Mammalian Cell Mutants Resistant to a Sphingomyelin-directed Cytolysin

GENETIC AND BIOCHEMICAL EVIDENCE FOR COMPLEX FORMATION OF THE LCB1 PROTEIN WITH THE LCB2 PROTEIN FOR SERINE PALMITOYLTRANSFERASE*

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Lysenin, a hemolytic protein derived from the earthworm *Eisenia fetida*, has a high affinity for sphingomyelin. Chinese hamster ovary (CHO) cells exhibited a high cytolytic sensitivity to lysenin, but treatment with sphingomyelinase rendered the cells resistant to lysenin. Temperature-sensitive CHO mutant cells defective in sphingolipid synthesis were resistant to lysenin, and this lysenin resistance was suppressed by metabolic complementation of sphingolipids. Selection of lysenin-resistant variants from mutagenized CHO cells yielded two types of sphingomyelin-deficient mutants, both of which showed less lysenin binding capability than wild-type cells. One mutant strain was severely defective in sphingomyelin synthesis but not glycosphingolipid synthesis, and another strain (designated LY-B) was incapable of de novo synthesis of any sphingolipid species and had no activity of serine palmitoyltransferase (SPT; EC 2.3.1.50) catalyzing the first step of sphingolipid biosynthesis. LY-B cells lacked the LCB1 protein, a component of SPT, and transfection of LY-B cells with the hamster LCB1 cDNA restored both SPT activity and sphingolipid synthesis to the cells. Expression of an affinity peptide-tagged LCB1 protein in LY-B cells caused the endogenous LCB2 protein to adsorb to a tag affinity matrix. In addition, an anti-hamster LCB2 protein antibody co-immunoprecipitated both SPT activity and the wild-type LCB1 protein with the LCB2 protein. Thus, cell surface sphingomyelin is essential for lysenin-induced cytolysis, and lysenin is a useful tool for isolation of sphingomyelin-deficient mutants. Moreover, these results demonstrate that the SPT enzyme comprises both the LCB1 and LCB2 proteins.

Sphingolipids are ubiquitous constituents of biomembranes in mammalian cells. The most abundant species of sphingolipid in mammalian cells is sphingomyelin (SM), which amounts to 5–20% of total phospholipids. Sphingolipid biosynthesis is initiated by condensation of t-serine with palmitoyl CoA, a reaction catalyzed by serine palmitoyltransferase (SPT; EC 2.3.1.50) to generate 3-ketodihydrosphingosine (see Ref. 1 for a review of sphingolipid biosynthesis). 3-Ketodihydrosphingosine is converted to dihydrosphingosine, which is N-acetylated and then dehydrogenated to form ceramide at the endoplasmic reticulum. After moving to the Golgi apparatus, ceramide is converted to sphingomyelin or glycosphingolipids, and these synthesized complex sphingolipids are translocated to the plasma membrane, where they are highly enriched. SPT is suggested to be a key enzyme for regulation of cellular sphingolipid content (1). Regulation of sphingolipid synthesis at the SPT step appears to be relevant to prevention of a harmful accumulation of metabolic sphingolipid intermediates including sphingoid bases and ceramide, since repression of other anabolic steps in the sphingolipid synthetic pathway may cause the intermediate accumulation. Genetic studies have shown that two different genes, LCB1 and LCB2, are required for expression of SPT activity in the yeast *Saccharomyces cerevisiae* (2–4). Recently, mammalian cDNA homologs of the LCB1 and LCB2 genes have been isolated (5–7). We have demonstrated that expression of the hamster LCB1 cDNA in SPB-1 strain, a temperature-sensitive Chinese hamster ovary (CHO) cell mutant defective in SPT, restored SPT activity to the mutant cells and that a hexahistidine (His6)-tagged hamster LCB1 protein but not an untagged protein produced in SPB-1 cells caused SPT activity to adsorb to a Ni2+-immobilized resin, indicating that the hamster LCB1 protein is a component of the SPT enzyme (7). On the other hand, another research group suggested that mammalian SPT did not require expression of an LCB1 protein, based on observations that overexpression of mouse LCB2 cDNA in human embryonic kidney 293 cells enhanced SPT activity in the cells even without co-expression of mouse LCB1 cDNA (6). It is thus currently controversial whether the SPT enzyme comprises two different subunits, namely the LCB1 and LCB2 proteins.

Lysenin, a 41-kDa protein derived from the coelomic fluid of the earthworm *Eisenia fetida*, induces hemolysis of mammalian red cells (8, 9). Recently, it has been shown that lysenin specifically binds to SM among various lipid types in cell-free systems (10). However, it remained unclear whether cell surface SM was essential for lysenin-induced cytolysis, since the

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§ The abbreviations used are: SM, sphingomyelin; SMase, sphingomyelinase; SPT, serine palmitoyltransferase; CHO, Chinese hamster ovary; PBS, phosphate-buffered saline; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; BSA, bovine serum albumin; GM3, N-acetylneuraminyl lactosylceramide.

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possibility was not eliminated that lysenin could also bind to other components of cells for inducing cytolysis even without cell surface SM. In the present study, we demonstrated that lysenin requires cell surface SM for its cytolytic activity in CHO cells and showed a novel rational method for isolation of SM-deficient cell mutants, which yielded two types of CHO cell mutant strains. By using one mutant strain lacking the LCB1 protein, we demonstrated, for the first time, direct evidence for complex formation of the LCB1 protein with the LCB2 protein in the SPT enzyme.

**EXPERIMENTAL PROCEDURES**

**Materials**—Lysenin purified from the coelomic fluid of *Eisenia fetida* (8) and rabbit anti-lysenin antiserum were gifts from Drs. Y. Sekizawa and H. Kobayashi (Zenyaku Kogyo Co., Tokyo), 3-((4,5-Dimethylthiazol-2-yl)-2,5-di-phenyltetrazolium bromide (MTT) was purchased from Sigma, and recombinant *Bacillus cereus* SSMase was from Hiagta Shogyo (Tokyo, Japan).

