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IDENTIFICATION OF THE MEMBRANE BINDING DOMAIN OF CTP:PHOSPHOCHOLINE CYTIDYLYLTRANSFERASE

by

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B.Sc., University of British Columbia, 1986

Thesis submitted in partial fulfillment of

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Identification of the Membrane Binding Domain of Rat Liver CTP: Phosphocholine Cytidylyltransferase

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ABSTRACT

Limited chymotrypsin proteolysis of CTP:phosphocholine cytidylyltransferase (CT; EC 2.7.7.15) produced several distinct fragments which were mapped to the N-terminus of CT using antibodies directed against the N and C terminus and the conserved central domain. A time course of chymotrypsin proteolysis showed a progression in digestion as follows: 42 → 39 → 35 → 30 → 28 → 26 kDa. Production of chymotryptic fragments correlated with a progressive loss of enzymatic activity.

The binding of CT and of the chymotryptic fragments to lipid vesicles was assessed by floatation analysis. The ability of the fragments to bind to activating vesicles correlated with the presence of a putative amphipathic alpha helix between residues 236 and 293. Undigested CT and the 39 and 35 kDa fragments contain the intact amphipathic helix and bound to all vesicle types tested. The 30, 28 and 26 kDa fragments lack some or all of the amphipathic helix and did not appear to bind to activating lipid vesicles. Chemical cleavage of rat CT using 2-nitro-5-thiocyanobenzoic acid, which cleaves peptide bonds at cysteine residues, resulted in the production of several fragments, all containing the putative amphipathic alpha-helix. All of these fragments were able to bind to all vesicle types tested.

The degree of resistance to chymotrypsin degradation increased when CT was bound to strongly activating lipid vesicles. Both the 39 and 35 kDa fragments persisted when CT was digested in the presence of phosphatidylcholine/oleic acid and phosphatidylcholine/phosphatidylglycerol vesicles but were rapidly

degraded in the absence of lipid or in the presence of weaker CT activators. These differences are likely a reflection of the different binding affinities of CT for each lipid, as was assessed by lipid floatation studies. The increased chymotrypsin-resistance of fragments containing the amphipathic helix when bound to activating lipids suggests that this helical domain is more shielded from solvent upon membrane binding. No change in sensitivity to chymotrypsin proteolysis was observed when CT was digested in the presence of its substrates, CTP and phosphocholine.

These results support the theory that CT interacts with membranes via its putative amphipathic helix which intercalates into the membrane bilayer of activating phospholipids.

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ABBREVIATIONS.

ATP	adenine triphosphate
β-ME	β-mercaptoethanol
cAMP	cyclic adenosine monophosphate
CDP	cytidine diphosphate
CPTcAMP	8-[4-chlorophenylthio]-adenosine-3':5'-monophosphate
CT	CTP:phosphocholine cytidylyltransferase
CTP	cytidine triphosphate
DAB	diaminobenzidine
DEAE	diethylaminoethyl
DG	diacylglycerol
DLPC	dilaurylphosphatidylcholine
DME	Dulbecco's Modified Eagle Medium
DPPC	dipalmitoylphosphatidylcholine
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
FCS	fetal calf serum
kDa	kilodaltons
Me_2SO	dimethyl sulfoxide
M_r	relative molecular mass
NTCB	2-nitro-5-thiocyanobenzoic acid
O	oleic acid
PBS	phosphate-buffered saline
PC	phosphatidylcholine
PG	phosphatidylglycerol
PE	phosphatidylethanolamine

PI	phosphatidylinositol
PMSF	phenylmethylsulfonyl fluoride
PS	phosphatidylserine
PVDF	polyvinylidinedifluoride
R_f	relative migration distance
SDS	sodium dodecylsufate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
Sph	sphingosine
SGPB	*Streptomyces griseus* protease B
TBS	Tris-buffered saline
TEMED	N,N,N',N'-tetramethylethylenediamine
TPA	12-*O*-tetradecanoylphorbol-13-acetate

PART 1: BACKGROUND

1.1 INTRODUCTION

CTP:phosphocholine cytidylyltransferase (CT; EC 2.7.7.15) is a key regulatory enzyme in the biosynthetic pathway of phosphatidylcholine in higher eukaryotes (Choy and Vance, 1978). CT catalyses the transfer of a cytidylyl-group from CTP to phosphocholine to form CDP-choline and PP_i (Figure 1). Phosphatidylcholine (PC) is the most abundant phospholipid in mammalian cells. It is the major lipid component of most animal cell membranes. In addition PC is the major phospholipid in mammalian lung surfactant and serum lipoproteins. Lastly, PC has been implicated as an important source of the second messengers diacylglycerol and arachidonic acid (Exton, 1990).

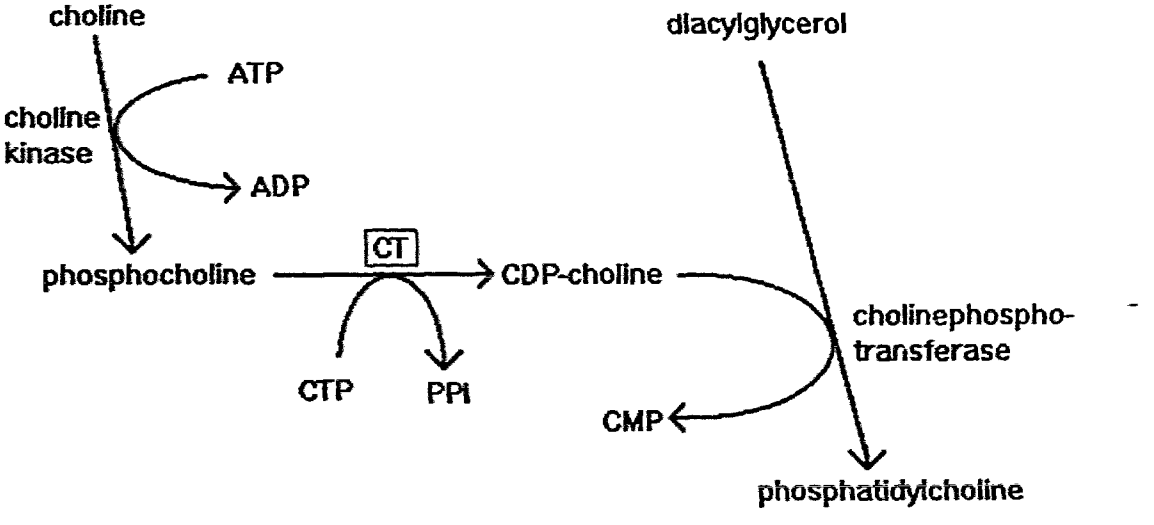


Figure 1: Major pathway for the synthesis of phosphatidylcholine in eukaryotes.

CT exists in an inactive soluble form and an active membrane-bound form (Vance and Pelech, 1984). Both forms appear to be homodimers of a 42 kDa monomer (Cornell, 1989; Weinhold *et al*, 1989). Two major types of regulation have been identified which affect the translocation of CT between the soluble and membrane compartments (Figure 2). *(i) Phosphorylation/ dephosphorylation.* Dephosphorylation accompanies the translocation of CT to membranes after treatment of cells with phospholipase C (Watkins and Kent, 1991) or fatty acids (Wang *et al*, 1993). Inhibitors of dephosphorylation result in accumulation of phosphorylated CT in the soluble fractions (Watkins and Kent, 1991; Hatch *et al*, 1990). *(ii) The lipid composition of the membrane.* Fatty acids (Pelech *et al*, 1984; Weinhold *et al*, 1984; Cornell and Vance, 1987a) and diacylglycerol (Cornell and Vance, 1987a; Jamil, 1992) stimulate membrane association of CT. Anionic phospholipids promote binding of CT to membranes *in vitro* (Cornell and Vance, 1989a; Cornell, 1991a). A decrease in the relative PC content of the membrane also stimulates CT translocation to the membranes (Yao *et al*, 1990).

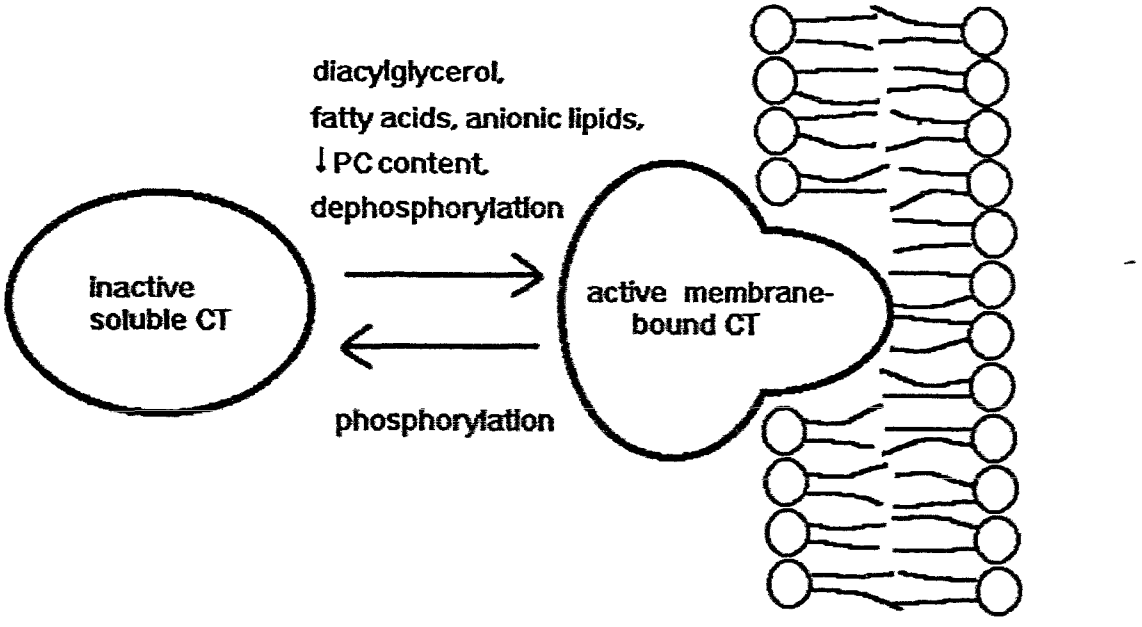


Figure 2: Regulation of translocation of CT between the membrane and the cytosol.

Despite extensive research very little direct structural information is available to explain the mechanisms of CT regulation. Determination of the amino acid sequence of rat CT (Kalmar *et al*, 1990) led to predictions of secondary structure which provided some insight into these mechanisms. The study reported in this thesis attempts to empirically verify some of these theoretical predictions. Elucidation of the mechanism of the interaction of CT with membrane lipids is central to an understanding of how CT and the PC biosynthetic pathway are controlled. I report the identification of the membrane-binding domain of rat liver CT and probe the nature of the mechanism of this interaction.

1.2 REGULATION OF CT

Translocation of CT from its soluble inactive form to its active membrane-bound form is controlled by phosphorylation, fatty acids, anionic phospholipids and diacylglycerol. Experimental evidence to support these conclusions is discussed here to provide background for the work presented in this thesis.

1.2.1 Subcellular Localization of CT

The soluble form of CT is an inactive reservoir which can be activated upon translocation and binding to a membrane surface. There has been some debate regarding the organelle with which CT associates. To study the subcellular localization of CT, the cytosolic and membrane fractions of homogenized cells are separated by differential and isopycnic centrifugation. Typically cells are homogenized and centrifuged at low speed (1000 x *g*) to remove nuclei and cell debris. The supernatant is then centrifuged at 100 000 x *g* to separate the cytosolic from the membrane fraction. This high speed pellet is referred to as the microsomal fraction and is a mixture of vesiculated fragments of

mitochondria, endoplasmic reticulum, Golgi, and plasma membranes. These membranes can be separated on Percoll or sucrose gradients and identified using marker enzymes. The amount of CT in each fraction can be quantified by assaying CT activity or by immunoprecipitation or immunoblotting. Assay of CT activity in the soluble fraction requires the addition of activating phospholipids.

CT is usually found in both the soluble and particulate fractions of homogenized cells. In cells stimulated with fatty acids or phospholipase C, soluble CT translocates to the particulate fraction. Until recently it was widely accepted that CT was a cytosolic protein which translocated to the endoplasmic reticulum (Tercé *et al*, 1988) and Golgi membranes (Vance and Vance, 1988). Tercé *et al* found that CT activity co-migrated with the enzyme markers in the endoplasmic reticulum and not the external cell membrane or any other cellular organelle in phospholipase C-stimulated Krebs II ascitic cells. Vance and Vance found that CT activity associated with the Golgi as well as the endoplasmic reticulum in rat hepatocytes. On the other hand several biochemical and immunofluorescent studies have produced compelling evidence that CT associates with the inner nuclear membrane of stimulated Chinese hamster ovary (CHO) cells. In these studies CT activity did not co-migrate with either the endoplasmic reticulum or Golgi marker enzymes in phospholipase C-treated CHO cells. 80% of the total CT activity co-migrated with a fraction containing a high concentration of nuclei (Morand and Kent, 1989). As there are no suitable markers for nuclear membrane, nuclei were identified by microscopy. The same group has immunolocalized CT to the inner nuclear membrane of choline-depleted CHO cells and oleate-treated HeLa cells and rat liver cells (Watkins and Kent; 1992, Wang *et al*, 1993a; Wang *et al*, 1993b). The soluble form of CT was immunolocalized to the nucleoplasm in untreated cells. An important

modification in the fractionation procedure used in the studies by Kent's group is that the Percoll gradient fractionation was performed on the whole cell homogenates, whereas the other groups used the low speed supernatant only and thus may have discarded the nuclear CT. Kent's group found that the nuclei could be pelleted from the cell homogenate using as little as 65 x *g* (Morand and Kent, 1989).

The enzyme which catalyses the step immediately following the CT reaction in the phosphatidylcholine pathway is cholinephosphotransferase which transfers the phosphocholine group on CDP-choline to diacyiglycerol to form the end product, phosphatidylcholine. This enzyme is bound to the endoplasmic reticulum and is often used as a marker to identify that membrane. It would seem logical to have CT on the same membrane as cholinephosphotransferase. In fact the endoplasmic reticulum is continuous with the nuclear envelope and it has been proposed that junctions between the nuclear and the endoplasmic reticulum membrane may be sites for *de novo* lipid biosynthesis (Watkins and Kent, 1992). On the other hand the product of the CT-catalysed reaction, CDPcholine, is soluble and could diffuse to the endoplasmic reticulum from the nuclear membrane making co-localization of the two enzymes unnecessary.

1.2.2 CT is a Member of a Class of Amphitropic Proteins

CT is among a class of newly recognised proteins called amphitropic proteins. These are proteins which have the ability to reversibly translocate from the cytosol to the membrane. Prominent examples of proteins in this class are protein kinase C, *Escherichia coli* pyruvate oxidase and arachidonate 5-lipoxygenase (Hannun *et al*, 1986; Grabau and Cronan, 1986; Rouzer and Kargmen, 1988).

Protein kinase C shares some regulatory features with CT. Prior to stimulation of a cell by phorbol esters or hormones, protein kinase C is in an inactive cytosolic form. Partitioning of protein kinase C into membranes requires diacylglycerol (DG), anionic phospholipids and Ca^{2+} (Hannun *et al*, 1986; Nishizuka 1986). The requirement for Ca^{2+} is not fully understood. It may form a bridge between the enzyme and the anionic phospholipids. Once protein kinase C is activated by DG or phorbol esters, which mimic DG, it can phosphorylate epidermal growth factor receptor and other enzymes involved in cell proliferation and differentiation.

Pyruvate oxidase binds its substrate and cofactor in the cytosol and undergoes a conformational change which exposes a lipid-binding domain (Grabau and Cronan, 1986). The catalytic activity is stimulated 50-fold in the presence of lipids, both anionic and cationic (Blake *et al*, 1978) and lipid-binding has both an electrostatic and a hydrophobic element (Grabau and Cronan, 1986).

Less is known about the regulation of arachidonate 5-lipoxygenase. It is found in both cytosolic and membrane fractions of human leukocytes (Rouzer *et al*, 1985). Its substrate is arachidonic acid, which is derived from hydrolysis of phospholipids. For the enzyme to bind its substrate it must concentrate near or on the membrane. The lipoxygenase requires ATP and Ca^{2+} for maximal activity (Yamamoto, 1989), and is activated *in vitro* by PC vesicles and whole cell lipids (Rouzer *et al*, 1985).

Other notable amphitropic proteins are synapsin, tubulin, lipases and phospholipases, blood coagulant factors, and apolipoproteins (Burn, 1988;

Kumar *et al*, 1981; Brzozowski *et al*, 1991; Van de Bosch, 1980; Mann, 1987; Segrest, *et al*, 1990). Although CT has features in common with many of the amphitropic enzymes it also has its own unique characteristics. Part of the study presented in this thesis attempts to better define similarities and differences between CT and other members in this class of proteins.

1.2.3 Signals for Translocation

A. Phosphorylation/Dephosphorylation

Phosphorylation was first recognised as playing a major role in CT activation over a decade ago when it was shown that addition of Mg^{2+} and ATP to rat liver cytosol resulted in inhibition of CT activity and that protein kinase inhibitors reduced this inhibition (Pelech *et al*, 1982). Several lines of evidence have led us to the current theory that soluble CT is a phosphoprotein which can be activated by dephosphorylation and translocated to membranes. This process is reversible and phosphorylation of the membrane-bound enzyme results in deactivation and translocation to the soluble fraction.

(i) CT is a Phosphoprotein *In Vivo*

Watkins and Kent (1990) showed that CT was phosphorylated in HeLa cells by immunoprecipitating cytosolic CT from cells grown in medium containing ^{32}Pi. The immunoprecipitate was separated by SDS-PAGE, blotted and subjected to autoradiography. A single 42 kDa band was observed, corresponding to the molecular weight of CT. Phosphoaminoacid analysis of the phosphorylated CT revealed that serine was the only residue phosphorylated.

(ii) Correlation Between Phosphorylation State and Subcellular Distribution

Recently the phosphorylation state of CT was directly correlated with its activation and subcellular distribution. Watkins and Kent (1991) found that dephosphorylation of soluble CT was necessary for translocation to the membrane fraction. An antibody generated against a peptide in the N-terminus of CT was used to analyse CT from the cytosol and particulate fraction of control and phospholipase C-treated CHO cells. Phospholipase C has been shown to stimulate PC synthesis in cultured cells (Kent, 1979). Western Blot analysis revealed a 42 kDa band in the soluble fraction of control cells and in the particulate fraction of phospholipase C-stimulated cells. A second, slightly slower migrating form of CT was present only in the soluble fraction of control cells. Treatment of the soluble fraction of control cells with Mg^{2+}, which activates protein phosphatases, abolished the slower migrating band, whereas treatment with Mg^{2+} and NaF, a general phosphatase inhibitor, had no effect on this band. Pretreatment of the CHO cells with okadaic acid, a potent inhibitor of type 1 and 2A phosphatases (Hescheler *et al*, 1988), increased the amount of CT in the soluble fraction. Phosphopeptide analysis showed that both bands from the soluble fraction of control cells were multiply phosphorylated whereas the particulate CT band from the phospholipase C treated cells had very little ^{32}P. They concluded that dephosphorylation of CT is required for phospholipase C-mediated translocation of CT from the soluble to the particulate fraction (Watkins and Kent, 1991).

Kent's group also showed that translocation of CT correlated with dephosphorylation of the enzyme in oleate-stimulated HeLa cells indicating that this phenomenon is not unique to CHO cells nor to phospholipase C-induced membrane translocation (Wang *et al*, 1993a).

(iii) Membrane Association is Reversible

Both okadaic acid and NaF inhibited PC biosynthesis and increased the level of CT in the soluble fraction, with a corresponding decrease in the particulate fraction compared to control cells (Hatch *et al*, 1992). Addition of fatty acids to okadaic acid-treated hepatocytes reversed the inhibition of PC synthesis. Phosphopeptide mapping showed an increase in the phosphorylation state of several peptides from CT in okadaic acid-treated cells compared with control cells. Addition of oleic acid to okadaic acid-treated hepatocytes reversed the okadaic acid effect and caused a progressive increase in PC synthesis. Initially it was assumed that the addition of fatty acids to these cells was the signal for translocation of CT to the membranes thus stimulating PC synthesis. However, determination of the fatty acid and DG levels of okadaic acid- and okadaic acid-oleic acid-treated cells revealed that the fatty acid level was not altered in either case relative to the controls. Hepatocytes can rapidly esterify fatty acids to DG and phospholipids. There was a 40% depletion in the DG levels of the okadaic acid-treated cells compared to controls. Addition of fatty acids resulted in increased DG levels and increased membrane CT activity. The authors concluded that okadaic acid inhibited PC synthesis by causing an increase in the phosphorylated state of CT and that addition of fatty acids overcame this effect by increasing the DG levels thus causing translocation of CT to membranes. There is considerable support for DG-mediated translocation of CT (Cornell and Vance, 1987a and b; Cornell, 1991 a and b; Slack *et al*, 1990; Kolesnick and Hemer, 1990).

(iv) Effect of cAMP on PC Synthesis

Short term treatment with glucagon and cAMP analogs have been shown to inhibit PC synthesis (Pelech *et al*, 1981). cAMP is a second messenger which

responds to the hormone glucagon or epinephrine in conditions of starvation, diabetes or stress, by curtailing fatty acid and glycogen synthesis. cAMP activates cAMP-dependent protein kinase which phosphorylates a number of proteins including acetyl Co A carboxylase, the regulatory enzyme in fatty acid biosynthesis (Witters *et al*, 1979), and a hormone-sensitive lipase (Steinberg, 1976). The mechanism for cAMP-induced inhibition of PC synthesis was investigated.

Pelech *et al* (1981) showed that the glucagon and cAMP analog inhibition of PC synthesis in cultured hepatocytes corresponded with a reduction in CT activity. The authors suggested that cAMP-dependent protein kinase may phosphorylate CT. This inhibition was overcome by supplementation with palmitate or oleate and with exogenously added DG (Pelech *et al*, 1983; Kolesnick and Hemer, 1990).

The amino acid sequence of CT contains a putative site for cAMP-dependent protein kinase between residues 61 and 64. CT from rat liver was shown to be weakly phosphorylated by cAMP-dependent protein kinase *in vitro* (Sanghera and Vance, 1989). However, treatment of rat hepatocytes with the cAMP analog, CPTcAMP, did not alter the phosphorylated state of CT or the subcellular distribution although it did result in an inhibition of PC biosynthesis (Jamil *et al*, 1992). It was subsequently determined that *DG levels* rather than CT activity were limiting PC synthesis in cAMP-treated cells, by substrate level inhibition of cholinephosphotransferase. Possible causes for DG depletion are the inhibition of acetyl CoA carboxylase and the stimulation of DG lipase, probably by cAMP-dependent protein kinase phosphorylation.

(v) Other Potential Phosphorylation sites on CT

The amino acid sequence of rat liver CT contains a number of other possible phosphorylation sites in addition to the cAMP-dependent protein kinase site (Figure 3). These sites include six potential phosphorylation sites for protein kinase C ([Ser]Thr-Xaa-Lys[Arg], Woodgett *et al*, 1986), two sites for calmodulin-dependent protein kinase (Arg-Xaa-Xaa-Thr[Ser], Kemp and Pearson, 1990) as well as a site at Ser-362 for casein kinase II (Asp-Ile-Ser-Glu-Asp-Glu-Glu-Asp, Murakami *et al*, 1990; Kuenzel *et al*, 1987). There are three Ser/Pro-rich motifs located in the C-terminus of CT which are potential sites for a cell cycle control protein kinase (cdc 2 kinase: Cisek and Corden, 1989; Morino and Nurse, 1990). Casein kinase II has been shown to phosphorylate wild type rat CT *in vitro* but not two mutant forms of CT, one having the putative casein kinase sequence deleted and one with the serine changed to an alanine (Kalmar *et al*, unpublished). Rat liver CT was not phosphorylated by protein kinase C *in vitro* (Sanghera and Vance, 1989). Both Sanghera and Vance (1989) and Watkins and Kent (1990) reported that only serine residues were phosphorylated on immunoprecipitated CT from cultured cells.

The above studies suggest that the phosphorylation state of CT is involved in activation of CT *in vivo*. Dephosphorylation is associated with translocation from the soluble fraction in cells to the membranes where it is active. CT has multiple phosphorylation sites, and dephosphorylation in response to a certain stimulus may occur on only one or two critical residues, thus not significantly changing the overall state (Watkins and Kent, 1990). Much more work is needed to identify the specific kinases which modulate CT activity.

