Expression of phosphatidylethanolamine *N*-methyltransferase-2 in McArdle-RH7777 hepatoma cells inhibits the CDP-choline pathway for phosphatidylcholine biosynthesis via decreased gene expression of CTP:phosphocholine cytidylyltransferase

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Phosphatidylethanolamine *N*-methyltransferase-2 (PEMT2) of rat liver was expressed in McArdle-RH7777 rat hepatoma cells, which lack endogenous PEMT activity. Expression of the enzyme was confirmed by assay of PEMT activity and immunoblotting. There was no change in the amount of phosphatidylcholine in the transfected cells [Cui, Houweling and Vance (1994) J. Biol. Chem. **269**, 24531–24533], even though the expression of PEMT2 caused an increased incorporation of [*methyl*-^{3}H]methionine and [^{3}H]ethanolamine into phosphatidylcholine. In contrast, [^{3}H]serine incorporation into phosphatidylcholine was only marginally enhanced by PEMT2 expression. Incorporation of [*methyl*-^{3}H]choline into phosphatidylcholine was decreased by greater than 60 %, suggesting that the CDP-choline pathway was inhibited as a result of PEMT2 expression. CTP:phosphocholine cytidylyltransferase (CT) activities in transfected cell lines were decreased in proportion to the level of expression of PEMT2. Immunoblot analyses showed a decrease in CT mass as a function of PEMT2 expression. In contrast, there was no change in the mass of protein disulphide-isomerase or the relative amounts of most proteins expressed in the PEMT2-transfected, compared with control, cells. Similarly, the expression of CT mRNA was decreased in PEMT2-expressing cells, whereas the mRNAs for protein disulphide-isomerase and actin were unchanged. When cell growth was slowed by incubating McArdle-RH7777 cells at 25 °C, compared with 37 °C, there was no difference in the specific activity of the CT. These results argue that PEMT2 expression down-regulates the CDP-choline pathway by decreasing the expression of the gene for the CT. The decreased activity of the CDP-choline pathway might contribute to the slower rate of cell division in PEMT2-transfected hepatoma cells.

INTRODUCTION

A unique feature of eukaryotic cells is the presence of phosphatidylcholine (PC) as the major cellular phospholipid component. All eukaryotic cells synthesize PC via the CDP-choline pathway which utilizes dietary or recycled choline as an initial substrate in the pathway. However, hepatocytes also have the ability to convert phosphatidylethanolamine (PE) into PC by three consecutive methylation reactions [1]. The methylations are catalysed by at least two PE methyltransferases: one is mainly localized on the endoplasmic reticulum (PEMT1); the other (PEMT2) is found on a mitochondria-associated membrane exclusively [2]. Both enzymes catalyse all three steps of methylation. The PEMT2 cDNA has recently been cloned from a rat liver cDNA library [2].

Traditionally, PE methylation is considered to be an alternative pathway for PC biosynthesis in liver, and PEMT activity is present only at very low levels in non-hepatic cells and tissues [1]. PE methylation also appears to be the major mechanism by which organisms synthesize the choline moiety which is utilized for PC and acetylcholine biosynthesis. The recent discoveries of methylation of phosphoethanolamine to phosphocholine in plants and animals [3–6] indicates an alternative cellular mechanism for the biosynthesis of the choline moiety. The relative contributions of PE methylation and phosphoethanolamine methylation for the generation of choline have not been determined. Thus, an argument can be made that PE methylation is critical for generation of choline which is essential for eukaryotic cell growth and cell division. However, this proposed function does not provide insight into why PE methylation is essentially a liver function in animals. Why has PE methylation in liver survived during evolution if choline were readily available in animal diets?

One approach that we have adopted to gain insight into the function of PEMT2 is transfection of cell lines with the cDNA that encodes for this enzyme. In preliminary transfection studies we discovered, to our surprise, that homogenates of a rat hepatoma cell line, McArdle-RH7777, had less than 2 % of the PEMT activity recovered in rat liver homogenates and primary rat hepatocytes [2]. Subsequent studies demonstrated very low PEMT activity in the human hepatoma cell line, HepG2. Since the McArdle-RH7777 cells had low PEMT activity and were of a rat liver origin, we chose these cells for construction of cell lines that stably expressed PEMT2. In our initial studies we found a 3-fold slower rate of McArdle-cell division as an unexpected consequence of PEMT2 transfection [7]. When primary liver cancer nodules were examined, there was a significant decrease in the amount of PEMT2 mass compared with normal liver [7]. Why should expression of PEMT2 decrease the rate of hepatoma cell division?

Expression of PEMT2 in cells might have increased the concentration of PC. However, in the hepatoma cells this was not the case [7], and we were curious why. We now report that stable expression of PEMT2 in these rat hepatoma cells feedback-

Abbreviations used: CT, CTP:phosphocholine cytidylyltransferase; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PEMT, phosphatidylethanolamine *N*-methyltransferase.

