protein kinase Cα, appear to regulate CT phosphorylation [12,13]. Recent studies have reported that distribution and enzyme activity is not exclusively determined by the phosphorylation state of CT [14] and that dephosphorylation of CT is not required for binding to membranes [15]. In contrast, Yang and Jackowski [16] recently provided evidence that phosphorylation of CT interferes with the activation of CT by lipids.

Secondary-structural analysis of rat CT [17] revealed that it lacks a hydrophobic domain of sufficient length to span a bilayer. In addition, no signal for covalent attachment of lipid was detected. CT contains a region between residues 236 and 315 that is predicted to be two α-helices with an intervening turn at residues 294–297 [17]. Protease-protection experiments [18] have recently identified the α-helical region between residues 236 and 293 of CT, as the activating membrane-binding domain. When displayed on a helical wheel, this region shows an asymmetrical distribution of polar residues on one face and hydrophobic residues on the other. It has been postulated that the hydrophobic face intercalates with the lipid bilayer [17]. Deletion mutants of CT have confirmed that the area between 236 and 314 is required to confer lipid regulation on CT [14,19]. The exact location of the membrane-binding domain within this region is, however, unknown.

In the present study, we mapped the lipid-binding domain of CT by assessing the binding of CT–glutathione S-transferase (GST) fusion proteins that contained deletions of various domains in this putative membrane-binding region to lipid bilayers and biological membranes. As an 11-mer motif is repeated three times within this region, we designed truncated CT proteins with one or more deletions of the 11-mer motif. We report that membrane binding of CT *in vitro* depends on the

Abbreviations used: CHO, chinese hamster ovary; CT, CTP–phosphocholine cytidylyltransferase; DPPC, dipalmitoylphosphatidylcholine; GST, glutathione S-transferase; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; LTR, long terminal repeat.

* To whom correspondence should be addressed.

presence of the region encompassing residues 267–277. The truncated CT proteins were also examined for their ability to compete with endogenous CT for intracellular membrane binding and their influence on PC synthesis in stably transfected A459 and L2 cells. These *in situ* studies confirmed that residues 267–277 constitute an essential region for lipid–CT interactions.

MATERIALS AND METHODS

Preparation of GST fusion protein constructs

Two truncated CT mutants were constructed by overlap extension using PCR [20]. For the truncated des-(267–277)-CT mutant, four primers (primer 1, 5′-TACATGGATCCACCCGCATTGTCCGTGAC-3′; primer 2, 5′-CTCCTCCCATTTCTGCACAAAATTCTTTCGACTT-3′; primer 3, 5′-GTGCAGAAATGGGAGGAGAAGTCCCGAGA-3′; primer 4, 5′-AGACTGAATTCCGTCCTCTTCATCCTCGCT-3′) were synthesized and PCR was carried out using UITma® DNA polymerase (Perkin–Elmer Cetus, Norwalk, CT, U.S.A.). The template was rat lung CT [21]. For the des-(256–266, 278–288)-CT deletion mutant, two segments of DNA containing the respective deletions and a common *Taq* I restriction site were constructed using two sets of primers: primer 1, 5′-TACATGGATCCACCCGCATTGTCCGTGAC-3′ and primer 2, 5′-ATGAGGTCGATGCTCTTCTCCTCCACATCTTTCACTTTC TTCTTTACCTTATCAACTCGT-3′; primer 3, 5′-GAAGAGCATCGACCTCATCCAGAAGTTCCTGGAAATGTTTGG TCCAGAAGGAGCGCTGAA-3′ and primer 4, 5′-AGACTGAATTCGTCCTC TTCATCCTCGCT-3′. Again the template was rat lung CT. The PCR programme was 95 °C for 1 min, 55 °C for 1 min and 72 °C for 1.5 min for 35 cycles. The PCR products were purified by electrophoresis on agarose gel, cut with *Taq* 1, ligated together and amplified using primers 1 and 4. The deletions (267–277) and (256–266, 278–288) of the PCR products were confirmed by DNA sequencing. All other GST fusion proteins were prepared by PCR amplification of the appropriate regions of rat lung CT cDNA [21]. All PCR products were subcloned into the *Bam*HI and *Eco*RI sites of PGEX-2TK (Pharmacia), essentially as described by Smith and Johnson [22]. The insert-containing plasmid was used to transform the HB101 strain of *Escherichia coli*. Expression of fusion proteins was induced with 0.2 mM isopropyl β-D-thiogalactoside, and bacteria were collected and lysed by sonication in PBS containing 1 mM PMSF, 20 μg/ml leupeptin, aprotinin and 100 mM EDTA. Fusion proteins were purified from bacterial lysate with glutathione–agarose beads, as described [22]. For the competition assays, fusion proteins were eluted from the glutathione–agarose beads with 5 mM GSH/50 mM Tris, pH 8.0.

CT antibodies

Rabbit polyclonal antibodies were raised against GST fusion proteins encompassing either the first 31 amino acids of the N-terminus or the last 45 amino acids of the C-terminus of rat lung CT [21]. Aliquots of the fourth bleed were purified by passing them over a column containing GST and then the IgG fractions were isolated on Immunopure® plus immobilized Protein A (Pierce). In addition, a rabbit polyclonal antibody was raised against a synthetic peptide corresponding to residues 164–176 of rat lung CT, as described by Jamil et al. [23]. An aliquot of the fifth bleed was affinity-purified by passing it first over a column containing Protein A and then a column with the CT portion coupled to CNBr-activated Sepharose. The IgG fraction of the antiserum recognized purified rat lung CT [24] and CT molecules of fetal type-II cells with molecular masses of approx. 42 kDa [21].

Expression of CT in insect cells

Rat lung CT cDNA [21] was subcloned in the transfer vector pBlueBac (Invitrogen) using convenient restriction sites in the polylinker region. Transfections, screening for recombinant viruses, cloning and propagation of recombinant viruses were performed as described by O'Reilly et al. [25]. A 60 ml volume of viral stock [$(1–2) \times 10^8$ plague-forming units/ml] was used to infect 750 ml of *Sf*9 cells (2×10^6 cells/ml), grown in suspension in spinner flasks, for a period of 4 days. The infected cells were harvested by centrifugation, washed with PBS, lysed in homogenization buffer using a Dounce homogenizer and purified essentially as described by MacDonald and Kent [26].