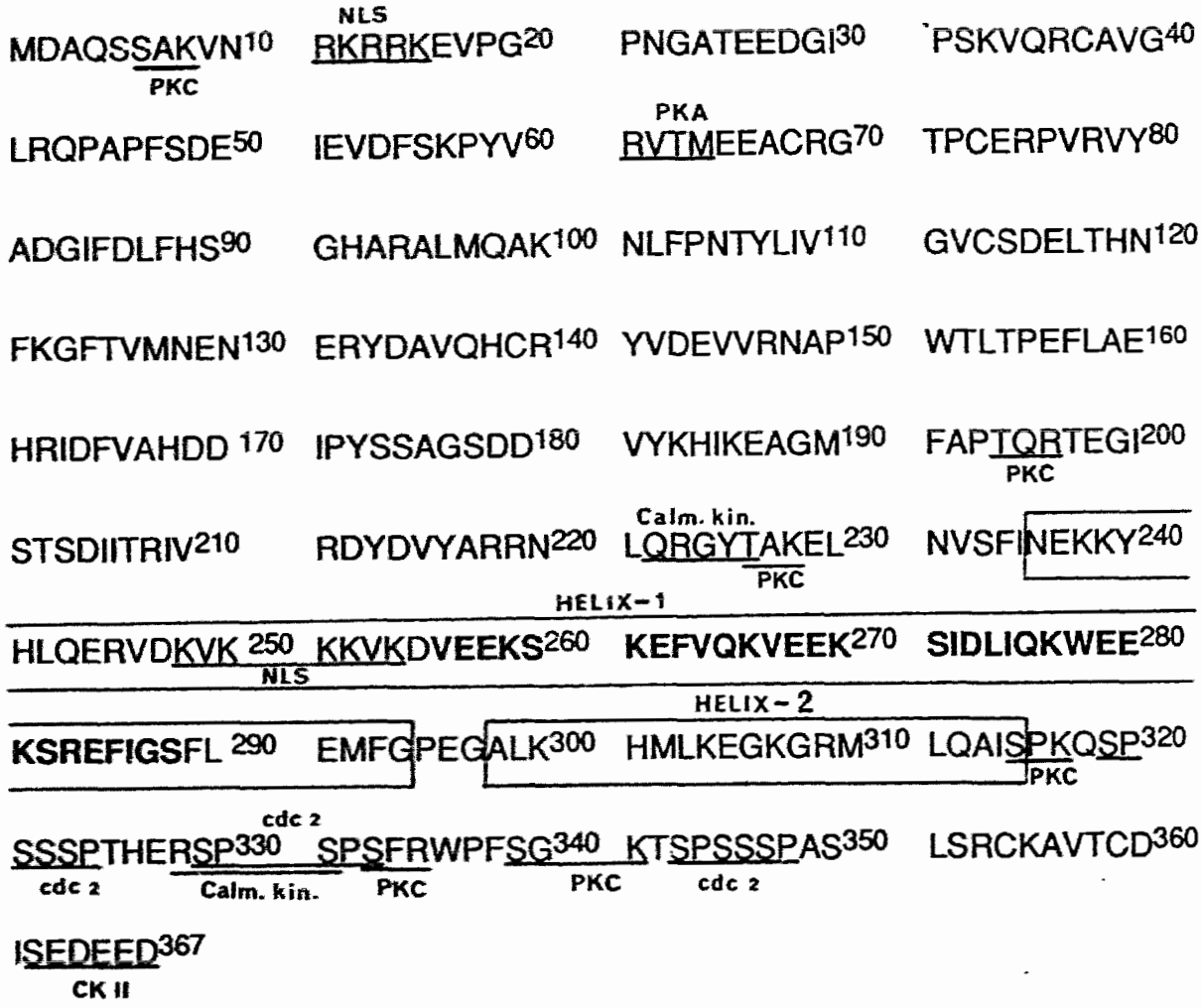


Figure 3. Amino acid sequence of rat CT indicating putative phosphorylation sites, amphipathic helices and nuclear localization sites. Abbreviations: PKC, protein kinase C; NLS, nuclear localization site; PKA, cAMP-dependent protein kinase A; Calm. Kin., calmodulin kinase; cdc 2, cell cycle control kinase; CK II, casein kinase II.

B. Lipid Regulators

A second component of CT activation is lipid regulation. Purified CT can be activated *in vitro* by specific lipids in the absence of any phosphatases. Early studies on rat liver and lung CT identified a low molecular weight inactive form (L-form) of CT in the cytosol which could be converted to a high molecular active weight form (H-form) by incubation with liposomes composed of total lipids from rat liver and lung cells (Choy *et al,* 1977; Choy and Vance, 1978; Feldman *et al,* 1978). Subsequent characterization of the lipid activators of CT has revealed two classes of activators: (i) fatty acids and anionic phospholipids such as phosphatidylserine, phosphatidylinositol, and phosphatidic acid; and (ii) lipids with small polar head groups such as mono- and diacylglycerol. Zwitterionic phospholipids such as PC and phosphatidylethanolamine are poor activators and positively charged sphingolipids are inhibitory (Johnson *et al,* 1992).

(i) Fatty Acids

A number of groups have demonstrated the stimulatory effects of fatty acids on PC biosynthesis and have been able to correlate increases in PC biosynthesis with the translocation of CT from the soluble fraction to the microsomal fraction. Pelech *et al* (1983) incubated rat hepatocytes with oleate or palmitate and demonstrated a two- to three-fold enhancement of [methyl-^{3}H]choline incorporation into PC. The amount of CT in the microsomal fraction doubled with a corresponding decrease in the soluble fraction. Cornell and Vance (1987a and b) observed rapid stimulation of phosphatidylcholine synthesis and a corresponding translocation of CT from the soluble to the membrane fraction in HeLa cells incubated with oleate, oleyl alcohol or palmitate (from 30% in control cells to 80% in stimulated cells). They found that the fatty acid-promoted

translocation of CT could be blocked by the detergent Triton X-100 but not by 1 M KCl suggesting that CT binds to membranes via a hydrophobic rather than an ionic interaction. Fatty acids were not able to activate CT in the absence of a membrane.

Acyl chain length and the degree of unsaturation of fatty acids is important in determining their ability to activate CT (Pelech *et al*, 1983; Cornell and Vance, 1987a and b). Both saturated and unsaturated fatty acids stimulated incorporation of [methyl-^{3}H]choline into PC in cultured rat hepatocytes, with unsaturated fatty acids being more effective than their saturated counterparts (Pelech *et al*, 1983). Short chain fatty acids ($n \leq 12$) were poor CT activators *in vitro* and in cultured cells (Pelech *et al*, 1983; Cornell and Vance, 1987a). In rat lung cytosol a variety of naturally occurring unsaturated fatty acids activated CT whereas saturated fatty acids had no effect (Feldman and Weinhold, 1981).

Fatty acid activation of CT has been demonstrated to be reversible (Weinhold *et al*, 1984; Cornell and Vance, 1987a). Addition of bovine serum albumin, a fatty acid binding protein, to postmitochondrial supernatants from fatty acid-treated cells caused a release of CT from the microsomes to the soluble fraction and a corresponding decrease in microsomal free fatty acid content. Fatty acid stimulation of CT may have physiological significance. A correlation was found between increased fatty acid content and the increased CT activity in the microsomal fraction of rat lung tissue immediately following birth (Weinhold, 1981).

(ii) Anionic Phospholipids

Early studies with crude cytosolic extracts showed that among the phospholipids, anionic or negatively-charged phospholipids were the most potent activators (Feldman *et al*, 1978). The effects of various lipids on purified rat CT activity have been demonstrated using Triton-mixed micelles (Cornell, 1991a). Most membrane proteins need to be reconstituted in detergents once purified in order to stabilize them in a hydrophobic environment. Purification of CT requires solubilization of the purified form in Triton X-100 micelles. CT is not active in Triton micelles but can be activated by addition of specific lipids. Only anionic lipids could activate CT when added as mixed micelles. The greater the negative charge of the lipid the lower the mol % required for activation. Maximal cardiolipin and phosphatidic acid (charge of -2) activation was achieved at 10 mol % lipid. 40 mol % fatty acid (charge of ~ -1/2) was needed for maximum activation. Phosphatidylglycerol (PG), phosphatidylinositol (PI) and phosphatidylserine (PS), (all with a charge of -1) required intermediate concentrations. The authors concluded that the activation of CT by anionic phospholipids is related the surface charge density of the membrane (Cornell, 1991a).

(iii) Diacylglycerol

Diacylglycerol in membranes has been shown to stimulate translocation and activation of CT *in vitro* and *in vivo.* Cornell (1991a) showed that activation by uncharged lipid activators such as DG, PC and oleyl alcohol in Triton X-100 micelles required much higher concentrations (> 80 mol %) than the charged lipid activators.

The role of DG in CT activation in cultured cells has been studied. CT in HeLa cell cytosol was activated with PC liposomes containing at least 20 mol% monooleoylglycerol or dioleoylglycerol demonstrating that a negative charge is not required (Cornell and Vance, 1987a). Pre-treatment of HeLa cell microsomes with phospholipase C caused cytosolic CT to translocate and correlated with a seven-fold increase in the DG content of the microsomes (Cornell and Vance, 1987a). Phospholipase C treatment of neuroblastoma cells caused translocation of CT to membranes with a concomitant increase in DG levels (Slack *et al*, 1990). The same response was seen when a soluble form of DG, dioctanoylglycerol, was added.

Phorbol esters are potent tumor promoters which activate protein kinase C by mimicking DG. Protein kinase C phosphorylates membrane-bound target proteins such as epidermal growth factor receptor and induces mitogenesis, cell cycle differentiation and exocytosis (Nishizuka, 1986). There have been numerous reports that phorbol esters stimulate CT translocation (Pelech *et al*, 1984) and PC production in HeLa cells (Paddon and Vance, 1980; Pelech *et al*,, 1984; Watkins and Kent, 1990), GH3 pituitary cells (Kolesnick, 1987; Paddon and Vance, 1980) and in rat skeletal myoblasts (Hill *et al*, 1984). The mechanism for phorbol ester-mediated CT translocation and stimulation of the PC pathway was investigated (Utal *et al*, 1991). A three-fold increase in membrane-associated CT and a corresponding decrease in cytosolic CT was observed in HeLa cells stimulated with 12-*O*-tetradecanoylphorbol-13-acetate. DG levels were elevated and there was a close temporal relationship between the production of DG and the binding of CT to membranes. The involvement of protein kinase C in translocation of CT was ruled out by down-regulating HeLa cells for protein kinase C and treating them with dioctanoylglycerol.

Translocation of CT was still observed. The authors concluded that phorbol esters enhance PC synthesis by increasing the levels of DG in the membranes and not by direct action of protein kinase C or phorbol esters on CT. The reason for the increase in DG levels in phorbol ester-treated cells may be due to protein kinase C activation of a PC-specific phospholipase C (Daniel et al, 1986). In another study, CT translocated to microsomes enriched in dioctanoylglycerol *in vitro* but phorbol esters failed to stimulate translocation (Kolesnick and Hemer, 1990).

(iv) PC Depletion of Membranes

Sleight and Kent (1980) proposed that changes in the membrane concentration of PC provide a sensitive mechanism of feedback regulation of PC synthesis by controlling the translocation of CT to the membrane. They lowered the PC content by treatment of fibroblasts with low concentrations of phospholipase C and found an increased rate of incorporation of [^{3}H]choline into lipid. Since DG is a product of the hydrolysis of PC by phospholipase C, it was necessary to exclude the possibility that an increased concentration of DG was signaling CT translocation to membranes. The levels of fatty acids, diacylglycerol and PC were analysed in hepatocytes grown in choline- and methionine-deficient medium (Yao *et al,* 1990). (Methionine is needed for synthesis of PC via methylation of phosphatidylethanolamine.) The hepatocytes were taken from both choline-deficient and choline-supplemented rats. There was no difference between the fatty acid levels and the diacylglycerol levels in cells grown with or without choline. However, the amount of CT in the membrane fraction of cells deficient in choline was inversely proportional to the concentration of PC in the membrane. There was a strong positive correlation between the amount of CT in the cytosol and the concentration of PC in the microsomal fraction in cells that

were supplemented with choline, methionine or lyso-PC. These results support the hypothesis that the level of PC in cells acts as a feedback regulator of CT activity.

(v) Effect of Cationic Lipids on CT Activity

Positively charged sphingolipids such as sphingosine inhibit CT activity in a reversible manner (Sohal and Cornell, 1990). Sphingosine and lysosphingolipids inhibited purified rat liver CT activation when added to 1:1 mixtures of egg PC and activating lipids oleic acid, PI, PG and PS. Adding increasing amounts of the activating lipids competitively reversed the inhibition by sphingosine. It was proposed that sphingosine acts to antagonize the effects of anionic lipids at the level of CT-membrane binding (Sohal and Cornell, 1990).

(vi) Mechanisms of Lipid Specific Activation

The diversity of the lipid structures which activate CT presents a challenge in trying to formulate a unifying hypothesis for the effects of lipids on CT activity and membrane binding. Cornell (1991 a, b) suggested that all activating lipids have the effect of loosening the membrane lipid packing, thus facilitating the intercalation of CT part of the way into the bilayer. Jamil and Vance (1993) suggested that promotion of non-bilayer phases is the unifying parameter for lipid activation.

It is not clear exactly how fatty acids induce translocation of CT from the soluble to the particulate fraction. One possibility is that they bind to CT and induce a conformational change which has a higher affinity for membranes. This mechanism is unlikely since pre-incubation of fatty acids with CT prior to addition of membrane vesicles does not affect activity (Cornell and Vance, 1987a). A

second possibility is that CT has a higher affinity for membranes containing fatty acids. Fatty acids increase the negative charge density of a membrane and may also disturb the lipid packing due to their small head groups. In this way fatty acids could facilitate intercalation of CT into the membrane. Vesicles made with a 1:1 mixture of PC and oleic acid are one of the most potent activators of pure CT.

The small head groups of diacylglycerol and oleyl alcohol may also cause packing defects facilitating the integration of CT into the bilayer. Similarly, anionic phospholipids may loosen the lipid packing by increased charge repulsions between neighboring molecules. A specificity toward the polar head group was not apparent in the Triton mixed micelle study (Cornell and Vance, 1987a) as PG, PI and PS have very different head groups but had similar activation curves.

Studies using preformed lipid vesicles showed that vesicles with a high curvature were more effective at activating CT than planar bilayers (Cornell, 1991b). Activation in vesicles was enhanced by induction of the gel-to-liquid crystalline phase transition. A correlation between CT translocation and the ratio of bilayer to non-bilayer forming lipids in model membrane systems has also been found (Jamil and Vance, 1993). All of these effects should result in a loosening of lipid-lipid interactions thus further supporting the theory that packing defects enhance CT-lipid interactions.

Several other proteins have been identified which interact specifically with anionic lipids. These include protein kinase C (Nishizuka, 1983), colicin A (Massotte *et al*, 1989) and synapsin (Benfenati *et al*, 1989). For these proteins a

two-step model has been proposed for membrane binding in which the anionic lipid surface promotes an electrostatic attraction between the protein and the membrane, followed by penetration of the protein into the hydrophobic core. CT has a cluster of positively charged residues in its proposed membrane-binding domain (Kalmar *et al*, 1990) which may serve to attach it to the surface of negatively charged membranes prior to intercalation.

1.3 STRUCTURAL FEATURES OF CT

Much effort has been invested over the past 25 years in defining the regulatory mechanisms of CT in an attempt to understand control of PC biosynthesis. Until the yeast CT and rat CT genes were cloned and sequenced (Tsukagoshi ,1987; Kalmar *et al*, 1990) very little attention was given to the study of CTs tertiary structure. Structural information about an enzyme can provide important clues to the mechanisms of its regulation.

Once the amino acid sequence for a protein has been obtained it can be analysed and compared with data bases of other protein sequences. Secondary structural predictions can be made based on homologies between the protein in question and proteins with known secondary structure based on X-ray-crystallographic data. Computer algorithms such as those of Chou-Fassman (1978) and Garnier *et al* (1978) analyse the amino acid sequence, looking at individual amino acids in the context of their neighboring residues and predict the most favourable interactions between neighboring residues based on the tendency to form a particular conformational state. Predictions can be made such as which regions will be α-helical, which will be β-sheets and where turns

and disulfide bonds may occur. The pattern of hydrophobicity of the protein can be assessed based on the total hydrophobicity value of a group of amino acids (Kyte and Doolittle, 1982). If a protein has any transmembrane sequences these may be identified by long stretches of hydrophobic residues. Speculations about the locations of sites for membrane-binding, substrate-binding, catalysis and regulation can be made before any empirical evidence is obtained.

1.3.1 Amino Acid Sequence and Secondary Structural Predictions of Rat CT

(i) Primary Structure

Rat liver CT is a 367 amino acid polypeptide. Overall it is hydrophilic and has a charge of -2 at pH 7. The amino acid sequence has several interesting features which were immediately obvious (see Figure 3). There is a Ser/Pro-rich domain near the C-terminal one-eighth of the sequence which is a potential site for a cell cycle control kinase (Cisek and Corden, 1989; Morino and Nurse, 1990) as well as several other putative phosphorylation sites. On the N-terminal side of the Ser/Pro-rich region lies an 11-amino acid sequence which is repeated three times in tandem. There are two clusters of positively charged residues; Arg-Lys-Arg-Arg-Lys (residues 12 to 16) and Lys-Val-Lys-Lys-Lys-Val-Lys (residues 248 to 254) which resemble nuclear localization signals (Roberts *et al,* 1987). Although CT is known to interact with membranes there are no hydrophobic regions in the sequence long enough to span a membrane (~30 residues). In fact there are no sequences with more than 5 hydrophobic residues in register. In addition, no region could be identified for covalent attachment of CT to lipid, such as a hydrophobic C-terminus for glycosyl-phosphatidylinositol, a C-terminal

Cys-Ala-Ala-Xaa for an isoprenyl group, or a glycine in position 2 for N-myristoylation (Caras *et al*, 1987; Hancock *et al*, 1989; Kamps *et al*, 1985).

(ii) Secondary Structure

The N-terminal two-thirds of rat liver CT is predicted by Garnier *et al* (1978) algorithms to be a tightly-folded globular domain as it contains a number of short α–helices and β–sheets, interrupted by proline-induced turns. The C-terminus is less structured, containing 8 prolines, and is predicted to be randomly coiled. The N- and C-terminal domains are linked by two long α–helical stretches which are predicted to be amphipathic. The two α–helices are interrupted by a proline and two glycines, indicative of a turn.

1.3.2 Comparison of Mammalian CT With Yeast CT and *Bacillus Subtilis* Glycerolphosphate Cytidylyltransferase

The sequence for CT in yeast was reported in 1987 (Tsukagoshi *et al*) (Figure 4). In 1990 a rat liver CT cDNA was the first mammalian CT cDNA to be isolated (Kalmar *et al*). Recently cDNAs from mouse and human origins have been cloned (Lachance, M.Sc. Thesis, 1993). The sequences are highly conserved. There is one non-conserved amino acid change between rat and mouse and 10 non-conserved changes between rat and human. The sequence of rat CT between residues 74 and 234 shares 63% identity with residues 101-260 on the yeast sequence. This region is also homologous to the active site in *B. subtilis* glycerolphosphate cytidylyltransferase between residues 6-94 and is predicted to be the catalytic domain. The N- and C-terminus of rat and yeast are less homologous, with only 25% and 37% identity respectively.

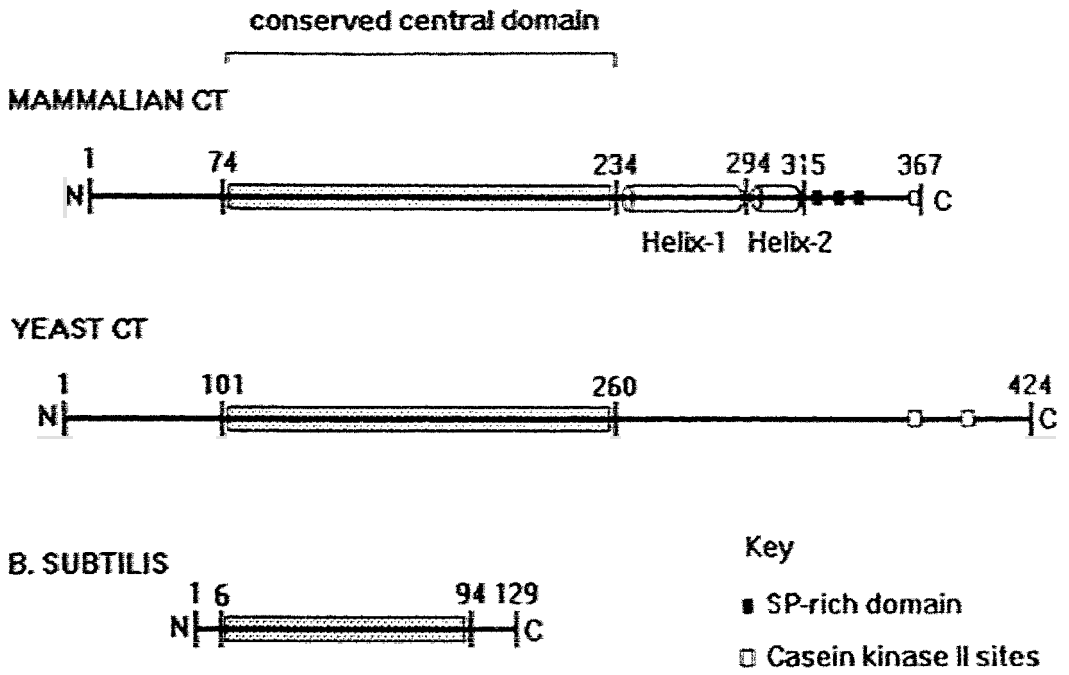


Figure 4: Comparison between the amino acid sequences of mammalian and yeast CT, and *B. subtilis* glycerolphosphate cytidylyltransferase.

1.3.3 The Amphipathic α–Helix as the Membrane-Binding Domain

As there are no long stretches of hydrophobic amino acids capable of spanning a bilayer, the amphipathic α-helix was proposed as a potential membrane binding domain (Kalmar *et al*, 1990). This region, between residues 236 and 315 of the rat CT (see Figure 4), is predicted to contain two α–helices with an intervening turn at residues 294-297. The N-terminal helix, Helix-1, is profoundly amphipathic as seen in a helical wheel representation (Figure 5) (Schiffer and Edmundson, 1967). There is an asymmetric distribution of polar and non-polar residues, with charged residues localized on one face of the helix and hydrophobic residues on the other face. Kalmar *et al* (1990) proposed that this helix could interact with membranes by intercalating into the lipid bilayer, with its

helical axis parallel to the membrane surface (Figure 6). The polar face of the helix would be exposed to the cytosol or other regions of CT and the hydrophobic face would interact with the hydrophobic core of the lipid bilayer. Within the Helix-1 between residues 256-288, an 11-mer motif is repeated three times in tandem: **VEEKSKEFVQK VEEKSIDLIQK WEEKSREFIGS**. There is a cluster of positively-charged amino acids at the polar-nonpolar interface of Helix-1, with a cluster of negatively charged residues along the center of the polar face. These alternating acidic and basic amino acids provide the potential for numerous salt bridges between every 3 to 4 residues, thus greatly stabilizing the α–helix. Helix-2, is also amphipathic though not to the extent of Helix-1. It is separated from Helix-1 by 2 glycines and a proline, characteristic of a turn.

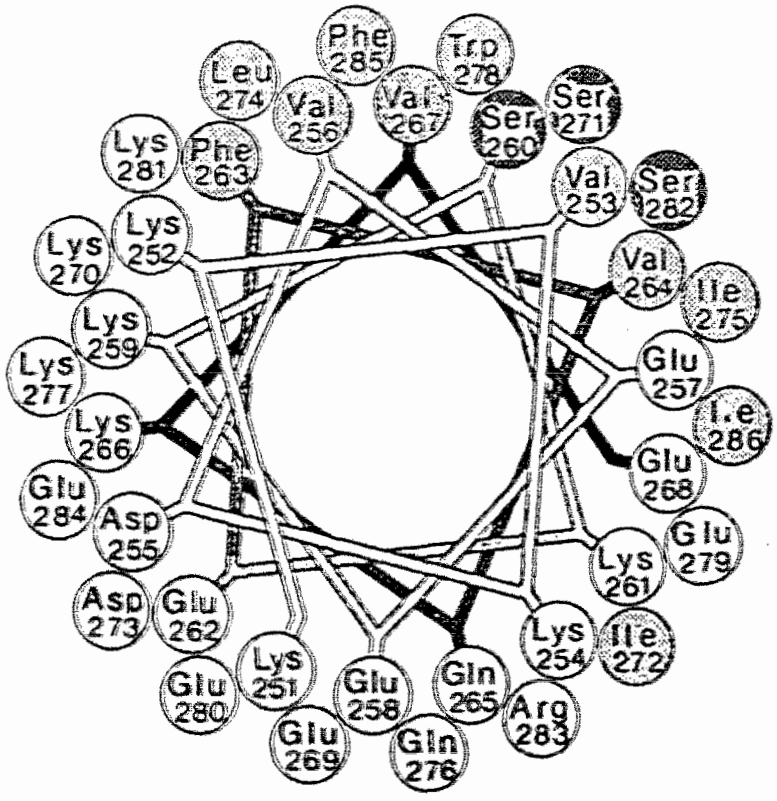


Figure 5: Helical wheel representation of the putative amphipathic helical region of Helix-1 between residues Lys-251 and Glu-268.