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inhibits the CDP-choline pathway for PC biosynthesis. Mechanistic studies demonstrate that the decreased enzyme activity is due to a decrease at the level of gene expression of the rate-limiting enzyme in the pathway, CTP: phosphocholine cytidylyltransferase (CT). Control studies indicate that the decreased activity is not simply a function of slower cell growth. Instead, it is possible that the down-regulation of the CDP-choline pathway may contribute to the decreased rate of cell division.

MATERIALS AND METHODS

Materials

All radioactive materials, including [*methyl*-^{3}H]choline, [*methyl*-^{3}H]methionine, L-[3-^{3}H]serine, [1-^{3}H]ethanolamine and [^{32}P]dCTP were from Amersham. The BCA protein assay kit was purchased from Pierce Chemicals. All tissue-culture media and G418 were from GIBCO–BRL.

Plasmids and establishment of hepatoma cell lines that stably express rat liver PEMT2

The construction of the PEMT2 expression plasmid has been described previously [2]. A 10 μg portion of the PEMT2 expression plasmid was co-transfected with 0.3 μg of pSV-neo [8] (American Type Culture Collection) into McArdle-RH7777 cells by calcium phosphate precipitation [9]. The neomycin-resistant colonies were selected with 0.6 μg/ml G418 in Dulbecco's modified Eagle's medium with 10% fetal-bovine serum and 10% horse serum. Colonies were picked and grown as individual cell lines in the presence of 0.3 μg/ml G418. Each cell line was assayed for PEMT activity to confirm PEMT2 expression. Control cells were transfected by the expression vector without the PEMT2 cDNA insert and had negligible PEMT activity.

Enzyme assays

CT assays were performed as described in [10] in the presence of PC:oleate vesicles, and PEMT activity was assayed as described by Ridgway and Vance [11].

Immunoblot analyses

The protein samples were separated on 12.5% polyacrylamide gels that contained 0.1% SDS [12] and transferred to nitrocellulose membranes by electrophoretic blotting [13]. The anti-PEMT2 specific polyclonal antibody was described previously [2], as was the antibody to CT [14]. Antibody to protein disulphide-isomerase was kindly provided by Dr. Marek Michalak, University of Alberta. The membranes probed with specific antibodies were revealed by enhanced chemiluminescence according to the manufacturer's instructions (Amersham).

Northern-blot analyses

Cells were grown to 70–80% confluency. mRNAs were prepared by Quick prep micro kit from Pharmacia. A 3 μg portion of mRNA from each sample was separated on a 1% agarose/formaldehyde gel and transferred to Hybond N$^+$ membranes (Amersham). The 1.3 kb CT cDNA (*Eco*RI/*Eco*RI) fragment [15] was labelled with ^{32}P by a random-priming kit from Bethesda Research Laboratories. The ^{32}P-labelled CT cDNA was hybridized to the membrane in the presence of 50% formamide at 42 °C for 24 h. The final washing stringency was 60 °C and 0.2 × SSC (1 × SSC = 0.15 M NaCl, 15 mM sodium citrate, pH 7.0). The membrane was exposed to X-ray film. Protein disulphide-isomerase cDNA was a gift from Professor Marek Michalak of the University of Alberta. Rat actin cDNA was purchased from the American Type Culture Collection.

Lipid extraction and TLC of phospholipids

The total lipids were extracted from the cultured cells as described by Folch et al. [16]. TLC of phospholipids was performed in the solvent system chloroform/methanol/acetic acid/formic acid/water (70:30:12:4:1, by vol.).

RESULTS

Expressed PEMT2 converts PE into PC

The PEMT2 expression plasmid was constructed by fusing the rat liver PEMT2 cDNA to the 3′ end of the cytomegalovirus promoter [2]. PEMT2 expression under this promoter is constitutive. The cell lines studied expressed PEMT specific activities that ranged from 130 to 790 pmol/min per mg of protein. The latter value is similar to that present in rat liver which is approx. 1 nmol/min per mg [11]. However, one difference is that in rat liver PEMT activity comprises both PEMT1 and PEMT2 [2]. Control cells were transfected by the expression vector without the PEMT2 cDNA insert and had negligible PEMT activity. The proteins from the homogenates from two cell lines, with specific activities of 230 and 790 pmol/min per mg respectively, were also probed by PEMT2-specific antibody on a Western blot and compared with control cells. The PEMT2 protein mass detected by immunoblotting correlated well with the enzyme activity from the corresponding cell lines. Hence the expressed PEMT2 proteins have been processed properly and function as mature enzymes.

