Identification of a Protein Complex between Choline-phosphate Cytidyltransferase and a 112-kDa Protein in Rat Liver*

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Antisera raised against purified cytidylyltransferase (CT) immunoprecipitated CT activity from liver cytosol and detected the M₄₅,000 subunit of CT on Western blots. Antisera detected a M₄₁₂,000 protein on Western blots of liver cytosol. This protein was not detected in purified CT and was not detected by preimmune serum. The 112-kDa antibodies, isolated by affinity chromatography, did not immunoprecipitate CT activity. Antiserum raised against an N-terminal sequence of CT and antibodies raised against an internal sequence of CT immunoprecipitated CT activity but did not detect the 112-kDa protein. These results showed that the 112-kDa protein was not a form of CT. We concluded that the antiserum probably contained anti-idiotypic antibodies that recognized CT binding sites on the 112-kDa protein. Purified CT that was conjugated to horseradish peroxidase bound to crude 112-kDa protein immobilized on nitrocellulose. The binding was competitively reduced by purified CT and by affinity-purified antibodies to the 112-kDa protein. CT and 112-kDa protein eluted from DEAE-Sepharose. When the putative 112-kDa protein-CT complex was chromatographed on a second DEAE-Sepharose column or on a Bio-Gel A-1.5m column, CT activity and 112-kDa protein were eluted together. Chromatography of the complex on hydroxylapatite dissociated most of the complex, producing CT free of the 112-kDa protein. We conclude that the 112-kDa protein is a CT-binding protein. The formation and/or dissociation of this complex may be important in the regulation of CT.

The biosynthesis of phosphatidylyceroline can be regulated at the step in the pathway catalyzed by CTP:choline-phosphate cytidylyltransferase. A translocation mechanism has emerged as a central feature in most regulatory models (for reviews, see Refs. 1–3). In this mechanism inactive enzyme in cytosol is activated by binding to membranes. Extensive studies have led to several suggestions concerning the signal(s) initiating translocation. For example, it has been proposed that translocation is initiated by decreases in the phosphatidylyceroline content in membranes (4–6), and by increases in cellular fatty acid concentrations (9–10). In addition, cytidylyltransferase can be phosphorylated (18–20). Indirect evidence suggests that phosphorylation may control the binding of cytidylyltransferase to membranes (19, 20). However, the involvement of phosphorylation is unclear because neither phorbol esters nor cAMP alters the overall extent of cytidylyltransferase phosphorylation although phosphatidylyceroline synthesis is changed (8, 18, 20).

In most instances, it has been assumed that translocation is initiated by modulation of membrane properties. Although this is a reasonable assumption, it is possible that the binding of cytidylyltransferase to membranes is initiated through alteration in the properties or forms of soluble enzyme. Moreover, there is evidence indicating that cytidylyltransferase can be activated in cytosol without apparent translocation (22–24). Other results have suggested the existence of a latent form of cytidylyltransferase (25).

In an attempt to clarify the function of cytosolic forms of cytidylyltransferase, we previously explored the relationships between the inactive dimer form of cytidylyltransferase (L-Form) and the active, lipid-aggregated form (H-Form) (26–28). The results from these studies suggest that the cytosolic L-Form binds to membranes thereby generating the active H-Form on the membrane. In some instances active H-Form may be released from membranes resulting in active cytidylyltransferase in cytosol. Although these results provide evidence for the potential importance of active cytidylyltransferase in cytosol, they do not clarify the signal mechanism(s) that initiated the activation of L-Form.

In the present studies, we used polyclonal antisem raised against purified cytidylyltransferase to detect forms of the cytidylyltransferase in cytosol. These antisem preparations detected a protein in liver cytosol with an apparent molecular mass of 112,000 daltons. Because this protein may have represented a new form of cytidylyltransferase, we proceeded to more fully characterize the immunodetection of the 112-kDa protein by the antisem. We present in this paper the results of these studies, demonstrating that the 112-kDa protein binds cytidylyltransferase and appears to be part of a protein complex form of cytidylyltransferase.

