Localization of the Membrane-associated CTP:Phosphocholine Cytidylyltransferase in Chinese Hamster Ovary Cells with an Altered Membrane Composition*  

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The subcellular localization of the membrane-associated CTP:phosphocholine cytidylyltransferase was determined in Chinese hamster ovary cells in which the phospholipid composition had been altered by growth in the presence of N-methylethanolamine or treatment with phospholipase C. Cell homogenates were fractionated on Percoll density gradients, and marker enzyme activities were used to determine the location of the cellular membrane fractions. The peak of cytidylyltransferase activity occurred in the gradient at a density intermediate to that of the peaks of endoplasmic reticulum and plasma membrane markers. The profile of cytidylyltransferase activity most closely resembled that of the Golgi membrane marker; however, upon sucrose gradient centrifugation, the profile of the Golgi apparatus was very different from that of cytidylyltransferase. Differential centrifugation suggested a nuclear membrane association of the enzyme. Cytidylyltransferase was associated with a membrane fraction that sedimented when subjected to very low speed centrifugation (65 × g, 5 min). From Percoll gradient fractions, nuclei were identified by microscopy, and they migrated with cytidylyltransferase activity. The data are consistent with a localization of cytidylyltransferase in the nuclear membrane.

CTP:phosphocholine cytidylyltransferase catalyzes the production of CDP-choline and plays a central role in maintaining cellular phosphatidylcholine levels (1–5). Two experimental systems, growth in the absence of choline and phospholipase C treatment, have been used to characterize the regulatory properties of this enzyme in Chinese hamster ovary (CHO) cells (2, 6–9). Cytidylyltransferase is found in both the cytosolic and particulate fractions of the cell. The rate of phosphatidylcholine synthesis is modulated by changes in the amount of membrane-associated cytidylyltransferase. In response to changes in phosphatidylcholine levels brought about by phospholipase C treatment, choline starvation, or supplementation of choline-starved cells with N-methylethanolamine (ME) or N,N-dimethylethanolamine, cytidylyltransferase activity in crude homogenates increases. This is accompanied by an increase in the total cytidylyltransferase activity that is associated with the membrane and a decrease in cytosolic activity.

The mechanism of the activation and translocation of cytidylyltransferase is not well understood. The results described here represent the first step in our investigation of the mechanism of translocation. Our primary objective was to determine to which cellular membrane(s) cytidylyltransferase binds. Abundant evidence has indicated that the endoplasmic reticulum is the main site of phospholipid synthesis. Localization of cytidylyltransferase in the microsomal fraction from rat liver suggests that cytidylyltransferase is associated with the endoplasmic reticulum (10–12). In addition, evidence that the enzyme is also associated with the Golgi apparatus from rat liver has been presented (11–13). Here we present evidence suggesting that the membrane-associated cytidylyltransferase in CHO cells is associated with the nuclear membrane.

EXPERIMENTAL PROCEDURES

Materials—Radiochemicals were purchased from the following companies: CDP-[methyl-14C]choline, phospho-[methyl-14C]choline, 3-hydroxy-3-methyl[3-14C]glutaryl-CoA (HMG-CoA), Amersham Corp.; [3H]thymidine, ICN Biomedicals, Inc.; [3H]imidazole, UDP-[3H]galactose, Du Pont-New England Nuclear. ME was from Aldrich. Aprotinin and α1-antichymotrypsin were from Boehringer Mannheim. Jack bean β-galactosidase (grade VII) and asialofetuin (type I) were purchased from Sigma. Octylglucoside was from Boehringer Mannheim.

Cell Culture—The CHO-K1 cell line was obtained from the American Type Culture Collection (ATCC CCL6). The cultures were maintained in 80-cm² flasks (Nunc) in F-12 medium containing 5% fetal bovine serum (M. A. Bioproducts) at 37 °C, 5% CO₂, and 100% relative humidity. The cells were removed from the flask with trypsin and routinely subcultured at a ratio of 1:6. The culture medium was replaced every other day. Cell stocks were discarded after 20 passages. CHO cells with an altered lipid composition were prepared by growing the cells in choline-free F-12 medium containing 100 µM ME (added from a 100 mM stock) and 5% fetal bovine serum that had been dialyzed against calcium- and magnesium-free phosphate-buffered saline (14). For experiments, cells were plated at a density of 5 × 10⁶ cells/100-mm dish or 1.2 × 10⁶ cells/150-mm dish.

