Interorganelle Trafficking of Ceramide Is Regulated by Phosphorylation-dependent Cooperativity between the PH and START Domains of CERT

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The synthesis and transport of lipids are essential events for membrane biogenesis. However, little is known about how intracellular trafficking of lipids is regulated. Ceramide is synthesized at the endoplasmic reticulum (ER) and transported by the ceramide transfer protein CERT to the Golgi apparatus, where it is converted to sphingomyelin. CERT has a phosphoinositide-binding pleckstrin homology (PH) domain and a lipid transfer START domain for intermembrane transfer of ceramide, and contributes to interorganelle metabolic and functional interactions. CERT movement for trafficking of ceramide proceeds at membrane contact sites (MCSs), at which ceramide from membranes and transferring it to acceptor membranes is predicted to form no globular domains but may have various crucial functions. Indeed, the MR has a short peptide motif named a FFAT motif, which interacts with VAMP-associated protein (VAP), an ER-resident type II membrane protein (7). In addition to the START domain, both the Golgi-targeting PH domain and ER-interacting FFAT motif of CERT are required for efficient trafficking of ceramide from the ER to the Golgi apparatus (5, 8).

CERT consists of three distinct regions. The amino-terminal ~120 residues of CERT form the phosphoinositide-binding pleckstrin homology (PH) domain. The PH domain of CERT serves to target the Golgi apparatus by recognizing phosphatidylinositol 4-monophosphate (PI4P) (5). The carboxyl-terminal ~230 residues form the lipid transfer START domain. The START domain of CERT is capable of specifically extracting ceramide from membranes and transferring it to acceptor membranes (5, 6). The middle region (MR), the ~250 amino acid residues between the PH and START domains, is predicted to form no globular domains but may have various crucial functions. Indeed, the MR has a short peptide motif named a FFAT motif, which interacts with VAMP-associated protein (VAP), an ER-resident type II membrane protein (7). In addition to the START domain, both the Golgi-targeting PH domain and ER-interacting FFAT motif of CERT are required for efficient trafficking of ceramide from the ER to the Golgi apparatus (5, 8).

The regulation of lipid metabolism is necessary for homeostasis in cells. Mechanisms underlying regulation of the synthesis of lipids have been elucidated mainly in terms of the transcriptional and post-transcriptional controls of lipid syntheses and their regulatory proteins, as best exemplified in the regulation of sterol synthesis (12). In addition, accumulating evidence that cells express various types of lipid transfer proteins raises the possibility that intracellular trafficking of lipids is another
key process in the regulation of the synthesis of lipids. However, little is known about whether and how lipid trafficking events are regulated.

In the present study, we show that the ER-to-Golgi trafficking of ceramide is down-regulated by hyperphosphorylation of a previously uncharacterized motif present in CERT and that this down-regulation requires an autoinhibitory interaction between the PH and START domains of CERT. In addition, perturbation of SM/cholesterol rafts at the plasma membrane induces dephosphorylation of CERT. These results revealed a novel mechanism for the regulation of the synthesis of lipids, in which a specific lipid trafficking process is controlled.

**EXPERIMENTAL PROCEDURES**

**Materials**—Anti-HA antibody was purchased from Roche Diagnostics, anti-GS28 was from StressGen. d-erythro-[3-3H]Sphingosine (20 Ci/mmol) and [palmitoyl-1-14C]N-palmitoyl-d-erythro-sphingosine (55 mCi/mmol) were purchased from American Radiolabeled Chemicals. λ-Phage protein phosphatase (APPase) was from New England Biolabs, and RCA120 was from Oil Mills (Tokyo, Japan). Lactosylceramide was from Matreya, and PI4P was from Wako Pure Chemical Industries (Osaka, Japan). ISP-1 was a gift from Dr. Tetsuro Fujita (Setsunan University, Hirakata, Japan). Anti-HA antibody-conjugated agarose, fumonisin B1, and methyl-β-cyclohexadextrin (MCD) were purchased from Sigma. Sphingomyelinase of Bacillus cereus was from Higeta Shoyu (Tokyo, Japan). A recombinant form of His-tagged CERT WT was expressed and purified from BL21(DE3) Escherichia coli as described previously (5). Rabbit polyclonal antibodies were generated against the recombinant protein of CERT (Scrum, Tokyo). Anti-CERT C-terminal polyclonal antibodies were raised by immunization of rabbits with a synthetic peptide (CTSYQVQETAKGPILF). These antibodies were affinity-purified using the corresponding antigens immobilized on SulfoLink Coupling Gel (Pierce).

