Ceramide is synthesized at the endoplasmic reticulum (ER) and transported to the Golgi apparatus by CERT for its conversion to sphingomyelin in mammalian cells. CERT has a pleckstrin homology (PH) domain for Golgi targeting and a START domain catalyzing the intermembrane transfer of ceramide. The region between the two domains contains a short peptide motif designated FFAT, which is supposed to interact with the ER-resident proteins VAP-A and VAP-B. Both VAPs were actually co-immunoprecipitated with CERT, and the CERT/VAP interaction was abolished by mutations in the FFAT motif. These mutations did not affect the Golgi targeting activity of CERT. Whereas mutations of neither the FFAT motif nor the PH domain inhibited the ceramide transfer activity of CERT in a cell-free system, they impaired the ER-to-Golgi transport of ceramide in intact and in semi-intact cells at near endogenous expression levels. By contrast, when overexpressed, both the FFAT motif and the PH domain mutants of CERT substantially supported the transport of ceramide from the ER to the site where sphingomyelin is produced. These results suggest that the Golgi-targeting PH domain and ER-interacting FFAT motif of CERT spatially restrict the random ceramide transfer activity of the START domain in cells.

The transport and sorting of lipids from the sites of their synthesis to their appropriate destinations are essential events for membrane biogenesis in cells. In the synthesis of sphingolipids in mammalian cells, ceramide is newly produced at the endoplasmic reticulum (ER) and transported from the ER to the trans-Golgi regions, where it is converted to sphingomyelin (SM) by the enzyme phosphatidylcholine:ceramide choline-phosphotransferase (SM synthase). Ceramide is also converted to glucosylceramide (GlcCer) presumably in the cis-Golgi regions or a subdomain of the ER. GlcCer is converted to more complex glycosphingolipids in the Golgi apparatus.

The major transport of ceramide from the ER to the Golgi apparatus for the synthesis of SM is an ATP- and cytosol-dependent pathway (1, 2) and is genetically impaired in a Chinese hamster ovary (CHO) mutant cell line named LY-A (1, 2). After functional gene rescue experiments, the factor mutated in LY-A cells was identified to be CERT (also known as Goodpasture antigen-binding protein), a 68-kDa cytosolic protein (3). CERT consists of three distinct regions. The N-terminal ~120 residues of CERT form a pleckstrin homology (PH) domain, which is a phosphoinositide-binding domain (4, 5). The C-terminal ~230 residues form a START domain, a putative lipid transfer domain (6). The middle region, the ~250 amino acid residues between the PH and START domains, is predicted to form no globular domains.

The PH domain of CERT serves to target the Golgi apparatus by recognizing phosphatidylinositol 4-monophosphate (PI(4)P) (3, 7). The START domain of CERT is capable of extracting ceramide from membranes and transferring it to acceptor membranes (3, 8). Based on these findings, we have proposed that CERT extracts ceramide from the ER and carries it to the Golgi apparatus. However, it remains unclear how CERT selectively interacts with the ER to extract newly synthesized ceramide in cells, although the middle region of CERT has a putative motif interacting with the ER (9).

VAP is an ER-resident type I membrane protein (10–12). Mammals have two VAPs, VAP-A (also known as VAP-33) (13) and VAP-B (VAP-B has a splicing variant named VAP-C with no putative membrane-spanning domain) (14). VAP-A and VAP-B, which have ~60% amino acid identity, form a homodimer and also a heterodimer (14) and are ubiquitously expressed in various tissues (10, 13, 14). Wyles et al. (15) initially showed that oxysterol-binding protein (OSBP) interacts with VAP-A, and, shortly after, Loewen et al. (9) showed that conserved short peptide motifs present in OSBP and its yeast relatives are crucial for the interaction of OSBPs with VAP-A. Based on the conserved sequence (EFFDAXE), the motifs are referred to as FFAT motifs (two phenylalanines in an acidic

The abbreviations used are: ER, endoplasmic reticulum; PH, pleckstrin homology; SM, sphingomyelin; GlcCer, glucosylceramide; CHO, Chinese hamster ovary; PI(4)P, phosphatidylinositol 4-monophosphate; VAMP, vesicle-associated membrane protein; VAP, VAMP-associated protein; OSBP, oxysterol binding protein; PBS, phosphate-buffered saline; GFP, green fluorescent protein; 25HC, 25-hydroxycholesterol.
truct) (9). Interestingly, a FFAT motif is present in the middle region of CERT (9) (Fig. 1).

