ELSEVIER

Biochimica et Biophysica Acta 1324 (1997) 273–284

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

An amphipathic α-helix is the principle membrane-embedded region of CTP:phosphocholine cytidylyltransferase. Identification of the 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine photolabeled domain

Joanne E. Johnson [a,1], Ruedi Aebersold [b], Rosemary B. Cornell [a,*]

[a] *Institute of Molecular Biology and Biochemistry and Department of Chemistry, Simon Fraser University, Burnaby, BC, Canada V5A 1S6*

[b] *Department of Molecular Biotechnology, University of Washington, Seattle, WA, USA 98195*

Received 8 May 1996; revised 9 September 1996; accepted 29 October 1996

Abstract

CTP:phosphocholine cytidylyltransferase (CT), the rate controlling enzyme in phosphatidylcholine biosynthesis, is activated by reversible membrane binding. To investigate the membrane binding mechanism of CT, we have used the photoreactive hydrophobic probe 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine ([^{125}I]TID). Association of CT with phosphatidylcholine/oleic acid (1:1) vesicles was first demonstrated by gel filtration analysis. Upon irradiation, CT was covalently labeled by [^{125}I]TID presented in phosphatidylcholine/oleic acid vesicles. This demonstrates an intercalation of part of the protein into the hydrophobic core of the membrane. To identify the membrane-embedded domain, the chymotrypsin digestion products of [^{125}I]TID labeled CT were analysed. Chymotrypsin digestion produced a set of previously defined N-terminal fragments (Craig, L., Johnson, J.E. and Cornell, R.B. (1994) J. Biol. Chem. 269, 3311), as well as several small C-terminal fragments which react with an anti-peptide antibody raised against the proposed amphipathic α-helix. All fragments containing the amphipathic helical region of the enzyme had [^{125}I]TID label associated, while the chymotryptic fragment which lacked this region was not highly labeled. Similar fragment labeling patterns were produced when [^{125}I]TID was presented in phosphatidylcholine/oleic acid or phosphatidylcholine/diacylglycerol vesicles, suggesting that the same domain of CT mediates binding to membranes containing either of the two lipid activators. A 62-residue synthetic peptide corresponding in sequence to the amphipathic helical region of CT was labeled with [^{125}I]TID, demonstrating its ability to intercalate independently of the rest of the protein. These results indicate a membrane-binding mechanism for cytidylyltransferase involving the intercalation of the amphipathic α-helix region into the hydrophobic acyl chain core of the activating membrane.

Keywords: Lipid photolabeling; Anionic lipid vesicle; Proteinase digestion; Peptide sequencing

Abbreviations: CT, CTP:phosphocholine cytidylyltransferase; [^{125}I]TID, 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine; PC, phosphatidylcholine; PMSF, phenylmethylsulfonyl fluoride; PVDF, poly(vinylidine difluoride); DPPC, dipalmitoylphosphatidylcholine; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; BSA, bovine serum albumin; MWCO, molecular weight cut off; [^{3}H]TUA, 11-[4-[3-(trifluoromethyl)diazirinyl]phenyl]-[2^{3}H]undecanoic acid

* Corresponding author. Fax: +1 604 2915583; E-mail: Rosemary_Cornell@sfu.ca

[1] Present address: Dept. of Pharmacology, University of California at San Diego, La Jolla, CA 92093, USA.

PII S0005-2736(96)00233-7

1. Introduction

CTP:phosphocholine cytidylyltransferase (CT), which catalyses a key regulatory step in the biosynthesis of phosphatidylcholine (PC) in mammalian systems, is an amphitropic enzyme that is regulated by reversible binding to membranes. PC is the major phospholipid component of animal cell membranes, and thus its breakdown and resynthesis must be coordinated [1–3]. Knowledge of how CT is regulated is central to the understanding of how cellular levels of PC are controlled in response to agonist induced PC turnover.

The exact mechanism for membrane binding and subsequent activation of CT is not known, although the key stimulus appears to be the lipid composition of the membrane. Membranes composed solely of PC poorly activate the enzyme while those containing anionic phospholipids and fatty acids [4–6] or neutral lipids with small polar head groups such as diacylglycerol [7–10] are potent activators. The presence of cationic lipids such as sphingosine inhibits enzyme activation [11]. Both electrostatic and hydrophobic interactions have been implicated in the binding process [6,10,12]. Cell culture studies have demonstrated a dephosphorylation event concurrent with or subsequent to membrane binding [13–16]. Dephosphorylation appears to be secondary to changes in lipid composition as a trigger for membrane binding [14,17].

On the basis of structural predictions and homologies of the primary sequence [18], we have proposed a tripartite model for the domain structure of CT (Fig. 1).

(i) The N-terminal two-thirds forms a discrete, proteinase insensitive domain [19]. This domain is proposed to house the catalytic site based on its sequence homology with yeast CT [20] and a related *B. subtilis* glycerol phosphate cytidylyltransferase [21,22], as well as a study of a mutant in this region defective in CTP binding [23]. (ii) The C-terminal 55 residues are rich in serine-proline motifs that are potential substrates for cyclin dependent kinases, and contain a potential site for casein kinase II. This domain is highly phosphorylated in vivo and in vitro [24–26], although the function of phosphorylation is not yet established. (iii) The ~ 60 residue region linking the catalytic and phosphorylation domain is predicted to be an amphipathic α-helix, which we have proposed to mediate membrane binding [18]. This type of structure mediates the membrane interactions of several other proteins, including apolipoproteins [27,28], prostaglandin H_2 synthase-1 [29], Factor VIII [30], and various lytic peptides [31].

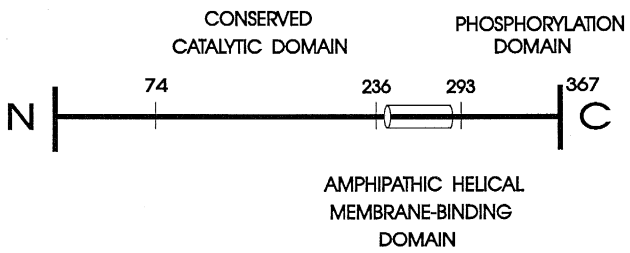

Fig. 1. Model for the domain structure of CT.

Evidence to support involvement of the putative amphipathic α-helix region of CT in membrane binding has emerged recently. (i) Chymotrypsin proteolysis of CT produced discrete fragments that were mapped to the N-terminus and were missing various amounts of the C-terminal region of the protein. Those fragments which lacked all or part of the putative amphipathic helical region were unable to bind to lipid vesicles [19]. (ii) Spectroscopic studies of a 33-residue synthetic peptide corresponding to the C-terminal half of the amphipathic helix domain demonstrated that the peptide was induced into a helical conformation by anionic lipids and intercalated into the acyl chain region of anionic lipid vesicles with a selectivity for anionic lipids [32]. (iii) CT deletion mutants lacking this domain that were expressed in COS or CHO cells, had a decreased association with the membrane fraction [25,33]. (iv) Antibodies directed against a 10 amino acid segment of this region inhibited membrane binding [34]. While there is evidence for a requirement for the amphipathic helical domain in CT-membrane interactions, the results of previous studies do not rule out an indirect effect of this helical region on membrane binding. Although synthetic peptides corresponding to the amphipathic helix domain bind to lipid vesicles, it had not been shown directly using the whole enzyme that this region contacts and intercalates into the membrane. Furthermore, the synthetic peptide bound only to vesicles containing anionic lipids, but not to vesicles containing the lipid activator, diacyl-

glycerol [32]. This raised the possibility that the amphipathic helix domain was not responsible for binding to vesicles containing DAG.

