Reconstitution of ATP- and Cytosol-dependent Transport of de Novo Synthesized Ceramide to the Site of Sphingomyelin Synthesis in Semi-intact Cells*

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Transport of ceramide synthesized at the endoplasmic reticulum to the Golgi compartment, where sphingomyelin (SM) synthesis exists, was reconstituted within semi-intact Chinese hamster ovary cells. When [3H]ceramide that had been produced from [3H]sphingosine at 15 °C in perforated cells was chased at 37 °C, [3H]ceramide-to-[3H]SM conversion occurred in a cytosol-dependent manner. In various aspects (i.e. kinetics, ATP dependence, and temperature dependence), [3H]ceramide-to-[3H]SM conversion in perforated cells was consistent with that in intact cells. The cerolysin from LY-A strain, a Chinese hamster ovary cell mutant defective in endoplasmic reticulum-to-Golgi transport of ceramide, did not support [3H]ceramide-to-[3H]SM conversion in perforated wild-type cells, whereas the wild-type cytosol rescued the conversion in perforated LY-A cells. Brefeldin A-treated cells, in which the endoplasmic reticulum and the Golgi apparatus were merged, no longer required cytosol for conversion of [3H]ceramide to [3H]SM. These results indicated that the assay of [3H]ceramide-to-[3H]SM conversion in semi-intact cells is a faithful in vitro assay for the activity of ceramide-dependent transport of ceramide and that LY-A cells are defective in a cytosolic factor involved in ceramide transport. In addition, conversion of [3H]ceramide to [3H]glucosylceramide in semi-intact cells was little dependent on cytosol, suggesting that ceramide reached the site of glucosylceramide synthesis by a cytosol-independent (or less dependent) pathway.

Transport and sorting of lipids from cellular sites of their synthesis to appropriate destinations are essential events for membrane biogenesis in cells. Various pathways for intracellular transport of newly synthesized lipids have been suggested. Phosphatidylycerine produced in the endoplasmic reticulum (ER) is converted to phosphatidylethanolamine, which is translocated to the cytoplasmic layer of the plasma membrane lipid bilayer. Cholesterol synthesized in the ER is likely delivered to the plasma membrane in an ATP-dependent manner by a non-Golgi pathway. In various aspects, sphingomyelin (SM) and glycosphingolipids are synthesized at the cytosolic surface of the ER, translocated from the ER to the Golgi apparatus, and then converted to SM by the enzyme phosphatidylcholine:ceramide cholinephosphotransferase (SM synthase) in the lumenal side of the Golgi apparatus or to glucosylceramide (GlcCer) on the cytosolic surface of the Golgi apparatus. After translocation into the Golgi lumen, GlcCer is further converted to lactosylceramide and more complex glycosphingolipids. SM and glycosphingolipids produced in the Golgi lumen are predominantly delivered to the ectoplasmic layer of the plasma membrane lipid bilayer. However, the mechanisms underlying transport and sorting of lipids are unknown.

In vitro analysis of lipid transport between different intracellular organelles by using isolated organelles or semi-intact cells permeable to macromolecules would be a powerful approach to determine whether cytosolic macromolecules are involved in this function. Although several attempts to reconstitute ER-to-Golgi apparatus transport of Cer in cell-free and semi-intact cell systems have been made, the findings from these studies with in vitro systems have varied. Some investigators reached the conclusion that Cer transport does not require ATP or cytosol, whereas others argued that fusion of ER-derived transport vesicles enriched in Cer with the Golgi membrane requires cytosol. The contradictory results most likely reflect the fact that the nature of intracellular transport of long chain Cer for de novo SM synthesis in intact cells was little known at that time, so that it was difficult to test how accurately the in vitro systems mimicked Cer trafficking events occurring in intact cells.

Recently, we have isolated several types of Chinese hamster ovary (CHO) cell mutants resistant to lysenin, an SM-directed cytolysin, and found that one strain, designated LY-A, is defective in transport of Cer from the ER to the Golgi compartment for SM synthesis. In vivo analysis of the mutant and wild-type CHO cells has revealed that the main pathway for Cer trafficking from the ER to the site of SM synthesis is the ATP-dependent pathway, which is impaired in LY-A cells, and synthesis is...
suggested that Cer reached GlcCer synthase by an ATP-independent (or less ATP-dependent) pathway in CHO cells (11). These findings, together with the observation that LY-A and wild-type cells were almost identical in the rate of processing of glycoproteins to endoglycosidase H-resistant forms (11), have suggested the existence of specific machinery involved in Cer trafficking. Recent advances in the study of intracellular Cer transport now allow us to achieve a faithful in vitro assay of Cer transport from its synthesis site to the site of SM synthesis after carefully evaluating the accuracy of the in vitro system.

In the present study, we devised an in vitro reconstitution system of Cer trafficking from the ER to the site of SM synthesis using perforated CHO cells. In various aspects (i.e. kinetics, temperature dependence, ATP dependence, and the phenotypic difference between wild-type and LY-A strains), the conversion of pulse-labeled Cer to SM in the in vitro system is consistent with Cer-to-SM conversion in intact cells. Analysis with this reconstitution system demonstrates that the ATP-dependent trafficking of Cer requires cytosol and that the phenotype of LY-A cells is due to the recessive deficiency of a heat-labile molecule of cytosol.