In the present study, we show that CERT actually interacts with VAPs via its FFAT motif. In addition, we show that, when CERT is not overproduced, both the PH domain and the FFAT motif are required for efficient ER-to-Golgi trafficking of ceramide in cells. By contrast, when excess CERT proteins exist, neither the FFAT motif nor the PH domain is essential for the transport of ceramide from the ER to the site where SM is produced. These results suggest that the Golgi-targeting PH domain and the ER-interacting FFAT motif of CERT spatially restrict the random ceramide transfer activity of the START domain in cells.

**EXPERIMENTAL PROCEDURES**

**Cells and Cell Cultures**—The LY-A cell line, a CHO-K1-derived mutant cell line defective in the CERT-mediated ER-to-Golgi transport of ceramide, was previously established by us (16). CHO cells were routinely maintained in Ham’s F-12 medium (Invitrogen) supplemented with 10% newborn bovine serum, 100 units/ml of penicillin G, and 100 μg/ml of streptomycin sulfate at 33 °C in a 5% CO2 incubator (17). HeLa-S3 cells were cultured in Dulbecco’s modified Eagle’s medium (Sigma) supplemented with 10% fetal bovine serum, 100 units/ml of penicillin G, and 100 μg/ml of streptomycin sulfate at 37 °C. Nutridoma medium (Ham’s F-12 containing 1% Nutridoma-SP (Roche Applied Science) and 25 μg/ml of gentamicin) was used as a serum-free medium.

**Introduction of Point Mutations or a Deletion into the CERT FFAT Motif**—DNA constructs encoding the various mutants of human CERT used in this study were made as described in supplementary data part 1.

**Cloning of Human VAP-A and VAP-B Genes**—The open reading frames of the human VAP-A and VAP-B genes fused with the HA epitope or the red fluorescent protein HcRed were constructed as described in supplementary data part 1.

**Metabolic Labeling of Lipids with [14C]Serine or [3H]-Sphingosine**—The metabolic labeling of CHO cells with radioactive serine or sphingosine was performed as described previously (1). In brief, subconfluent cell monolayers in 6-cm dishes were incubated in 1.5 ml of Nutridoma medium containing 18.5 kBq of l-[U-14C]serine (Amersham Biosciences) at 37 °C or 37 kBq of d-erythro-[3-3H]sphingosine (American Radiolabeled Chemicals, Inc.) at 33 °C for 2 h. After two washes with 1 ml of cold phosphate-buffered saline (PBS), labeled lipids extracted from the cells (18) were separated on TLC plates (solvent system; methyl acetate, n-propanol, chloroform, methanol, 0.25% KCl, 25/25/25/10/9, v/v, for the [14C]serine-labeling; chloroform, methanol, water, 65/25/4, v/v, for the [3H]sphingosine-labeling) and analyzed with BAS image analyzers (Fuji Film Inc.).

**Preparation of Recombinant CERT Proteins**—Escherichia coli BL21(DE3) cells (Stratagene) were transformed with the bacterial expression plasmid pET28a(+) encoding a His6-tagged version of CERT or its mutants. Recombinant CERT proteins were purified from the transformed bacteria cells as described previously (3).

**Assay of Ceramide Trafficking in Semi-intact CHO Cells**—The transport of ceramide from the ER to the site where SM is synthesized was assayed in semi-intact CHO cells as described previously (2). In brief, perforated LY-A cells were incubated with [3H]sphingosine and palmitoyl-CoA at 15 °C for 30 min to produce [3H]ceramide at the ER. The prelabeled perforated cells were incubated with 40 μg of protein at 37 °C for 30 min. After the reaction was stopped, the lipids were extracted from the reaction mixture and separated by TLC. Radioactive lipids separated on the TLC plates were detected with a BAS1800 image analyzer (Fuji Film Inc.). The radioactivity of lipids in the control experiments, in which perforated LY-A cells were pulse-labeled with [3H]sphingosine in the absence of palmitoyl-CoA and chased, was regarded as background activity derived from imperforated cells existing among the semi-intact cells; thus this value was subtracted from the radioactivity of each lipid produced in the standard counterparts.

