Thermolabile CDP-Choline Synthetase in an Animal Cell Mutant Defective in Lecithin Formation*

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In previous studies, we have reported the isolation of a Chinese hamster ovary cell mutant (strain 58), which is temperature-sensitive for growth and defective in the biosynthesis of phosphatidylcholine (Esko, J. D., and Raetz, C. R. H. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 5192-5196). We now present a detailed biochemical and enzymatic analysis of strain 58 and several spontaneous temperature-resistant revertants. When mutant 58 is shifted from 33 to 40 °C, the level of phosphatidylcholine declines immediately, while all other major lipid species, including sphingomyelin, continue to be made for about 20 h. The selective inhibition of phosphatidylcholine synthetase is accompanied by a 5- to 10-fold drop in the intracellular CDP-choline fraction, which in Chinese hamster ovary cells consists of about 70% CDP-choline and 30% dCDP-choline. In cell extracts, the specific activity of the CDP-choline synthetase (phosphocholine cytidylyltransferase) is reduced 15- to 100-fold, and the residual activity is thermolabile, arguing in favor of a structural gene mutation. Since the formation of CDP-choline and dCDP-choline is defective both in vitro and in vivo, the same enzyme appears to be responsible for the synthesis of both metabolites. Two other enzymes of phosphatidylcholine synthetase (choline kinase and choline phosphotransferase) are present with virtually the same specific activity in mutant as in the parental line. Most spontaneous temperature-resistant revertants of mutant 58 regain a nearly normal phospholipid composition at 40 °C and have CDP-choline synthetase activity comparable to the parental cells. Taken together, the present work provides strong evidence that a single mutation, most likely in a structural gene, is responsible for the biochemical and phenotypic properties of strain 58.

The biochemical mechanisms that regulate the synthesis of membrane phospholipids and coordinate lipid metabolism with membrane protein synthesis have not been elucidated (1, 2). In simple prokaryotic organisms (1), the availability of ATP and membrane phospholipids and coordinate lipid metabolism with membrane protein synthesis have not been elucidated (1, 2). In simple prokaryotic organisms (1), the availability of ATP and membrane phospholipids and coordinate lipid metabolism with membrane protein synthesis have not been elucidated (1, 2). In simple prokaryotic organisms (1), the availability of ATP and membrane phospholipids and coordinate lipid metabolism with membrane protein synthesis have not been elucidated (1, 2). In simple prokaryotic organisms (1), the availability of ATP and membrane phospholipids and coordinate lipid metabolism with membrane protein synthesis have not been elucidated (1, 2). In simple prokaryotic organisms (1), the availability of ATP and membrane phospholipids and coordinate lipid metabolism with membrane protein synthesis have not been elucidated (1, 2). In simple prokaryotic organisms (1), the availability of ATP and membrane phospholipids and coordinate lipid metabolism with membrane protein synthesis have not been elucidated (1, 2). In simple prokaryotic organisms (1), the availability of ATP and membrane phospholipids and coordinate lipid metabolism with membrane protein synthesis have not been elucidated (1, 2).
Temperature Sensitivity and Viability of Mutant 58 at 40 °C—Under permissive conditions (33 °C), mutant 58 grew at about 80% of the parental rate, doubling every 24-30 h. Upon being shifted to 40 °C, the cell mass increased 2-4-fold, depending on the lot of fetal calf serum used, and thereafter further growth was inhibited. Although cells of mutant 58 that were shifted to 40 °C retained greater than 80% viability for at least 30 h, visible cell lysis began to occur after 36 h. Continuous incubation at 40 ± 0.2 °C resulted in the complete inhibition of colony formation.

Rapid Selective Inhibition of Phosphatidylinositol Synthesis in Mutant 58 Shifted to 40 °C—As shown previously, the relative amount (expressed as a percentage of the total phospholipid) of phosphatidylinositol dropped 2-fold when mutant 58 was shifted to 40 °C for 20 h (3). To determine the absolute amount of each lipid and the time course of the change in lipid composition, the experiments in Figs. 1 and 2 were carried out. For cells maintained at 33 °C, the amount of phosphatidylinositol increased above the level observed for the wild-type (Fig. 1, left panel) but decreased below the level observed for the wild-type at 40 °C (Fig. 1, right panel). In contrast to phosphatidylinositol, the amount of phosphatidylcholine remained constant over the entire period of incubation at 40 °C. The overall error in these determinations was approximately ±10%.
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FIG. 1. Phospholipid, phosphatidylcholine, and sphingomyelin contents of mutant 58 at 33 °C and after a shift to 40 °C. The mutant was incubated at 33 °C in the presence of 32P (2 μCi/ml) for several generations to label the phospholipids to constant specific radioactivity (19). The cells were then harvested and dispensed at 3 × 10^5 cells/100-mm diameter dish in medium containing 32P with the same specific radioactivity. After incubation at 33 °C for 1 day, some cultures were shifted to 40 °C. At the indicated times, duplicate cultures were harvested with trypsin and centrifuged at 600 × g for 5 min to remove residual medium. The cell pellets were resuspended, and a portion was extracted by the Bligh-Dyer method with carrier phospholipid (18, 19). The lipid extracts were analyzed by two-dimensional thin layer chromatography as described previously (19), and the radioactivity in each spot was quantitated by liquid scintillation spectrometry. The relative content of each phospholipid was used to calculate the total amount of each radioactive phospholipid in each culture dish. Solid lines refer to the mutant at 33 °C; broken lines to the mutant at 40 °C. Left panel: content of phosphatidylcholine (PC) and sphingomyelin (SPH).

