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Expression of Wild-Type and Mutant Rat Liver CTP: Phosphocholine Cytidylyltransferase in a Cytidylyltransferase-Deficient Chinese Hamster Ovary Cell Line[1]

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The strain 58 Chinese hamster ovary (CHO) mutant defective in CTP:phosphocholine cytidylyltransferase was characterized as an expression system for exogenous cytidylyltransferase. Strain 58 cells express less than 5% of the wild-type level of cytidylyltransferase protein at the permissive temperature even though the steady-state messenger RNA levels were found to be similar to those in the parental CHO-K1 cell line. A point mutation from arginine to histidine at amino acid 140 was identified in the strain 58 protein. Rat liver cytidylyltransferase was stably expressed in strain 58 cells and shown to be active, targeted to the nucleus, phosphorylated, and activated by methylethanolamine supplementation or phospholipase C treatment. Thus, the mechanisms by which cytidylyltransferase is processed and regulated in CHO-K1 cells are intact in strain 58 cells. The heterologously expressed protein complemented the strain 58 defects in both temperature-sensitive growth and phosphatidylcholine biosynthesis, consistent with a single lesion in the structural gene for cytidylyltransferase being responsible for both phenomena. Overexpression of cytidylyltransferase activity at levels up to eightfold higher than those in CHO-K1 cells did not appreciably affect phosphatidylcholine metabolism. A putative casein kinase II phosphorylation site was altered by site-directed mutagenesis and expressed in the strain 58 cells. Alteration of this site did not affect expression and regulation of cytidylyltransferase activity.

The reaction catalyzed by CTP:phosphocholine cytidylyltransferase (EC 2.7.7.15) is the major point of control of *de novo* phosphatidylcholine biosynthesis in mammalian cells (1). The enzyme is present as an inactive, soluble, nucleoplasmic form (2) which becomes activated in response to numerous stimuli (1) and translocates (3) to the nuclear envelope (4, 5). The mechanistic features of the activation and translocation processes are not understood in detail, but may involve direct interaction with lipids (6, 7), protein–protein interaction (8), and phosphorylation–dephosphorylation (9–12).

In order to elucidate the mechanistic details of catalysis and regulation by this enzyme, it is necessary to express normal and mutant cDNAs for the enzyme in a suitable system. Kalmar *et al.* (13) cloned a cDNA encoding the rat liver enzyme and demonstrated enzymatic activity of the encoded protein by expression in a COS-1 cell line. Cytidylyltransferase activity in the transfected cells was 10-fold higher than the endogenous activity found in untransfected COS-1 cells, indicating that this system would be suitable for some applications, such as determining how overexpression of the enzyme affects cytidylyltransferase regulation and phosphatidylcholine metabolism. The COS cell system, however, would not be suitable for expressing mutant cytidylyltransferase constructs, since the endogenous COS cell cytidylyltransferase would mask the effects of the mutant enzymes.

A desirable feature of an expression system for a protein is the absence of expression of the homologous, endogenous protein in the system. Cytidylyltransferase, however, as a housekeeping enzyme, is expected to be expressed in all nucleated mammalian cells. Fortunately, a Chinese hamster ovary (CHO)[3] cell line with unusually low levels of cytidylyltransferase has been established. The strain

[1] The nucleotide sequence for CHO-K1 cytidylyltransferase has been submitted to GenBank and assigned Accession No. L13244.

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[3] Abbreviations used: CHO, Chinese hamster ovary; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PLC, phospholipase C; poly(A)$^+$, polyadenylation-selected.

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58 CHO mutant, isolated by Esko and co-workers (14, 15), is deficient in and temperature-sensitive for cytidylyltransferase. The mutant cell line was isolated by mutagenizing with ethyl methane sulfonate and subsequently screening for colonies deficient in incorporation of choline into phospholipids. The mutant cell line is temperature-sensitive for both growth and choline incorporation into lipid, and cytidylyltransferase activity is temperature-sensitive *in vitro*. Reversion rate analysis suggests that a single point mutation in the structural gene for cytidylyltransferase is responsible for the observed phenotype. The strain 58 cells are able to maintain phosphatidylcholine levels at 80–90% of wild-type levels at the permissive temperature.

In the present study we have characterized the defect in strain 58 in more detail and expressed rat liver cytidylyltransferase in strain 58 cells. The defect in the host strain does not interfere with expression, targeting, phosphorylation, translocation, or activation of a heterologously expressed cytidylyltransferase. Furthermore, Chinese hamster ovary cells expressing total cytidylyltransferase activity over a 160-fold range do not have significant differences in phosphatidylcholine metabolism, suggesting that these cells are able to compensate for excess enzyme by decreasing enzymatic activity. We have used the strain 58 cell line as a host cell to characterize a mutant cytidylyltransferase altered by site-directed mutagenesis.

EXPERIMENTAL PROCEDURES

Materials. All radiochemicals were purchased from Amersham. Electrophoresis grade reagents for polyacrylamide gel electrophoresis were purchased from Bio-Rad. All general molecular biology reagents were purchased from GIBCO-BRL unless otherwise stated. Synthetic oligonucleotides were prepared by the Laboratory for Macromolecular Structure, Purdue University. Electron microscopy grade formaldehyde (20%) was purchased from Ladd Research Industries, Inc. Methanol and acetone were purchased from Baxter. All other chemicals were reagent grade from Sigma unless otherwise stated. The pCMV4 plasmid was a gift of Dr. David Russell, University of Texas, Southwestern Medical Center.

Cell culture. CHO-K1 (ATCC CCL6) cells were obtained from the American Type Culture Collection. CHO strain 58 (14, 15) was obtained from Dr. C. R. H. Raetz. All cells were cultured in F12 (Ham's) medium containing 10% fetal bovine serum (Whittaker Bioproducts) as described (4).

Cell fractionation. Cell fractionation with digitonin was performed as described previously (16) with 0.4 mg/ml digitonin and protease inhibitors (1 μg/ml leupeptin, 2 μg/ml antipain, 10 μg/ml benzamidine, 1 μg/ml chymostatin, 1 μg/ml pepstatin, and 1 mM phenylmethyl sulfonyl fluoride) included in the release buffer.

Enzyme and miscellaneous assays. Cytidylyltransferase activity was measured as described previously (17). All cytidylyltransferase assays contained 0.2 mM phosphatidylcholine plus oleic acid (1:1 molar ratio), 10 mM $MgCl_2$, and 6 mM ADP (18), unless otherwise noted. Protein was assayed by the modified Lowry method (DC protein assay; Bio-Rad), as indicated.

