CERT Mediates Intermembrane Transfer of Various Molecular Species of Ceramides*

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Ceramide produced at the endoplasmic reticulum is transported to the Golgi apparatus for conversion to sphingomyelin. The main pathway of endoplasmic reticulum-to-Golgi transport of ceramide is mediated by CERT, a cytosolic 68-kDa protein, in a nonvesicular manner. CERT contains a domain that catalyzes the intermembrane transfer of natural C16-ceramide. In this study, we examined the ligand specificity of CERT in detail by using a cell-free assay system for intermembrane transfer of lipids. CERT did not mediate the transfer of sphingosine or sphingomyelin at all. The activity of CERT to transfer saturated and unsaturated diacylglycerols, which structurally resemble ceramide, was 5–10% of the activity toward C16-ceramide. Among four stereoisomers of C16-ceramide, CERT specifically recognized the natural 1-erythro isomer. CERT efficiently transferred ceramides having C14, C16, C18, and C20 chains, but not longer acyl chains, and also mediated efficient transfer of C16-dihydroceramide and C16-phytoseracemide. Binding assays showed that CERT also recognizes short chain fluorescent analogs of ceramide with a stoichiometry of 1:1. Moreover, (1R,3R)-N-(3-hydroxy-1-hydroxymethyl-3-phenylpropyl)dodecamide, which inhibited the CERT-dependent pathway of ceramide trafficking in intact cells, was found to be an antagonist of the CERT protein. These results indicate that CERT can mediate transfer of various types of ceramides that naturally exist and their close relatives.

The intracellular transport of lipids from the sites of their synthesis to their appropriate destinations must occur, because various steps in lipid biosynthesis occur in different intracellular compartments. The trafficking of integral membrane proteins in eukaryotic cells is mediated by transport vesicles, which load the desired set of proteins and deliver them to the correct organelles. By contrast, many types of lipid synthesized in the endoplasmic reticulum (ER)† have been suggested to be sorted to other organelles by nonvesicular mechanisms, although some lipid flux routes such as the endocytosis of plasma membrane lipids occur by vesicle-mediated mechanisms (1–3). In mammalian cells, ceramide is synthesized at the ER and translocated to the Golgi compartment for conversion to sphingomyelin (4). There are at least two pathways by which ceramide is transported from the ER to the Golgi site for the synthesis of sphingomyelin: an ATP- and cytosol-dependent major pathway and an ATP- or cytosol-independent (or less dependent) minor pathway (5–7). The major pathway is impaired in a Chinese hamster ovary (CHO) mutant cell line, LY-A, without any deficiency in the ER-to-Golgi transport of proteins (5–7).

We have identified CERT as a factor defective in LY-A cells by functional rescue experiments, and we have shown that CERT mediates the ATP-dependent pathway of ER-to-Golgi trafficking of ceramide in a nonvesicular manner (8).

CERT is a tripartite cytosolic protein (~600 amino acids in length (8–10). The amino-terminal region of ~120 amino acids is a phosphatidylinositol 4-phosphate (PtdIns4P)-binding pleckstrin homology domain, which can target the Golgi apparatus (11). The next region of ~250 amino acids (referred to as the middle region) contains coiled-coil motifs (9), which might play a role in homo- or hetero-oligomerization, and a motif that may participate in association with the ER (12). The carboxyl terminus of ~230 amino acids is a steroidogenic acute regulatory protein (STAR)-related lipid transfer (START) domain.

START domains were initially recognized as putative lipid-binding domains of ~210 amino acid residues, which exist in various types of proteins implicated in intracellular lipid transport, lipid metabolism, and signal transduction (13, 14). Although more than 200 proteins have been nominated so far as proteins having START domains in data bases (for example, see smart.embl-heidelberg.de), only a few have been experimentally shown to bind or transfer specific lipids. For example, START and MLN64 proteins recognize cholesterol (14–17), and phosphatidylcholine (PtdCho)-transfer protein is capable of intermembrane transfer of PtdCho in vitro (18, 19). The silkworm Bombyx mori larvae produce a carotenoid-binding START domain (20). We demonstrated previously that the START domain of CERT can efficiently extract natural long chain C16-ceramide but not other types of lipids, including sphingosine, sphingomyelin, PtdCho, and cholesterol, from phospholipid bilayers (8). We have also shown that the START domain of CERT greatly facilitated intermembrane transfer of C16-cer-

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amide in a cell-free system (8). However, many details as to the substrate specificity of the START domain of CERT remain undefined, although various molecular species of ceramide exist in mammalian cells.