**EXPERIMENTAL PROCEDURES**

Materials and Analytical Methods—We obtained [¹⁴CCH₃]phosphocholine, CDP-[¹⁴CCH₃]choline, and ECL peroxidase detection reagent from Amersham Corp. Phosphatidylyceroline, oleic acid, insoluble protein A (lyophilized *Staphylococcus aureus* cell powder, P 951), N-
octyl-b-glucopyranoside (octyl glucoside), goat anti-rabbit IgG conjugated to horseradish peroxidase, and rabbit IgG were obtained from Sigma. Hydroxylapatite and Affi-Prep 10 were purchased from Bio-Rad. Cytidylyltransferase was purified from rat liver as described previously (29). Antiserum (N-terminal) raised against a synthetic peptide corresponding to an N-terminal sequence of CT (31) was provided by Dr. Claudia Kent, University of Michigan. Antibody (CT peptide antibody) raised against an internal sequence of CT (20) was provided by Dr. Dennis Vance, University of Alberta, Edmonton, Alberta, Canada.

Cytidylyltransferase activity was determined by measuring the formation of radioactive CDP choline formed from [14CH_3]phosphocholine as previously described (29). Protein was determined by the Markwell modification of the Lowry assay (30) using bovine serum albumin as the standard.

Preparation of Cytidylyltransferase Antiserum—We purified cytidylyltransferase from 100 g of rat liver. Two of these preparations were combined and concentrated by vacuum dialysis against 50 mM Tris, pH 7.5, containing 0.15 M NaCl, and 0.05% Triton X-100. The solution was dried in a Savant vacuum centrifuge. We dissolved the residue in 0.5 ml of water. The sample contained about 200 μg of protein. The enzyme solution was mixed with 1.5 ml of complete Freund's adjuvant and injected subcutaneously into a rabbit. After 18 h at 4°C, the mixture subcutaneously in multiple sites on the back of a young female rabbit. After 6 weeks, we injected the rabbit with 50 μg of enzyme protein mixed with complete Freund's adjuvant for additional times at 3-week intervals. Two weeks after the last injection (after the original injection), blood was drawn and serum was prepared. We stored the serum at −70°C. This antiserum was designated CT antiserum II.

We also raised antiserum against cytidylyltransferase that was purified from the first hydroxylapatite column (29). This cytidylyltransferase was not completely pure (32). It contained a significant residue in 0.5 ml of water. The sample contained about 200 pg of cytidylyltransferase activity was determined in the supernatant. The suspension of insoluble protein A was prepared by homogenizing 1.0 g of liver in 5.0 ml of Buffer A.

Approximately 600 nmol/min enzyme activity in 600 μl of elution buffer was mixed with 1.5 mg of Immunopur-activated peroxidase (Pierce Chemical Co.). We adjusted the pH to 9.5 with 25 mM NaOH. The reaction mixture was incubated overnight at 4°C. The mixture was quenched with 100 ml of 50 mM Tris, pH 7.5, containing 0.05% Triton X-100, at room temperature. The reaction mixture was diluted 20-fold with 25 mM Tris, pH 7.5, containing 75 mM NaCl and 1 mM EDTA and applied onto a column of DEAE-Sepharose (500-pl bed volume) equilibrated with 0.1 M NaCl, 1 mM EDTA, and 0.05% Triton X-100. The column was washed with an additional 12-15 ml of equilibration buffer. CT-HRP was eluted with 300 mM potassium phosphate, pH 7.5, containing 150 mM NaCl, 1 mM EDTA, and 0.05% Triton. We eluted the column with a flow rate of 2 ml/h into siliconized glass tubes. Approximately 600 nmol/min enzyme activity in 600 μl of elution buffer was mixed with 1.5 mg of Immunopur-activated peroxidase (Pierce Chemical Co.). We adjusted the pH to 9.5 with 25 mM NaOH. The reaction mixture was incubated overnight at 4°C. The mixture was quenched with 100 ml of 50 mM Tris, pH 7.5, containing 0.05% Triton X-100, at room temperature. The reaction mixture was diluted 20-fold with 25 mM Tris, pH 7.5, containing 75 mM NaCl and 1 mM EDTA and applied onto a column of DEAE-Sepharose (500-pl bed volume) equilibrated in the same buffer. The sample was applied at a flow rate of 8 ml/h. Unreacted horseradish peroxidase was removed by the column. The column was washed with an additional 12-15 ml of equilibration buffer. CT-HRP was eluted with 0.30 M potassium phosphate, pH 7.5, containing 0.15 M NaCl, 1 mM EDTA, and 0.05% Triton, at a flow rate of 2 ml/h. Column fractions were collected into siliconized glass tubes. Peroxidase activity was determined by measuring the rate of oxidation of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (35).