We then determined whether the expressed PEMT2 was capable of converting PE into PC via methylation in these cell lines. Radioactive precursors [*methyl*-^{3}H]methionine and [^{3}H]ethanolamine were used to label the phospholipids of these cells. Methionine is the precursor of *S*-adenosylmethionine, which is the methyl-group donor for all three steps of PE methylation. Figure 1(a) shows a linear relationship between the amount of radioactivity incorporated into PC from [^{3}H]methionine and the corresponding PEMT activity in these cell lines. The incorporation of [^{3}H]methionine into PC was 5 times greater in the cell line expressing the highest amount of PEMT2 activity (790 pmol/min per mg of protein) than in cells transfected with vector lacking the PEMT2 insert. Among the phospholipids labelled, over 90% of the radioactivity was in PC. Labelling of lysoPC and sphingomyelin was insignificant, indicating that conversion of PE into PC is the primary methylation event among phospholipids. The basal level of PC labelling by methionine in control cells was low (9300 d.p.m./mg of protein), but noticeable in comparison with sphingomyelin and lysoPC. We speculate that this might be due to the impurity of the labelling material, or other methylation pathway(s) in these cells, even though we cannot detect any PEMT activity under our experimental conditions.

Incorporation of radioactivity from [^{3}H]ethanolamine into PC is indicative of methylation of PE derived from the CDP-ethanolamine pathway. In control cells incubated for 19 h with [^{3}H]ethanolamine, 86% of the label in phospholipids was in PE (1.55×10^6 d.p.m./mg of protein) and 12% of the label was incorporated into PC (0.25×10^6 d.p.m./mg of protein) (Figure 1b). Labelling of PC from [^{3}H]ethanolamine showed a linear relationship between 0.25×10^6 d.p.m./mg of protein in control cells to 1.8×10^6 d.p.m./mg of protein in cells with PEMT activity of 790 pmol/min per mg. This represented a change of radioactive PC among total labelled phospholipids from 12% in control cells

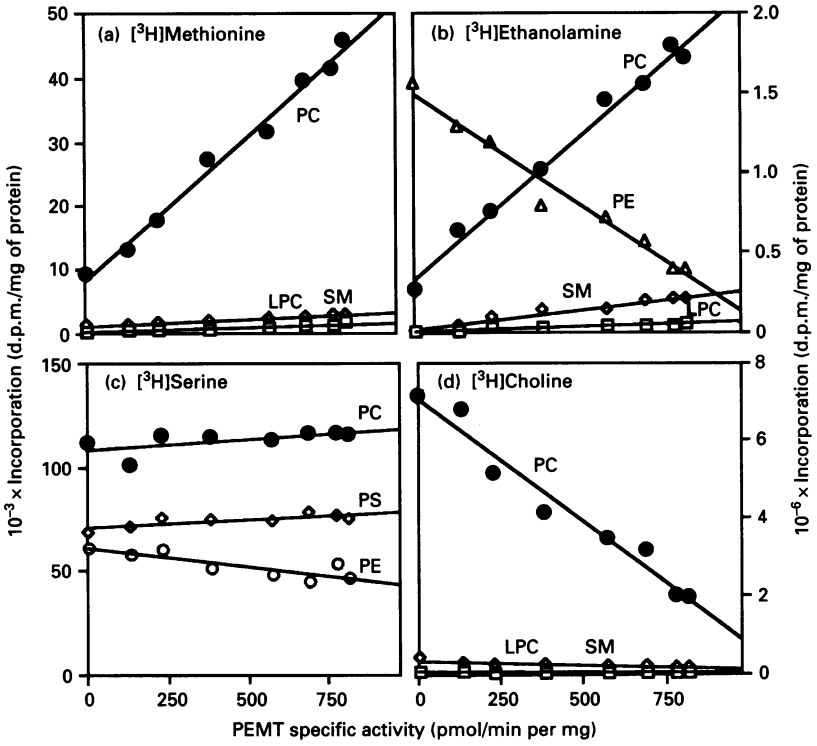

Figure 1 Incorporation of radioactive precursors into the major phospholipids of McArdle-RH7777 hepatoma cells as a function of PEMT specific activity

The PEMT activity for each individual cell line was measured in cellular homogenates. Equal numbers of cells from each cell line were plated on 10 cm Petri dishes at 40% confluency. The cells were incubated for 4 h to allow the attachment to the surface of the plate. The labelled precursor was added to the growth medium and the cells were incubated for 19 h. The cells were washed three times with PBS and then scraped from the plates. Samples were taken for protein measurements. Total lipids were extracted and phospholipids separated on thin-layer plates. Bands were revealed by iodine vapour, scraped, and radioactivity was determined. In (a) 30 μCi of [*methyl*-^{3}H]methionine was added to each dish. In (b) 5 μCi of [^{3}H]ethanolamine was added to each dish. In (c) 25 μCi of [^{3}H]serine was added to each dish. In (d) 10 μCi of [*methyl*-^{3}H]choline was added to each dish. Abbreviations: SM, sphingomyelin; LPC, lysophosphatidylcholine. This experiment was repeated three times with similar results. In addition, similar results were obtained when the incubations were done for only 9 h.

to 64% in hepatoma cells that expressed PEMT2. Labelling of sphingomyelin and lysoPC was also increased as a result of PEMT2 expression, but was quite minor in comparison with PC (Figure 1b). The apparent labelling of PC in cells with no PEMT activity was half due to co-migration of lysoPE with PC.