Marker Assays—Ouabain-sensitive Na⁺K⁺-ATPase, β-hexosaminidase, NADP⁺-cytochrome c reductase, and cholinephosphotransferase activities were determined as described previously (15) and are expressed as nmol/min. Galactosyltransferase activity with ovalbumin or agalactofetuin as acceptor was measured using the conditions described (15). Agalactofetuin was prepared by incubating 10 mg of asialofetuin and 3 units of jack bean β-galactosidase in 25 mM sodium citrate, pH 4.0, at 37 °C for 40–48 h. The agalactofetuin was dialyzed against 10 mM Tris-HCl, 0.02% sodium azide, pH 7.5, and used at a final concentration of 1 mg/ml in the assay. When agalactofetuin was the acceptor, the galactosyltransferase reaction was stopped by the addition of 10% (w/v) trichloroacetic acid, 2% (w/v) phosphotungstic acid. The labeled product was isolated on glass fiber filters (Whatman, GF/A) which were washed with cold 5% (w/v) trichloroacetic acid.
1% (w/v) phosphotungstic acid. Unless indicated otherwise, galactosyltransferase activity is expressed as pmol/min. HMG-CoA reductase activity was determined in a 75-μl incubation mixture containing 33.3 mM potassium phosphate, 1.68 mM MgCl₂, 20 mM Na₃EDTA, 51.3 mM KCl, 0.67 mM dithiothreitol, 33 μM [¹⁴C]HMG-CoA (12 cpm/pmol, PH 7.4). The incubation was stopped with 25 μl of 6 N HCl containing [³H]mevalonate (500 cpm/μl). The sample was then centrifuged at 2,000 g in a microcentrifuge. The amount of mevalonate produced during the assay was calculated from the specific radioactivity of the [¹⁴C]HMG-CoA used, a sample of which was applied to a 0.5-inch section of a silica gel sheet and counted under identical conditions as the assay mixtures, and from recovery of [³H]mevalonate. Routinely, greater than 80% of the standard mevalonate was recovered. Activity is expressed as pmol/min. Cytidylyltransferase activity was measured as described previously (6) with the exception that the charcoal pellet was washed twice with water instead of three. All assays were performed in the presence of 0.2 mM phosphatidylcholine, 0.2 mM oleic acid. The lipids were prepared in a 15-ml conical glass tube. The solvents were evaporated, and the lipids were resuspended in 10 mM Tris-HCl, 0.25 M sucrose, pH 7.4, and scraped into 0.5-inch sections and the radioactivity counted following a 1-h incubation in scintillation mixture (Budget-Solve, Research Products International Corp.). The amount of mevalonate produced during the assay was calculated from the specific radioactivity of the [¹⁴C]HMG-CoA used, a sample of which was applied to a 0.5-inch section of a silica gel sheet and counted under identical conditions as the assay mixtures, and from recovery of [³H]mevalonate. Routinely, greater than 80% of the standard mevalonate was recovered. Activity is expressed as pmol/min.

Preparation of Cell Homogenates for Membrane Fractionation—All procedures for the preparation and fractionation of cell homogenates were carried out at 0–4°C. Cultures to be fractionated were harvested with 10 mM Tris-HCl, 0.5 M sucrose, pH 7.4, and scrapped from the dishes with a rubber policeman. The cell suspension was homogenized in a Dounce homogenizer with 20 strokes of a B-type pestle. Unless otherwise indicated, homogenates were treated with micrococcal nuclease for 30 min as described (15). Differential Centrifugation—Centrifugation at 65 × g was carried out in a TOMY refrigerated microcentrifuge with a TMA-4 rotor (1,000 rpm). To isolate the crude nuclear pellet, CHO cell homogenates in 13 × 100-mm borosilicate glass tubes were subjected to centrifugation at 2,000 × g, 5 min in a Beckman JA-20 rotor. Total particulate and cytosolic fractions were prepared by centrifugation of the homogenate at 105,000 × g, 60 min. Pellets were resuspended in the homogenization buffer.