Cytidylyltransferase Binding to 112-kDa Protein—We measured the binding of cytidylyltransferase conjugated with horseradish peroxidase (CT-HRP) to protein samples containing the 112-kDa protein.
tein. We used fractions collected from the DEAE-Sepharose columns as a source of 112-kDa protein. We selected fractions that contained 112-kDa protein with low amounts of cytidylyltransferase activity (fractions 125–128 from the DEAE-Sepharose column shown in Fig. 10). Samples were diluted to 0.2 ml with 25 mM Tris, 0.5 M NaCl pH 7.4 (TBS), and were applied to nitrocellulose using a slot blot device (Bio-Rad). The blot was removed from the device, cut into 6 mm strips, and blocked for 2 h in 100 ml of TBS containing 5% milk. The nitrocellulose strips were then incubated with CT-HRP in 2.0 ml of TBS containing 3% milk, 0.1% bovine serum albumin, and 0.1% Tween 20, in a siliconized Plexiglass incubation tray (Bio-Rad). The strips were washed five times in TBS containing 0.1% Tween 20. Peroxidase activity was detected with ECL reagent.

RESULTS

Immunodetection of a 112-kDa Protein by Cytidyltransferase Antiserum—All three antiserum immunoprecipitated cytidylyltransferase activity in liver cytosol with nearly equivalent titer (Fig. 1). The three cytidylyltransferase antisera also immunoprecipitated cytidylyltransferase activity in the cytosol fraction from lung, heart, brain, and kidney and preparations of purified cytidylyltransferase (data not shown). The antiserum raised toward the 38-kDa protein did not immunoprecipitate cytidylyltransferase activity.

We compared the ability of the four antisera to detect the 45-kDa subunit of cytidylyltransferase on Western blots prepared from SDS-polyacrylamide gels (Fig. 2). All three cytidylyltransferase antisera detected the 45-kDa subunit in purified cytidylyltransferase. Antiserum I1 immunoprecipitated cytidylyltransferase activity from liver cytosol (Fig. 3R). Thus, the CT-Affi-Prep column preferentially adsorbed a population of antibodies that were specific for cytidylyltransferase. To test this possibility, we fractionated antiserum II on a cytidylyltransferase affinity column (CT-Affi-Prep) and on a 112-kDa protein affinity column (112-Affi-Prep). We collected the effluent material and IgG fraction from both columns. These preparations were used to immunoprecipitate cytidylyltransferase activity from liver cytosol and to immunostain purified cytidylyltransferase and partially purified 112-kDa protein on native slot blots. The serum proteins that did not bind to the CT-Affi-Prep (CT effluent) failed to immunoprecipitate cytidylyltransferase activity (Fig. 3A) and did not detect purified cytidylyltransferase on slot blots (Fig. 4). The antibodies in the CT effluent detected the 112-kDa protein on native slot blots (Fig. 4). The antibody fraction that was eluted from the CT-Affi-Prep (CT IgG) immunoprecipitated cytidylyltransferase activity in liver cytosol (Fig. 3B). Thus, the CT-Affi-Prep column preferentially adsorbed a population of antibodies that were specific for purified cytidylyltransferase. On the other hand, the serum proteins that did not bind to the 112-Affi-Prep (112 effluent) immunoprecipitated cytidylyltransferase activity (Fig. 3A) and detected purified cytidylyltransferase (Fig. 4).

Fig. 1. Immunoprecipitation of enzyme activity in liver cytosol by cytidylyltransferase antisera. Samples of liver cytosol containing 0.38 nmol/min enzyme activity were incubated with varied aliquots of rabbit antisera as described under “Experimental Procedures.”
Cytidylyltransferase Binding to 112-kDa Protein

![Graph 1](image1)

**Fig. 3.** Immunoprecipitation of cytidylyltransferase activity by fractions from a cytidylyltransferase affinity column and a 112-kDa affinity column. Cytidylyltransferase antiserum II was incubated overnight at 4 °C with either Affi-Prep coupled to purified cytidylyltransferase (CT-Affi-Prep) or Affi-Prep coupled to partially purified 112-kDa protein (112-kDa Affi-Prep). In each case, 500 μl of coupled Affi-Prep was mixed with 200 μl of antiserum (diluted to 2.0 ml with TBS) in a 0.8 × 8-cm column on a rotary mixing wheel. The Affi-Prep was then allowed to settle and the column was eluted. The original volume in the mixture was collected as the CT effluent or 112-kDa effluent. The columns were then washed and bound IgG was eluted as described under "Experimental Procedures." A, immunoprecipitation of cytidylyltransferase activity in liver cytosol by CT effluent and 112-kDa effluent. B, immunoprecipitation of cytidylyltransferase activity in liver cytosol by antibodies bound to either the CT affinity column or the 112-kDa affinity column. The antibody preparations were compared on the basis of IgG concentrations. The IgG was determined by a slot blot immunoaasay using anti-rabbit IgG for detection of IgG. After development with ECL reagent, the densities of the bands on autoradiographic film were measured. The amount of IgG was calculated from a standard curve of rabbit IgG.

