Protein Phosphatase 2Cε Is an Endoplasmic Reticulum Integral Membrane Protein That Dephosphorylates the Ceramide Transport Protein CERT to Enhance Its Association with Organelle Membranes*

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Satoko Saito,1 Hiroyuki Matsui,1 Miyuki Kawano, Keigo Kumagai,2 Nario Tomishige,1 Kentaro Hanada,1 Seishi Echigo,1 Shiri Tamura1,1, and Takayasu Kobayashi1,2

From the 1Department of Biochemistry, Institute of Development, Aging and Cancer, Tohoku University, 4-1 Seiryo-machi, Aoba-ku, Sendai 980-8575, Japan, 2Division of Oral Surgery, Graduate School of Dentistry, Tohoku University, 4-1 Seiryo-machi, Aoba-ku, Sendai 980-8575, Japan, and 3Department of Biochemistry and Cell Biology, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan

Protein phosphatase 2Cε (PP2Cε), a mammalian PP2C family member, is expressed in various tissues and is implicated in the negative regulation of stress-activated protein kinase pathways. We show that PP2Cε is an endoplasmic reticulum (ER) transmembrane protein with a transmembrane domain at the amino terminus and the catalytic domain facing the cytoplasm. Yeast two-hybrid screening of a human brain library using PP2Cε as bait resulted in the isolation of a cDNA that encoded vesicle-associated membrane protein-associated protein A (VAPA). VAPA is an ER resident integral membrane protein involved in recruiting lipid-binding proteins such as the ceramide transport protein CERT to the ER membrane. Expression of PP2Cε resulted in dephosphorylation of CERT in a VAPA expression-dependent manner, which was accompanied by redistribution of CERT from the cytoplasm to the Golgi apparatus. The expression of PP2Cε also enhanced the association between CERT and VAPA. In addition, knockdown of PP2Cε expression by short interference RNA attenuated the interaction between CERT and VAPA and the sphingomyelin synthesis. These results suggest that CERT is a physiological substrate of PP2Cε and that dephosphorylation of CERT by PP2Cε may play an important role in the regulation of ceramide trafficking from the ER to the Golgi apparatus.

Vesicle-associated membrane protein-associated protein A (VAPA) is an endoplasmic reticulum (ER)-resident type II transmembrane protein with homologs widely distributed from yeast to human (1–3). Recently, evidence has accumulated that in mammalian cells VAPA participates in the regulation of inter-organelle transport of membrane lipids by recruiting lipid transfer proteins to the ER membrane. VAPA associates with a short, conserved peptide sequence termed the “two phenylalanines in an acidic tract” (FFAT) motif that is found in several lipid transfer proteins including ceramide transport protein CERT, oxysterol-binding protein, Opi1 protein, and PITP/Nir/rdgB families (4–11). VAPA is composed of two conserved domains, an amino-terminal immunoglobulin-like β sheet responsible for FFAT motif binding and a carboxyl-terminal transmembrane domain (8). In addition to its role in recruiting FFAT motif-targeted proteins to ER membranes, VAPA has been proposed to function in vesicle trafficking (1, 12–14), in the organization of the microtubule network (10, 15), and in the replication of hepatitis C virus RNA (16, 17).

In mammalian cells ceramide is synthesized in the ER and transported to the Golgi apparatus where it is converted to sphingomyelin (SM). The ceramide transport protein CERT plays a key role in the ER-to-Golgi trafficking of ceramide (18–20). CERT consists of several distinct domains including a Steroidogenic acute regulator-associated lipid transfer (START) domain capable of specifically extracting ceramide from membrane, a pleckstrin homology (PH) domain that serves to target the Golgi apparatus by recognizing phosphatidylinositol 4-monophosphate, and a FFAT motif, which interacts with VAPA. In addition to these functional domains, CERT possesses a regulatory sequence referred to as a serine repeat (SR) motif between the PH domain and FFAT motif (21). The Ser/Thr residues of the SR motif are phosphorylated in vivo. These phosphorylation sites match the typical consensus motif for protein kinase CK1 in which Ser-132 would serve as priming phosphorylation site for sequential phosphorylation of downstream Ser/Thr residues within the motif. Enhanced phosphorylation of these sites results in down-regulation of CERT activity; under such conditions ceramide transport from the ER to the Golgi apparatus diminishes as a result of repression of both the ceramide transfer activity of the START domain and the phosphatidylinositol 4-monophosphate binding activity of the ceramide transfer activity of the START domain and the phosphatidylinositol 4-monophosphate binding activity of the ceramide transfer activity of the START domain.
PH domain (21). A loss of SM and cholesterol from the plasma membrane induces dephosphorylation of the SR motif, resulting in activation of CERT. However, the protein phosphatase(s) responsible for dephosphorylation of CERT has not yet been identified.