SDS/PAGE and Western blot

CT–GST fusion proteins were separated on a 10% (w/v) polyacrylamide gel using the method of Laemmli [27]. Samples were boiled for 5 min in Laemmli sample buffer. Gels were run at constant voltage (85 V) and stained with Coomassie Blue. For Western-blot analysis of CT from *Sf*9 cells, gels were electrophoretically transferred to a nitrocellulose membrane [28]. Non-specific binding was blocked by incubating the nitrocellulose membrane with 3% (w/v) dry skimmed milk in PBS at 4 °C for 60 min, then 1:200 diluted rabbit anti-CT antiserum was added to detect 500 ng of full-length CT, purified from transfected *Sf*9 insect cells. After overnight incubation at 4 °C, the nitrocellulose membrane was washed three times with PBS, and this was followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:20000) for 60 min at 4 °C. The membranes were then thoroughly washed with cold PBS (2×10 min), immersed in chemiluminescence detection reagents for 1 min and exposed to Amersham Hyperfilm-ECL for 10 s in an X-ray cassette.

Preparation of lipid vesicles

Lipids (Avanti Polar Lipids) in chloroform were spiked with 1 μCi of [^{3}H]dipalmitoylphosphatidylcholine ([^{3}H]DPPC) (specific radioactivity 62 Ci/mmol) from a toluene/methanol (1:1, v/v) stock. The lipid solution was dried in a 5 ml round-bottomed tube under a stream of nitrogen. The dried film was resuspended in 1 ml of 25 mM Hepes, pH 7.0, and sonicated with a Branson sonicator, at 0–4 °C with a fine titanium probe at 50 W output, for 5 min. The sonicated lipid vesicles were centrifuged at 20000 ***g*** for 1 min to remove titanium particles and residual multilamellar vesicles. In some cases, sonicated lipid vesicles were sequentially extruded through polycarbonate filtration membranes with pore sizes of 0.8, 0.4 and 0.1 μm. The sequential filtrates contained vesicles with average diameter of < 0.8, < 0.4 and < 0.1 μm.

Lipid-binding assay

CT fusion proteins captured on glutathione–agarose beads were incubated in 100 μl of 25 mM Hepes, pH 7.0, containing 25 nmol of ^{3}H-labelled PC/oleic acid vesicles [specific radioactivity 1600 d.p.m./nmol of PC/oleic acid (1:1 molar ratio)] for 5 min at 37 °C in a 1.5 ml centrifuge tube with gentle agitation. The

reaction was stopped by centrifugation at 3000 ***g*** for 2 min to pellet out the fusion-protein-bound ^{3}H-labelled vesicles. Residual unbound vesicles were removed by washing the CT–GST fusion protein twice with 1 ml of Hepes solution. The samples were counted for tritium ([^{3}H]DPPC) in an LKB liquid-scintillation counter. Competition studies using fusion proteins bound to agarose beads and increasing concentrations of various eluted fusion proteins were used to determine the specificity of the CT fragments for binding of ^{3}H-labelled lipid vesicles.

Microsomal-membrane-binding assay

The membrane lipid binding of recombinant CT and CT fusion proteins was evaluated using microsomes isolated from adult rat lung. Microsomes were isolated as described previously [6]. Recombinant CT was iodinated with Iodogen (Pierce). To determine specific CT binding, microsomes (32 μg of protein) were incubated at 37 °C in 100 μl of 145 mM NaCl/50 mM Tris/HCl, pH 7.4, with 10 ng of ^{125}I-CT (specific radioactivity 2397 d.p.m./ng of protein) in the absence or presence of excess amounts of unlabelled recombinant CT. The reaction was terminated after 5 min by centrifugation at 250000 ***g*** for 30 min. Residual unbound ^{125}I-CT was removed by washing the microsomal pellet with 1 ml of buffer. The samples were transferred to scintillation vials for γ counting. To characterize the lipid-binding domain of CT, binding of ^{125}I-CT to microsomes was competed for with increasing amounts of eluted CT fusion proteins.

Plasmids and establishment of lung epithelial cell lines that stably express CT mutant proteins

For expression of CT mutant proteins in lung epithelial cells, the deletion cDNA constructs containing *Bam*HI and *Hin*dIII sites were inserted into the glucocorticoid-responsive pLKneo vector [29], which was cleaved with *Bam*HI and *Hin*dIII. The resultant plasmids, pLKneo–CT constructs, were purified and transfected into human pulmonary adenoma A549 cells and rat lung L2 cells (American Type Culture Collection), using cationic liposomes (Lipofectamine; Gibco) and plasmid DNA at a ratio of 10:1. Cells transfected with the pLKneo vector alone were used as control. The neomycin-resistant clones were selected with 0.5 mg/ml G418 in F12 medium with 10 % (v/v) fetal bovine serum. The colonies were assayed by reverse transcriptase-PCR to confirm mRNA expression of CT mutant protein. Non-transfected and transfected cells were incubated overnight in serum-free F12 medium and then exposed to serum-free medium with and without 1 μM cortisol. At the end of the incubation period, the medium was removed and the cells were washed with PBS, and RNA was extracted. The primers for amplification of mRNA of CT mutant proteins were a sense primer (5′-GTGG-AGGAGAAGAGCATC-3′) complementary to nucleotides 863–880 and an antisense primer (5′-GTCCTCTTCATCCT-CGCT-3′) complementary to nucleotides 1148–1165 of rat lung CT [21]. The reverse transcriptase-PCR using this primer set will also amplify endogenous CT mRNA. Therefore endogenous CT mRNA was specifically amplified using the same sense primer with an antisense primer (5′-GCCCCGTTTGTCTGTTCTT-TGTA-3′) complementary to nucleotides 1266–1288 of the 3′-untranslated region of rat lung CT [21]. Positive colonies of cortisol-induced mRNA expression of CT mutant proteins were identified by comparing the reverse transcriptase-PCR products from cortisol-treated transfectants and non-transfectants as well as from cells transfected with pLKneo vector alone.

Assay of PC synthesis

Positive transfectants, non-transfectants and cells transfected with pLKneo vector alone were grown in 48-well culture plates in F12/10 % (v/v) fetal bovine serum containing 0.2 mg/ml G418. At confluence, cells were washed, incubated in serum-free medium and then exposed to fresh medium with and without 1 μM cortisol. After a 24 h incubation, cells were pulsed for 5 h with 5 μCi/ml [^{3}H]choline to assess PC synthesis [6].