**EXPERIMENTAL PROCEDURES**

**Materials**—Creatine phosphate, creatine phosphokinase, UDP-glucose, fatty acid-free bovine serum albumin (BSA), fumonisin B₁, apyrase, and brefeldin A (BFA) were purchased from Sigma. N-7-[7-Nitro-benzo-2-oxa-1,3-diazol-4-yl]amino(caproyl)-N-erythro-sphingosine (C₂₀-NBD-Cer) was from Molecular Probes, Inc. 1-<sup>3</sup>H-sphingosine (20 Ci/mmol) and 1-<sup>4</sup>H-sphingosine (10 Ci/mmol) were from American Radiolabeled Chemicals, Inc.; nonradioactive N-erythro-sphingosine was from Matreya Inc.; and palmitoyl CoA (free acid form) was from Doosan Serdary Research Laboratories (Kyungki-Do, South Korea). High performance TLC (Silica gel 60) was purchased from Merck.

**Cells and Cell Culture**—The CHO-K₁ cell line was obtained from the American Type Culture Collection (ATCC CCL 61). Strain LY-A, a CHO-K₁-derived mutant cell line, was previously established by us (10). Cells were routinely maintained in Ham’s F-12 medium (Life Technologies, Inc.) supplemented with 10% newborn bovine serum, penicillin G (100 units/ml) and streptomycin sulfate (100 µg/ml) at 33 °C in 5% CO₂ atmosphere.

**Preparation of Cytosol Fraction**—CHO cells were cultured in ES medium (Nissui Co., Tokyo, Japan) supplemented with 5% fetal bovine serum, 2 mM l-glutamine, 0.1% sodium hydrogen carbonate, and 10 mM Hepes-NaOH, pH 7.4, at 37 °C in spinner bottles to a density of 1.2 x 10⁶ cells per ml. The cell homogenate was centrifuged (900 g) for 5 min. The supernatant fluid was centrifuged (105,000 g) for 1 h. The supernatant fluid was used as the standard counterparts for correction of the background.

**Preparation of Semi-intact Cells**—Semi-intact CHO cells were prepared as reported previously (12), with several modifications. Cells harvested by trypsinization were seeded in 10 ml of F-12 medium containing 10% newborn bovine serum at a density of 0.3 x 10⁶ cells per 10-cm dish (Corning) and cultured at 37 °C overnight to reach subconfluence. Hereafter, all manipulations were carried out at 4 °C or on ice. The subconfluent cell monolayer was washed twice with a hypotonic buffer (10 mM Hepes-KOH, pH 7.2, 15 mM KCl, 0.1 mM MgCl₂) and incubated for 10 min in 5 ml of the same buffer. The medium was then replaced with 5 ml of H/KCl buffer (25 mM Hepes-KOH, pH 7.2, 115 mM KCl), and the cell monolayer was secretary release of ceramide was collected by low speed centrifugation (250 x g for 5 min). The pellet as semi-intact cells was washed with 5 ml of H/KCl buffer and resuspended in 100 µl of H/KCl buffer. Before using the semi-intact cells for the in vitro assay of Cer transport, the protein concentration of the suspension was determined as described below. The protein concentration of the semi-intact suspension was 2.5–3.0 mg/ml. Trypan blue permeability was optically determined under a phase contrast microscope after the mixing of the suspension of the semi-intact cells with 0.3% of solution of trypan blue in phosphate-buffered saline. Note that the presence of MgCl₂ in the hypotonic buffer was important for efficient perforation of CHO-K₁ and LY-A cells, and that 10–20% of cells remaining unperforated after superfusion with hypotonic buffer for perforation of CHO-15B cells does not contain MgCl₂ (12). For preparation of BFA-treated semi-intact cells, the subconfluent CHO cell monolayer was washed twice with 5 ml of serum-free F-12 medium and incubated in 5 ml of Nutridoma medium (serum-free F-12 medium supplemented with 1% Nutridoma-SP (Roche Molecular Biochemicals) and 10 mM Hepes-NaOH, pH 7.4 containing 1 µg/ml BFA at 37 °C for 30 min. Then, the semi-intact cells were prepared as described above. Trypan blue permeability analysis showed that more than 95% of the BFA-treated cells were perforated by this method.

**In Vitro Assay of Transport of Cer from the ER to the Site of SM Synthesis**—<sup>2</sup>H-sphingosine (20 Ci/mmol, 500 µCi/ml) in ethanol was dried under a nitrogen gas stream and dispersed in H₂O at 320 µCi/ml by mixing and sonication. Semi-intact cells (1.6 mg of protein/ml) were incubated in H/KCl buffer containing 10 µM palmitoyl-CoA and 1.6 µM [³H]sphingosine (32 µCi/ml) for 30 min at 15 °C for pulse labeling of Cer. Then, after addition of 2 mM fumonisin B₁ to a final concentration of 20 µM, the labeled semi-intact cells were incubated on ice for 15 min to block Cer synthesis. Standard chase incubations were conducted in a final total volume of 90 µl in a transport reaction mixture: 20 mM Hepes-KOH (pH 7.0), 70 mM KCl, 2.5 mM MgOAc, 250 µM GTP, 0.2 mM dithiobiotrethiol, 0.5 mM UDP-glucose, and an ATP regenerating system (50 µM ATP (sodium form, neutralized with NaOH), 2 mM creatine phosphate, 8 units/ml creatine phosphokinase, 20 µM fumonisin B₁ (final concentrations), 100 µg/ml of cytosol, and 40 µg of the fumonisin B₁-treated semi-intact cells prelabeled. To start each transport reaction, 25 µl (40 µg of protein) of the suspension of the fumonisin B₁-treated semi-intact cells prelabeled was added to 65 µl of a 1.4-fold concentrated transport reaction medium, and the mixture was incubated at 37 °C for 30 min. The reaction was stopped by addition of 700 µl of chloroform/methanol (1:2, by volume). Then, 230 µl of chloroform and 420 µl of 0.1 M KCl were added to the sample for phase separation to isolate lipids (13). Lipids extracted were separated on high performance TLC plates with a solvent system of chloroform/methanol/H₂O (65:25:4, by volume). Radioactive lipids separated on the plates were detected with a BAS1800 image analyzer (Fuji Film Inc., Tokyo, Japan), and after gels were collected from the plates by scraping, the radioactivity of each lipid was determined by liquid scintillation counting in a toluene/water mixture. As a positive control, radioactive lipids from perforated cell-derived activity, we routinely carried out background control experiments, in which semi-intact cells were pulse-labeled with [³H]sphingosine in the absence of palmitoyl-CoA and chased. The radioactivity incorporated to Cer, SM, and GlcCer in the control experiments, which was regarded as background activity derived from unperforated cells existing in the semi-intact cell preparation, was subtracted from the radioactivity of each lipid produced in the standard counterparts for correction of the background.