In this report, we have applied the technique of hydrophobic photolabeling [35] to directly determine the regions of the CT protein that intercalate into the hydrophobic core of the membrane. The hydrophobic probe 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine ([^{125}I]TID) partitions specifically into the hydrophobic membrane core [36,37]. Photoactivation of the diazirine functional group produces a highly reactive carbene species which has the potential to react non-selectively with any molecule in the vicinity. Thus, any regions of the protein which are embedded in the hydrophobic core of the membrane should become labeled by the probe. This technique has been used to identify membrane interacting regions of integral membrane proteins, such as bacteriorhodopsin [38], a light-harvesting protein B870 [39], the nicotinic acetylcholine receptor [40]; as well as amphitropic intercalating proteins such as synapsin I [41], Sec A [42] and a soluble fragment of hemagglutinin [43,44]. Using a combination of photolabeling, proteolysis and amino acid sequencing, we have determined that a region of membrane-bound CT does indeed penetrate into the hydrophobic core, and that this region corresponds to the amphipathic helical region of the protein.

2. Experimental procedures

2.1. Materials

A 62 residue peptide corresponding to the putative amphipathic helix region of rat liver CT (residues 238 to 299) was synthesized and HPLC purified by Dr. Ian Clark-Lewis (University of British Columbia) as described [45]. The peptide was $>97\%$ pure as assessed by analytical reverse-phase HPLC. Its amino acid composition was consistent with its expected sequence. [^{125}I]TID, [*methyl*-^{3}H]DPPC and [1-^{14}C]oleic acid were from Amersham, and [*phenyl*-^{3}H]Triton X-100 was from Dupont/NEN. Egg PC was from Avanti. *sn*-1,2-diacylglycerol was generated by digestion of egg PC using phospholipase C as described [46]. Oleic acid, α-Chymotrypsin (Type II: from bovine pancreas), Ovalbumin, Sephacryl-S400-HR, and the goat-anti-rabbit horseradish peroxidase antibody were from Sigma. PVDF was from Biorad. BSA (fraction V, fatty acid free) was from Calbiochem. Rat liver CT recombinant baculovirus, *Trichopusia ni* (*T. ni*) cells and antiserum raised against a C-terminal peptide of CT were kindly provided by Dr. Claudia Kent (U. Michigan). Apolipoprotein A-I was kindly provided by Dr. R.J. Cushley (Simon Fraser U.) Rabbit antiserum against the N-terminus of CT was prepared as described [25]. Antiserum against the amphipathic helix was generated as follows: A synthetic peptide corresponding to residues 256–288 with an additional N-terminal cysteine was coupled to keyhole limpet hemocyanin (KLH) using succimidyl 4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate (Pierce; [47]). Rabbit antiserum was generated by monthly subcutaneous injections with 50 μg KLH conjugated peptide in Freunds incomplete adjuvent. The blood was collected from the ear vein 10 days post-injection. Electrophoresis reagents were Molecular Biology Grade purchased from BRL or Sigma.

2.2. Methods

Purification of CT: Recombinant rat liver CT was expressed in *T. ni* insect cells using a baculovirus expression system and purified as described [48] with some revisions [25]. The pure enzyme was stored in a stabilizing buffer containing 0.05% Triton X-100.

2.2.1. [^{125}I]TID photolabeling

[^{125}I]TID (0.2 to 2 mol% of total lipid) was preincubated 10 min at room temperature with sonicated PC/oleic acid (1:1) vesicles or PC/diacylglycerol (4/1) vesicles (ref. 10) in 10 mM Tris, pH 7.4, 1 mM EDTA, 1 mM DTT. CT ($\sim$20 μg) was added so that the final Triton X-100 concentration was <20 mol% of the total lipid, and the CT:lipid molar ratio was 1:300. For photolabeling of the peptide, [^{125}I]TID and PC/oleic acid were preincubated in 10 mM Tris, pH 7.4, 1 mM EDTA, and the peptide was added (1:200, peptide/lipid molar ratio). After a 10 min incubation at room temperature, the protein-lipid mixture was transferred to a 1 ml quartz cuvette. All samples were irradiated for 10 min at room temperature using a 100 W Hg lamp with a filter to exclude light <300 nm.

2.2.2. Partial digestion of CT with chymotrypsin

After photolysis, the CT-lipid mixture was partially digested with chymotrypsin as previously described [19]. Samples were incubated with proteinase at the indicated chymotrypsin:CT mass ratio for 10–15 min at 37°C, at which time the reaction was terminated by addition of PMSF to a final concentration of 2 mM.

2.2.3. Gel electrophoresis

Samples containing whole or digested CT were prepared for electrophoresis by extensive dialysis (MWCO of 3500) against 10 mM Tris, pH 7.5, 0.5 mM EDTA, 0.005% SDS, 2 mM DTT, 0.05% BSA (fatty acid free). For chymotrypsin-digested samples, 0.5 mM PMSF was included in the dialysis buffer. Samples were concentrated in a Savant Speed-Vac evaporator prior to addition of sample buffer. CT and large fragments were separated on a 12% polyacrylamide gel by the method of Laemmli [49]. Small CT fragments were separated on a 16.5%T/3%C Tricine gel system [2] by the method of Schagger and von Jagow [50]. In both cases, the portion of the gel containing the bromphenol blue dye-front was excised, as it contained labeled lipid present in the sample.

Samples containing peptide were delipidated prior to electrophoresis by precipitation with cold acetone at −80°C for 3–8 h, followed by a wash with cold diethyl ether. The resulting precipitate was resuspended in sample buffer and electrophoresed overnight at 4°C on a 16.5%T/6% C polyacrylamide gel with a 1 cm 10%T/3%C spacer between the stacking and separating gels [50].

2.2.4. Protein and radioactivity quantitation

For autoradiography, ^{125}I-containing gels were Coomassie stained, dried, and exposed to preflashed Reflections film (Dupont) with an intensifying screen (Dupont) at −80°C for the indicated time. Protein mass associated with the bands was estimated using a Microtek Scanmaker densitometer with NIH imaging software. ^{125}I-labeling was quantified in excised bands by gamma-counting.

[2] T refers to the total percent (w/v) concentration of both acrylamide monomers C refers to the percent of total acrylamide that is the crosslinker bisacrylamide.

2.2.5. Immunoblotting

After separation by electrophoresis as described above, proteins were transferred to PVDF membrane using a Pharmacia LKB Novablot system at 2 mA/cm^2 for 1 to 1.5 h as described [19]. Immunoreactions were as described [25].