Cytidylyltransferase assay

After 24 h incubation with and without cortisol, transfectants, non-transfectants and cells transfected with pLKneo vector alone were collected by scraping into homogenization buffer (145 mM NaCl, 50 mM Tris/HCl, pH 7.4, 50 mM NaF and 2.5 mM EDTA). Post-mitochondrial membrane (P1), microsomal membrane (P2) and cytosolic (S) fractions were obtained as previously described [6]. CT activity was assayed in the forward direction by measuring the rate of incorporation of [*methyl*-^{14}C]phosphocholine into CDP-choline [6].

Statistical analysis

Statistical significance was determined by one-way analysis of variance followed by assessment of differences using Student–Newman–Keuls test for non-paired groups. Significance was defined as $P < 0.05$.

RESULTS AND DISCUSSION

CT fusion proteins

Analysis of the C-terminal region of CT revealed three repeated 11-mer motifs (256–266, 267–277 and 278–288) in a region that has been postulated to mediate CT binding to membranes [14,17–19]. To determine which of these repeats is responsible for binding to lipid bilayers, a series of constructs (CT–GST fusion proteins) was generated which encompassed one, two or all three repeats, and/or flanking sequences (Figure 1, top). The calculated masses of the constructs (I–IX) were 44.6, 38.7, 37.3, 28.6, 56.4, 55.1, 43.1, 36.0 and 30.9 kDa respectively. The GST fusion proteins ran at similar positions on SDS/PAGE (Figure 1, bottom). GST ran at approx. 26 kDa. Furthermore all GST fusion proteins, except fragment IV, which lacked the C-terminus and N-terminus, were detected by antibodies directed against either the C- or N-terminus (not shown).

Lipid-vesicle-binding assay

The membrane-binding ability of the GST fusion proteins was assessed using ^{3}H-labelled lipid vesicles. To measure vesicle binding, fusion proteins, attached to glutathione–agarose beads, were incubated with radiolabelled lipid vesicles. Vesicles bound to the fusion proteins were separated from unbound vesicles by centrifugation. Glutathione–agarose beads alone demonstrated no significant lipid binding. We optimized the binding assay with construct I, which contained the complete putative membrane-binding region (236–293) of CT [18], as well as flanking sequences. Construct IX or GST alone was used as control. The assay was optimized for temperature, quantity of [^{3}H]PC/oleic acid vesicles, number of washes and duration (Figure 2A). PC/oleic acid binding of GST was in the same range as that observed with

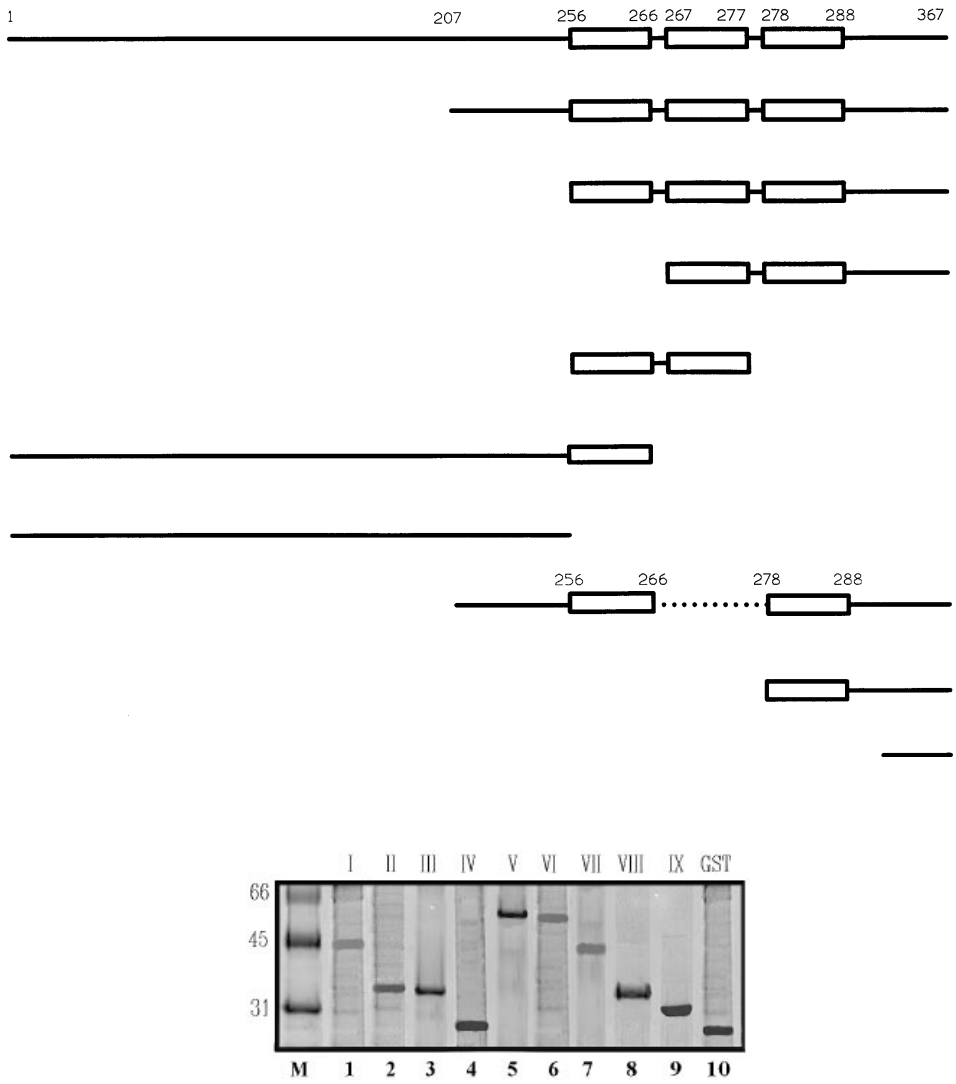

Figure 1 Identification of the specific sequence in CT that binds to membranes

Top, Schematic representation of the various constructs prepared from the different regions of CT: construct I encompasses residues 207–367, construct II 256–367, construct III 267–367, construct IV 256–277, construct V 1–266, construct VI 1–256, construct VII 207–367 des-(266–277), construct VIII 278–367 and construct IX 322–367. Bottom, Coomassie Blue-stained SDS/polyacrylamide gel of GST fusion proteins of the different constructs depicted at the top, and of GST.