**Cell Culture and Plasmid Construction**—HeLa-S3 cells were maintained in Dulbecco’s modified Eagle’s medium, supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. HA-tagged CERT immunopurified from the cells as described above was subjected to SDS-PAGE, and the gel was stained with Coomassie Brilliant Blue. The doublet bands of HA-tagged CERT, which were estimated to be totally ~20 pmol equivalent by densitometric analysis with known amounts of bovine serum albumin for calibration, were excised together and trypsindized in gel as described previously (15). The trypsindized CERT were extracted from the gel and concentrated to 5–10 μl by evaporation. The peptides were dissolved in 50 μl of 5% acetic acid, and the solution was applied to a Ga3+-chelated resin (phosphopeptide isolation kit, Pierce). The resin was washed: twice with 50 μl of 0.1% acetic acid, and once with 20 pmol equivalent by densitometric analysis.
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**A**

Expression

| Type I | Type II | Type III |
|-------|---------|----------|
| +     | +       | -        |
| -     | +       | +        |

**B**

![Western blot analysis of CERT protein expression](image)

**C**

![MALDI-TOF mass spectrum](image)

**D**

| Peak | Observed peptide mass | Calculated peptide mass | Residue number | Inferred peptide sequence |
|------|-----------------------|-------------------------|----------------|--------------------------|
| p1+10Pi | 3744.4 | 3744.1 | 147-175 | HGS(Mox)VLVSGASGYSATTSFFKKEKSHL+10Pi |
| p1+9Pi | 3653.9 | 3654.1 | 147-175 | HGS(Mox)VLVSGASGYSATTSFFKKEKSHL+9Pi |
| p1+8Pi | 3584.3 | 3584.1 | 147-175 | HGS(Mox)VLVSGASGYSATTSFFKKEKSHL+8Pi |
| p2+6Pi | 3270.4 | 3269.7 | 146-170 | HGS(Mox)VLVSGASGYSATTSFSSFKK+6Pi |
| p2+7Pi | 3190.0 | 3188.7 | 146-170 | HGS(Mox)VLVSGASGYSATTSFSSFKK+7Pi |

**FIGURE 1. CERT expressed in HeLa Cells is phosphorylated at multiple Ser/Thr residues.** A, effect of λPPase on M of CERT. HA-tagged CERT WT stably expressed in HeLa cells and His$_{16}$-tagged CERT expressed in E.coli cells were purified. These purified CERT proteins were incubated with or without λPPase at 30 °C for 15 min and analyzed by Western blotting with anti-CERT polyclonal antibodies and anti-HA antibody. B, HA-tagged CERT WT purified from HeLa cells was treated with or without λPPase and analyzed by Western blotting with anti-phosphoamino acid antibodies and anti-HA antibody. C, mass spectrometric analysis of phosphopeptides of CERT WT. The purified HA-tagged CERT WT was trypsinized in gel, and the phosphopeptides isolated from the trypsinized CERT were analyzed by MALDI-TOF MS in a linear mode. p1 and p2 correspond to the peptides indicated in D, and P$_i$ corresponds to phosphate. D, list of the phosphopeptides indicated in Fig. 1C. The calculated peptide mass values represent average molecular mass. M(ox), oxidized methionine.

