Identification of a putative membrane-interacting domain of CTP: Phosphocholine cytidylyltransferase from rat liver

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Identification of a putative membrane-interacting domain of CTP:phosphocholine cytidylyltransferase from rat liver

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Abstract

A putative membrane-interacting domain of CTP:phosphocholine cytidylyltransferase (CT) was identified using two peptide-specific antibodies. One antibody (SA2) was raised against the N-terminus of CT (amino acid residues 1–17) and the other antibody (SA209) against an α-helical domain of the enzyme (amino acid residues 247–257). Both antibodies quantitatively immunoprecipitated CT from rat liver cytosol and showed specificity towards CT when octylglucoside extracts of rat liver cytosol were assessed by Western blot analysis. However, further experiments revealed that the antibodies had different characteristics. Whereas the antibody directed against the N-terminus of CT (SA2) did not influence CT/membrane interaction, the new antibody (SA209) against the α-helical domain of the enzyme interfered with this interaction. Our results provide experimental evidence that the α-helical domain (amino acid residues 228–287) of CT may serve as a membrane-interacting domain.

Key words: CTP:phosphocholine cytidylyltransferase; Membrane binding; Peptide-specific antibody

1. Introduction

In mammals, CTP:phosphocholine cytidylyltransferase (EC 2.7.7.15) (CT) is the rate-limiting enzyme in the biosynthesis of phosphatidylcholine (PC) via the CDP-choline pathway [1–4]. Its activity is regulated by translocation of the enzyme between membranes (active form) and cytosol (inactive form) [5]. Regulation of the translocation process by changes in the lipid composition of cellular membranes seems to be one of the major mechanisms for the control of PC biosynthesis [6–14]. To investigate the translocation process of CT in more detail antibodies were produced using tryptic fragments of CT as the immunogen [15]. Furthermore, two peptide-specific antibodies against different domains of CT were introduced [16,17]. Recently, the cDNA for rat liver CT was cloned and sequenced. The sequence did not contain any signal for covalent linkages to lipids or any hydrophobic domains necessary for a membrane-spanning helix. Only an extensive amphipathic α-helix (amino acid residues 228–287), which contains three 11-mer repeats in tandem, could serve as a membrane interacting domain [18].

To test this hypothesis, we used a peptide-specific antibody against one part of the postulated membrane-binding domain (amino acid residues 247–257). In the present study, we investigated CT/membrane interaction by using this peptide-specific antibody in two experimental systems, the indirect membrane interacting sandwich ELISA and the activation of purified CT by liposomes. Both systems showed that the antibody against amino acid residues 247–257 (SA209) influenced CT/membrane interaction. Our results confirm the hypothesis that the amphiphathic α-helix represents the membrane-binding domain of CT.

2. Materials and methods

2.1. Materials

[*methyl*-^{14}C]Phosphocholine (2.04 GBq/mmol) and the ECL immunoblotting detection reagent were from Amersham (Braunschweig, Germany). The BCA kit for protein determination was from Pierce (Weiskirchen, Germany). Phosphatidylcholine, oleic acid, octylglucopyranosid, *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester, phenylenediamine and protein A–Sepharose were obtained from Sigma (München, Germany). Peroxidase-conjugated swine anti-rabbit antibody was from Dakopatts (Hamburg, Germany) and the nitrocellulose membrane (0.45 μm) was from Schleicher & Schüll (Dassel, Germany). Silicagel 60 high-performance thin layer chromatography plates and reagents were purchased from Merck (Darmstadt, Germany). For quantification of radioactivity a thin-layer chromatography scanner (Berthold LB2821 HR, Wildbad, Germany) was used.

2.2. Peptide synthesis and characterization

The peptide MDAQSSAKVNSRKRRKE (CT peptide 1–17), corresponding to the amino-terminal 17 amino acids of rat liver CT, and the peptide DKVKKKVKDVE (CT peptide 247–257), corresponding to amino acids 247–257 of rat liver CT, both with a cysteine added for coupling to a carrier, were synthesized by automated solid-phase peptide synthesis (Model 431A, Applied Biosystems, Weiterstadt,

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Abbreviations: BCA, bicinchoninic acid; BSA, bovine serum albumin; CT, CTP:phosphocholine cytidylyltransferase; ECL, enhanced chemiluminescence; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; PC, phosphatidylcholine; SA2, polyclonal peptide-specific antibody against cytidylyltransferase residues 1–17; SA209, polyclonal peptide-specific antibody against cytidylyltransferase residues 247–257; S.D., standard deviation.