construct IX (Figure 2A). Based on these results, the ability of GST fusion proteins to bind to lipid vesicles was standardized by incubating GST fusion proteins with 25 nmol of ^{3}H-labelled lipid vesicles in 100 μl of Hepes, pH 7.0, at 37 °C for 5 min followed by centrifugation and two washes with Hepes buffer. The assay was linear up to 2 nmol of GST fusion protein. We then analysed the ability of construct I to bind various lipid vesicles. Construct I bound vesicles composed of PC/oleic acid and PC/PG but not PC/PE (Figure 2B). The highest affinity was noted with PC/oleic acid (1:1) and PC/PG (1:1) vesicles. Both PC/oleic acid and PC/PG vesicles have been shown to be strong activators of CT activity [18,30,31]. This suggests that construct I has similar lipid-binding characteristics to native CT. GST alone displayed no binding preference for any of the lipid vesicles. As it has been reported that vesicle binding by CT increases with decreasing vesicle size [32], the influence of vesicle size on binding to construct I was also measured. Vesicle binding by construct I was similar for vesicles extruded through polycarbonate filters of 0.1 and 0.4 μm (Figure 2C). A slight but not significant decrease in vesicle binding was observed when the vesicles were extruded through a 0.8 μm filter. Thus increasing the surface area of the vesicles appears not to influence vesicle binding by CT. However, it should be noted that we did not accurately size the lipid vesicles. It is possible that vesicles extruded through the 0.8 μm filter contained mainly liposomes with a diameter of less than 0.1 μm. Further, we cannot exclude the possibility that the surface curvature of the vesicles affects CT activity as reported previously. Unilamellar vesicles generated by sonication were used in subsequent experiments.

Lipid-vesicle-binding ability of CT fusion proteins

The membrane-binding ability of the various constructs was determined. GST fusion proteins I–IV, which all contained residues 267–277, bound equally well to [^{3}H]PC/oleic acid vesicles (Figure 3A). Vesicle binding increased linearly with increasing

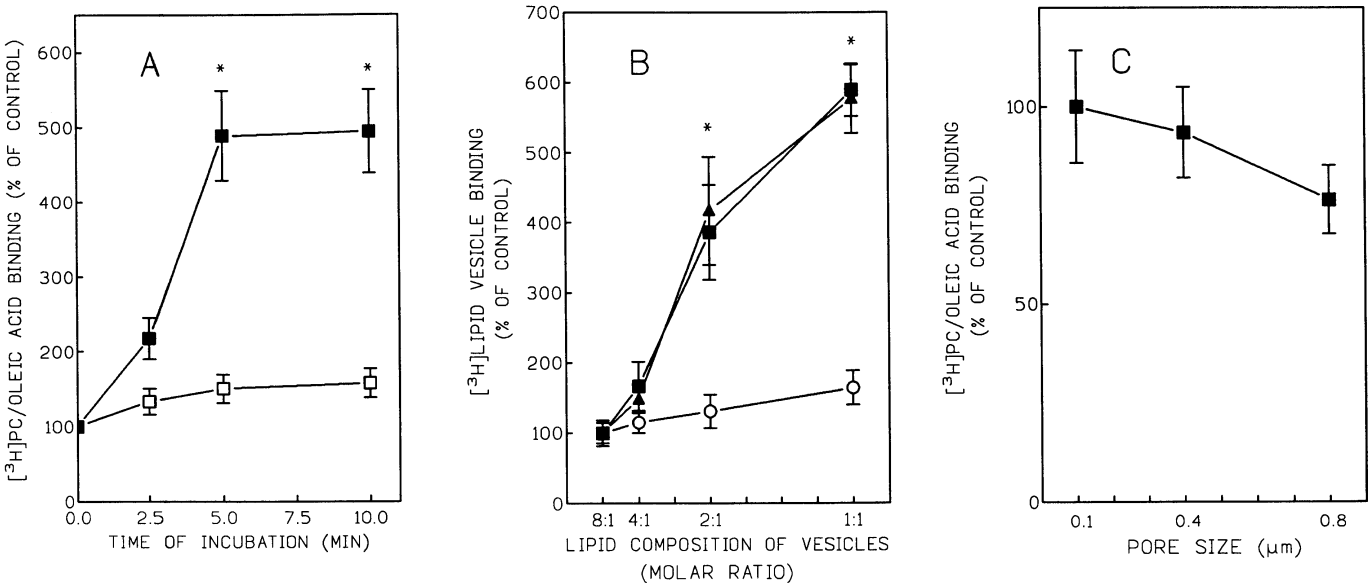

Figure 2 Lipid vesicle binding of CT–GST fusion proteins as a function of incubation time, lipid composition and vesicle size

(**A**) Agarose-bound CT constructs (0.5 nmol) were incubated for various time periods with 25 nmol of [^{3}H]PC/oleic acid (1:1 molar ratio) vesicles at 37 °C. At the end of the incubation, radiolabelled vesicles bound to the fusion proteins were isolated by centrifugation, washed and counted. ■, CT construct (I); □, construct (IX). Control (100%): construct I 227 ± 36 d.p.m. and construct IX 344 ± 28 d.p.m. Data are means ± S.D. for three separate experiments carried out in triplicate. (**B**) Agarose-bound construct I was incubated with ^{3}H-labelled lipid vesicles of various composition for 5 min at 37 °C. PC/oleic acid, PC/phosphatidylglycerol (PG) or PC/phosphatidylethanolamine (PE) vesicles were tested at PC/lipid molar ratios of 8:1, 4:1, 2:1 and 1:1 respectively. Results are expressed as percentage of binding using PC/lipid vesicles with a molar ratio of 8:1. Control (100%): 215 ± 38 d.p.m. PC/oleic acid; 193 ± 21 d.p.m. PC/PG; 131 ± 12 d.p.m. PC/PE. Data are means ± S.D. for three separate experiments carried out in triplicate. ■, PC/oleic acid; ▲, PC/PG; ○, PC/PE. (**C**) Agarose-bound construct I was incubated with [^{3}H]PC/oleic acid (1:1 molar ratio) vesicles, which were sized by sequential extrusion through polycarbonate filters with pore sizes ranging from 0.8 to 0.1 μm. Results are expressed as percentage of binding using vesicles extruded through a filter with 0.1 μm pore size (100%). Control (100%): 1249 ± 212 d.p.m. Data are means ± S.D. for three separate experiments carried out in triplicate.