FIG. 2. Content of individual phospholipids in mutant 58 growing at 33 °C or after a shift to 40 °C. Conditions were the same as in the legend to Fig. 1. Left panel: content of phosphatidylinositol (PI), cardiolipin (CL), and phosphatidylglycerol (PG); right panel: content of phosphatidylethanolamine (PE) and phosphatidylserine (PS).

synthesis of dCDP-choline as well as CDP-choline.

Enzymes of Phosphatidylcholine Synthesis in Extracts of Mutant 58—The altered pattern of choline metabolites present in strain 58 strongly suggested a defect in the enzymatic synthesis of CDP-choline. This was verified conclusively by preparing extracts of parental and mutant cells (both grown at 33 °C) and then determining the specific activity of choline kinase, CDP-choline synthetase, and choline phosphotransferase at 40 °C. As shown in Table I, there was no significant difference between mutant and wild type in the specific activities of choline kinase and choline phosphotransferase, while CDP-choline synthetase was reduced over 100-fold at 40 °C in this experiment. The fact that the CDP-choline synthetase has the highest specific activity in the parental cells (Table I) is of no special significance, since the assay conditions for the choline kinase and phosphotransferase have not been optimized for CHO cells.

Further enzymatic studies revealed that the residual CDP-choline synthetase activity present in extracts of mutant 58 was thermolabile relative to that of wild-type cells. The Q_10 of the parental enzyme was approximately 2.5 under the assay conditions employed, while the specific activity of the mutant was considerably higher at 33 °C than at 40 °C. Attempts to stabilize this residual enzymatic activity by addition of 10% glycerol, 10-30% sucrose, 0.1-0.3 M KCl, or 0.6 mg/ml of lysophosphatidylethanolamine were not successful. Alternative methods of cell lysis (Dounce homogenization or hypotonic lysis) resulted in the same pattern shown in Table I. Under the best conditions, the level of synthetase in the mutant was not higher than 7% of wild type at 33 °C and only 1-2% at 40 °C. In addition, the conversion of dCTP to dCDP-choline was similarly defective in such extracts. The parental enzyme utilized CTP and dCTP with approximately equal efficiency (data not shown).

The Effect of Cytidine Derivatives on the Mutant—In initial studies of mutant 58, we observed that the temperature-sensitive phenotype was accentuated somewhat when dialyzed fetal bovine serum was employed (data not shown). When we tested the ability of various compounds involved in phosphatidylcholine synthesis (choline, phosphorylcholine, CDP-choline, and dCDP-choline) to suppress the temperature-sensitive phenotype of the mutant, we discovered that CDP-choline at 30-100 μM permitted the cells to grow considerably longer at 40 °C. Similar results were obtained by supplementing the
FIG. 3. Anion exchange chromatography of water-soluble choline metabolites from cells shifted to 40 °C. Conditions for labeling mutant and wild type cells with [methyl-3H]choline and quantitating cellular choline metabolites by anion exchange chromatography have been described previously (3, 16). Adsorbed metabolites (phosphorylcholine and CDP-choline) were sequentially eluted from the resin with a nonlinear formic acid gradient (16). Five-ml fractions were collected (0.5 ml/min) and analyzed for radioactivity. Recovery of radioactivity and carrier CDP-choline was greater than 95% in all experiments. ■, radioactivity from cell extracts; □, absorbance of added carrier CDP-choline at 280 nm (pH 2). The insets are expansions of the CDP-choline region, fractions 20-35.

FIG. 4. Separation of CDP-choline and dCDP-choline extracted from parental cells. The fractions containing radioactivity in the CDP-choline region obtained from parental cells (as in Fig. 3) were pooled and lyophilized to remove residual formic acid. The sample was redissolved in water and adjusted to pH 9 with dilute sodium hydroxide. Approximately 10^6 cpm of authentic [methyl-3H]dCDP-choline (see under “Experimental Procedures”) was added and the sample was chromatographed as described in the legend to Fig. 3, except that the eluting gradient consisted of 0.2 M ammonium formate (pH 9.4) and uniformly contained 5 mM sodium tetaborate. After chromatography, 1.0 ml of each fraction was analyzed by liquid scintillation spectrometry, and the amounts of H (□) and 14C (▲) were quantitated. Next, 0.1 ml of 10 N HCl was added to the remainder of each fraction, and the absorbance at 280 nm (pH 2) was measured (×). The ultraviolet light absorbing material which migrated with the authentic [methyl-3H]dCDP-choline standard resulted from the low specific radioactivity of this material (see under “Experimental Procedures”). The recovery of radioactivity and carrier was greater than 95%.