2.2.6. Sequence analysis

For N-terminal sequencing, chymotryptic fragments of CT were electrophoresed in the Tricine gel system, transferred to PVDF membrane and stained with Amido Black. The detected bands were cut out and subjected to N-terminal sequencing in an Applied Biosystems model 477A protein sequencer equipped with an on-line model 120A PTH analyzer following standard operating procedures.

2.2.7. Gel filtration analysis of CT binding to PC/oleic acid vesicles

The association of CT with PC/oleic acid vesicles was analysed by gel filtration [6]. CT was incubated with PC/oleic acid vesicles (1:350 protein to lipid molar ratio, 4 mM total lipid) or with Triton X-100 micelles (10 mM) for 10 min at room temperature. [^{3}H]DPPC, or [^{14}C]oleic acid and [^{3}H]Triton X-100 were included in trace amounts. [^{3}H]Triton X-100 was vortexed with preformed vesicles, while [^{3}H]DPPC or [^{14}C]oleic acid was sonicated with the unlabeled lipids during vesicle preparation. The sample was loaded on a Sephacryl-S400 column (44.5 cm × 1 cm) that had been pre-equilibrated with column buffer (10 mM Tris, pH 7.5, 1 mM EDTA, 0.5 mM Triton X-100, 2 mM DTT) and run at 0.3 ml/min. Fractions (∼0.9 ml) were collected and samples analysed (1) for ^{3}H/^{14}C-labeled lipid by scintillation counting; and (2) for CT by gel electrophoresis and subsequent densitometry of the Coomassie-stained gels. The void volume and total volume of the column were identified using Blue-Dextran and [^{3}H]glycerol, respectively.

3. Results

3.1. Gel filtration analysis of CT binding to PC/oleic acid vesicles

Our objective was to photoreact CT with [^{125}I]TID under conditions where all of the CT was in the

membrane-bound form. The purified CT was obtained bound to Triton X-100 micelles, to which we added egg PC/oleic acid vesicles containing traces of [^{125}I]TID. The resulting PC/oleic acid/Triton X-100 ratio was 2:2:1. This mixture forms vesicles that fully activate CT [6]. Sephacryl S400 gel filtration chromatography was used to analyse the degree of binding of CT to the vesicles under photolysis conditions (Fig. 2). In the presence of Triton X-100 micelles alone, CT eluted slightly ahead of the peak of Triton X-100 radioactivity (Fig. 2A). Since Triton X-100 was in large excess (10 mM), the bulk of the detergent was in the form of a protein-free micelle (90 000 daltons). CT associates with Triton X-100 as a 84 kDa dimer [51], thus the few micelles containing protein would be significantly bigger and elute earlier. The size of Triton X-100 micelles containing a CT molecule was previously estimated at $\sim 2 \cdot 10^5$ daltons, by Sephacryl S-400 chromatography [6].

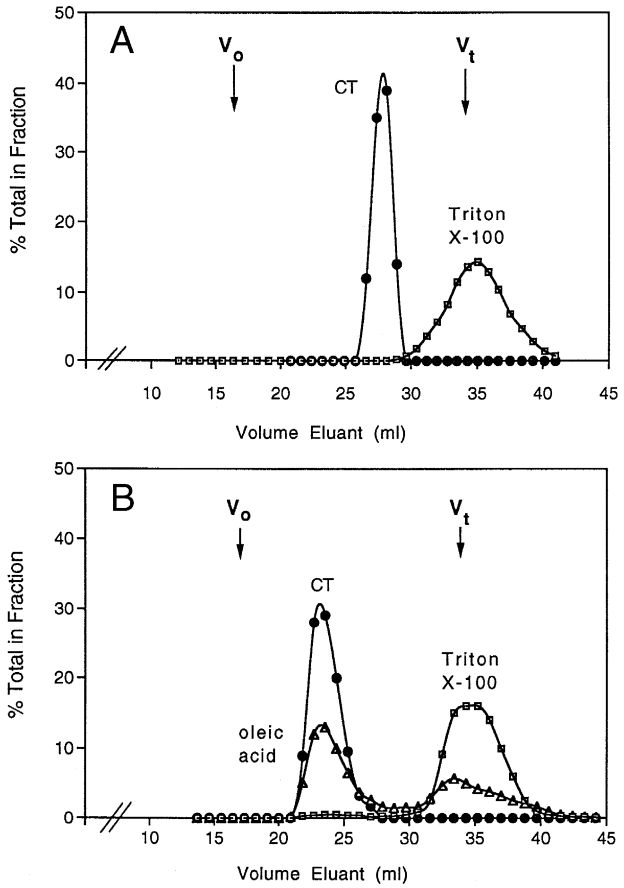

Fig. 2. Gel filtration analysis of the binding of CT to PC/oleic acid vesicles. The samples applied to the Sephacryl-S400 column were prepared and chromatographed, and fractions were analysed for radioactivity and CT as described in Section 2.2. [^{14}C]oleic acid was incorporated into the vesicles and [^{3}H]Triton X-100 was added to the samples in trace amounts. (A) Chromatography of CT plus 10 mM Triton X-100. (B) Chromatography of CT plus 4 mM PC/oleic acid (1:1) vesicles. The lipid to protein molar ratio in this sample was 350:1; the Triton X-100 content was 20 mol% of the total amphiphile. CT protein (●), [^{3}H]Triton X-100 (□), [^{14}C]oleic acid (△).

In the presence of PC/oleic acid/Triton X-100 (2:2:1), the conditions used for the photolabeling reaction, CT eluted earlier than in the presence of Triton X-100 alone (Fig. 2A and B). [^{14}C]Oleic acid was incorporated into the vesicles as a tracer. In some experiments, [^{3}H]DPPC was incorporated into the vesicles instead of [^{14}C]oleic acid, and eluted at the same volume as did the oleic acid, demonstrating that the two lipids remain complexed. [^{14}C]Oleic acid or [^{3}H]DPPC, along with a small amount of the Triton X-100, eluted early in the column presumably as large vesicles in the absence (data not shown) or presence of CT (Fig. 2B). In the presence of PC/oleic acid, CT eluted from the column as a single peak corresponding exactly to the peak of radiolabeled [^{14}C]oleic acid, and much earlier than the peak of ^{3}H-labeled Triton X-100 present in the sample. No CT protein eluted in fractions between 26 to 29 ml where CT was found when mixed with Triton X-100 only (Fig. 2A). We conclude that 100% of the CT in the sample was bound to PC/oleic acid vesicles under these conditions, which were the same as those used in the photolabeling experiment.