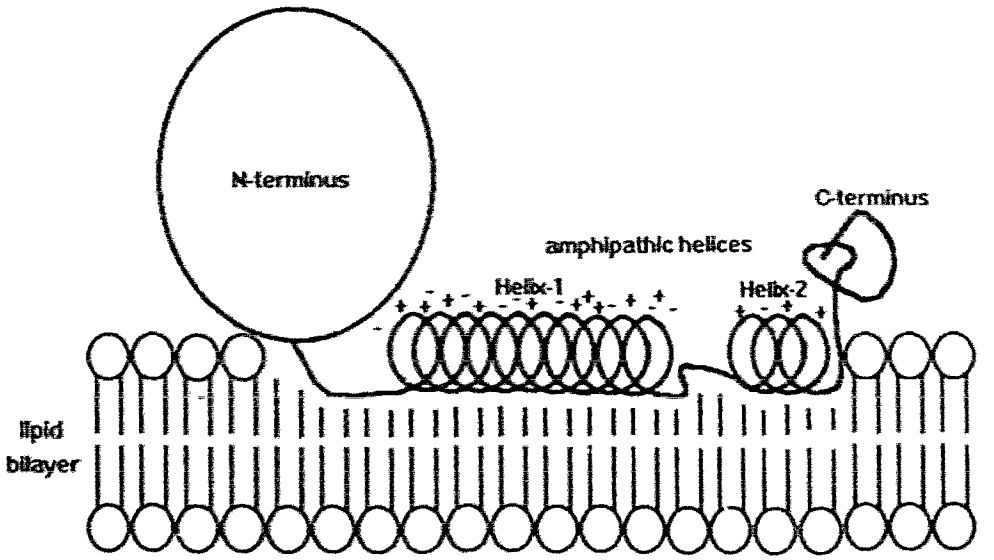


Figure 6: Model of teriary conformation and membrane interaction of rat CT based on secondary structural analysis.

1.3.4 Evidence for Membrane Binding Role of Amphipathic Helices

Amphipathic helices are very common secondary structures in most soluble and membrane-associated proteins. An extensive list of soluble proteins that interact with membranes and contain amphipathic α–helices has been compiled based on a detailed analysis of their physical, chemical and structural properties (Segrest *et al*,1990). There is much evidence, both direct and indirect, that these helices mediate interactions between polar and hydrophobic structures, including protein-membrane interactions and inter- and intramolecular protein-protein interactions.

X-ray analysis and CD studies on a number of small hormones and toxins (eg. β-endorphin, diphtheria toxin, cholera toxin and mellitin) are consistent with these

peptides interacting with hydrophobic surfaces via an amphipathic helix. X-ray crystal studies of bee venom mellitin have revealed that this peptide is an amphipathic α–helix, and associates as a tetramer in solution which shields its hydrophobic face. Integration of this helix into membranes is predicted to disrupt the membrane to such an extent that cell lysis occurs. There is X-ray crystallographic evidence for inter- and intramolecular protein-protein interactions involving amphipathic helices for such proteins as glucagon (Sasaki, 1975) and triacylglycerol lipase (Brzozowski, 1991). The amphipathic α–helix in triacylglycerol lipase is interesting in that it is part of a "lid" region which obscures the active site of the enzyme. The hydrophobic face of the helix is exposed to the hydrophobic active site and the polar face interacts with the external solvent. Upon activation the lid region shifts, exposing a large hydrophobic surface area to bind to triacylglycerol and the polar surface of the lid becomes associated with the external polar surface of the protein.

There is a precedent for amphipathic helices having an orientation parallel to the surface of a membrane bilayer, both in studies on apolipoproteins and synthetic peptides.

(i) Apolipoproteins: The interaction of CT with membranes is modeled on the apolipoproteins. These are the protein components of plasma lipoproteins and they provide a hydrophobic environment for transport of lipids in the bloodstream. Apolipoprotein A_1 and A_2 are capable of moving from one lipoprotein particle to another. All apolipoprotein A's contain from 1 to 13 copies of a 22-mer repeat, each repeat having the periodicity of an amphipathic α–helix, having a distinct polar and nonpolar face. At the interface between the polar and nonpolar faces there is a clustering of positively charged lysines and arginines with charged acidic residues running down the center of the helix similar to that

seen in CT. Each 22-mer is punctuated by a proline (Segrest *et al*, 1974). The apolipoprotein A's are believed to lie parallel to the surface of lipoproteins with their prolyl residues providing bends thus allowing the continuing α–helix to follow the curvature of the lipoprotein particle (Segrest *et al*, 1990).

(ii) Synthetic peptide studies: Chung *et al* (1992) used circular dichroism (CD), maximum fluorescence emission wavelength and fluorescence quenching to study the orientation of a synthetic 21-residue peptide in diphytanoylphosphatidylcholine (diPhy-PC) vesicles. This peptide associates with membranes and can be induced to form a transmembrane channel upon application of a voltage gradient. They synthesized seven 21-mers with the sequence (LSSLLSL)3. A tryptophan was substituted in each of the seven positions of the central hexamer and its fluorescence shift and quenching by CsCl were studied. The peptide was found to be a helix in both aqueous and hydrophobic environments. The average orientation of the peptide in the presence of lipid vesicles was with its helical axis parallel to the surface of the membrane and a few angstroms below the polar head groups. This would allow the polar serines to interact with the polar head groups of the diPhy-PC and the hydrophobic leucines to associate with the fatty acid chains of the phospholipids. Application of a transmembrane voltage caused the peptide to switch from a surface to a transmembrane orientation, forming a channel with other peptides.

1.3.5 CT Associates as a Dimer

Glycerol density gradient analysis of cytosolic CT revealed that it was dimeric (Weinhold *et al*, 1989). Rat CT runs at approximately 42 kDa on SDS-PAGE. A second minor band is also visible on gels at 84 kDa. Chemical cross-linking studies were used to determine whether purified rat liver CT was associated as a

dimer (Cornell, 1989). Chemical cross-linkers such as glutaraldehyde and the thiol-cleavable reagents dithiobis(succinimidyl propionate) and dimethyl-3,3'-dithiobispropionimidate covalently join proteins which are in close vicinity of each other (<12 Å; Middaugh, 1983).

Reaction of CT with any of these cross-linkers generated an 84 kDa band. Reduction of cross-linked CT with DTT and electrophoresis in the second dimension showed that the 84 kDa protein was a dimer of the 42 kDa protein. These results were obtained using purified CT bound to a Triton micelle or to a membrane vesicle. Cross-linking removed CT activity and DTT reduction partially reactivated the enzyme. From the study the author concluded that CT associated as a non-covalently bound dimer on membranes (Cornell, 1989).

1.4 PROTEOLYSIS AS A TOOL TO STUDY CT

Proteolysis has been chosen as a tool to study both the tertiary structure and the membrane-binding domain of rat CT. Proteases preferentially cleave proteins at hinge regions between structural domains as these sites are more accessible than sites buried within the protein. Thus protease fragmentation patterns can help to identify structural domains. In the same way proteolysis can be used to assess the degree of compactness of a protein; a more tightly folded domain will expose fewer cleavage sites and hence will be more resistant to proteolysis. Domains of integral membrane proteins which are buried within the membrane will be inaccessible to proteases.

Proteolysis has been widely used in studying membrane protein topology and in mapping functional domains on enzymes. By applying limited proteolysis to some enzymes, their various domains can be isolated and studied independently of each other, allowing identification of catalytic, regulatory and membrane-binding domains. In addition proteolysis has been used to identify conformational changes of proteins upon interaction with substrates and cofactors as well as membranes. Some examples of this technique are described below.

1.4.1 Skeletal muscle sodium channel - topology of a membrane-bound protein.

The tertiary structure of the α-subunit of rat skeletal muscle sodium channel (SkM-1) was probed with several proteases of widely varying specificities (Zwerling *et al*, 1991). Proteolytic fragments were identified using a number of antibodies directed against synthetic peptides from SkM-1. Limited proteolysis generated a highly reproducible temporal sequence of fragments. The C-terminus and regions linking a number of homologous repeats were found to be protease-sensitive whereas the homologous repeats themselves and the N-terminus were found to be protease-resistant. These results support the current structural model for SkM-1 which predicts the homologous repeats to span the membrane and are organised into four discrete domains. The regions linking the four domains are also protease-sensitive. The N-terminus, which has been shown to be cytoplasmic (Vassilev *et al*, 1988) is predicted to be compact and globular while the C-terminus (also cytoplasmic) is extended. These studies were performed in both membranes and detergent micelles and similar fragmentation patterns were found in both systems.

1.4.2 ADP/ATP carrier protein - topology of membrane-bound protein.

The ADP/ATP carrier protein is located on the inner mitochondrial membrane. The orientation of the protein in the membrane was investigated by determining its accessibility to various proteases using mitochondria devoid of outer membrane (mitoplasts) and inside out submitochondrial particles (Marty *et al*, 1992). During transport the carrier adopts two different conformations that can be trapped by two different inhibitors, carboxyatractyloside (CATR) and bongkrekic acid (BA). CATR and BA are able to bind to the outer face and the inner face of the carrier respectively. The ability of several proteases to cleave the carrier in its two different conformations and in its two different membrane orientations was determined by SDS-PAGE and immunoblotting. From these studies the authors proposed a model of the orientation of the ADP/ATP carrier protein in membranes.

1.4.3 Bovine factor Va - characterization of the membrane-binding domain using proteolysis.

Digestion of bovine factor Va with trypsin, chymotrypsin and elastase resulted in two protected proteolytic fragments which were sequenced by N-terminal analysis (Kalafatis *et al*, 1990). These fragments were found to lie on the light chain of factor Va, between residues ~1657-1791 (peptide 1) and ~1546-1656 (peptide 2). The precise location depended on the protease used. Only peptide 1 was able to bind to phospholipid vesicles. Factor Xa is an endogenous protease in the blood coagulation cascade and cleaves factor Va between residues 1765 and 1766 on the light chain producing an N-terminal fragment which has membrane-binding ability (Krishnaswamy and Mann, 1988). Knowing this the authors were able to map the membrane binding site to a region between residues 1667 and 1765.

1.4.4 Pyruvate oxidase - identification of lipid binding site.

Escherichia coli pyruvate oxidase is an amphipathic protein that is dramatically activated by lipids *in vitro*. The lipid-binding site was determined using chymotrypsin proteolysis (Recny *et al*,1985). In the presence of its substrate pyruvate and its cofactor thiamine pyrophosphate (TPP) pyruvate oxidase undergoes a conformational change, exposing a lipid binding site and a chymotryptic cleavage site. Binding to lipid results in activation of the oxidase. Pyruvate oxidase can be activated in the absence of lipids by limited proteolysis in the presence of pyruvate and TPP, which results in the removal of a C-terminal 2.6 kDa peptide. Further activation by lipid is not observed. Binding of pyruvate oxidase to lipids protects the 2.6 kDa cleavage site suggesting intercalation of this site into the membrane. Proteolysis in the absence of pyruvate and TPP results in cleavage at a different site and inactivation of the enzyme. Identical results were obtained using a C-terminal truncated protein produced by recombinant DNA techniques (Grabau and Cronan, 1986). Secondary structural predictions based on amino acid sequence analysis of the 2.6 kDa peptide suggest an amphipathic α–helix. These results implicate the C-terminus in lipid binding.

1.4.5 Tubulin - increased protease-resistance upon binding to membranes.

Soluble tubulin binds to dipalmitoylPC vesicles at the lipid phase transition (Kumar *et al*, 1981). The β subunit of tubulin is preferentially protected from trypsin digestion by vesicle interaction. Tryptophan fluorescence and CD studies suggest that tubulin has an altered conformation and a higher helical content in a lipid environment.

1.5 OBJECTIVES

The primary objective of this thesis was to determine the domain structure of rat CT, in particular the lipid-binding domain, using chymotrypsin proteolysis. Fragments from the proteolytic digestion were mapped using antibodies directed against peptides in the N- and C-terminus and the conserved central domain.

A lipid floatation assay was used to determine whether proteolytic fragments containing the putative amphipathic helix had membrane-binding capability. The potential for fragments to bind to several different lipid species was analysed. A second proteolytic agent, NTCB, was also used to identify the membrane-binding site. NTCB cleaves at cysteine residues and should produce a number of large fragments containing the putative amphipathic helix. These fragments were assessed for their ability to bind to membranes.

The proteolytic fragmentation pattern of soluble CT was compared to that of membrane-bound CT in an attempt to identify regions protected from proteolysis by the membrane, and also to detect any conformational changes which may occur upon membrane-binding. The interactions between CT and different lipids with varying activating potentials was compared.

I have attempted to elucidate the mechanism by which CT binds to membranes by first identifying the membrane-binding region and then by probing the nature of this interaction. This research should contribute to the vast effort to elucidate control of PC biosynthesis.

PART 2. MATERIALS AND METHODS

2.1 MATERIALS

COS-1 cells (Glutzman, 1981) and the pAX111 expression vector (Kay, 1991) were gifts from Dr. Robert Kay (University of British Columbia). Diacylglycerol was generated by digestion of egg PC with phospholipase C as described (Weinhold *et al*, 1991). All other phospholipids were from Avanti Polar Lipids except the [methyl-^{3}H]DPPC which was from DuPont-New England Nuclear. Tissue culture supplies were from Corning. Fetal calf serum was from GIBCO/Life Technologies. Electrophoresis reagents were from Bio-Rad. PVDF was from Immobilon. SGPB and subtilisin were gifts from Dr. Thor Borgford (Simon Fraser University). Chymotrypsin, trypsin and proteinase K were from Sigma. The antibody directed against the conserved central domain of CT was a gift from Drs. Harris Jamil and Dennis Vance (University of Alberta). The antibodies directed against the N- and C-terminus of CT were generously donated by Dr. Claudia Kent (University of Michigan). Goat anti-rabbit horseradish peroxidase and diaminobenzidine were from Sigma. Tris was from ICN. [^{3}H]Phosphocholine was synthesized from [^{3}H]choline (DuPont-NEN) as described (Cornell, 1989). Counting scintillant was from Amersham. Other chemicals were from Sigma or BDH.

2.2 BUFFERS, REAGENTS AND MEDIA

2.2.1 COS Cell Transfection

TD: 25 mM Tris-HCl, 0.5 mM Na_2HPO_4, 140 mM NaCl, 5 mM KCl, pH 7.5.

TS: TD + 1 mM $MgCl_2$, 1 mM $CaCl_2$

DME: 3.75 g $NaHCO_3$, 1 package Dulbecco's Modified Eagle Medium in 1litre pH 7.1.

DME/FCS: DME + 5% FCS.

DEAE-Dextran: 1 mg/ml

Trypsin solution: 0.05% trypsin, 0.53 mM EDTA, in PBS.

2.2.2 CT isolation

Homogenization buffer: 10 mM Tris-HCl, 1 mM EDTA, 3 mM $MgCl_2$, 1 mM PMSF, 2 mM DTT, pH 7.4.

Homogenization buffer + PO_4: homogenization buffer + 0.2 M K_2HPO_4, pH 7.4.

Phosphate-buffered saline (PBS): 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, 3 mM KCl,150 mM NaCl, pH 7.4.

PBS/EDTA: 2.5 mM EDTA in PBS.

Dialysis buffer: 10 mM Tris-HCl, 2 mM DTT, 0.01% (W/V) SDS, 1 mM PMSF, 2mM DTT, pH 7.4.

2.2.3 CT Assay

Carrier: CDPcholine, 10 mg/ml; phosphocholine, 60 mg/ml.

2.2.4 Protein Assay

Dye reagent: 10 mg Coomassie Blue, 5 ml 95% ethanol, 10 ml H_3PO_4 in 100 ml dH_2O.

2.2.5 SDS-PAGE

Separating gel buffer (4X conc.): 1.5 M Tris-HCl, 0.4% SDS, pH 8.7.

Stacking gel buffer (4X conc): 0.5 M Tris-HCl, 0.4% SDS, pH 6.8.

Sample buffer: 0.13 M Tris-HCl, 8% SDS, 2% β-mercaptoethanol, 20% glycerol, Bromophenol Blue, 0.1 mg/ml, pH 6.8.

Reservoir buffer (5X conc): 0.125 M Tris-HCl, 0.96 M glycine, 0.5% SDS.

Acrylamide stock: 30% acrylamide, 0.8% bis-acrylamide.

2.2.6 Silver Staining

Fixer: 40% methanol, 10% acetic acid.

2.2.7 Western Blots

Tris-buffered saline (TBS): 20 mM Tris-HCl, 150 mM NaCl, pH 7.4.

Transfer buffer: 39 mM Glycine, 48 mM Tris-HCl, 0.0375% SDS, pH 8.7.

Blocking buffer: 3% FCS in TBS.

Washing buffer: 0.1% FCS in TBS.

Anti-sera buffer: 1% FCS, 0.05% Tween-20 in TBS.

DAB buffer: 45 mM Tris-HCl, 0.15% $NiCl_2$.

Stripping buffer: 62.5 mM Tris-HCl, 100 mM βME, 2% SDS, pH 6.7.

2.3 METHODS

2.3.1 COS Cell transfection

COS cells were transfected with pAX111 expression vector containing full length rat liver CT cDNA (Kalmar), using the method of Hammarskjold (1986). COS cells were grown on 10 cm plates in DME/FCS at 37°C, 5% CO_2. Cells were

subcultured 24 h prior to transfection. Solutions were warmed to room temperature. Media was removed by aspiration and washed with 10 ml TS, then 10 ml TD. DNA/DEAE-Dextran (1 µg DNA/ml DEAE-Dextran) was added and cells were left at room temperature for 10 min and then at 37°C, 5% CO_2 for 40 min. After incubation DNA/DEAE-Dextran was aspirated and 4 ml TS/20% glycerol was added for 2 min. TS/20% glycerol was aspirated off and cells were washed with 10 ml TS then 10 ml DME. Chloroquine diphosphate (10 ml of 0.2 mM solution) was added in DME/FCS and cells were incubated for 2.5 h at 37°C, 5% CO_2. Chloroquine was aspirated off and 10 ml DME/FCS was added and cells were incubated at 37°C, 5% CO_2. Cells were transferred to fresh buffer after 24 h to 15 cm plates and CT was harvested 64-68 h after DNA was added.

2.3.2 Subculturing COS cells

Solutions were warmed to room temperature. Media was aspirated and cells were washed with 10 ml PBS. Trypsin solution (1 ml) was added for approx. 2 min and then 1 ml DME/FCS was added. Cells were rinsed from the plate with a flame-polished Pasteur pipet, added to 20 ml DME/FCS in a 15 cm plate and incubated at 37°C, 5% CO_2.

2.3.3 Harvesting Rat CT From COS Cells

Cells from 15 cm plates were harvested 64-68 h after transfection. The following steps were carried out at room temperature. Media was aspirated and cells were washed 3 times with 5 ml PBS. PBS/EDTA (5 ml) was added and cells were incubated for 5 min at 37 °C to loosen cells. Cells were rinsed from plates with another 5 ml of PBS using a flame-polished Pasteur pipet. Cells were pelleted at 1000 *g* for 5 minutes at room temperature and washed with 1 ml

homogenization buffer. The following steps were carried out at 4°C. Cells were homogenized in 1 ml of homogenization buffer with 32 strokes of a Teflon grinder. K_2HPO_4 (pH 7.4) was added from a 2 M stock in 5 equal portions, vortexing after each addition, to a final concentration of 0.2 M. The homogenate was centrifuged at 3000x *g* for 4 minutes. The supernatant was centrifuged at 100 000x *g* for 1 hour. The supernatant of this high speed spin was aliquoted and stored at -70°C and thawed only once. Samples of the supernatant were assayed for protein concentration using the Bradford assay (Section 2.2.4) and for CT activity (Section 2.2.3) in the presence of 0.5 mM PC/oleic acid (1:1) vesicles. CT was digested at various protein-to-chymotrypsin ratios (300:1 to 100:1) and analysed by SDS-PAGE and immunoblotting to determine the optimum chymotrypsin concentration which would generate all of the proteolytic fragments within 10 minutes.

2.3.4 Purification of CT from Rat Liver Cytosol

The purification scheme for rat liver CT is described in Weinhold *et al* (1986) with modifications (Cornell, 1989).

2.3.5 CT Assay

The reaction mixture contained 50 mM Tris-HCl, pH 7.4, 0.1 M NaCl, 10 mM $MgCl_2$, 3 mM CTP, 1.5 mM , PC/oleic acid vesicles (0.25 mM/0.25 mM) and 5 µg enzyme sample in a total volume of 50 µl. Samples were assayed in duplicate. [^{3}H]phosphocholine was added to start the reaction and samples were incubated in a 37 °C shaking water bath and stopped after 30 min with 30 µl methanol/ammonia, 9:1 (V:V). Samples were vortexed and centrifuged at 3000 *g* for 30 sec. Carrier (12.5 µl) was spotted on Merck plastic silica plates followed by 30 µl of sample. Plates were developed in methanol/0.6% NaCl/ammonia

(5:5:1, V:V:V), dried and sprayed with 0.02% dichlorofluorescein. CDPcholine bands were visualized by UV (254 nm) and scraped into plastic mini-vials containing 0.5 ml H_2O. Counting scintillant (4.5 ml) was added to the samples and they were counted for 2 min in a Beckman Model LS600 Liquid Scintillation Counter.

2.3.6 Preparation of Small Unilamellar Vesicles

Lipids in chloroform stocks were dried in a 5 ml round bottom flask on a rotary evaporator. The dried film was resuspended in homogenization buffer and sonicated with a Branson sonicator at 0-4^{o}C with a fine titanium probe at 80% output until the turbid solution had clarified (approx. 5 minutes). The sonicated vesicles were centrifuged at 15 000 rpm for 1 minute to pellet titanium and any residual multilamellar vesicles. PC/DG and dilaurylPC suspensions were sonicated and centrifuged at room temperature. Lipids for floatation assays were spiked with radiolabeled lipid. [Methyl-^{3}H]DPPC (1 µCi) from a toluene:methanol (1:1) stock was added to unlabeled lipid prior to evaporation and sonication.

2.3.7 Protein Assay

The protein content of COS cell extract was assayed using the method of Bradford (1976). A standard curve was prepared using 1-100 µg ovalbumin. Samples were made to 100 µl with H_2O, 3 ml protein dye reagent was added and samples were incubated at 37 ^{o}C for 15 min. Absorbance was determined at 595 nm.

2.3.8 SDS-PAGE

(i) Gel preparation: Proteins were separated on a 12% polyacrylamide gel using the method of Laemmli (1970). The gels were prepared by the following method: Separating buffer was diluted to a 1X conc with H_2O. Acrylamide solution was added to 12%, and then 0.1% TEMED (V/V) and 0.05% ammonium persulfate (W/V) were added. This solution was poured into a custom-built gel apparatus and carefully overlaid with 1 ml of reservoir buffer. The gel was allowed to polymerize (15 min). Stacking buffer was diluted to 1X conc in H_2O. Acrylamide solution was added to a final concentration of 4% and then TEMED and ammonium persulfate were added (to final concentrations of 0.1% and 0.05% respectively) just before pouring. The reservoir buffer was washed off the separating gel and the stacking gel solution was poured over the separating gel then a 10-tooth comb was inserted in the top to form 10 wells. The stacking gel was allowed to polymerize (20 min) and the comb was removed and the wells were washed out with H_2O.

(ii) Sample preparation: Samples, including Bio-Rad prestained protein standards, were concentrated in a Savant Speed Evaporator and boiled for 5 minutes in Sample Buffer before loading them on the gels. Reservoir buffer was added to the gel apparatus so that it was in contact with both the top and the bottom of the gel. Gels were run at constant voltage (200 V) until the dye front reached the bottom of the gel.

2.3.9 Silver Staining

Gels were soaked 3 times for 20 min in Fixer, 3 times for 20 min in 10% EtOH/5% acetic acid, 3 times for 20 min in H_2O, and for 30 min in 0.012 M silver nitrate with gentle agitation. Gels were rinsed in H_2O and developed in 0.28 M Na_2CO_3, 0.05% formaldehyde. Once bands were visible the reaction was

stopped with 2.3 M citric acid, rinsed in H_2O, soaked in reservoir buffer containing 50% glycerol, and dried on a Bio-Rad Model 583 Gel Dryer.

2.3.10. Western Blots (Immunoblotting)

After separating proteins by SDS-PAGE, bands were visualized using the method of Towbin *et al* (1979).