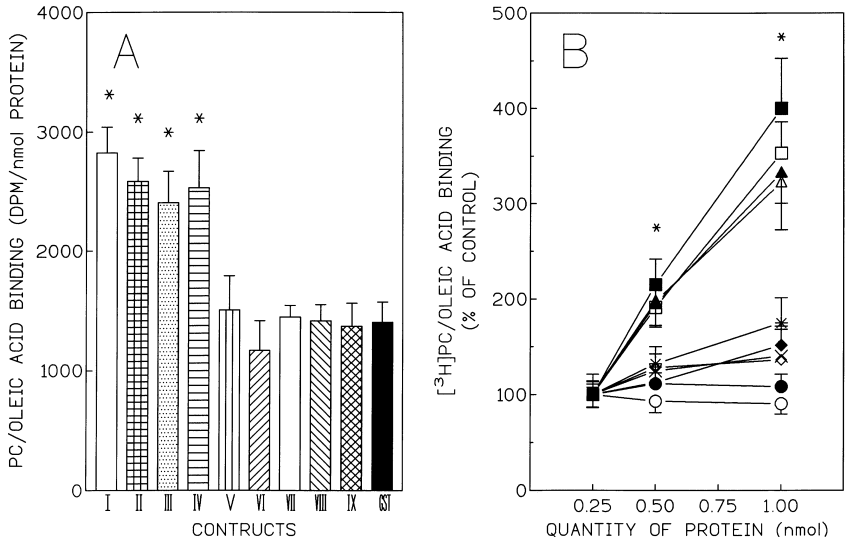

Figure 3 Binding of GST–CT constructs to PC/oleic vesicles

(**A**) Agarose-bound GST fusion proteins (0.5 nmol) of the various constructs depicted in Figure 1 were incubated with 25 nmol of [^{3}H]PC/oleic acid (1:1 molar ratio) vesicles for 5 min at 37 °C. The lipid vesicles bound to the CT–GST constructs were separated from unbound vesicles by centrifugation. Data are means ± S.D. for at least three separate experiments carried out in triplicate. *$P < 0.05$ compared with GST. (**B**) Binding as a function of amount of GST fusion protein. Data are expressed as % of binding using 0.25 nmol of CT–GST fusion protein. Data are means ± S.D. for at least three separate experiments carried out in triplicate. ■, Construct I; □, construct II; △, construct III; ▲, construct IV; ◆, construct V; ◇, construct VI; ×, construct VII; *, construct VIII; ○, construct IX; ●, GST. *$P < 0.05$ compared with control (0.25 nmol of fusion protein) for constructs I–IV.

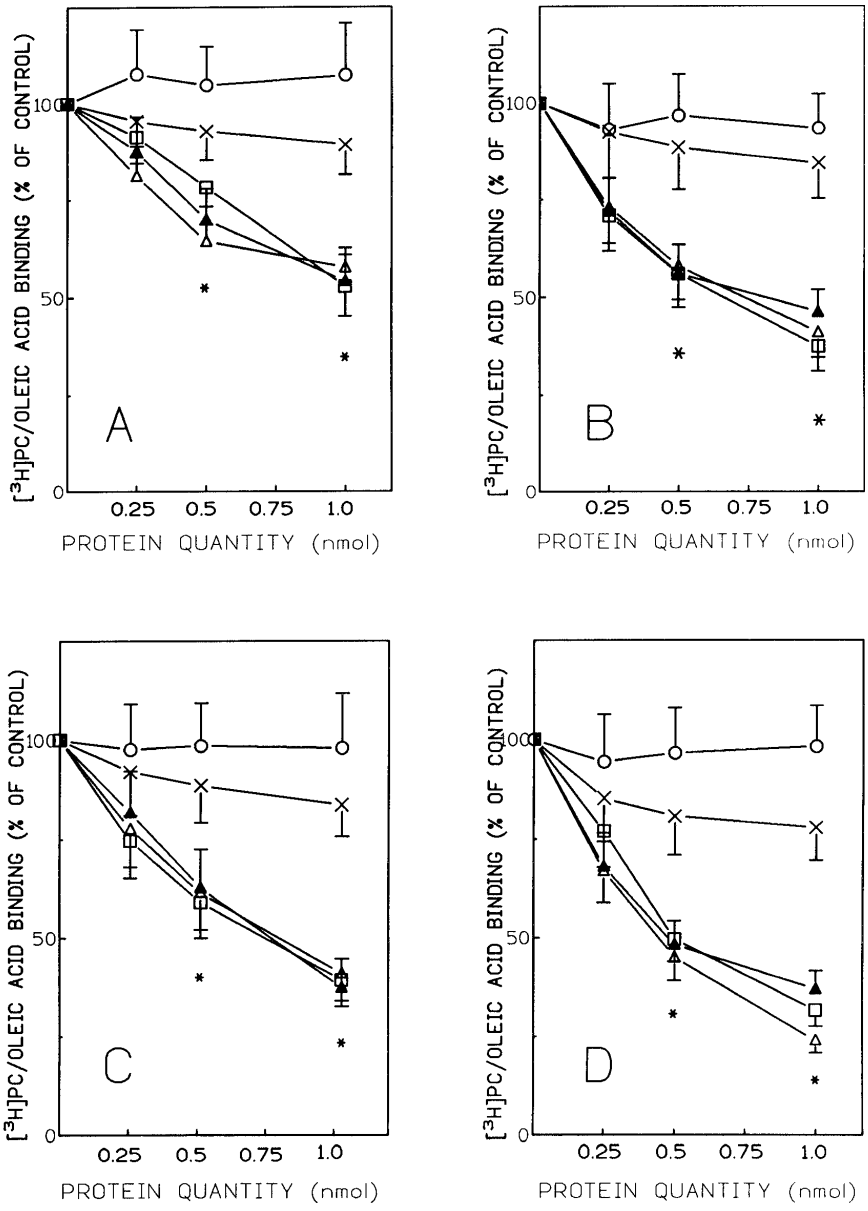

Figure 4 Cross-competition for PC/oleic acid vesicle binding to agarose-bound CT constructs, using eluted GST fusion proteins