3.2. [^{125}I]TID labeling of CT in the presence of PC/oleic acid vesicles

Purified CT was photoreacted with [^{125}I]TID incorporated into PC/oleic acid vesicles (1:1). Irradiation of the enzyme under the conditions described did not affect the CT activity (data not shown). Electrophoresis was utilized to separate the protein from lipid, which migrated at the dye front and contained $>$ 99% of the label. As seen in Fig. 3, CT was significantly labeled by [^{125}I]TID. CT contained 0.03 to 0.05% of the total label added. When 0.2 mol% [^{125}I]TID was used, approximately 1 in 5000 CT molecules became labeled, while at 1 mol% [^{125}I]TID, an estimate of 1 in 650 CT monomers had label associated. The lack of stoichiometric labeling of CT

does not imply that 100% of the molecules present are not bound and intercalated in the membrane. TID photolabeling is a trace labeling technique. When TID is activated by irradiation, the carbene formed reacts quickly and with low specificity with any molecule in the vicinity. It can react with methylene groups of the acyl chains of lipid and, as the lipid is in large excess, the bulk of the label becomes lipid associated. To demonstrate the selectivity for membrane interacting proteins, a soluble protein, ovalbumin, was subjected to photolabeling under the same conditions as CT. Unlike CT, ovalbumin did not co-elute with lipid vesicles when analyzed by Sephacryl S-400 chromatography. Ovalbumin was not significantly labeled by the probe (Fig. 3B, lane 2). Apolipoprotein A-I, which associates with PC [32], is also thought to interact with membranes via an amphipathic helix [27]. When reacted with [^{125}I]TID in the presence of PC vesicles under the same conditions as CT, this protein was labeled to approximately the same extent as CT (Fig. 3B, lane 3).

In a similar set of experiments, vesicle-bound CT was photolabeled with a distinct hydrophobic probe, 11-[4-[3-(trifluoromethyl)diazirinyl]phenyl]-[2^{3}H]undecanoic acid ([^{3}H]TUA). This photoreactive

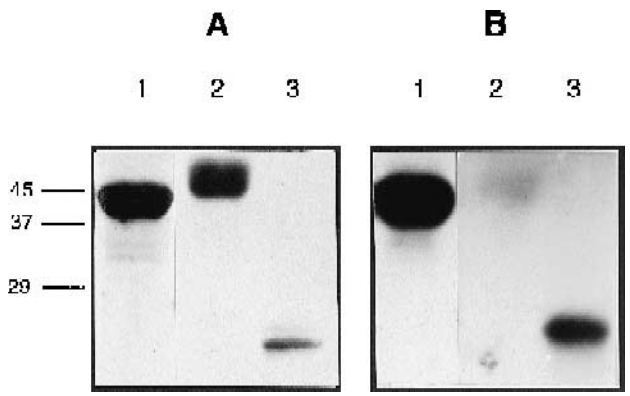

Fig. 3. [^{125}I]TID labeling of CT and control proteins. Proteins were labeled with [^{125}I]TID (0.2 mol%, 3 μCi) in vesicles as described in Section 2.2. Samples were electrophoresed on a 12% acrylamide gel. (A) Gel stained with Coomassie blue. (B) Autoradiograph of dried gel, exposed for 20 h. Lane 1: CT (20 μg) labeled in the presence of 0.75 mM PC/oleic acid (1:1). Lane 2: ovalbumin (12 μg) labeled in the presence of 0.75 mM PC/oleic acid (1:1). Lane 3: apolipoprotein A-I (11 μg) labeled in the presence of 0.5 mM PC. The positions of molecular weight standards are shown.

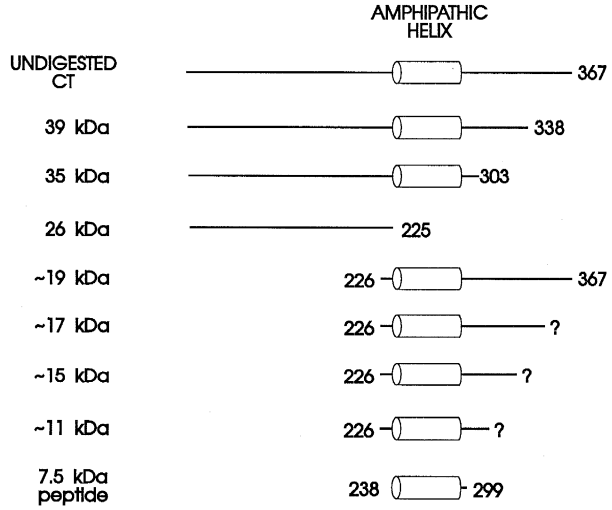

Fig. 4. Proposed CT fragments generated by chymotryptic digestion. The large 39, 35 and 26 kDa fragments were mapped by Craig et al. (1994). The N-terminal residues of the low molecular weight fragments of $M_{ap} = 19$, 17, 15 and 11 kDa were determined by N-terminal sequencing. The 19 kDa fragment contains the C-terminal residues (determined by antibody mapping, Fig. 6), and has a calculated molecular weight of 16200. The exact molecular weights of the smaller fragments remain undetermined. All low molecular weight fragments contain the amphipathic helix region as determined by antibody mapping.

fatty acid also labels membrane-embedded regions of proteins [52]. CT bound to PC/oleic acid vesicles was labeled by this probe, as was the control protein, apolipoprotein A-I. Ovalbumin was not labeled by [^{3}H]TUA (data not shown). Thus, the results obtained using two distinct membrane probes confirm that the membrane-bound form of CT is embedded in the hydrophobic core of activating membranes.

3.3. Chymotrypsin fragmentation pattern of CT

The products of partial proteolysis of CT by chymotrypsin have been previously characterized [19]. Under those conditions, CT was digested from the C-terminus to produce discrete fragments of $\sim$39, 35, 30, 28, and 26 kDa which contained the N-terminal domain but were missing different lengths of the C-terminal region, as mapped using antibodies directed against these regions. The C-terminal residue of each fragment was estimated (Fig. 4) based on its apparent molecular weight and chymotrypsin's selectivity for large, hydrophobic amino acids. The two

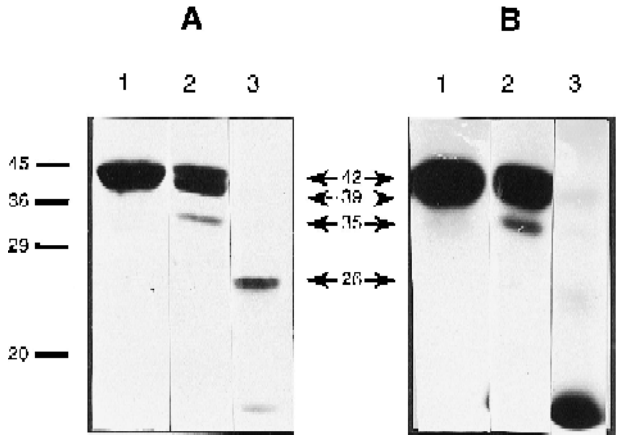

Fig. 5. Analysis of the high molecular weight fragments derived from [^{125}I]TID-labeled CT. CT (20 μg per lane) was photolabeled with [^{125}I]TID ($\sim$4 μCi) in the presence of 0.75 mM PC/oleic acid. Lane 1: undigested sample. Lane 2: digestion by an endogenous protease. Lane 3: digestion by chymotrypsin at a mass ratio of 100:1 (CT:chymotrypsin). Proteins were separated on a 12% gel as described in Section 2.2. (A) Gel stained with Coomassie blue. (B) Autoradiograph of dried gel, exposed for 20 h. The position of molecular weight standards is shown.

largest fragments contained the amphipathic helix region of CT, while the $\sim$30, 28 and 26 kDa fragment were missing most or all of this region. Under the current digestion and electrophoresis conditions, fragments of 39, 35 and one in the 30 to 26 kDa region were produced (Fig. 5A) [3], along with a faint band migrating immediately behind the dye front (Fig. 5A, lane 3).