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Germany) using 9-fluorenylmethoxycarbonyl (Fmoc) chemistry with [2-(1-H-benzotriazol-1-yl)-1,1,3,3-tetramethyl-uronium hexafluorophosphate] (HBTU) activation of amino acids. The purity of the peptides was assessed on an analytical reverse-phase column (Eurospher 80 C 18, 5 μm; Knauer Säulentechnik, Berlin, Germany). A 60-min linear gradient from 0 to 30% acetonitrile was used for separation and the absorbance at 215 nm was monitored. The purity of the peptides was > 90%.

The peptides were coupled to keyhole limpet hemocyanin, through their additional cysteine residues with *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester as described [17].

2.3. Antibody production

Antibodies against CT peptide 1–17 (SA2) and against CT peptide 247–257 (SA209) were prepared by Eurogentec (Seraing, Belgium) using a standard protocol [16]. Briefly, the synthetic peptide fragments of CT conjugated with keyhole limpet hemocyanin (1,000 μg each) were injected at several sites into two rabbits, initially with complete Freund's adjuvant followed by injection of peptide conjugates (500 μg each) in incomplete Freund's adjuvant every 2 weeks. 10 days after the third booster injection the blood of the two rabbits was taken and sera were obtained by centrifugation at 8,000 × *g* for 20 min. 100 μl aliquots of the sera were stored at −20°C.

Preimmune sera were taken from the rabbits before they were immunized with CT peptide 1–17 or with CT peptide 247–257 and likewise stored at −20°C. In the experiments of this study, preimmune serum of the rabbit which was immunized with CT peptide 247–257 was used as a negative control.

2.4. Purification of rat liver cytidylyltransferase

CT was partially purified from rat liver cytosol as described [19] using acetic acid precipitation, octylglucoside extraction followed by hydroxylapatite column chromatography. Purified CT was stored at −70°C.

2.5. Cytidylyltransferase assay

Prior to determination of CT activity, 50 μl of purified enzyme preparation and 50 μl of rabbit serum SA2, rabbit serum SA209 or of preimmune serum were mixed and incubated for 3 h at 4°C. 50 μl buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 10 mM $MgCl_2$) was used as a control. Then, CT activity was measured by a modified method of Sohal and Cornell [20]. The reaction mixture contained 50 mmol/l Tris-HCl, pH 7.4, 0.03% Triton X-100, 100 mmol/l NaCl, 10 mmol/l $MgCl_2$, 3 mmol/l CTP, 1.5 mmol/l [*methyl*-^{14}C]phosphocholine (spec. act. 20 Bq/nmol), liposomes (0.4 mM oleic acid/0.4 mM PC) and 10 μl of enzyme preparation in a final volume of 55 μl. After incubating for 30 min at 37°C the reaction was stopped by freezing the samples in liquid nitrogen. The samples were lyophilized, dissolved in 20 μl methanol/water (1:1, v/v) and applied to high-performance thin layer chromatography plates. After developing the plates in a solvent system containing methanol/0.6% NaCl/25% aqueous NH_3 (8:5:1, by vol.), the radioactivity was determined by radioscanning. One unit of enzyme activity is defined as 1 nmol of CDP-choline formed per min.

2.6. Immunoprecipitation of cytidylyltransferase

Immunoprecipitation of CT from rat liver cytosol was performed as described [16]. Briefly, 100 μl cytosol was incubated for 2 h at 4°C with different volumes of the SA2 and SA209 sera or preimmune serum as control. 5 mg of protein A–Sepharose was added to each sample to precipitate the CT–antibody complexes, and the samples were incubated overnight at 4°C. The protein A–Sepharose was sedimented at 13,000 × *g* for 2 min and CT activity in the supernatant was assessed as described above.

2.7. Immunoblotting

30 μl of the octylglucoside extract of rat liver cytosol or 10 μl of purified CT were mixed with SDS sample buffer and separated by 10% SDS-PAGE [21]. After transfer to nitrocellulose membrane the blot was incubated with 5% non-fat dry milk in PBS for 1 h at room temperature to block non-specific binding. The membrane was incubated with serum SA2 (1:1,000), serum SA209 (1:1,000 or 1:3,000) or preimmune serum (1:1,000) in PBS containing 0.1% Tween-20 overnight at 4°C. After washing with PBS/Tween-20 three times for 5 min, bound antibody was detected using horseradish peroxidase-conjugated anti-rabbit immunglobulin antibodies and the ECL detection reagents according to the manufacturer's instructions.