Agarose-bound CT fusion proteins (0.5 nmol) were incubated for 5 min at 37 °C with 25 nmol of [^{3}H]PC/oleic acid (1:1 molar ratio) vesicles in the absence or presence of increasing concentrations of eluted CT fusion proteins. Vesicles bound to the agarose-bound CT fusion proteins were separated from unbound vesicles by centrifugation. (**A**), (**B**), (**C**) and (**D**) show the competition between agarose-bound CT constructs I, II, III and IV and various eluted CT fragments respectively. Data are expressed as percentage of control values (mean ± S.D. for at least three separate experiments carried out in triplicate). Control (100%): construct I, 1402 ± 137 d.p.m. (**A**); construct II, 1283 ± 124 d.p.m. (**B**); construct III, 1223 ± 159 d.p.m. (**C**); construct IV, 1253 ± 167 d.p.m. (**D**). Eluted CT fusion proteins: construct II (□); construct III (△); construct IV (▲); construct VII (×); construct IX (○). *$P < 0.05$ compared with control for constructs II–IV.

quantities of constructs I–IV (Figure 3B). In contrast, truncated CT proteins V–IX (all lacking residues 267–277) were not able to bind to lipid vesicles when compared with GST, independent of concentration (Figures 3A and 3B). These data suggest that PC/oleic acid binding does not require an intact C-terminus. Construct IV (residues 256–277) had no C-terminal sequences but showed optimal vesicle binding. This finding is consistent with the observation that a proteolytic 35 kDa fragment of rat CT, missing the C-terminus, bound to lipid vesicles [18]. Besides the C-terminus, construct IV also lacked the third 11-mer repeat (residues 278–288), suggesting that this motif is not essential for lipid binding. The finding that construct VIII, which contained the third 11-mer repeat and the C-terminal region, displayed no PC/oleic acid vesicle binding makes it also unlikely that the third 11-mer is required for lipid binding. CT–GST fusion protein VI included the N-terminus but demonstrated no specific lipid binding. Thus it appears that the N-terminal region encompassing residues 1–256 does not participate in PC/oleic acid binding. Deletion of the domain N-terminal to residue 256 did not affect the lipid-binding capacity of construct II, confirming that this region does not contribute to the CT–vesicle interaction. Besides the N-terminal region, CT–GST fusion protein V contained the first 11-mer repeat. However, the fusion protein demonstrated no significant PC/oleic acid vesicle binding, suggesting that the first

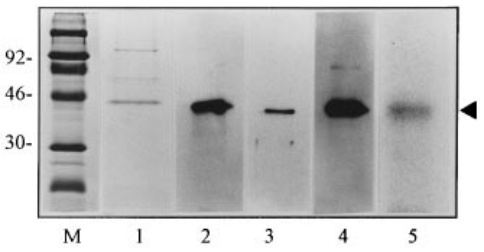

Figure 5 Characterization of recombinant CT

Rat CT was expressed and purified in recombinant baculovirus-infected insect cells. The recombinant CT was analysed by SDS/PAGE and Western blotting. Lane 1, Coomassie Blue-stained SDS/polyacrylamide gel of CT; lane 2, immunoblot with N-terminal antibody; lane 3, immunoblot with antibody raised against conserved region of CT; lane 4, immunoblot with C-terminal antibody; lane 5, iodinated recombinant CT.

1s1-mer motif (residues 256–266) is also not required for lipid binding. The observation that deletion of the first 11-mer motif (construct III) did not decrease the vesicle binding supports the idea that the domain N-terminal to residue 266 is not essential for CT-membrane interactions. Proteolytic cleavage experiments have also suggested that the N-terminal domain is not required for lipid binding [18]. GST fusion proteins III, IV and VII contained two helices. Construct VII, lacking residues 267–277, but containing the first and third 11-mer repeat, showed no lipid binding, consistent with both motifs playing no major role in vesicle binding. In contrast, CT–GST fusion proteins III and IV, comprising the second 11-mer repeat (residues 267–277) and either the first or third motif, displayed optimal lipid binding, suggesting that the CT region encompassing residues 267–277 is essential for binding to membranes. To verify further that this region is involved in lipid vesicle binding, we performed competition studies for [^{3}H]PC/oleic acid vesicle binding to agarose-bound constructs I–IV. As shown in Figure 4, increasing concentrations of eluted fusion proteins II, III and IV competed equally with the agarose-bound constructs for lipid binding. We did not observe any increase in lipid binding with the eluted GST proteins, suggesting that the CT–agarose beads were saturated with the GST fusion protein. To exclude the possibility that eluted GST–protein binds to sites still available on the glutathionine beads, we pretreated the agarose-bound constructs with GST before the competition assay. Similar results to those observed without pretreatment were obtained for constructs I–IV. The eluted des-(267–277)-CT mutant (construct VII) did not significantly inhibit vesicle binding to constructs I–IV (Figure 4). Neither construct IX (Figure 4) nor constructs V, VI and VIII (not shown) competed with GST fusion proteins I–IV for vesicle binding. These data are compatible with the concept that residues 267–277 are required for the interaction of CT with lipid bilayers.

Microsomal membrane binding of full-length CT and CT fusion proteins

The ability of CT to bind to a biological membrane (microsomal) was also investigated. Rat CT was expressed in recombinant baculovirus-infected insect cells. The purified recombinant CT ran at 42 kDa on SDS/PAGE (Figure 5, lane 1) and was recognized by antibodies raised against the N- (lane 2) and C-terminus (lane 4) of CT as well as by an antibody directed against the conserved central domain of the enzyme (lane 3). The iodinated CT ran also at approx. 42 kDa on the gel (lane 5). In preliminary experiments, ^{125}I-CT binding to lung microsomes was optimized for incubation time and amount of microsomes. Maximal binding of ^{125}I-CT to microsomes (approx. 125 pg of CT/μg of microsomal protein) was observed within 5 min of incubation. Binding of ^{125}I-CT to microsomes was significantly reduced with excess amounts of unlabelled recombinant CT (Figure 6A). In contrast, BSA did not compete with ^{125}I-CT for microsomal binding. This suggests that iodinated CT binds to