Protein phosphatase 2C (PP2C) is a member of major protein serine/threonine phosphatase superfamily (PP1, PP2A, PP2B, and PP2C) conserved among eukaryotes. Thus far, 13 distinct PP2C homologs (PP2CA, PP2CB, PP2CG, FIN13, PP2CA/LKAP, PP2CC, PP2Cz, PP2Cη, PP2Cκ, Wip1, CaMKP/hFEM2/POPX2, CaMKP-N/POPX1, NERP-2C, and SCOP/PHLPP) have been identified in mammalian cells (22–27). In vivo and in vitro studies have shown that PP2Cs play important roles in the regulation of cellular functions such as stress response, cell cycle, cytoskeleton organization, mRNA splicing, and Wnt signaling (22, 23).

PP2Ce was originally identified by us as a negative regulator of stress-activated protein kinase signaling pathways (28, 29). Ectopic expression of PP2Ce in mammalian cells represses the activity of transforming growth factor β-activated kinase 1 and apoptosis-regulating kinase 1, two mitogen-activated protein kinase kinase kinases. PP2Ce keeps these kinases in an inactive state in quiescent cells by associating with and dephosphorylating them. PP2Ce associates with transforming growth factor β-activated kinase 1 and apoptosis-regulating kinase 1 in quiescent cells, but the association was transiently suppressed in response to treatment of the cells with interleukin-1 and H2O2, respectively, which activate these respective kinases. On the basis of these results we proposed that PP2Ce regulates transforming growth factor β-activated kinase 1 and apoptosis-regulating kinase 1 pathways by a common regulatory mechanism (28, 29).

PP2Ce has a unique hydrophobic region composed of 60 amino acids at the amino terminus whose function is not yet known. During the course of studies to elucidate the functional role of the amino-terminal region of PP2Ce, we noticed that PP2Ce is an ER resident integral membrane protein and identified VAPA as a binding partner of PP2Ce on ER. Furthermore, we obtained evidence suggesting that PP2Ce regulates CERT function through dephosphorylation of its SR motif.

**EXPERIMENTAL PROCEDURES**

*Materials*—Restriction enzymes and other modifying enzymes used for DNA manipulation were obtained from New England Biolabs (Beverly, MA). Lipofectamine 2000 was purchased from Invitrogen. Glutathione Sepharose-4B, protein G-agarose beads, polyclonilidene difluoride membrane, ECL plus kit and [U-14C]serine were obtained from GE Healthcare. Horseradish peroxidase-labeled secondary antibody was obtained from Cell Signaling (Beverly, MA). Anti-hemagglutinin (HA), anti-GST, anti-GS28, and anti-protein disulfide isomerase (PDI) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-GM130 antibody was purchased from BD Biosciences. Alexa 488-conjugated anti-rabbit IgG and Alexa 568-conjugated anti-mouse IgG were obtained from Molecular Probes (Eugene, OR). Complete protease mixture was obtained from Roche Applied Science. Methyl-β-cyclodextrin was obtained from Sigma-Aldrich. All other reagents were purchased from Wako Pure Chemicals (Osaka, Japan).

*Production of Anti-PP2Ce Antibserum*—Polyclonal antibodies recognizing PP2Ce were raised in rabbits against a mixture of the peptides QHLQDYEKDENSVL and CPLSHDHD- PYQLKERKR (corresponding to residues 149–163 and 225–241 of mouse PP2Ce, respectively). Antibodies were affinity-purified on NSH-Sepharose covalently coupled to these peptides.