**Cell Culture**—Ham’s F-12 medium supplemented with 10% newborn calf serum, penicilllin G (100 units/ml), and streptomycin sulfate (100 μg/ml) was used as the normal culture medium. Nutridoma medium (F-12 medium containing 1% Nutridoma-SP (Boehringer Mannheim) and gentamicin (10 μg/ml)) and Nutridoma-BO medium (Nutridoma medium supplemented with 0.1% fetal calf serum and 10 μM sodium olate complexed with bovine serum albumin (BSA)) were used as sphingolipid-deficient culture media (11). CHO cells were routinely maintained in the normal culture medium in a 5% CO2 atmosphere at 33 °C. For culture in sphingolipid-deficient medium, CHO cells were incubated in the normal culture medium at 33 °C for several hours and washed twice with phosphate-buffered saline (PBS), and then cell monolayers were cultured in Nutridoma medium or Nutridoma-BO medium. In some experiments, Nutridoma-BO medium was supplemented with ω-erythro-sphingosine (Matreya) or egg SM (Avanti Polar Lipids) as described previously (11).

**Viability of Cells Exposed to Lysenin**—CHO cell monolayers were incubated in 0.5 ml of F-12 medium containing various concentrations of lysenin in 35-mm dishes at 37 °C for 30 min as described in the legends for Figs. 1 and 2. As a background control, cells were incubated in F-12 medium containing 0.1% SDS. After the addition of 0.1 ml of MTT (5 mg/ml in PBS) to the dishes, the cell monolayers were incubated at 37 °C for 1 h, and then the MTT-containing medium was removed. Formazan produced in the cell monolayers was dissolved in 0.5 ml of 40% acetic acid/pH 3.0, and the absorbance at 570 nm of the solution was measured with a spectrophotometer (12). The values of the absorbance at 570 nm were corrected by subtraction of the value obtained from the background control, and the corrected values of the absorbance at 570 nm were used as a measure of viability of cells, which was expressed relative to that of lysenin-unexposed control cells as 100%.

**Isolation of Lysenin-resistant CHO Variants**—CHO-K1 cells (ATCC CCL-61) were propagated with ethidium bromide (RNGYVRSSSTATAAAAGGQIH) corresponding to the amino-terminal region of the LCB2 protein were used as antigens in rabbits. The specific antibodies were purified from the antisera by affinity chromatography with 21-mere peptides (TEEELEAARSTIREAQAVQLC for the LCB1 protein and RNGYVRSSSTATAAAAGGQIHHC for the LCB2 protein) linked to a matrix (SulfoLinkTM Coupling Gel, Pierce). For Western blot analysis, protein samples were incubated in SDS-sample buffer at 37 °C for 15 min, separated by SDS-polyacrylamide gel electrophoresis (10% acrylamide), and transferred to a polyvinylidene difluoride membrane (Bio-Rad). After blocking the blot membrane with 10% skim milk in PBS containing 0.1% Tween 20, the LCB1 and LCB2 proteins on the membrane were detected by using the anti-LCB1 antibody and anti-LCB2 antibody (0.5 μg/ml, respectively, as the primary antibody and a horseradish peroxidase-conjugated goat anti-rabbit IgG (ICN Pharmaceuticals, Inc.; 1:200 dilution in 0.2% BSA/PBS) for 1 h. After washing with PBS, the antibodies were observed under a Zeiss Axioscope fluorescence microscope.

**Immunofluorescence Staining of Lysenin Bound to CHO Cells**—All manipulations were done at room temperature unless noted otherwise. CHO cells were grown on glass coverslips in Nutridoma-BO medium at 40 °C for 2 days to about 50% confluence. After washing with PBS, the monolayers were fixed with 3% formaldehyde in PBS for 20 min, washed with PBS, incubated with 0.1 M NH4Cl in PBS for 20 min, and then washed twice with 2% BSA/PBS. For the monolayers incubated with lysenin (400 ng/ml in 0.2% BSA/PBS) for 1 h and washed three times with PBS for 5 min with gentle shaking. The monolayers were incubated with anti-lysenin antiserum (1: 500 dilution in 0.2% BSA/PBS) for 1 h and washed with PBS, followed by incubation with a fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (ICO Pharmaceuticals, Inc.; 1:200 dilution in 0.2% BSA/PBS) for 1 h. After washing with PBS, the monolayers were observed under a Zeiss Axioscope fluorescence microscope.

**Asay of SPT Activity in Cell Lysates**—SPT activity in CHO cell lysates was determined as described previously (16) and corrected for protein.

**Transfection of LY-B Cells with lCB1 or lCB2 cDNA—**pSV-cLCB1 and pSV-cLCB2, which are lCB1- and lCB2-expressing plasmids, respectively, with a G418-resistance determinant (7), were linearized by digestion with PvuII. LY-B cells were transfected with linearized pSV-cLCB1 or pSV-cLCB2 by lipofection with Lipofectamine74 reagent (Life Technologies Inc.). After selection for G418 resistance (400 μg/ml), the drug-resistant transfectants were seeded to prepare replicate colonies on polystyrene dishes, and the replicated colonies were assayed for SPT activity (13). When cells transfected with pSV-cLCB1, about 50% of the G418-resistant colonies recovered SPT activity. One SPT-positive colony of the transfectants transfected with pSV-cLCB1 was purified with a cloning cup and was designated the LY-B/cLCB1 clone.