Western blot analysis. Indicated amounts of protein were applied to 10% Laemmli gels (19) and electrophoresed for 1 h at 200 V, constant voltage. The proteins were electroblotted to Immobilon-P membranes (Millipore) and processed as described (8). Rabbit antiserum to the amino terminus of rat liver cytidylyltransferase (5) was used at a dilution of 1:1000, and horseradish peroxidase-conjugated goat anti-rabbit IgG (GIBCO-BRL) was used at a dilution of 1:3000. Immune complexes were detected by the chemiluminescence detection method (ECL, Amersham) with Hyperfilm-ECL (Amersham). Relative intensity of immunoreactive protein bands was quantitated on a Zeineh Soft Laser Scanning Densitometer at 520 nm.

Immunofluorescence. Cells were plated and processed for immunofluorescence as described (2). Affinity-purified antibody (5) was used at a dilution of 1:400 and fluorescein-conjugated, horse anti-rabbit IgG (Vector) was used for detection at a dilution of 1:200.

Ribonuclease protection assay. Polyadenylation-selected (poly(A)$^+$) RNA was prepared using the FastTrack mRNA Isolation Kit (Invitrogen). Ribonucleotide probes were prepared using the MAXIscript In Vitro Transcription Kit (Ambion) with [α-^{32}P]dUTP. The RPA II Ribonuclease Protection Assay Kit (Ambion) was used to measure levels of cytidylyltransferase mRNA from CHO-K1 cells and strain 58 cells. Following autoradiography, bands were cut from the gel, added to 5 ml Ecolume liquid scintillant, and counted in a Beckman LS1701 scintillation counter. Cytidylyltransferase mRNA levels were normalized to the β-actin mRNA levels of each cell type.

Cloning and sequencing. A partial cDNA for CTP:phosphocholine cytidylyltransferase was cloned from CHO-K1 cells by the polymerase chain reaction (PCR). Poly(A)$^+$ RNA was isolated from 5×10^7 cells using the FastTrack kit (Invitrogen). First-strand cDNA synthesis was performed with the cDNA Cycle kit (Invitrogen) and used directly for PCR. PCR mixtures contained 1× PCR Buffer (Cetus), 2.5 mM dATP, 2.5 mM dCTP, 2.5 mM cGTP, 2.5 mM dTTP, and 5′-ATCGATCTAGAATGGAYGCNCARAGYTCNGCNAA-3′ and 5′-ATCGATGTCGACTCYTCYTCRTCYTCRCT(AGT)ATRTCRCA-3′ as primers. Reactions were annealed for 1 min at 53°C, extended for 2 min at 72°C, and melted for 30 s at 94°C. The amplified fragments were cut with *XbaI* and *SalI*, cloned into pBluescript II KS+ and M13mp18, and sequenced in both directions using four internal primers in each direction. Five clones derived from three distinct CHO-K1 mRNA preparations and four clones derived from two distinct strain 58 mRNA preparations were analyzed. Each segment of the cDNA was sequenced at least three times in each direction.

The 5′ and 3′ ends of the coding sequence and the adjacent untranslated regions of the cDNAs were obtained using the RACE (rapid amplification of cDNA ends) method (20) as described with the kit materials (GIBCO-BRL) and sequenced directly following gel purification. For generation of the 5′ end, the gene-specific primer 5′-AGTCAAATATTCCATCCGC-3′ was used, and for generation of the 3′ end, the gene-specific primer 5′-ATCGATCTAGACACTGCCGTTACGTAGACGAGGTGGTG-3′ was used.

Computer-based sequence analysis was performed on the Clinical Research Center VAX computer (University of Michigan Hospital) using the Genetics Computer Group Sequence Analysis Software Package, version 7.1 (21). The nucleotide sequence for CHO-K1 cytidylyltransferase (Accession No. L13244) has been submitted to the GenBank Database.

Stable transfection of CHO strain 58 cells. A cDNA encoding rat liver cytidylyltransferase (22) was inserted into the pCMV4 cloning vector (23) using the *HindIII* and *XbaI* restriction sites, resulting in the plasmid pCMV4-RCCT. The vector was prepared as described (24) and purified through two cesium chloride gradients. CHO strain 58 cells were plated at density of 5×10^5 cells/100-mm dish for transfection. Twenty micrograms of pCMV4-RCCT was mixed with 1 μg of pWL-neo (Stratagene) and 50 μg Lipofectin (GIBCO-BRL) and incubated for 20 min at room temperature. The cells were washed three times with 5 ml of calcium- and magnesium-free PBS and fed 5 ml serum-free F12 medium. The DNA–Lipofectin mixture was added dropwise and left on the cells for 5 h. The cells were then fed 10 ml F12 medium with 10% fetal bovine serum and grown for 48 h at 34°C. The cells were subcultured to 150-mm culture dishes at a split ratio of 1:12 and after 24 h were fed fresh F12 medium containing 10% fetal bovine serum, 0.4 mg/ml geneticin, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. The cells were grown

at 34°C until visible colonies could be isolated with cloning cylinders and subcultured as described (25). Cell lines were screened for expression of cytidylyltransferase by activity assays, Western blot analysis, and immunofluorescence.

Phosphopeptide mapping. Cells were plated and labeled as described (11) with modification. Cells were labeled for 8 h at 34°C in the presence of 2.5 mCi/ml of carrier-free $^{32}P_i$. Cells were washed with PBS and scraped in 0.2 ml lysis buffer (40 mM $NaHPO_4$, pH 7.4, 100 mM NaF, 1% Nonidet P-40, 400 mM NaCl, 10 mM EDTA, 0.2 mM dithiothreitol, and 0.4 mg/ml digitonin) with protease inhibitors and incubated for 5 min at room temperature. The cell ghosts were pelleted by centrifugation at 15,000*g* for 15 min at 4°C and the soluble fraction was transferred to a fresh tube containing 2 μl of anti-rat liver cytidylyltransferase rabbit serum (5). The antibody–homogenate mixture was incubated for 6 h at 4°C with constant mixing. Protein A–Sepharose was prepared by swelling the dry support for 2 h in wash buffer (50 mM Tris–Cl, pH 8.3, 150 mM NaCl, 1 mM EDTA, and 0.5% Nonidet P-40). The resin was washed twice with wash buffer containing 1.5% bovine serum albumin to block nonspecific binding interactions, washed five times with wash buffer alone to remove excess albumin, resuspended in an equal volume of wash buffer, and stored at 4°C. Forty microliters of the protein A–Sepharose slurry was added to the antibody–homogenate mixture and incubated at 4°C for 1 h with constant mixing. Immune complexes were collected by centrifugation, eluted, and separated by polyacrylamide gel electrophoresis as described (11). Membranes were exposed to X-ray film (Kodak) at room temperature for 0.5–4 h. Digestion with trypsin of cytidylyltransferase and peptide separation was performed essentially by the method of Aebersold *et al.* (26) with minor modifications (11).