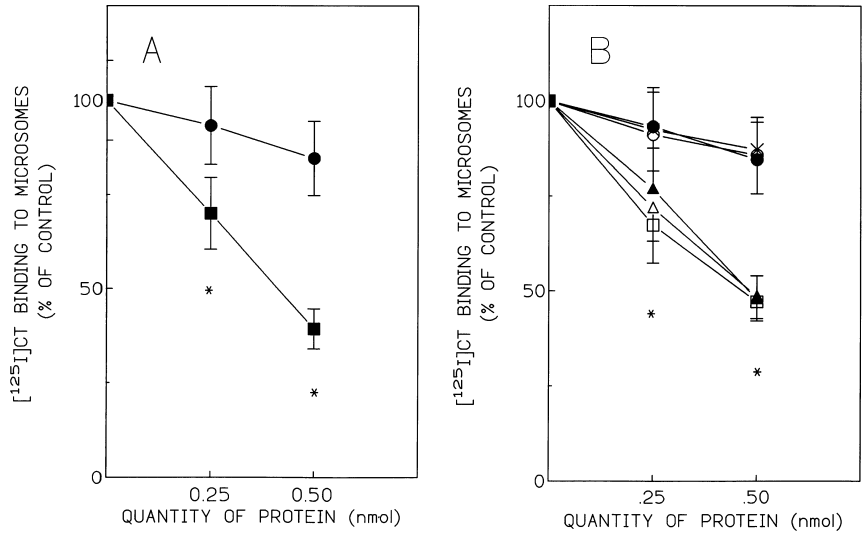

Figure 6 Cross-competition for ^{125}I-CT binding to lung microsomes, using recombinant CT and GST fusion proteins

(**A**) ^{125}I-CT (10 ng) was incubated for 5 min at 37 °C with 32 μg of lung microsomes in the absence or presence of increasing concentrations of unlabelled recombinant CT (■) or BSA (●). Iodinated CT bound to the microsomal membranes was separated from unbound ^{125}I-CT by centrifugation. (**B**) ^{125}I-CT (10 ng) was incubated for 5 min at 37 °C with 32 μg of lung microsomes in the absence or presence of increasing concentrations of eluted GST fusion proteins. Iodinated CT bound to the microsomal membranes was separated from unbound ^{125}I-CT by centrifugation. □, Construct II; △, construct III; ▲, construct IV; ×, construct VII; ○, construct IX; ●, GST. Data are means ± S.D. for at least three separate experiments carried out in triplicate and are expressed as percentage of control values (290 ± 17 d.p.m./μg of microsomal protein). *$P < 0.05$ compared with control for CT and constructs II–IV.

Table 1 Constructs encompassing residues 267–277 inhibit PC synthesis in A549 cells

A549 cells, stably transfected with cDNA for various CT deletion mutants under the control of glucocorticoid-inducible LTR promoter, were stimulated with cortisol and then processed for PC synthesis. Data are means ± S.D. for three separate experiments carried out in quadruplicate and are expressed as percentage of PC synthesis of cells transfected with pLKneo vector alone. $^*P < 0.05$ compared with control (pLKneo) and constructs VII, VIII and IX.

Construct	Reduction in PC synthesis (%)
pLKneo	–
II	50 ± 5*
III	47 ± 6*
VII	4 ± 1
VIII	4 ± 1
IX	6 ± 1

Table 2 Constructs encompassing residues 267–277 inhibit endogenous CT activity in 549 cells

A549 cells, stably transfected with cDNA for various CT deletion mutants under the control of glucocorticoid-inducible LTR promoter, were stimulated with cortisol and then processed for measurement of CT activity. Data are means ± S.D. for three separate experiments carried out in duplicate. $^*P < 0.05$ compared with control, cortisol-treated pLKneo, cortisol-treated construct VII and cortisol-treated construct VIII.

	Total CT activity (nmol/min)	
Construct	Control (F12)	Treatment (cortisol)
pLKneo	16.58 ± 2.06	16.88 ± 2.04
II	15.83 ± 1.78	12.10 ± 1.33*
III	16.27 ± 1.51	11.81 ± 1.42*
VII	16.26 ± 2.39	16.77 ± 1.33
VIII	16.42 ± 1.59	17.33 ± 2.05

microsomal membranes in a specific manner. We then analysed whether the GST constructs were able to compete with full-length CT for microsomal membrane binding. Again, constructs II, III and IV competed with ^{125}I-CT for microsomal binding, whereas GST and constructs VII and IX were not able to displace labelled CT binding to the microsomal membranes (Figure 6B). GST fusion proteins V, VI and VIII also did not alter ^{125}I-CT binding to microsome (not shown). Thus GST constructs containing residues 267–277 appear to have similar microsomal-binding characteristics to native CT, suggesting that the CT region of the constructs may display a similar α-helical conformation to the native enzyme. A recent study has shown that a 33-mer corresponding to the three 11-mer repeats (residues 256–288) can form an α-helix in the presence of vesicles composed of PC and anionic lipids [33]. The ability of the second 11-mer alone to form an α-helix in the presence of anionic lipid bilayers remains to be established.

In situ competition for CT–lipid binding

To verify that residues 267–277 are critical for lipid regulation of CT activity, we stably transfected the cDNA for CT constructs II, III, VII, VIII and IX under the control of a glucocorticoid-inducible long terminal repeat (LTR) promoter in pulmonary epithelial A549 cells. Transfectants, non-transfectants and cells transfected with pLKneo vector alone were exposed to cortisol to induce CT mutant protein expression. The truncated CT proteins containing residues 267–277 were expected to act as dominant inhibitors by competing for physiological CT–membrane interactions. Basal PC synthesis was similar for transfectants and non-transfectants and cortisol treatment did not change PC synthesis in non-transfectants [control: 14.28 ± 1.81 ($\times 10^3$) d.p.m./10^6 cells; cortisol: 14.95 ± 1.82 ($\times 10^3$) d.p.m./10^6 cells] or A549 cells transfected with pLKneo vector alone [control: 15.17 ± 1.02 ($\times 10^3$) d.p.m./10^6 cells; cortisol 16.14 ± 2.12 ($\times 10^3$) d.p.m./10^6 cells]. Exposure of A549 cells transfected with constructs II and III to cortisol, however, reduced PC synthesis when compared with cortisol-treated A549 cells transfected with vector alone (Table 1). Induction of des-(267–277)-CT mutant (construct VII) in A549 cells had no effect on PC synthesis. Mutant proteins VIII and IX also did not affect PC formation in A549 cells. Similar results were obtained with pulmonary epithelial L2 transfectants (not shown). Thus it appears that residues 267–277 in CT mutants are required to compete with endogenous CT for lipid binding in cells. Induction of mutant proteins II and III, but not CT mutant VII, indeed resulted in a decrease in total CT activity (Table 2), which was accompanied by a shift in CT activity from membrane to soluble fraction (Table 3). CT mutant protein VII, which does not contain residues 267–277, affected neither total CT activity nor subcellular distribution of CT activity in A549 cells.