In most types of mammalian cells, the hydrophobic moiety of complex sphingolipids is mainly composed of ceramide but also includes dihydroceramide and phosphoceramide at low levels (21–23). Notably, dihydroceramidase is abundant in human lens membranes (24, 25). Moreover, the length of the amido acyl chain of the ceramide moiety is diverse; C16-C26 acyl chains are observed in natural sphingomyelin. Notably, C18 and C24:1 ceramide is predominant for sphingomyelin in the brain (21, 26, 27), whereas C16-ceramide is predominant in many other tissues (22, 28–30). Such structural diversity in the ceramide moiety may affect the nature of membranes where complex sphingolipids are abundant. The physiological importance of the diversity of the ceramide structure has also been recognized, based on differences in bio-modulation activity between ceramide and dihydroceramide (31) or between natural long chain ceramide and unnatural short chain ceramide (32). Hence, it should be of biological significance to determine whether CERT can catalyze the intermembrane transfer of various species of ceramide and its relatives in addition to C18-ceramide.

In the present study, we show that CERT is capable of mediating the intermembrane transfer of various types of ceramides that naturally exist in its START domain-dependent manner. In addition, we show that an inhibitor of ER-to-Golgi transport of ceramide is an antagonist of CERT.

**EXPERIMENTAL PROCEDURES**

**Materials**—Palmitic acid, stearic acid, arachidic acid, behenic acid, lignoceric acid, nervonic acid, *O*-ribiophosphorylsphingosine, and *Clostridium perfringens* phospholipase C were purchased from Sigma. D-erythro-Sphingosine, L-erythro-sphingosine, L-threo-sphingosine, B-erythro-dihydroxyphosphorylsphingosine, and porcine-derived lactosylceramide were from Matreya Inc., and egg phosphatidylcholine (PtdCho) and egg phosphatidylethanolamine (PtdEtn) were from Avanti Polar Lipids Inc. N-palmitoyl-1-14C-Palmitoyl-d-erythro-sphingosine (55 mCi/mmol), [1-14C]palmitoyl-(4,4-difluoro-5,7-dimethyl-4-bora-2,4-diazol-3-yl)glycerol produced was then purified by TLC as described under “Preparation of [14C]-Dipalmitoylglycerol—[1-14C]Dipalmitoylglycerol was prepared by phospholipase C treatment of [1-14C]Dipalmitoyl-Ptd-Cho. For the preparation of [1-14C]Dipalmitoylphosphatidylcholine, 50 nmol of [14C]Dipalmitoylglycerol was added to 10 mM buffer A (20 mM NaHCO3, 0.1% sodium deoxycholate, and 0.1% Triton X-100 in HEPES-NaCl buffer containing 50 mM sodium succinate) were diluted to 1 nmol/ml (71.8 and 45.7 pmol/ml for the H9262 and H9251 strains, respectively) with buffer C (20 mM Hepes-NaCl buffer (pH 7.4) containing 50 mM sodium succinate, and 0.1% Triton X-100). Donor vesicles per assay consist of 32 nmol of egg PtdCho, 8 nmol of egg PtdEtn, 4 nmol of porcine lactosylceramide, and 0.5 nmol of radioactive ceramide (27.5 nCi for [14C]ceramides or 10 nCi for [3H]ceramides). Acceptor vesicles per assay consist of 320 nmol of egg PtdCho and 80 nmol of egg PtdEtn. Note that the excess amount of acceptor ceramide to donor ceramides is crucial to minimize donor-to-acceptor transfer of ceramide, which interferes with the donor-to-acceptor transfer reaction. We previously demonstrated that the addition of acceptor ceramide (PtdCho 125 nCi per assay) as a nonexchangeable lipid marker. According to the number of assays, appropriate amounts of lipids dissolved in organic solvents were mixed in a polypropylene tube (Eppendorf) and dried under a nitrogen gas stream. After addition of buffer C, phospholipid vesicles were prepared by sonication with a probe-type sonicator (model UP-50H, Dr. Hirschel GmbH, Teltow, Germany) at 80% output and 50% cycle for 10 min in a water bath at room temperature. The...
volume of buffer C that should be added at this step was 20 μl per assay in donor vesicles and 60 μl per assay in acceptor vesicles (note that at least 200 μl of the buffer is required for the sonication step). To remove lipid aggregates, the sonicated samples were centrifuged at 20,000 × g for 30 min at 4 °C, and the supernatant fraction was collected as small vesicles. The radioactivity of the supernatant was determined by liquid scintillation counter for assessing the recovery yields after pre-centrifugation. In some cases, the recovery of lipids in the supernatant fraction was also assessed by the lipid phosphorous quantification method (37). Both assessments showed that over 90% of lipids were reproducibly recovered in the supernatant fraction. The prepared small vesicles were used for intermembrane ceramide transfer assay as follows. In typical experiments, 18 μl of buffer C, 60 μl of acceptor vesicles, and 2 μl of recombinant CERT or CERT-ST (1 nmol/ml in buffer C) were mixed in a 1.5-ml polypropylene tube. Then 20 μl of donor vesicles was added to the tube to start the ceramide transfer reaction. After tapping the tube quickly, the reaction mixture was incubated for 10 min at 37 °C. For mock incubation, buffer C as the vehicle buffer was added in place of the recombinant proteins. After adding 2 μl of 0.1 mM ethanolic stock solution of C5-DMB-ceramide or C6-NBD-ceramide and 1 μM drug, the chilled mixture was centrifuged (20,000 × g, 3 min, 4 °C), and 960 μl of supernatant fluid was determined by liquid scintillation counting.