**Cell-free Assay of Intermembrane Transfer of Ceramide**—Recombinant wild-type and mutant CERTs were purified by using a Talon Co2⁺ affinity column (Clontech) (3), and the transfer of ceramide between artificial phospholipid vesicles was assayed in a cell-free system as described previously (3, 8).

**Co-immunoprecipitation of Epitope-tagged VAP with CERT**—CHO-K1 cells cultured in dishes 100 mm in diameter were transiently co-transfected with 1.0 μg of pcDNAhyg/kzHA-VAP-A and 1.0 μg of various pcDNA/FLAG-CERT constructs by using Lipofectamine Plus™ reagent (Invitrogen). The cells grown at 37 °C overnight were rinsed twice with 5 ml of cold PBS. Thereafter, the cells and samples were handled at 4 °C.
CERT Interacts with VAP-A in a FFAT Motif-dependent Manner—To examine whether CERT interacts with VAPs, we performed a co-immunoprecipitation analysis using epitope-tagged human VAP-A and CERT constructs. After their transfection with expression plasmids encoding FLAG-tagged CERT and HA-tagged VAP-A, CHO-K1 cells were lysed with the mild detergent digitonin, and the supernatant fluid obtained by high speed centrifugation of the lysate was used as a solubilized cell extract. When the FLAG M2 monoclonal antibody was used, virtually all of the FLAG-tagged CERT present in the cell extract was precipitated (Fig. 2A, right panels, lane 3), and ~1% of the HA-tagged VAP-A present in the extract was also precipitated (Fig. 2A, left panels, lane 3). Far less HA-tagged VAP-A was precipitated in the absence of FLAG-tagged CERT, although the expression of HA-tagged VAP-A was not affected by the co-expression of FLAG-tagged CERT (Fig. 2A, left panels, lanes 2 and 3). Note that the 33-kDa doublet bands observed in the anti-HA blots were identified as the HA-tagged VAP-A protein, because such bands were absent in the extract of control cells not transfected with the HA-tagged VAP-A expression plasmid (Fig. 2A, left panels, lane 1). Collectively, these results indicated that VAP-A is co-immunoprecipitated with CERT.

We next determined whether the co-immunoprecipitation of VAP-A with CERT was dependent on the FFAT motif of CERT. For this, we made two mutants of human CERT: CERT(D324A) and CERT ΔFFAT (Fig. 1). The CERT(D324A) mutant has an aspartic acid residue thought to be crucial to the function of the FFAT motif (9) replaced with an alanine residue, whereas the CERT ΔFFAT mutant is missing the entire motif. Neither mutant was co-immunoprecipitated with VAP-A, although levels of the CERT mutants were similar to the level of the wild-type CERT (Fig. 2A, lane 3 versus lanes 4 and 5). These results indicated that the interaction of CERT with VAP-A is dependent on the FFAT motif.

We previously showed that the mutation G67E in CERT destroys the PI4P binding activity of the PH domain and that the CERT(G67E) mutant cannot mediate ER-to-Golgi trafficking of ceramide because of impaired Golgi targeting (3). Interestingly, a larger amount of VAP-A was co-immunoprecipitated with CERT(G67E) than with the wild-type CERT control (Fig. 2A, lane 3 versus lanes 4 and 5), suggesting that CERT(G67E) interacted with VAP-A more effectively than the wild-type CERT.