2.8. Indirect membrane interacting sandwich ELISA

The ELISA technique was performed essentially as described [22]. 100 μl of antibody SA2 (1:500 in PBS) were incubated in 96 well ELISA plates for 1 h at 37°C and fixed with 0.25% glutardialdehyde for another 30 min at room temperature. After washing with PBS, the wells were saturated with PBS alone, PBS/0.05% Tween-20 or PBS/1% BSA to block non-specific binding. The wells were incubated with different concentrations of rat liver cytosol in a total volume of 200 μl overnight at 4°C. After washing, wells were incubated for 1 h at 37°C with liposomes (0.2 mM oleic acid/0.2 mM PC in H_2O) or H_2O as a control. Then, CT was detected using SA209 antibody; CT was incubated for 1 h at 37°C with 100 μl of SA209 antibody (1:250 in PBS, 1:250 in PBS/0.05% Tween-20 or 1:250 in PBS/1% BSA as described above) in the presence or in the absence of liposomes followed by incubation for 1 h at 37°C with a peroxidase-conjugated swine anti-rabbit antibody (1:2,000 in PBS). The wells were thoroughly washed and the amount of peroxidase-conjugated swine anti-rabbit antibody was assessed using 200 μl substrate solution (0.8 mg/ml phenylenediamine, 0.015% H_2O_2 in 0.1 M citrate buffer, pH 6.0). After 10 min at 37°C the reaction was stopped with 50 μl 1.25 M sulfuric acid and the absorbance was measured at 492 nm. The specific absorbance of each sample (E_{492}) was calculated as the difference between the absorbance in the presence and the absorbance in the absence of rat liver cytosol. The assay was linear in the range of 10–100 μl of cytosol.

In some experiments, the SA209 antibody was coated to the wells and the SA2 antibody was used to detect antibody SA209-bound CT in the presence or in the absence of liposomes.

2.9. Other procedures

Rat liver cytosol was prepared by sequential centrifugation of the homogenate of 10 g liver in 50 ml buffer A (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1.0 mM EDTA, 2.0 mM dithiothreitol, 0.025% sodium azide) at 10,000 × *g* for 20 min and 100,000 × *g* for 60 min. The protease inhibitor, phenylmethylsulfonylfluoride, was added to achieve a 1.0 mM final concentration. Liposomes were prepared as described [20] by sonication for 3 min at 4°C using a 375 W sonicator (Heat Systems, Plainview, NY, USA) at 20% power output. Vesicles prepared in this way were small and unilamellar [20]. Protein was determined by the BCA assay [23] using BSA as standard. Statistical comparisons were made in these studies by Student's *t*-test.

3. Results and discussion

3.1. Characterization of the antibodies

To characterize the new antibody against amino acid residues 247–257 of CT (SA209) Western blots of octylglucoside extracts of rat liver cytosol were analysed. Fig. 1 shows that the SA209 antibody in dilutions of 1:1,000 and 1:3,000 specifically reacts with CT. As a positive control we used the antibody against amino acids 1–17 of CT (SA2) as described by Watkins and Kent [17]. The SA2 antibody is not as specific as the SA209 antibody. Especially, an additional 50 kDa protein is detected (Fig. 1). This is in accordance with results using another antibody against amino acids 1–17 of CT that was made available by Dr. C. Kent (Ann Arbor, USA) (data not shown).

Recently, the nuclear localization of CT was reported using this antibody against amino acids 1–17 of CT [24], and there exists some controversy about the hypothesis that most of the enzyme resides in the cytosol [25]. Because of its specificity, the SA209 antibody will be a useful tool to immunolocalize CT by electron microscopy.

The SA209 antibody quantitatively precipitated CT

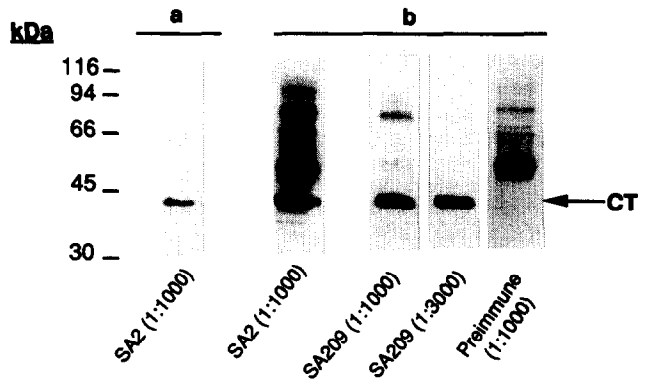

Fig. 1. Western blot analysis of cytidylyltransferase. Proteins obtained from the partially purified preparation of CT after hydroxylapatite column chromatography (a) or octylglucoside extraction (b) were separated on 10% SDS-gels and subjected to Western blot analysis as described in section 2. The blots were probed with either SA2 serum (1:1,000), SA209 serum (1:1,000 and 1:3,000) or preimmune serum as indicated in the figure.