Serine is a versatile precursor which can be incorporated into phosphatidylserine (PS), which is decarboxylated to PE and the PE methylated to PC. Figure 1(c) shows the profile of radioactivity incorporated into the major phospholipids labelled by serine. Expression of PEMT2 did not influence the incorporation of [^{3}H]serine into PC or PS. Labelling of PE from serine decreased slightly as a result of PEMT2 expression. These results contrast with labelling of PE by [^{3}H]ethanolamine, which was decreased by 62% as a result of PEMT2 expression (Figure 1b).

The high incorporation of radioactivity into PC from [^{3}H]serine in cells transfected with the cytomegalovirus vector alone was due to labelling of the glycerol and fatty acid moieties of PC. When PC from these samples was digested with phospholipase C, 87% of the radioactivity was in the diacylglycerol backbone and 13% in the choline head group. In the cells that expressed PEMT2 activity of 790 pmol/min per mg of protein, 25% of the [^{3}H]serine label was in the headgroup and 75% in the diacylglycerol moiety. This is consistent with enhanced conversion of PS-derived PE into PC in the PEMT2-transfected cells. However, the doubling of methylation of serine-derived PE was much lower than the 7-fold increase in the conversion of PE derived from CDP-ethanolamine into PC (Figure 1b). Apparently, PE derived from ethanolamine, rather than PE derived from PS, was preferentially converted into PC as a result of PEMT2 expression.

The results reported in Figure 1 were after 19 h incubations with the labelled precursor. Similar results were obtained when the incubations were done for 9 h.

PEMT2 expression inhibits the incorporation of [*methyl*-^{3}H]choline into PC in McArdle-RH7777 cells

Once the functional expression of rat liver PEMT2 in these rat hepatoma cells was established, we examined the capacity of the CDP-choline pathway to make PC by labelling phospholipids with [*methyl*-^{3}H]choline. In control cells, over 95% of the radioactivity in phospholipid was in PC (7.2×10^6 d.p.m./mg of protein) with minimal labelling of sphingomyelin and lysoPC (Figure 1d). In the cells expressing PEMT2 activity (790 pmol/min per mg), compared with control cells, the labelling of PC by [*methyl*-^{3}H]choline was decreased by 65%, to 2×10^6 d.p.m./mg of protein. A linear relationship was observed between decreased labelling of PC from [*methyl*-^{3}H]choline and the degree of PEMT2 expression.

Expression of CT is decreased in hepatoma cells that express PEMT2

CT is usually regarded as the rate-limiting enzyme for the CDP-choline pathway [17–20]. The major mode for regulation of CT

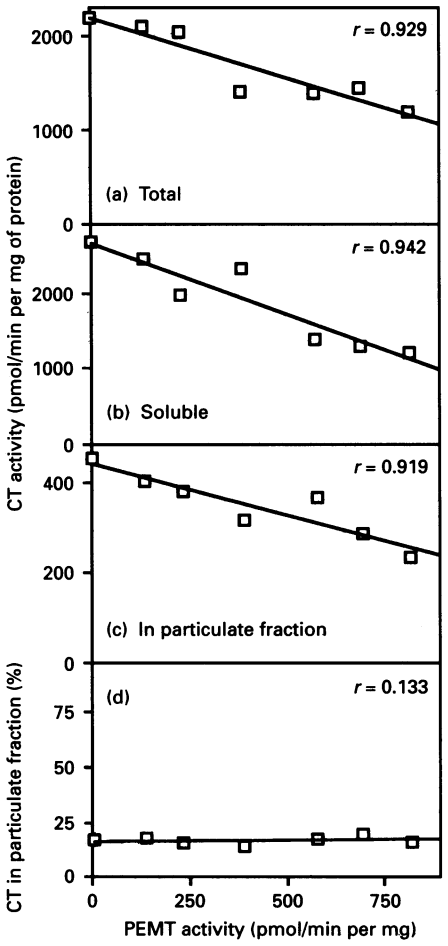

Figure 2 Activity of CT as a function of PEMT expression in McArdle-RH7777 hepatoma cells

The cells were grown on 10 cm dishes to 80% confluency. The plates were washed three times with PBS, and cells were then harvested and homogenized with a Polytron homogenizer. Unbroken cells were removed by centrifugation at 600 *g* for 5 min. (**a**) The specific activities of CT and PEMT were measured in the 600 *g* supernatant. This experiment was repeated twice with similar results. (**b–d**) The supernatants were centrifuged at 350000 *g* for 15 min and CT activities in soluble fractions (**b**) were measured. The pellets were washed three times with PBS and resuspended for CT activity measurements (**c**). The percentage of CT in the particulate fraction was calculated (**d**). This experiment was repeated once with similar results. The *r* value was calculated by the Cricket Graph III program.