To further investigate this band, we used a Tricine gel system, which effectively separates low molecular weight proteins/peptides. Separation revealed four previously uncharacterized low molecular weight CT fragments with apparent molecular weights of $\sim$19, 17, 15 and 11 kDa. (Fig. 6A). The largest of the fragments reacted with an antibody directed against the C-terminal 13 residues of CT (Fig. 6B) and all fragments reacted with an antibody directed against a 33 residue peptide of the amphipathic helix domain (Fig. 6C). Sequence analysis revealed that all four fragments have the same N-terminal sequence, beginning at Thr226. As the largest fragment (apparent mass = 19 kDa) reacted with the C-terminal antibody, it is assumed to comprise the entire C-terminal region, giving it a calculated molecular weight of 16 200. The apparent discrepancy in electrophoretic mobility may be attributed to its highly phosphorylated state [24,25]. The three smaller fragments appear to be produced by progressive digestion of the C-terminus from the 16.2 kDa fragment. The molecular weights of these fragments have been estimated at $\sim$14, 12 and 9 kDa based on potential cleavage sites. The lowest molecular weight fragment would comprise the amphipathic helix region with a $\sim$10 amino acid extension at the N-terminus. The exact C-terminal residue is not known. Thus, in addition to the susceptible C-terminal cleavage sites which yield the 39 and 35 kDa fragments, a main site for cleavage by chymotrypsin is at Tyr225, which produces an N-terminal 26 kDa fragment and a series of low molecular weight fragments derived from the C-terminal portion containing the amphipathic helix domain.

3.4. Localization of the [^{125}I]TID label on the CT protein

To establish which region of CT becomes membrane embedded, the protein was labeled with

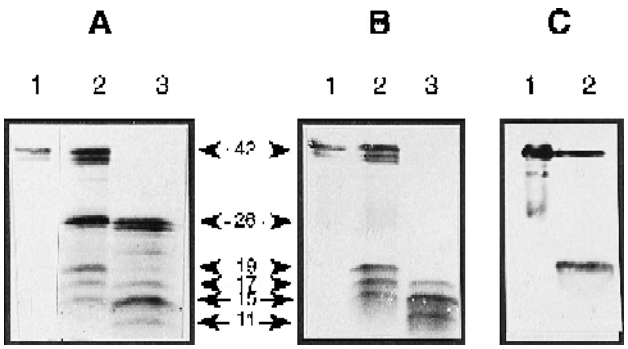

Fig. 6. Antibody mapping of the low molecular weight chymotryptic fragments of CT. Undigested CT (4 μg, lane 1), CT (12 μg) digested by chymotrypsin at 1:10 (chymotrypsin:CT) mass ratio (lane 2) or 1:8 ratio (lane 3), were electrophoresed on 16.5% T/3% C Tricine gel system as described in Section 2.2. The gel was Coomassie-stained (A), or transferred to PVDF membrane and immunoreacted with a 1:200 dilution of antibody directed against the amphipathic helix region (B), or a 1:1250 dilution of antibody directed against the C-terminal region (C).

[3] The purified CT contains an endogenous proteinase which partially digests the protein during the incubation at 37°C (Fig. 5A, lane 2). A number of different proteinases have been shown to produce similar fragmentation patterns for CT, suggesting that there are a few very sensitive sites in the native protein (Craig and Cornell, unpublished observation).

[^{125}I]TID in the presence of PC/oleic acid vesicles followed by digestion with chymotrypsin. The chymotryptic fragments were separated by electrophoresis and the label analysed by autoradiography (Fig. 5). Low proteinase concentration produced the 39 and 35 kDa fragments, both of which were labeled with [^{125}I]TID (Fig. 5A and B, lane 2). At high chymotrypsin/CT ratios, the 26 kDa fragment was the dominant fragment produced (Fig. 5A, lane 3). This fragment, which lacks the entire amphipathic helix region, had only a small amount of label associated (Fig. 5B, lane 3). The protein band near the bottom of the gel in Fig. 5A, lane 3, corresponds to the low molecular weight fragments of CT, and was highly labeled by [^{125}I]TID (Fig. 5B, lane 3).

The low molecular weight fragments of CT, photolabeled in the presence of PC/oleic acid vesicles, were separated using the Tricine gel system. Autoradiography revealed that the fragments at 19, 17, 15 and 11 kDa contained relatively high levels of [^{125}I]TID label (Fig. 7B). These fragments encompass the amphipathic helix region of CT. In a separate experiment, the label associated with each CT fragment was quantified (Table 1). The 39 and 35 kDa fragments contained as much or more label as the whole CT (110–170 cpm/μg), while the dominant Coomassie-stained 26 kDa fragment contained less label (70 cpm/μg). The low molecular weight fragments were more enriched in label than the whole CT molecule (500–1200 cpm/μg). This pattern is consistent with the amphipathic helix region, contained in the low molecular weight fragments but lacking in the 26 kDa fragment, playing the key role in membrane intercalation.

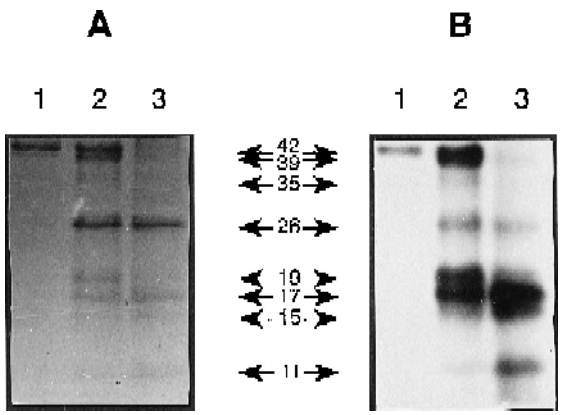

Fig. 7. Analysis of the low molecular weight fragments derived from [^{125}I]TID labeled CT. CT (20 μg) was photolabeled with [^{125}I]TID (0.2 mol%, ~3 μCi) in the presence of 0.75 mM PC/oleic acid. Lane 1: undigested sample (~1.5 μg). Lane 2: digestion by chymotrypsin at a mass ratio of 100:1 (CT/chymotrypsin). Lane 3: chymotrypsin/CT ratio of 1:30. Proteins were separated on a 16.5%T/3%C Tricine gel as described in Section 2.2. (A) Gel stained with Coomassie blue. (B) Autoradiograph of dried gel, exposed for 3 days.