Metabolite analysis. Cell cultures were incubated with radioactive choline and phospholipase C as described for each experiment. The cultures were harvested by washing the monolayers three times with ice-cold PBS, and scraping the cells from the dishes in 0.5 ml distilled water. The cell suspensions were extracted with chloroform–methanol as described (27), using distilled water instead of saline. The organic phases were evaporated under N_2 and counted in a Beckman LS1701 liquid scintillation counter. The aqueous phases were evaporated under reduced pressure and the metabolites quantitated as described (28).

Methylethanolamine treatment. Cells were seeded in duplicate dishes at a density of 2×10^5 cells/60-mm culture dish in choline-free F12 medium containing 10% dialyzed fetal bovine serum and supplemented with 100 mM choline. The cells were allowed to recover for 8 h at 34°C. The cells were then washed twice with 5 ml of PBS and fed fresh choline-free F12 medium containing 10% dialyzed fetal bovine serum and either 100 μM choline or 100 μM methylethanolamine and returned to 34°C for growth. The cells were routinely grown for 48 h prior to harvesting with fresh changes of medium each day.

Phospholipase C treatment. Phospholipase C was purified from the medium of *Clostridium perfringens* cultures through the ion-exchange chromatography step as described (29). The purified enzyme was diluted into calcium- and magnesium-free PBS plus 1 mg/ml bovine serum albumin and stored in liquid nitrogen. Enzyme activity was determined by the simultaneous assay as described (30). The cells were washed twice with calcium- and magnesium-free PBS and the enzyme was added from the frozen stocks to a final concentration of 10 mUnits/ml in F12 medium containing 5% fetal bovine serum. Phospholipase C treatments were routinely 3 h in duration, immediately prior to harvest of the cells.

Site-directed mutagenesis. Site-directed mutagenesis was performed as described with the Oligonucleotide-Directed In Vitro Mutagenesis System, version 2.0 (Amersham). The primers used for mutagenesis were 5'-GTGACATCTGCGAGGATGA-3' (Ser^{362} to Cys^{362}) and 5'-GTGACATCGCCGAGGATGA-3' (Ser^{362} to Ala^{362}).

RESULTS

Characterization of Cytidylyltransferase in Strain 58

The strain 58 cell line isolated by Esko and co-workers (14, 15) is deficient in and temperature-sensitive for cytidylyltransferase activity. At the permissive temperature, 34°C, the mutant cells contain approximately 20-fold less cytidylyltransferase activity than the CHO-K1 cells, while at the nonpermissive temperature, 40°C, the difference is approximately 100-fold (15, 17). Western blot analysis of the two cell lines with a polyclonal rabbit antibody to the amino terminus of cytidylyltransferase (5) demonstrates that, even at the permissive temperature, strain 58 cells contains less than 5% of the cytidylyltransferase protein present in the CHO-K1 cells (2). This result was also observed with an additional rabbit antibody raised against recombinant rat liver cytidylyltransferase produced in a baculovirus[4] overexpression system[4] (22). Furthermore, indirect immunofluorescence of the mutant cell line with the anti-amino terminus antibody (5) shows a marked absence of nuclear staining in comparison to the CHO-K1 cells (2). It appears, therefore, that the low activity measurable in strain 58 cells at 34°C reflects the low level of enzyme protein present.

Although evidence suggests that the lesion in strain 58 cells is in the cytidylyltransferase gene, it was also possible that a regulatory mutation accounts for the low levels of cytidylyltransferase. Messenger RNA levels for cytidylyltransferase were, therefore, compared for the two cell lines by ribonuclease protection assay. When normalized to β-actin mRNA levels in each cell type, the ratio of cytidylyltransferase mRNA in strain 58 cells relative to the CHO-K1 cells was 0.90 ± 0.13. The fact that the steady-state mRNA levels were similar in the two cell types is consistent with previous data (15) suggesting that a single point mutation is responsible for the observed defect in strain 58 cells. The fact that cytidylyltransferase protein levels were so low in strain 58 is probably due to enhanced degradation of the mutant protein.

In order to identify the lesion in strain 58 cytidylyltransferase, it was necessary to determine the deduced protein sequences of the enzyme from both wild-type CHO-K1 and strain 58 cells. The cDNA sequence encoding CHO-K1 cytidylyltransferase was obtained and compared to the rat liver[5] (13) and mouse testis[6] cDNA sequences (Fig. 1). Cytidylyltransferase is highly conserved between these species with 97% sequence identity at the

[4] Y. Wang, J. I. S. MacDonald, and C. Kent, unpublished observations.

[5] Discrepancies from the reported rat liver cDNA sequence (13) are reported here at positions 91 and 114. The identities of these residues were deduced from independent cDNA clones for the rat liver enzyme (22), and conservation of the residue at these positions in the CHO-K1 (Fig. 1), mouse testis,[6] and *Saccharomyces cerevisiae* (35) was deduced from amino acid sequences. In addition, a glycine residue was confirmed at position 91 by partial peptide sequence of tryptic peptides of purified rat liver cytidylyltransferase (T. D. Sweitzer and C. Kent, unpublished observations).

[6] M. S. Rutherford, C. O. Rock, N. A. Jenkins, D. J. Gilbert, T. G. Tessner, N. G. Copeland, and S. Jackowski submitted for publication. The sequence is available from GenBank, Accession No. Z12302.