Percoll Density Gradient Fractionation—Approximately 1 × 10⁸ cells/10 ml were harvested for each gradient. The gradients had a height of 11.5 ml and were subjected to centrifugation for 2.5 h at 90,000 × g in a SW 41 rotor. Centrifugation was halted without the use of a brake. Fractions of approximately 1 ml were collected by hand from the top of the gradient, and marker enzyme activities were measured immediately. Cytidylyltransferase activity was inhibited up to 60% at the concentrations of sucrose used in the gradient. The data shown in Fig. 2 were not corrected for this inhibition.

Cytospin Preparations—Cell homogenates and Percoll gradient fractions were diluted with 20 mM Tris-HCl, 0.25 M sucrose, 1.5 mM MgCl₂, pH 7.4, 1:10 and 1:5, respectively. Samples (200 μl) were spun onto glass slides using a Skanodan Southern Instrument (Cytospin 2 apparatus at 700 rpm for 5 min. Cytospin preparations were stained with modified Wright’s stain using an automatic slide stainer (Amer Hema-Tek slide stainer). The samples were photographed with a Leitz Neofluor objective on a Leitz Technikan Pan 2415 film and the print developed with Kodak HC-110 developer (dilution B).

Phospholipase C Treatment—Phospholipase C was purified from the culture fluid of Clostridium perfringens through chromatography on QAE-Sephadex (17). Enzyme activity was determined as described (18); 1 unit is expressed as 1 amol of phosphatidylcholine hydrolyzed/min. Enzyme stocks were stored in liquid nitrogen and thawed immediately before use. On the day of the experiment, the culture medium was removed from the cells and replaced with fresh medium containing 5 milliliters/ml phospholipase C. The cells were harvested 3 h later for 20,000 × g supernatants, and the activity of cytidylyltransferase was determined using the method of Lowry et al. (19) with bovine serum albumin as a standard. Cell number was determined with a Coulter Counter following removal of the cells from the culture dish with trypsin. Cells were labeled with [³H]myristic acid as described (15).

RESULTS

Translocation of Cytidylyltransferase in ME-supplemented CHO Cells—For purposes of localizing the membrane-associated cytidylyltransferase, we chose to supplement the cells with ME. Under these growth conditions, most of the cytidylyltransferase activity is found in the particulate fraction of the cell (7). The time of growth in supplemented medium prior to the translocation of cytidylyltransferase was determined. In choline-supplemented cells, about 20% of the enzyme was in the total particulate fraction. Within 12 h after the removal of choline, the enzyme activity in the membrane fraction was increased by 2.5-fold. By 24 h, the enzyme was maximally translocated with greater than 80% of the activity in the particulate fraction. The cytosolic fraction always contained a portion of the enzyme activity. Routinely, subcellular fractionation studies utilized cells supplemented with ME for at least 48 h.

Effect of Growth in Choline-free Medium on Membrane Density—Although the membrane lipid composition of cells grown in ME-supplemented medium is altered (7), it seemed unlikely that a change in the lipid headgroup from choline to ME would alter the overall density of membrane organelles. To test this, homogenates from control and ME-supplemented cells were fractionated on self-forming Percoll density gradients. A comparison of the migration of marker enzyme activities confirmed that there were no large changes in the densities of the membranes. In addition, the specific activities of the membrane enzymes for the markers used in this study were identical from choline and ME-supplemented cell homogenates (data not shown).

Cytidylyltransferase Is Not Associated with the Golgi Apparatus or Plasma Membrane in CHO Cells—To determine where in the cell the membrane-associated cytidylyltransferase was localized, membranes from ME-supplemented CHO cells were fractionated on Percoll density gradients. Marker enzyme activities were used to determine the location of the various cellular membrane fractions. The profile of cytidylyltransferase activity across the gradient most closely resembled that of galactosyltransferase, a trans Golgi marker (Fig. 1). The peak of cytidylyltransferase activity occurred in the gradient at a density intermediate to the peak of the endoplasmic reticulum and plasma membrane markers.

When homogenates from control cells were fractionated on Percoll density gradients, cytidylyltransferase activity and other cytosolic components were evenly distributed across the gradient (data not shown). This was expected since in untreated CHO cells approximately 80% of the enzyme activity is cytosolic, and the density gradients were prepared by mixing cell homogenates with Percoll prior to centrifugation. The centrifugation conditions employed to form the gradient (28,000 × g, 1 h) do not affect the soluble proteins. They remain evenly distributed throughout the gradient.