![Graph 2](image2)

**Fig. 4.** Immunodetection of cytidylyltransferase and 112-kDa protein by fractions eluted from either a CT affinity column or a 112-kDa affinity column (see Fig. 3 for details). Purified cytidylyltransferase (0.3 nmol/min) or crude 112-kDa protein (0.84 μg of protein) was applied to nitrocellulose membranes with a slot blot device. Slot blots containing cytidylyltransferase or 112-kDa proteins were probed with: A, antiserum II (6.5 μg/ml protein); B, CT effluent (7.8 μg/ml protein); C, 112-kDa effluent (6.1 μg/ml protein); D, 112-kDa IgG (0.3 μg/ml IgG).

![Graph 3](image3)

**Fig. 5.** Immunoprecipitation of cytidylyltransferase activity in liver cytosol by N-terminal antiserum and C-peptide antibody. The incubations with N-terminal antiserum contained 0.43 nmol/min cytidylyltransferase activity. Incubation with C-peptide antibody contained 0.07 nmol/min cytidylyltransferase activity.

The 112-kDa effluent did not detect the 112-kDa protein (Fig. 4). The antibody fraction that was eluted from the 112-Affi-Prep column (112-kDa IgG) did not immunoprecipitate cytidylyltransferase activity (Fig. 3B). The 112-kDa IgG detected the 112-kDa protein and weakly detected purified cytidylyltransferase (Fig. 4). These data suggested that the 112 affinity column preferentially adsorbed a population of antibodies that were specific for the 112-kDa protein. The inability of affinity-purified 112-kDa antibodies and the CT effluent to immunoprecipitate cytidylyltransferase activity coupled with the fact that both preparations detected the 112-kDa protein indicated that the 112-kDa protein was not an active form of cytidylyltransferase.

The 112-kDa protein could be an inactive, precursor form of cytidylyltransferase. If this were true, the 112-kDa protein would include the amino acid sequence of the 45-kDa cytidylyltransferase protein. To assess this possibility, we evaluated the ability of antibody preparations, raised toward synthetic peptides corresponding to amino acid sequences in purified cytidylyltransferase, to detect the 112-kDa protein. Both antibodies (N-terminal and C-peptide) immunoprecipitated cytidylyltransferase activity in liver cytosol (Fig. 5). The N-terminal antiserum exhibited a 20-fold greater titer toward cytidylyltransferase activity than antiserum II. Both the N-terminal antiserum and the CT-peptide antibody detected the 45-kDa subunit of cytidylyltransferase in liver cytosol (Fig. 6, lanes 2 and 4) and in purified cytidylyltransferase (Fig. 6, lanes 3 and 5). Antiserum II detected the 112-kDa protein in liver cytosol (Fig. 6, lane 1). Neither N-terminal antiserum nor CT-peptide antibody detected the 112-kDa protein in liver cytosol (Fig. 6, lanes 2 and 4). The results argue strongly against the possibility that the 112-kDa protein was an inactive form of cytidylyltransferase. Furthermore, the results support the conclusion, derived from the CT-Affi-Prep and 112-Affi-Prep columns, that the 112-kDa protein was not an active form of cytidylyltransferase.

Since the 112-kDa protein was not an active or inactive form of cytidylyltransferase and was not present in purified cytidylyltransferase, we considered the possibility that the
Western blot immunodetection of cytidylyltransferase and 112-kDa protein by cytidylyltransferase antibodies raised against an N-terminal cytidylyltransferase peptide (N-terminal antiserum) and an internal peptide of cytidylyltransferase (CT-peptide antibody). Liver cytosol (0.21 nmol/min cytidylyltransferase activity and 60 μg of protein) was applied to lanes 1, 2, and 4. Purified cytidylyltransferase (0.20 nmol/min activity) was applied to lanes 3 and 5. Lane 1 was probed with antiserum I (1:50000). Lanes 2 and 3 were probed with N-terminal antiserum (1:50000). Lanes 4 and 5 were probed with CT peptide antibody (1:100).