Table 1
Extent of [^{125}I]TID labeling of the fragments of CT

Fragment	%Distribution of protein	CPM/μg protein	TID/protein molar ratio
Undigested CT	–	110	1: 4600
39	21	170	1: 3300
35	8	160	1: 4100
26	48	70	1:12000
19	2	1200	1: 1100
17	10	510	1: 3200
15	4	490	1: 3700
11	7	790	1: 3200
peptide	–	870	1: 3100

CT or peptide was photolabeled in the presence of PC/oleic acid with 0.2 mol% [^{125}I]TID. An aliquot of the CT sample was removed (undigested CT), the remainder digested with chymotrypsin, and the fragments separated by electrophoresis as described in Section 2.2. Protein was quantified by densitometry of the Coomassie stained gel, normalized to a CT standard. The percent distribution of protein mass among the fragments is given. ^{125}I was quantified by gamma-counting of the excised bands. Mol CT was estimated from the quantified mass of protein using the estimated true molecular weights for each fragment of 16, 14, 12 and 9 kDa. Moles of TID were calculated from CPM and specific activity of the [^{125}I]TID.

3.5. [^{125}I]TID Photolabeling of CT in the presence of PC/diacylglycerol vesicles or Triton X-100 micelles

In addition to anionic lipids, typified by PC/oleic acid vesicles, CT is also activated by membranes containing diacylglycerol. As synthetic peptide corresponding to the three 11-mer repeats [32] did not bind to PC vesicles containing diacylglycerol. This raised the possibility that the diacylglycerol-sensor is outside of the amphipathic helix. To probe the interaction of whole CT with diacylglycerol-containing membranes, [^{125}I]TID labeling of CT was attempted in the presence of egg PC/diacylglycerol vesicles

(4:1), using conditions for complete membrane association established for PC/oleic acid vesicles (Fig. 2). We also photolabeled CT in its Triton X-100 micelle-bound state (Triton:CT ratio ≅ 100). Photolabeling efficiencies were similar to that obtained using PC/oleic acid vesicles (data not shown). CT was subsequently digested with chymotrypsin and the fragments separated on the Tricine gel system. The fragmentation pattern of CT and [^{125}I]TID labeling patterns obtained in the presence of PC/diacylglycerol or Triton (data not shown) were identical to those obtained in the presence of PC/oleic acid (Fig. 7). The whole enzyme and the large 39 and 35 kDa fragments contained associated label. The low molecular weight fragments containing the amphipathic helix region of the enzyme contained the large majority of the label, while the 26 kDa fragment lacking this region was not highly labeled. Thus, the intercalation of CT into PC/diacylglycerol membranes or pure Triton micelles involves the same amphipathic helix region involved in interaction with anionic membranes.

3.6. Photolabeling of a peptide corresponding to the entire amphipathic helical domain

Analysis of digested, photolabeled CT supports our hypothesis for the amphipathic helix region as the membrane-binding domain. To further substantiate this model, we obtained a 62 residue peptide corresponding in sequence to the entire proposed amphipathic helical domain of CT (residues 238–299). This peptide has a molar partition coefficient for PC/oleic acid (1:1) vesicles of 2×10^5, and adopts an α-helical conformation in response to lipid vesicles or detergent micelles, as analysed by CD and 2D-NMR [66]. This peptide was photoreacted with [^{125}I]TID in presence of PC/oleic acid and was separated from lipid on a 16.5%T, 6% C Tricine gel system. The peptide was highly labeled by [^{125}I]TID (Table 1). The efficiency of [^{125}I]TID incorporation into the peptide was 0.05 to 0.08%, slightly higher than that obtained for the whole enzyme. The radioactivity per μg of peptide was similar to that obtained for the low molecular weight fragments of CT (Table 1). At 0.2 mol% [^{125}I]TID, 1 in 3100 peptide molecules became associated with [^{125}I]TID. These results confirm that this region of the protein can intercalate into membranes independently of the rest of the protein. We attempted N-terminal sequencing of [^{125}I]TID labeled peptide, as well as the low molecular weight chymotrypsin fragments, to identify the labeled amino acids, but the degree of labeling was insufficient, and all cycles in the Edman degradation contained background levels of radioactivity.

4. Discussion

4.1. The CT-membrane interaction involves intercalation into the hydrophobic membrane core

In this report, we have used the photoreactive probe [^{125}I]TID to investigate the mechanism of the CT-membrane interaction. The fact that [^{125}I]TID significantly labels CT suggests a mechanism of membrane interaction whereby the protein intercalates into the hydrophobic membrane core. It is well accepted that CT interacts with membranes, but the mechanism of membrane interaction is not known. There are several lines of evidence to suggest that the binding is not solely a peripheral charge interaction between protein and lipid head groups, but that an actual penetration to the hydrophobic core is involved. While ionic strength effects the enzyme activity [6], high salt is insufficient to extract enzyme from the membrane; detergents are needed [8]. Intercalation was postulated due to dependence of activity on a fluid membrane phase, enhancement of activity at the phase transition and dependence on bilayer curvature [10]. Intercalation has been demonstrated for synthetic peptides corresponding in sequence to a proposed membrane-binding region of the protein [32]. *Thus, although previous studies had suggested that the protein is embedded in the hydrophobic core of the membrane, this had never been demonstrated directly using the purified native enzyme.*

The degree of labeling with [^{125}I]TID is consistent with an interaction of CT involving membrane intercalation. While the [^{125}I]TID labeling efficiency depends on the mol percent and type of protein in the membrane as well as the reaction efficiency of TID under the irradiation conditions used, a range of 0.5–0.01% of the [^{125}I]TID generally becomes incor-

porated into membrane-embedded proteins [35]. The [^{125}I]TID labeling efficiency of CT, 0.03 to 0.05%, falls within this range. A bromelain-released soluble fragment of hemagglutinin lacking the trans-membrane anchor (BHA2) was [^{125}I]TID labeled with an efficiency of 0.025% using conditions similar to ours [43]. The key region of BHA2 labeled by [^{125}I]TID was a small membrane-interacting region proposed to be involved in fusion. Both CT and Apo A-I were labeled to a similar extent with [^{125}I]TID, and both are postulated to bind as amphipathic helices oriented with helix axes parallel to the membrane surface.

4.2. The amphipathic helix of CT is the key region involved in intercalation

The results obtained using proteolysis of CT in combination with photolabeling demonstrate the key role of the amphipathic helix region in the membrane intercalation of the protein. The 39 and 35 kDa N-terminal fragments containing this region were associated with the hydrophobic photolabel, while the 26 kDa fragment lacking this region contained a low level of labeling. We have identified low molecular weight fragments that had not been previously characterized [19] due to the gel conditions and antibodies used for detection in that study. These fragments, with molecular weights in the range of $\sim$9 kDa to 16 kDa, comprise the entire amphipathic helix region as well as various lengths of the C-terminal domain. The persistence of these fragments demonstrates the resistance of the amphipathic helix region to digestion, consistent with a membrane-bound location where it would be inaccessible to the proteinase. The high [^{125}I]TID labeling of the low molecular weight fragments comprising the amphipathic helix region demonstrates the key role for this region in CT membrane binding. A synthetic peptide corresponding in sequence to the amphipathic helix region became labeled to the same extent as these fragments, substantiating our conclusion that this region functions as the key membrane binding site for CT. This finding also validates the use of synthetic peptides as models for CT binding to anionic lipids.

