A Temperature-sensitive Mammalian Cell Mutant with Thermolabile
Serine Palmitoyltransferase for the Sphingolipid Biosynthesis*

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We devised an in situ assay method for the activity of serine palmitoyltransferase (SPT) that catalyzes the first step in sphingolipid biosynthesis and isolated a temperature-sensitive mutant of Chinese hamster ovary cells with thermolabile SPT. This mutant stopped growing at 40 °C after several generations, although the cells grew at 33 and 37 °C at rates similar to those of the parent. The SPT activity in cell homogenates of the mutant grown at low temperatures was 4–8% of that in the parent homogenates. When the cells were cultured for several generations at 40 °C, the activity in the mutant homogenate became negligible. When cell homogenates were incubated at 45 °C before enzyme assay, mutant SPT was more markedly inactivated than parental SPT, indicating that mutant SPT had become thermolabile. The rates of de novo synthesis of sphingolipids in the mutant were much slower at 40 °C than at lower temperatures, in contrast to those in the parent. The sphingomyelin content in the mutant cultivated at 40 °C for several generations was also less than that at low temperatures. These results indicate that SPT functions in the main pathway for sphingolipid biosynthesis. The temperature-sensitive growth of the mutant defective in sphingolipid synthesis suggests that sphingolipid(s) plays an essential role in cell growth.

Sphingolipids, which are widely present in animal, plant, and microorganism cells, are a major family of biological membrane lipids (1). The structural backbone of sphingolipids is a sphingoid base (long-chain base). In a proposed pathway for sphingolipid biosynthesis (Fig. 1), serine palmitoyltransferase (SPT) catalyzes the first step reaction, in which L-serine condenses with palmitoyl-CoA to produce 3-ketodihydrosphingosine (3-KDS). 3-KDS is enzymatically converted to dihydrosphingosine, subsequently to sphingosine, and then to ceramide. Alternatively, dihydrosphingosine may be converted to N-acetyldihydrosphingosine, which is then changed to N-acetylsphingosine (ceramide) (2–5). Ceramide is further converted to sphingomyelin and glycosphingolipids. However, the pathway for sphingolipid biosynthesis is controversial and several alternative pathways have also been proposed (2–5).

Several biological roles of sphingolipids have been suggested. Sphingoid bases and lysosphingolipids are proposed to be physiological repressors of protein kinase C from the fact that these compounds inhibit protein kinase C activity in vitro and in vivo (6, 7). Sphingomyelin may influence transport or metabolism of cholesterol in cells because depletion of cellular sphingomyelin alters intracellular distribution of cholesterol (8), and mutant cells deficient in lysosomal sphingomyelinase accumulate not only sphingomyelin but also cholesterol abnormally (9). Glycosphingolipids present at the cell surface are suggested to play roles in defining the specificity of cell-cell recognition, from the fact that the composition of cellular glycosphingolipids changes strikingly during the differentiation and development of higher eukaryotes (10) and that particular glycosphingolipids specifically interact with each other (11).

To elucidate the metabolic mechanism and the physiological roles of sphingolipids, mutant cells defective in sphingolipid biosynthesis would be useful. However, so far no mammalian cell mutant deficient in sphingolipid synthesis has been isolated. In this paper, we describe the isolation and characterization of a temperature-sensitive Chinese hamster ovary (CHO) cell mutant with thermolabile SPT.

MATERIALS AND METHODS

Cell Culture—The CHO-K1 cell line was obtained from the American Type Culture Collection (ATCC CCL 61). CHO cells were routinely maintained in coated polystyrene dishes (100 mm diameter; CorningTM) containing 10 ml of Ham's F 12 medium supplemented with 10% newborn calf serum (Flow Laboratories), penicillin G (100 units/ml), streptomycin sulfate (100 μg/ml), and NaHCO3 (1.176 g/liter) in a 5% CO2 atmosphere at 100% humidity and at either 33 or 37 °C (12).