detection of 112-kDa protein by the cytidylyltransferase antiserum was due to the presence of anti-idiotypic antibodies. These antibodies could have been generated toward the cytidylyltransferase binding region of the CT IgG. The binding domains on these anti-idiotypic antibodies would mimic regions in the cytidylyltransferase molecule and therefore may recognize cytidylyltransferase binding sites on proteins that bind to cytidylyltransferase. This scenario is analogous to the binding of hormone anti-idiotypic antibodies to hormone receptors (36, 37). Anti-idiotypic antibodies have been reported to arise in response to immunization with a primary antigen (38, 39). If the detection of the 112-kDa protein by our antiserum is due to the presence of anti-idiotypic antibodies, the 112-kDa protein may be a protein that specifically binds cytidylyltransferase.

Cytidylyltransferase Binding to the 112-kDa Protein—Positive evidence for direct cytidylyltransferase binding to the native form of the 112-kDa protein was obtained using cytidylyltransferase conjugated with horseradish peroxidase (CT-HRP). CT-HRP bound to protein fractions containing partially purified 112-kDa protein, applied to slot blots on nitrocellulose membrane (Fig. 7). The amount of bound CT-HRP increased with increasing amounts of 112-kDa protein applied to the membrane. The 112-kDa protein samples contained some cytidylyltransferase activity. To control for the possibility that CT-HRP was binding to the cytidylyltransferase activity in the sample, we applied purified cytidylyltransferase (0.1–1.0 nmol/min) to nitrocellulose and probed with CT-HRP. There was no binding of CT-HRP (data not shown). The amount of CT-HRP bound to the 112-kDa protein was competitively reduced by addition of purified cytidylyltransferase to the incubation mixture (Fig. 8). If the 112-kDa IgG was generated toward the binding region of the cytidylyltransferase IgG, then the 112-kDa IgG should also competitively reduce the binding of CT-HRP to the 112-kDa protein. The results in Fig. 9 show that the amount of CT-HRP bound to the membrane was competitively reduced by addition of 112-kDa IgG to the incubation mixture. These results provided evidence that the native form of the 112-kDa protein binds purified cytidylyltransferase.

We attempted to detect binding of CT-HRP to the 112-kDa protein by Western blots from SDS-electrophoretic gels. Samples of partially purified 112-kDa protein were collected from a DEAE-Sepharose column. (Peak 2, Fig. 10). Aliquots of 112-kDa protein preparation were applied to a nitrocellulose membrane using a slot blot device, as follows: lane 1, 1 μg of protein and 0.2 nmol/min enzyme activity; lane 2, 2.5 μg and 0.5 nmol/min; lane 3, 5 μg and 1.0 nmol/min. Each row of samples was cut into a separate strip of membrane. After blocking, the strips were incubated with the following amounts of cytidylyltransferase-horseradish peroxidase conjugate (CT-HRP). Row A, 0.36 absorbance units of peroxidase; row B, 0.91 units; row C, 2.8 units. Row D was incubated with 9.0 units peroxidase carried through the coupling procedure without cytidylyltransferase.

kDa protein on Western blots from SDS-electrophoretic gels. Samples of partially purified 112-kDa protein were collected from a DEAE-Sepharose column. Protein samples were fractionated by SDS-polyacrylamide gel electrophoresis and electrotransferred to PVDF membranes on nitrocellulose. After blocking, the membranes were probed with CT-HRP. The CT-HRP did not detect the 112-kDa protein. We repeated...
the experiment using purified cytidylyltransferase to probe the PVDF membranes. The possible binding of cytidylyltransferase to the 112-kDa protein was assessed with N-terminal antiserum to detect bound cytidylyltransferase. Cytidylyltransferase binding to the 112-kDa protein also could not be demonstrated using this experimental design. Apparently cytidylyltransferase cannot bind to denatured 112-kDa protein or the binding requires the association of two or more 112-kDa subunits.

**Isolation of Protein Complexes of Cytidylyltransferase from Liver Cytosol**—The standard procedure for purification of cytidylyltransferase from liver cytosol begins with the addition of phosphatidylcholine mixed with oleic acid (0.5 mM phosphatidylcholine and 1.0 mM oleic acid). This is followed by precipitation by acidification to pH 5.0 with acetic acid. The enzyme activity is resolubilized with octyl glucoside and fractionated by chromatography on DEAE-Sepharose, followed by chromatography on hydroxylapatite.