The same region of CT is labeled by [^{125}I]TID whether the lipid vesicles contain oleic acid or diacylglycerol. Since the amphipathic peptide does not bind to diacylglycerol-containing vesicles [53] some region other than the amphipathic helix (residues 238–299) may be needed to impart sensitivity to diacylglycerol. Despite this difference, the same domain mediates intercalation into membranes containing either oleic acid or diacylglycerol. The amphipathic helix was also the key photolabeled domain of Triton X-100-bound CT. Since the enzyme is not activated by Triton X-100, this finding suggests that mere intercalation of the amphipathic helix into a hydrophobic environment is not sufficient for conversion to the active conformation.

All previous observations in addition to the present data are consistent with a mode of interaction involving an amphipathic helix domain lying with its axis parallel to the surface of membrane, with the non-polar face embedded to the acyl chain region of the membrane, and polar face interacting with lipid head groups, the aqueous solution or a distinct part of the protein. The amphipathic helix domain does not form a specific 'binding pocket' designed to interact with a specific head group or with lipid monomers as in the case of the C1 domain of protein kinase C [54] or the lipid binding sites of lipases [55,56], but rather forms a *relatively* non-specific, low affinity hydrophobic surface. With a structural reorganization, an amphipathic helix has the potential to convert between a structure compatible with a soluble or a membrane-bound form. For example, the solution form of insect apolipophorin is a five amphipathic-helix bundle with the hydrophobic faces in the interior which, in a reversible manner, spreads open on the membrane surface to expose the hydrophobic faces of the helices to the membrane without disrupting the secondary structure [57]. Similarly, the pore-forming amphipathic helix bundles of diphtheria toxin, colicin A, endotoxin, and exotoxin turn 'inside-out' to expose non-polar residues to the surface of membranes [58]. A tertiary structural reorganization that retains the secondary structure of the amphipathic helix could account for the conversion of CT between membrane-bound and soluble forms. The amphipathic helix region has been proposed to form an autoinhibitory domain, which would be displaced from the catalytic domain upon membrane binding, thereby activating the enzyme [33]. Unfortunately, the lipid free form of purified CT forms insoluble aggregates [51], which make it unamenable to TID photolabeling.

4.3. [^{125}I]TID weakly labels the catalytic domain of CT

There was some label found in the 26 kDa fragment, which appeared to be more than just background. [^{125}I]TID has a partition coefficient for egg PC or red cell membranes of 4×10^4 [37], which in our system (500 μl solution volume, $\sim$0.17 mg lipid) would result in 93% of the TID partitioning into the vesicles, and 7% into the aqueous phase. Lack of significant labeling of ovalbumin suggests that this small amount of free TID does not contribute to the labeling of soluble proteins. CT is not a soluble protein, and the proximity of the N-terminal region to the vesicle may contribute to the small amount of labeling. When labeling the integral F_o subunits of *Escherichia coli* ATP synthase, a peripheral F_1 subunit (β) became associated with a low level of label, suggesting that [^{125}I]TID can react with peripheral membrane proteins [59]. While the N-terminal region of CT does not associate with membranes on its own [19,25,33], a small portion could penetrate the membrane as this region is held in close proximity to the membrane surface by the amphipathic helix.

Non-membrane proteins have been photolabeled with [^{125}I]TID in the absence of lipid. It is thought that [^{125}I]TID probes for hydrophobic patches or pockets in the soluble proteins. Hydrophobic sites in calmodulin [60], troponin C [61] and lactalbumin [62] involved in the interaction with target proteins have been labeled by [^{125}I]TID in a Ca^{2+} dependent manner in the absence of lipids. A cytochrome *P*-450 [63], prostaglandin endoperoxide H synthase-1 [64], and an H^+-ATPase from yeast [65] were labeled in their membrane-embedded regions, and also in soluble domains containing hydrophobic substrate-binding pockets. While the active sites of cytochrome *P*-450 and PGHS-1 would be designed to accommodate hydrophobic substrates, the active site for the H^+-ATPase may more closely resemble the active site of CT, since both catalyze nucleotide reactions. Little is known about the environment of the active site of CT [23]. There may exist a hydrophobic pocket involved in substrate binding, or interaction with the hydrophobic portion of the amphipathic helix when the protein is in its soluble form.

In summary, the binding of CT to membranes involves an intercalation of the amphipathic α-helical region into the hydrophobic core of the membrane. The structural changes involved in this reversible interaction, and the mechanism whereby these changes activate the enzyme are now key questions in the elucidation of the regulation this enzyme.

Acknowledgements

We thank Dr. Adrienne Drobnies for preparation of the antibodies raised against the amphipathic helix, Dr. Ian Clark-Lewis and Philip Owen for the peptide synthesis, Dr. Steven Holdcroft for use of the mercury lamp, and Dr. Claudia Kent and James MacDonald for providing *T. ni* cells, CT recombinant baculovirus and antibody raised against the C-terminal region of CT. We thank Dr. J. Brunner for providing the [^{3}H]TUA photolabeling probe. This work was funded by a grant from the Medical Research Council of Canada (to R.B.C.) and by the National Science Foundation (USA) Science and Technology Center for Molecular Biotechnology (to R.A.). J.E.J. was supported by a Medical Research Council of Canada Studentship.

References

[1] Pelech, S.L. and Vance, D.E. (1989) Trends Biochem. Sci. 14, 28–30.
[2] Walkey, C.J., Kalmar, G.B. and Cornell, R.B. (1994) J. Biol. Chem. 269, 5742–5749.
[3] Tronchere, H., Planat, V., Record, M., Terce, F., Ribbes, G. and Chap, H. (1995) J. Biol. Chem. 270, 13138–13146.
[4] Pelech, S.L., Pritchard, P.H., Brindley, D.N. and Vance, D.E. (1983) J. Biol. Chem. 258, 6782–6788.
[5] Feldman, D.A. and Weinhold, P.A. (1987) J. Biol. Chem. 262, 9075–9081.
[6] Cornell, R.B. (1991) Biochemistry 30, 5873–5880.
[7] Cornell, R. and Vance, D.E. (1987) Biochim. Biophys. Acta 919, 26–36.
[8] Cornell, R. and Vance, D.E. (1987) Biochim. Biophys. Acta 919, 37–48.
[9] Slack, B.E., Breu, J. and Wurtman, R.J. (1991) J. Biol. Chem. 266, 24503–24508.
[10] Cornell, R.B. (1991) Biochemistry 30, 5881–5888.
[11] Sohal, P.S. and Cornell, R.B. (1990) J. Biol. Chem. 265, 11746–11750.
[12] Arnold, R.S. and Cornell, R.B. (1996) Biochemistry, 35, 9177.