from rat liver cytosol (Fig. 2). There seemed to be a substantial activation of CT at lower amounts of added serum. However, this effect was not significant ($P < 0.2$). In contrast, the addition of 50 μl or 100 μl preimmune serum to rat liver cytosol did not influence CT activity in the supernatant after precipitation of antibodies with protein A–Sepharose. Similar results were obtained using the SA2 antibody (data not shown).

3.2. Measurement of cytidylyltransferase/membrane interaction by sandwich ELISA technique

The interaction of CT with membranes was investigated using liposomes in a sandwich ELISA. CT from rat liver cytosol was bound to SA2 antibody coated to microtitre wells. Then, CT was detected with SA209 antibody and a peroxidase-conjugated swine anti-rabbit antibody. As shown in Fig. 3, the binding of SA209 was significantly reduced in the presence of liposomes (0.2 mM oleic acid, 0.2 mM PC). When non-specific binding was blocked with PBS or with PBS/0.05% Tween-20 there was a reduction of the signal by 90% and 45%, respectively, as compared with controls. The reduced binding of SA209 indicates that the epitope represented by amino acids 247–257 is masked in the presence of liposomes, supposedly by specific binding of these liposomes to the α-helical domain. On the other hand, when non-specific binding was blocked with PBS/1% BSA the binding of antibody SA209 was not affected. This is not surprising since it has been reported that oleic acid-stimulated translocation of CT to membranes is reversed by the addition of excess BSA [24,26].

In another set of experiments, CT was bound to SA209 antibody coated to microtitre wells and detected with antibody SA2. As shown in Fig. 3, the detection of CT with antibody SA2 was not influenced by liposomes, indicating that the effect of oleic acid-containing liposomes is not due to non-specific interactions with the surface of the protein.

With higher concentrations of lipid activators, e.g. concentrations of 1 mM oleic acid/1 mM PC, the effect of liposomes on the detection of CT, as assessed by the sandwich ELISA technique, was no longer specific for the SA209 antibody (data not shown).

Table 1
Influence of SA2 and SA209 antibodies on liposome-activated cytidylyltransferase activity

Pre-incubation	Activation of CT (%)
Buffer (control)	147 ± 16
Serum SA2	134 ± 14
Serum SA209	91 ± 5*
Preimmune serum	131 ± 6

CT (50 μl) partially purified by hydroxylapatite chromatography from rat liver cytosol was pre-incubated for 3 h at 4°C with 50 μl of SA2 serum, 50 μl of SA209 serum, 50 μl of preimmune serum or 50 μl of buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 10 mM $MgCl_2$) as a control. Then, CT activity of the different probes was measured in the presence of liposomes (0.4 mM oleic acid, 0.4 mM PC). The values are given in % activation of unstimulated CT (in the absence of liposomes) and were calculated as [(stimulated CT activity)−(unstimulated CT activity)] × [100/(unstimulated CT activity)]. The data represent the mean of three determinations ± S.D. Unstimulated CT activity was 3.45 ± 0.7 U/ml and stimulated CT activity under control conditions was 8.53 ± 0.9 U/ml. *Significantly different from controls at $P < 0.01$.