Percoll density fractionation suggested that cytidylyltransferase is associated with the Golgi apparatus. There is evidence that rat liver Golgi membranes contain cytidylyltrans-
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To determine if, under conditions of choline starvation, cytidyltransferase as well as cholinephosphotransferase activity (11, 12). To determine if, under conditions of choline starvation, cytidyltransferase from CHO cells was truly associated with the Golgi apparatus, nuclease-treated CHO homogenates were fractionated on sucrose density gradients by the technique of Balch et al. (16) in which the homogenate is mixed with dylyltransferase from CHO cells was truly associated with the sucrose to a final concentration of 1.4 M and is loaded in the middle of a step gradient. The homogenates were treated with nuclease to allow for fractionation of whole cell homogenates instead of only postnuclear supernatant material. With this type of gradient, the 0.8/1.2 M sucrose interface contained plasma membrane marker activity (not shown) as well as the marker for the Golgi apparatus (Fig. 2). Galactosyltransferase activity in this fraction represented as much as a 15-fold enrichment of the homogenate activity. The plasma membrane and Golgi membranes were well separated from cholinephosphotransferase activity, the endoplasmic reticulum marker, which migrated to the 1.4/1.6 M sucrose interface. In each experiment, a portion of the galactosyltransferase activity did not migrate from the 1.4 M sucrose. Similar results have been observed by others (16). The amount of galactosyltransferase activity in this fraction varied and may result from minor differences in homogenization conditions between experiments. Damage to the relatively fragile Golgi apparatus would result in release of the luminal galactosyltransferase, which would remain in the 1.4 M sucrose.

When homogenates from ME-supplemented cells were fractionated by this technique, cytidyltransferase activity did not migrate with the Golgi membranes (Fig. 2). Under these conditions, cytidyltransferase activity migrated with the endoplasmic reticulum at the 1.4/1.6 M sucrose interface. From this experiment, it appears that cytidyltransferase is not associated with either the Golgi apparatus or the plasma membrane.

Cytidyltransferase Is Not Associated with the Bulk Endoplasmic Reticulum in CHO Cells—The possible localization of cytidyltransferase in the endoplasmic reticulum was reexamined after concluding that the enzyme was not in the Golgi apparatus or the plasma membrane from ME-supplemented cells. Results from the sucrose gradient experiment suggested that the enzyme was associated with the endoplasmic reticulum; however, fractionation of the membranes in Percoll argued against this. These data could be reconciled if the enzyme were associated with a portion of the endoplasmic reticulum. On a sucrose step gradient, all of the membranes of endoplasmic reticulum origin may accumulate at the appropriate interface. However, in a Percoll density gradient, these membranes, whose densities differ only slightly, could be separated in the shallow gradient. In addition, the size, density, and composition of the endoplasmic reticulum vesicles could differ in an environment of Percoll versus sucrose. Finally, the validity of cholinephosphotransferase as a marker enzyme for the endoplasmic reticulum would be critical.

To address these points, homogenates from ME-supplemented CHO cells were fractionated on a series of Percoll density gradients of various starting densities. In addition to cholinephosphotransferase activity, HMG-CoA reductase and NADPH-cytochrome c reductase activities were measured as markers for the endoplasmic reticulum. HMG-CoA reductase and choinephosphotransferase activities comigrated in the gradients (Fig. 3). NADPH-cytochrome c reductase was localized with the other endoplasmic reticulum markers (data not shown); however, a portion of this activity is soluble in CHO cells, making it a less suitable marker (8, 15). The distribution of the endoplasmic reticulum depended on the starting Percoll density. With increasing Percoll concentrations, the peak of marker activities broadened; and at 12.2% Percoll, two peaks were observed (Fig. 3). In each of the gradients, the pattern of cytidyltransferase activity differed.

**Fig. 1.** Percoll density gradient profile of cytidyltransferase and membrane markers. A CHO cell homogenate from ME-supplemented cells was mixed with 15% Percoll and fractionated as described under "Experimental Procedures." Density decreases from left to right. Marker enzyme activities are expressed as units/fraction. ER, endoplasmic reticulum; PM, plasma membrane.