[13] Wang, Y., MacDonald, J.I.S. and Kent, C. (1993) J. Biol. Chem. 268, 5512–5518.
[14] Houweling, M., Jamil, H., Hatch, G.M. and Vance, D.E. (1994) J. Biol. Chem. 269, 7544–7551
[15] Weinhold, P.A., Charles, L. and Feldman, D.A. (1994) Biochim. Biophys. Acta 1210, 335–347.
[16] Shiratori, Y., Houweling, M., Zha, X. and Tabas, I. (1995) J. Biol. Chem. 270, 29894–29903.
[17] Wang, Y. and Kent, C. (1995) J. Biol. Chem. 270, 17843–17849.
[18] Kalmar, G.B., Kay, R.J., Lachance, A., Aebersold, R. and Cornell, R.B. (1990) Proc. Natl. Acad. Sci. 87, 6029–6033.
[19] Craig, L., Johnson, J.E. and Cornell, R.B. (1994) J. Biol. Chem. 269, 3311–3317.
[20] Tsukagoshi, Y., Nikawa, J. and Yamashita, S. (1987) Eur. J. Biochem. 169, 477–486.
[21] Mauel, C., Young, M. and Karamata, D. (1991) J. Gen. Microbiol. 137, 929–941.
[22] Park, Y.S., Sweitzer, T.D., Dixon, J.E. and Kent, C. (1993) J. Biol. Chem. 268, 16648–16654.
[23] Veitch, D.P. and Cornell, R.B. (1996) Biochemistry 35, 10743–10750.
[24] MacDonald, J.I.S. and Kent, C. (1994) J. Biol. Chem. 269, 10529–10537.
[25] Cornell, R.B., Kalmar, G.B., Kay, R.J., Johnson, M.A., Sanghera, J.S. and Pelech, S.L. (1995) Biochem. J. 310, 699–708.
[26] Yang, W. and Jackowski, S. (1995) J. Biol. Chem. 270, 16503–16506.
[27] Segrest, J.P., Garber, D.W., Brouillette, C.G., Harvey, S.C. and Anantharamaiah, G.M. (1994) Adv. Protein Chem. 45, 303–369.
[28] Rozek, A., Buchko, G.W. and Cushley, R.J. (1995) Biochemistry 34, 7401–7408.
[29] Picot, D., Loll, P.J. and Garavito, R.M. (1994) Nature 367, 243–249.
[30] Gilbert, G.E. and Baleja, J.D. (1995) Biochemistry 34, 3022–3031.
[31] Epand, R.M., Shai, Y., Segrest, J.P. and Anantharamaiah, G.M. (1995) Biopolymers (Peptide Science) 37, 319–338.
[32] Johnson, J.E, and Cornell, R.B. (1994) Biochemistry 33, 4327–4335.
[33] Wang, Y. and Kent, C. (1995) J. Biol. Chem. 270, 18948–18952.
[34] Wieder, T., Geilen, C.C., Wieprecht, M., Becker, A. and Orfanos, C. (1994) FEBS Lett. 345, 207–210.
[35] Brunner, J. (1993) Annu. Rev. Biochem. 62, 483–514.
[36] Brunner, J., Senn, H. and Richards, F.M. (1980) J. Biol. Chem. 255, 3313–3318.
[37] Brunner, J. and Semenza, G. (1981) Biochemistry 20, 7174–7182.
[38] Brunner, J., Franzusoff, A.J., Luscher, B., Zugliani, C. and Semenza, G. (1985) Biochemistry 24, 5422–5430.
[39] Meister, H., Bachofen, R., Semanza, G. and Brunner, J. (1985) J. Biol. Chem. 260, 16326–16331.
[40] Blanton, M.P. and Cohen, J.B. (1992) Biochemistry 31, 3738–3750.
[41] Benfenati, F., Greengard, P., Brunner, J. and Bahler, M. (1989) J.Cell Biol. 108, 1851–1862.
[42] Ulbrandt, N.D., London, E. and Oliver, D.B. (1992) J. Biol. Chem. 267, 15184–15192.
[43] Harter, C., Bachi, T., Semenza, G. and Brunner, J. (1988) Biochemistry 27, 1856–1864.
[44] Harter, C., James, P., Bachi, T., Semenza, G. and Brunner, J. (1989) J. Biol. Chem. 264, 6459–6464.
[45] Clark-Lewis, I., Moser, B., Walz, A., Baggiolini, M., Scott, G.J. and Aebersold, R. (1991) Biochemistry 30, 3128–3135.
[46] Myher, J. and Kuksis, A. (1979) Can. J. Biochem. 57, 117–124.
[47] Ziltener, H., Clark-Lewis, I., Hood, L., Kent, S. and Schrader, J. (1987) J. Immunol. 138, 1099–1104.
[48] MacDonald, J.I.S. and Kent, C. (1993) Prot. Express. Purif. 4, 1–7.
[49] Laemmli, U.K. (1970) Nature 227, 680–685.
[50] Schagger, H. and Von Jagow, G. (1987) Anal. Biochem. 166, 368–379.
[51] Cornell, R.B. (1989) J. Biol. Chem. 264, 9077–9082.
[52] Brunner, J. (1989) FEBS Lett. 257, 369–372.
[53] Johnson, J.E. (1995) PhD dissertation.
[54] Zhang, G., Kazaniets, M., Blumberg, P. and Hurley, J. (1995) Cell 81, 917–924.
[55] Van Tilbeurgh, H., Sarda, L., Verger, R. and Cambillau, C. (1992) Nature 359, 159–162.
[56] Dennis, E.A. (1994) J. Biol. Chem. 269, 13057–13060
[57] Breiter, D.R., Kanost, M.R., Benning, M.M., Wesenberg, G., Law, J.H., Wells, M.A., Rayment, I. and Holden, H.M. (1991) Biochemistry 30, 603–608.
[58] Parker, M.W. and Pattus, F. (1993) Trends Biochem. Sci. 18, 391–395.
[59] Hoppe, J., Brunner, J. and Jorgensen, B.B. (1984) Biochemistry 23, 5610–5616.
[60] Krebs, J., Buerkler, J., Guerini, D., Brunner, J. and Carafoli, E. (1984) Biochemistry 23, 400–403.
[61] Buerkler, J. and Krebs, J. (1985) FEBS Lett. 182, 167–170.
[62] Van Ceunebroeck, J.C.L., Krebs, J., Hanssens, I. and Van Cauwelaert, F. (1986) Biochem. Biophys. Res. Comm. 138, 604–610.
[63] Frey, A.B., Kreibich, G., Wadhera, A., Clarke, L. and Waxman, D.J. (1986) Biochemistry 25, 4797–4803.
[64] Otto, J. C. and Smith, W. L. (1996) J. Biol. Chem. 271, 9906–9910.
[65] Seto-Young, D., Monk, B.C. and Perlin, D.S. (1992) Biochim. Biophys. Acta 1102, 213–219.
[66] Dunne, S., Cornell, R.B., Johnson, J.E., Glover, N. and Tracey, A. (1996) Biochemistry 35, 11975–11984.