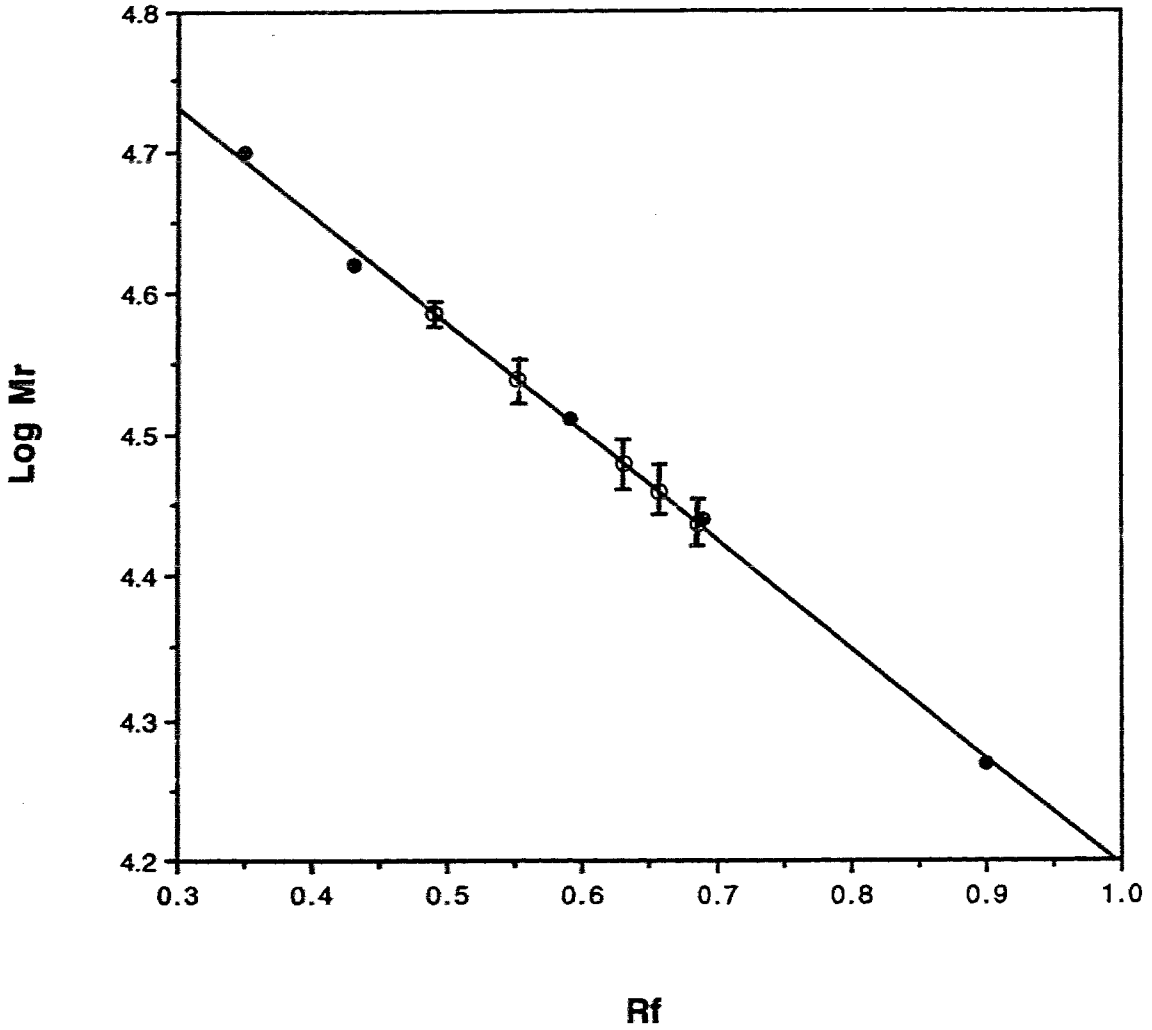
(i) Transfer: The protein bands on gels were transferred to PVDF membrane immediately after electrophoresis using an LKB 2117 Multiphor II Electrophoresis Unit. The graphite electrodes, the PVDF filters and the filter paper were all soaked in transfer buffer for 15 min prior to transfer. The PVDF membrane was first wetted with methanol, rinsed with water and then soaked in transfer buffer. Once electrophoresis was complete the gel was laid on the PVDF filter on the anode, with two pieces of filter paper on top and two underneath. The cathode was carefully placed on top of the stack. Proteins were transferred at 1.2 mA/cm^2 for 1.5 to 2 hours.

(ii) Blotting: After transfer the PVDF filters were soaked in blocking buffer for 1 hour at room temperature with gentle agitation or at 4^oC overnight. The filters were washed for 30 min in washing buffer and then incubated at room temperature in antisera buffer with a 1/1000 dilution of anti-CT antibody for two hours. The wash step was repeated and the filters were then incubated at a 1/1000 dilution of goat anti-rabbit horseradish peroxidase for 1 1/2 hours. After a final wash the filters were developed with 1.6 mM diaminobenzidine in DAB buffer with 0.03% hydrogen peroxide. The reaction was stopped with a large excess of water. The molecular weight of each fragment was determined by plotting the R_f's vs. log M_r of prestained standards and is considered accurate to within 1 kDa.

(iii) Stripping and reblotting: In some cases, immunoblotted filters were stripped of antibody by incubation at 60°C in stripping buffer until bands were no longer visible (1 to 3 hours). Stripped filters were blotted with new antibodies as described above.

2.3.11 Determination of the Relative Molecular Mass of the Proteolytic Fragments

Bio-Rad prestained standards (Coomassie-labeled) were run on every SDS gel and the relative mobility (R_f) of each standard was plotted against the log of the apparent molecular masses (log M_r) to obtain a standard curve. The R_fs were calculated by taking the ratio of the distance of migration of each protein (measured from the stacking gel/separating gel interface to the protein) to the distance of migration of the dye front (measured from the stacking gel/separating gel interface to the dye front). The standards were phosphorylase B, M_r=106 kDa; bovine serum albumin, M_r=80 kDa; ovalbumin, M_r=49.5 kDa; carbonic anhydrase, M_r=32.5 kDa; soybean trypsin inhibitor, M_r=27.5 kDa and lysozyme, M_r=18.5. The M_r of undigested CT and the proteolytic fragments was obtained by measuring the R_fs and interpolating from the standard curve. A representative standard curve is shown in Figure 7 along with the M_r determinations for the chymotryptic fragments averaged from three Western blots. M_r generally fall within 1 kDa of the M_r for which the chymotryptic fragments are named, ie. 39, 35, 30, 28 and 26 kDa.



● standards

o chymotryptic fragments

Log M_r	M_r (kDa)	Corresponds to:
4.59	39.2	39
4.54	34.7	35
4.48	30.1	30
4.46	28.6	28
4.43	27.1	26

Figure 7: Determination of the Relative Molecular Mass of the Chymotryptic Fragments.

2.3.12 Chymotrypsin Digestion

Chymotrypsin diluted in homogenization buffer + phosphate was added to COS cell cytosolic extract at a mass ratio of 200:1 (mg cytosolic protein:mg chymotrypsin) unless otherwise specified. When chymotrypsin digestion was done in the presence of lipid the COS cell extract was pre-incubated with lipid at 37°C for 5 min. The sample was incubated in a shaking 37°C water bath. Aliquots were removed at specific time intervals and stopped with 1.6 mM PMSF (100 mM PMSF in Me_2SO diluted to 1.6 mM PMSF in homogenization buffer + phosphate or H_2O) giving a final concentration of 1 mM PMSF. The samples were incubated 1 minute further at 37°C and were then concentrated in a Savant Speed-Vac Evaporator. The proteins were separated by SDS-PAGE at 200 V and then immunoblotted. An alternative to varying the time of chymotrypsin digestion was to vary the concentration of chymotrypsin and to maintain a constant digestion time of 10 minutes.

2.3.13 CT Assay of Chymotrypsin Digestion Products

CT was digested with chymotrypsin at a mass ratio of 400:1 as described. Aliquots were removed at various time points from 0 to 20 min and quenched with PMSF in homogenization buffer + phosphate. Protein (5 μg) from each time point was assayed for CT activity in duplicate and the remainder was concentrated and analysed by SDS-PAGE and immunoblotting.

2.3.14 NTCB Digestion

CT from COS cell extract was denatured in 8 M urea for 30 min at 37°C. NTCB (100 mM, pH 7.4) was added to COS cell extract to a final concentration of 25 mM NTCB. The sample was incubated in darkness at 37°C for 2 h. An alternative to urea denaturation was to digest CT for longer periods - 48 to 72 h -

at 37°C. The reaction was stopped with 1% β-mercaptoethanol and the sample was dialysed in dialysis buffer at room temperature overnight, until most of the orange colour was removed. The sample was concentrated in a Savant Speed Evaporator and analysed by SDS-PAGE and immunoblotting. Samples which were analysed for lipid binding ability were loaded directly on a sucrose density gradient and dialysed after centrifugation.

2.3.15 Lipid Floatation of Undigested CT - Sucrose Density Gradient Centrifugation

The lipid floatation assay was adapted from Cornell and Vance (1987b). COS cell extract was incubated with [^{3}H]DPPC-labeled vesicles for 5 minutes at 37°C in a 2 ml Beckman centrifuge tube (Figure 8). Dry sucrose was added to 50% (W/V) (ρ=1.23 g/ml) and the solution was gently vortexed to dissolve the sucrose. The mixture was carefully overlaid with a 13% (W/V) (ρ=1.06 g/ml) sucrose solution in homogenization buffer +phosphate (with fresh 0.5 mM PMSF and 1 mM DTT). The gradients were centrifuged in a TLS55 swinging bucket rotor in a Beckman TL80 Ultracentrifuge at 45 000 rpm for 14 to 20 hours at 15°C. The gradients were then divided into four or five equal fractions and collected from the top of the tube with an elongated Pasteur pipet. An aliquot from each fraction was analysed for ^{3}H by liquid scintillation counting. The remainder of each fraction was then dialysed overnight with 200 volumes of dialysis buffer and concentrated prior to analysis by SDS-PAGE and immunoblotting (Figure 8).

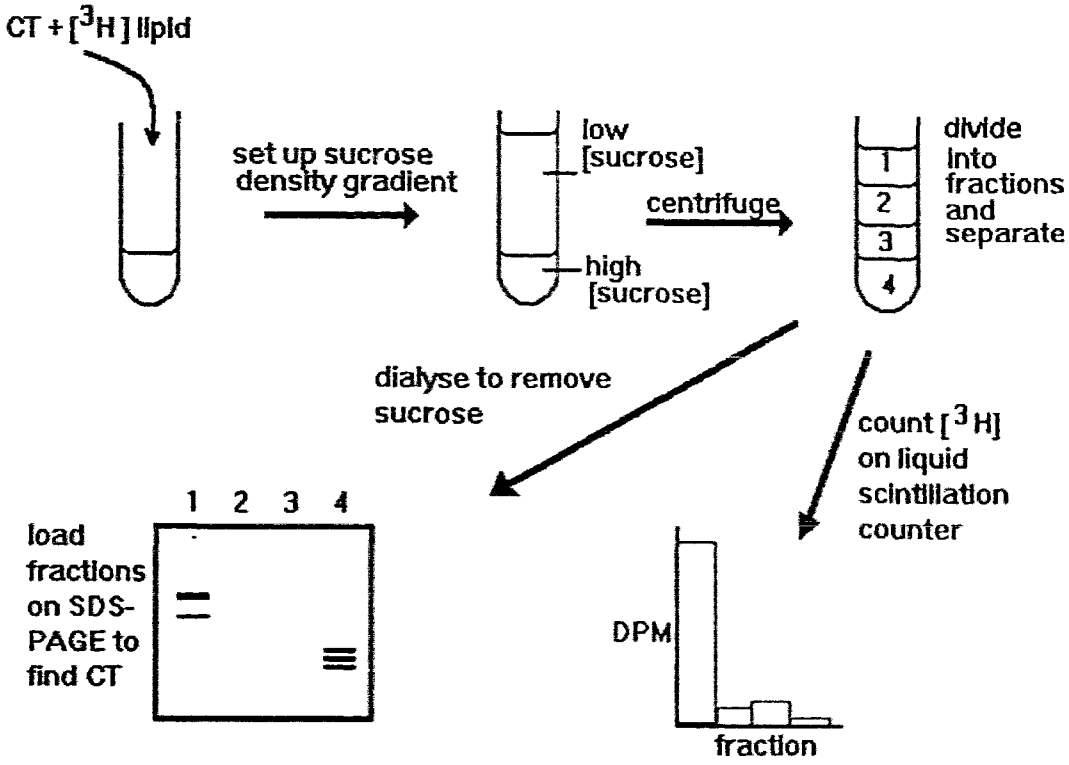


Figure 8: Lipid floatation assay.

2.3.16 Lipid Floatation of Partially Digested CT

COS cell extract was incubated with [^{3}H]DPPC-labeled vesicles for 5 minutes at 37^{o}C in a 2 ml Beckman centrifuge tube and then digested with chymotrypsin at ratio of 200:1 at 37^{o}C. Aliquots were removed at various time points, quenched with 1.6 mM PMSF (final concentration, 1 mM PMSF), and loaded on a sucrose density gradient as described above.

PART 3. RESULTS

3.1 PROTEOLYSIS

3.1.1 Limited Digestion of CT with *Streptomyces griseus* protease B, Trypsin, Subtilisin and Chymotrypsin.

The molecular weight of rat CT, calculated from the amino acid sequence, is 41.7 kDa (Kalmar *et al*, 1990). CT migrates at approximately 42 kDa on SDS-PAGE. Often this band is resolved into a doublet which represents two different phosphorylation states in the cell (Watkins and Kent 1991). One or two bands are also seen at approximately 84 kDa representing the CT dimer (Cornell 1989) (Figure 9, Lane 1). This dimer persists even when 8 M urea is present in the Sample buffer. CT was digested with a variety of proteases having different specificities, in an attempt to gain insight into its tertiary structure. Limited digestion of purified rat liver CT with *Streptomyces griseus* protease B (SGPB), trypsin, subtilisin and chymotrypsin resulted in the production of one to three fragments which migrated on an SDS gel between 25 and 30 kDa (Figure 9, Lanes 2-5) as detected by Silver Staining (Section 2.3.9). CT was digested with SGPB for 16 h at a protein:protease mass ratio of 10:1. Two bands were produced, at 28 and 26 kDa (Lane 2). CT was digested with trypsin for 10 min at a mass ratio of 10:1, producing two bands at 26 and 25 kDa (Lane 3). Digestion with subtilisin for 10 min at a mass ratio of 5:1 resulted in one band at 26.5 kDa (Lane 4). The 27.5 kDa band is subtilisin. Chymotrypsin digestion for 10 min at a mass ratio of 12:1 produced 2 bands at 27 and 26 kDa (Lane 5). It appears that there is a region in CT where protease cleavage sites are highly susceptible to attack, producing fragments of 25 to 30 kDa. From these results we speculated that CT is comprised of a protease-resistant domain composed of

approximately two thirds of the protein (~26 kDa) and a protease-sensitive domain of approximately one third of the protein.

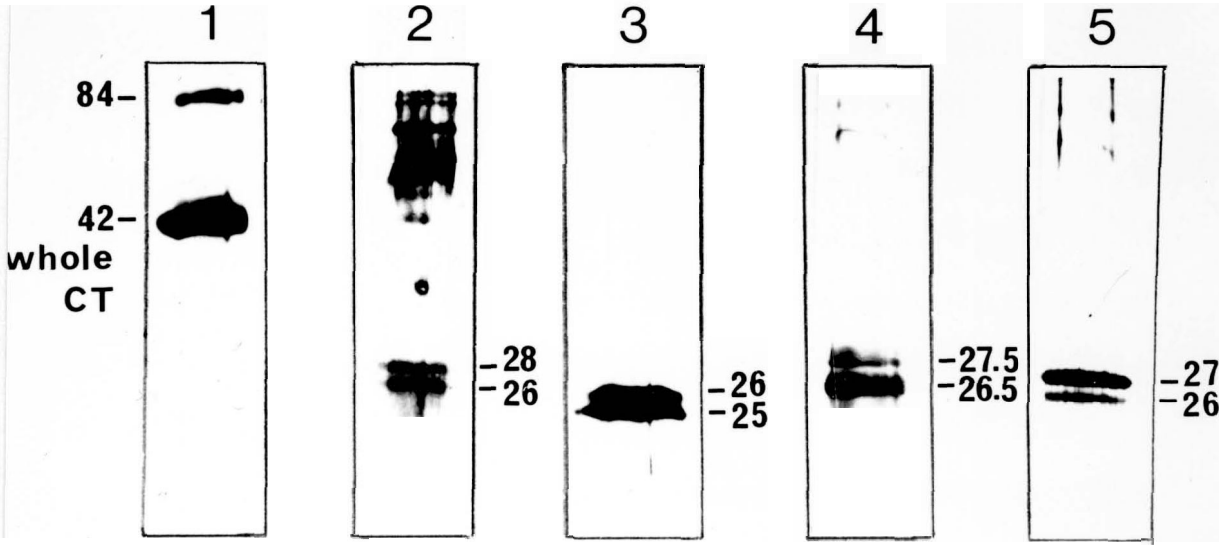


Figure 9. Limited Digestion of Purified Rat Liver CT with SGPB, Trypsin, Subtilisin and Chymotrypsin. 2.4 μg purified rat liver CT was incubated with the following mass ratios of CT:protease: SGPB, 10:1; trypsin, 10:1: subtilisin, 5:1; and chymotrypsin 12:1. Samples were digested with trypsin, subtilisin or chymotrypsin for 10 min at 37°C or with SGPB for 16 h on ice. Digestion reactions were quenched with 1 mM PMSF. Samples were analysed by SDS-PAGE and Silver Staining (Sections 2.3.8 and 2.3.10). Lane 1, 1 μg undigested CT; Lane 2, SGPB digest; Lane 3, trypsin digest, Lane 4, subtilisin digest; Lane 5, chymotrypsin digest. Numbers to the right of each lane represent the M_r of each band in kDa as determined from a plot of Log M_r of prestained standards vs. the R_f on SDS-PAGE and are accurate to within 1 kDa. The 27.5 kDa band in Lane 4 is subtilisin and not a digestion product of CT.

3.1.2 Chymotrypsin Fragmentation Pattern

The pattern of protease digestion was analysed by varying the time of digestion or the concentration of protease. Several proteolytic intermediates were observed by this analysis. Chymotrypsin digestion was chosen to further investigate the domain structure of CT because it was commercially available, inexpensive, it was completely inactivated by PMSF, and it migrated well below the chymotryptic fragments of CT on an SDS gel.

(i) Purified rat liver CT: A Western Blot of undigested purified rat liver CT is shown in Figure 10A, Lane 1, showing the 42 kDa monomer and the 84 kDa dimer. A typical profile of the fragmentation pattern of purified rat liver CT partially digested with chymotrypsin is shown in Figure 10A, Lane 2. CT was digested with chymotrypsin at a protein:chymotrypsin mass ratio of 50:1 for 10 min. Fragments of the following molecular weights were reproducibly observed: 39, 35, 30, 28 and 26 kDa, along with the 42 kDa undigested CT. Bands also appeared at approximately 70 kDa and 60 kDa; these bands are dimers of the chymotryptic fragments. Weak bands at 39 and 35 kDa can occasionally be seen in undigested CT due to digestion by endogenous proteases. CT bands were detected with affinity purified antibody directed against CT, as well as by Coomassie and silver stain.

(ii) CT from COS cell extract: COS cells were transfected with rat liver CT cDNA resulting in a 30-fold overexpression of CT. Analysis by SDS-PAGE and immunoblotting revealed a 42 kDa monomer and an 84 kDa dimer (Figure 10B, Lane 2), as is seen with purified rat liver CT. When the cytosolic extract from COS cells was partially digested with chymotrypsin at a mass ratio of 200:1 five fragments were generated (Figure 10B, Lane 1) identical to those of purified CT. None of these bands were apparent when extracts from COS cells transfected with the vector alone were digested and blotted (data not shown). COS cells

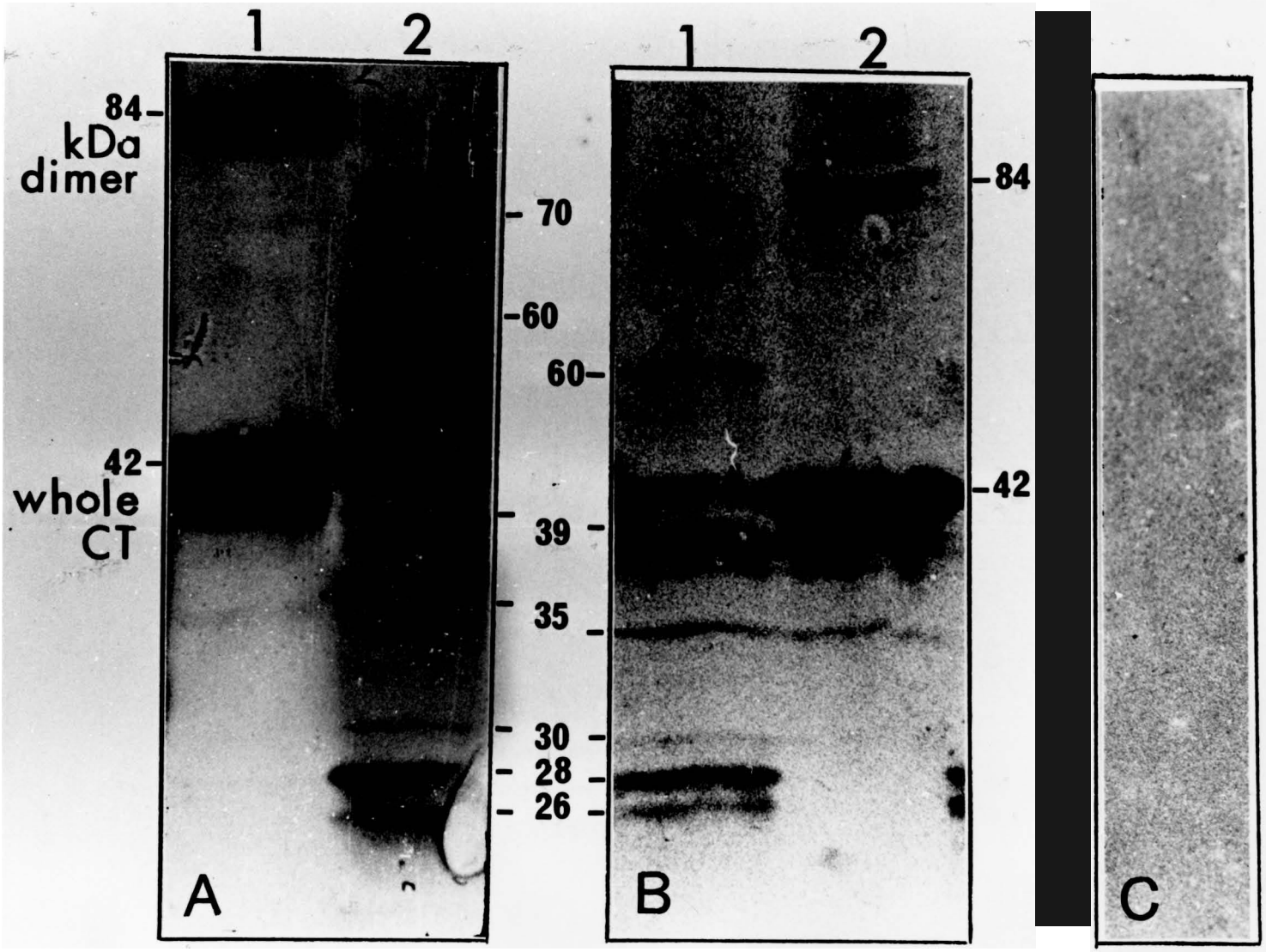
provide a more concentrated source of CT than does the rat liver preparation; thus COS cell extract was utilized for all the remaining experiments described in this thesis.

(iii) Urea-denatured CT: When purified rat liver CT was denatured with 6 M urea prior to chymotrypsin digestion, complete digestion was observed (Figure 10C). This suggests that in native CT fragments of 39, 35, 30 28 and 26 kDa were resistant to chymotrypsin due to the tertiary structure of the enzyme.

3.1.3 Time Course of Chymotrypsin Digestion

A time course for the chymotryptic digestion of COS cell CT is shown in Figure 11A. CT and the chymotryptic fragments were detected on a Western Blot using an antibody against a peptide in the conserved central domain of CT, anti-P1 (Section 2.3.10). Molecular weight estimations are considered accurate to within 1 kDa. At 1 min the 39 and 35 kDa fragments were visible along with faint bands at 30 and 28 kDa. At 2 min these bands were intensified. At 5 min most of the whole CT and the 30 kDa fragment had been digested to smaller fragments. The 28 kDa band had increased in intensity and a 26 kDa band had appeared. At 10 min only the 39, 28 and 26 kDa fragment were still visible. After 20 min no bands could be detected by the anti-P1 antibody. These results suggest the following progression of digestion: 42 → 39 → 35 → 30 → 28 → 26 kDa. Given that CT has at least 50 potential sites for chymotrypsin cleavage it appears that many of these sites within each fragment are inaccessible to proteolysis.