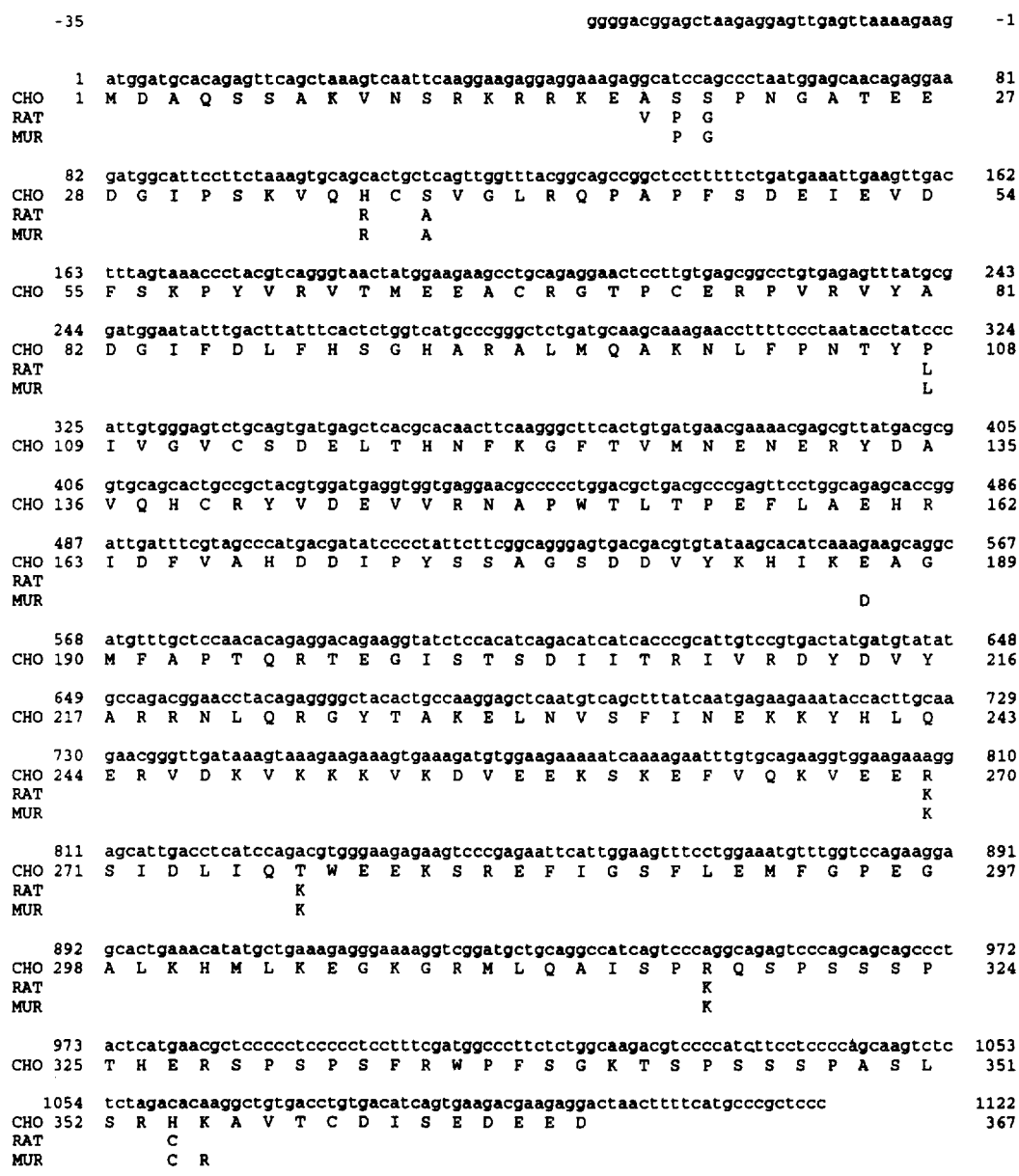

FIG. 1. Nucleotide and deduced amino acid sequence of a partial cDNA encoding CHO-K1 cytidylyltransferase and alignment with the rat and mouse protein sequences. The cDNA was cloned and sequenced as described under Experimental Procedures. The deduced amino acid sequence is given for the CHO-K1 sequence, whereas only the differences are shown for the rat liver[4] (RAT) and mouse testis (MUR) sequences.

amino acid level and 94% sequence identity at the nucleotide level between the hamster and either of the other two rodent sequences. Next, the sequence of the strain 58 cDNA was determined. The only change in the cDNA sequence of strain 58 cytidylyltransferase was a guanine to adenine point mutation corresponding to nucleotide 419 in the CHO-K1 sequence (Fig. 1). The altered codon encodes a histidine instead of an arginine at position 140 in the deduced amino acid sequence. Apparently this defect is responsible for the observed destabilization of the cytidylyltransferase protein and activity *in vitro* and *in vivo*.

Expression of Rat Liver Cytidylyltransferase in Strain 58 Cells.

To determine if strain 58 cells were a suitable system for heterologous cytidylyltransferase expression, a cDNA fragment encoding rat liver cytidylyltransferase (22) was cloned into the pCMV4 mammalian expression vector (23). This vector utilizes the human cytomegalovirus immediate early promoter and an alfalfa mosaic virus enhancer to express foreign genes at high levels. When this construct was used in transient expression assays, rat liver cytidylyltransferase was expressed in the nucleus of strain

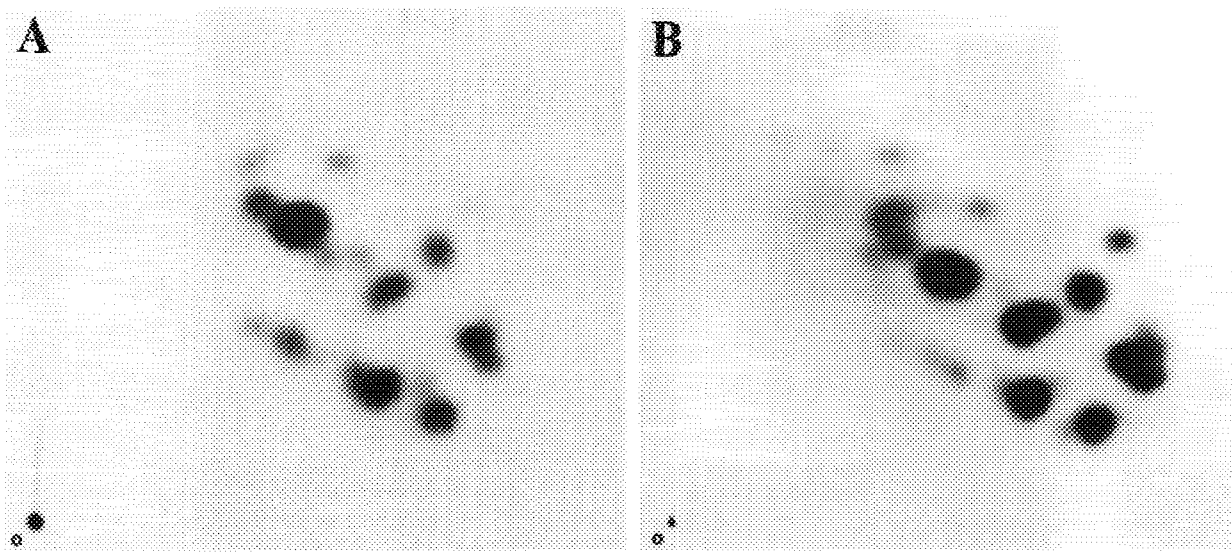

FIG. 2. Phosphopeptide mapping of ^{32}P-labeled cytidylyltransferase. Cytidylyltransferase was immunoprecipitated from the cytosolic fraction of CHO cells, separated by SDS–PAGE, blotted, and digested with trypsin as described under Experimental Procedures. Two-dimensional chromatography/electrophoresis on cellulose thin-layer plates was used to resolve phosphopeptides from CHO-K1 cells (A) and strain 58 cells expressing the rat liver enzyme (B). Electrophoresis was from left (cathode) to right (anode), and chromatography was from bottom to top. The origin (*o*) is indicated.