We used antiserum II to detect the 112-kDa protein in fractions eluted from DEAE-Sepharose columns during the purification of cytidylyltransferase. Preliminary studies suggested that the 112-kDa protein eluted coincident with cytidylyltransferase activity. We modified the DEAE-Sepharose elution procedure to optimize the separation of apparent forms of cytidylyltransferase. This procedure resulted in the separation of four peaks of cytidylyltransferase activity (Fig. 10). Each of the cytidylyltransferase peaks also contained 112-kDa protein. Peak 1 contained low cytidylyltransferase activity (not readily observed in Fig. 10 but apparent if the data were plotted on a smaller scale). Peak 2 was eluted at the end of a 0.15-0.3 M NaCl gradient. Peak 3 was eluted with a gradient of 0.3-0.4 M NaCl. The largest amount of cytidylyltransferase activity and 112-kDa protein was eluted with 0.4 M NaCl, 50 mM octyl glucoside. These results suggested the existence of a 112-kDa protein-cytidylyltransferase complex.

We obtained additional evidence for the existence of stable 112-kDa protein-cytidylyltransferase complex by fractionation of Peak 2 on a second column of DEAE-Sepharose. All of the detectable cytidylyltransferase activity and 112-kDa protein in Peak 2 was recovered at nearly the same elution position found on the original DEAE-Sepharose column (Fig. 11A). The data showed that the complex in Peak 2 was stable and was not converted to the other observed forms of the complex. The addition of phosphatidylcholine-oleic acid to Peak 2 caused both proteins to become more tightly adsorbed on a second DEAE-Sepharose column (Fig. 11B). The two proteins were eluted simultaneously by 50 mM octyl glucoside. The elution position coincided with that of Peak 4 on the initial DEAE-Sepharose column. The data provided additional support for the existence of a specific protein complex containing cytidylyltransferase. Gel filtration chromatography on Bio-Gel A-1.5m agarose was used to further characterize the relationship between the two proteins in Peak 2 from the DEAE-Sepharose column. The 112-kDa protein and cytidylyltransferase activity eluted as a high molecular weight complex at an elution position corresponding to a molecular mass of approximately 600,000-650,000 daltons (Fig. 12). The 112-kDa protein was detected only in the fractions containing cytidylyltransferase activity. The results provided additional evidence supporting the existence of a stable 112-kDa protein-cytidylyltransferase complex. The results also suggested that the complex may exist either as a high molecular weight aggregate or in a highly asymmetric form.

**Separation of 112-kDa Protein-Cytidylyltransferase Complex on Hydroxylapatite**—In the purification of cytidylyltransferase, hydroxylapatite chromatography of Peak 4 from the DEAE-Sepharose column results in nearly pure enzyme. The purified enzyme is eluted by Triton X-100 after a series of salt and octyl glucoside gradient elutions. As shown in Fig. 10, Peak 4 contained both cytidylyltransferase activity and 112-kDa protein. We separated Peak 4 on hydroxylapatite. The 112-kDa protein and 45-kDa subunit of cytidylyltransferase were determined by Western blot immunodetection. Cytidylyltransferase activity was measured by direct assay. Triton X-100 eluted over 90% of the cytidylyltransferase activity and 45-kDa subunit recovered from the column (Fig. 13). This was expected from previous results on the purification of cytidylyltransferase (29). The purified enzyme contained no detectable 112-kDa protein, consistent with the results shown in Fig. 2. The 112-kDa protein was eluted in four peaks. The first three peaks contained cytidylyltransferase (determined by both activity and Western blot immunodetection). These three peaks apparently contained 112-kDa protein-cytidylyltransferase complex. The fourth peak of 112-kDa protein appeared to consist of only 112-kDa protein.
Cytidylyltransferase Binding to 112-kDa Protein