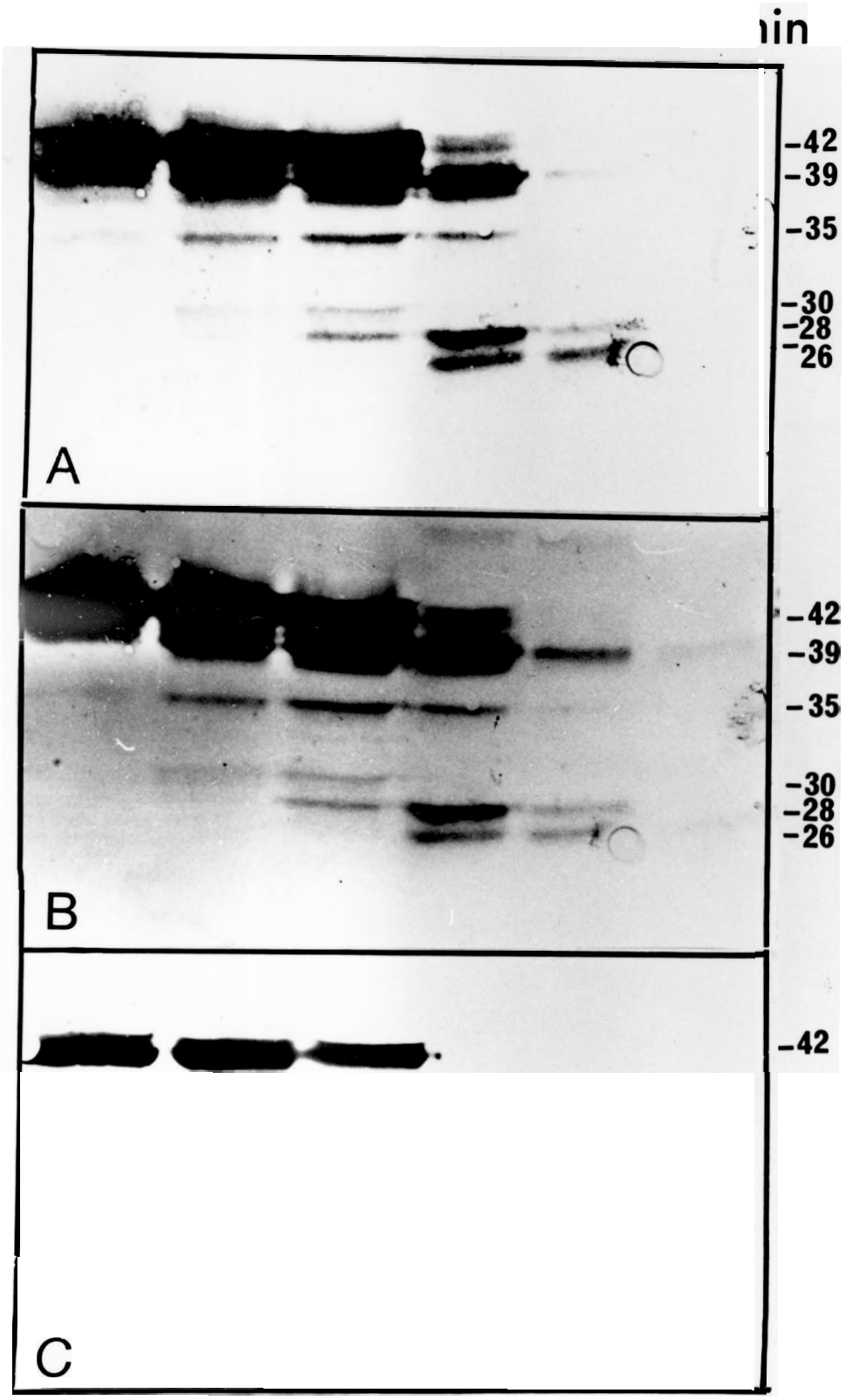
Figure 10: Chymotrypsin fragmentation pattern. (A) 7.5 µg purified rat liver CT was digested at 37°C for 0 min (Lane 1) and 10 min (Lane 2) at a protein to chymotrypsin ratio of 50:1. (B) 45 µg CT from from COS cell extract was digested for 0 min (Lane 2) and for 10 min (Lane 1) at a protein to chymotrypsin ratio of 200:1. (C) Purified rat liver CT was denatured in 6 M urea for 30 min at 30°C and then digested for 10 min at a protein to chymotrypsin ratio of 300:1. The quenched reactions were analysed by SDS-PAGE and immunoblotting with anti-P1 antibody as described in Sections 2.3.8 and 2.3.10.


1
2
84
kDa
dimer
42
whole
CT
70
60
39
35
30
28
26
A
1
2
60
84
42
B
C

3.1.4 Antibody Mapping of the Chymotryptic Fragments

Based on secondary structural predictions we suspected that the protease-resistant fragments contained the N-terminus. To test this we stripped the anti-P1 antibody from the PVDF filter shown in Figure 11A and reblotted the filter with antibody raised against an N-terminal peptide of CT (anti-N, Figure 11B) (Section 2.3.10). A pattern very similar to that from the anti-P1 antibody emerged indicating that each of the chymotryptic fragments contained the N-terminal region of CT. To confirm that the fragments did not contain any of the C-terminus the anti-N antibody was stripped from the filter, which was reblotted with antibody directed against a peptide in the C-terminus of CT (anti-C). As expected only whole CT was detected by the anti-C antibody (Fig 11C). These findings provided evidence that the chymotryptic fragments were N-terminal. From the molecular weight estimations and the specificity of chymotrypsin (Phe, Tyr, Trp, Met and Leu) the most likely cleavage sites for the five prominent chymotryptic fragments have been proposed in Figure 12 and are illustrated on the CT model in Figure 13.

Figure 11. Antibody mapping of the chymotryptic fragments. 225 µg of CT from COS cell extract was digested at a protein to chymotrypsin ratio of 200:1. Aliquots were removed at the indicated time points and quenched with 1 mM PMSF. (A) Samples were analysed by SDS-PAGE and immunoblotting with the anti-P1 antibody. (B) The filter from *A* was stripped and reblotted with the anti-N antibody as described in Section 2.3.10. (C) The filter from *B* was again stripped and reblotted, this time with the anti-C antibody.


-42
-39
-35
-30
-28
-26
A
-42
-39
-35
-30
-28
-26
B
-42
C

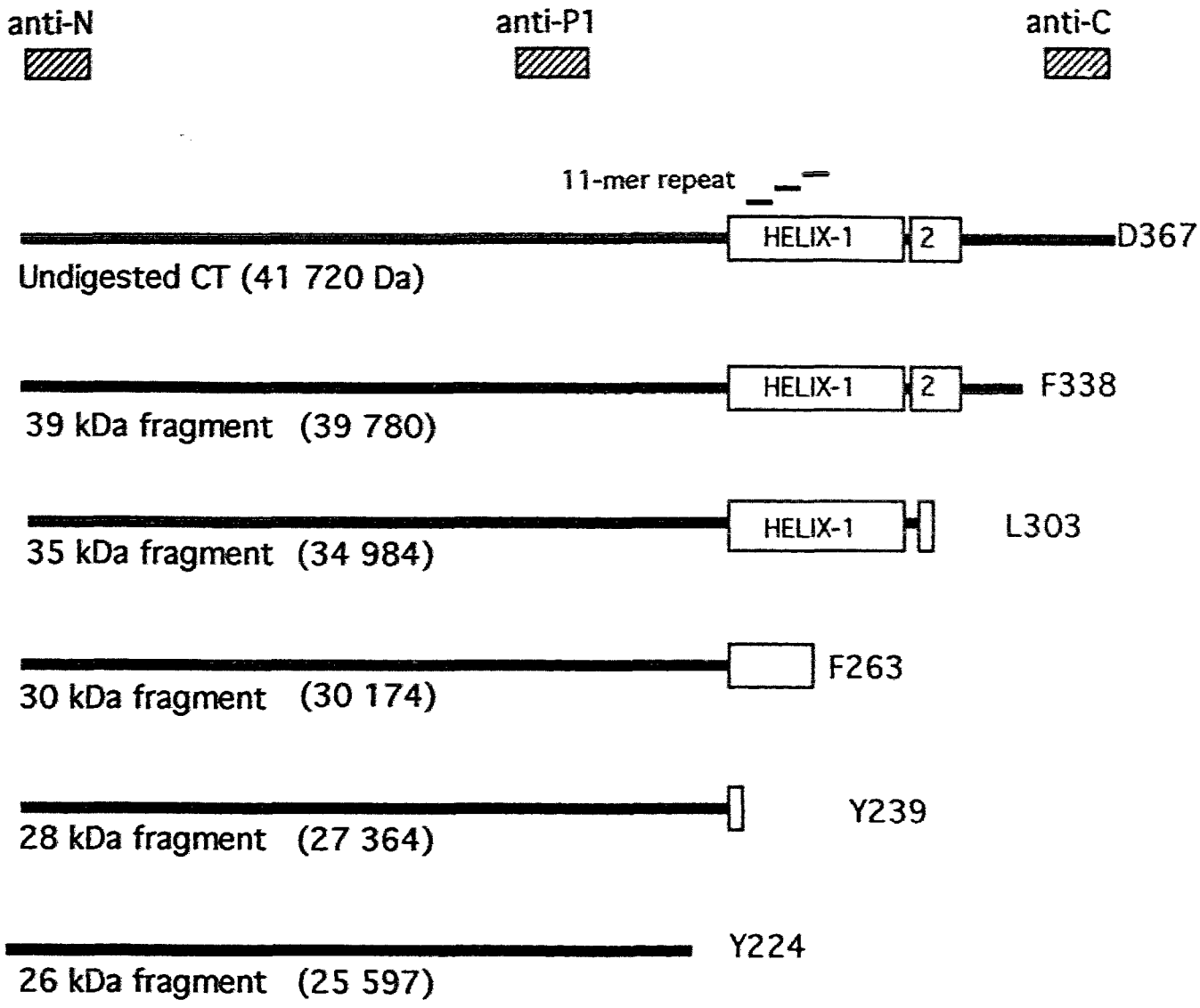


Figure 12: Proposed sites for chymptrypsin digestion of CT. The proposed sites for chymotrypsin cleavage are those sites C-terminal to Phe, Tyr, Trp, Met and Leu which generate theoretical fragments with molecular weights closest to that of the fragments determined by SDS-PAGE.

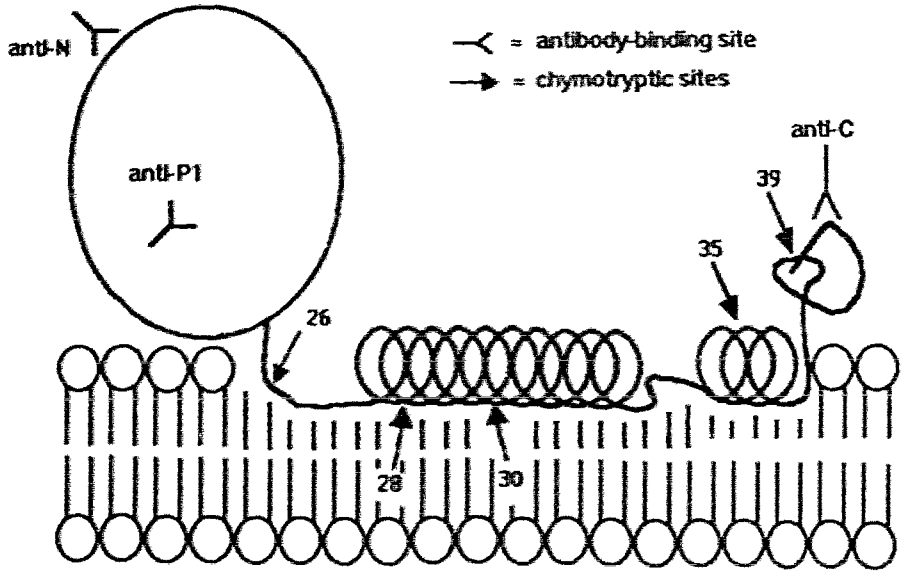


Figure 13: Proposed chymotryptic cleavage sites indicated on the CT model.

3.1.5 Activity of the Chymotryptic Fragments

CT from COS cell extract was digested with chymotrypsin for various time intervals over a 10 min period. The activity at each time point was determined and is shown in Figure 14A, along with the chymotryptic digestion profile detected by the anti-C and the anti-P1 antibodies (14B). As whole CT was digested to its smaller fragments the activity of the sample gradually decreased. Undigested CT had a specific activity of 33.2 ± 0.9 units/mg. After chymotrypsin digestion for 2 minutes the 39 kDa band predominated and whole CT was barely detectable by either the anti-P1 or anti-C terminal antibody. At this time point the activity had dropped to 4.8 units/mg, about 15% of the original activity. After chymotrypsin digestion for 2.5 min there was still an intense 39 kDa band but no whole CT remained. The activity was just 1.8 units/mg or 6% of the original

activity. These results show that a substantial loss of activity occurs when as few as 30 amino acids are removed from the C-terminus.

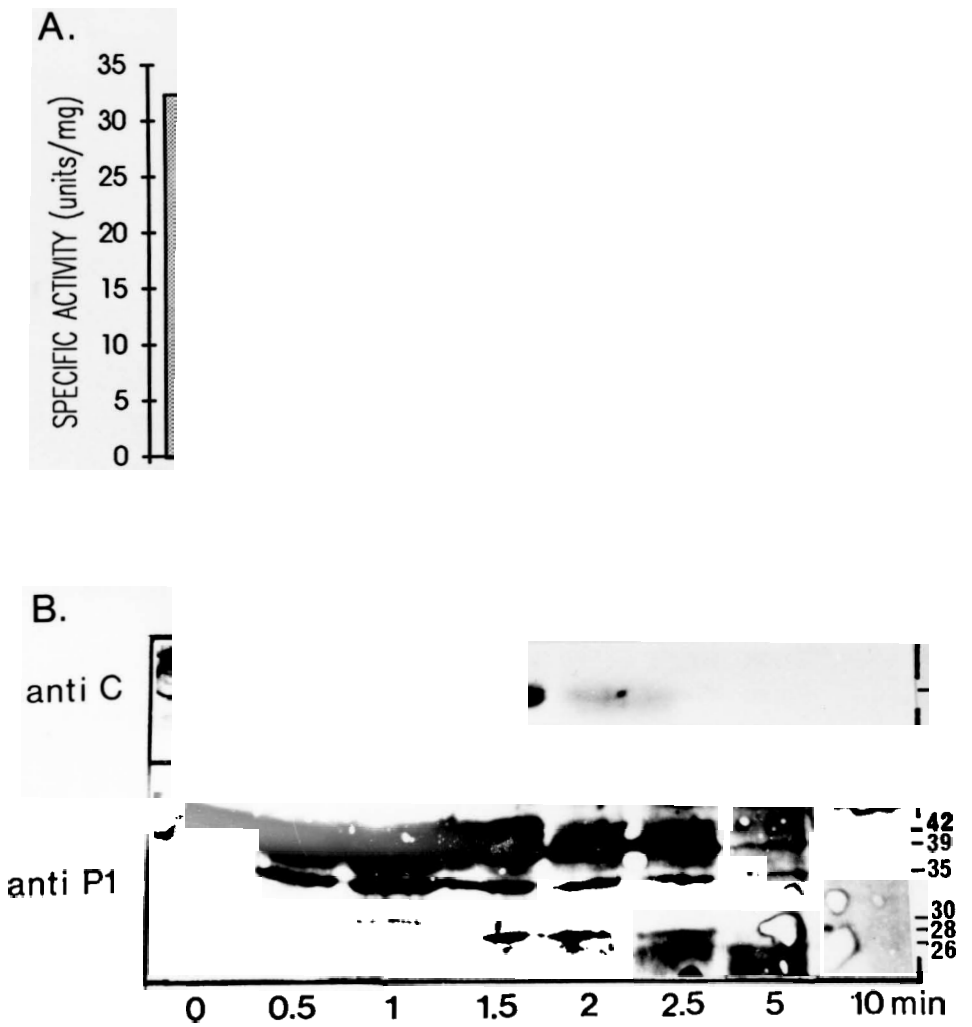


Figure 14. Activity of chymotryptic fragments. 500 µg of CT from COS cell extract was digested at a protein to chymotrypsin ratio of 400:1. Aliquots were removed at the indicated time points and quenched with 1 mM PMSF. 5 µg of each aliquot was assayed in duplicate and the remainder was concentrated in a Savant Speed Evaporator and analysed by SDS-PAGE and immunoblotting. The immunoblot filters were first blotted with the anti-P1 antibody then stripped and reblotted with the anti-C terminal antibody. For the 0 time point, chymotrypsin was added directly to the PMSF. (A) Activity of samples at given time points (1 unit = 1 nmol CDPcholine/min). (B) Western Blot of samples at given time points as detected with the anti-C terminal antibody and the anti-P1 antibody.

3.2 LIPID BINDING

3.2.1 Activation of CT by PC/oleic acid, PC/PG, PC/DG, dilaurylPC and PC/sphingosine

The activity of CT in the COS cell cytosolic extract was assayed in the presence of 0.5 mM PC/oleic acid (1:1), PC/PG (1:1), PC/DG (3:1), dilaurylPC and PC/sphingosine (1:1). The results are shown in Table I. PC/oleic acid and PC/PG vesicles were strong CT activators. The PC/DG (3:1) vesicles were weaker activators and dilaurylPC did not activate CT. PC/sphingosine was inhibitory. These data were consistent with previously published results (Cornell 1991a and b, Weinhold *et al* 1986, Feldman and Weinhold, 1987, Johnson *et al* 1992, and Sohal and Cornell 1990).

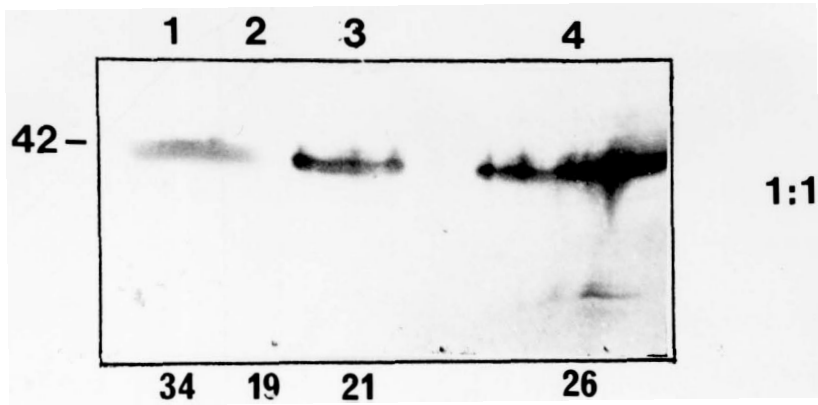
LIPID (0.5 mM)	SPECIFIC ACTIVITY (units/mg protein)
no lipid	33 ± 8
PC/oleic acid (1:1)	139 ± 47
PC/PG (1:1)	151 ± 47
PC/DG (3:1)	80 ± 23
dilaurylPC	36 ±1
PC/sphingosine (1:1)	22 ± 5

Table I: Activation of CT by PC/oleic acid, PC/PG, PC/DG, dilaurylPC and PC/sphingosine vesicles. 5 µg of COS cell extract was assayed in duplicate in the presence of 0.5 mM lipid vesicles as described in Materials and Methods. Data are duplicated determinations. Experiments were repeated with similar results.

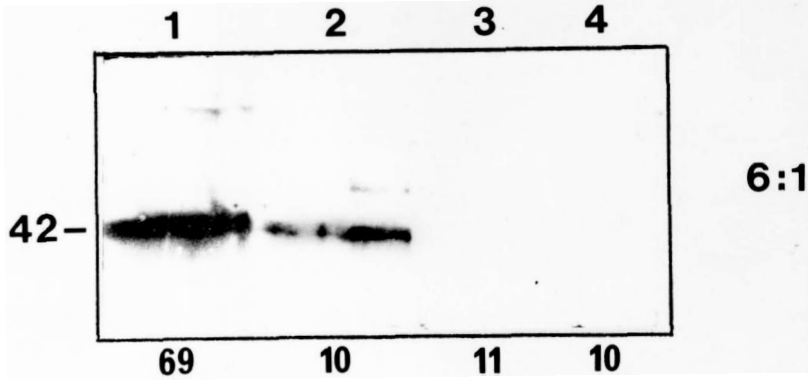
3.2.2 Ability of Undigested CT to Bind to PC/Oleic Acid Vesicles

PC/oleic acid vesicles are strong CT activators. The ability of CT to bind to these vesicles was determined using a lipid floatation assay. CT from COS cell extract was pre-incubated with radiolabeled PC/oleic acid (1:1) vesicles at various lipid-to-protein mass ratios: 1:1, 6:1, and 16:1. The radiolabel was 0.1 μCi of [^{3}H-methyl]DPPC. The samples were centrifuged overnight in a sucrose density gradient and each fraction was dialysed and analysed by SDS-PAGE and immunoblotting (Sections 2.3.8, 2.3.9 and 2.3.14). The majority of the lipid and any lipid-associated protein floated due to their low density whereas unbound protein remained in the bottom fraction. The goal was to float all of the vesicle-bound CT to the top fraction of the sucrose gradient. This was achieved only at the highest lipid:protein mass ratio tested: 16:1 (13 mM PC/oleic acid, Figure 15). When lower lipid-to-protein ratios were used the protein-lipid vesicle complexes were found throughout the gradient. This is presumably due to a higher protein-to-vesicle ratio, resulting in a higher density of each complex. Figure 15 demonstrates the capacity of intact CT to bind to PC/oleic acid vesicles.

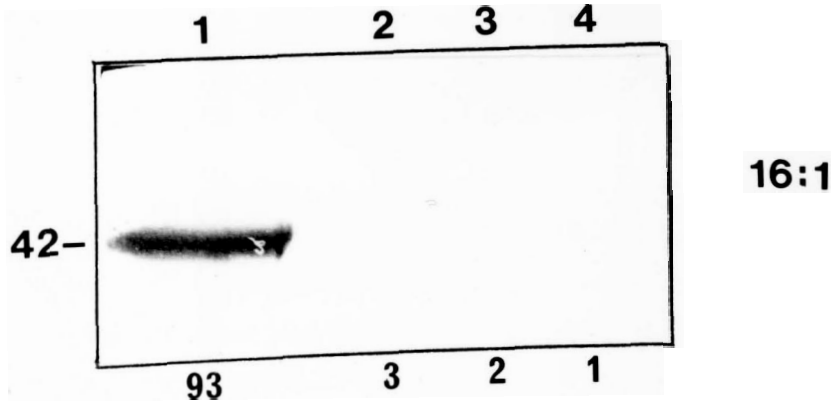
Figure 15. Binding of undigested CT to PC/oleic acid vesicles. 135 μg of CT from COS cell extract was incubated with 0.1 μCi [methyl-^{3}H]DPPC-labeled PC/oleic acid vesicles in the following concentrations for 5 minutes at 37^{o}C: (A) 1 mM; (B) 5 mM; or (C) 13 mM. CT bound to lipid was separated from unbound CT by sucrose density gradient centrifugation (Section 2.3.14) and the fractions were analysed by SDS-PAGE and immunoblotting with the anti-P1 antibody. Lanes 1 to 4 represent fractions 1 to 4 respectively, with fraction 1 being the top fraction and fraction 4 being the bottom fraction. The % of radiolabeled lipid in each fraction is shown at the bottom of each lane. Ratios to the right of each Western Blot represent the approximate lipid:protein mass ratios.



A.



B.



C.

3.2.3 Ability of Undigested CT to Bind to PC/Oleic acid, PC/PG, PC/DG, dilaurylPC and PC/Sphingosine

Several other lipids with varying CT-activating potentials were tested for their ability to bind CT. Unbound CT was separated from CT bound to lipid vesicles by floatation in a sucrose density gradient as described in Section 2.3.14. A high lipid-to-protein ratio was chosen - 20:1 - to enable vesicles with bound protein to float to the top fraction. In Figure 16 virtually all of the CT was found in the top fraction with >90% of the lipid when CT was incubated with radiolabeled PC/oleic acid, PC/PG or PC/sphingosine vesicles (16A,B and E). When PC/DG and dilaurylPC vesicles were used only 50% of the CT was seen in the top fraction, even though more than 92% of the lipid floated to the top (16C and D), indicating a weaker affinity of CT for these vesicles. The lower affinity of CT for PC/DG and dilaurylPC was also apparent when using lower lipid-to-protein ratios. The proportion of CT in the top fraction was reduced at 4 mM and 1 mM PC/DG and dilaurylPC compared to PC/oleic acid, PC/PG and PC/sphingosine (data not shown). In the absence of lipid CT remained in the bottom fraction. It is clear that CT is capable of binding to a variety of lipids in varying degrees *in vitro*. CT binds with a higher affinity to strong activators than to weak activators and binds tightly to the inhibitory lipid PC/sphingosine as well.