**FIG. 1.** Structures of synthesized ceramides used in this study. Various types of radiolabeled ceramides were prepared by the SCDase reaction as described under "Experimental Procedures." Stereochemical structures and production yields of these ceramides are listed. The positions of radioactive atoms are also specified in the structure. The conversion yield of each ceramide product shown in the SCDase reaction is represented as the mol % of its radiolabeled starting material.
"elute fraction." A 3.75-fold volume of chloroform/methanol (1:2, v/v) was then added to each retrieved fraction, mixed, and centrifuged (20,000 × g, 10 s). In addition, to retrieve fluorophores that were nonspecifically bound to the resin and tube, 170 μl of TBS and 750 μl of chloroform/methanol (1:2, v/v) were added to the tube containing the resin used, mixed, and centrifuged (20,000 × g, 10 s). The supernatant was retrieved as the "residual fraction." The DMB (excitation at 480 nm; emission at 515 nm) and NBD (excitation at 470 nm; emission at 530 nm) fluorophores in these fractions were quantified with a fluorescence spectrophotometer (model F-3000, Hitachi, Tokyo, Japan). When the binding specificity to CERT and ceramide was analyzed, some modifications were made, because the amount of ceramide must be in excess to that of CERT for this analysis. Briefly, various concentrations of C12-DMB-ceramide were mixed with 40 pmol of recombinant CERT or CERTΔST and 15 μl of 50% slurry of TALON metal affinity resin, and then the mixture (the volume of which was 155 μl) was incubated at 37 °C for 30 min. The amounts of the recombinant proteins distributed to the elute fraction were estimated by densitometric analysis after a portion of the fraction was subjected to SDS-PAGE and Comassie Blue® staining, using calibration patterns made with known amounts of CERT and CERTΔST. The amount of C12-DMB-ceramide in the elute fraction was quantified as described above.

Binding of LPS Alexa Fluor® 488 to recombinant proteins was assessed by essentially the same procedures except that the fluorescent intensity of LPS Alexa Fluor® 488 (excitation at 488 nm, emission at 538 nm) distributed to each fraction was measured without organic solvent extraction.