**Metabolic Labeling of Sphingolipids in Intact Cells with [³H]Sphingosine**—For preparation of a stock solution containing 160 µM [³H]sphingosine (330 Ci/mmol) and 320 µCi BSA, 32 µl of ³H-erythro-[³H]sphingosine (20 Ci/mmol, 500 µCi/ml) and 14.4 µl of 1 mg/ml nonradioactive ³H-erythro-sphingosine in ethanol were put in a 1.5-ml tube (Eppendorf), dried under a stream of nitrogen gas, and dispersed in 300 µl of phosphate-buffered saline containing 21 mg/ml fatty acid-free BSA by mixing and brief sonication. Cells were seeded in 5 ml of F-12 medium containing 10% newborn bovine serum at a density of 1.2 x 10⁶ cells per 6-cm dish and cultured in the normal culture medium overnight at 37 °C. After two washes with 2 ml of serum-free F-12 medium, the cell monolayer was incubated in 1.5 ml of Nutridoma medium containing 1.6 µM [³H]sphingosine (330 Ci/mmol) complexed with BSA for 30 min at 15 °C. The pulse-labeled cells were washed twice with 2 ml of serum-free F-12 medium and incubated in 1.5 ml of Nutridoma medium in the presence of 20 µM fumonisin B₁ for 15 min on ice. Then, the cells were incubated for 30 min at various temperatures for chase. After chase, the cells were washed twice with cold phosphate-buffered saline with 1 ml of cold 0.1% SDS, and 800 and 20 µl each of the lysates were used for lipid extraction (13) and for determination of protein concentration, respectively. For metabolic labeling of BFA-treated cells, CHO cells were washed twice with 2 ml of serum-free F-12 medium and incubated in 1.5 ml of Nutridoma medium containing 1 µg/ml BFA at 37 °C for 30 min. Then, after addition of [³H]sphingosine to the BFA-containing medium, cells were incubated
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Fig. 1. Transport of Cer from its synthesis site to the site of SM synthesis in semi-intact cells. Semi-intact (SI) cells were pulsed with [3H]sphingosine in the presence (+) or absence (−) of 10 μM palmitoyl-CoA at 15 °C for 30 min. This was followed by chase in the transport mixture containing cytosol at 37 °C for 30 min. Semi-intact cells and cytosol were harvested by the indicated strains.

Lipids were extracted and separated on a high performance TLC plate with a solvent of chloroform/methanol/H2O (65:25:4, by volume). For zero chase-time experiments, lipids were extracted from the pulse-labeled semi-intact cells without chase. Radioactive lipids on the TLC plate were visualized by radioactive imaging analysis. Mobilities of standard lipids are shown at the left.

for 30 min at 15 °C for metabolic labeling. After separation of extracted lipids by high performance TLC, the radioactivity of each lipid was determined as described above, and normalized to cell protein. Enzyme Assays—Enzyme assays for SM and GcCer synthetases with C2-NBD-Cer as the enzyme substrate were performed as described previously (14) with minor modifications. Briefly, enzyme sources were incubated in H/KCl buffer containing 100 mM C2-NBD-Cer and 0.5 mM UDP-glucose for 20 min at 37 °C. When the glucose concentration in the pulse reaction mixture reduced [3H]Cer by 85%, indicating that [3H]Cer was produced largely in perforated cells but not unperforated cells under the standard pulse conditions that we used (Fig. 1).

Transmission electron microscopic analysis indicated that the electron-dense cytosol observed in intact cells was not present in perforated cells but that various intracellular organelles including the ER, Golgi apparatus, mitochondria, and nucleus were maintained in the perforated cells, although the ER structure found in the perforated cells was swollen (data not shown), consistent with a previous study (12). Considering these observations, we refer to the perforated cells, which retain ER- and Golgi apparatus-bound enzymes but not cytosolic soluble proteins, as semi-intact cells henceforth.