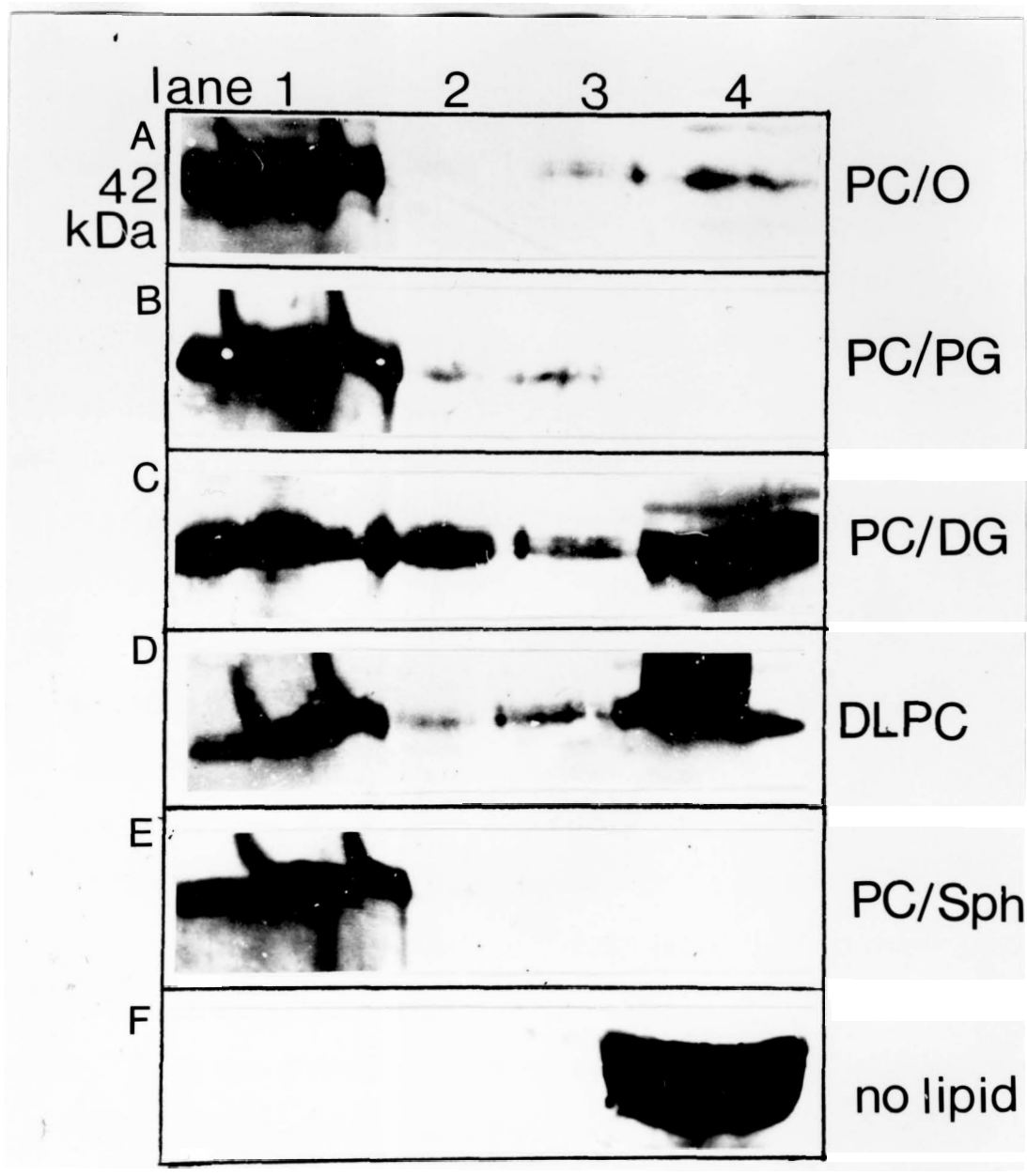
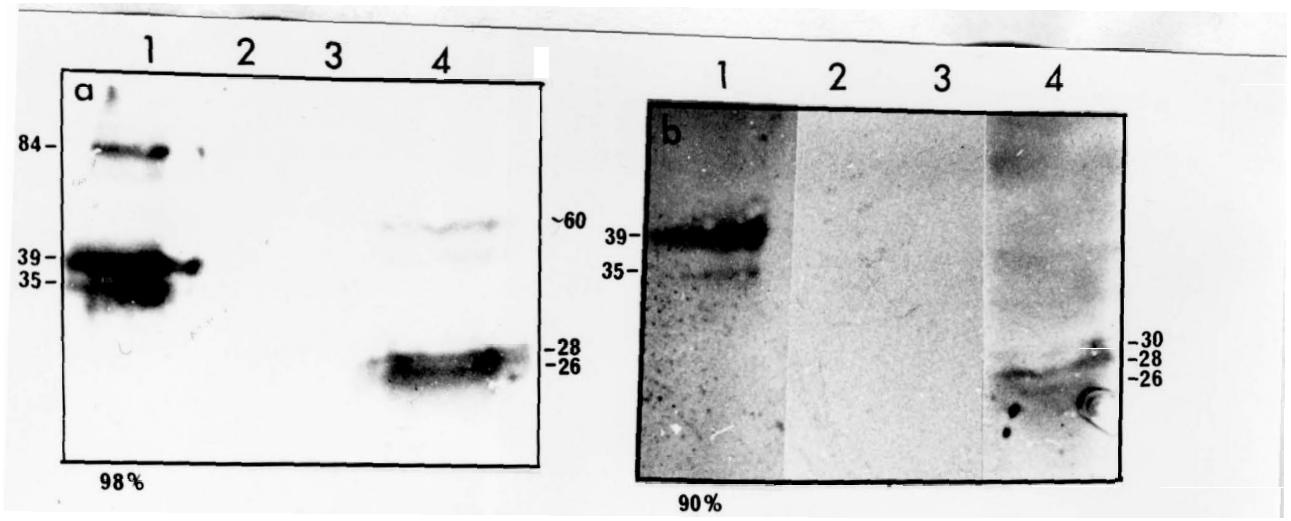


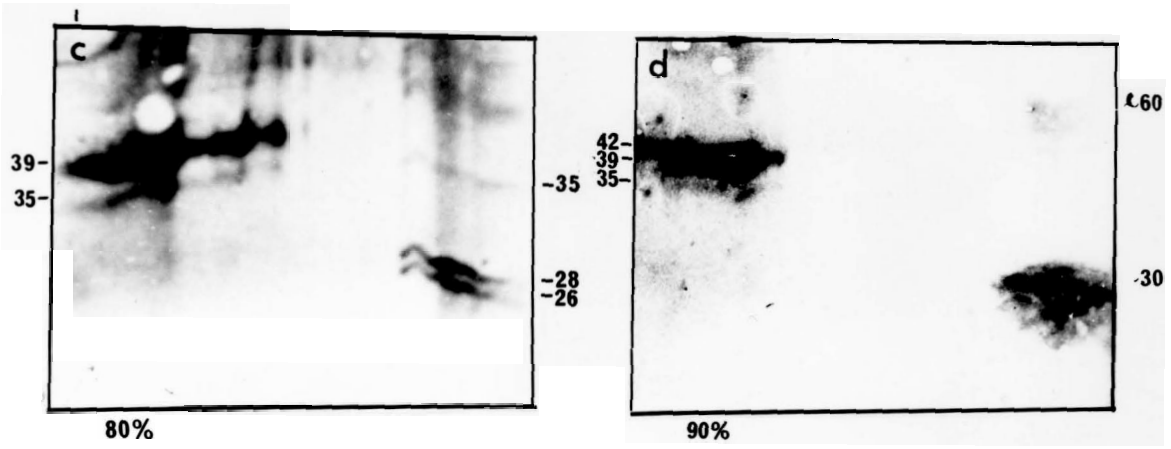
Figure 16. Binding of undigested CT to lipid vesicles. 33 µg of CT from COS cell extract was incubated with each of the following for 5 minutes at 37°C: (A) PC/oleic acid vesicles (1:1); (B) PC/PG (1:1); (C) PC/DG (3:1); (D) dilaurylPC; (E) PC/sphingosine (1:1) or (F) homogenization buffer + phosphate. Lipid vesicles were radiolabeled with 0.1 µCi [methyl-^{3}H]DPPC. The final lipid concentration for *A* to *E* was 15 mM. CT bound to lipid was separated from unbound CT by sucrose density gradient centrifugation and the fractions were analysed by SDS-PAGE and immunoblotting with the anti-C antibody. Lanes 1 to 4 represent fractions 1 to 4 respectively. The % of radiolabeled lipid found in the top fraction for each gradient is as follows: PC/O, 96%; PC/PG, 89%; PC/DG, 93%; dilaurylPC, 98%; PC/sphingosine, 96%.

3.2.4 Ability of the Chymotryptic Fragments to Bind to Lipid Vesicles

The ability of the chymotryptic fragments to bind to various lipid vesicles was assessed using floatation in a sucrose density gradient. Vesicles were composed of PC/oleic acid, PC/PG, PC/DG, dilaurylPC and PC/sphingosine. CT was pre-incubated with radiolabeled lipid vesicles and then partially digested with chymotrypsin and centrifuged in a sucrose density gradient (Section 2.3.15). Fractions were dialysed to remove the sucrose and analysed by SDS-PAGE and immunoblotting. The 39 and 35 kDa fragments floated to the top fraction along with the majority of the lipid in all cases (Figure 17a-e). The 30, 28 and 26 kDa fragments did not bind to PC/oleic acid, PC/PG, PC/DG or dilaurylPC (Figure 17a-d). In the absence of lipid all the proteolytic fragments remained in the bottom fraction (Figure 17f). These findings are consistent with our hypothesis that CT binds to membranes via its amphipathic helix since the 39 and 35 kDa fragments retain this portion and the 26-30 kDa fragments have lost some or all of this region. The diffuse band at ~60 kDa in Figures 17a and c represents dimerization of the 26-30 kDa fragments. As the fragments have not lost their ability to dimerize, the N-terminal two-thirds of CT must contain the dimerization domain. The 30, 28 and 26 kDa fragments *did bind* to PC/sphingosine (Figure 17e) which is suggestive of CT interacting with PC/sphingosine via an N-terminal domain rather than exclusively via the amphipathic helix.

Figure 17. Ability of the chymotryptic fragments to bind to lipid vesicles. 460 μg of CT from COS cell extract was incubated with the each of the following for 5 minutes at 37°C: (a) PC/oleic acid (1:1) vesicles; (b) PC/PG (1:1); (c) PC/DG (3:1); (d) dilaurylPC; (e) PC/sphingosine (1:1) vesicles; f) homogenization buffer + phosphate. Lipid vesicles were radiolabeled with 0.1 μCi [methyl-^{3}H]DPPC. The final lipid concentration for *a* to *e* was 4 mM. The samples were digested with chymotrypsin at a protein to chymotrypsin ratio of 200:1. Aliquots of each sample were removed at 1, 3 and 6 minutes, quenched with 1 mM PMSF and then combined to give a range of chymotryptic fragments. Fragments bound to lipid were separated from unbound fragments by sucrose density gradient centrifugation and the fractions were dialysed and analysed by SDS-PAGE and immunoblotting with the anti-P1. Lanes 1 to 4 represent Fractions 1 to 4 respectively. The % of radiolabeled lipid found in the top fraction is indicated under Lane 1 of each Western Blot.


1 2 3 4
a
84-
39-
35-
~60
-28
-26
98%
1 2 3 4
b
39-
35-
-30
-28
-26
90%


c
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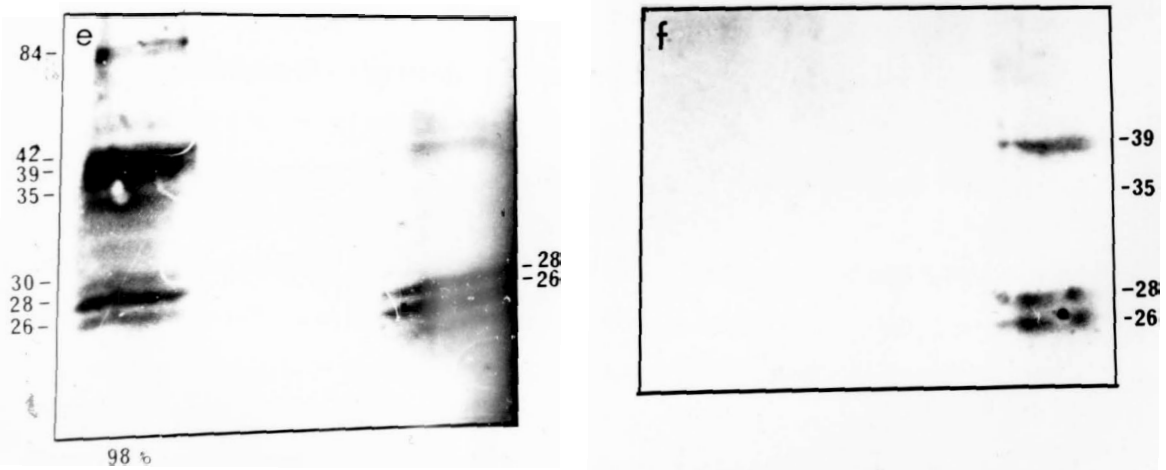
35-
-35
-28
-26
80%
d
42-
39-
35-
~60
-30
90%


e
84-
42-
39-
35-
30-
28-
26-
-28
-26
98 %
f
-39
-35
-28
-26

3.3 NTCB DIGESTION

3.3.1 NTCB Digestion Pattern

Another proteolytic agent, 2-nitro-5-thiocyanobenzoic acid (NTCB), was used to fragment CT. NTCB cleaves peptide bounds at cysteine residues and can generate a number of CT fragments, all of which will contain the intact amphipathic helices. CT was digested by NTCB and the membrane-binding ability of the fragments was analysed.

CT from COS cell extract was urea-denatured and partially digested in NTCB (Section 2.3.13). The sample was analysed by SDS-PAGE and immunoblotting (Figure 18). Lane 1 of Figure 18 shows the 42 kDa undigested CT. Lane 2 shows the NTCB digestion pattern detected by an antibody directed against whole CT (anti-CT). Seven fragments were generated, which migrated at approximately 42, 41, 40, 36, 33, 30 and 26 kDa on a 15% SDS gel. The molecular weight estimations are considered accurate to within 1 kDa. The Western Blot was stripped and reblotted with the anti-C terminal antibody (Figure 18, Lane 3). This antibody detected only 3 bands, the 36, 33 and 30 kDa bands. The weaker bands seen in Lane 3 are bands that were detected by the anti-CT antibody and were not completely stripped from the filter.

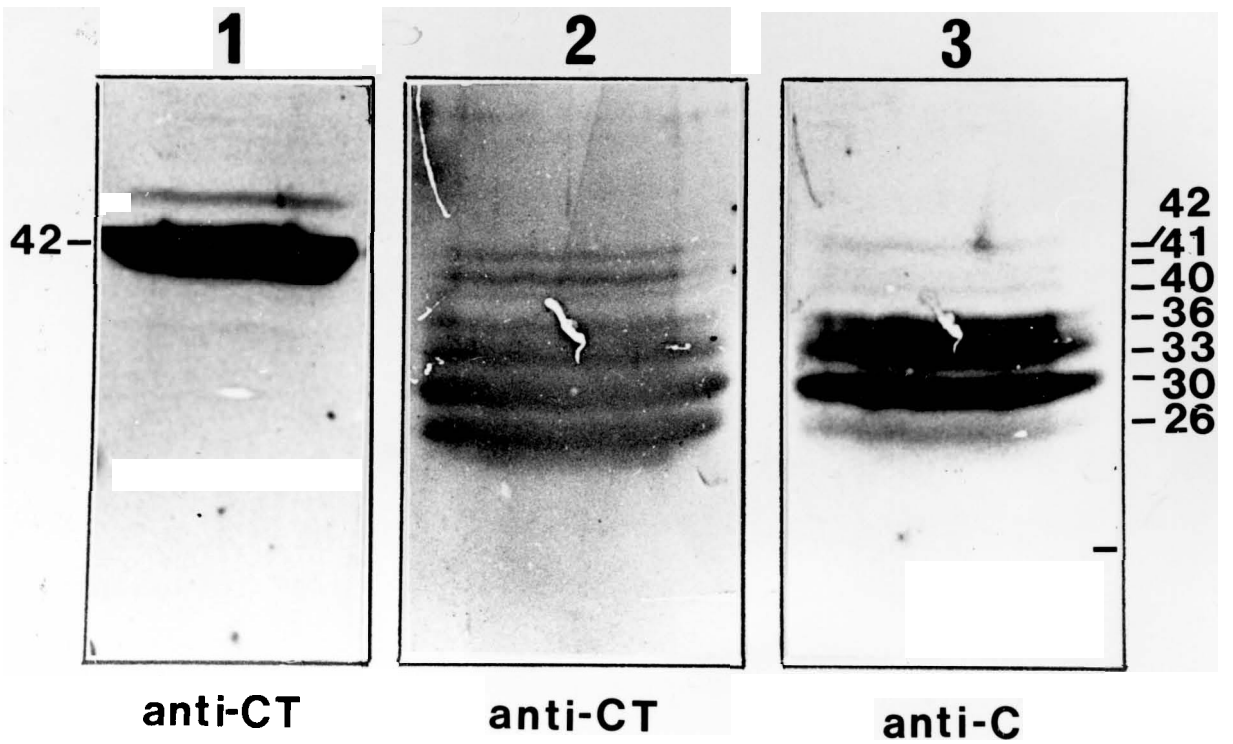


Figure 18: NTCB digestion pattern. 87 μg of CT from COS cell extract was denatured in 8 M urea, 2 mM DTT, pH 8.5 for 2 h at 37°C. NTCB was added to a final concentration of 25 mM, pH 8.5 and the sample was incubated at room temperature for 15 min, then at 37°C for 2 h. The digestion was quenched with 1% β-mercaptoethanol (V/V), dialysed in 400 volumes of dialysis buffer for 16 h and analysed by SDS-PAGE and immunoblotting. NTCB-digested CT was detected with a polyclonal antibody against whole CT and is shown in Lane 2. The blot was stripped and reblotted with anti-C antibody and is shown in Lane 3. Lane 1 shows urea-denatured CT which was treated the same as the NTCB-digested sample, but substituting homogenization buffer + phosphate for NTCB. It was detected with antibody against whole CT.

NTCB cleaves peptide bonds at the N-terminal side of cysteine residues. CT has 7 cysteines; 5 in the N-terminal region and 2 very close to the C-terminus. Figure 19 indicates schematically the location of the cysteine residues on the amino acid sequence of CT, and indicates the possibilities for generation of fragments by NTCB. Cleavage at the C-terminal cysteines would result in fragments slightly smaller than whole CT. These fragments should be detected on a Western Blot by antibodies directed against whole CT (anti-CT), the conserved central domain (anti-P1) and the N-terminal domain (anti-N) but not by the anti-C terminal antibody. Cleavage at the N-terminal cysteines would theoretically produce five distinct fragments between 38 and 26 kDa. These fragments should be detected by the anti-CT, anti-P1 and anti-C terminal antibodies but not by the anti-N terminal antibody. Subsequent cleavage of these fragments at the C-terminal cysteines would decrease the molecular weight of each fragment by 1 to 2 kDa, prohibiting detection by the anti-C terminal antibody. Complete digestion of CT with NTCB should result in one central fragment, between residues 140 and 354, which should run at approximately 25 kDa on an SDS gel, and several peptides too small to be detected by SDS-PAGE. This central fragment, as well as any larger fragments, encompass the putative amphipathic α-helix. If the amphipathic α-helix is in fact the membrane-binding domain, then these fragments should have the capacity to bind to membranes.

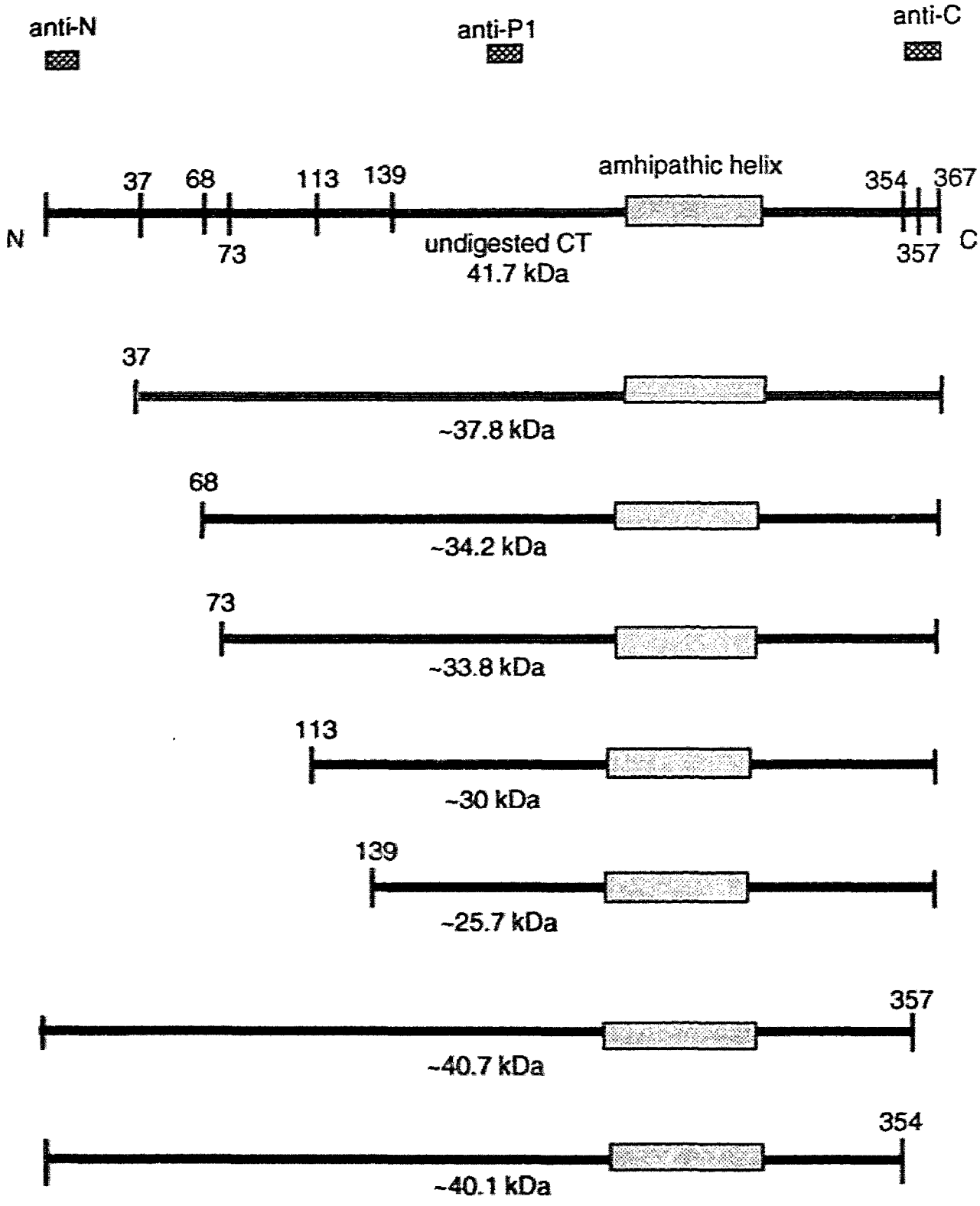


Figure 19: Theoretical NTCB digestion products. Based on CT containing 7 cysteines; at positions 37, 68, 73, 113, 139, 354 and 357.

3.3.2 Ability of the NTCB-generated Fragments to Bind to PC/Oleic Acid Vesicles

Since all of the NTCB fragments greater than 25 kDa contain the putative amphipathic α-helix we examined their ability to bind to the strongly activating PC/oleic acid vesicles. For the lipid binding analysis CT was not denatured with urea prior to NTCB digestion because it was important to maintain CT in a conformation as close to the native state as possible. CT from COS cell extract was partially digested with NTCB in the absence or presence of 5 mM PC/oleic acid vesicles as described in Section 2.3.13 and centrifuged in a sucrose density gradient. Fractions were dialysed to remove the sucrose and NTCB and analysed by SDS-PAGE and immunoblotting. In the absence of lipid all of the NTCB fragments were found in the bottom fraction (Figure 20A, Lane 5). When PC/oleic acid vesicles were present during NTCB digestion all of the fragments were found in the top fraction with 89% of the lipid (20B, Lane 2). These results add further support to our hypothesis that CT binds to membranes via its amphipathic helix.