Effect of (1R,3R)-HPA-12 on Metabolic Labeling of Lipids with [14C]Serine in CHO Cells—LY-A, a CHO-K1-derived mutant cell line, is defective in the trafficking of ceramide from the endoplasmic reticulum to the Golgi apparatus because of a mutation in the CERT gene (6, 8). The LY-A2 cell line is a stable transformant of LY-A expressing the CERT cDNA (8). For a concentrated stock, (1R,3R)-HPA-12 was dissolved in dimethyl sulfoxide at 10 mM. Cells were seeded at a density of 1.0 × 106 per 6-cm dish in 5 ml of Ham’s F-12 medium supplemented with 10% newborn bovine serum, penicillin G (100 units/ml), and streptomycin sulfate (100 μg/ml) and cultured for 16 h at 33 °C in a 5% CO2 atmosphere. After two washes with 2 ml of serum-free Ham’s F-12 medium, the cells were incubated in 1.5 ml of Nutridoma medium (Ham’s F-12 medium supplemented with 1% Nutridoma-SP (Roche Diagnostics) and 25 μg/ml gentamicin) containing 1 μCi (1R,3R)-HPA-12 or the vehicle dimethyl sulfoxide for 15 min on ice and, after the addition of 1-μl [14C]serine (0.75 μCi) to the medium, were incubated for 2 h at 33 °C. The metabolically labeled lipids were then analyzed as described previously (38).

Determination of Protein Concentration—Protein concentrations were determined using the bicinchoninic acid protein assay kit (Pierce) with bovine serum albumin as the standard.

RESULTS

Intermembrane Transfer of Lipids by CERT—We showed previously (8) that CERT efficiently extracts ceramide, but not nonceramide lipids, from phospholipid vesicles. In addition, we showed that CERT greatly facilitates intermembrane transfer of natural long chain C16-ceramide (8). However, the lipid substrate specificity of CERT-mediated intermembrane transfer remained unexplored. Thus, we tested the substrate specificity of CERT-mediated intermembrane transfer in a cell-free assay system. Because lipid transfer between artificial membranes might be nonspecifically enhanced by proteins, we used the START domain-deleted CERTΔST recombinant in control assays. As expected, the START domain-dependent transfer of lipids was accurately. The lipid transfer assays showed that CERT catalyzes the efficient intermembrane transfer of ceramide, but not sphingosine, sphingomyelin, PtdCho, or cholesterol (Fig. 2), in a START domain-dependent manner. This pattern was consistent with the substrate specificity of the lipid extracting activity of CERT (8). The activity to transfer dioleoylglycerol and dipalmitoylglycerol was −5 and −10%, respectively, of the activity toward ceramide (Fig. 2), raising the possibility that CERT has the potential to transfer diacylglycerol in intact cells (see "Discussion").

Stereochemical Specificity of CERT-mediated Transfer of C16-Ceramide—Because ceramide has two chiral carbon atoms at positions C-2 and C-3 of the sphingosine backbone, there can be four stereochemical isomers of C16-ceramide, among which d-erythro is the natural configuration (Fig. 1). To examine the stereochemical selectivity of ceramide recognition by CERT, we synthesized the four isomers of C16-[14C]ceramide by SCDase-catalyzed in vitro N-palmitoylation of sphingosines. The four isomers could be prepared in radioactively pure forms, although production yields of the unnatural isomers were much less than the yield of the natural isomer (Fig. 1).

In lipid transfer assays using the synthesized stereochemical isomers of C16-[14C]ceramide, CERT catalyzed the efficient transfer of d-erythro C16-ceramide, but not the unnatural l-erythro-, d-threo-, or l-threo-types (Fig. 3A). Thus, CERT recognizes only the natural isomer among the four stereochemical isomers of C16-ceramide.

Recognition of Dihydroceramide and Phytoceramide by CERT—Although ceramide is the predominant hydrophobic backbone of complex sphingolipids in mammalian cells, some sphingolipids also contains dihydroceramide and phytoceramide (21–25). In vitro lipid transfer assays using synthesized radioactive substrates demonstrated that CERT is capable of catalyzing the intermembrane transfer of C16, dihydroceramide and C16-phytoceramide with ~40% efficiency of the C16-ceramide transfer (Fig. 3B).

CERT-mediated Transfer of Ceramides Having Fatty Acyl Chains of Various Lengths—To examine the effects of different acyl chain lengths of ceramide on CERT-mediated lipid transfer, we prepared d-erythro-[14C]ceramides having C14-, C16-, C18-, C20-, C22-, and C24-saturated acyl chains and also a C24:1-monounsaturated acyl chain (Fig. 1). In vitro lipid transfer assays showed that the efficiency of the CERT-mediated transfer of ceramide is dependent on its acyl chain length (Fig. 4). Among the molecular species having different acyl chain lengths, C14-, C16-, C18-, C20-, and C22- ceramides were similarly effective. When compared with the amount of C16-ceramide transferred, the transfer efficiency of C20- and C24:1-ceramide was ~40%. The transfer of C20-ceramide was less (Fig. 4).