**Western Blot Analysis—**Multiple antigenic peptide forms (17) of a 20-mer peptide (TEEELEAARSTIREAQAVQLC) corresponding to the amino-terminal region of the LCB1 protein with ethidium bromide (RNGYVRSSSTATAAAAGGQIHHC) corresponding to the amino-terminal region of the LCB2 protein were used as antigens in rabbits. The specific antibodies were purified from the antisera by affinity chromatography with 21-mere peptides (TEEELEAARSTIREAQAVQLC for the LCB1 protein and RNGYVRSSSTATAAAAGGQIHHC for the LCB2 protein) linked to a matrix (SulfoLinkTM Coupling Gel, Pierce). For Western blot analysis, protein samples were incubated in SDS-sample buffer at 37 °C for 15 min, separated by SDS-polyacrylamide gel electrophoresis (10% acrylamide), and transferred to a polyvinylidene difluoride membrane (Bio-Rad). After blocking the blot membrane with 10% skim milk in PBS containing 0.1% Tween 20, the LCB1 and LCB2 proteins on the membrane were detected by using the anti-LCB1 antibody and anti-LCB2 antibody (0.5 μg/ml, respectively, as the primary antibody and a horseradish peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad; 1:2500 dilution) as the secondary antibody with an enhanced chemiluminescence kit (Amersham Pharmacia Biotech), unless otherwise noted.

**N-Methylation Affinity Chromatography**—After transfection of LY-B cells with plasmid pSV-HT/cLCB1, which encodes a His6-tagged cLCB1 protein (7), transfection of SPT was confirmed in an in situ SPT assay, and one purified clone was designated LY-B/HT/cLCB1. All manipulations were done at 4 °C or on ice unless noted otherwise. Membranes were prepared from CHO cells cultured in spinner bottles at 37 °C as described previously (18). Stock suspension of 1% soybean phospholipids (soyole; Wako Pure Chemicals, Osaka, Japan) was prepared by sonication. The membranes (2 mg of protein) were incubated at 37 °C with 100 μg of lyso-lPC in 1 ml of 100 mM Tris-HCl (pH 7.5), 2 mM MgCl2, 2 mM CaCl2, and 10% glycerol for 1 h. Samples were applied to a column of nickel-nitrilotriacetic acid agarose (Qiagen) and washed extensively with 20 ml of 1 M Tris-Cl (pH 7.5), 200 mM MgCl2, and 100 mM CaCl2. After washing, the column was eluted with a 100-ml linear gradient of 0 to 4 M imidazole in 150 mM Tris-Cl (pH 7.5), 200 mM MgCl2, and 100 mM CaCl2. Fractions (0.5 ml) were collected, and each fraction was assayed for SPT activity.
bated in 1 ml of a solubilization buffer consisting of 0.1 M sodium phosphate buffer (pH 8.0), 50 mM NaCl, 10 mM imidazole, 0.1% soybean phospholipids, and 1% sucrose monolaurate (Mitsubishi Kasei Shokuhin Inc., Tokyo, Japan) for 10 min. After centrifugation (105 × g, 30 min) of the sample, 0.6 ml of the supernatant fluid was incubated with 50 μl of nickel-nitrilotriacetic acid agarose (Qiagen) for 1 h with gentle shaking. The resin was precipitated by centrifuging for 1 min at 2000 × g, and the supernatant fluid was recovered as an unadsorbed fraction. After washing three times with 1 ml of the solubilization buffer, the resin was incubated with 0.6 ml of an elution buffer consisting of 0.1 M sodium phosphate buffer (pH 8.0), 1 mM imidazole, 0.1% soybean phospholipids, and 1% sucrose monolaurate for 10 min with gentle shaking. After precipitating the resin, the supernatant fluid was recovered as an adsorbed fraction. The recovered fractions were assayed for SPT activity and analyzed for distribution of the LCB1 and LCB2 proteins by Western blotting.

**Immunoprecipitation of the Wild-type LCB1 Protein with the Anti-clCB2 Antibody**—All manipulations were done at 4 °C or on ice unless noted otherwise. A suspension (10 μl of bed volume) of protein A-Sepharose CL-4B (Amersham Pharmacia Biotech) in 0.3 ml of PBS containing 0.1% BSA and 0.1% Na2S3O4 was incubated with 5 μg of the anti-clCB2 antibody or a control preimmune IgG overnight. The resultant resin was washed four times with PBS containing 0.1% BSA and 0.1% Na2S3O4 and three times with 50 mM Hepes-Na (pH 7.5) containing 1% sucrose monolaurate, prior to immunoprecipitation assay. Membranes (0.8 mg of protein) prepared from CHO-K1 cells were incubated in 0.4 ml of 50 mM Hepes-Na (pH 7.5) containing 1% sucrose monolaurate for 10 min, and after centrifugation (105 × g, 30 min) the supernatant fluid was recovered as the solubilized membrane fraction. A portion (0.2 ml) of the solubilized membrane fraction was incubated with the pretreated resin for 3 h with shaking. After precipitating the resin (105 × g, 10 s), the supernatant fluid was recovered as the nonimmunoprecipitable fraction. The precipitated resin was washed four times with 0.5 ml of 50 mM Hepes-Na (pH 7.5) containing 1% sucrose monolaurate and incubated in 0.2 ml of SDS-sample buffer at 95 °C for 5 min. After precipitating the resin, the supernatant fluid was recovered as the immunoprecipitable fraction. The solubilized membrane and nonimmunoprecipitable fractions were assayed for SPT activity and also analyzed by Western blotting as described above. Western blot analysis of the immunoprecipitable fractions was performed by using biotinylated antibodies as the primary antibodies and horseradish peroxidase-conjugated streptavidin (Pierce) as the secondary probe for the enhanced chemiluminescence detection. The blot membrane was blocked with 5% BSA in PBS containing 0.1% Tween 20. Biotinylation of the anti-clCB1 and anti-clCB2 antibodies was performed with EZ-Link™ sulfo-NHS-biotin (Pierce), according to the manufacturer’s instructions.