*Yeast Two-hybrid Screening*—A cDNA encoding a dominant negative mutant of mouse PP2Ce(D302A) was cloned into the pGBK-T7 to produce “bait” vector. This construct was used to screen a human brain cDNA library in the pACT2 vector. The dominant negative mutant was used because it was expected to associate with its substrate more stably than the wild type in the cells (28). In a screen of 1 × 10⁶ library clones, 5 independent clones encoding human VAPA were isolated.

*Plasmid Constructs*—Human VAPA and human CERT cDNAs were obtained by reverse transcriptase-PCR from total RNA of HEK293 cells. Plasmids expressing GST-VAPA and HA-CERT were prepared by inserting these cDNAs into pCX-GST-MS and pcDNA-HA-MS vectors, respectively. Plasmids expressing PP2Ce and PP2Ce-FLAG were constructed by inserting the PP2Ce cDNA into pcDNA-3 and pcDNA-MS-FLAG vectors, respectively. Plasmids expressing ΔN-PP2Ce and FLAG-ΔN-PP2Ce were constructed by inserting the PP2Ce cDNA encoding amino acids 58–360 into pcDNA-3 and pcDNA-FLAG-MS vectors, respectively. Site-directed mutagenesis was carried out to generate deletion mutants of PP2Ce and VAPA using the directed PCR method. To produce GFP fusion proteins, pEGFP-N3-M vector was used in which an inherent initiating ATG codon was mutated to GTG to prevent non-fused GFP protein from being produced.

*Cell Culture and Transfection*—HEK293 cells and HeLa cells were grown in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% (v/v) fetal bovine serum. At ~90% confluency, the cells were transfected using Lipofectamine 2000. The total amount of DNA used for transfection was 1.6 μg per well of a 12-well plate. After transfection, the cells were cultured for 24–48 h before harvest. Cells transfected with the indicated expression plasmids were washed twice with phosphate-buffered saline (PBS) and lysed with ice-cold lysis buffer containing 50 mM Tris-HCl, pH 7.5, 1% (v/v) Triton X-100, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1 mM sodium orthovanadate, 50 mM NaF, 10 mM β-glycerophosphate, 5 mM sodium pyrophosphate, and a Complete protease inhibitor mixture. Cell lysate containing 200 μg of protein was incubated for 0.5 h with glutathione-Sepharose beads (5 μl). After washing the beads 5 times with lysis buffer, the bound protein was subjected to 10% (w/v) SDS-PAGE and then transferred onto polyvinylidene difluoride membranes. The membranes were incubated with primary antibody for 1 h at 25 °C followed by incubation with horseradish peroxidase-conjugated secondary antibody for 0.5 h at 25 °C and developed by chemiluminescence using ECL plus kit. Dilution of antibody was 1:500 for anti-GST antibody, 1:1000 for anti-PDI, anti-HA, and anti-PP2Ce antibodies, and 1:2000 for horseradish peroxidase-conjugated secondary
Isolation of Microsomal Membrane from Cultured Cells—The membrane fraction of cultured cells was prepared as described by Nohturfft et al. (30). Briefly, cells were scraped into ice-cold PBS and resuspended in buffer B (10 mM HEPES-NaOH, pH 7.5, 0.25 M sorbitol, 10 mM potassium acetate, 1.5 mM magnesium acetate, and Complete protease inhibitor mixture). The suspension was passed through a 23-gauge needle 25 times and centrifuged at 1000 × g for 5 min. The resulting supernatant was centrifuged at 16,000 × g for 20 min. The pellet was resuspended in buffer C (50 mM HEPES, pH 7.5, 0.25 M sorbitol, 70 mM potassium acetate, 2.5 mM magnesium acetate, 5 mM EGTA, and protease inhibitor) and centrifuged again at 16,000 × g for 3 min. The resulting precipitates were resuspended in buffer C to obtain the microsomal fraction.

Fractionation by Triton X-114—Triton X-114 was added to the microsomal fraction at 4 °C to a final concentration of 2% (v/v) to solubilize the membrane proteins. The Triton X-114-treated sample was layered over a cushion of 0.25 M sucrose (v/v) to solubilize the membrane proteins. The Triton X-114-solubilized membranes were centrifuged at 100,000 × g for 90 min to produce cytosol and microsomal fraction. The microsomal fraction was homogenized in solution A, and membrane proteins were extracted by the addition of 2% (v/v) Triton X-114.