There might be the possibility that the same amount of different acyl chain ceramides was not incorporated into donor vesicles, thereby resulting in differences in the transfer effi-
ciency. To rule out this possibility, we performed another control experiment. Donor vesicle preparations containing different acyl chain \([3H]\)ceramides were centrifuged at 20,000 \(\times g\) for 3 min in the presence or absence of Ricinus communis lectin. Regardless of the differences in the acyl chain lengths, most (>99%) of the radioactivity added to each preparation was precipitated in the presence of the lectin, whereas none (<1%) of the radioactivity was precipitated in the absence of the lectin. These results indicated that nearly 100% of these radioactive ceramides added to vesicle preparations were actually incorporated into donor vesicles.

CERT Recognizes Fluorescent Short Chain Analogs of Ceramide—The fluorescent analogs of ceramide C5-DMB-ceramide and C6-NBD-ceramide have been widely used as probes mimicking natural ceramide in intact cells and in cell-free systems (6, 39, 40). Because these short chain fluorescent analogs of ceramide spontaneously transfer between membranes (6, 40, 41), it was difficult to determine accurately the CERT-dependent transfer of C5-DMB-ceramide and C6-NBD-ceramide in our cell-free transfer assay system. Therefore, to examine if CERT recognized these lipids, we performed a binding assay. CERT could clearly bind both C5-DMB-ceramide and C6-NBD-ceramide (Fig. 5, A and B).

We were also interested in testing if CERT recognizes the endotoxin LPS, because LPS has a moiety that may be structurally similar to ceramide (42). However, we detected no binding of a fluorophore-conjugated LPS to CERT (Fig. 5C).

Binding Stoichiometry of CERT and Ceramide—We next attempted to determine a binding stoichiometry of CERT and ceramide. For this, we used C5-DMB-ceramide as a ceramide ligand, because binding assays at various concentrations of C5-DMB-ceramide were feasible under liposome-free conditions. The molar ratio of C5-DMB-ceramide bound to CERT in the presence of large excess C5-DMB-ceramide was estimated to be about 0.8 (Fig. 5D). These results most likely indicated that the binding stoichiometry of CERT and ceramide is 1:1. The binding assays also suggested that the apparent dissociation constant between CERT and C5-DMB-ceramide was about 200 nM (Fig. 5D).

Inhibition of CERT-mediated Transfer of Ceramide by (1R,3R)-HPA-12 in Vitro—(1R,3R)-HPA-12, a chemically synthesized artificial compound, acts as a selective inhibitor of the transport of ceramide from the ER to the site of sphingomyelin synthesis (38). Because (1R,3R)-HPA-12 has structural similarity to \(\beta\)-erythro-ceramide, we hypothesized that CERT might be a target of (1R,3R)-HPA-12. To test this hypothesis, we examined whether (1R,3R)-HPA-12 inhibited CERT-mediated transfer of C16-\(\beta\)-erythro-ceramide in the cell-free assay system. As shown in Fig. 6, (1R,3R)-HPA-12 inhibited CERT-mediated transfer of ceramide with a 50% inhibitory concentration of \(~0.5\) \(\mu\)M. In contrast, its stereochemical isomers and methoxy derivatives, which are inactive as inhibitors of in vivo

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**Fig. 3.** CERT transfers various natural isomers of C16-ceramide. The intermembrane transfer of various isomers of C16-ceramide was assayed with purified recombinant CERT (closed bars) or CERT\(\Delta ST\) (open bars). The amounts of radioactive substrates transferred to acceptor vesicles are shown. Results are means ± S.D. from triplicate experiments. A, substrates are four stereoisomers of C16-ceramide. B, substrates are C16-\(\beta\)-erythro-ceramide, C16-\(\beta\)-erythro-dihydroceramide, and C16-\(\beta\)-ribo-phytoceramide.