mutant with CTP, CDP, CMP, and cytidine. These findings suggested that the accentuation of the phenotype of the mutant in the presence of dialyzed serum may have been caused by the removal of cytidine (or cytidine nucleotides) from the serum. Interestingly, when experiments like those shown in Figs. 3 and 4 were carried out with cells growing in the presence of 30 μM cytidine, the levels of CDP-choline (plus dCDP-choline) were 3-4-fold higher when normalized to the cell density both in CHO-K1 and mutant 58. However, in the parental CHO-K1 line, this did not significantly alter the phospholipid composition, while in mutant 58, there was a partial restoration of the phosphatidylcholine content (data not shown). These results suggested that cytidine might be acting to raise the intracellular pool of CTP, which has been suggested to limit the rate of phosphatidylcholine synthesis in animal cells (8). Higher levels of CTP in the in vitro assay of CDP-choline synthetase (5-50 nm) did not elevate the specific activity of the mutant relative to that of the wild type, arguing against reduction in the affinity of the enzyme for CTP in the mutant.

Biochemical Properties of Spontaneous, Temperature-resistant Revertants of Strain 58—In order to determine whether the conditional lethality of mutant 58 and the reduced CDP-choline synthetase were the result of separate unrelated mutations, spontaneous temperature-resistant revertants were isolated. This was done by placing 1-3 x 10^6 cells in a 100-mm diameter tissue culture dish in the presence of 10% dialyzed fetal bovine serum and incubating them at 40°C for 9 days. The growth medium was replaced every 3-4 days, and under these conditions, the incidence of revertants that grew at approximately the parental rate was about 1 in 10^6. Altogether, 6 colonies were repurified and shown to grow normally at 40°C.

Revertants 1-5, isolated in this way, regained nearly normal levels of CDP-choline synthetase in vitro, as shown for strains 1 and 4 in Table I. Revertant 6 had only 2-3 times more enzymatic activity than mutant 58, presumably because it was not a true revertant or represented a bypass mutation. Indeed, the Q10 of the synthetase in revertant 6 between 33 and 40°C was negative, like that of mutant 58.

The phospholipid compositions of CHO-K1, mutant 58, and the three revertants are presented in Table II. Revertants 1 and 4 are virtually the same as CHO-K1 in this regard, consistent with restoration of a nearly normal CDP-choline
The table II

| TABLE II | Phospholipid composition of mutant 58, wild type CHO-K1, and temperature-resistant revertants at 40 °C |
|----------|--------------------------------------------------------------------------------------------------|
| Strain   | Percentage of total phospholipid* |
|----------|-----------------------------------|
| Mutant 58|                                    |
| Wild type CHO-K1 | PC | SPH | PE | PI | PS | PG | Other |
| Mutant 58 | 51.3 | 10.2 | 21.3 | 7.4 | 6.3 | 0.5 | 3.0 |
| Revertant 1 | 50.2 | 10.1 | 22.1 | 7.8 | 3.6 | 0.6 | 3.6 |
| Revertant 4 | 40.7 | 11.1 | 34.2 | 7.0 | 7.5 | 0.5 | 5.9 |

*The abbreviations used are: PC, phosphatidylcholine; SPH, sphingomyelin; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PG, phosphatidylglycerol.

An intriguing aspect of lipid metabolism in mutant 58 is shown in the results in Figs. 1 and 2. Although the inhibition of phosphatidylcholine synthesis in this mutant is selective and immediate, the synthesis of all other phospholipids does not cease until after 20 h of incubation under nonpermissive conditions. This occurs at a time when macro molecular synthesis is still continuing (3) and viability is high. One possibility is that phosphatidylcholine depletion leads to the inhibition of total phospholipid synthesis, perhaps at the level of the glycerol-3-phosphate acyltransferase or at the level of de novo fatty acid synthesis. This possibility could be examined in extracts prepared from mutant 58 after incubation under nonpermissive conditions for about 20 h.

Some uses of strains like mutant 58 deserve mention. In conjunction with existing cytogenetic techniques, mutant 58 should facilitate the mapping of the CDP-choline synthetase gene on human chromosomes (3). Although mutant 58 is probably defective in a structural gene, the possibility of altered regulation or posttranslational modification cannot be entirely eliminated. This can be explored effectively through the isolation of additional mutants and the purification of the mutant enzyme. Furthermore, the well defined temperature-sensitive phenotype of mutant 58 may allow for the isolation of genomic DNA capable of restoring temperature resistance using the recently developed techniques of animal cell transformation (32, 33). Finally, it will be of great interest to determine the relationship of the CDP-choline synthetase gene to other genes involved in phosphatidylcholine and phospholipid metabolism, as well as genes for sterol, triglyceride, and lipoprotein biogenesis, since control mechanisms unique to membrane assembly may emerge.

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