Interaction of CERT with VAP-B—The human genome encodes two isoforms of VAP, VAP-A and VAP-B, at different gene loci (14), and VAP-A and VAP-B are suggested to form a homodimer and also a heterodimer (14, 15). We next examined whether CERT also interacts with VAP-B. In the extract of cells expressing both FLAG-tagged CERT and HA-tagged VAP-A, HA-VAP-A was clearly co-immunoprecipitated with FLAG-CERT by the anti-FLAG antibody, when compared with the control using the extract of cells expressing HA-VAP-A but not FLAG-CERT (Fig. 2B, lanes 2 and 3), being consistent with our previous results (Fig. 2A). HA-VAP-B was also significantly co-immunoprecipitated with FLAG-CERT, compared with the FLAG-CERT-absent control (Fig. 2B, lanes 4 and 5). In our co-immunoprecipitation experiments, both VAPs were detected by Western blotting for the HA epitope with the anti-HA antibody. Nevertheless, the band of VAP-B in the co-immunoprecipitated fraction was fainter than that of VAP-A, whereas the band of VAP-B
in the cell extract (input fraction) was intenser than that of VAP-A (Fig. 2B, lanes 3 and 5). These results suggested that CERT could interact with VAP-B, but less efficiently than with VAP-A.

It should also be noted that we could not observe significant co-immunoprecipitation of VAPs with CERT when the cell lysate was diluted more or when the widely used non-ionic detergent Triton X-100 was employed in place of digitonin in our co-immunoprecipitation experiments unless cells were pretreated with a membrane-permeable chemical cross-linker (data not shown). These observations might reflect a weak and transient interaction of CERT with VAPs in cells.

**Mutation in the FFAT Motif Affects the Synthesis of SM in Intact Cells**—The CHO mutant LY-A cell line is defective in the ER-to-Golgi trafficking of ceramide for the synthesis of SM, because of the mutation G67E in the endogenous CERT gene (3). To minimize possible artifacts caused by extreme overproduction after transient transfection, we obtained stable transformants of LY-A cells with an expression plasmid encoding wild-type human CERT, CERT(G67E), or CERT(D324A) and analyzed these transformants. To compare expression levels, Western blotting of CERT was performed using anti-COL4A3BP antibody (Genway Biotech), which was raised by immunization of a chicken with the recombinant human CERT protein. Although the anti-CERT antibody was virtually mono-specific to the endogenous human CERT and its splicing variant CERTL in HeLa-S3 cells, they cross-reacted with an unknown protein with a $M_r$ of ~65,000 in addition to the endogenous hamster CERT and CERTL in CHO cells (Fig. 3A). Despite the difficulty with mono-specific detection of CERT in CHO cells, Western blotting showed that the levels of exogenously expressed human CERT are similar among the LY-A transformants designated LY-A/CERT, LY-A/CERT(D324A), and LY-A/CERT(G67E), and about five times the level of the endogenous CERT in HeLa cells (Fig. 3A).

For analysis of the de novo synthesis of sphingolipids, we performed metabolic labeling of lipids with radioactive serine in CHO cells. The amount of radioactivity incorporated into SM during 2 h of labeling in LY-A cells was ~10% of the level in parental CHO-K1 cells. Although the rate at which SM was synthesized in LY-A/CERT cells was near the parental level, the rate in LY-A/CERT(D324A) cells was ~35% of the parental level, indicating that CERT(D324A) was less active than the wild-type CERT in the ER-to-Golgi trafficking of ceramide in CHO cells (Fig. 3B). The amount of SM produced in LY-A/CERT(G67E) cells was ~20% of the parental level, like in non-transfected LY-A cells.

De novo synthesis of sphingoid bases appears to be down-regulated when the consumption of ceramide is inhibited, although its mechanism is unknown. Thus, for a more specific