Fig. 11. Elution of cytidylyltransferase peak 2 complex on DEAE-Sepharose column. Panel A, cytidylyltransferase activity that eluted from a DEAE-Sepharose column during the addition of 0.50 M NaCl (Peak 2) was diluted 1:1 with 50 mM Tris, 1 mM EDTA, 2 mM DTT, pH 7.5. The sample, containing 280 nmol/min enzyme activity, was applied to a column of DEAE-Sepharose (1.6 x 6 cm) equilibrated with 50 mM Tris, 150 mM NaCl, 1 mM EDTA, 2 mM DTT, pH 7.5 (Buffer A). The column was eluted with 75 ml of Buffer A, followed by a 200-ml linear gradient of NaCl (0.15–0.40 M) in Buffer A. At fraction 68, the column was eluted with Buffer A containing 0.40 M NaCl. At fraction 88, the column was eluted with Buffer A containing 30 mM octyl glucoside (OG) and 0.40 M NaCl. Each fraction contained 2.75 ml. The inset below panel A shows the 112-kDa protein immunodetected by antiserum II on Western blots. Panel B, DEAE Sepharose Peak 2 was diluted 1:1 with 50 mM Tris, 1 mM EDTA, 2 mM DTT, pH 7.5, and incubated with a mixture of phosphatidylycholine and oleic acid at a final concentration of 0.30 and 0.60 mM, respectively. The sample, contained 1340 nmol/min enzyme activity and was applied to a column of DEAE-Sepharose (2.6 x 11.5 cm), equilibrated with 50 mM Tris, 150 mM NaCl, 1 mM EDTA, 2 mM DTT, pH 7.5 (Buffer A). The column was eluted with a 400-ml linear gradient of NaCl (0.15–0.30 M) in Buffer A. At fraction 80, the column was washed with 300 ml of Buffer A containing 0.30 M NaCl. At fraction 140, the column was eluted with Buffer A containing 30 mM octyl glucoside and 0.30 M NaCl. The inset below panel B shows the immunodetection of 112-kDa protein as described above.

DISCUSSION

In this study we have identified a protein in liver cytosol that binds cytidylyltransferase. The evidence supporting this conclusion is derived from three independent types of experimental results: immunological detection, direct binding of cytidylyltransferase-horseradish peroxidase conjugate, and coincident elution of the 112-kDa protein and cytidylyltransferase during chromatography on DEAE-Sepharose, hydroxylapatite, and Bio-Gel A-1.5m.

The immunodetection of the 112-kDa protein by cytidylyltransferase antiserum presented the first indication that the 112-kDa protein might bind cytidylyltransferase. The immunodetection was specific for the cytidylyltransferase antiserum, since the 112-kDa protein was not detected by preimmune serum or by antiserum raised against another protein isolated in the final step of cytidylyltransferase purification. The antibodies for the 112-kDa protein appeared to be a response to cytidylyltransferase and not to 112-kDa protein contamination in the purified cytidylyltransferase used to raise the antiserum. The response to the booster injection of cytidylyltransferase (disproportionate induction of cytidylyltransferase antibodies compared to 112-kDa antibodies) and the affinity isolation of antibodies both showed that the 112-kDa antibodies were distinct from the cytidylyltransferase antibodies. The results from the chromatography of antiserum on cytidylyltransferase and 112-kDa affinity columns and the inability of N-terminal and CT-peptide antibodies to detect the 112-kDa protein suggested that the 112-kDa protein was not an active or inactive species of cytidylyltransferase. One remaining possibility was that the antibodies raised against the 112-kDa protein were anti-idiotypic antibodies produced against the anti-cytidylyltransferase IgG. We reasoned that if this were true, the 112-kDa protein may be a cytidylyltransferase-binding protein. With this premise, we proceeded to develop methods to directly assess whether the 112-kDa protein would specifically bind cytidylyltransferase. Simultaneously, we determined the distribution of the 112-kDa protein during the purification of cytidylyltransferase. Both approaches provided data suggesting that the 112-kDa protein bound cytidylyltransferase. The HRP-labeled cytidylyltransferase bound to a partially purified preparation of 112-kDa protein. The observation that the binding was competitively decreased by cytidylyltransferase showed a specificity toward cytidylyltransferase. The fact that the binding was also competitively reduced by 112-kDa IgG suggested that the binding sites for cytidylyltransferase and 112-kDa IgG were similar. This result is consistent with the premise that the 112-kDa IgG is an anti-idiotypic antibody that mimics a binding site on cytidylyltransferase.

The 112-kDa protein coeluted with cytidylyltransferase from DEAE-Sepharose. These results suggested that a 112-kDa protein-cytidylyltransferase complex exists. The coelution of cytidylyltransferase and 112-kDa protein upon subsequent chromatography on DEAE-Sepharose, Bio-Gel A-1.5m agarose and hydroxylapatite provided further support. Taken together, the results provided convincing evidence that a protein with a molecular mass of 112,000 daltons (determined under denaturing conditions) binds cytidylyltransferase and forms a stable protein complex.