In Situ Colony Assay for SPT and Isolation of Mutants Defective in the Enzyme—CHO-K1 cells were treated with ethyl methanesulfonate (400 μg/ml) at 33 °C for 18 h, as described previously (12). After incubation in the culture medium for 4 days at 33 °C, the mutagenized cells were harvested and then seeded at 200–400 colonies per dish at 33 °C. After 3 days, the cells in each dish were overlaid with a polyester disc, a filter paper, and glass beads in that order, followed by incubation at 33 °C. After 20 days, the polyester disc was transferred to a dish containing 5 ml of the culture medium, followed by incubation at 40 °C for 1 day. The disc was then washed twice with 10 ml of phosphate-buffered saline, immersed in 2 ml of 50 mM HEPES-Na (pH 7.5) containing 5 mM EDTA and 5 mM diithiothreitol, and finally frozen at −70 °C for 90–60 min. The frozen disc was thawed at 40 °C, and transferred to a dish containing 2 ml of 50 mM HEPES-Na (pH 7.5), 5 mM EDTA, 5 mM diithiothreitol, 50 μM pyridoxal phosphate, 0.1 mM palmitoyl-CoA (Sigma), and 60 μM L-[3-14C]serine (57 mCi/mmol, 1 Ci = 37 GBq; ICN Radiochemicals). After incubation at 40 °C for 30 min, the disc was blotted onto a paper towel, transferred to another dish containing 5 ml of 10% trichloroacetic acid, and then maintained at room temperature for 30 min. The disc was washed three times with 40 ml of 5% trichloroacetic acid, dried, and exposed to X-ray film with an intensifying screen at −70 °C for 2 weeks. The master dishes were supplemented with 8 ml of culture medium containing 10% dimethyl sulfoxide, and stored at −70 °C throughout these manipulations. After autoradiography, colonies on discs were stained with Coomassie Blue (13). Mutant clones were identified as colonies showing faint spots on the autoradiogram.

The abbreviations used are: SPT, serine palmitoyltransferase; CHO, Chinese hamster ovary; 3-KDS, 3-ketodihydrosphingosine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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and retrieved from the master dish (13). A mutant clone, SPB-1, isolated in this way was purified by three cycles of retrieval and finally by limited dilution to yield only one colony per well. The mutant cell line was maintained at 33 °C under the standard culture conditions as described above.

Enzyme Assays—CHO cells maintained at 33 °C were seeded and cultivated for 3-4 days at various temperatures. CHO monolayers cultured to subconfluence were washed with 10 ml of phosphate-buffered saline twice and then harvested in phosphate-buffered saline with a rubber policeman. After centrifugation (500 × g, 5 min, 4 °C), the cells were suspended in 50 mM HEPES-Na (pH 7.5) containing 1 mM EDTA and 5 mM dithiothreitol to a final concentration of about 1 mg of protein/ml. The suspension was sonicated three times for 5 s at 1-min intervals on ice, and the resultant homogenate was used for enzyme assays. SPT activity was assayed by the method of Merrill (14) with a modification. Cell homogenates (0.1 ml) were added to 0.1 ml of reaction medium comprising 50 mM HEPES-Na, 20 mM KCl, 0.1 mM pyridoxal phosphate, 0.4 mM palmitoyl-CoA, and 0.2 mM L-[G-3H]serine (100 mCi/mmol; ICN Radiochemicals), followed by incubation for 5-30 min at the indicated temperatures. The methods for stopping the reaction and extraction of 3-KDS were essentially the same as previously described (14). In the assay for phosphatidylserine synthase (serine-exchange enzyme) (15), cell homogenates (0.1 ml) were added to 0.1 ml of 50 mM HEPES-Na (pH 7.5) containing 20 mM CaCl₂ and 0.2 mM L-[3H]serine, followed by incubation for 10 min. The reaction was stopped by the addition of 2.3 ml of chloroform/methanol (1:2, v/v), and the product was extracted by the method of Bligh and Dyer (16). In the assay for glycerol-3-phosphate acyltransferase (17), homogenates (0.1 ml) were added to 0.1 ml of 50 mM HEPES-Na (pH 7.5) containing 10 mM CaCl₂, 0.4 mM palmitoyl-CoA, and 1 mM [3H]glycerol 3-phosphate (1 mCi/mmol; ICN Radiochemicals), followed by incubation for 10 min. The methods used for stopping the reaction and extraction of the product were the same as previously described (17). Radioactivity in the chloroform-soluble fraction in these assays was determined in a toluene scintillation mixture with a counter (Model Ls 3801, Beckman). The radioactivity in the reaction mixture without palmitoyl-CoA in the SPT and glycerol-3-phosphate acyltransferase assays, and in that without CaCl₂ in the phosphatidylserine synthase assay was subtracted as the background.