**Fig. 2.** Sucrose density gradient profile of cytidyltransferase and membrane markers. A CHO cell homogenate from ME-supplemented cells was fractionated by sucrose gradient centrifugation as described under "Experimental Procedures." Marker enzyme activities are expressed as units/fraction.
from the pattern of the marker activities for the endoplasmic reticulum. When the endoplasmic reticulum migrated as two peaks, cytidylyltransferase activity overlapped with the less dense peak. Clearly, cytidylyltransferase is not associated with the bulk of the endoplasmic reticulum. This point is further illustrated by the experiment shown in Fig. 4. In this experiment, a cell homogenate prepared from ME-supplemented CHO cells was divided into two fractions by low speed centrifugation (2,000 × g, 5 min), and the membranes in each fraction were separated on Percoll density gradients. Most of the endoplasmic reticulum, as indicated by HMG-CoA reductase and cholinephosphotransferase activities, was in the low speed supernatant fraction (Fig. 4A). Cytidylyltransferase activity, however, was found in the low speed pellet (Fig. 4B). Fractionation of the low speed pellet in 12.2% Percoll resulted in cytidylyltransferase migrating with the less dense of the two peaks of endoplasmic reticulum marker activities (Fig. 4B). The endoplasmic reticulum marker activity in this peak represented 15–18% of the total cellular activity. The data are consistent with cytidylyltransferase being associated with a specific portion of the endoplasmic reticulum.

**Cofractionation of Cytidylyltransferase with Nuclei.—** Since cytidylyltransferase activity from ME-supplemented CHO cells was highly enriched in the pellet from low speed centrifugation, it was possible that the enzyme was associated with the nuclear membrane. An investigation of the membrane composition of the low speed pellet was consistent with this hypothesis. The low speed pellet contained approximately 30% of the endoplasmic reticulum, 40% of the plasma membrane, and less than 20% of the Golgi apparatus and lysosomes (Table 1). However, this pellet contained 50–70% of the total cytidylyltransferase activity. In addition, at all times before and after the removal of choline from the culture medium, at least 80% of the total membrane-associated activity was in the low speed pellet.

When we isolated nuclei by existing techniques involving centrifugation of nuclei through sucrose in the absence of detergents (20, 21), cytidylyltransferase activity was associated with the nuclei; however, the nuclei were contaminated with endoplasmic reticulum, and the yields were extremely low. Treatment of the cell homogenate with 0.04% Nonidet P-40 resulted in the recovery of 90% of the total nuclei. Analysis of the detergent-treated nuclei by electron microscopy revealed that the nuclei were not contaminated with other cellular membranes; however, the outer nuclear membrane was not intact. Furthermore, under these conditions, membrane-associated cytidylyltransferase activity was solubilized. These results are consistent with cytidylyltransferase being associated with the outer membrane of the nuclear envelope.

If cytidylyltransferase is associated with the nuclear envelope, Percoll gradient fractions containing enzyme activity should contain these structures. Since there are no suitable enzyme markers for the nuclear membrane, these membranes must be identified by microscopy. We first attempted to
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Table I
Composition of crude nuclear pellet

| Activity in crude nuclear pellet | CT | PM, ATPase | ER, CPT | ER, HMG-R | Lysosomes | β-HEX | Golgi, GT | Nuclei, [%] thymidine |
|-------------------------------|----|------------|---------|-----------|-----------|------|----------|---------------------|
| Choline                       | 10 ± 8 (7) | 32 ± 10 (14) | 28 ± 8 (16) | ND | 14 ± 6 (3) | 14 ± 1 (2) | ND |
| ME                            | 59 ± 20 (11) | 49 ± 10 (6) | 32 ± 14 (7) | 38 ± 16 (2) | 14 ± 6 (2) | 18 (1) | 87 ± 10 (2) |
| Phospholipase C               | 62 ± 22 (6) | 38 ± 19 (4) | 35 ± 19 (5) | ND | 7 (1) | 3 (1) | ND |