**Fig. 4.** CERT-mediated transfer of ceramides having various fatty acyl chain lengths. The intermembrane transfer of ceramides having various fatty acyl chain lengths was assayed with purified recombinant CERT (closed bars) or CERT\(\Delta ST\) (open bars). The amounts of radioactive substrates transferred to acceptor vesicles are shown. Results are means ± S.D. from triplicate experiments.
ceramide trafficking (33), did not affect the in vitro ceramide transfer even at 4 μM (Fig. 6). Thus, the inhibition of CERT-mediated intermembrane transfer of ceramide by (1R,3R)-HPA-12 was not due to possible nonspecific events such as drug-induced denaturing of proteins or lipids. Collectively, these results indicated that (1R,3R)-HPA-12 is an antagonist of CERT.

(1R,3R)-HPA-12 Inhibits CERT-mediated Trafficking of Ceramide in Intact CHO Cells—To see if (1R,3R)-HPA-12 really inhibits CERT-mediated trafficking of ceramide from the ER to the Golgi site for sphingomyelin synthesis, we examined the effect of the drug on de novo synthesis of sphingomyelin in various CHO cell lines. In wild-type CHO-K1 cells, (1R,3R)-HPA-12 inhibited de novo synthesis of C16-ceramide in vitro. Donor vesicles containing 100 pmol of C16-[14C]ceramide were preincubated with purified recombinant CERT (2 pmol) and various drugs at the indicated concentrations for 5 min at 37 °C. The intermembrane transfer reaction was then started by adding acceptor vesicles to the mixture and incubated for 30 min at 37 °C. The amounts of radioactive ceramide transferred to acceptor vesicles are shown. Results are means ± S.D. from triplicate experiments.

Fig. 6. Inhibition of CERT-mediated ceramide transfer by (1R,3R)-HPA-12. A, structure of (1R,3R)-HPA-12 and its derivatives. B, effects of (1R,3R)-HPA-12 and its derivatives on CERT-mediated transfer of C16-ceramide in vitro. Donor vesicles containing 100 pmol of C16-[14C]ceramide were preincubated with purified recombinant CERT (2 pmol) and various drugs at the indicated concentrations for 5 min at 37 °C. The intermembrane transfer reaction was then started by adding acceptor vesicles to the mixture and incubated for 30 min at 37 °C. The amounts of radioactive ceramide transferred to acceptor vesicles are shown. Results are means ± S.D. from triplicate experiments.
transfected with the human CERT cDNA, de novo synthesis of sphingomyelin was restored to the wild-type level (8), and the restored activity of sphingomyelin synthesis in CERT/LY-A2 cells was found to be again sensitive to (1R,3R)-HPA-12 (Fig. 7). These results confirmed that (1R,3R)-HPA-12 inhibits CERT-mediated trafficking of ceramide in intact CHO cells.

**DISCUSSION**

In the present study, we explored the substrate selectivity of CERT-mediated lipid transfer reactions in a cell-free assay system, and we showed that CERT is capable of mediating the efficient intermembrane transfer of various ceramide molecular species, including ceramide having C14-C20 saturated acyl chains, C16-dihydroceramide, and C16-phytoceramide, that naturally exist in mammalian cells (Figs. 3 and 4). In mammalian tissues, “dihydrophosphingomyelin” (phosphocholine dihydroceramide) widely exists in smaller amounts than sphingomyelin (21, 25). Mammalian tissues might also have “phytosphingomyelin” (phosphocholine phytoceramide) in very small amounts (23, 43). A homology search with publicly available tools and data bases predicts that mammals have no additional isoforms of CERT, except for a large splicing variant of CERT. These results suggest that CERT and its splicing variant CERTL mediate the transport of various ceramide molecular species from the ER to the Golgi site, where sphingomyelin and its isoforms are synthesized.

Different members of the Lag1-related family have been suggested to regulate de novo synthesis of different molecular species of ceramide (44–46). Notably, C18-ceramide synthesized in human embryonic kidney 293 cells overproducing UOG1, a Lag1-related family member, is selectively used for the synthesis of glucosylceramide, but not of sphingomyelin (44). The UOG1-dependent channeling of C18-ceramide to glucosylceramide synthesis is unlikely due to a possible selectivity of ceramide species by CERT, because CERT catalyzes the efficient transfer of C18-ceramide as well as C16-ceramide (Fig. 4). However, it remains unclear whether different interactions of different Lag1-related family members with CERT might affect destinations of ceramide species synthesized de novo.