The assay. Liposomes consisting of 1.6 μmol of egg phosphatidylcholine, 0.4 μmol of egg phosphatidylethanolamine, 0.2 μmol of lactosylceramide, and various amounts of PI4P per ml of buffer C (20 mM HEPES-NaOH (pH 7.4), 50 mM NaCl, 1 mM Na$_2$EDTA) were prepared by sonication. The liposomes (10 μl) were mixed with 200 fmol of purified CERT in the buffer (40 μl) containing 0.3 mg/ml bovine serum albumin, 25 mM Tris-HCl (pH 7.4), 0.05 mM Na$_2$EDTA, 2.5 mM dithiothreitol, and 0.005% Brij 35, and the mixtures were incubated at 25 °C for 1 h. After 15 μl of 2.5 mg/ml RCA120 was added, the mixture was incubated on ice for 10 min to aggregate liposomes. Then, liposomes were precipitated by centrifugation at 20,000 × g for 3 min at 4 °C. The amounts of CERT in the supernatant and the pellet fractions were determined by Western blotting with the anti-HA antibody, and the ratio of the precipitated to the total amount (pellet/pellet + supernatant) was calculated.

**Other Methods**—The reconstitution of ceramide transport from the ER to the Golgi in semi-intact cells was performed as described previously (4). The transfer of ceramide between artificial phospholipid vesicles was done as described (5). Co-immunoprecipitation of VAP with CERT is described in supplementary data part 2. The treatment of TEV protease (Invitrogen) was carried out according to the manufacturer's instructions unless specific conditions were indicated.

**RESULTS**

**CERT Is Phosphorylated at Serine and Threonine Residues**—We expressed HA epitope-tagged human wild-type CERT (HA-CERT WT) in HeLa cells and purified it using affinity chromatography. Purified CERT appeared as doublet bands upon SDS-PAGE: for simplicity, the form with a higher molecular weight ($M_r$) (~77,000) is hereafter referred to as type I, and the form with a lower $M_r$ (~73,000) is referred to as type II (Fig. 1A). When treated with the protein phosphatase λPPase, the doublet bands of HA-CERT WT were converted to a single band (referred to as type III), the $M_r$ of which was slightly lower than that of the type II form. Similar patterns were observed when HA-CERT WT was expressed in human embryonic kidney 293 cells and Chinese hamster ovary cells (data not shown), whereas the $M_r$ of a recombinant protein of CERT WT expressed in bacteria was not affected by λPPase treatment (Fig. 1A). These results are consistent with previous studies showing that CERT/GPBP26 and its splicing variant CERT$_2$/GPBP are phosphorylated in mammalian cells (16, 17). The type I form clearly reacted with both anti-phosphoserine (Ser(P)) and anti-phosphothreonine (Thr(P)) polyclonal antibodies, while the type II form showed less reactivity with the anti-Ser(P) antibody and no appreciable reactivity with the anti-Thr(P) antibody (Fig. 1B). λPPase treatment of CERT almost completely abolished the reactivity with these antibodies. None of the type I-III forms appreciably reacted with an anti-phosphotyrosine (Tyr(P)) antibody (Fig. 1B).

**Identification of a Motif for Hyperphosphorylation of CERT**—To determine the phosphorylation sites of CERT, we stably expressed HA-CERT WT in HeLa cells and purified the expressed protein. The protein was separated by SDS-PAGE, and the bands were excised and digested by trypsin. Then, phosphopeptides purified from the trypsin-digested CERT were analyzed by MALDI-TOF MS. Several pairs of signals with a 16-mass split, which is due to methionine oxidation, were...
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The Effects of Mutations at the SR Motif on the Phosphorylation and the Activity of CERT. A. The SR motif and its mutants. In the sequence of CERT S132A and CERT 10E, the mutated residues are marked by bold letters. PH, PH domain; SR, serine-repeat motif; F, FFAT motif; ST, START domain. B. Analysis of phosphorylation of CERT WT, CERT S132A, and CERT 10E. CERT WT and CERT mutants were transiently expressed in HeLa S3 cells, and the cells were lysed. The lysate was incubated with or without APPase and analyzed by Western blotting. C. Ceramide trafficking activity of purified CERT in semi-intact cells. CERT WT, CERT S132A, and CERT 10E transiently expressed in HeLa cells were purified. When indicated, CERT proteins were treated with APPase in the purification process. Then, purified CERT proteins were analyzed for the activity to mediate ER-to-Golgi trafficking of ceramide in semi-intact LY-A cells. The data shown are the means ± S.D. from three experiments.