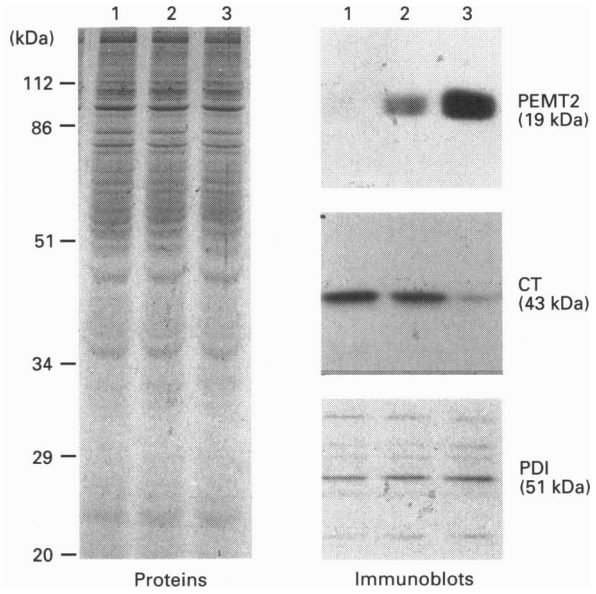

Figure 3 Total cellular proteins and immunoblot analyses of CT, PEMT2 and protein disulphide-isomerase in PEMT2-transfected cell lines

Samples (50 μg) of total cellular proteins from each cell line were separated on 12.5% polyacrylamide gels (0.1% SDS) and stained with Coomassie Blue (left panel). Unstained proteins were transferred to a nitrocellulose membrane and probed with specific antibodies to PEMT2, CT and protein disulphide isomerase (PDI) (right panels). The blots were revealed by enhanced chemiluminescence. Lane 1, McArdle-RH7777 cells co-transfected with pCMV-5 vector plasmid and pSV-neo plasmid. PEMT specific activity was not detectable. Lane 2, McArdle-RH7777 cells co-transfected with pCMV-5/PEMT2 vector plasmid and pSV-neo plasmid. PEMT specific activity was 232 pmol/min per mg of protein. Lane 3, McArdle-RH7777 cells co-transfected with pCMV-5/PEMT2 vector plasmid and pSV-neo plasmid. PEMT specific activity was 790 pmol/min per mg of protein. This experiment was repeated once with similar results. Molecular masses (kDa) of marker proteins are indicated.

activity is by the reversible translocation of the enzyme between soluble (inactive) and particulate (active) fractions of the cell. More recently, several conditions have been identified in which CT activity was governed by the level of gene expression [21,22]. The following experiments were designed to determine whether or not inhibition of the CDP-choline pathway by PEMT2 expression was mediated by an effect on CT activity. If so, was CT being regulated by translocation of the membrane-bound active form to a soluble, inactive, form, or was the expression of the CT gene down-regulated?

Total cellular CT and PEMT activities were measured in control McArdle-RH7777 cells and in all PEMT2-expression cell lines. The specific activities of both CT and PEMT were plotted against each other. Figure 2(a) shows an inverse correlation between PEMT and CT activities in all cell lines. In cells with the highest level of PEMT2 expression (790 pmol/min per mg of protein), CT activity was decreased by approx. 50% compared with controls. The magnitude of the decrease in CT specific activity was very similar to the decrease in PC labelling by [*methyl*-^{3}H]choline, suggesting that the decreased cellular CT activity may be responsible for the inhibition of the CDP-choline pathway. In order to reveal if translocation of CT from membranes (active) into the soluble (inactive) portion of the cells was involved in the inhibition of CT, the cellular homogenates from various cell lines were separated into soluble and particulate fractions. CT specific activity was measured and plotted against PEMT activity for each cell line. Figures 2(b) and 2(c) show the results for soluble and particulate fractions respectively. In both fractions, the decreases in CT specific activities were very similar to that in total cellular homogenates. The percentage of CT activity associated with the particulate fraction plotted against PEMT activity remained at approx. 17% (Figure 2d) in all cell lines regardless of the level of PEMT activity expressed. Therefore it is unlikely that the translocation of CT from an active to an inactive form is involved in the inhibition of the CDP-choline pathway.