RESULTS

PP2Ce Dephosphorylates CERT

PP2Ce Is Localized in the ER—Unlike PP1, PP2A, and PP2B, whose subcellular localization and activities are controlled by separate regulatory subunits, the members of PP2C family are monomeric proteins and, therefore, are thought to contain distinct domains that influence the subcellular localization or function. In this context we noticed that PP2Ce has an amino-terminal non-catalytic region composed of about 60 amino acids in addition to the carboxyl-terminal catalytic domain composed of about 300 residues. Because the amino-terminal region was hydrophobic, we speculated that this region might function as a membrane association domain. To test this possibility we transfected HEK293 cells with a plasmid for either full-length PP2Ce or PP2Ce lacking the amino-terminal 57 amino acids (AN-PP2Ce) and separated the postnuclear supernatant of the cell extracts into cytosolic and membranous fractions. Western blot analysis using anti-PP2Ce antibody revealed that the full-length PP2Ce localized to the membrane fraction, whereas AN-PP2Ce was recovered in the cytosolic fraction (Fig. 1a). These results suggest that PP2Ce is a membrane-associated protein and that the amino-terminal hydrophobic region is required for its membrane association.

PP2Ce Is Transiently Localized to the ER—The results of the Western blot analysis were confirmed by indirect immunofluorescence. Cells were transfected with plasmids encoding AN-PP2Ce or full-length PP2Ce and incubated for 24 h with media containing [14C]serine. As expected, the AN-PP2Ce was found in the cytosol (Fig. 1b). In contrast, the full-length PP2Ce was found in the membrane fraction (Fig. 1c). The intensity of the band was quantified by imaging analyzer (FLA-7000, Fuji Film, Tokyo, Japan).

PP2Ce mRNA is Reduced upon Knockdown of Human PP2Ce mRNA—To determine the effect of PP2Ce knockdown on human PP2Ce expression, we used an siRNA against human PP2Ce (31). HeLa cells transfected with siRNAs were incubated with 10 mM methyl-β-cyclodextrin for 30 min and then incubated in 1.5 ml of serum free medium containing 37 kBq of [1-14C]serine at 37 °C for 2 h. Labeled lipids extracted from the cells were separated on TLC plate (methyl acetate, n-propanol, chloroform, methanol, 0.25% CaCl2 = 25/25/25/10/9) and quantified by FLA-7000 imaging analyzer.
To determine the subcellular localization of endogenous PP2Cε, we separated the postnuclear supernatant of mouse brain extracts into cytosolic, mitochondrial, and microsomal fractions. Western blot analysis demonstrated that endogenous PP2Cε was mainly localized in the microsomal fraction (Fig. 1b). We then carried out a sucrose density gradient centrifugation of the homogenates prepared from HEK293 cells expressing PP2Cε. As shown in Fig. 1c, PP2Cε was co-fractionated with PDI, an ER resident protein, but not with GS28, a Golgi marker protein. Furthermore, PP2Cε-GFP fusion proteins expressed in HEK293 cells exhibited a diffuse reticular pattern that overlapped with staining of PDI when observed by confocal microscopy (Fig. 1d). These results suggest that PP2Cε is an ER resident protein.

**PP2Cε Is a Type 1 Integral Membrane Protein of the ER**—We next determined whether PP2Cε was an ER transmembrane protein, a peripheral membrane protein that associates with transmembrane proteins or a luminal protein that resides in the lumen of ER as a soluble protein. To this end, the microsomal fraction prepared from HEK293 cells expressing PP2Cε was treated with high concentration of salt, alkali, or Triton X-100. Western blot analysis indicated that, whereas PP2Cε remained associated with the membrane fraction under a high salt concentration or alkaline conditions (Fig. 2a, lanes 2 and 3), it was solubilized upon treatment with Triton X-100 (Fig. 2a, lane 4), suggesting that PP2Cε is either a transmembrane or luminal protein. To discriminate between these two possibilities, the microsomal fraction prepared from HEK293 cells expressing PP2Cε was treated with Triton X-114, and the solubilized proteins were subjected to phase separation at 30 °C. PP2Cε was recovered in the detergent phase, which contains transmembrane proteins, but not in the hydrophilic phase, which is expected to contain luminal proteins (Fig. 2b). Thus, PP2Cε is likely to be a transmembrane (TM) protein.