Reconstitution of ATP-dependent Transport of Long Chain Cer from Its Synthesis Site to the Site of SM Synthesis in Semi-intact CHO Cells—To determine whether transport of Cer from the ER to the site of SM synthesis was able to be reconstituted in semi-intact CHO cells, we first developed pulse-labeling conditions, where radioactive Cer was formed in semi-intact cells. De novo sphingolipid biosynthesis is initiated by condensation of serine and palmitoyl-CoA to generate ketohydrosphingosine, which is converted to dihydrosphingosine (for a review of sphingolipid biosynthesis, see Ref. 17). Acyl transfer from acyl-CoA to the amino group of dihydrosphingosine produces dihydroceramide, which is desaturated to generate ceramide. These reactions are catalyzed by ER-embedded enzymes, the catalytic sites of which face the cytosol (18–20). Because the dihydrosphingosine-N-acyltransferase enzyme catalyzes acylation of sphingosine as well as dihydrosphingosine (21), production of Cer from sphingosine at the ER occurs under sphingosine-supplied conditions. In addition, conversion of metabolically labeled Cer to SM in intact cells can be blocked at low temperatures (22). Thus, radioactive Cer was specifically produced in semi-intact cells of both wild-type and LY-A strains by incubation with 10 μM palmitoyl-CoA and 1.6 μM Δ9-erythro-[3H]sphingosine at 15 °C for 30 min, whereas the pulse reaction produced only a very small amount of [3H]SM (Fig. 1). Note that neither ATP cytosol nor UDP-glucose was added to the pulse reaction mixture. Thus, no [3H]GlcCer was produced by the pulse reaction (Fig. 1), although [3H]Cer-to-[3H]GlcCer conversion in semi-intact cells did not require ATP or cytosol (see below). Omission of palmitoyl-CoA from the pulse reaction mixture reduced [3H]Cer production by ~85%, indicating that [3H]Cer was produced largely in perforated cells but not unperforated cells under the standard pulse conditions that we used (Fig. 1).

We next developed chase conditions in which pulse-labeled [3H]Cer was converted to [3H]SM in semi-intact cells at a rate similar to that observed in intact cells. The prelabeled semi-intact cells (40 μg of protein) were incubated with the cytosol fraction (100 μg of protein) and an ATP-regenerating system at 37 °C for 30 min. Fumonisin B1, an inhibitor of sphingosine-N-
acyltransferase (or dihydrophosphoglycine- N-acyltransferase) (23), was added to the chase reaction mixture to block de novo synthesis of $[^3H]$Cer. When prelabeled semi-intact cells of the wild-type strain were chased in the presence of the cytosol fraction from wild-type CHO cells, $[^3H]$Cer was efficiently converted to $[^3H]$SM and $[^3H]$GlcCer (Fig. 1). The time course of the $[^3H]$Cer conversion in semi-intact wild-type cells reached a plateau after a 60-min chase, by which time $\sim 50$% and $\sim 15\%$ of pulse-labeled $[^3H]$Cer was converted to $[^3H]$SM and $[^3H]$GlcCer, respectively (Fig. 2A). The half-time of $[^3H]$SM formation from $[^3H]$Cer in semi-intact cells was $\sim 20$ min, which was almost equal to the half-time of $[^3H]$SM formation from $[^3H]$Cer in intact wild-type CHO cells prelabeled with $[^3H]$phosphoglycine (Fig. 2, A and C). Semi-intact LY-A cells with the cytosol fraction from LY-A cells were defective in conversion of $[^3H]$Cer to $[^3H]$SM but not to $[^3H]$GlcCer (Fig. 2B), which is consistent with the phenotype of intact LY-A cells (11). Conversion of $[^3H]$Cer to metabolites other than $[^3H]$SM and $[^3H]$GlcCer was negligible in both LY-A and wild-type cells under the in vitro conditions, because the sum of the radioactivity of $[^3H]$Cer, $[^3H]$SM, and $[^3H]$GlcCer was nearly constant throughout chase (Fig. 2, A and B).

Depletion of ATP in the chase reaction mixture by using apyrase reduced the conversion of $[^3H]$Cer to $[^3H]$SM in wild-type cells to the level in LY-A cells in vitro, whereas the ATP depletion did not affect conversion of $[^3H]$Cer to $[^3H]$GlcCer (Fig. 2D). Assay for SM and GlcCer synthases with the enzyme substrate $C_p$-NBD-Cer, which moves spontaneously between and across membranes (5), indicated that neither enzyme activities of semi-intact cells were ATP-dependent (Table 1).

**Temperature Dependence of the $[^3H]$Cer Metabolism in Intact and Semi-Intact Cells**—Pulse-labeling of semi-intact cells with $[^3H]$phosphoglycine and palmitoyl CoA at $15\,^\circ C$ produced a substantial amount of $[^3H]$SM but not $[^3H]$Cer (Fig. 3A). Efficient production of $[^3H]$Cer but not $[^3H]$SM was also observed when intact cells were incubated in a serum-free culture medium containing $[^3H]$phosphoglycine at $15\,^\circ C$ (Fig. 3B). The absence of efficient Cer-to-SM conversion at $15\,^\circ C$ was due to an inactive SM synthase at this temperature, because BFA-treated cells, in which the ER and the Golgi apparatus were merged (24), produced 14-fold more $[^3H]$SM without any increase in the $[^3H]$Cer level, compared with the BFA-untreated control (Fig. 3). Therefore, the inability of cells to convert Cer to SM at $15\,^\circ C$ indicated that transport of Cer from its synthesis site to the site of SM synthesis occurred little, if at all, in CHO cells at $15\,^\circ C$. BFA treatment enhanced conversion of $[^3H]$Cer to $[^3H]$GlcCer at $15\,^\circ C$ in intact cells to $\sim 2$-fold of the BFA-untreated control level (Fig. 3B). The weak effect of BFA on enhancement of $[^3H]$GlcCer production compared with $[^3H]$SM at $15\,^\circ C$ will be discussed below.