Since the lower CT activity could result from decreased enzyme mass, the proteins from two PEMT-expression cell lines, plus control cells, were probed with CT-specific antibody by immunoblot analyses. As shown in Figure 3, a significant decrease in CT mass was observed as the result of PEMT2 expression. The decrease in protein mass reflected the decrease in total cellular CT activity in these cell lines (Figure 2). To evaluate if this decrease in the protein mass of CT were specific, we measured the protein contents in these cell lines. The variations in the amount

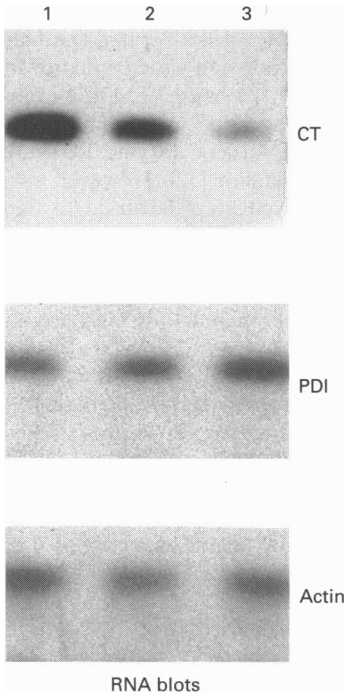

Figure 4 Northern-blot analysis of CT, actin and protein disulphide-isomerase (PDI) mRNAs in McArdle-RH7777 cells that express PEMT2

The cells were grown to 80% confluency. mRNAs were extracted by a mRNA extraction kit from Pharmacia; 3 μg of mRNA was loaded in each lane and separated on a 1.0% agarose/formaldehyde gel. The RNAs were transferred to a Hybond N^+ membrane and probed with ^{32}P-labelled CT, PDI or actin cDNAs. The washing stringency was 60 °C with 0.2 × SSC (SSC = 0.15 M NaCl/0.015 M sodium citrate) for 30 min. The blots were exposed to X-ray film. Lane 1, McA-RH7777 cells co-transfected with pCMV-5 vector plasmid and pSV-neo plasmid. PEMT specific activity was not detectable. Lane 2, McArdle-RH7777 cells co-transfected with pCMV-5/PEMT2 vector plasmid and pSV-neo plasmid. PEMT specific activity was 232 pmol/min per mg of protein. Lane 3, McArdle-RH7777 cells co-transfected with pCMV-5/PEMT2 vector plasmid and pSV-neo plasmid. PEMT specific activity was 790 pmol/min per mg of protein. This experiment was repeated once with similar results.

of protein in cell lines with different growth rates were very small, ranging from 1.3 to 1.6 ng/cell. Therefore, all the enzyme activities normalized to mg of protein not only reflected the specific activities but also represented the relative activities per cell. This result was verified by revealing the protein bands of equal numbers of cells from three different cell lines on SDS/PAGE (Figure 3). All three cell lines showed very similar patterns and intensities of protein bands. That down-regulation of CT expression was not part of a general phenomenon was further illustrated by immunoblot experiments with an antibody specific to protein disulphide-isomerase, an enzyme that is presumably not directly related to phospholipid metabolism (Figure 3). Thus it appears that, in comparison with most cellular proteins, the decrease of CT mass was a relatively specific result of PEMT2 expression.

To determine whether the decrease in CT mass was accompanied by a decreased amount of CT mRNA in these cell lines, equal amounts of mRNA were probed with ^{32}P-labelled rat liver CT cDNA fragments. As is apparent in Figure 4, the level of CT mRNA was decreased to approximately the same extent as CT protein mass in the PEMT2-expressing cells (Figure 3). Since the levels of total cellular mRNAs in the three cell lines and the recoveries of mRNA preparations were unknown, the RNA blot was hybridized with probes to actin and protein disulphide-isomerase mRNAs. Both mRNAs were present at similar concentration in all three cell lines. Therefore, the decrease in CT mRNA was not a result of a general down-regulation of mRNA in PEMT2-expressing cells. This result indicates that the expression of the CT gene is inhibited by PEMT2 or as a consequence of PE methylation.

Growth of hepatoma cells is slowed at 25 °C without a concomitant down-regulation of CT activity

It was possible that the lower expression of CT activity in the PEMT2-transfected cells could be a result of the slower growth rate. Although this scenario seemed unlikely to us, since the activity was normalized per mg of protein, we chose an alternative approach to slow cell growth. McArdle-RH7777 cells were grown for 1–3 days at either 25 °C or 37 °C. The rate of growth was assessed and the activity of CT in homogenates was measured after 3 days. As expected, the growth rate at the lower temperature was one third of that at 37 °C, yet the specific activity of CT when assayed at 37 °C (2.64 nmol/min per mg of protein) was the same regardless of the growth temperature. Thus a slower growth rate does not necessarily result in down-regulation of CT. This contrasts with PEMT2-transfected McArdle-RH7777 cells, which exhibited a slower growth rate [7] and major down-regulation of the expression of the CT gene.

DISCUSSION

PEMT2-mediated inhibition of the CDP-choline pathway occurs because of diminished gene expression of CT

The major conclusion from this study is that expression of PEMT2 in McArdle-RH7777 hepatoma cells down-regulates the CDP-choline pathway by decreasing the expression of the CT gene. One explanation is that an unknown feedback-control mechanism exists between the level of PC supplied by PEMT2 in the transfected cells and the expression of CT. Most examples of short-term regulation of CT activity involve the translocation of CT between soluble (inactive) and membrane-bound (active) forms [17–20]. However, two examples in the literature document that the expression of CT can be increased at the level of gene expression. Murine macrophage-derived cells (BAC1.2F5), arrested in the G1 phase of the cell cycle, were stimulated by the addition of colony-stimulating factor, which induced a 4-fold increase in the level of CT mRNA [21]. In another study, partial hepatectomy activated CT by enhanced expression of its mRNA [22,23]. The studies reported herein point to a completely novel mechanism for regulation of CT expression that appears to be independent of growth factors.