Miscellaneous—Protein was determined by the method of Lowry et al. (18) with bovine albumin as a standard. (2S)-N-t-Butoxycarbonyl-3-KDS was provided by Drs. I. Kitagawa, H. Shibuya, and K. Kawashima (University of Osaka, Japan). N-t-Butoxycarbonyl-3-KDS was treated with trifluoroacetic acid and thiophenol at room temperature for 1 min, and the resultant 3-KDS was concentrated by evaporation and immediately used as the standard for TLC. Other lipids used were commercially available.

RESULTS

Isolation of Mutants Defective in SPT—To isolate mutant cells defective in SPT activity, we first devised an in situ assay method for SPT in CHO cells grown on polyester discs. Radioactive 3-KDS, produced by cell colonies permeabilized by freezing and thawing, was precipitated in colonies with trichloroacetic acid so that 3-KDS generated in situ was detectable by autoradiography (Fig. 2). Since the background spot was faint, as in the case of the absence of palmitoyl-CoA (Fig. 2A), the intensity of autoradiographic spots was used as a quantitative measure of generated 3-KDS in situ. It should be noted that 3-KDS was the main lipid product derived from radioactive serine under our assay conditions (data not shown, also see Refs. 14 and 19) since phosphatidylserine synthase activity is completely repressed in the absence of Ca²⁺ (20).

Selection was carried out under the conditions to isolate temperature-sensitive mutants, since the lesion in SPT activity could have lethal effects on cells. We obtained one mutant clone defective in SPT activity by screening about 80,000 colonies of mutagenized cells.

Growth of the mutant, designated as SPB-1 (sphingoid base biosynthesis), in culture medium containing 10% newborn calf serum was examined at various temperatures. When cultivated at 40 °C, SPB-1 cells grew slowly for several generations and then stopped growing, although they could grow at 33 and 37 °C with generation times 1.3-fold longer than those of the parent (Fig. 3). Consistently, the colony size of the mutant cultivated at 40 °C was much smaller than that of
were seeded at 2000 cells/dish (100 mm diameter), each containing at the indicated temperatures were used for the SPT reaction. The temperatures for the reaction were the same as the respective growth medium for the indicated times, radioactive 3-KDS produced was determined as described under "Materials and Methods." Cell culture and SPT assay temperatures:

<table>
<thead>
<tr>
<th>Cells</th>
<th>Temperature</th>
<th>Specific activities</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>°C</td>
<td>SPT*</td>
</tr>
<tr>
<td>SPB-1</td>
<td>33</td>
<td>2.1 ± 0.9</td>
</tr>
<tr>
<td>37</td>
<td>1.5 ± 0.7</td>
<td>80 ± 1</td>
</tr>
<tr>
<td>40</td>
<td>&lt;0.5</td>
<td>112 ± 11</td>
</tr>
<tr>
<td>CHO-K1</td>
<td>33</td>
<td>26 ± 1</td>
</tr>
<tr>
<td>37</td>
<td>36 ± 7</td>
<td>88 ± 1</td>
</tr>
<tr>
<td>40</td>
<td>52 ± 15</td>
<td>95 ± 1</td>
</tr>
<tr>
<td>Mixture†</td>
<td>37</td>
<td>18 ± 1</td>
</tr>
</tbody>
</table>

* SPT, phosphatidylserine (PS) synthase, and glycerol-3-phosphate acyltransferase (GPAT) activities are expressed as picomole of 3-KDS, phosphatidylserine, and mono- (or di-) acylglycerophosphate produced, respectively, per mg of protein/min.
† Initial velocities of the SPT reaction were determined from the results shown in Fig. 5.
† A mixture of equal volumes of the homogenates of SPB-1 and CHO-K1.