The elution of apparent 112-kDa protein-cytidylyltransferase complexes in multiple, distinct peaks from both DEAE-Sepharose and hydroxylapatite suggests that different forms of the complex may exist. This could result from differences in the relative amounts of 112-kDa protein and cytidylyltransferase or the presence of other proteins and/or lipids. The fact that the complex in Peak 2 from DEAE-Sepharose was induced to elute in the Peak 4 region by the addition of phosphatidylycholine-oleic acid provided evidence for the involvement of lipids with the complex. During the development
Fig. 12. Elution of 112-kDa protein-cytidylyltransferase complex from a Bio-Gel A-1.5m gel filtration column. The 112-kDa protein-cytidylyltransferase complex eluted from a second DEAE-Sepharose column (Fig. 11A) was concentrated to 0.5 ml by centrifugal ultrafiltration (Centricon 10, Amicon). A 0.4-ml sample (88 nmol/min cytidylyltransferase activity) was applied to a Bio-Gel column (1.6 × 46 cm) equilibrated in 50 mM Tris, 150 mM NaCl, 1 mM EDTA, 2 mM DTT, pH 7.5. The column was eluted in the equilibration buffer. The 112-kDa protein was detected on Western blots by immunostaining with antiserum I. The relative amount of 112-kDa protein was determined by densitometric analyses of autoradiograms. The elution position for the void volume (V₀) and internal volume (Vₗ) of the column are shown with arrows along with the elution positions of thyroglobulin (TG), Immunoglobulin (IgG), and ovalbumin (Oval).

Fig. 13. Elution of 112-kDa protein-cytidylyltransferase complex from hydroxylapatite. Peak 4 from a DEAE-Sepharose column (Fig. 10) was diluted with 2.5 volumes of 50 mM Tris, 150 mM NaCl, 1 mM EDTA, 2 mM DTT, pH 7.5 (Buffer A). The sample contained 3400 nmol/min cytidylyltransferase activity and 3.5 mg of protein and was applied to a column of hydroxylapatite (1.6 × 4.7 cm), equilibrated with Buffer A. Fraction collection was begun with sample application. The column was eluted as follows: A, 100 ml of 150 mM potassium phosphate; B, 100 ml of 200 mM potassium phosphate; C, 100-ml gradient of 0–50 mM octyl glucoside in 200 mM potassium phosphate; D, 50 ml of 50 mM octyl glucoside in 200 mM potassium phosphate; E, 50 ml of 100 mM octyl glucoside in 200 mM potassium phosphate; F, 60 ml of 0.03% Triton X-100 in 200 mM potassium phosphate (collected into siliconized glass tubes). All solutions were made in Buffer A. Cytidylyltransferase activity was assayed in selected fractions. The 112-kDa protein and 45-kDa protein were detected on Western blots of SDS-polyacrylamide gels by immunostaining with antiserum II or N-terminal antiserum, respectively. The concentration of the two proteins was estimated by densitometric analysis of the x-ray film, after exposure of the Western blot to ECL reagent.
of the purification procedure for cytidylyltransferase, we observed that the addition of phosphatidycholine-oleic acid microemulsions early in the procedure was required for subsequent binding of cytidylyltransferase to DEAE-Sepharose in a form that could only be eluted by detergent. Conceivably, the purification of cytidylyltransferase by this method may depend upon the stabilization of the 112-kDa protein-cytidylyltransferase complex by the addition of lipids. The association of lipids with the complex may impart hydrophobic properties that allow the complex to be separated from the bulk of other proteins. The dissociation of the complex by octyl glucoside coupled with the removal of lipids during hydroxylapatite chromatography left the nascent, hydrophobic cytidylyltransferase tightly bound to the hydroxylapatite to be finally eluted by Triton X-100.

The metabolic significance of the 112-kDa protein cytidylyltransferase complex is unknown. One possibility is that the 112-kDa protein is a chaperone type protein for cytidylyltransferase. The concept of chaperone proteins has been increasingly applied to cell biology problems involving protein assembly and transport (40). Chaperone proteins are defined as a family of proteins that bind partially unfolded proteins until the polypeptide chains can fold into active proteins (either in a partially unfolded, inactive form or perhaps a single inactive monomeric subunit) and protect cytidylyltransferase from nonspecific hydrophobic interactions during translocation to a membrane location. Alternatively, the 112-kDa protein may direct cytidylyltransferase to a specific membrane location by selecting specific binding sites on membranes. It is also possible that the 112-kDa protein inhibits translocation by competing with membranes for the binding of cytidylyltransferase. The formation and dissociation of the complex could be an important part of the regulatory mechanism for cytidylyltransferase.

REFERENCES