The Function of CERT to Mediate Intracellular Trafficking of Ceramide Is Down-regulated by Phosphorylation of the SR Motif—We next examined the effects of the phosphorylation of the SR motif on CERT-mediated intracellular trafficking of ceramide. HA-tagged CERT proteins were transiently expressed in HeLa cells and purified to near homogeneity (supplemental data part 4 and Fig. S2), and the activity of the purified proteins to traffic ceramide from the ER to the Golgi was analyzed by using a reconstitution system within semi-intact cells. To assess the activity of exogenously added CERT, semi-intact cells were prepared from LY-A cells, in which endogenous CERT is impaired (5). The purified CERT WT protein, which was composed of the type I and II forms of HA-CERT WT in approximately equivalent amounts (supplemental data part 4 and Fig. S2), was active as expected (Fig. 2C). Interestingly, the activity of CERT S132A was ~3-fold that of CERT WT, while the activity of CERT 10E was only 25% of the CERT WT level (Fig. 2B). On the basis of these results, we expected CERT S132A to act as a mimic of the type II form of CERT WT. In addition, for a mimic of the type I form of CERT WT, we made the mutant CERT 10E, in which all ten Ser/Thr residues in the SR motif were replaced with glutamic acid residues, because glutamic acid often mimics Ser(P)/Thr(P) (19). Of note, the M, of CERT 10E was also slightly reduced by APPase treatment, like that of CERT S132A and the type II form of CERT WT (Fig. 2B), which indicated that one or a few phosphorylation sites other than the SR motif exist in CERT, although we have not identified the other site(s).

With further analysis using MS, we confirmed that the type I form of CERT WT is highly phosphorylated at the SR motif and that the type II form of CERT WT and CERT S132A are far less phosphorylated or not phosphorylated at the motif (supplemental data part 3 and Fig. S1).

The phosphorylation of the SR motif reduces the affinity of CERT for PI4P—How does the phosphorylation of the SR motif down-regulate the ceramide trafficking activity of CERT? To answer this question, we examined phosphorylation of CERT in HeLa cell lysate with APPase. The activity of the type III form of CERT WT was slightly less phosphorylated or not phosphorylated at the motif (supplemental data part 3 and Fig. S1). The APPase-induced shift in the $M_\text{r}$ of CERT S132A was slight, similar to that of the type II form of CERT WT (Fig. 2B). On the basis of these results, we expected CERT S132A to act as a mimic of the type II form of CERT WT. In addition, for a mimic of the type I form of CERT WT, we made the mutant CERT 10E, in which all ten Ser/Thr residues in the SR motif were replaced with glutamic acid residues, because glutamic acid often mimics Ser(P)/Thr(P) (19). Of note, the $M_\text{r}$ of CERT 10E was also slightly reduced by APPase treatment, like that of CERT S132A and the type II form of CERT WT (Fig. 2B), which indicated that one or a few phosphorylation sites other than the SR motif exist in CERT, although we have not identified the other site(s). With further analysis using MS, we confirmed that the type I form of CERT WT is highly phosphorylated at the SR motif and that the type II form of CERT WT and CERT S132A are far less phosphorylated or not phosphorylated at the motif (supplemental data part 3 and Fig. S1).
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In this question, we analyzed subfunctions of CERT in vitro. The PH domain of CERT has PI4P binding activity, which is important for Golgi-targeting of CERT (5, 8). We determined the PI4P binding activity of CERT by a pull-down assay with artificial phospholipid vesicles ("liposomes"). The purified CERT WT protein from HeLa cells was co-precipitated with liposomes in a PI4P-dependent manner (Fig. 3A). Interestingly, the type II form, but not type I form, of CERT WT co-precipitated preferentially with PI4P-containing liposomes (Fig. 3A). CERT S132A, a mimic of the type II form of CERT WT, efficiently interacted with PI4P; nearly 100% of CERT S132A was co-precipitated with vesicles containing 0.5 mol % PI4P (Fig. 3B). By contrast, CERT 10E, a mimic of the type I form of CERT WT, did not exhibit any PI4P-dependent co-precipitation (Fig. 3B). APPase pretreatment resulted in a 4-fold increase in the PI4P binding activity of CERT WT (Fig. 3A, 3B, and 3C). The mutant CERT G67E has no PI4P-binding activity due to the mutation of a conserved glycine residue in the PH domain (5). As expected, CERT G67E exhibited no PI4P-dependent co-precipitation with liposomes regardless of APPase pretreatment, and a CERT mutant with G67E/S132A double mutations also showed no affinity for PI4P (Fig. 3B). The requirement of a functional PH domain for the enhancement of the PI4P binding activity of CERT S132A ruled out the possibility that the S132A mutation generated a new PI4P-binding site other than the PH domain and then enhanced the PI4P binding activity. PI4P could not be replaced by phosphatidylinositol 3-monophosphate (PI3P) for the co-precipitation of CERT, confirming the specificity of this assay (Fig. 3B). Collectively, these results demonstrated that the phosphorylation of the SR motif represses the PI4P-binding subfunction of the PH domain of CERT.