In contrast to the broad specificity of CERT for the ceramide substrate, CERT mediates no transfer of sphingosine, sphingomyelin, cholesterol, and PtdCho (Fig. 2). Nevertheless, small but significant levels of CERT-mediated transfer of diacylglycerols were reproducibly observed (Fig. 2), consistent with our previous result showing that CERT could extract dioleoylglycerol from artificial membranes even at a much lower efficiency than C16-ceramide (8). When one molecule of sphingomyelin is newly synthesized by the PtdCho:ceramide phosphocholine transfer reaction catalyzed by sphingomyelin synthase, one molecule of PtdCho-derived diacylglycerol should be generated. The generated diacylglycerol might cause a feedback inhibition of sphingomyelin synthesis, because diacylglycerol can inhibit the activity of this enzyme in vitro (47). Although the metabolic fate of diacylglycerol generated during the de novo synthesis of sphingomyelin is unknown, a previous study (48) suggested that diacylglycerol generated during the resynthesis of sphingomyelin was rapidly metabolized to triacylglycerol in baby hamster kidney cells treated with extracellular sphingomyelincase. Triacylglycerol synthesis is likely to occur predominantly at the ER (49). Thus, it would be interesting to hypothesize that CERT transports ceramide from the ER to the Golgi and, in turn, transports diacylglycerol from the Golgi to the ER.

The intracellular redistribution of C5-DMB-ceramide from the ER to the Golgi region is impaired in LY-A cells having a mutation in the CERT gene and also in energy-poisoned wild-type CHO cells (6, 8). Therefore, we have proposed that C5-DMB-ceramide may be a good probe for the CERT-mediated pathway of ceramide in cells (8). This proposal was further supported by the present study showing that CERT actually binds C5-DMB-ceramide (Fig. 5A). Binding assays with C5-DMB-ceramide allowed us to estimate the binding stoichiometry of CERT and ceramide to be 1:1 (Fig. 5D). This is consistent with the START domain of the PtdCho-transfer protein that harbors a single PtdCho molecule in the crystal structure of their complex (50).

Notably, although no clear impairments of the ER-to-Golgi redistribution of C5-NBD-ceramide were observed in LY-A cells nor in energy-poisoned cells (6), the cell-free binding assay showed that CERT is also capable of binding C5-NBD-ceramide (Fig. 5B). Previous studies with model membranes have shown that C5-NBD-ceramide undergoes spontaneous intermembrane transfer at a much faster rate than C5-DMB-ceramide (half-times of transfer equilibration (t1/2) for C5-NBD-ceramide and C5-DMB-ceramide are ~0.4 and ~7 min, respectively) (40, 41). The t1/2 of natural C16-ceramide has been estimated to be in the order of days (51), and CERT enhances the t1/2 of C16-ceramide to the order of minutes (7). Thus, the rapid spontaneous transfer of C5-NBD-ceramide likely masks the CERT-mediated transfer of C5-NBD-ceramide in intact cells, even when the latter process occurs.

(1R,3R)-HPA-12 is an inhibitor of an ATP- and cytosol-dependent transport of ceramide from the ER to the site of sphingomyelin synthesis (38). We also demonstrated here that (1R,3R)-HPA-12 inhibited CERT-mediated transfer of ceramide in a cell-free assay system (Fig. 6). HPA-12 derivatives incapable of inhibiting the CERT-dependent transport pathway in cells also did not affect the CERT-mediated intermembrane transfer of ceramide in the cell-free system (Fig. 6). Collectively, we conclude that (1R,3R)-HPA-12 is an antagonist of CERT. Bioinformatic studies have shown that the human genome encodes numerous proteins with putative lipid transfer domains such as START domains (13, 14), Sec14-related domains (52), and oxysterol-binding protein-related domains (53), although for most of the postulated lipid transfer proteins, their actual ligands remain unknown. Because various inhibitors of lipid metabolism and lipid-mediated signaling have been used as clinical drugs, inhibitors of lipid transfer processes are
also candidates for new types of medicines. Our studies showing (1R,3R)-HPA-12 as an antagonist of a ceramide transfer protein will hopefully open the way to the development of antagonists of specific lipid transfer proteins.

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