We examined whether the apparent low SPT activity of the mutant was due to an alteration of SPT itself. The activities of phosphatidylserine synthase and glycerol-3-phosphate acyltransferase, which utilize serine and palmitoyl-CoA, respectively, as a substrate (15, 17), were quite similar in the SPB-1 and parent cells (Table I), eliminating the possibilities of enhanced substrate degradation in the SPT reaction and of nonspecific inhibition of protein synthesis in the mutant. It was unlikely that diffusible inhibitory factors (including stimulatory factors of 3-KDS degradation) were present in SPB-1 cells, because the specific activity of SPT in a mixture of the homogenates of the SPB-1 and parent CHO-K1 cells was about half the sum of their individual activities (Table I). The SPT activity of the mutant at 40 °C was less than that at lower temperatures, whereas the activity of the parent at 40 °C was higher than that at low temperatures (Fig. 5 and Table I), suggesting that mutant SPT was thermolabile. To test this possibility, homogenates from cells grown at 33 °C were preincubated at a high temperature (45 °C) for various times and then the residual SPT activity was determined by

**Fig. 4.** Colony formation of SPB-1 and CHO-K1 cells. Cells were seeded at 2000 cells/dish (100 mm diameter), each containing 10 ml of culture medium, and then cultivated for 10 days at the indicated temperatures. Colonies were stained with Coomassie Blue.

**Fig. 5.** Time course of SPT reaction in cell homogenates from SPB-1 and CHO-K1 cells. Homogenates of cells cultivated at the indicated temperatures were used for the SPT reaction. The temperatures for the reaction were the same as the respective growth temperatures. After incubation of cell homogenates in SPT assay medium for the indicated times, radioactive 3-KDS produced was determined as described under "Materials and Methods." Cell culture and SPT assay temperatures: ●, 33 °C; □, 37 °C; △, 40 °C.

**Fig. 6.** Products in SPT assay of cell homogenates from SPB-1 and CHO-K1. Cell homogenates from the indicated cells grown at 33 °C were incubated in SPT assay medium containing 0.1 mM L-[3-14C]serine (57 mCi/mmol) in the presence (+) or absence (−) of 0.2 mM palmitoyl-CoA (Pal-CoA) for 30 min at 33 °C. The radioactive product was extracted and subjected to TLC analysis with a solvent system (chloroform, methanol, 2 N ammonia = 80/20/2, v/v/v). An autoradiogram of the TLC plate is shown and the position of standard 3-KDS visualized with iodine vapor is marked. Minor radioactive compounds may be degraded products of 3-KDS which is highly unstable (35).

The chloroform-soluble and radioactive product of the reaction using SPB-1 cell homogenate was confirmed to be 3-KDS by TLC analysis (Fig. 6).

**Table I**

Activities of SPT, phosphatidylserine synthase, and glycerol-3-phosphate acyltransferase in cell homogenates of SPB-1 and CHO-K1 cells

Enzymes were assayed in homogenates of cells cultured for 3–4 days at the indicated temperatures. Assay temperatures were the same as those of respective cultivation. Data shown are the means ± S.D. for at least two independent experiments.
enzyme assay at 33 °C. SPT of the mutant homogenate was indicated to be more thermolabile, indicating that the mutant SPT had been altered and had become thermolabile.

**Sphingolipid Synthesis in Mutant SPB-1**—To examine the effects of the defective SPT activity on sphingolipid synthesis, the *de novo* synthetic rates of sphingolipids in mutant cells were analyzed by metabolically labeling lipids with [14C]serine. In mammalian cells, sphingomyelin, phosphatidylserine, and phosphatidylethanolamine are the major lipids derived from serine. Serine is incorporated into phosphatidylserine through the serine-base exchange reaction catalyzed by phosphatidylserine synthase and phosphatidylethanolamine is produced through the decarboxylation of phosphatidylserine.