**Protein Determination**—Protein concentrations were determined by the method of Lowry et al. (19) using BSA as the standard.

**RESULTS**

**Cytolytic Sensitivity of CHO-K1 Cells to Lysenin Is Repressed after SMase Treatment of Cells**—CHO-K1 fibroblasts were exposed to various concentrations of lysenin at 37 °C for 30 min, and then their viability was determined. The cells were killed by lysenin in a dose-dependent manner; the LD50 of lysenin was about 35 ng/ml, and exposure to lysenin at more than 200 ng/ml resulted in nearly complete loss of the viability (Fig. 1A). We next attempted to isolate SM-deficient mutants by selecting lysenin-resistant variants of SPB-1 strain. To examine whether the effect of SMase treatment on reduction of SM level without accumulation of ceramide increased the resistance of cells to lysenin. For this, we used a CHO mutant strain (SPB-1 strain) in which de novo sphingolipid synthesis stops at nonpermissive temperatures because of thermolabile SPT activity (11, 13). When cells were cultured in a sphingolipid-deficient medium at 40 °C for 2 days, SM and ceramide levels in SPB-1 cells were reduced to about 30% and less than 5%, respectively, of the wild-type levels (Fig. 2A). After culture under the sphingolipid-deficient conditions, the cells were exposed to lysenin at 200 ng/ml for 30 min. More than 70% of SPB-1 cells exposed to lysenin survived, compared with less than 10% of the wild-type cells (Fig. 2B). When the SM level in SPB-1 cells was restored by the addition of sphingosine to the culture medium for metabolic utilization (11, 20), the lysenin resistance of the mutant cells was suppressed (Fig. 2B). Collectively, these results demonstrated that reduction of SM level in cells conferred lysenin resistance to the cells.

**A Novel Selection Method for Isolation of Cell Mutants Defective in Sphingolipid Biosynthesis**—We next attempted to isolate SM-deficient mutants by selecting lysenin-resistant variants of CHO cells. Mutagenized CHO-K1 cells were cultured in a sphingolipid-deficient medium for 1–2 days and then exposed to lysenin (100 ng/ml) for 1 h. More than 95% of the cells were killed in the initial exposure to lysenin. After propagation of the survivors in the same medium, the cells were propagated by the lysenin treatment two more times, and clones of the survivors were purified. We screened a total number of about 2 × 106 mutagenized cells and eventually isolated 27 lysenin-resistant clones. During the screening, cells were propagated at 33 °C and exposed to lysenin at 37 or 40 °C, because lysenin-resistant variants might be temperature-sensitive for growth.

**Lysenin Resistance of CHO Mutant Cells Defective in Sphingolipid Biosynthesis**—We determined whether reduction of SM level without accumulation of ceramide increased the resistance of cells to lysenin. For this, we used a CHO mutant strain (SPB-1 strain) in which de novo sphingolipid synthesis stops at nonpermissive temperatures because of thermolabile SPT activity (11, 13). When cells were cultured in a sphingolipid-deficient medium at 40 °C for 2 days, SM and ceramide levels in SPB-1 cells were reduced to about 30% and less than 5%, respectively, of the wild-type levels (Fig. 2A). After culture under the sphingolipid-deficient conditions, the cells were exposed to lysenin at 200 ng/ml for 30 min. More than 70% of SPB-1 cells exposed to lysenin survived, compared with less than 10% of the wild-type cells (Fig. 2B). When the SM level in SPB-1 cells was restored by the addition of sphingosine to the culture medium for metabolic utilization (11, 20), the lysenin resistance of the mutant cells was suppressed (Fig. 2B). Collectively, these results demonstrated that reduction of SM level in cells conferred lysenin resistance to the cells.

**A Novel Selection Method for Isolation of Cell Mutants Defective in SM Metabolism**—We next attempted to isolate SM-deficient mutants by selecting lysenin-resistant variants of CHO cells. Mutagenized CHO-K1 cells were cultured in a sphingolipid-deficient medium for 1–2 days and then exposed to lysenin (100 ng/ml) for 1 h. More than 95% of the cells were killed in the initial exposure to lysenin. After propagation of the survivors in the same medium, the cells were propagated by the lysenin treatment two more times, and clones of the survivors were purified. We screened a total number of about 2 × 106 mutagenized cells and eventually isolated 27 lysenin-resistant clones. During the screening, cells were propagated at 33 °C and exposed to lysenin at 37 or 40 °C, because lysenin-resistant variants might be temperature-sensitive for growth.
However, none of the lysenin-resistant clones obtained in this study exhibited stringent temperature sensitivity for growth (data not shown; see also below). De novo synthetic rates of sphingolipids in these lysenin-resistant mutants were compared by metabolic labeling of lipids with [14C]serine at 40 °C for 2 h. Based on the activity of de novo sphingolipid synthesis, we tentatively classified the lysenin-resistant clones into three types, as summarized in Table I. In type I mutants (clones 1–11), the de novo synthetic rate of SM was less than 20% of the wild-type level, while the synthetic rate of glucosylceramide was more than 50% of the wild-type level. Type II mutants (clones 13, 15, 16, 17, 20, and 21) could not appreciably synthesize SM, glucosylceramide, or ceramide. In type III mutants (clones 14, 18, 23, 24, 28, 32, 33, 35, 37, and 40), the synthetic rate of SM was nearly normal (more than 70% of the wild-type level).

The type III mutants consisted of clones originated from at least two different mutant progenitors, because they were obtained from two different batches of mutagen treatment (Table I). On the other hand, all of the type I mutant clones were derived from one batch of mutagen treatment, suggesting that the type I clones were siblings of a mutant progenitor. Likewise, the type II mutant clones were probably siblings of another mutant progenitor. For further analysis, we chose clones 6 and 16 as representatives of the type I and II mutants, respectively, and hereafter refer to the lysenin-resistant clones 6 and 16 as LY-A and LY-B, respectively.