3.3. Inhibition of liposome-stimulated cytidylyltransferase activity

In a different approach to investigate the CT/mem-

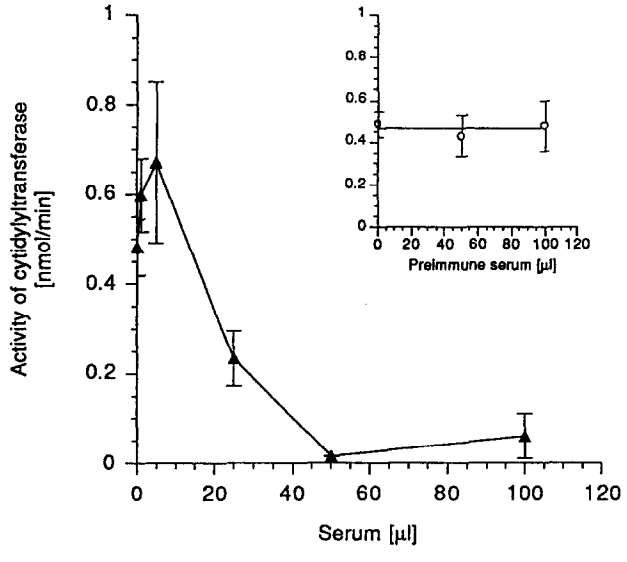

Fig. 2. Immunoprecipitation of cytidylyltransferase from rat liver cytosol. 100 μl of rat liver cytosol were incubated with increasing amounts of SA209 serum or preimmune serum in a final volume of 200 μl. Then, CT was precipitated as described in section 2 and the remaining CT activity in the supernatant was measured. Each point represents the mean of three determinations ± S.D.

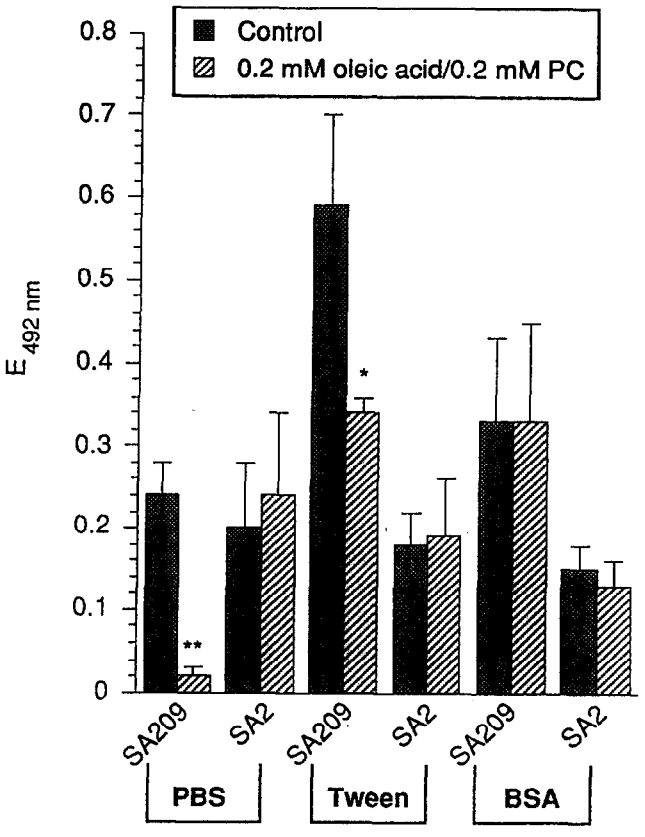

Fig. 3. Cytidylyltransferase/membrane interaction determined by sandwich ELISA technique. 50 μl of rat liver cytosol were used in a sandwich ELISA as described in section 2. CT was detected using SA209 antibody (1:500) or SA2 antibody (1:500) in the absence (control) or in the presence of liposomes (0.2 mM oleic acid/0.2 mM PC). The assay was performed under different blocking conditions (PBS, PBS/0.05% Tween-20 or BPS/1% BSA) as indicated in the figure. The data represent the mean of three determinations ± S.D.; *significantly different from controls at $P < 0.02$; **significantly different from controls at $P < 0.001$. The experiment was repeated, and similar results were obtained.

brane interaction, purified CT was pre-incubated with SA2 serum, SA209 serum, preimmune serum or buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 10 mM $MgCl_2$) as a control. Afterwards, CT was activated using liposomes (0.4 mM oleic acid/0.4 mM PC). Only preincubation with SA209 serum significantly reduced activation of CT by liposomes (Table 1). Interestingly, the SA209 antibody inhibited the activation of CT by liposomes but did not affect the basal activity of unstimulated CT (data not shown).

In conclusion, our data substantiate the hypothesis of Kalmar et al. [18] that an unbroken α-helix from Lys-228 to Glu-287, or a part thereof, may constitute the membrane-binding domain of rat liver CT. During the preparation of this manuscript, the membrane-binding domain of CT was further characterized using chymotryptic proteolysis [27]. The data presented were fully consistent with our findings. In this context, the SA209 antibody, which is directed against CT-peptide 247–257, will be a useful tool to investigate the interaction of CT with membrane lipids.

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