When intact wild-type CHO cells prelabeled at $15\,^\circ C$ were chased in the presence of fumonisin B1 at various temperatures ($25\,^\circ C, 30\,^\circ C$, and $37\,^\circ C$), more than $15\%$ of the pulse-labeled $[^3H]$Cer was converted to $[^3H]$SM at $\geq 25\,^\circ C$, whereas less than $5\%$ was converted at $< 15\,^\circ C$ (Fig. 4A). A similar pattern of temperature dependence of $[^3H]$Cer-to-$[^3H]$SM conversion was observed when semi-intact cells prelabeled at $15\,^\circ C$ were chased at various temperatures under the cytosol- and ATP-supplied conditions (Fig. 4B).

**Cytosol Is Required for Transport of Long Chain Cer from Its Synthesis Site to the Site of SM Synthesis**—Conversion of $[^3H]$Cer to $[^3H]$SM in semi-intact cells was ATP-dependent (Table I). The activities of semi-intact cells were derived from wild-type CHO cells (A and LY-A cells (B), C, intact wild-type cells, which had been prelabeled with $[^3H]$phosphoglycine at $15\,^\circ C$ for 30 min, were chased at $37\,^\circ C$ for various time periods. Lipids were extracted from semi-intact and intact cells after chase, and the radioactivity of each lipid was determined. WT, wild-type. D, semi-intact cells pulsed with $[^3H]$phosphoglycine were chased at $37\,^\circ C$ for $30$ min in the complete transport reaction mixture ($\pm$) or an ATP-depleted transport reaction mixture ($-$), which contained apyrase (11 units/ml) in place of ATP. Semi-intact cells and cytosol were from the indicated strains. After chase, the radioactivity of each sphingolipid indicated was determined. The data (means $\pm$ S.D. from three experiments) are expressed as a percentage of the mean values of the wild-type control, in which wild-type semi-intact cells (40 $\mu$g of protein) were chased in the complete transport reaction mixture containing wild-type cytosol (100 $\mu$g of protein).

**Table 1** Effects of cytosol and ATP on SM and GlcCer synthase activities in semi-intact cells

| Reaction mixture | Wild-type | LY-A |
|------------------|-----------|------|
|                  | $C_p$-NBD-SM | $C_p$-NBD-GlcCer | $C_p$-NBD-SM | $C_p$-NBD-GlcCer |
| Complete$^a$     | 100       | 100   | 100   | 100       |
| Cytosol$^b$      | 98 $\pm$ 1 | 113 $\pm$ 6 | 101 $\pm$ 12 | 93 $\pm$ 7 |
| $^\Delta$ATP$^c$| 96 $\pm$ 1 | 99 $\pm$ 7  | 98 $\pm$ 13  | 95 $\pm$ 16 |

$^a$ Percentage of complete reaction mixture control.
$^b$ Complete reaction mixture consisted of the transport reaction mixture (see under “Experimental Procedures”) supplemented with $10\, \mu$M $C_p$-NBD-Cer complexed with 0.1% BSA.
$^c$ The cytosol fraction was not added to the reaction mixture.

$\Delta$ 11 units/ml apyrase in place of ATP was added to the reaction mixture.

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3H]Cer to [3H]SM in semi-intact wild-type cells required the cytosol fraction prepared from wild-type cells. The level of [3H]SM produced in the presence of 300 μg/ml BFA for 30 min was 10-fold the cytosol-negative control level, and the cytosol dependence reached a plateau around 100 μg/ml BFA for 30 min, after which they were pulsed with [3H]sphingosine at 15 °C for 30 min. After pulse, the radioactivity of each sphingolipid was determined. The data are shown as means ± S.D. from three experiments.

Fig. 3. Effects of BFA on conversion of [3H]Cer to [3H]SM and [3H]GlcCer in semi-intact and intact cells. A, semi-intact wild-type cells, prepared from cells pretreated with (+) or without (−) 1 μg/ml BFA for 30 min, were pulsed with [3H]sphingosine at 15 °C for 30 min in the presence (+) or absence (−) of 1 μg/ml BFA. SI, semi-intact. B, intact wild-type cells were pretreated with or without 1 μg/ml BFA for 30 min, after which they were pulsed with [3H]sphingosine at 15 °C for 30 min. After pulse, the radioactivity of each sphingolipid was determined.

Fig. 4. Temperature dependence of [3H]Cer metabolism in intact and semi-intact cells. A, intact wild-type cells, which had been prelabeled with [3H]sphingosine, were chased for 30 min at various temperatures. B, semi-intact wild-type cells pulsed with [3H]sphingosine were chased for 30 min at various temperatures in the transport reaction mixtures containing wild-type cytosol. Lipids were extracted from chased semi-intact and intact cells, and the radioactivity of each lipid was determined.