**SM Level and Lysenin Binding Capability of LY-A and LY-B Cells**—After culture of cells in a sphingolipid-deficient medium for 2 days at 40 °C, the phospholipid composition of the cells was determined. SM content in LY-A and LY-B cells was less than 30% of the wild-type level, although there was no appreciable difference in the contents of other major phospholipids except for phosphatidylcholine (Table II). Phosphatidylcholine content in the two mutants was slightly higher than the wild-type level (Table II), in agreement with the expectation that defects in SM formation resulted in accumulation of phosphatidylcholine, which donates its phosphorylcholine moiety for SM formation (1). The content of GM3, the main ganglioside in CHO cells (21, 22), was also determined. GM3 content in LY-B cells was about 30% of the wild-type level, while that in LY-A cells was slightly higher than that in CHO-K1 cells (Table II). There was no significant difference in the content of free cholesterol among wild-type, LY-A, and LY-B cells (data not shown). These results confirmed that LY-B cells were defective in de novo synthesis of all sphingolipid species and that LY-A cells were defective in SM synthesis but not glycosphingolipid synthesis.

We next compared lysenin binding capability among wild-type, LY-A, and LY-B cells. After culture in Nutridoma-BO medium at 40 °C for 2 days, CHO cell monolayers were fixed with formaldehyde and incubated with lysenin, and lysenin bound to the cell surface was detected by indirect immunofluorescence staining. LY-A and LY-B cells exhibited far less fluorescence than wild-type cells (Fig. 3). Lysenin specificity of the immunofluorescence staining was confirmed by the observation that wild-type cells were not appreciably labeled when incubated without lysenin (data not shown). These observations showed that lysenin binding capability is lower in these SM-deficient mutants than in wild-type cells.

**LY-B Strain Is Defective in SPT Activity**—The inability of LY-B cells to synthesize any sphingolipid species de novo suggested that the initial step of sphingolipid biosynthesis was defective in the mutant cells. Determination of SPT activity in cell lysates showed that the activity in LY-B cells was less than 5% of that in CHO-K1 cells (Table III). Interestingly, almost complete loss of both SPT activity and de novo sphingolipid synthesis in LY-B cells was observed irrespective of whether the cells were cultured at 33 or 40 °C (Table III and Fig. 4, lanes 2 and 6), whereas complete loss of these activities in the previously isolated SPB-1 cells occurred after exposure of cells to 40 but not 33 °C (Table III and Fig. 4, lanes 4 and 8; see also

Fig. 2. Sphingolipid-deficient mutant cells are resistant to lysenin. A, CHO-K1 and SPB-1 cells were cultured in Nutridoma-BO medium at 40 °C for 2 days, and then lipids were extracted from the cells. After separation of the extracted phospholipids by TLC, the amount of SM was determined by lipid phosphorus quantification. The amount of ceramide in the extracted lipids was determined by a diacylglycerol kinase method. The mean values from duplicate experiments are shown. B, after cultivation in Nutridoma-BO medium at 40 °C for 2 days, CHO-K1 and SPB-1 cells were incubated in F-12 medium containing lysenin (200 ng/ml) at 37 °C for 30 min. When indicated, 2 mM sphingosine-BSA complex was added daily to Nutridoma-BO medium during the cultivation (the addition of sphingosine alone did not affect the viability of CHO-K1 or SPB-1). Viability of the cells was determined by an MTT method. Each value shown is the mean of duplicate experiments.

### Table I

| Mutagen treatment<sup>a</sup> | Lysenin-resistant clone number | Sphingolipid synthesis<sup>b</sup> | Mutant type<sup>c</sup> |
|------------------------------|--------------------------------|-----------------------------------|------------------------|
| Batch 1                      | 1, 2, 3, 4, 5, 6 (LY-A), 7, 8, 9, 10, 11 | SM: +, GleCer: +, Ceramide: + | Type I                  |
| Batch 2                      | 13, 15, 16 (LY-B), 20, 21             | SM: +, GleCer: +, Ceramide: + | Type II                 |
| Batch 2                      | 14, 18                              | SM: +, GleCer: +, Ceramide: + | Type III                |
| Batch 3                      | 23, 24, 27, 28, 32, 33, 35, 37, 40   | SM: +, GleCer: +, Ceramide: + | Type III                |
| None                         | Wild type CHO-K1                    | SM: +, GleCer: +, Ceramide: + |                        |

<sup>a</sup> Batch number of mutagen treatment from which the lysenin-resistant variants were selected.

<sup>b</sup> Glucosylceramide.

<sup>c</sup> The lysenin-resistant clones 6 and 16 are also referred to as LY-A and LY-B, respectively.
LY-B Cells Lack the LCB1 Protein—The yeast *S. cerevisiae* requires two genes, *LCB1* and *LCB2*, for SPT activity (2–4). Chinese hamster cDNA homologs of the yeast *LCB1* and *LCB2* genes (the Chinese hamster cDNA homologs were designated cLCB1 and cLCB2, respectively) have also been isolated (7). We determined whether transfection of LY-B cells with a recombinant plasmid expressing cLCB1 or cLCB2 complemented the defect of SPT activity. When LY-B cells were transfected with the cLCB1-expressing plasmid, about 50% of transformant colonies showed recovery of SPT activity, whereas none of the colonies transfected with the cLCB2-expressing plasmid did so. For further analysis, one SPT-positive clone of the cLCB1-transfected LY-B colonies was purified and designated LY-B/cLCB1.