To determine whether the carboxyl-terminal catalytic domain of PP2Cε projects into the cytosol or the ER lumen, we treated the microsomal fraction prepared from HEK293 cells expressing PP2Cε with proteinase K with or without Triton X-100. Our expectation was that, if the carboxyl terminus of PP2Cε faces the cytosol like SREBP1, it would be digested by proteinase K even in the absence of Triton X-100. However, PP2Cε would be digested by proteinase K only in the presence of Triton X-100 if its carboxyl-terminal region projects into ER lumen like PDI because it is protected from digestion by proteinase K. We found that PP2Cε was readily digested by proteinase K irrespective of the presence of Triton X-100, as was observed with SREBP1. This finding suggests that PP2Cε exists as a transmembrane protein whose carboxyl-terminal catalytic domain faces the cytoplasm (Fig. 2c).

To further confirm the conclusion described above, we carried out the protease protection assay using the microsomal fraction prepared from the cells expressing GST-PP2Cε in which GST tag was fused to the amino terminus of PP2Cε.

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PP2Ce Dephosphorylates CERT

Although the majority of GST-PP2Ce expressed in the cells was recovered in the cytosolic fraction (data not shown), a significant amount of GST-PP2Ce was localized to the microsomal fraction (Fig. 2d, lanes 1 and 4). When immunoblotting was carried out using the anti-PP2Ce antibody after the protease digestion of the microsomal fraction, the bands of GST-PP2Ce were found to disappear upon the protease treatment (Fig. 2d, lanes 5 and 6). In contrast, a faster migrating band whose molecular mass was 27 kDa appeared in accordance with the disappearance of the full-length GST-PP2Ce when stained with anti-GST antibody, indicating that GST portion (~26.5 kDa) was protected from digestion by protease (Fig. 2d, lanes 2 and 3). These results suggest that the GST portion of the expressed GST-PP2Ce was localized in the microsomal lumen, whereas the carboxyl-terminal portion of the GST-PP2Ce existed outside of the microsome, supporting the conclusion that the amino-terminal region of PP2Ce functions as the TM domain.

We were interested in identifying the TM domain of PP2Ce. To this end we expressed a series of amino-terminal deletion mutants of PP2Ce (Fig. 3a, Δ1–Δ7) in HEK293 cells and examined their subcellular localization. Of these mutants, Δ1, Δ5, Δ6, and Δ7 were localized only to the membrane fraction (Fig. 3b, lanes 2, 6, 7, and 8). Two mutants (Δ2 and Δ4) distributed equally to membranous and cytosolic fractions (Fig. 3b, lanes 3 and 5). Most of mutant Δ3, which lacked amino acids between 26 and 45, exhibited cytosolic localization (Fig. 3b, lane 4). Because the region between amino acids 26 and 45 is predicted to form an α-helix (data not shown) and is rich in hydrophobic amino acids, it may function as a TM domain (Fig. 3a). To directly show that the amino-terminal region of PP2Ce is involved in the membrane localization, we constructed three GFP fusion proteins, N1-, N2-, and N3-GFP, which were composed of the amino-terminal regions of PP2Ce with three different lengths fused to GFP (Fig. 3a). These proteins were expressed in HEK293 cells, and their subcellular localization was determined. All fusion proteins were localized to the membrane fraction (Fig. 3c), indicating that amino-terminal region containing amino acids between 26 and 45 is involved in the membrane localization. The amino acid sequence surrounding the TM domain of mouse PP2Ce is conserved in orthologs in insect and sea urchin genomes, suggesting that membrane localization of PP2Ce is widely conserved (Fig. 3d).