[3H]Cer to [3H]SM in semi-intact wild-type cells required the cytosol fraction prepared from wild-type cells. The level of [3H]SM produced in the presence of 300 μg of protein of the cytosol fraction was ~10-fold the cytosol-negative control level, and the cytosol dependence reached a plateau around 100 μg of protein/assay (Fig. 5A). In contrast, the activity of Cer-to-SM conversion in semi-intact LY-A cells was little enhanced by the cytosol fraction from LY-A cells, and the activity in semi-intact LY-A cells with 300 μg of protein of the LY-A cytosol remained at ~20% of the wild-type level (Fig. 5B). Conversion of [3H]Cer to [3H]GlcCer in semi-intact wild-type and LY-A cells was less dependent on cytosol, and enhancement of [3H]GlcCer production by cytosol was maximally ~1.5-fold, compared with the control level (Fig. 5A and B). BFA-treated semi-intact cells did not require cytosol for conversion of Cer to SM (Fig. 5C), suggesting that a rate-determining step in the cytosol-dependent conversion of [3H]Cer to [3H]SM in semi-intact cells was intermembrane transport, but not intramembrane transport, of Cer. The cytosol fraction, which was the supernatant fluid obtained by high speed centrifugation of a postnuclear supernatant fraction of cells, had no appreciable activities of SM synthase, GlcCer synthase, or sphingosine-N-acyltransferase. In addition, enzyme assays showed that activities of SM and GlcCer synthases within semi-intact cells were not affected by cytosol (Table I). The activity of the wild-type cytosol to support Cer-to-SM conversion in semi-intact cells was inactivated by heat treatment (Table II). This activity was little affected by dialysis at 4 °C for 16 h, compared with the control cytosol kept for the same time without dialysis (Table II). Treatment of the wild-type cytosol (100 μg) with trypsin (5 μg) at 30 °C for 30 min resulted in almost complete loss of the cytosol activity. These results revealed that transport of Cer from its synthesis site to the site of SM synthesis requires a heat-labile cytosol protein(s) and suggested that Cer reached the site of GlcCer synthesis by a cytosol-independent (or less dependent) pathway.

When semi-intact cells were pulse-labeled with d-erythro-[3H]dihydrosphingosine instead of d-erythro-[3H]sphingosine, we obtained results similar to those of pulse and chase experiments with [3H]sphingosine, except that radioactive sphingolipid metabolites after [3H]dihydrosphingosine-labeling in-
hatched bars

wild-type cells, prepared after pretreatment with 1 mM points

the reaction mixture. For no chase, lipids were extracted from the

pulsed with [3H]sphingosine and chased at 37 °C for 30 min in the

from wild-type (Table II). Dialysis of the wild-type cytosol did not

show any activity to support Cer-to-SM conversion to semi-

intact cells (Table II). Dialysis of the wild-type cytosol did not

affect the activity to complement the LY-A cytosol, compared with

the nondialyzed control level (Table II). These results

indicated that the phenotype of LY-A cells defective in Cer-

to-SM conversion is due to the recessive defect in a heat-labile

macromolecule of cytosol. This conclusion was supported by in vivo

metabolic labeling experiments using hybrid cells; the

conversion rate of Cer to SM in wild-type × LY-A hybrids was an

intermediate value between the levels in wild-type × wild-

type hybrids and LY-A × LY-A hybrids.2

**DISCUSSION**

To address the question of whether cytosolic macromolecules

were involved in intracellular transport of Cer, we here attempted to

reconstitute transport of Cer from its synthesis site to

the site of SM synthesis within semi-intact CHO cells, which

retain the ER and Golgi apparatus but not cytosolic soluble

proteins. In the reconstitution system that we devised, [3H]Cer

is pulse-labeled with d-erythro-[3H]sphingosine and palmitoyl

taoCoA in semi-intact cells at 15 °C, and the prelabeled semi-

intact cells are chased in the presence of the cytosol fraction

and an ATP-regeneration system at 37 °C to convert [3H]Cer to

[3H]SM. Several lines of evidence indicate that the assay of

[3H]Cer-to-[3H]SM conversion using the semi-intact cell system

is a faithful in vitro assay for the activity of Cer transport from

the ER to the site of SM synthesis. First, long chain [3H]Cer

produced by metabolic conversion of [3H]sphingosine in semi-

intact cells is expected to be embedded in the membrane in the

same way as natural Cer synthesized at the ER in intact cells. Second,

the conversion rates of [3H]Cer to [3H]SM in intact and semi-intact cells are similar (Fig. 2, A and C). Third, Cer-to-SM conversion in semi-intact cells is dependent on ATP (Fig. 2D),

**TABLE II**

Effects of heat treatment and dialysis of cytosol on the activity to

support Cer-to-SM conversion in semi-intact cells

| Cytosol (protein amount in μg) | % of control |
|-------------------------------|-------------|
| None                          | 16 ± 6      |
| WT (100)                      | 100 ± 18    |
| WT heated* (100)              | 21 ± 5      |
| WT dialyzed* (100)            | 73 ± 2      |
| WT nondialyzed* (100)         | 85 ± 5      |
| WT (100) + LY-A (100)         | 93 ± 8      |
| WT heated* (100) + LY-A (100) | 32 ± 3      |
| WT dialyzed* (100) + LY-A (100) | 71 ± 6   |
| WT nondialyzed* (100) + LY-A (100) | 79 ± 4 |
| LY-A (100)                    | 27 ± 2      |

* The cytosol fraction was incubated at 95 °C for 5 min prior to assay.

+ The cytosol fraction was dialyzed against 10 mM Tris-HCl, pH 7.4,

containing 0.25 M sucrose with a Slide-A-Lyzer® MINI dialysis unit (a

nominal molecular mass limit of 10 kDa; Pierce) at 4 °C for 16 h, prior

to assay.