Cells maintained at 33 °C were seeded, cultivated for 2 days at various temperatures, and incubated in the presence of [14C]serine for 2 h. When lipids in the parent CHO-K1 cells were labeled under these conditions, sphingomyelin, phosphatidylserine, and phosphatidylethanolamine were heavily labeled, while ceramide and ceramide monohexoside were weakly labeled (Table II). The amount of the radioactivity of labeled lipids increased in proportion to the pulse time within, at least, 4 h (the time course of sphingomyelin labeling was shown in Fig. 8). Sphingoid bases (3-KDS, dihydrosphingosine, and sphingosine) were not appreciably detected under our experimental conditions, possibly because of rapid conversion of these compounds to ceramide or complex sphingolipids in the cells. Indirect labeling (e.g., via radioactive fatty acids derived from metabolites of [14C]serine) was negligible, since phosphatidylcholine and phosphatidylinositol were not labeled under the present conditions (data not shown).

The rates of synthesis of ceramide in mutant SPB-1 were 34, 8.6, and less than 3% of those in CHO-K1 at 33, 37, and 40 °C, respectively (Table II). SPB-1 cells also showed a similar temperature-dependent defect in the synthesis of ceramide monohexoside (Table II). The synthetic rates of sphingomyelin in the mutant 33, 37, and 40 °C were 90, 62, and only 4%, respectively, of those in the parent (Table II). The low activity of sphingolipid synthesis in the mutant cells was not due to a defect in the uptake of radioactive serine, because SPB-1 and CHO-K1 cells showed similar rates of metabolic labeling of both phosphatidylserine and phosphatidylethanolamine (Table II).

**Change in Sphingomyelin Content in SPB-1**—To examine the effects of the sphingolipid synthesis deficiency on the cellular sphingolipid content, the relative content of sphingomyelin (a major sphingolipid in CHO cells) was analyzed by labeling cellular phospholipids with 32P, for several generations. Under these experimental conditions, the total radioactivity incorporated into phospholipids was virtually identical between the mutant and the parent cells (data not shown). As shown in Table III, the sphingomyelin contents of SPB-1 grown at 33 and 37 °C were about 75 and 50%, respectively, of those of the parent. When cells were cultured at 40 °C, the sphingomyelin content of the mutant was reduced to one-third that of the parent. The slight increase in the phosphatidylinositol content of SPB-1 at 37 or 40 °C (Table III) is possibly due to a reduced synthesis of sphingomyelin because depletion of ceramide in SPB-1 cells might block the conversion from phosphatidylcholine to sphingomyelin, which is catalyzed by phosphatidylcholine:ceramide cholinephosphotransferase. There were no significant differences in the content of other phospholipids between SPB-1 and the parent at 33, 37, or 40 °C.

**DISCUSSION**

Braun and Snell (24), and Stoffel et al. (25) initially demonstrated that the synthesis of sphingoid bases proceeded through 3-KDS produced by SPT in a particulate fraction of yeast, *Hansenula ciferri*, cells. Thereafter, the SPT activity was detected in cell-free systems of many types of cells, including mouse brain (26), rabbit aorta (27), CHO (14), and various rat tissues (28). Here we isolated a temperature-sensitive CHO cell mutant (SPB-1) with thermolabile SPT. From the fact that exposure of cell homogenate to a high temperature inactivated mutant SPT to a greater extent than parental SPT (Fig. 7), we suggest that the structural SPT gene is mutated in the mutant cells, rendering the SPT thermolabile. The SPT activity in homogenate of the mutant cultivated at 40 °C was distinctly lower than that cultivated at low temperatures (Fig. 5 and Table I), and the *de novo* synthesis of sphingolipids in the mutant also exhibited a defect depending on culture temperature (Table II). From these facts, we conclude that the thermolabile SPT of the mutant causes the temperature-dependent defect in the sphingolipid biosynthesis, and that the SPT functions in the main pathway for sphingolipid synthesis in CHO cells.