Phosphorylation of the SR Motif Reduces Ceramide Transfer Activity of CERT—We next examined the effects of the phosphorylation of the SR motif on the activity for the intermembrane transfer of ceramide, which is catalyzed by the START domain of CERT. In a cell-free assay system to determine the activity to transfer ceramide between liposomes, CERT S132A exhibited 2-fold more activity than CERT WT, and CERT 10E exhibited less than 30% that of CERT WT (Fig. 3C). Although APPase pretreatment did not significantly affect the ceramide...
transfer activity of CERT S132A or CERT 10E, it enhanced the activity of CERT WT to the level of CERT S132A. The strong activity of CERT S132A for ceramide transfer was not due to its enhanced activity for PI4P binding, because (i) the in vitro ceramide transfer assay system was free of PI4P, (ii) the ceramide transfer activity of CERT G67E was almost identical to that of CERT WT, and (iii) dephosphorylation of CERT G67E, like CERT WT, enhanced the ceramide transfer activity to the level of CERT S132A. These results indicated that phosphorylation of the SR motif represses the ceramide transfer subfunction of the START domain.

Effect of the Phosphorylation of the SR Motif on the CERT-VAP Interaction via a FFAT Motif—Another known subfunction of CERT is to interact with the ER resident protein VAP via the FFAT motif. To examine the interaction of CERT with VAP, we transiently expressed them in HeLa cells, treated the cells with a membrane-permeable chemical cross-linker, and then subjected the lysate of the cells to co-immunoprecipitation. CERT WT, but not the FFAT motif mutant CERT D324A, was co-immunoprecipitated with VAP (Fig. 3D), consistent with our previous study (8). Both CERT S132A and CERT 10E were co-immunoprecipitated with VAP, like CERT WT. These results indicated that the phosphorylation of the SR motif does not greatly affect the FFAT motif-dependent interaction of CERT with VAP.

Cooperative Regulation of PH and START Domains of CERT by Phosphorylation of the SR Motif—As described above, the phosphorylation of the SR motif of CERT repressed subfunctions of both the PH and START domains, although the two domains are separated by ~250 amino acids. The findings led us to question whether the down-regulation of one domain would require the existence of another domain in the same molecule. To answer this question, we constructed two types of CERT 10E derivatives: one having a TEV protease-cleavable site after the PH domain (named CERT 10E TEV-A) and the other before the START domain (named CERT 10E TEV-B) (Fig. 4A). When these proteins purified from HeLa cells were digested with TEV protease, they split into two segments as predicted: CERT 10E TEV-A was completely split into the PH domain segment and the MR (having the 10E mutations)-linked START domain segment, while CERT 10E TEV-B was split into the PH domain-linked MR (having the 10E mutations) segment and the START domain segment (Fig. 4B). Before TEV treatment, the PI4P binding activity of both new constructs was very weak, like that of the parental CERT 10E (Fig. 4C). However, TEV treatment dramatically enhanced the PI4P-binding activity of both CERT 10E TEV-A and TEV-B, while it did not affect the activity of the parental CERT 10E. Notably, when CERT 10E TEV-B was treated with TEV protease, the PH-MR segment having the 10E mutation restored the PI4P binding activity even in the presence of an equimolar amount of the split START domain (Fig. 4C). These results demonstrated that the down-regulation of the PI4P binding activity of the PH domain in the CERT 10E construct is dependent on the existence of the START domain in the same molecule. Furthermore, TEV treatment of CERT 10E TEV-A (which produced MR-START and PH fragments) enhanced the ceramide transfer activity to the levels of CERT S132A and the dephosphorylated CERT WT (Figs. 3C and 4D). Collectively, these results indicated that the repression of the PH domain by the phosphorylation of the SR motif requires the START domain, and the repression of the START domain in turn requires the PH domain. Hence, the
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phosphorylation of the SR motif cooperatively regulates both the PH and START domains.