Another mechanism that might have inactivated CT without affecting the level of gene expression would have been increased phosphorylation of CT. It is known that the enzyme in the soluble form (which is inactive) is highly phosphorylated compared with the active form of CT on the membranes [24–29]. This apparently is not an issue in the PEMT-transfected McArdle-RH7777 cells, since the percentage of total CT on membranes has not changed and the decreased CT activity can be fully accounted for by the decreased mass of enzyme.

The actual mechanism for the down-regulation of the expression of CT is unknown. It is reasonable to suggest that activation of PEMT2 has in some way altered a *trans*-acting factor that modulates the expression of the CT gene. It is known that the gene for CT is located on chromosome 16 of the mouse been identified. One hypothesis might be that a PC-binding protein modulates the expression of the CT gene (Figure 5). When there is an abundant supply of PEMT2-derived PC, it

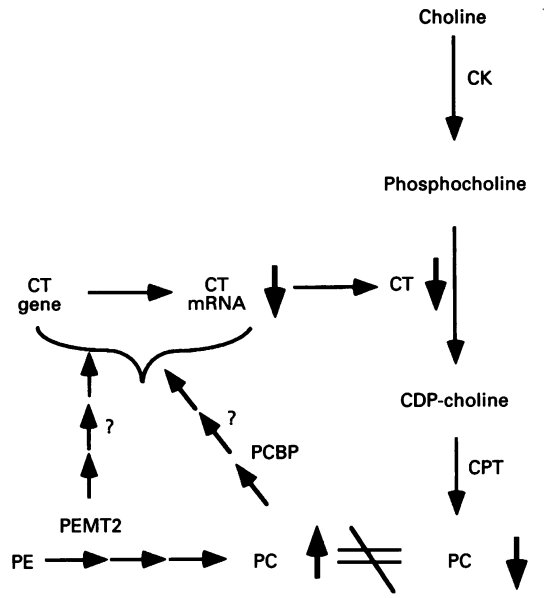

Figure 5 Postulated mechanisms for the down-regulation of the CT gene and the non-equivalency of the PCs derived from PE methylation and the CDP-choline pathway

PEMT or its product somehow causes the down-regulation of CT. Possibly there is a sensor PC-binding protein (PCBP) that determines the concentration of PEMT2-derived PC in membranes. When occupied with PC, PCBP might down-regulate the CT gene. Alternatively, PEMT2 may methylate an unknown substrate that in turn causes the CT gene to be expressed at a lower level. Since PEMT2-derived PC will not substitute for the CDP-choline pathway in mutant CHO cells defective in CT [41,44], it is possible that PC derived from the methylation of PE is not equivalent to PC originating from the CDP-choline pathway. Abbreviations: CK, choline kinase; CPT, CDP-choline:1,2-diacylglycerol cholinephosphotransferase.

could bind to this protein, move to the nucleus and inhibit gene expression. If the PC levels fell in the cellular membranes, less PC might bind to this putative protein, which would no longer bind to a regulatory element on the CT gene. This proposal gained some plausibility recently. Skinner et al. [31] have reported that a protein in yeast, the phosphatidylinositol/PC exchange protein, directly inhibits CT activity when PC, but not phosphatidylinositol, is bound to the protein. An effect on gene expression was not reported. The results from our studies in McArdle-RH7777 cells should facilitate the identification of one or more proteins that are involved in regulation of CT expression in hepatocytes.

Alternatively, PEMT expression might alter CT gene expression by some other mechanism. The possibility that PEMT2 might be methylating another compound in the hepatoma cells which alters CT gene expression cannot be eliminated, but there is no evidence for an alternative methylating activity associated with PEMT2.

Reciprocal regulation of PC biosynthesis via PE methylation and the CDP-choline pathway in liver cells

A clear reciprocal relationship exists between PEMT activity and the activity of the CDP-choline pathway in the McArdle-RH7777 hepatoma cells. PE originating largely from the CDP-ethanolamine pathway is converted into PC, and this decreases the biosynthesis of PC via the CDP-choline pathway. Thus the normal concentration of PC required by the hepatoma cells can apparently be supplied by either pathway. Surprisingly, there was not a large increase in the conversion of PS-derived PE into PC in the PEMT2-transfected cells. We had postulated an opposite result: that PEMT2 expression would enhance conversion of PS into PE and then into PC, because PEMT2 is exclusively localized on a mitochondria-associated membrane in liver [2] which is enriched in the PS-biosynthetic enzyme, PS synthase, compared with endoplasmic reticulum [32]. However, in the transfected cells PEMT2 is not exclusively localized to the mitochondria-associated membrane, but is also found on endoplasmic reticulum (Y.-J. Shiao and J. E. Vance, unpublished work). Thus the expressed PEMT2 appears to have significant preference for PE derived from the CDP-ethanolamine pathway in the McArdle-RH7777 cells.