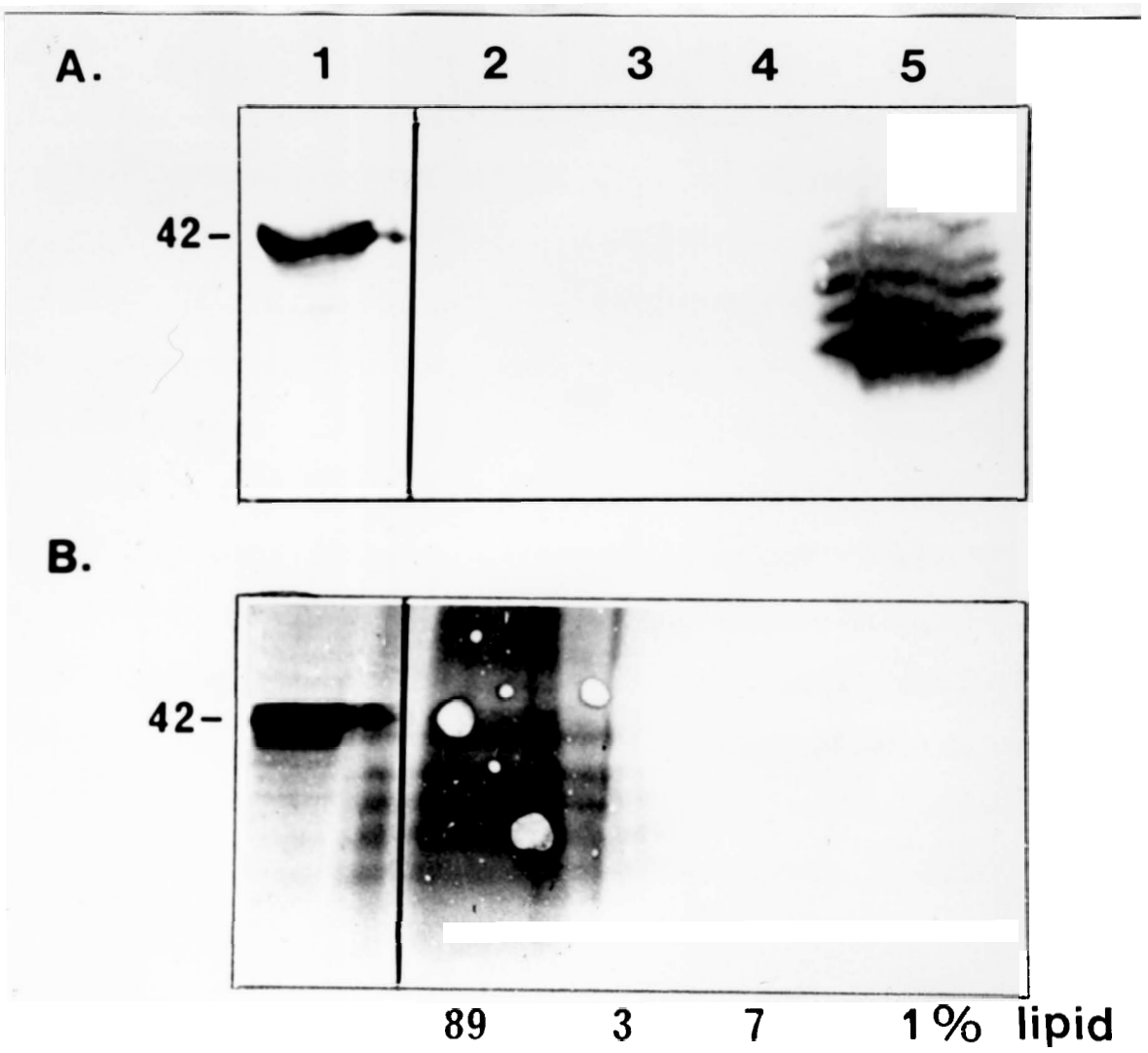
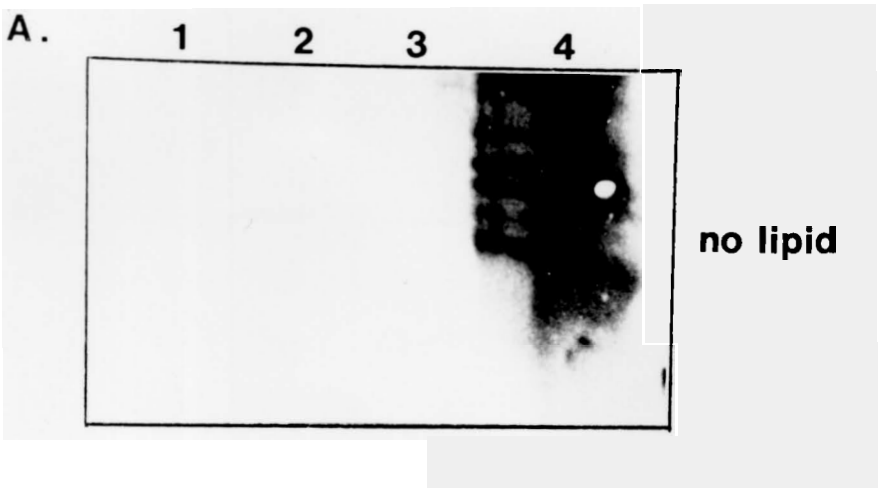


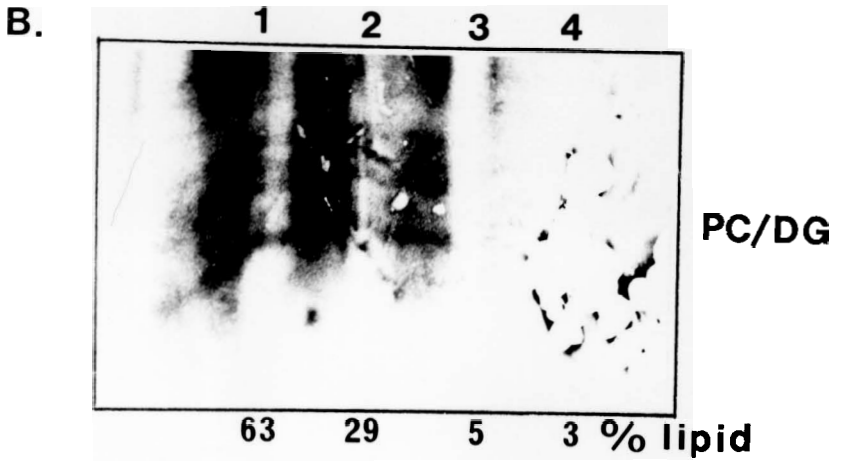
Figure 20: Ability of NTCB-generated fragments to bind to PC/oleic acid vesicles. 300 μg of CT from COS cell extract was incubated in (A) liposome buffer or (B) 5 mM PC/oleic acid for 5 min and digested in 25 mM NTCB, pH 7.5 for 52 h at 37°C. PC/oleic acid vesicles were radiolabeled with 0.1 μCi [methyl-^{3}H]DPPC. The digestion was quenched with 1% β-mercaptoethanol (V/V) and centrifuged on a sucrose density gradient and the fractions were dialysed and analysed by SDS-PAGE and immunoblotting with anti-P1 antibody. Lane 1 in A and B is undigested CT. Lanes 2 to 5 represent fractions 1 to 4 respectively. The % of radiolabeled lipid found in each fraction is indicated under each lane in B.

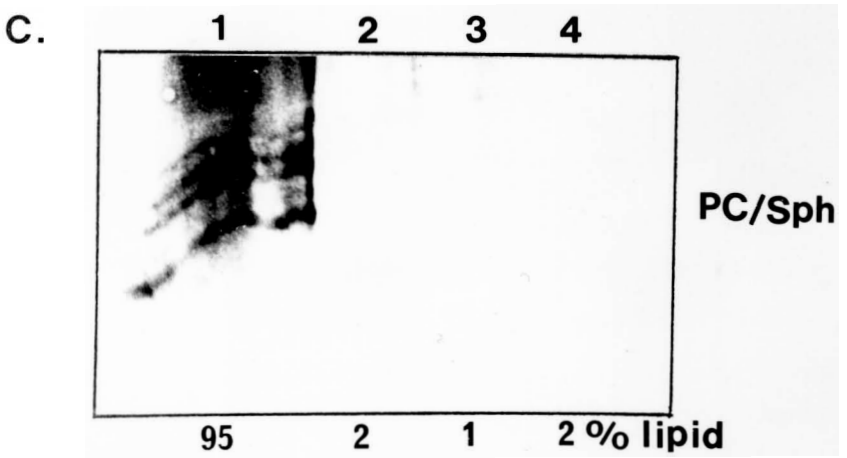
3.3.3 Ability of NTCB-generated Fragments to Bind to PC/DG and PC/Sphingosine.

The ability of the NTCB fragments to bind to a weak activator, PC/DG, and an inhibitory lipid vesicle, PC/sphingosine, was assessed using the lipid floatation assay. CT from COS cell extract was digested by NTCB in the absence of lipid or in the presence of 10 mM PC/DG or 10 mM PC/sphingosine vesicles and centrifuged in a sucrose density gradient. Fractions were dialysed to remove the sucrose and NTCB and analysed by SDS-PAGE and immunoblotting. In the absence of lipid all of the NTCB fragments were found in the bottom fraction (Figure 21A). When PC/DG vesicles were present during NTCB digestion the fragments were found in the top two fractions with 63% and 29% of the lipid respectively (Figure 21B). When PC/sphingosine vesicles were present during NTCB digestion all of the fragments were found in the top fraction along with 95% of the lipid (Figure 21C).

Figure 21. Binding of NTCB-generated fragments to PC/DG and PC/sphingosine. 270 µg of CT from COS cell extract was incubated in (A) liposome buffer, (B) 10 mM PC/DG or (C) 10 mM PC/sphingosine for 5 min and digested in 25 mM NTCB, pH 7.5 for 52 h at 37°C. Lipid vesicles were radiolabeled with 0.1 µCi [methyl-^{3}H]DPPC. The digestion was quenched with 1% β-mercaptoethanol (V/V) and centrifuged on a sucrose density gradient and the fractions were dialysed and analysed by SDS-PAGE and immunoblotting with anti-P1 antibody. Lanes 1 to 4 represent fractions 1 to 4 respectively. The % of radiolabeled lipid found in each fraction is indicated under each lane in B and C.


A.
1
2
3
4
no lipid


B.
1
2
3
4
PC/DG
63
29
5
3 % lipid


C.
1
2
3
4
PC/Sph
95
2
1
2 % lipid

3.4 COMPARISON OF THE CHYMOTRYPSIN SENSITIVITY OF FREE CT, LIPID-BOUND CT AND SUBSTRATE-BOUND CT

3.4.1 Effect of PC/Oleic Acid on the Chymotrypsin Sensitivity of CT

We compared the chymotryptic digestion patterns for free CT and CT bound to PC/oleic acid vesicles to determine whether the binding of an activating lipid vesicle would alter the sensitivity of CT to proteolysis. CT from COS cell extract was incubated with 0.5 mM PC/oleic acid vesicles or with buffer and then partially digested with chymotrypsin at two different protein:chymotrypsin ratios: 125:1 and 100:1. The samples were analysed by SDS-PAGE and immunoblotting. Figure 22 shows the chymotrypsin digestion pattern for CT in the absence of lipid and in the presence of PC/oleic acid vesicles. When lipid was absent (Lane 2 and 3) CT was rapidly digested to its 30-28 kDa fragments. However when CT was digested in the presence of PC/oleic acid vesicles the 39 and 35 kDa bands were very intense, appearing to be protected from chymotrypsin proteolysis (Lanes 4 and 5). The presence of PC/oleic acid vesicles did not affect the digestion pattern, as four of the five fragments, 39, 35, 30 and 28 were produced. The 26 kDa fragment was also observed when CT was digested in the presence of PC/oleic acid vesicles (see Figure23B). Thus binding of CT to PC/oleic acid vesicles protects it from digestion by chymotrypsin but does not alter the conformation so as to expose new cleavage sites.

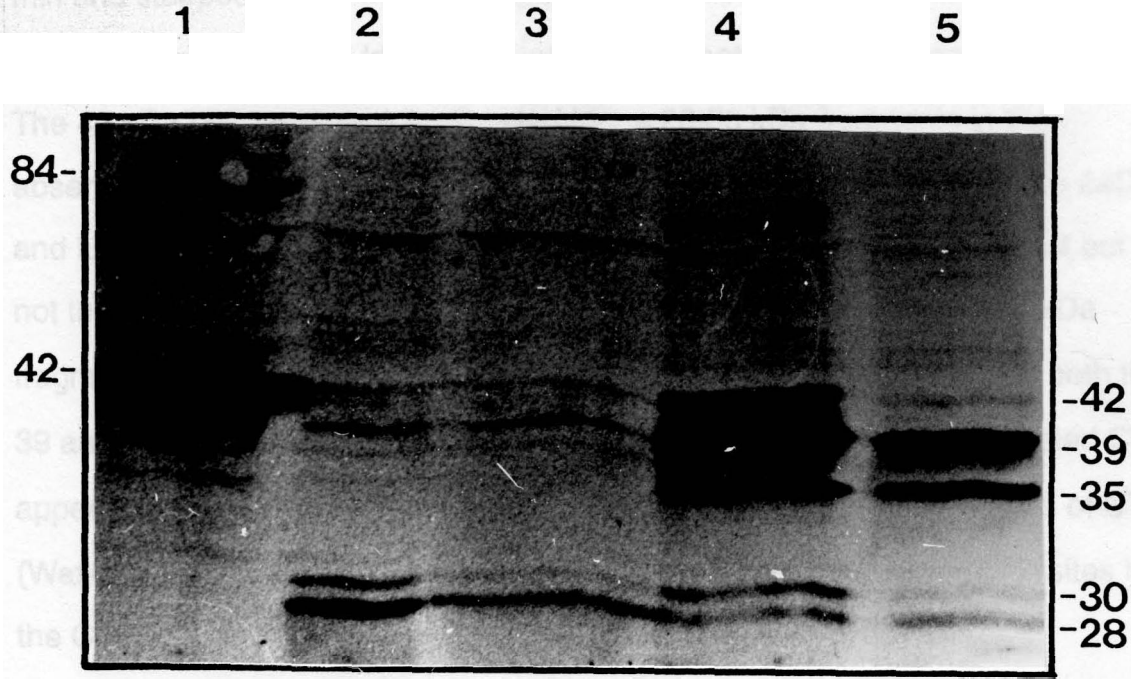


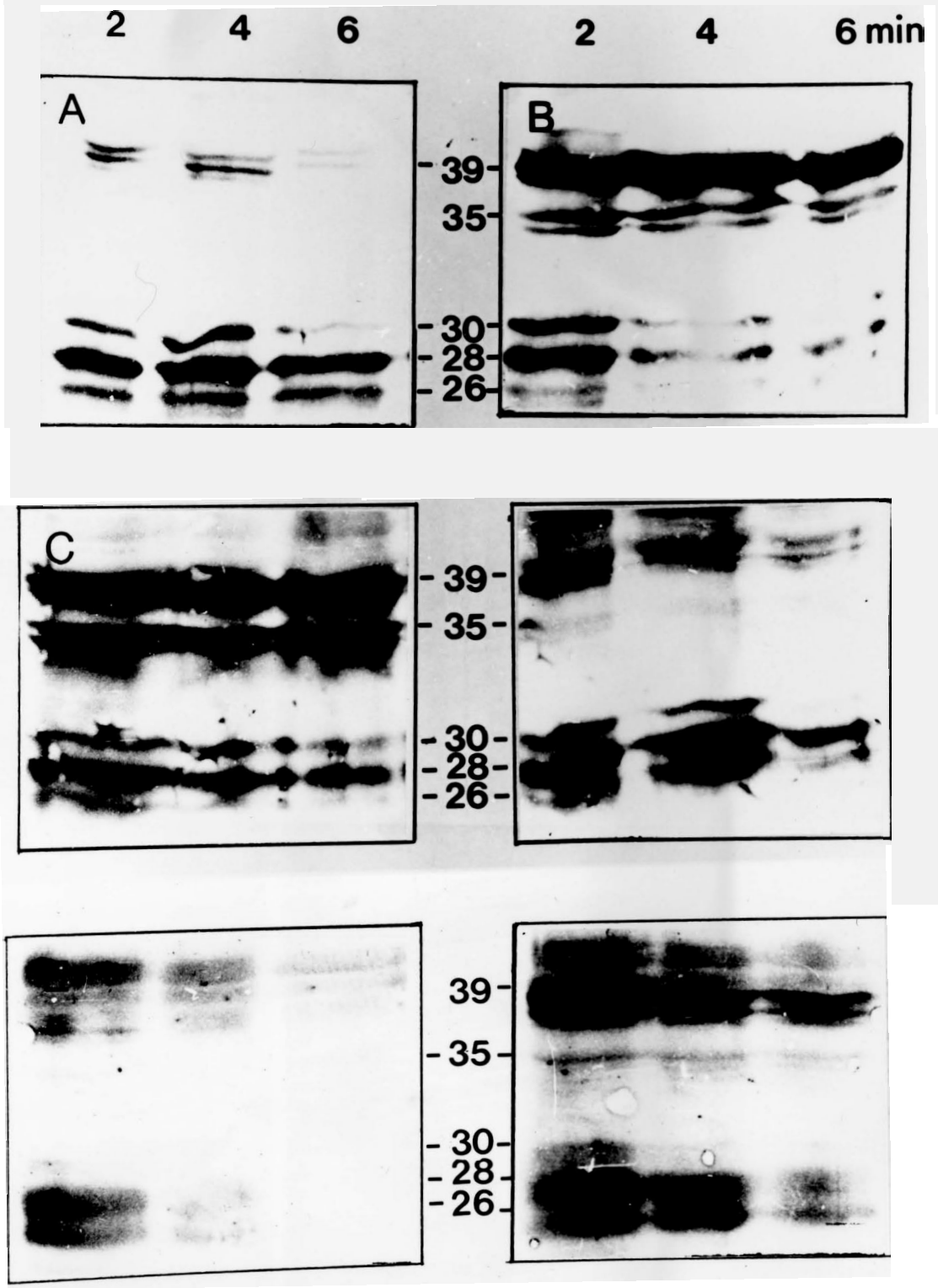
Figure 22 Effect of PC/oleic acid on the chymotrypsin-sensitivity of CT. 20 μg of CT from COS cell extract was digested with chymotrypsin for 10 min in the absence of lipid (Lane 2 and 3) or in the presence of 0.5 mM PC/oleic acid (1:1) (Lane 4 and 5). The protein:protease mass ratio was 125:1 in Lanes 2 and 4 and 100:1 in Lanes 3 and 5. Undigested CT is shown in Lane 1.

3.4.2 Effect of PC/PG, PC/DG, DilaurylPC and PC/Sphingosine on the Chymotrypsin Sensitivity of CT

To determine whether another strong activator, PC/PG, was capable of protecting CT from chymotrypsin digestion and whether weaker activators or inhibitors would also have an effect on chymotrypsin sensitivity, the chymotrypsin digestion patterns of CT were compared in the absence of lipid and in the presence of 4 mM PC/oleic acid, PC/PG, PC/DG, dilaurylPC and

PC/sphingosine vesicles. CT from COS cell extract was digested for 2, 4 and 6 min and stopped with 1 mM PMSF (Figure 23). Both PC/oleic acid and PC/PG protected the 39 and 35 kDa fragment from chymotrypsin digestion (23B and C). The 42 kDa band was rapidly digested to the 26-30 kDa fragments in the absence of lipid (23A) and in the presence of PC/DG or dilaurylPC (Figure 24D and E). PC/sphingosine appeared to partially protect the 39 kDa fragment but not the 35 kDa fragment (Figure 23F). Thus protection of the 39 and 35 kDa fragments appears to be exclusive to strong CT activators. In Figure 23 both the 39 and 35 kDa fragments appear as doublets. Often the 42 kDa undigested CT appears as a doublet and represents two different phosphorylation states of CT (Watkins and Kent 1991). As there are very few chymotrypsin digestion sites in the C-terminus, the doublets at 39 and 35 may represent different phosphorylation states. These doublets appear simultaneously and with equal intensity, unlike the other fragments which appear progressively over time, with one band disappearing as another band appears.

Figure 23: Effect of PC/PG, PC/DG, dilaurylPC and PC/sphingosine on chymotrypsin sensitivity of CT. 160 µg of CT from COS cell extract digested with chymotrypsin at a mass ratio of 100:1 in the presence of: (A) homogenization buffer + phosphate; (B) PC/oleic acid (1:1); (C) PC/PG (1:1); (D) PC/DG (3:1); (E) dilaurylPC; and (F) PC/sphingosine (1:1) vesicles. The final lipid concentration for B to F was 4 mM. Aliquots of each sample were removed at the indicated time points and quenched with 1 mM PMSF. Samples were analysed by SDS-PAGE and immunoblotting with the anti-P1 antibodies.


2
4
6
2
4
6 min
A
B
39
35
30
28
26
C
39
35
30
28
26
39
35
30
28
26

3.4.3 Effect of Substrate Binding on Chymotrypsin Sensitivity of CT

Saturating concentrations of substrate can stabilize the conformation of the active form of an enzyme (Vogel and Bridger, 1981). Substantial differences in conformation induced by substrate saturation of the active site could be reflected in altered protease sensitivity. CT from COS cell extract was digested with chymotrypsin in the absence of substrate, in the presence of 10 mM CTP or 10 mM phosphocholine, or both CTP and phosphocholine, and analysed by SDS-PAGE and immunoblotting. A chymotrypsin time curve for each sample from 0 to 10 min is shown in Figure 24. There was no apparent difference between CT digested in the absence of substrate and digestion in the presence of CTP, phosphocholine or CTP and phosphocholine, suggesting that CT does not undergo a major conformational change upon binding to its substrates.

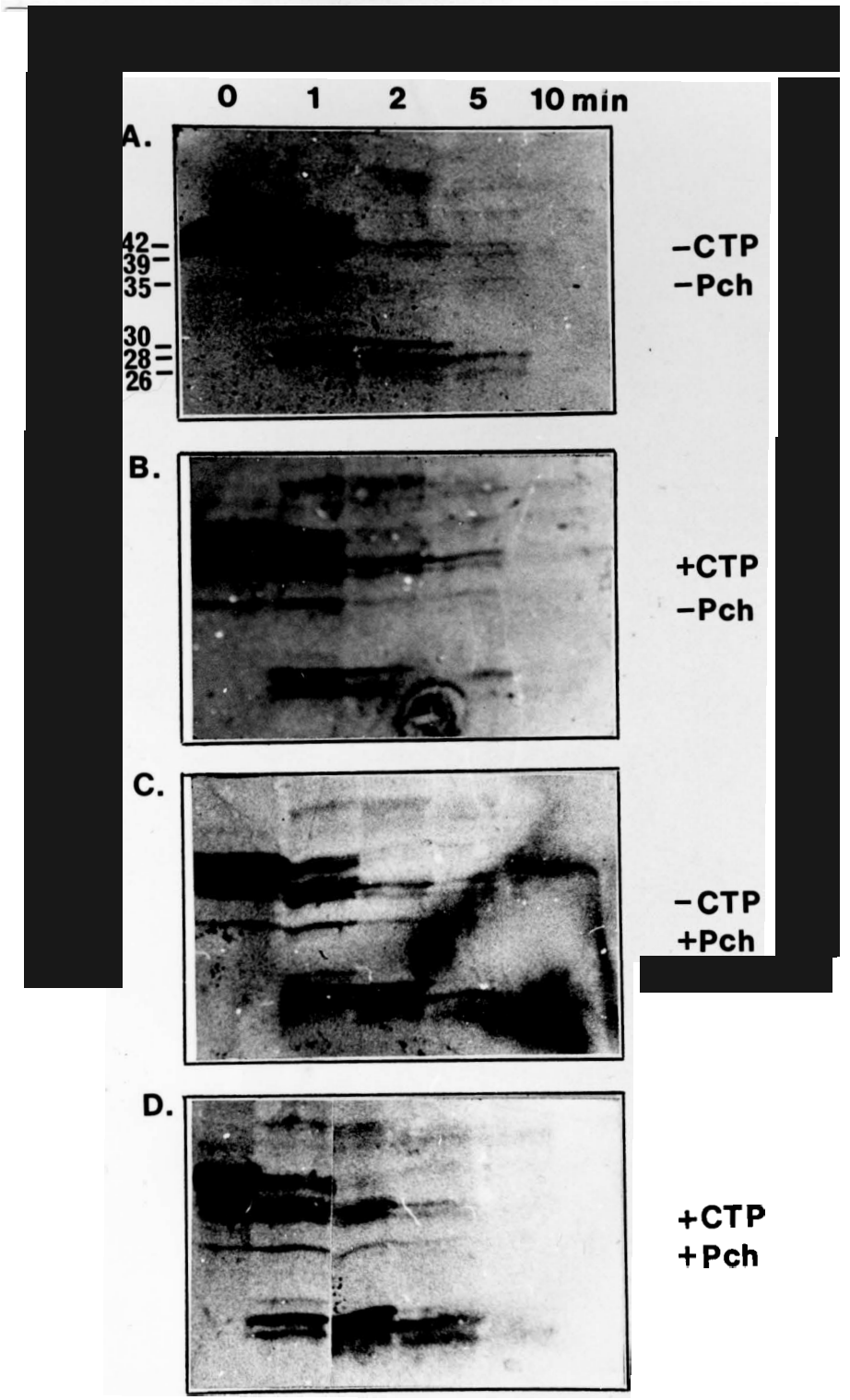


Figure 24: Effect of substrate binding on chymotrypsin sensitiviy of CT. 250 μg of CT from COS cell extract was digested with chymotrypsin at a mass ratio of 200:1 in the presence of (A) homogenization buffer + phosphate; (B) 10 mM CTP; (C) 10 mM phosphocholine (Pch); or (D) 10 mM CTP and 10 mM phosphocholine.

3.4.4 Effect of Substrate Binding in the Presence of PC/Oleic Acid Vesicles on Chymotrypsin-Sensitivity

Since CT has a lipid requirement for activation its sensitivity to chymotrypsin digestion was investigated in the presence of PC/oleic acid vesicles. CT may not readily bind substrates in the absence of lipid. CT from COS cell extract was incubated with 0.5 mM PC/oleic acid vesicles and then digested in the absence of substrate and in the presence of 10 mM CTP, 10 mM phosphocholine or both CTP and phosphocholine. Samples were digested with chymotrypsin for 0 to 10 min and analysed by SDS-PAGE and immunoblotting with the anti-P1 antibody. The chymotryptic digestion patterns of CT in the absence and presence of substrate looked very similar (Figure 25). At 10 min only a faint doublet at 39 kDa remained in the absence of substrate (25A) or in the presence of phosphocholine alone (25C). This doublet was more intense in the presence of CTP (25B) or both CTP and phosphocholine (25D), suggesting a more tightly folded conformation for CT when CTP is bound. This conformation may protect the chymotryptic site N-terminal to the 39 kDa cleavage site.