3 As a nondialyzed control, the wild-type cytosol fraction kept at 4 °C

for 16 h without dialysis was used.

Inclined saturated dihydro forms in addition to desaturated

forms as assessed by TLC analysis of sphingoid bases liberated

by acid hydrolysis.2

LY-A Cells Are Deficient in a Cytosolic Factor Involved in Cer

Transporting for SM Synthesis—To address the question of whether the impairment of Cer-to-SM conversion in LY-A cells was due to deficiency of a cytosolic soluble factor, we carried out cytosol exchange experiments using the in vitro system. When prelabeled semi-intact cells of the wild-type strain were chased in the presence of the cytosol fraction of LY-A cells instead of the wild-type cytosol, the activity of Cer-to-SM conversion was ~20% of the complete wild-type control level (Fig. 6). More interestingly, when semi-intact LY-A cells were chased with the wild-type cytosol, the activity was ~100% of the control level (Fig. 6). Exchange of the cytosol fraction did not affect conversion of Cer to GlcCer (Fig. 6). These results demonstrated that LY-A cells are deficient in a cytosolic factor involved in transport of Cer for SM synthesis.

The Deficiency of the LY-A Cytosol Is Recessive—We next examined whether the deficiency of the LY-A cytosol was recessive or dominant to the wild-type cytosol. For this, we car-

![Fig. 5. Cytosol dependence of conversion of [3H]Cer to [3H]SM and [3H]GlcCer in the semi-intact cell system.](image)

A and B, semi-intact cells pulsed with [3H]sphingosine were chased at 37 °C for 30 min in the transport reaction mixture containing various amounts of the cytosol fraction. After chase, the radioactivity of each sphingolipid was determined. Both semi-intact (SI) cells and cytosol fractions were derived from wild-type (WT) CHO cells (A) and LY-A cells (B). C, semi-intact wild-type cells, prepared after pretreatment with 1 μg/ml BFA, were pulsed with [3H]sphingosine and chased at 37 °C for 30 min in the complete transport reaction mixture in the presence of 1 μg/ml BFA. After chase, the radioactivity of each sphingolipid was determined. In the experiments without cytosol (−), no cytosolic activity was added to the reaction mixture. For no chase, lipids were extracted from the transport reaction mixture containing the pulse-labeled semi-intact cells without chase. The bars each show the mean of duplicate experiments, with actual activities for individual experiments indicated by points. Open bars, no chase; gray bars, chase in the presence of cytosol; hatched bars, chase in the absence of cytosol.

2 S. Yasuda, M. Fukasawa, T. Funakoshi, M. Nishijima, and K. Hanada, unpublished observations.
being consistent with our previous observation that ATP-depletion in intact CHO-K1 cells by energy inhibitors inhibits ER-to-Golgi apparatus trafficking of Cer (11). Fourth, a rate-determining step for production of [3H]SM from [3H]Cer in vitro as well as in vivo is Cer transport but not enzyme reaction of SM synthase, because efficient conversion of Cer to SM occurs in BFA-treated intact and semi-intact cells even under low temperature and ATP-depleted conditions, unlike in untreated controls (Fig. 3A). Furthermore, the semi-intact cell system reproduces the phenotype of mutant LY-A cells defective in Cer trafficking from the ER to the site of SM synthesis; the rate of Cer-to-SM conversion in semi-intact LY-A cells is only ~20% of the wild-type level, whereas the rate of Cer-to-GlcCer conversion in semi-intact LY-A cells is near the wild-type level (Fig. 2B). From these observations, we conclude that the in vitro system reconstitutes intracellular Cer transport events occurring in intact CHO cells for de novo SM synthesis.

Analysis with the in vitro reconstitution system demonstrated that the ATP-dependent conversion of Cer to SM requires cytosol (Fig. 5A). In addition, cytosol exchange and cytosol mixing experiments demonstrated that the phenotype of LY-A cells defective in Cer-to-SM conversion results from the recessive deficiency of the LY-A cytosol (Figs. 6 and 7). The activity of the wild-type cytosol to rescue the LY-A cytosol in terms of Cer-to-SM conversion in semi-intact cells is heat-labile and is not permeable by a dialysis membrane (Table II). SM synthase activity itself, which was assessed with C6-NBD-Cer as the enzyme substrate, was not dependent on ATP or cytosol (Table I). Collectively, these results indicate that ATP-dependent transport of Cer from the ER to the site of SM synthesis in CHO cells requires cytosol and that LY-A cells are defective in a heat-labile cytosol macromolecule involved in the Cer transport. BFA treatment rendered Cer-to-SM conversion in semi-intact cells independent of cytosol (Fig. 5C). Our preferred interpretation of this observation is that cytosol is required for intermembrane transport of Cer from the ER to the Golgi compartment for SM synthesis. However, because catalytic sites of SM synthase and GlcCer synthase have been suggested to exist in the luminal and cytoplasmic sides, respectively, of the Golgi complex (25–29), there is the alternative interpretation that cytosol is required for transport of Cer across the Golgi membrane but that cytosol-independent transbilayer movement of Cer occurs in the merged organelle formed by BFA treatment. Unfortunately, the effects of BFA treatment on transbilayer movement of natural Cer are unknown. Thus, at this time, the latter interpretation is conceivable, and it is also possible that both transbilayer and intermembrane transport of Cer for de novo SM synthesis require cytosol.