Determination of SPT activity in cell lysates showed that the activity in LY-B/cLCB1 cells was restored to the wild-type level (Table III). As expected, the sphingolipid synthetic rate in LY-B/cLCB1 cells was similar to that in wild-type CHO-K1 cells (Fig. 4, lanes 1 and 5 versus lanes 3 and 7), the contents of SM and G₃₄₃ were restored to the wild-type levels (Table II), and the cells exhibited similar lysenin binding capability and lysenin sensitivity to the wild-type cells (data not shown).

Membrane fractions were prepared from cells cultured at 33 or 40 °C, and the level of the LCB1 protein in the fractions was examined by Western blot analysis with a polyclonal antibody raised against the carboxyl-terminal peptide of the cLCB1 protein. In agreement with the prediction that the cLCB1 cDNA encodes a 53-kDa protein with a membrane-spanning domain (7), this antibody recognized a 53-kDa protein in the membrane fraction of wild-type CHO-K1 cells (indicated by an arrow in Fig. 5A), while no 53-kDa protein was detectable in the membrane fraction of LY-B cells cultured at either 33 or 40 °C (Fig. 5A, lanes 3 and 4). We identified the 53-kDa protein as the Chinese hamster LCB1 protein because LY-B/cLCB1 cells overproduced it to 2–3-fold the wild-type level (Fig. 5A, lanes I and 2 versus lanes 5 and 6). Calibration for the densities of the 53-kDa protein in the Western blot showed that the expression level of the LCB1 protein in LY-B cells was less than 10% of the wild-type level (Fig. 5B). The 53-kDa protein was hardly detected in the cytosol fractions of CHO-K1, LY-B, or LY-B/cLCB1 cells (data not shown).

Expression of an Affinity Peptide-tagged LCB1 Protein in LY-B Cells—The yeast *S. cerevisiae* encodes a 53-kDa protein with a membrane-spanning domain (7), this antibody recognized a 53-kDa protein in the membrane fraction of wild-type CHO-K1 cells (indicated by an arrow in Fig. 5A), while no 53-kDa protein was detectable in the membrane fraction of LY-B cells cultured at either 33 or 40 °C (Fig. 5A, lanes 3 and 4). We identified the 53-kDa protein as the Chinese hamster LCB1 protein because LY-B/cLCB1 cells overproduced it to 2–3-fold the wild-type level (Fig. 5A, lanes I and 2 versus lanes 5 and 6). Calibration for the densities of the 53-kDa protein in the Western blot showed that the expression level of the LCB1 protein in LY-B cells was less than 10% of the wild-type level (Fig. 5B). The 53-kDa protein was hardly detected in the cytosol fractions of CHO-K1, LY-B, or LY-B/cLCB1 cells (data not shown).

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white ovalbumin (45 kDa), and carbonic anhydrase (31 kDa). Mass standards used were bovine serum albumin (66 kDa), hen egg analysis for detection of the LCB1 protein.

 Cultured LY-B cells and CHO-K1 cells were subjected to Western blot indicated protein amounts of membrane fractions prepared from 33 °C cLCB1 cells. The arrow indicates the 53-kDa LCB1 protein. Molecular mass standards used were bovine serum albumin (66 kDa), hen egg white ovalbumin (45 kDa), and carbonic anhydrase (31 kDa). B, the indicated protein amounts of membrane fractions prepared from 33 °C cultured LY-B cells and CHO-K1 cells were subjected to Western blot analysis for detection of the LCB1 protein.

A Tag Affinity Matrix—To address the unresolved question of whether the SPT enzyme contained both LCB1 and LCB2, we determined whether the chromatographic behavior of the hammer LCB2 protein was affected by affinity peptide tagging of the LCB1 counterpart. For this, LY-B cells were ideal host cells, because competitive effects of the tagged LCB protein with the endogenous LCB1 protein could be avoided.

Membranes prepared from LY-B/cLCB1 cells and LY-B transformants expressing an NH₂-terminally His₆-tagged cLCB1 protein (designated the LY-B/HTcLCB1 strain) were solubilized with sucrose monolaurate, a nonionic detergent, and the solubilized membranes were incubated with a Ni²⁺-immobilized resin that has a high affinity for the His₆ sequence. Unadsorbed proteins and adsorbed proteins were fractionated, and distribution of the LCB1 and LCB2 proteins between the fractions was examined by Western blot analysis. Most of both the 54-kDa His₆-tagged cLCB1 and the endogenous 63-kDa LCB2 proteins derived from LY-B/HTcLCB1 cells were distributed in the adsorbed fraction, while none of the 53-kDa cLCB1 or 63-kDa LCB2 proteins derived from LY-B/cLCB1 cells were distributed in this fraction (Fig. 6A). In agreement with the distribution patterns of the LCB1 and LCB2 proteins, about 70% of the SPT activity from LY-B/HTcLCB1 cells adsorbed to the Ni²⁺-immobilized resin, while none of the SPT activity from LY-B/cLCB1 cells did so (Fig. 6B), consistent with our previous study (7).

Co-immunoprecipitation of the Wild-type LCB1 Protein with the LCB2 Proteins—The anti-cLCB1 antibody used in this study failed to immunoprecipitate the LCB1 protein or SPT activity solubilized with sucrose monolaurate (data not shown). In contrast, the anti-cLCB2 antibody, which was raised against an NH₂-terminally region of the LCB2 protein as the antigen, was able to precipitate both the LCB2 protein and SPT activity of solubilized membranes (see below). Because the amino acid sequence of the cLCB2-derived antigen peptide has no significant homology to any region of the cLCB1 protein, the anti-cLCB2 antibody does not recognize the hammer LCB1 protein directly. Thus, to show complex formation of the LCB1 and LCB2 proteins in wild-type cells, we determined whether the anti-cLCB2 antibody co-immunoprecipitated the wild-type LCB1 protein.