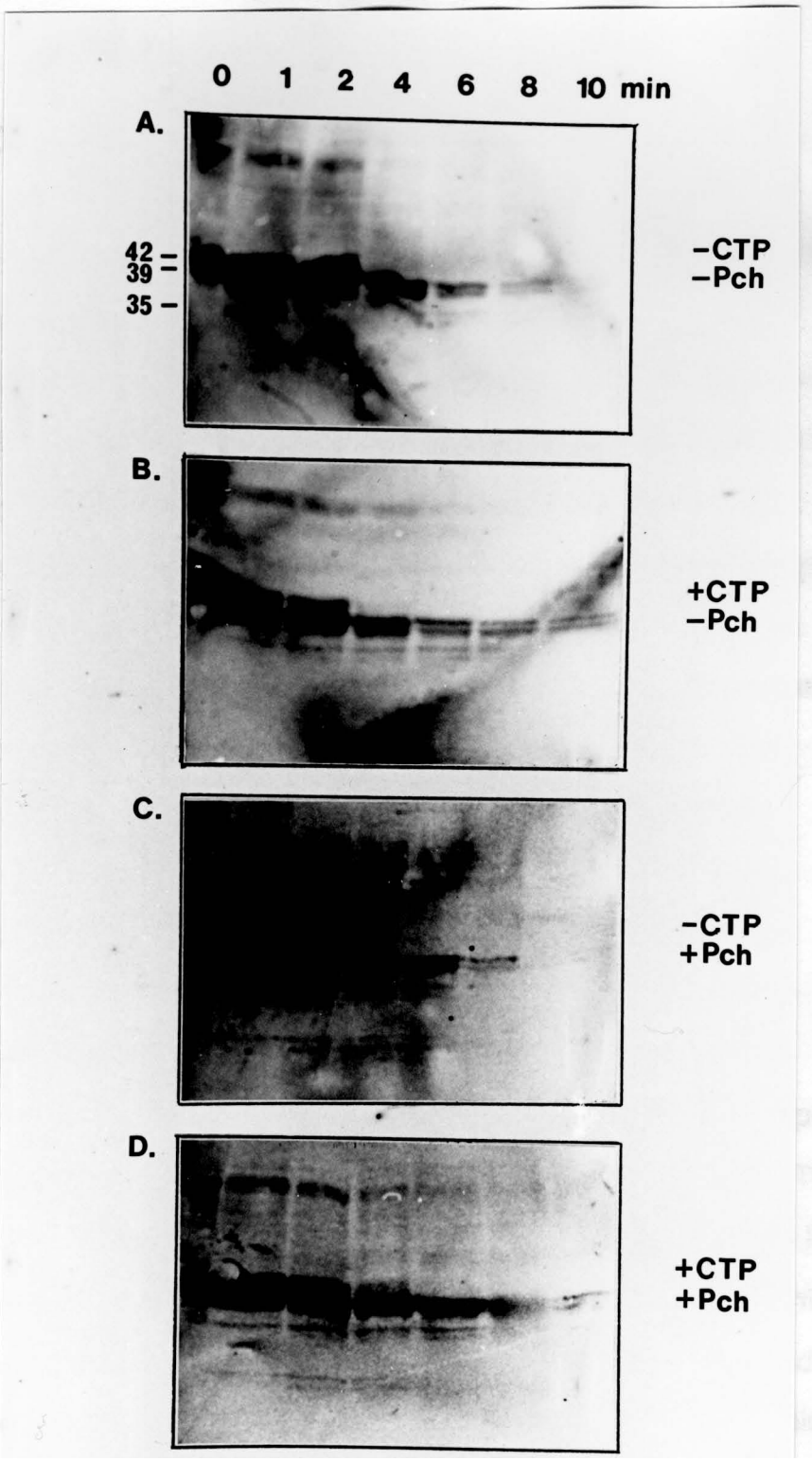


Figure 25: Effect of substrate on chymotrypsin sensitivity of CT in the presence of PC/oleic acid vesicles. CT was incubated with 0.5 mM PC/oleic acid vesicles for 5 min at 37°C and digested with chymotrypsin at a mass ratio of 200:1 in the presence of (A) homogenization buffer + phosphate; (B) 10 mM CTP; (C) 10 mM phosphocholine (Pch); or (D) 10 mM CTP and 10 mM phosphocholine.

PART 4. DISCUSSION

The results of our research have lead us to four major conclusions: *(i)* CT has a bipartite structure with a tightly folded globular N-terminal domain of approximately 26 kDa, linked to a more loosely folded and protease-accessible C-terminal domain; *(ii)* the amphipathic α-helix mediates binding to activating membranes; *(iii)* strongly activating lipids protect CT from digestion by chymotrypsin; *(iv)* CT binds to strongly activating lipid vesicles with a higher affinity than to weakly activating lipid vesicles. Binding of CT to vesicles containing sphingosine appears to be mediated by a central or N-terminal region rather than the amphipathic α-helix.

4.1 DOMAIN STRUCTURE OF RAT CT

4.1.1 Antibody Characterization of the Chymotryptic Fragments

The globular nature of the N-terminal domain (residues 1-235) was proposed based on the secondary structural predictions of CT (Lachance, M.Sc. Thesis). The Garnier algorithm predicted six short α-helices alternating with five β-sheets, each interrupted by a proline-induced turn. The two putative amphipathic α-helices between residues 236 and 315 are interrupted by 2 glycines and a proline at residue 294-297. The sequence C-terminal to the amphipathic α-helices (residues 316-367) contains 8 prolines and is predicted to be random-coiled.

CT was partially digested with four proteases: SGPB, trypsin, subtilisin and chymotrypsin. CT contains a large number of potential cleavage sites for each

protease. If CT was denatured with urea prior to proteolysis, complete digestion was observed. On the other hand proteolytic cleavage of the native enzyme in every case resulted in the production of 1 or 2 fragments between 25 and 30 kDa. This suggests that there is a region in CT which is highly accessible to proteases and is preferentially cleaved and that protection of the majority of the proteolytic sites on CT is imparted by the tertiary folded structure. The secondary structural predictions for the N-terminus of CT were interpreted to suggest a globular, tightly folded domain which should be somewhat resistant to proteolysis. The region immediately C-terminal to this domain was a potential hinge region between two domains - the globular N-terminal "head" and the extended α-helical "tail" - and was therefore likely to be accessible to proteolysis. SGPB has a specificity for small aromatic amino acids, particularly phenylalanine. Limited digestion of CT with SGPB produced a 28 and a 26 kDa fragment. Cleavage at Phe-234 or Tyr-240 would result in an N-terminal fragment of approximately 26 kDa. Cleavage at Phe-262 would produce a 30 kDa fragment. Trypsin, which is specific for the basic amino acids lysine and arginine, generated a 26- and a 25 kDa fragment. The 25 kDa fragment could have arisen from cleavage at Arg-217 or Arg-219, and the 26 kDa fragment could have been generated by cleavage at Lys 227. Subtilisin is also specific for aromatic amino acids and valine, alanine and glycine. Limited proteolysis of CT with subtilisin produced a 26 kDa fragment. A peptide of this molecular weight could be generated by cleavage at Tyr-240. Chymotrypsin cleaves at aromatic amino acids and at leucine and methionine. Chymotrypsin digestion of CT resulted in two bands at approximately 27 and 26 kDa. There are four sites between residues 224 and 240 which could produce fragments of these molecular weights.

Chymotrypsin was the chosen protease for further analysis of CT's tertiary structure. CT contains at least 50 sites for chymotrypsin proteolysis. Conditions of limited digestion in which only the most accessible sites would be cleaved produces five fragments from 39-26 kDa. The progression of proteolysis indicates that the 42 kDa undigested CT is cleaved first to the 39 kDa fragment and subsequently to the 35, 30, 28 and 26 kDa fragments. Each of these fragments was mapped to the N-terminus of CT using antibodies directed at the N-terminus and the conserved central domain. Thus chymotrypsin digestion proceeds from the more accessible C-terminus, which was predicted to be loosely folded. The molecular weight of each fragment is considered accurate to within 1 kDa. Given this error factor there are several possible cleavage sites for the 35 kDa fragment between residues 299 and 311. All of these sites fall within Helix-2 and would result in disruption of that helix. Similarly there are several possible cleavage sites for the 26-30 kDa fragments, all of which are very close to the sites proposed in Figure 12. The chymotryptic sites on CT are illustrated schematically in Figure 12 and in the model of CT's tertiary structure in Figure 13.

4.1.2 Activity of the Chymotryptic Fragments

Although we believe that the central conserved region houses the active site of CT, we have shown that *none* of the chymotryptic fragments studied have significant activity. Removal of 30 amino acids from the C-terminus, producing the 39 kDa fragment, reduces the activity of CT to 6% of the original activity. C-terminal deletion mutants of rat CT expressed from the truncated cDNAs in COS cells have confirmed that a loss of as little as 20 amino acids from the C-terminus results in a 90% reduction in CT activity (Kalmar, McDonald and Cornell, unpublished). The C-terminal domain of CT may be required for activity

because it is essential for correct folding or stability of the N-terminal domain, or because the C-terminus itself participates in catalysis. If the C-terminal domain of CT is in fact a regulatory domain then CT is unlike many other enzymes whose regulatory domains have an autoinhibitory function. These enzymes are *activated* upon removal of their regulatory domains (Soderling, 1990).

4.1.2 Dimerization Potential of the Chymotryptic Fragments

In addition to the production of the 39-29 kDa fragments, chymotrypsin digestion often results in the appearance of higher molecular weight bands on SDS gels (Figure 10A, Lane 2; Figure 17a and d). These bands migrate at molecular weights of 80, 70 and 50-60 kDa on SDS gels and correlate with the appearance of the 39, 35 and 30-26 kDa fragments. These higher molecular weight bands have been detected by the anti-N and anti-P1 antibodies but not by the anti-C antibody, suggesting that they are dimers of the 39, 35 and 30-26 kDa fragments respectively. This suggests that the domain responsible for dimerization is present on the N-terminal two-thirds of CT. We had suggested that the amphipathic helix might modulate dimerization by acting as a coiled-coil (Kalmar et al, 1990). These observations rule out a role for the amphipathic helix as being soley responsible for dimerization.

4.2 MEMBRANE BINDING ABILITY OF UNDIGESTED CT AND THE PROTEOLYTIC FRAGMENTS

4.2.1 Undigested CT

Whole CT was shown to bind in varying degrees to all vesicle types tested, regardless of their activating potential. CT had a higher binding affinity for

PC/oleic and PC/PG vesicles than for PC/DG and dilaurylPC. Our studies showed that 1:1 ratios of PC/oleic acid and PC/PG are strong activators of CT *in vitro*, whereas PC/DG (3:1) is a weak *in vitro* activator and dilaurylPC does not activate CT. PC/DG vesicles were made in a 3:1 ratio because of the tendency of DG to form hexagonal structures at higher concentrations. The lower ratio of DG in these vesicles may be responsible for the weaker activation and weaker binding affinity of CT. Both oleic acid and PG are negatively charged which may also account for the higher partitioning of CT into these vesicles. CT binds to PC/DG vesicles but requires a higher lipid to protein mass ratio than with anionic phospholipids. A lipid to protein ratio of 20:1 was required to bind 50% of the total CT to PC/DG vesicles (Figure 16C) whereas a lipid to protein ratio mass ratio of only 6:1 was sufficient to bind the majority of CT to PC/oleic acid vesicles (Figure 15C).

Differences in binding affinity may help to explain the ability of certain lipids to activate CT. However, binding to a lipid vesicle is not sufficient for activation. CT was shown to bind to dilaurylPC vesicles which do not activate CT. This suggests that the chemical nature of the vesicles, and *not* the number of lipid vesicles available to CT, is the factor regulating activity.

The observation that CT binds to lipids which do not activate it *in vitro* presents the possibility that CT can exist bound to membranes in an inactive state *in vivo*. This observation may not have any physiological significance as the concentrations of lipid used in these studies are well in excess of physiological levels. However there is a precedent set for CT binding in an inactive state *in vivo* in lung Type II cells where membrane-bound CT is inactive during development and is rapidly activated after birth (Stern *et al*, 1976). In addition

protein kinase C has an inactive membrane-bound state as well as a bound active state (Bazzi and Nelsestuen, 1989).

CT was shown to bind readily to PC/sphingosine vesicles. This is not unexpected, as sphingosine was able to competitively inhibit CT activity when added to anionic lipid vesicles (Sohal and Cornell, 1990). Since sphingosine is positively charged, we propose that CT interacts with PC/sphingosine vesicles via a different mechanism than with anionic and neutral lipids, possibly via an ionic interaction between sphingosine and the numerous acidic residues on CT. Binding to positively charged lipid vesicles results in an inactive conformation.

4.2.2 Chymotryptic Fragments

The ability of the chymotryptic fragments to bind to lipid vesicles correlates with the presence of Helix-1. Both the 39 and 35 kDa fragments contain the entire Helix-1 and can bind to all the vesicles tested. A portion of Helix-2 is missing in the 35 kDa fragment. No fragment smaller than 35 kDa was seen to bind to PC/oleic acid, PC/PG, PC/DG or dilaurylPC vesicles which is consistent with these fragments having lost the lipid-binding domain. According to the molecular weight approximations the 30 kDa fragment contains ~50% of the amphipathic Helix-1 and only the first few residues of the first 11-mer repeat. The 28 kDa fragment contains none of the 11-mer repeats and very little of Helix-1. The 26 kDa fragment does not contain any of Helix-1 or Helix-2. These results suggest that the full Helix-2 is not required for membrane binding and the N-terminal half of Helix-1 is insufficient for binding. However firm identification of the cleavage sites by C-terminal analysis is needed before we can define the exact domain required for membrane binding.

All of the chymotryptic fragments were seen to bind to PC/sphingosine. We infer that this interaction is not mediated via Helix-1 but via the N-terminal domain, possibly by an ionic interaction since the domain N-terminal to Helix-1 (residues 1-235) has a net charge of -3.

4.2.3 NTCB-Generated Fragments

Incomplete digestion of CT with NTCB produced several fragments between 42 and 26 kDa, all of which possessed membrane binding ability and contained the intact amphipathic α-helices. Helix-1 has been shown to be unnecessary for binding of the chymotryptic fragments to PC/sphingosine. The 26 kDa fragment (beginning at residue 139) bound to PC/sphingosine indicating that the N-terminal 138 amino acids are not necessary for binding to PC/sphingosine.

In intact CT, the NTCB-generated fragments, and the 39 and 35 kDa chymotryptic fragments all contain the central region of CT and Helix-1. All of these fragments are able to bind to membranes. Lipid binding studies were not done at low lipid concentrations to determine if the NTCB-generated fragments had a higher specificity for anionic phospholipids. The 30-26 kDa chymotryptic fragments have lost some or all of Helix-1 and do not have membrane-binding ability (except with PC/sphingosine). We conclude that CT interacts with uncharged and anionic membranes via Helix-1.

Recently direct evidence for an α-helix in CT has been obtained by analyzing the conformation of synthetic peptides derived from the region of the three 11-mer repeats in Helix-1. Circular dichroism studies show that a 22-mer (residues 267-288) and a 33-mer (residues 256-288) can be induced to form α-helices in the presence of PC/oleic acid, PC/PG and cardiolipin which are all anionic, but not

PC/sphingosine (Johnson and Cornell, unpublished). Thus lipid specificity for the induction of helicity in the peptides correlates with lipid specificity for activation of the whole enzyme.

4.3 COMPARISON OF THE CHYMOTRYPSIN SENSITIVITY OF FREE CT, LIPID-BOUND CT AND SUBSTRATE-BOUND CT

4.3.1 Effect of Lipid Binding on the Chymotrypsin Sensitivity of CT

When CT was digested by chymotrypsin in the presence of PC/oleic acid and PC/PG vesicles, the 39 and 35 kDa fragments were more resistant to degradation than CT digested in the absence of lipid. This resistance was not seen when CT was digested in the presence of PC/DG and dilaurylPC. Protection of the 39 and 35 kDa fragments could be imparted by: *(a)* a change in the conformation of CT to a more tightly folded structure upon binding to activating membranes; *(b)* dimerization; or *(c)* the membrane itself presenting a physical barrier to chymotrypsin. The same set of fragments are generated by chymotrypsin digestion in the absence or presence of all lipid vesicles tested suggesting that the conformation is not altered so as to expose new cleavage sites or conceal others. If binding to membranes triggers a conformational change it not likely a dramatic change. The role of dimerization in the protection of the 39 and 35 kDa fragments cannot be ruled out. CT has been shown to exist as a dimer in its membrane-bound form (Cornell, 1989). Binding of CT to membranes may shift the two monomers with respect to each other resulting in a more compact structure. However since the dimerization domain is believed to be N-terminal, dimerization is more likely to protect N-terminal regions whereas

protection of the 39 and 35 kDa fragments requires protection of sites lying in the amphipathic helix.

It is therefore probable that protection of these fragments from proteolysis is due to steric hindrance by the membrane. The 39 and 35 kDa fragments are the only chymotryptic fragments capable of binding to PC/oleic acid and PC/PG vesicles. If Helix-1 is intercalated into the lipid bilayer the sites for chymotrypsin digestion, which are primarily the large hydrophobic amino acids Phe, Trp and Tyr, may be buried within the hydrophobic core and thus less accessible to the binding site of chymotrypsin. Other proteins that have altered sensitivity to proteases upon membrane-binding include Sec A (Ulbrandt, 1992), protein kinase C (Newton and Koshland, 1989) and tubulin (Kumar *et al*, 1981). Sec A and protein kinase C both become more sensitive to proteolysis whereas tubulin becomes more resistant to proteolysis upon membrane binding.

The observation that PC/DG and dilaurylPC did not protect the 39 and 35 kDa fragments is partially explained by the weaker affinity of CT for these lipids. Since fewer CT molecules are bound to these vesicles during digestion, protection is less evident. However protection studies were done at two different lipid concentrations, 1 mM and 4 mM. It was expected that the 39 and 35 kDa fragments would be more protected in the presence of higher concentration of lipid, but this was not evident. This observation, together with the fact that CT is not activated by dilaurylPC and only weakly by PC/DG, suggests that CT interacts with these vesicles in a slightly different manner than with anionic lipid vesicles. This may mean that the amphipathic helix does not intercalate into the membrane bilayer as deeply, or that the conformation of CT is slightly different when bound to different lipids.

CT has a higher affinity for PC/sphingosine vesicles than for PC/DG or dilaurylPC and some protection of the 39 kDa fragment is observed when CT is digested in the presence of 4 mM PC/sphingosine. Since we have determined that CT binds to PC/sphingosine via an N-terminal or central domain it is not surprising that PC/sphingosine does not protect CT to the extent of the strongly activating vesicles. However since PC/sphingosine protects the 39 kDa fragment, this suggests that digestion sites N-terminal to the 39 kDa cleavage site are less accessible to chymotrypsin upon binding to PC/sphingosine. It is not clear whether this protection is due to protein folding or steric hindrance by the membrane itself.

4.3.2 Effect of Substrate Binding on the Chymotrypsin Sensitivity of CT

The chymotryptic digestion patterns for free CT and CT in the presence of its substrates were indistinguishable. Inclusion of PC/oleic acid vesicles in the reaction medium resulted in a slight increase in the resistance of the 39 kDa doublet in the presence of CTP or CTP and phosphocholine. This suggests that when CT is saturated with CTP, it either becomes more tightly folded or intercalates deeper into the membrane in the presence of CTP, thus protecting the chymotryptic cleavage sites N-terminal to the 39 kDa cleavage site.

4.4 CONCLUSIONS

We have shown that intact CT can bind *in vitro* to a variety of lipid species, both activating and inhibitory. In our system CT's association with lipid is not a transient one as it remains bound to lipid throughout 14-20 hour centrifugations.

We saw that CT bound weakly to PC/DG and dilaurylPC but bound with a high affinity to PC/O and PC/PG vesicles which are strong activators. CT fragments generated by NTCB and chymotrypsin were shown to bind to lipid vesicles, provided that they contain the amphipathic α-heiix domain. PC/DG and dilaurylPC did not protect CT from chymotrypsin digestion, which may result from the low affinity of CT for vesicles of these compositions.

4.4.1 CT Model

This research has provided strong support for our original model of CT which was based solely on secondary structural predictions. Our current model of CT's tertiary structure is illustrated in Figure 26, with CT shown as a dimer. Chymotrypsin digestion and antibody mapping confirmed that CT has a tightly folded globular N-terminal domain and a more loosely folded C-terminal domain. These domains are linked by two amphipathic helices. Since the production of the 39-26 kDa chymotryptic fragments corresponds with production of the fragment dimers we propose that CT monomers are aligned parallel to each other as shown in Figure 26, with the chymotryptic cleavage sites on each monomer being equally accessible to proteolysis.

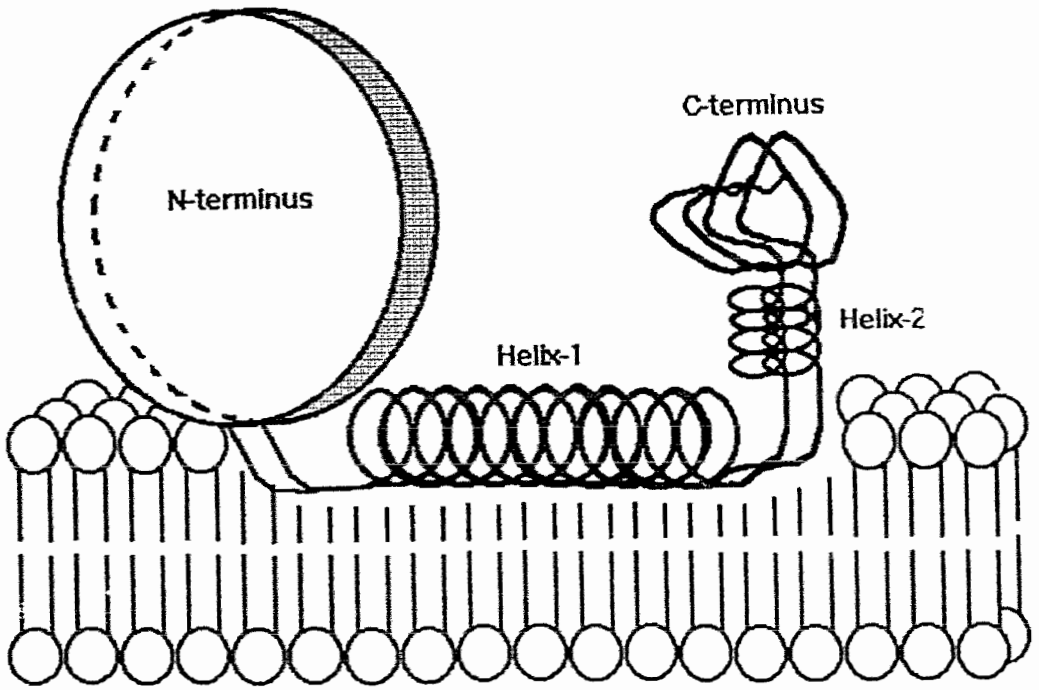


Figure 26: Current model for tertiary structure of CT and interation with lipid bilayer.

Membrane binding studies of both the chymotryptic and NTCB-generated fragments have implicated Helix-1 as the membrane binding domain. This was supported by the observation that strongly activating lipid vesicles were able to protect chymotryptic cleavage sites along Helix-1. At this time we cannot predict the depth to which the helix is intercalated into the lipid bilayer. Its hydrophobic amino acid side chains probably interact with the fatty acid moieties of the lipid. If intercalation is deep the polar side chains would interact with the phospholipid head groups. Shallow intercalation would mean that the polar side chains would interact with surrounding cytosol or with a polar surface of CT itself. Helix-2 was not found to be necessary for membrane binding and is not predicted to interact with the lipid bilayer in our current model. As Helix-2 is predicted to be

amphipathic, its non-polar surface may interact with other hydrophobic residues on CT or with the membrane. However Helix-1-lipid interactions are sufficient for membrane binding. These studies have brought us a step closer to understanding the mechanism of lipid regulation of CT and therefore regulation of the PC biosynthetic pathway.

4.5 FUTURE STUDIES

There is still much to be learned regarding the interaction between CT and lipid bilayers. Lipid photolabeling and peptide studies which are currently in progress should provide definitive proof that CT interacts with membranes via Helix-1 (Johnson and Cornell). CT has already been photolabeled by a hydrophobic probe present in PC/oleic acid vesicles. The next stage is to attempt to photolabel the chymotryptic fragments to isolate the membrane-binding region. Results from studies with synthetic 22-mer and 33-mer peptides of the 11-mer repeat in Helix-1 showed that CT-activating phospholipids induce α-helicity in the peptide whereas non-activating lipids do not. In the presence of anionic lipid vesicles fluorescence of a tryptophan on the helix was quenched by a hydrophobic quencher but not an aqueous quencher. Several different PCs have been obtained which are brominated at various carbons on the acyl chain. Their ability to quench tryptophan in the 33-mer peptide should provide information on the depth of intercalation of the helix into the membrane.

Many questions remain unanswered regarding the relationship between the structure of CT and its function as an enzyme. What is the basis for differences in affinity between different classes of lipid? How does interaction with lipids

induce changes in the catalytic site? How does phosphorylation/ dephosphorylation affect the conformation of CT? Mutant studies are currently underway to identify phosphorylation sites important in control of CT activity. Studies with radiolabeled substrates should help to identify the catalytic region and provide information about the catalytic mechanism.

Future work (in our lab) will continue to focus on the CT-membrane interaction, not only to define the mechanism of lipid regulation of CT, but hopefully to gain a clearer understanding of the regulation of the amphitropic class of proteins.

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