58 cells (2) and was active. To characterize the expressed protein in more detail, strain 58 cell lines stably expressing the rat liver enzyme were isolated and studied.

Sixteen clonal cell lines were isolated that expressed cytidylyltransferase activity over a range of 0.2- to 10-fold that of the wild-type CHO-K1 cell line. Western blot analysis of cell homogenates reflected the wide range of activities expressed in the clonal cell lines, and the expressed protein migrated with the same mobility as the CHO-K1 enzyme by SDS–polyacrylamide gel electrophoresis (data not shown).

K_m values of the expressed enzyme were determined from secondary plots of families of curves obtained at several fixed concentrations of one substrate, with the other substrate varied. The expressed rat liver enzyme had K_m values for both substrates similar to those determined for the endogenous CHO-K1 enzyme and the rat liver enzyme expressed in a baculovirus overexpression system (22). The K_m values for CTP were 0.41 ± 0.05, 0.47 ± 0.09, and 0.51 ± 0.04 mM for the strain 58-expressed rat liver enzyme, CHO-K1 enzyme, and baculovirus-produced enzyme, respectively. The K_m values for phosphocholine were 0.47 ± 0.15, 0.43 ± 0.05, and 0.39 ± 0.09 mM for the strain 58-expressed enzyme, CHO-K1 enzyme, and baculovirus-produced enzyme, respectively.

A major mechanism for regulation of cytidylyltransferase activity involves phosphorylation and dephosphorylation (9–12). To determine if the expressed rat liver enzyme was phosphorylated in a fashion similar to that for the endogenous CHO-K1 enzyme, unstimulated cells

TABLE I

Stimulation of Cytidylyltransferase Translocation by Methylethanolamine Treatment and Phospholipase C Treatment

		Methylethanolamine treatment		Phospholipase C treatment	
Cell type	Fraction	+Choline	+Methylethanolamine	−PLC	+PLC
CHO-K1	Soluble	20.8 ± 0.71 (85.7)	2.4 ± 0.06 (8.0)	19.2 ± 0.52 (82.4)	7.5 ± 0.12 (30.0)
	Particulate	3.5 ± 0.16 (14.3)	27.8 ± 1.05 (92.1)	4.1 ± 0.04 (17.6)	17.5 ± 0.11 (70.0)
RL-wt	Soluble	14.7 ± 0.04 (67.4)	11.6 ± 0.45 (37.3)	16.6 ± 0.56 (70.3)	12.9 ± 0.42 (41.9)
	Particulate	7.1 ± 0.21 (32.6)	19.5 ± 0.68 (62.7)	7.0 ± 0.50 (29.7)	17.9 ± 0.63 (58.1)

Note. Activity is expressed as nmol CDP–choline synthesized/min/mg protein. Values represent the mean specific activity ± range for duplicate dishes. Percentage of total activity is given in parentheses. Cells were grown for 2 days in the presence of 100 μM choline or 100 μM methylethanolamine in the absence of choline or treated with 10 mU/ml phospholipase C from *C. perfringens* for 3 h as described under Experimental Procedures. Cells were fractionated with digitonin and cytidylyltransferase activity was determined in the soluble and particulate fractions.