As shown above, PI4P binding and ceramide transfer activities of the TEV-cleavable CERT 10E constructs were restored by TEV treatment (Fig. 4, C and D). Furthermore, FFAT motif-dependent activity of CERT 10E was similar to that of CERT WT (Fig. 3D). These results suggest that the PH domain, the START domain, and the FFAT motif of CERT 10E are properly folded.

Our previous analysis of non-phosphorylated CERT and its domain deletion mutants expressed in E. coli has shown that the ceramide transfer activity of PH domain-deleted CERT was similar to that of wild-type CERT and that there is no difference in the affinity for PI4P between START domain-deleted CERT and wild-type CERT (5). Thus, unlike phosphorylated CERT (as mimicked by the 10E mutation), non-phosphorylated CERT exhibits no dramatic effects of START domain deletion and PH domain deletion on PI4P binding activity and ceramide-transfer activity, respectively. It should be also pointed out that the ceramide transfer activity of the START domain separated from CERT 10E TEV-B is 3–4-fold than that of CERT S132A and the dephosphorylated CERT (Figs. 3C and 4D). When we analyzed CERT derivatives expressed in E. coli, the ceramide transfer activity of the START domain alone was also greater than that of full-length CERT and of PH domain-deleted CERT (5). Thus, the ceramide transfer function of the START domain appears to be repressed also by the MR domain, independently of the PH domain or phosphorylation of the SR motif.

CERT Is Dephosphorylated by Perturbing SM/Cholesterol Rafts—To seek a trigger to affect the phosphorylation of CERT, we examined the impact of a reduction in the amount of SM in cells. When HeLa cells were incubated with a B. cereus-derived recombinant sphingomyelinase (bSMase) for up to 2 h to hydrolyze SM of the plasma membrane, about 50% of total CERT had shifted from the type I form to the type II form within 1 h of the initiation of bSMase treatment (Fig. 5A). SM preferentially interacts with cholesterol, and these two lipids have been suggested to form relatively detergent-resistant membrane microdomains, so-called SM/cholesterol rafts, in the plasma membrane (20–22). Hence, we next tested whether the depletion of cholesterol from cells also affected the phosphorylation of CERT. When cells were incubated with the cholesterol adsorbent MCD, a similar shift of CERT WT occurred (Fig. 5A). No loss in the viability of cells was observed after treatments with bSMase and MCD under the conditions we used (data not shown). The fact that neither bSMase nor MCD affected the $M_c$ of CERT S132A and CERT 10E suggested that the shift in $M_c$ was due to dephosphorylation of the SR motif (Fig. 5A).

HA-tagged CERT WT and CERT S132A were stably expressed in HeLa cells, and their intracellular distribution was analyzed by indirect immunostaining with an anti-HA antibody and an antibody against GS28, a Golgi marker protein. CERT WT was distributed throughout the cytoplasm/nucleus with partial concentration at the Golgi region under normal culture conditions, and the treatment of cells with bSMase greatly redistributed CERT WT to the Golgi region (Fig. 5B). In contrast, CERT S132A localized to the Golgi region under normal culture conditions, and the bSMase treatment did not affect the localization of CERT S132A (Fig. 5B). These results are consistent with enhancement of the PI4P binding activity of CERT by dephosphorylation at the SR motif (Fig. 3B).
To see whether the loss of de novo synthesis of SM affected the phosphorylation of CERT in cells, we used ISP-1/myriocin, a specific inhibitor of serine palmitoyltransferase that catalyzes the first step in the biosynthesis of sphingolipids (23, 24). In HeLa cells stably expressing HA-CERT WT, ~80 and ~20% of the CERT exists as the type I and type II form, respectively, under ISP-1-ununtreated control conditions (Fig. 5C). When the cells were incubated with ISP-1 for up to 24 h, the type I form of CERT gradually shifted to the type II form (Fig. 5C). The shift in $M_2$ induced by ISP-1 treatment did not occur for CERT S132A or CERT 10E. The ISP-1–induced shift indeed resulted from the inhibition of de novo synthesis of sphingolipids, because incubation of cells with exogenous sphingosine after 24-h treatment with ISP-1 gradually increased the amount of the type I form of CERT WT in parallel with the disappearance of the type II form (Fig. 5D). Relatively long term (~24 h) incubation with ISP-1 was required for accumulation of the type II form, suggesting that the balance of type I/type II forms of CERT responded to the amount of SM, rather than the rate of the synthesis of SM, in cells.