**FIGURE 2. Interaction between CERT and VAPs in co-immunoprecipitation experiments.** A, CHO-K1 cells were transiently transfected with HA-tagged VAP-A and FLAG-tagged wild-type (WT) or mutant CERT and solubilized with 1% digitonin. After incubation of the solubilized fraction with an anti-FLAG antibody-coupled gel, fractions unbound (Sup) and bound (Ppt) to the gel were separated by centrifugation and analyzed by Western blotting for the HA epitope (left panels) and FLAG epitope (right panels) as described under "Experimental Procedures." Input, 7% equivalent of the solubilized fraction applied to the anti-FLAG antibody-coupled gel was analyzed as an input control. For SDS-polyacrylamide gel electrophoresis, acrylamide concentrations were 10% (w/v) for VAP and 7.5% (w/v) for CERT. B, digitonin extracts from CHO-K1 cells transiently expressing HA-tagged VAP-A or VAP-B and FLAG-tagged CERT were subjected to immunoprecipitation assays as described above.
analysis of the conversion of ceramide to SM, we also performed metabolic labeling with radioactive sphingosine as a precursor, which bypasses the synthesis of sphingoid bases to label complex sphingolipids. When cells were incubated with \(^{[3}H\)sphingosine for 2 h, slightly less \(^{[3}H\)SM was produced in LY-A/CERT(D324A) than in LY-A/CERT cells, although more \(^{[3}H\)ceramide accumulated in the LY-A/CERT-(D324A) cells. Again, LY-A/CERT-(G67E) cells showed a more severe deficiency of \(^{[3}H\)SM formation, like nontransfected LY-A cells. The activity of SM synthase in LY-A cells is similar to or slightly higher than that in parental CHO-K1 cells (1). These results indicate that the mutation D324A in the FFAT motif of CERT impairs the ER-to-Golgi ceramide trafficking function of CERT. Although we failed to obtain stable LY-A transformants with the CERT/H9004 FFAT mutant, we could examine the activity of the mutant protein in semi-intact cells as shown below.

Of note, when cells were labeled with \(^{[14}C\)serine, the levels of \(^{[14}C\)ceramide and \(^{[14}C\)GlcCer produced in LY-A cells were \(\approx 100\) and \(\approx 60\)%, respectively, of those in LY-A/CERT cells (Fig. 3B). In contrast, when labeled with \(^{[3}H\)sphingosine, LY-A cells produced 2.5 times more \(^{[3}H\)ceramide, and 1.2–1.5 times more \(^{[3}H\)GlcCer and \(^{[3}H\)GM3 than LY-A/CERT cells (Fig. 3C), indicating that wild-type CERT is not essential for the synthesis of GlcCer. The mild reduction of \(^{[14}C\)serine-derived GlcCer in LY-A cells is probably due to down-regulation of \textit{de novo} synthesis of sphingoid bases. But there is another mutually nonexclusive possibility; \textit{de novo} synthesis of GlcCer would partially depend on CERT, so that the formation of \(^{[14}C\)serine-derived GlcCer might be mildly reduced in LY-A cells. In both cases, a ceramide flow without wild-type CERT likely has a capacity of attaining the enhanced formation of \(^{[3}H\)sphingosine-derived glycosphingolipids.

**Effects of the Mutations in FFAT Motif on ER-to-Golgi Trafficking of Ceramide within Semi-intact Cells**—The trafficking of ceramide from the ER to the site where SM is produced \textit{de novo} can be reconstituted within semi-intact CHO cells consisting of perforated cells, isolated cytosols, and other supplements such as ATP (2). In the reconstitution system, the
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**FIGURE 4. CERT-mediated trafficking of ceramide within semi-intact cells.** Perforated LY-A cells were incubated with [3H]sphingosine and palmitoyl-CoA at 15 °C for 30 min to produce [3H]ceramide. The prelabeled perforated cells (40 μg) were then chased in Buffer A with or without the cytosol (100 μg) of CHO-K1 cells or LY-A cells without or with 2 ng (A) or 100 ng (B) of wild type (WT) and D324A, ΔFFAT, and G67E mutants of the recombinant CERT at 37 °C for 30 min as described under “Experimental Procedures.” After the reaction was stopped, the radioactivity of [3H]SM produced during the chase was determined. Expression levels of the CERT in the assay mixture were analyzed by Western blotting using anti-CERT antibody. The level of CERT was reduced to 80% or more of the wild-type level (Fig. 4B). These results ruled out the possibility that the mutations in the FFAT motif or PH domain of CERT impaired the intermembrane transfer activity catalyzed by the START domain, thereby affecting the ER-to-Golgi trafficking of ceramide.