Binding of des-(256–266, 278–288)-CT deletion mutant to lipid vesicles

Finally, to confirm that only residues 267–277 of the putative membrane-binding region (236–293) of CT [14,18,19] are essential for membrane binding, we synthesized a CT–GST construct that

Table 3 Constructs encompassing residues 267–277 alter distribution of endogenous CT activity in A549 cells

A549 cells, stably transfected with cDNA for various CT deletion mutants under the control of glucocorticoid-inducible LTR promoter, were stimulated with cortisol and then homogenized and fractionated. Enzyme activity was measured in the different subcellular fractions: post-mitochondrial membrane (P1), microsomal membrane (P2) and cytosolic (S) fractions. Data are means ± S.D. for three separate experiments carried out in duplicate. $^*P < 0.05$ compared with control, cortisol-treated pLKneo, cortisol-treated construct VII and cortisol-treated construct VIII.

	Control (F12) (% of total activity)			Treatment (cortisol) (% of total activity)		
Construct	S	P1	P2	S	P1	P2
pLKneo	47.1 ± 3.9	31.3 ± 3.0	21.5 ± 3.9	47.6 ± 2.9	30.6 ± 3.3	21.6 ± 3.1
II	46.9 ± 5.5	30.6 ± 2.2	22.3 ± 2.6	60.0 ± 5.4*	26.9 ± 2.3	12.9 ± 1.5*
III	47.0 ± 5.1	31.4 ± 3.6	21.5 ± 1.9	58.5 ± 4.7*	27.0 ± 3.1	14.4 ± 1.3*
VII	47.9 ± 4.8	29.2 ± 3.1	22.8 ± 1.3	48.5 ± 4.9	28.9 ± 2.9	22.5 ± 1.6
VIII	49.0 ± 2.6	28.6 ± 2.2	22.2 ± 2.3	49.2 ± 5.9	29.0 ± 2.8	21.3 ± 1.6

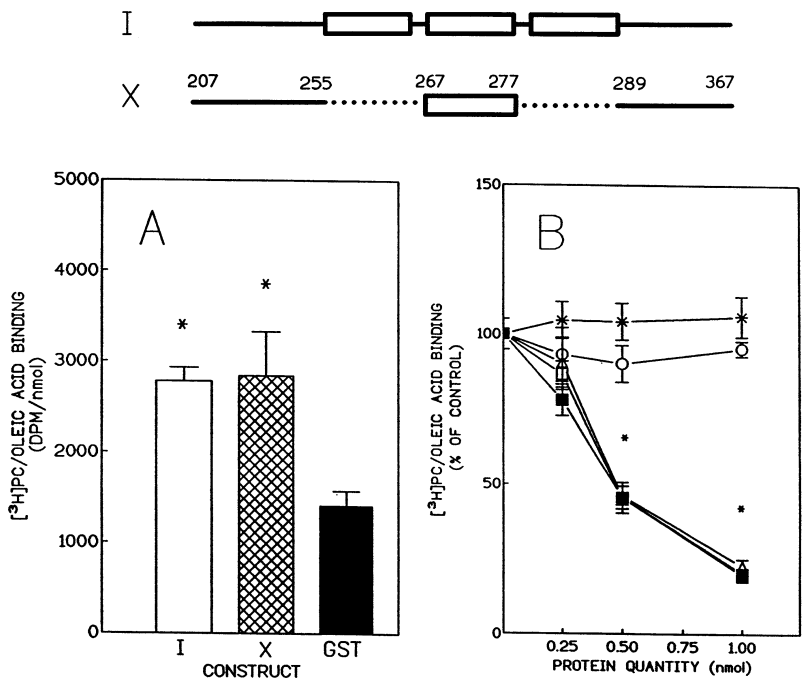

Figure 7 Binding of des-(256–266, 278–288)-CT deletion mutant to PC/oleic vesicles

(**A**) Agarose-bound GST fusion proteins were incubated with [^{3}H]PC/oleic acid vesicles as described in Figure 3. Data are means ± S.D. for at least three separate experiments carried out in triplicate. *$P < 0.05$ compared with GST. (**B**) Cross-competition for PC/oleic acid vesicle binding to agarose-bound CT construct X, using eluted GST fusion proteins, was carried out as described in Figure 4. ■, Construct I; □, construct II; △, construct III; ○, construct IX; *, construct VIII. *$P < 0.05$ compared with control for constructs I–III.

lacked residues 256–266 and 278–288 (Figure 7; construct X) and compared its lipid-vesicle-binding ability with that of a construct encompassing all three 11-mer repeats (construct I). The des-(256–266, 278–288)-CT deletion mutant displayed similar lipid-binding characteristics to CT construct I (Figure 7A). Vesicle binding increased linearly with increasing quantities of CT mutant X (not shown), and increasing concentrations of eluted fusion proteins I, II and III competed equally with the agarose-bound construct X for lipid binding (Figure 7B). In contrast, construct VIII and IX did not compete with construct X for lipid binding. These data are consistent with residues 267–277 of CT being essential for CT–lipid interactions.

In conclusion, the present study suggests that the CT region located between amino acid residues 267 and 277 from the N-terminus is required for the interaction of CT with membrane lipids. A putative membrane-binding domain had been predicted to reside in the region of rat CT encompassing residues 236–293 [18]. This was recently verified by limited proteolysis of rat CT with chymotrypsin, where the region located between residues 236 and 293 was shown to contain the activating membrane-binding domain [18]. Also, antibodies against residues 247–257 have been shown to interfere with CT–membrane association, providing further evidence that this region of CT is involved in lipid–protein interactions [34]. Finally, CT mutants in which the putative membrane-binding region was deleted have confirmed that residues 237–314 constitute a lipid-binding segment [12,14,19]. The sequence that we mapped to be required for membrane binding (residues 267–277) is within this region. The contribution of the individual amino acids in this region to the lipid binding remains to be elucidated.

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