Solvulbilized membranes from CHO-K1 cells were incubated with the anti-cLCB2 antibody or a control preimmune IgG bound to protein A Sepharose, followed by separation of the immunoprecipitable and nonimmunoprecipitable fractions. More than 80% of both the LCB1 and LCB2 proteins was precipitated with the anti-cLCB2 antibody, while none of these proteins was precipitated with the control IgG (Fig. 7A). Consistent with this, more than 80% of the SPT activity disappeared from the solubilized membrane fraction after immunoprecipitation with the anti-cLCB2 antibody but not the control IgG (Fig. 7B). Note that more than 95% of proteins of the solubilized membrane fraction were not precipitated with the anti-cLCB2 antibody, eliminating the minor possibility that the membranes had not been completely solubilized, so that the anti-cLCB2 antibody precipitated all membrane proteins.

Growth Phenotype of LY-B Cells—To ascertain the importance of sphingolipids for cell proliferation, we examined the growth of LY-B cells in a sphingolipid-deficient medium, Nutridoma-BO, at various temperatures. LY-B cells hardly grew in Nutridoma-BO at 33, 37, or 40 °C, although LY-B/cLCB1 cells as well as wild-type CHO-K1 cells grew logarithmically at these temperatures (Fig. 8A). Under sphingolipid-deficient conditions, LY-B cells were capable of dividing two or three times but thereafter began to die (Fig. 8A), probably by apoptosis, because DNA fragmentation, a hallmark of apoptosis, occurred in LY-B cells but not CHO-K1 or LY-B/cLCB1 cells during prolonged cultivation (data not shown). When SM or sphingosine was added to Nutridoma-BO medium, growth of LY-B cells was restored to the level of LY-B/cLCB1 cells, while that of LY-B/cLCB1 and CHO-K1 cells was not significantly affected (Fig. 8B). LY-B cells were able to grow in the normal culture medium (F-12 medium containing 10% newborn calf serum) (Fig. 8B), through metabolic utilization of serum sphingolipids by cells, as discussed below. In contrast to LY-B cells, LY-A cells were able to grow logarithmically in Nutridoma-BO medium at a slower rate than the wild-type rate (not shown), although it remains unknown whether the defect of SM synthesis in LY-A cells is responsible for the slower growing phenotype of LY-A cells.
conjugated streptavidin as the secondary probe for enhanced chemiluminescence detection. lane 1, solubilized membrane fraction; lanes 2 and 3, nonimmunoprecipitable fractions with the anti-cLCB2 antibody and the control IgG, respectively; lanes 4 and 5, immunoprecipitable fractions with the anti-cLCB2 antibody and the control IgG, respectively. B, the solubilized membrane and nonimmunoprecipitable fractions were assayed for SPT activity. Residual SPT activity in each nonimmunoprecipitable fraction with the indicated antibody is shown as a percentage of SPT activity of each nonimmunoprecipitable fraction from the mean value of the activity of the solubilized membrane fraction from three experiments.

**FIG. 7.** Immunoprecipitation of SPT activity and the LCB1 protein with an anti-cLCB2 antibody. Solubilized membrane fraction derived from CHO-K1 cells was incubated with an anti-cLCB2 antibody or a control preimmune IgG bound to protein A-conjugated streptavidin as the secondary probe for enhanced chemiluminescence detection. Lane 1, solubilized membrane fraction; lanes 2 and 3, nonimmunoprecipitable fractions with the anti-cLCB2 antibody and the control IgG, respectively; lanes 4 and 5, immunoprecipitable fractions with the anti-cLCB2 antibody and the control IgG, respectively. B, the solubilized membrane and nonimmunoprecipitable fractions were assayed for SPT activity. Residual SPT activity in each nonimmunoprecipitable fraction with the indicated antibody is shown as a percentage of SPT activity of each nonimmunoprecipitable fraction from the mean value of the activity of the solubilized membrane fraction from three experiments.

**DISCUSSION**

Lysenin Requires Cell Surface SM for Its Cytolytic Activity—It has recently been shown by using various cell-free systems, such as TLC-immunostaining and liposome lysis assay, that lysenin, a cytolsin, binds specifically to SM among various lipid types (10). However, it remained to be determined whether SM of target cells was essential for lysenin-induced cytolyis, since there was the possibility that lysenin could also bind to a nonlipidic component(s) of cells for inducing cytolyis even without cell surface SM. In the present study, we provided several lines of evidence that lysenin requires cell surface SM for its cytolytic activity. CHO-K1 cells were highly sensitive to lysenin but SMase treatment, which degrades SM at the cell surface, rendered the cells resistant to lysenin (Fig. 1). Lysenin resistance was caused by depletion of SM rather than accumulation of ceramide, because CHO mutant cells with reduced levels of both SM and ceramide were resistant to lysenin (Fig. 2). The resistance of the mutant cells was suppressed by restoration of the cellular SM level via metabolic utilization of exogenous sphingosine (Fig. 2B), eliminating the possibility that the lysenin resistance of the mutant cells was caused by background mutation unrelated to sphingolipid synthesis. In addition, selection of lysenin-resistant CHO cell variants yielded two types of CHO cell mutants defective in SM synthesis, both of which displayed far less lysenin binding capability than wild-type cells (Fig. 3). These findings together with the previous finding that lysenin has a high affinity for SM (10) indicate that cell surface SM serves as a predominant receptor for lysenin.

The earthworm *E. foetida* produces at least two lysenin-like proteins in addition to lysenin itself, and these three proteins have a high amino acid sequence identity (9, 23). Another research group has recently found that a cytolsin (named eiseniapore) purified from the earthworm *E. foetida* recognizes SM (24). Interestingly, eiseniapore binds to both SM and galactosylceramide (24), whereas lysenin shows no significant cross-reaction with galactosylceramide (10), and the specific product of the lysenin cDNA binds specifically to SM,2 suggesting that eiseniapore is a distinct molecule from lysenin. Eiseniapore may be a lysenin-like protein with a broader substrate specificity than lysenin, although confirmation of this possibility should await cDNA cloning of eiseniapore.