**FFAT Motif Mutants Have the Activity for the Intermembrane Transfer of Ceramide**—To test the possibility that mutations in FFAT motif affected the ceramide transfer activity of the CERT START domain, we examined the activity for the intermembrane transfer of ceramide between artificial phospholipid vesicles in a cell-free assay system. In the presence of wild-type CERT, the amount of ceramide transferred to acceptor membranes increased in a time-dependent manner at least up to 20 min, in agreement with our previous study (3). Similar kinetics was observed in the presence of the PH domain mutant CERT(G67E) and the FFAT motif mutant CERT(D324A) (Fig. 5A). When compared at a 10-min point of reaction time, the ceramide transfer activity of these CERT mutants and another FFAT motif mutant, CERT ΔFFAT, was ~80% or more of the wild-type level (Fig. 5B). These results ruled out the possibility that the mutations in the FFAT motif or PH domain of CERT impaired the intermembrane transfer activity catalyzed by the START domain, thereby affecting the ER-to-Golgi trafficking of ceramide.

**The FFAT Motif Mutant CERT(D324A) Retains the Golgi Targeting Activity**—To examine the subcellular distribution of CERT and VAP-A, we performed an immunofluorescence analysis using HeLa cells, in which various CERT constructs fused with GFP and VAP-A fused with the red fluorescent protein HcRed were transiently expressed. The wild-type CERT-GFP was distributed throughout the cytosol with preferential localization to the Golgi apparatus, whereas CERT(G67E) impaired this localization (Fig. 6A, panels a and i, and Fig. S2, panels a and g), being consistent with our previous results (3). CERT(D324A)-GFP also concentrated in the Golgi apparatus, similar to wild-type CERT-GFP (Fig. 6A, panel e, and Fig. S2, panel d). These results indicated that the FFAT motif mutants retain the Golgi targeting function.

When expressed in HeLa cells, HcRed-fused VAP-A displayed a cytoplasmic reticular distribution that well merged with ER-tracker™ (Fig. 6B), in agreement with previous studies showing that VAPs are ER-resident membrane proteins (10, 11). The cytoplasmic reticular distribution of HcRed-VAP-A was not appreciably affected by co-expression of CERT(G67E) or CERT(D324A)-GFP constructs (Fig. 6A, panels f and j). Nevertheless, when HcRed-VAP-A was co-expressed with wild-type CERT-GFP, a small amount of HcRed-VAP-A was redistributed to perinuclear regions, where CERT-GFP was colocalized (Fig. 6A, panels a–d).

**DISCUSSION**

Various proteins including OSBP, ORP, Nir1–3, Opi1p, and CERT ( alternatives, Goodpasure antigen-binding protein) trafficking of ceramide in LY-A cells recommenced when the LY-A cytosol was replaced with parental CHO-K1 cytosol or purified CERT was added to the LY-A cytosol (2, 3). We examined whether various recombinant CERT proteins purified from a bacterial expression system could restore the ER-to-Golgi trafficking of ceramide in semi-intact LY-A cells. When a small amount (2 ng/assay) of purified wild-type CERT was added to the reconstituted LY-A cells, the level of ceramide-to-SM conversion was almost identical to the level attained by using the CHO-K1 cytosol in place of the LY-A cytosol. In contrast, 2 ng of CERT(D324A) or CERT ΔFFAT restored the ceramide-to-SM conversion to only 25% or less of the wild-type level (Fig. 4A). CERT(G67E) showed even less activity. Western blotting using anti-CERT antibody showed that the level of exogenously added human CERT when a small amount was used in the reconstitution system is comparable with the level of endogenous CERT in the CHO cytosol and HeLa cell lysate (Fig. 4A, lower panel). Based on these findings together with the results of metabolic labeling in intact cells, we concluded that not only the PI4P-recognizing PH domain but also the VAP-binding FFAT motif is important for the ER-to-Golgi trafficking function of CERT in cells with a normal expression level of CERT.

When a large amount (100 ng/assay) of wild-type CERT was used, the rate at which ceramide was converted to SM was higher (~1.7 times) than that attained with the CHO-K1 cytosol (Fig. 4B). Under such conditions, CERT(D324A), CERT ΔFFAT, and CERT(G67E) could all restore the rate of conversion to the level attained with the CHO-K1 cytosol. These results showed that, when highly overproduced, CERT substantially supports the transport of ceramide from the ER to the site of the synthesis of SM even without the PI4P-recognizing PH domain or the VAP-binding FFAT motif.

5B). These results ruled out the possibility that the mutations in the FFAT motif or PH domain of CERT impaired the intermembrane transfer activity catalyzed by the START domain, thereby affecting the ER-to-Golgi trafficking of ceramide.