Fig. 5. Analysis of membrane fractions from CHO cells by light microscopy. Cytospin preparations of CHO cell homogenates (A and B) and fractions from a Percoll density gradient (fraction 1, C and D; fraction 6, E; fraction 3, F) were prepared as described under "Experimental Procedures." Bright field (A and C) and phase contrast images (B, D–F) of specimens stained with Wright’s stain. Bar, 10 μm.

identify nuclei in whole cell homogenates. When Cytospin preparations of micrococcal nuclease-treated homogenates were stained for examination by light microscopy, very few nuclei were observed. These nuclei were swollen in appearance, probably due to a change in the condensation state of the chromatin (22). Preparation of the cell homogenate in the presence of 1.5 mM MgCl₂ appeared to stabilize the nuclei, and following micrococcal nuclease treatment, nuclei were still clearly visible (Fig. 5, A and B). Cellular material was associated with the nuclei prepared in this manner; however, we could distinguish between nuclei and unbroken cells. A homogenate from ME-supplemented CHO cells, prepared in the presence of magnesium, was fractionated on a Percoll density gradient (Fig. 6). Two differences, attributed to the addition of magnesium, were observed. First, the apparent density of the endoplasmic reticulum was increased. Cholinephosphotransferase activity migrated in the 12.2% Percoll gradient as one major peak with a shoulder instead of two peaks (Fig. 6 versus Fig. 3C). Second, two peaks of cytidylyltransferase activity were observed (Fig. 6). One peak of activity migrated to the very bottom of the gradient. The major peak of cytidylyltransferase activity (fractions 5–8) was shifted to a higher density than when fractionation was carried out in the absence of magnesium (Fig. 6 versus Fig. 3C). To identify nuclei, Cytospin preparations of the fractions from the gradient shown in Fig. 6 were examined by light microscopy. Fraction 1 contained unbroken cells and pieces of membranes (Fig. 5, C and D). Notably, the unbroken cells all contained mitotic figures. Apparently, in the presence of magnesium, mitotic cells are not lysed under the homogenization conditions employed. Most importantly, nuclear envelopes ghosts migrated with cytidylyltransferase activity in the middle of the gradient (Fig. 5E). The nuclear envelopes did not contain darkly stained chromatin and nucleoli as did the nuclei from whole cell homogenates. The nuclear ghosts varied in size; however, the size of the structures was within the size range of intact nuclei from CHO cells. Also visible in

Fig. 6. Distribution of Percoll density gradients of cytidylyltransferase and cholinephosphotransferase from CHO cell homogenates in the presence of magnesium. CHO cells were grown for 3 days in choline-free F-12 medium, 5% dialyzed fetal bovine serum plus 100 μM ME. The cells were broken by homogenization in 10 mM Tris-Cl, 0.25 M sucrose, 1.5 mM MgCl₂, pH 7.4. The homogenate was mixed with Percoll and fractionated as described under “Experimental Procedures.” The starting Percoll concentration was 12.2%. Density decreases from left to right. Marker enzyme activities are expressed as units/fraction.
the fractions containing cytidylyltransferase activity and the nuclear membranes was a small number of unidentified structures that appeared tubular in nature. These structures were most abundant in fraction 3 (Fig. 5F), and this suggests that they are not enriched for cytidylyltransferase activity.

The Cytospin slides used for identification of nuclei by microscopy were prepared by very low speed centrifugation (55 g, 5 min). If cytidylyltransferase is associated with the nuclear membrane, one would expect that the enzyme activity would pellet under similar conditions. A homogenate from ME-supplemented CHO cells was subjected to centrifugation at 65 g for 5 min, and the activity remaining in the supernatant fraction was measured. Remarkably, only 35% of the total cytidylyltransferase activity remained in the supernatant fraction (not shown), indicating that 65% of the total cytidylyltransferase activity sedimented under these conditions. These data are consistent with a localization of cytidylyltransferase in the nuclear membrane.

In the fractionation procedures used here, sucrose was used as an osmotic stabilizer. It was possible that under conditions of low salt, cytidylyltransferase became bound artifactually to the altered nuclear membrane of ME-supplemented cells. To test this possibility, homogenates were prepared in 0.15 M KCl, 10 mM Tris, 1.5 mM MgCl₂, pH 7.4 and subjected to centrifugation at very low speed (65 g) to remove nuclei, as well as high speed (100,000 × g) to separate total membranes from cytosol. The use of salt rather than sucrose resulted in more cytidylyltransferase remaining in the cytosolic fraction (39% versus 18%). With either sucrose or salt in the homogenates, however, 80% of the membrane-associated cytidylyltransferase was removed by the 65 g centrifugation. These results indicate that cytidylyltransferase is associated with the nuclear fraction at physiological salt concentrations.