**DISCUSSION**

In the present study, we found that the SR motif in CERT is phosphorylated at multiple Ser/Thr residues and that the hyperphosphorylation results in the repression of both the PI4P binding activity of the PH domain and the ceramide transfer activity of the START domain. When the SR motif is hyperphosphorylated, the conversion of ceramide to SM is indeed reduced in semi-intact cells. We also found that a loss of SM and cholesterol from the plasma membrane induces the dephosphorylation of the SR motif to activate CERT. On the basis of these results, we concluded that SM/cholesterol rafts affect the phosphorylation of CERT, thereby controlling functions of both the PH and START domains cooperatively and regulating the intracellular trafficking of ceramide to control de novo synthesis of SM. Further studies are needed to elucidate how SM/cholesterol rafts affect the phosphorylation of CERT.

The repression of the PH domain by the phosphorylation at the SR motif requires the START domain in CERT, and likewise, the repression of the START domain requires the PH domain. These results suggest that the hyperphosphorylation of the SR motif induces autoinhibitory interaction between the PH and START domains and that the phosphorylation/dephosphorylation of the SR motif switches the conformation of CERT between a closed resting state and an open active state (Fig. 6).

When CERT WT was dephosphorylated by perturbation of SM/cholesterol rafts, it was redistributed from the cytosol/nucleus to the Golgi region. Golgi-targeted CERT retains the activity to interact with the ER-resident protein VAP (this study and Ref. 6). These results suggest that the open active form of CERT, but not the closed resting form, predominantly accesses the ER-Golgi MCSs and traffics ceramide from the ER to the Golgi with high efficiency (Fig. 6).

The simultaneous inhibition of both the PH and START domains for resting can be an effective way to prevent possible harmful situations where only one domain is active. Ceramide produced at specific organelles by the action of SMase modulates various signaling events (25). Such events may be perturbed if de novo synthesized ceramide is randomly transferred by an active START domain deficient of Golgi-targeting ability. The accumulation of ceramide in mitochondria has been suggested to cause pores to form in the outer membrane and the subsequent induction of apoptosis (26). Moreover, an active PH domain without an active START domain might abortively compete with other proteins utilizing PI4P in various cellular events.

The transient generation and elimination of specific phosphoinositides at the targeting compartments control the recruitment of various proteins with PH domains (27). The recruitment also depends on other factors such as GTP-binding proteins in various cases (28, 29). In addition, there is the possibility that membrane recruitment of PH domains can be controlled by altering the affinity of the domains for phosphoinositides (30), although this possible mechanism has not been well investigated. Phosphorylation-dependent masking of a PH domain with another intramolecular domain shown in the current study represents a good example of the third mechanism.

We previously demonstrated that CERT–mediated transport of ceramide from the ER to the site of SM synthesis requires ATP (3–5). Our current study indicated that the ATP requirement for ceramide trafficking cannot be attributed to the phosphorylation of the SR motif of CERT, because CERT S132A acts as a constitutive active form and CERT 10E as a constitutive inactive form. A recent study has shown that the formation of PI4P at the Golgi complex by phosphatidylinositol 4-kinase IIIβ is required for Golgi targeting of CERT (31). Thus, the ATP-dependent step for ceramide trafficking might be attributable to the synthesis of PI4P.
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