**DISCUSSION**

Various proteins including OSBP, ORP, Nir1–3, Opi1p, and CERT (alternatively, Goodpasure antigen-binding protein)
have FFAT motifs (9). For some of them, physical interactions with VAPs have been demonstrated (9, 15, 20, 21). We here showed for the first time that the FFAT motif of the ceramide transfer protein CERT is actually important for the ER-to-Golgi trafficking activity of CERT.

Supposed physiological roles of FFAT-containing proteins are diverse: OSBP controls two different types of protein phosphatases in a sterol-dependent manner (22). OSBP also affects the synthesis of SM in an oxysterol-dependent manner as discussed below. Nir2 having a phosphatidylinositol-transfer domain regulates vesicular trafficking from the trans-Golgi network compartment (23, 24). The yeast Op1p, a transcription factor sensing phosphatidic acid, regulates phospholipid metabolism (25). CERT mediates the ER-to-Golgi trafficking of ceramide (3). Despite such functional diversity, FFAT-containing proteins have a common feature capable of binding specific types of lipids (3, 8, 24, 26, 27). The association of FFAT motifs with the ER-resident membrane protein VAPs presumably facilitates lipid sensing at or lipid extraction from the ER membrane by the FFAT-containing proteins. VAPs also interact with various proteins that appear to have no FFAT motif: for example, SNAREs (13, 28), occludin (29), nonstructural proteins of hepatitis C virus (30–32), and Norwalk virus (33). VAPs may serve as wide-ranging adaptors for recruiting various cytosolic soluble proteins to the ER and also for the association of the ER membranes with other membranes.

Our analysis of the membrane topology of an N-terminally HA-tagged VAP-A by immunostaining under selective membrane permeabilization conditions showed a cytoplasmic orientation of the N terminus of the HA-tagged VAP-A in the ER (supplementary data part 4 and Fig. S3), in agreement with previous studies on VAP-B (12). Thus, the FFAT motif of CERT can interact with the N-terminal hydrophilic domain of VAP-A on the cytosolic surface of the ER.

VAP-A was more efficiently co-immunoprecipitated with the Golgi-targetless CERT(G67E) mutant than with the wild-type CERT (Fig. 2A). This might reflect that CERT(G67E) can interact with VAP-A distributed throughout the ER, whereas the Golgi-targeted population of wild-type CERT can probably interact only with VAP-A molecules residing at the ER very close to the Golgi membrane. Actually, we observed that CERT(G67E)-GFP is uniformly distributed throughout the cytosol (Fig. 6A, panel i), whereas a substantial proportion of the wild-type control is localized to the perinuclear Golgi region (Fig. 6A, panel a), consistent with our previous study (3). In addition, HcRed-VAP-A is almost uniformly distributed throughout the ER membranes (Fig. 6B), also consistent with previous studies (10, 15). Interestingly, when HcRed-VAP-A was co-expressed with wild-type CERT-GFP, a small amount of HcRed-VAP-A tended to co-localize with CERT-GFP in perinuclear regions (Fig. 6A, panel d). Neither CERT(G67E)-GFP nor CERT(D324A)-GFP induced such a perinuclear redistribution of HcRed-VAP-A (Fig. 6A, panels h and l), although the G67E mutant retains the activity to interact with VAP (Fig. 2A), and the D324A mutant retains the activity to interact with the Golgi region (Fig. 6A, panel e, and Fig. S2, panels d–f). These results suggest that Golgi-associated CERT has the potential to interact with VAP-A residing in the ER in an FFAT motif-dependent manner.

We previously demonstrated that the PI4P-recognizing PH domain of CERT is important for Golgi targeting in cells (3) and that the START domain of CERT extracts ceramide with a one-to-one ratio from donor phospholipid membranes and transfers it to acceptor membranes (8). Based on our previous results together with those of the present study, we propose the following model for CERT-mediated ER-to-Golgi trafficking of ceramide (Fig. 7). CERT efficiently recruits to the ER via an FFAT motifs-dependent interaction with VAPs, and then extracts ceramide from the ER membrane. The CERT-ceramide complex targets the Golgi apparatus, depending on the PI4P-recognizing PH domain of CERT, and releases ceramide there. As discussed above, Golgi-localized CERT is likely capable of interacting with VAPs. Narrow cytoplasmic gaps called membrane contact sites, at which two organelles come into close apposition within ~10 nm, is speculated to contribute to interorganelle metabolic and functional interactions (34, 35). At the ER-Golgi membrane contact site, CERT might rapidly shuttle the short distance between the ER and Golgi membranes for...
CERT FFAT Motif for Ceramide Trafficking