The mutant SPB-1 could grow at low temperatures, but stopped growing at 40 °C (Figs. 3 and 4), at which the *de novo* synthesis of sphingolipids in the mutant also stopped (Table II). Furthermore, the sphingomyelin content in the mutant was reduced to one-third of that in the parent when the cells are cultured for several generations at 40 °C (Table III). Thus, exogenous sphingolipids seem not to provide enough sphingolipids for the growth of the mutant, although CHO cells may uptake sphingolipids associated with lipoproteins from the culture medium via a receptor-mediated pathway for sphingolipid synthesis in CHO cells.

CHO cells produce GM3 (sialyl lactosylceramide) as their major GM3 (ganglioside) (38). When the relative content of the ganglioside in intact cells was examined by means of metabolic labeling with N-acetyl-[3H]mannotosamine, the ganglioside content in SPB-1 cells grown at 33 or 37 °C was identical with that in CHO-K1 cells, but the content in SPB-1 cells cultured at 40 °C for 3 days was 40% of that in CHO-K1 cells (K. Hanada, unpublished result).
TABLE II

Labeling of lipids with [14C]serine in intact cells of SPB-1 and CHO-K1

<table>
<thead>
<tr>
<th>Cells</th>
<th>Temperature</th>
<th>Ceramide</th>
<th>CMH</th>
<th>SM</th>
<th>PS</th>
<th>PE</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPB-1</td>
<td>33°C</td>
<td>1.2 (33%)</td>
<td>1.0 (42%)</td>
<td>9.9 (90%)</td>
<td>33 (85%)</td>
<td>11 (110%)</td>
</tr>
<tr>
<td>CHO-K1</td>
<td>33°C</td>
<td>3.5</td>
<td>2.3</td>
<td>11</td>
<td>38</td>
<td>10</td>
</tr>
<tr>
<td>SPB-1</td>
<td>37°C</td>
<td>0.5 (8.6%)</td>
<td>0.7 (20%)</td>
<td>6.2 (62%)</td>
<td>5/ (102%)</td>
<td>18 (106%)</td>
</tr>
<tr>
<td>CHO-K1</td>
<td>37°C</td>
<td>5.8</td>
<td>3.5</td>
<td>10</td>
<td>56</td>
<td>17</td>
</tr>
<tr>
<td>SPB-1</td>
<td>40°C</td>
<td>&lt;0.2 (&lt;3%)</td>
<td>&lt;0.2 (&lt;4%)</td>
<td>1.0 (4.2%)</td>
<td>81 (95%)</td>
<td>20 (83%)</td>
</tr>
<tr>
<td>CHO-K1</td>
<td>40°C</td>
<td>15</td>
<td>5.3</td>
<td>24</td>
<td>85</td>
<td>24</td>
</tr>
</tbody>
</table>

Table III

Compositions of phospholipids in SPB-1 and CHO-K1 cells

As shown in Fig. 5 and Table II, the difference in the rate of biosynthesis of ceramide between SPB-1 and parent CHO-K1 is consistent with that of SPT activity in cell homogenates, suggesting that the step catalyzed by SPT is rate-determining in the biosynthesis of sphingoid bases and ceramide. In contrast, the rate of sphingomyelin synthesis in SPB-1 at 37°C was 62% of that in CHO-K1, although SPT activity in SPB-1 was only 4% of that in CHO-K1. These results imply that sphingomyelin synthesis is regulated so as to maintain the sphingomyelin content at a constant level or at a constant proportion among the total lipids within cells; namely, the net synthesis of sphingomyelin in the parent cells is repressively regulated so as not to overproduce sphingomyelin, whereas sphingomyelin synthesis in SPB-1 operates at almost the full level with an inadequate supply of the precursor ceramide. Concerning regulation of sphingomyelin metabolism, Nelson and Murray (31) reported that the administration of dexamethasone stimulated phosphatidylethanolamine, ceramide cholinophosphotransferase in mouse fibroblasts, and there have been some other reports (32-34) that various stimuli enhanced either the synthesis or degradation of sphingomyelin. Nevertheless, the molecular mechanism underlying the regulation of sphingolipid metabolism is little understood. SPB-1 cells may contribute to the study of the metabolism.
A CHO Mutant Defective in Sphingolipid Synthesis

and the physiological roles of sphingolipids in mammalian cells.

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