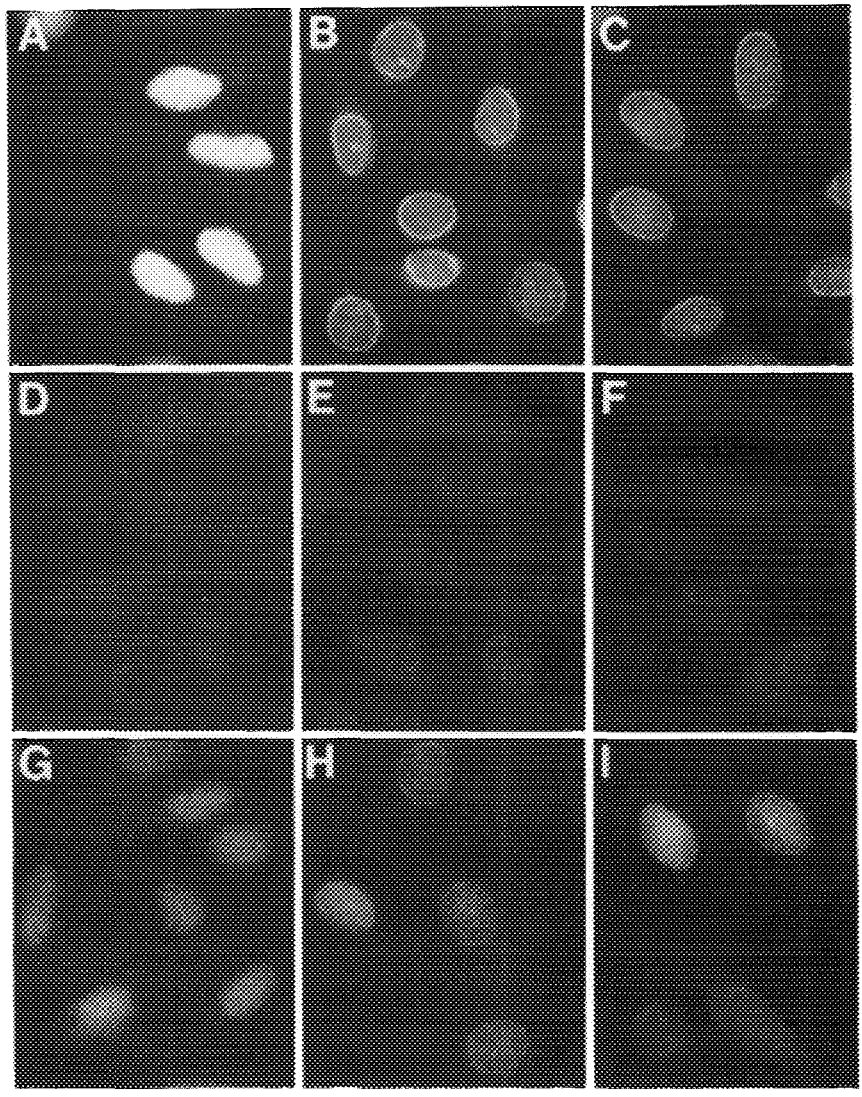

FIG. 3. Stimulation of cytidylyltransferase translocation to the nuclear envelope by treatment with methylethanolamine or phospholipase C. Cells were treated as described in the legend to Table 1 and processed for immunofluorescence as described under Experimental Procedures. CHO-K1 cells (A–C), strain 58 cells (D–F), and strain 58 cells expressing rat liver cytidylyltransferase (G–I) were left untreated (A, D, and G), treated with methylethanolamine (B, E, and H), or treated with phospholipase C (C, F, and I).

were grown for 8 h in the presence of $^{32}P_i$, and the soluble cytidylyltransferase was immunoprecipitated and subjected to two-dimensional tryptic peptide mapping. The phosphorylation pattern of the expressed rat liver cytidylyltransferase was quite similar to the CHO-K1 protein phosphorylation pattern (Fig. 2).

Response to Stimuli

Translocation and activation of cytidylyltransferase in CHO-K1 cells can be stimulated by growth in medium lacking choline and supplemented with monomethylethanolamine (31) or by treatment of cells with phospholipase C from *C. perfringens* (17). Growth of cells expressing the rat liver enzyme in choline-free medium supplemented with monomethylethanolamine resulted in a 2.7-fold increase in cytidylyltransferase activity associated with the particulate fraction (Table I). (For these experiments we used a clone that expressed the rat liver enzyme at a level similar to that of CHO-K1.) Similarly, treatment with phospholipase C resulted in an 2.5-fold increase in activity associated with the particulate fraction (Table I). The translocation was qualitatively observed by indirect immunofluorescence as a transition from a diffuse nuclear staining pattern in control cells to a sharp ring around the nucleus in stimulated cells (Fig. 3).

Complementation of the Strain 58 Phenotype

To determine if expression of rat liver cytidylyltransferase could overcome the temperature-sensitive growth of strain 58, growth curves of strain 58, CHO-K1, and

strain 58 cells expressing the rat liver enzyme were performed. Cells were grown for 24 h at 34°C and then shifted to 40°C (Fig. 4). As observed by Esko *et al.* (15), the strain 58 cells continued to grow for 24–36 h after the temperature shift and then stopped growing, while the parental CHO-K1 cells continued to grow. Strain 58 cells expressing the rat liver enzyme also continued to grow at the nonpermissive temperature.

Expression of rat liver cytidylyltransferase also overcame the defect in the mutant in phosphatidylcholine biosynthesis. The rate of choline incorporation into lipid was increased in cells expressing the rat liver enzyme when they were shifted from 34 to 40°C (Fig. 5). Cells expressing the rat liver enzyme incorporated 60% more choline in a 5-h period at 40°C than at 34°C, similar to the 50% increase in the rate of incorporation observed in the CHO-K1 cells under the same conditions. Choline incorporation into lipid in strain 58 cells, however, decreased by more than 95% when shifted to the nonpermissive temperature. Thus, expression of rat liver cytidylyltransferase overcomes the defects in strain 58 in both growth and phosphatidylcholine biosynthesis.

Phosphatidylcholine Metabolism in Cells Overexpressing Cytidylyltransferase

In order to ask if overexpression of heterologous cytidylyltransferase perturbs phosphatidylcholine metabolism in CHO cells, a clonal cell line overexpressing the rat liver enzyme was selected and analyzed. The activity of cytidylyltransferase, assayed *in vitro*, in this cell line was 57.5 nmol/min/mg of cell protein compared to the wild-type CHO-K1 activity of 7.65 nmol/min/mg protein. Even though a large excess of cytidylyltransferase activity was available in these cells, the phosphatidylcholine content of the cells was similar to that of CHO-K1 cells (Table II). The flux through the biosynthetic pathway was estimated by determining the turnover rate of phosphocholine, the metabolic intermediate immediately prior to the rate-determining step for the pathway (32). The phosphocholine pool was larger in cells expressing the rat liver enzyme than that in the CHO-K1 cells; however, the fractional turnover rate, or the fraction of the phosphocholine pool turned over per unit time (32), was lower (Table II). Therefore, the net turnover rate of phosphocholine, which is simply the product of the fractional turnover rate times the pool size, was similar in the two cell lines. The flux through the pathway, therefore, was also similar in the two cell lines. The fact that the CDP–choline pool size (Table II) was no higher in cells expressing the rat liver enzyme is further evidence for the lack of increased cytidylyltransferase activity in these cells.

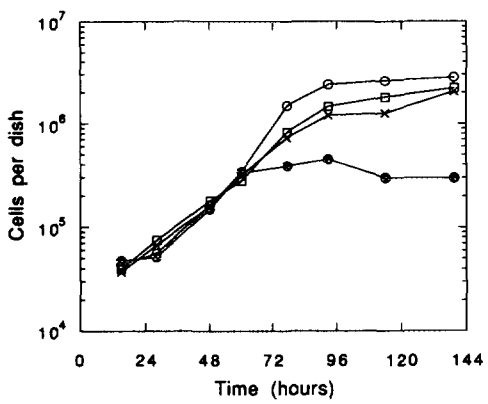

FIG. 4. Growth of CHO-K1 cells, strain 58 cells, and cells expressing rat liver cytidylyltransferase at 40°C. Multiple 60-mm dishes were inoculated with approximately 2.5 × 10^4 cells and incubated at 34°C. Twenty-eight hours after the start of the experiment, the cultures were transferred to 40°C. At the indicated times cells were dispersed with trypsin, diluted 1:1 with 0.05% eosin B, and counted on a hemacytometer. ○, CHO-K1 cells; ●, strain 58 cells; ×, strain 58 cells expressing the rat liver enzyme; and □, cells expressing the S362A enzyme.