We have previously isolated a CHO cell mutant defective in SPT (SBP-1 strain) after screening by an *in situ* SPT assay (13). In the present study, we showed that selection of lysenin-resistant variants is another rational approach for isolation of SM-deficient mutants. The new method yielded not only another SPT-defective mutant (LY-B strain) but also a novel type of SM-deficient mutant (LY-A strain). LY-A cells appear to be specifically defective in SM synthesis, because the content and synthetic rate of SM in LY-A cells were 30% or less of the wild-type levels, while those of glycosphingolipids in LY-A cells were almost normal (Tables I and II). Our ongoing characterization of the LY-A strain suggests that LY-A cells possess a normal activity of SM synthase but are defective in intracellular translocation of ceramide for SM synthesis, as will be reported elsewhere.3 We also obtained another type of lysenin-resistant variant, designated type III, that appears to produce

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2 A. Yamaji and M. Umeda, unpublished observations.

3 M. Fukasawa, M. Nishijima, and K. Hanada, submitted for publication.
SM normally (Table I). Although it is currently unknown why the type III mutants are resistant to lysenin, they might be defective in unknown host factor(s) responsible for the lysenin-dependent cytolyis or be somehow protected from the cytolyis.

Complex Formation of the LCB1 Protein with the LCB2 Protein in the SPT Enzyme—LY-B cells are almost completely defective in SPT activity irrespective of the culture temperature, so that they are unable to produce sphingolipids de novo at 40 or 33 °C (Table III and Fig. 4). Expression of the LCB1 protein in LY-B cells is below the detectable level by Western blot analysis (Fig. 5), and transfection of LY-B cells with the Chinese hamster LCB1 cDNA restores both SPT activity and sphingolipid synthesis along with expression of the LCB1 protein (Table III and Figs. 4 and 5). These results indicate that the deficiency of SPT activity in LY-B cells is primarily due to lack of the LCB1 protein and confirm our previous conclusion that the LCB1 protein is responsible for SPT activity in CHO cells (7). In addition, we showed here that expression of the His6-tagged LCB1 protein in LY-B cells caused the endogenous LCB2 protein to adsorb to the Ni2+-immobilized affinity matrix (Fig. 6), demonstrating for the first time that the LCB1 forms a complex with the LCB2 protein in the SPT enzyme. Moreover, when the solubilized membrane fraction from wild-type cells was incubated with an anti-LCB2 antibody, nearly complete co-immunoprecipitation of the LCB1 protein with the LCB2 protein was observed along with the disappearance of most SPT activity from the nonprecipitable fraction (Fig. 7), indicating that the complex of the LCB1 and LCB2 proteins is relevant to the predominant form of the SPT enzyme under physiological expression conditions.

Our finding that the lack of the LCB1 protein resulted in loss of SPT activity in CHO cells argues strongly that an LCB1 protein is essential for expression of SPT activity in mammalian cells, in agreement with our previous study showing that the LCB1 protein is a component of the SPT enzyme (7) and previous genetic studies showing that the yeast S. cerevisiae requires both LCB1 and LCB2 genes for the expression of SPT activity (2–4). Nevertheless, mammalian SPT has recently been reported not to require expression of an LCB1 protein, based on observations that overexpression of mouse LCB2 cDNA in human embryonic kidney 293 cells enhanced SPT activity in the cells even without co-expression of mouse LCB1 cDNA (6). One possible explanation for this discrepancy is that, because human embryonic kidney 293 cells express the human LCB1 protein endogenously, the overexpressed mouse LCB2 protein might form a functional complex with the endogenous LCB1 protein if the pool of the endogenous LCB1 protein is large enough to form such heterologous complex. Alternatively, considering that there is about 30% amino acid identity between mammalian LCB1 and LCB2 proteins (5–7), the overexpressed LCB2 protein might form a homo-oligomer with some degree of SPT activity, although a complex of the LCB1 and LCB2 proteins is predominantly relevant to physiological SPT. Further studies will be needed to determine the entire subunit composition of this enzyme.

Sphingolipids Play an Important Role(s) in Growth of CHO Cells over a Wide Range of Physiological Temperature—We previously demonstrated that temperature-sensitive SPB-1 cells defective in SPT require exogenous sphingolipids for growth at a nonpermissive temperature (39 °C) (11). However, recent studies have shown that sphingolipids are involved in heat stress responses in yeast cells (25–27), raising the possibility that sphingolipids are essential for growth of mammalian cells only at high temperature. In the present study, we demonstrated that LY-B cells hardly grew in a sphingolipid-deficient medium at 33–40 °C and that growth of the cells was rescued by the genetic complementation of the SPT defect in LY-B cells (Fig. 8A). Moreover, when SM or sphingosine was supplied externally to the culture medium, LY-B cells were able to grow in the medium (Fig. 8B). These results indicate that CHO cells require sphingolipids for growth over a wide range of physiological temperature.

It should be pointed out that LY-B cells could be maintained in the normal culture medium, F-12 medium containing 10% serum (see also Fig. 8D), despite their inability to synthesize sphingolipids de novo. Mammalian sera contain both SM and glycospingolipids (28–30), and the normal culture medium we used contains about 20 μg SM (11). LY-B cells maintain their growth capability in the normal culture medium presumably by utilizing exogenous sphingolipids, although we cannot exclude the possibility that their growth capability is supported by an unknown serum factor(s) in addition to sphingolipids. Nevertheless, the cellular sphingolipid level in LY-B cells can easily be reduced (maximally to the level causing cell death) by cultivating cells in a sphingolipid-deficient medium, providing a good model cell system for examination of the effects of reduction of sphingolipid levels on various functions of cells.

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