Efficient trafficking of ceramide (Fig. 7, inset). If CERT simultaneously binds the Golgi membranes (via its PH domain) and the ER membranes (via its FFAT motif), neck-swinging conformational changes of the START domain might catalyze the ER-to-Golgi transfer of ceramide very rapidly (Fig. 7, inset).

Under nonoverproducing conditions, neither the PH domain mutant nor the FFAT motif mutant of CERT could support efficient trafficking of ceramide from the ER to the site where SM is produced in intact and semi-intact cells (Figs. 3 and 4A). In contrast, both mutants substantially supported the synthesis of SM in semi-intact cells under overproducing conditions (Fig. 4B). These results indicated that these CERT mutants do not act as dominant-negative mutants. Rather, under overproducing conditions, the intermembrane transfer of ceramide catalyzed by the CERT START domain appears to support the synthesis of SM in a random transfer fashion without specific targeting to the ER or the Golgi apparatus.

Exposure of CHO cells to 25-hydroxycholesterol (25HC) stimulates the synthesis of SM (36), and the stimulation is further enhanced by overproduction of OSBP (37). During preparation of the present manuscript, a fast on-line version of a paper by Perry and Ridgway appeared (38), in which they showed that OSBP regulation of SM synthesis involves CERT. RNA interference experiments revealed that OSBP and VAP are required for stimulation of the CERT-dependent transport of ceramide and synthesis of SM by 25HC (38). They also demonstrated the co-immunoprecipitation of VAP and CERT, although they did not distinguish between VAP-A and VAP-B in the co-immunoprecipitation experiments (38). In our study, the cells were not treated with 25HC. Thus, it is likely that CERT/VAP interaction is important for basal SM synthesis as well as the OSBP/25HC-enhanced synthesis of SM.

GlcCer synthase is broadly distributed in the Golgi apparatus and possibly also in other organelles (39, 40). How ceramide

![Figure 6. Intracellular distribution of CERT and VAP-A. A, HeLa-S3 cells co-transfected with HcRed-VAP-A and the indicated CERT-GFP constructs were fixed and observed by confocal microscopy. Arrowhead in panel b, HcRed-VAP-A co-localized with CERT-GFP in the perinuclear region. A high magnification of the selected regions (boxes in panels c, g, and k) is shown in the right panels (panels d, h, and l). B, HeLa-S3 cells transfected with a HcRed-VAP-A expression plasmid were incubated with ER-Tracker™ in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and washed. Then the specimens were fixed and observed by confocal microscopy. Bars, 10 μm.](image-url)
synthesized at the ER reaches GlcCer synthase is unclear. The CERT-defective LY-A cells produce higher levels of labeled ceramide and GlcCer with a reduced level of SM in metabolic labeling with \[^{3}H\]sphingosine, compared with the corrected revertant LY-A/CERT cells (Fig. 3C) (3). Thus, we conclude that de novo synthesized ceramide can flow to the main site for the synthesis of GlcCer without wild-type CERT. Ceramide might reach the site of de novo GlcCer synthesis, independently of CERT. Alternatively, CERT(G67E), which LY-A cells endogeneously produce, might play a sufficient role in intermembrane trafficking of ceramide for the synthesis of GlcCer. It remains undetermined whether CERT interacts with SM synthase 1, which resides in the trans-Golgi region (41). It is also an interesting question whether CERT interacts directly with either dihydroceramide synthase or dihydroceramide desaturase (or with both) in the ER, because CERT acquires ceramide after the lipid is synthesized in the ER and is capable of transferring both dihydroceramide and ceramide (3, 8). To date, various isoforms of dihydroceramide synthases (42) and dihydroceramide desaturases (43) have been identified. Further studies are needed to address these unresolved questions.

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