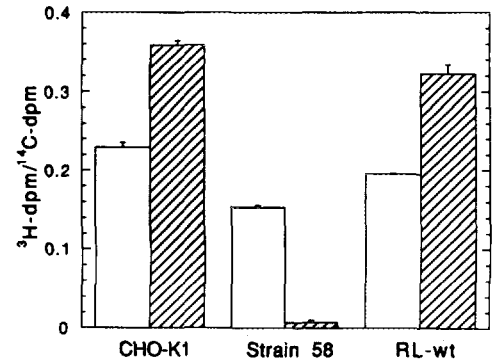

FIG. 5. Choline incorporation of CHO-K1 cells, strain 58 cells, and cells expressing rat liver cytidylyltransferase at 40°C. Cells were labeled for 2 days in the presence of [^{14}C]choline. To initiate the experiment, the cells were fed fresh medium containing [^{14}C]choline and [^{3}H]choline. Half of the dishes were returned to 34°C (open bars) and the remaining dishes were shifted to 40°C (hatched bars) for 5 h. Cells were harvested and the lipid fraction was extracted as described under Experimental Procedures. Data are expressed as the ratio of [^{3}H]choline to [^{14}C]choline associated with the lipid fraction.

Expression of Cytidylyltransferase with an Altered Casein Kinase II Site

A comparison of the deduced amino acid sequences for the mammalian and yeast cytidylyltransferases shows that a putative casein kinase II site at the carboxy terminus of the rat enzyme is highly conserved over a wide evolutionary range. This high degree of conservation suggests that this site is critical for cytidylyltransferase function. Preliminary studies with a synthetic peptide corresponding to the C-terminal 17 amino acids of the rat liver enzyme demonstrated that the peptide was phosphorylated by purified casein kinase II *in vitro*,[7] indicating that this sequence is a suitable casein kinase II substrate.

[7] Y. Wang and C. Kent, unpublished observations.

TABLE II

Phosphocholine Turnover and Choline-Labeled Pool Levels of CHO-K1 Cells, Strain 58 Cells, and Cells Expressing Rat Liver Cytidylyltransferase

	Phosphocholine			CDP-choline	Phosphatidylcholine
Cell type	Fractional turnover (h^{-1})	Pool size (pmol/μg)	Turnover rate (pmol/h)	Pool size (pmol/μg)	Pool size (pmol/μg)
CHO-K1	0.562	3.58 ± 0.22	2.01	0.39 ± 0.15	52.2 ± 4.3
Strain 58	0.299	5.89 ± 0.3	1.76	0.15 ± 0.03	41.5 ± 3.9
RL-wt	0.277	8.57 ± 1.05	2.37	0.37 ± 0.10	45.5 ± 1.3

Note. The rate of metabolism of intracellular phosphocholine was determined by labeling the cells for 2 days with [^{14}C]choline and [^{3}H]choline, followed by a chase of growth medium containing [^{14}C]choline alone as described under "Experimental Procedures". The change in the ratio of ^{3}H to ^{14}C in the phosphocholine pool was followed for 1 hour to determine the fractional turnover rate of phosphocholine. The R values for determination of the fractional turnover rate ranged from 0.992–0.994. Values for the pool sizes are averages ± standard deviations of five (phosphocholine) or six (CDP-choline and phosphatidylcholine) samples.

Moreover, studies with baculovirus-expressed rat liver cytidylyltransferase have shown that this site is phosphorylated *in vivo*.[8] To investigate whether this site is involved in the regulation of cytidylyltransferase activity in CHO cells, we altered the casein kinase II site and determined the effects of the alteration on enzyme activity and phosphorylation.

Oligonucleotide-directed mutagenesis was used to alter Ser^{362} to either Ala or Cys, and the altered proteins were expressed and characterized in CHO strain 58 cells. The properties of the S362A enzyme were identical to those of S362C. Several properties of the mutant enzyme were similar to those of the wild-type rat liver enzyme (data not shown): (i) the altered protein had K_m values for both CTP and phosphocholine similar to those measured for the wild-type enzyme; (ii) the altered enzyme was nuclear and translocated to the nuclear envelope in response to phospholipase C treatment or supplementation with methylethanolamine; (iii) the altered enzyme complemented the strain 58 phenotype for choline incorporation at 40°C, and (iv) phosphocholine pool sizes and turnover rates in cells expressing the altered enzyme were similar to those measured in cells expressing the wild-type enzyme. Finally, the mutant cytidylyltransferase was as capable as the wild-type enzyme at complementing the mutant phenotype with respect to growth at 40°C (Fig. 4).

The apparent lack of effect of the altered casein kinase II site on phosphatidylcholine biosynthesis led us to wonder if the site in the wild-type enzyme was actually phosphorylated *in vivo*. If not, then the tryptic phosphopeptide patterns of normal and altered cytidylyltransferase might be expected to be identical, because cytidylyltransferase is phosphorylated only on Ser (10) and there are no other serines in the tryptic peptide containing this site. Instead, phosphopeptide mapping of the altered cytidylyltransferase protein gave complex results (Fig. 6). The peptide containing the casein kinase II site was identified from our baculovirus work[8] as the lower of the two peptides farthest to the right of each panel, indicated by the arrowhead in Fig. 6A. As expected, this peptide was diminished in the S362A mutant. Presumably the remaining low level of this peptide arises from the endogenous strain 58 cytidylyltransferase. Other qualitative changes in the phosphopeptide maps were seen, however. Several phosphopeptides from the altered protein had an increased intensity while other spots decreased in intensity. This suggests that mutation of the putative casein kinase II site had additional effects on other phosphorylation sites.

DISCUSSION

Properties of Strain 58

From their studies on strain 58, Esko *et al.* (15) concluded that a single genetic defect is responsible for the temperature sensitivity of growth, conditionally defective phosphatidylcholine biosynthesis, and conditional decrease in cytidylyltransferase activity. They proposed that the mutation is most likely in the structural gene for cytidylyltransferase, because cytidylyltransferase activity is thermolabile *in vitro*, although they could not rule out a defect in regulation or post-translational modification. We have presented several lines of evidence that argue strongly that the defect is in the structural gene for cytidylyltransferase: (i) The ability to correct the mutant phenotypes in growth, choline incorporation, cytidylyltransferase activity, and immunocytochemical staining (11) by expressing exogenous cytidylyltransferase demonstrates that post-translational processing is not defective in the mutant. (ii) The fact that the mRNA level for cytidylyltransferase in strain 58 is the same as that in wild-type cells rules out a regulatory mutation affecting expression of cytidylyltransferase. (iii) We have identified a single base change in cytidylyltransferase cDNA from strain 58, which result in conversion of Arg^{140} to His. It is unlikely that this change is due to clonal variation be-

[8] J. I. S. MacDonald and C. Kent (1994) *J. Biol. Chem.*, in press.