The precise sites of SM synthase and GlcCer synthase in Golgi subcompartments are unknown. If GlcCer is synthesized in more proximal Golgi subcompartments compared with SM, the possibility exists that transport of Cer from the ER to the proximal Golgi subcompartment, where GlcCer but not SM is synthesized, does not require ATP or cytosol but that intra-Golgi apparatus transport of Cer from the proximal Golgi subcompartment to the subcompartment for SM synthesis requires ATP and cytosol. We cannot currently exclude this possibility. Wattenberg (30) previously developed a cell-free assay system of intra-Golgi apparatus transport of glycosphingolipid by using donor and acceptor Golgi membranes prepared...
from two types of CHO cell mutants defective in sugar metabolism. From analysis with this system, he has shown that ATP and cytosol are required for transport of glycosphingolipid from the Golgi subcompartment for galactosylation to the subcompartment for sialylation in GlcCer synthesis (30). LY-A cells are normal in de novo synthesis of GlcCer and also in processing of glycoproteins to endoglycosidase H-resistant forms (11). Therefore, the cytotoxic factor that is impaired in LY-A cells appears to be specifically responsible for Cer trafficking, even if LY-A cells are defective in intra-Golgi apparatus, but not ER-to-Golgi apparatus, transport of Cer.

Phosphatidylinositol-transfer protein β isofrom (PI-TPβ) is able to transfer not only phosphatidylinositol and phosphatidylcholine but also SM between membranes in cell-free systems (31, 32). Interestingly, when PI-TPβ is overexpressed in mouse NIH3T3 fibroblast cells, replenishment of SM in the plasma membrane upon degradation by bacterial sphingomyelinase is accelerated, suggesting that PI-TPβ is involved in membrane or lipid flow between the plasma membrane and the Golgi apparatus (33). Nevertheless, varying the expression levels of PI-TPβ in cells has no effects on de novo synthesis of SM (33), whereas both de novo synthetic rate and steady-state content of SM are lower in LY-A cells than in wild-type CHO cells (11). Thus, the transport pathway of Cer from the ER to the Golgi apparatus for de novo SM synthesis appears to be distinct from the PI-TPβ-dependent pathway.

One might expect that if both SM and GlcCer synthases use the same pool of Cer, dramatic elevation of GlcCer synthesis should occur when SM synthesis is blocked. However, conversion of [3H]Cer to [3H]GlcCer in LY-A cells is similar to the wild-type level in both intact and semi-intact cell systems (this study and see also Ref. 11). One possible explanation is that during, [3H]Cer that has a final destination of the site of SM synthesis becomes incapable of being redistributed to the site of GlcCer synthesis. For example, Cer used for SM synthesis might be first translocated into the lumen of the ER or to a post-ER compartment devoid of GlcCer synthase.

In contrast to Cer-to-SM, Cer-to-GlcCer conversion in vitro was little dependent on ATP or cytosol (Figs. 2D and 5). The ATP independence (or less ATP dependence) is consistent with our previous observation that depletion of intracellular ATP by energy inhibitors affected conversion of Cer to GlcCer in vivo by only a little (11). These in vitro and in vivo observations suggested that the access of de novo synthesized Cer to the site of GlcCer synthesis was largely independent of ATP and cytosol in CHO cells. Some populations of GlcCer synthase might exist transiently or permanently in the ER, and so intermembrane transport of Cer may not be required for production of GlcCer, because a previous study has suggested that GlcCer synthase was not strictly localized in the Golgi apparatus but was more widely distributed among the microsome (28). Alternatively, transport of Cer from the ER to the Golgi compartment for GlcCer synthesis might not require ATP or cytosol. Protein transport from the ER to the ER-Golgi intermediate compartment is not blocked at 15 °C, but this process requires cytosol (for reviews, see Refs. 34 and 35). Under assay conditions for Cer transport in semi-intact cells, pulse reaction was carried out at 15 °C without any supply of cytosol. Therefore, the lack of any obvious requirement of cytosol for Cer-to-GlcCer conversion in pulse and chase experiments within semi-intact cells suggests that if GlcCer synthesis occurs at the ER-Golgi intermediate compartment, the mechanism for Cer transport from the ER to this compartment for GlcCer synthesis differs from the protein transport mechanism.

Kok et al. (8) previously concluded that conversion of metabolically labeled Cer (with [14C]serine) to SM as well as to GlcCer did not require ATP or cytosol by analysis using streptolyxin O-permeabilized HT29 G+ cells (8). A possible explanation for the discrepancy between their conclusion and ours is that the streptolysin O-permeabilized cell system reconstitutes the ATP- or cytosol-independent pathway but not the ATP- and cytosol-dependent pathway of Cer transport. Another possibility is that the HT29 G+ strain, a permanent cell line derived from human colonic carcinoma, naturally lacks the ATP- and cytosol-dependent pathway of Cer transport, although we previously showed that conversion of metabolically labeled Cer (with [3H]phosphinosine and [3H]dihydrosphingosine) to its SM metabolites was inhibited by depletion of intracellular ATP in HeLa cells and normal human skin fibroblasts as well as in CHO cells (11).

The in vitro reconstitution system described in this study has revealed that cytosol is required for intracellular transport of long chain Cer and that the phenotype of LY-A cells defective in Cer transport results from the recessive defect in a cytosolic factor. Thus, the in vitro reconstitution system will also be a useful assay system for purification of the cytosolic factor that rescues the deficiency of LY-A cells.

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Cytosol-dependent Trafficking of Ceramide