Localisation of Cytidylyltransferase in Phospholipase C-Treated CHO Cells—It has been proposed that cytidylyltransferase binds to phosphatidylcholine-deficient membranes (6, 7). All of the cellular membranes may be phosphatidylcholine deficient under conditions of choline starvation; however, as we have just described, cytidylyltransferase binds to a specific membrane population. When the cells are treated with phospholipase C, the phosphatidylcholine is being degraded only at the outer leaflet of the plasma membrane. Under these conditions, cytidylyltransferase may bind to the plasma membrane, to the nuclear membrane as in ME-supplemented cells, or to a different membrane fraction. When membranes from phospholipase C-treated cells were fractionated, the results were identical to those discussed previously for ME-supplemented cells. As shown in Fig. 7, cytidylyltransferase activity from phospholipase C-treated cells migrated on a Percoll density gradient between the peaks of endoplasmic reticulum and plasma membrane. Characterization of a crude nuclear pellet suggested that the enzyme was not in the Golgi apparatus (Table I). The nuclear pellet contained 60% of the total cytidylyltransferase activity and only 3% of the trans Golgi marker. Furthermore, cytidylyltransferase activity sedimented when subjected to centrifugation at 65 × g for 5 min. At 5 milliunits/ml phospholipase C, 38% of the cytidylyltransferase was removed from the homogenate. At 10 milliunits/ml, 56% was removed. It appears that the enzyme binds to the same membrane in response to phospholipase C treatment or choline starvation. These experiments suggest that in response to an altered membrane phospholipid composition, cytidylyltransferase translocates to the nuclear membrane in CHO cells.

**DISCUSSION**

The results presented here suggest that the membrane form of cytidylyltransferase is associated with the nuclear envelope. First, the enzyme is associated with a membrane fraction that sediments when subjected to low speed centrifugation. As much as 80% of the membrane-associated activity sediments under these conditions. The centrifugation conditions employed are not sufficient to pellet other organelles and microsomal membrane components such as lysosomes, endoplasmic reticulum, Golgi apparatus, plasma membrane, and endosomes. Second, cytidylyltransferase does not migrate on density gradients with other cellular membranes. Fractionation of a whole cell homogenate on a sucrose step gradient eliminates the Golgi apparatus and plasma membrane as sites for cytidylyltransferase association. The enzyme does not migrate with the bulk of the endoplasmic reticulum in Percoll density gradients. Fractionation of the crude nuclear pellet (2000 × g, 5 min) on a Percoll density gradient most strikingly illustrates that only a small portion of the total endoplasmic reticulum migrates with cytidylyltransferase (Fig. 4). Third, nuclei have been identified in Percoll gradient fractions, and these organelles migrate with cytidylyltransferase activity. Cytidylyltransferase is probably membrane associated because it can be solubilized with low concentrations of detergent.

The data presented are consistent with a localization of cytidylyltransferase in the nuclear membrane, yet one cannot eliminate the possibility that the enzyme associates with a region of the endoplasmic reticulum. On density gradients, the endoplasmic reticulum markers and cytidylyltransferase activity were never completely separated. If the enzyme binds to the endoplasmic reticulum, clearly this occurs at a distinct location on this membrane. The endoplasmic reticulum is a complex tubular network, and based on morphology, distinguishable regions of this organelle have been described (23–25). In addition, the endoplasmic reticulum is continuous with the outer nuclear membrane (26–28). Therefore, it may prove difficult to separate the outer nuclear membrane and the endoplasmic reticulum by current fractionation techniques. More importantly, defining a region of this membrane as being of nuclear or endoplasmic reticulum origin, by biochemical analysis, may be an inaccurate way to describe this cellular organelle. The outer nuclear membrane and the endoplasmic reticulum are functionally related as indicated by the association of certain enzyme activities and ribosomes with both structures (29–33). If cytidylyltransferase binds to
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