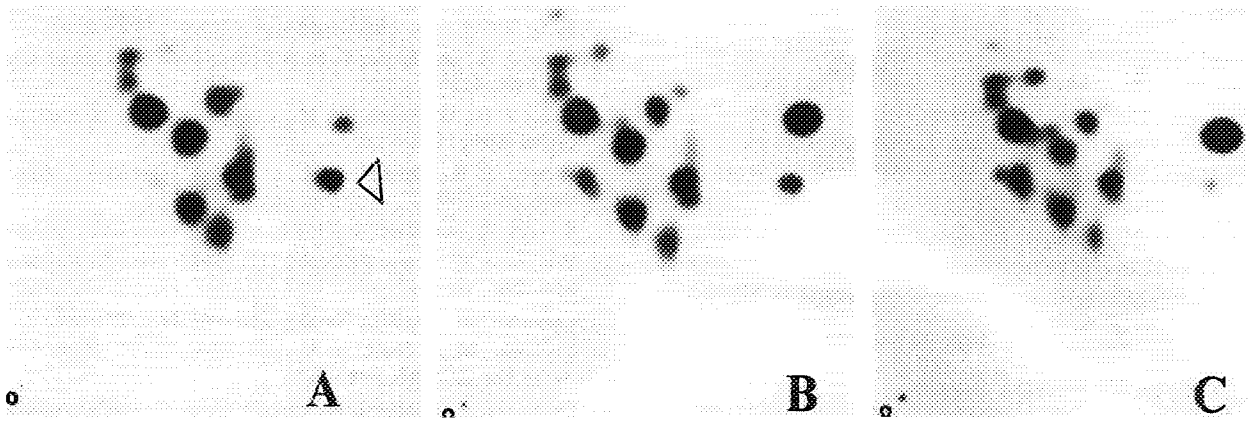

FIG. 6. Phosphopeptide mapping of altered cytidylyltransferase. Cells were labeled and phosphopeptides separated as described in the legend to Fig. 2. A, cytidylyltransferase from strain 58 cells expressing the wild-type enzyme; B, a mixture of equal levels, in dpm, of phosphopeptides from cells expressing wild type enzyme and those from cells expressing S362A enzyme; C, cytidylyltransferase from strain 58 cells expressing the S362A enzyme.

cause Arg^{140} is highly conserved throughout evolution. In fact, the sequence R^{140}YVDEV is found in a bacterial glycerol phosphate cytidylyltransferase (33), arguing that this Arg is critical to cytidylyltransferase structure and/or function. Preliminary evidence indicates that conversion of this Arg to Ala in the bacterial cytidylyltransferase results in a considerable loss in activity.[9]

Effects of Overexpression of Cytidylyltransferase on Phosphatidylcholine Metabolism

In this paper we have shown that overexpression of cytidylyltransferase in CHO cells has remarkably little effect on the CDP–choline pathway. In a sense, this enzyme is already overexpressed in normal cells; i.e., most of the enzyme is inactive. The high level of inactive enzyme seems to serve as a reservoir of enzyme that can be rapidly activated. Strain 58 cells, having only 5% of wild-type enzyme levels, can get along quite well at 34°C with only a slight reduction in growth rate. This indicates that the inactive reservoir is not needed under routine culture conditions. A deleterious effect of the strain 58 mutation can be seen only upon further reduction of cytidylyltransferase activity by temperature shift to 40°C or upon treatment of the cells with phospholipase C, a situation which necessitates activation of the enzyme in the reservoir (34). The cytidylyltransferase in *Saccharomyces cerevisiae* has been overexpressed about 114-fold. This very high level of expression results in only a sixfold increase in CDP–choline levels and no change in the rate of incorporation of labeled choline into phosphatidylcholine (35).

Rat liver cytidylyltransferase has also been over expressed in transient assays in COS cells (36). As was found in the present studies, the extent of stimulation of phosphatidylcholine biosynthesis by overexpressed cytidylyltransferase is not as great as the level of overexpression. Unlike the present studies, however, overexpression in COS cells results in a three- to fivefold increase in phosphatidylcholine biosynthesis, accompanied by a large increase in the CDP–choline pool. The rate of degradation of phosphatidylcholine in the COS system is also increased by about threefold, apparently to prevent accumulation of excess phosphatidylcholine. Thus it appears that mammalian cells can react very differently to overexpression of a critical enzyme, with the CHO system maintaining a tighter control on the activity of the overexpressed cytidylyltransferase.

In general, however, overexpression of enzymes of lipid metabolism has little effect on lipid metabolism and composition. When CHO phosphatidylserine synthase is overexpressed 15-fold, no change is seen in either the rate of serine incorporation into lipid or the lipid composition (37). Phospholipid composition remains unchanged in *Escherichia coli* upon 15- to 20-fold overexpression of phosphatidylserine synthase (38) or 10-fold overexpression of phosphatidylglycerolphosphate synthetase (39). As much as a 750-fold overexpression of cardiolipin synthase results in only a 4-fold increase in cardiolipin levels in *E. coli*, but this change in composition results in deleterious membrane changes (40). Thus it seems that these organisms tightly regulate lipid composition, and only extreme levels of overexpression affect lipid composition. On the other hand, multiple lipid changes are seen in *S. cerevisiae*, in which phosphatidylserine synthase is overexpressed about 10-fold: phosphatidylserine levels are double, phosphatidylethanolamine levels are halved, phosphatidylinositol decreases by 25% and phosphatidylcholine increases by about 40% (41). This organism seems unusually tolerant to changes in phospholipid composition, however, compared to bacterial and mammalian cells (42).

[9] Y. S. Park and C. Kent, unpublished observations.

Expression of S362 Mutant

The conservation of the C-terminal casein kinase II site between the yeast and rat cytidylyltransferase sequences, and the lack of conservation of other obvious phosphorylation sites between these sequences, argues that this site is important for some aspect of enzyme structure and/or function. The function of this site remains elusive at present, however. Clearly the presence of a serine at this site is not required for catalytic activity, nuclear localization, or ability to translocate to the membrane.

Strain 58 as an Expression System

Strain 58 cells have been shown to be an effective host cell system for the study of recombinant cytidylyltransferase proteins. Rat liver cytidylyltransferase was synthesized normally in the strain 58 cells, underwent normal phosphorylation, and maintained normal catalytic activity. The heterologously expressed rat liver cytidylyltransferase localized to the nucleus in these cells, as does the enzyme in normal CHO cells and in rat liver (2). These cells, then, should facilitate studies on the structure, activity, location, and regulation of cytidylyltransferase, as well as its possible role in coordinating membrane turnover.

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