Earlier studies with rat liver and primary hepatocytes showed a reciprocal relationship between PE methylation and the CDP-choline pathway. For example, in rats receiving a choline-deprived diet, PC biosynthesis via the CDP-choline pathway was inhibited in rat liver, whereas a near doubling of PEMT activity was observed [33]. Subsequent studies, performed after antibody to pure PEMT became available, demonstrated that induction of PEMT activity by choline deficiency was due to an increase in PE levels, and that no change in the amount of PEMT mass was detected by immunoblotting [34]. Choline deficiency has also been recently shown to stimulate the conversion of PE into PC in HepG2 cells, as monitored by isotope incorporation [28]. In another series of experiments, PE methylation was blocked by incubation of rat hepatocytes with 3-deaza-adenosine [35]. Under these conditions, there was a stimulation of the CDP-choline pathway which was ascribed to increased binding of CT to microsomal membranes. Furthermore, supplementation of rat hepatocytes with fatty acids activated PC biosynthesis via the CDP-choline pathway and concomitantly inhibited PE methylation [36]. Also, a rat hepatoma cell line that grew in the absence of choline, serum or exogenous lipid showed an induction of PE methylation and a 3-fold increase in PEMT activity [37]. However, the induced PEMT activity was still very low (1.44 ± 0.29 pmol/min per mg of protein) compared with rat liver (1 nmol/min per mg of protein).

A reciprocal relationship between the methylation and CDP-choline pathways has not always been observed since glucagon and cyclic AMP analogues inhibit PC biosynthesis via both pathways [14,38,39]. Since cyclic AMP and glucagon signal a limited supply of energy, it is reasonable to expect a decrease in the rate of PC biosynthesis via both pathways. Reciprocal regulation of the CDP-choline and PE methylation pathways will be restricted to hepatocytes, since PE methylation is not quantitatively a significant pathway in other cell types [1].

Two implications for biology emerge from the apparent reciprocal relationship between the methylation and CDP-choline pathways. (a) There seems to be a liver-specific regulatory mechanism which co-ordinates PC biosynthesis from PE methylation and the CDP-choline pathway. (b) The opposite relationship between growth and the activities of these two pathways suggest very different biological functions (Figure 5).

CDP-choline pathway and cell division

In experiments recently reported, we have shown that transfection of the McArdle-RH7777 hepatoma cells with PEMT2 results in a 3-fold lower rate of cell division [7]. Control experiments eliminated trivial explanations, such as the possibility that expression of a foreign protein might inhibit cell division. The current experiments suggest that inhibition of the CDP-choline pathway, rather than the expression of PEMT2 itself, might be the reason for a decreased rate of cell division in the transfected

hepatoma cells. A requirement for the CDP-choline pathway for normal cell growth has been known for 4 decades [40]. This was affirmed when Esko et al. [41] isolated a temperature-sensitive Chinese-hamster-ovary (CHO) cell mutant that would not grow at 40 °C and had almost no CT activity at the restrictive temperature. More recent data have suggested that PC is required for cells to progress from the G1 to the S phase of the cell cycle. When colony-stimulating factor was added to a macrophage cell line synchronized in the G1 phase, there was a rapid induction of CT mRNA and cell division progressed [21]. Subsequent experiments demonstrated this induction of CT was necessary to maintain the supply of PC in the cellular membranes in G1 when there was a marked increase in PC catabolism [27]. Tercé et al. [42] have also observed a block in the G1 phase of cell division in C3H/10T1/2 cells that are choline deficient. Thus there seems to be a requirement for PC for cells to progress beyond the G1 phase of cell division.

We have recently provided additional support for the idea that there is some special function of the CDP-choline pathway in cell division that cannot be compensated by PE methylation. Esko et al. [41] reported the isolation of a temperature-sensitive mutant of CHO cells that was defective in CT activity and would not grow at the restrictive temperature of 40 °C. Transfection of these cells with CT restored PC biosynthesis and eliminated the temperature-sensitive phenotype [43,44]. In contrast with our expectations, transfection of the mutant CHO cells with PEMT2 did not rescue the growth phenotype, even though the PC levels were restored to normal values at 40 °C [44]. Thus, as depicted in Figure 5, it appears that PC derived from PEMT2 methylation is not equivalent to PC derived via the CDP-choline pathway. Perhaps the PC provided via the CDP-choline pathway (molecular species or subcellular location) has a special property that is not provided by the methylation pathway. Alternatively, some other unknown requirement, such as modification of a protein by reaction with CDP-choline, might be involved.

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