PP2Ce Interacts with VAPA in Vivo—To identify the substrate(s) or the protein(s) responsible for regulation of PP2Ce activity, we carried out a yeast two-hybrid screen for interaction partners. The coding region of full-length PP2Ce(D302A) (a mutant of PP2Ce deficient in phosphatase activity) was fused to the GAL4 DNA binding domain and used as bait. In a screen of 1 × 10⁶ human brain library clones, VAPA was identified as an interacting partner of PP2Ce. VAPA interacted with PP2Ce but not with GAL4 DNA binding domain, PP2Ca, PP2Cb, PP2Cd, or PP2Cβ in the yeast two-hybrid system, suggesting that the interaction between VAPA and PP2Ce is specific (data not shown). To examine whether this interaction also occurs in mammalian cells, a GST fusion protein of VAPA was expressed with PP2Ce-FLAG in HEK293 cells, and the lysates from the cells were subjected to a GST pulldown assay. As shown in Fig. 4a, VAPA in fact interacted with PP2Ce but not with PP2Cb, suggesting that the interaction between PP2Ce and VAPA in cells is specific. ΔN-PP2Ce did not interact with VAPA, indicating that the amino-terminal non-catalytic region of PP2Ce is required for this interaction (Fig. 4a, lane 3 in the upper panel). We next attempted to identify the region of PP2Ce that is required for the interaction with VAPA. GFP fusion proteins containing full-length PP2Ce (FL-GFP) or various lengths of the amino-terminal region of PP2Ce (N1-GFP, N2-GFP, and N3-GFP in Fig. 3a) were expressed in HEK293 cells, and interaction with GST-VAPA was examined. Of these GFP fusion proteins, only FL-GFP and N3-GFP (the fusions bearing residues 1–68 of PP2Ce) interacted with GST-VAPA (Fig. 4b, lanes 4 and 5 in the upper panel). These results suggested that the hydrophilic region adjacent to the TM domain is also required for the interaction of PP2Ce with VAPA (see Fig. 3a).
To identify the region of VAPA that interacts with PP2Ce, we constructed GST fusion proteins containing either a VAPA mutant that lacks its TM domain (GST-VAPA-1) or the TM domain of VAPA without the rest of the protein (GST-VAPA-2) (Fig. 4c). GST-VAPA-2 but not GST-VAPA-1 was able to interact with PP2Ce, suggesting that the TM domain is responsible for the interaction with PP2Ce (Fig. 4d).

**PP2Ce Dephosphorylates CERT in a VAPA-dependent Manner**—Recently, we have reported that the ceramide transport protein CERT interacts with VAPA in the ER (11). This interaction is required for CERT to extract ceramide from the ER before transportation of ceramide to the Golgi apparatus. We also showed that the SR motif in CERT is phosphorylated at multiple Ser/Thr residues and that hyperphosphorylation of these sites results in down-regulation of ceramide trafficking function of CERT (21). We, therefore, were interested in determining whether PP2Ce associated with VAPA regulates CERT function through dephosphorylation. We first tested the possibility that PP2Ce dephosphorylates CERT in vivo. HA-CERT was expressed in HEK293 cells with or without GST-VAPA in the presence or absence of co-expressed PP2Ce-FLAG, PP2Ce(D302A)-FLAG which is a phosphatase activity-deficient mutant of PP2Ce or FLAG-PP2Ceβ. As judged by a shift in SDS-PAGE mobility, PP2Ce-FLAG, but not PP2Ce(D302A)-FLAG, caused dephosphorylation of HA-CERT when GST-VAPA was co-expressed in the cells (Fig. 5, lanes 3, 4, 7, and 8 in the upper panel). In contrast, HA-CERT remained phosphorylated when it was co-expressed only with PP2Ce-FLAG (Fig. 5, lanes 5 and 6 in the upper panel). FLAG-PP2Ceβ did not dephosphorylate HA-CERT even in the presence of GST-VAPA (Fig. 5, lanes 9 and 10 in the upper panel). These results suggest that PP2Ce specifically dephosphorylates CERT in a manner depending on VAPA expression.

**Expression of PP2Ce Induces Redistribution of CERT to the Golgi Apparatus**—We have previously shown that the treatment of cells with sphingomyelinase resulted in redistribution of CERT from the cytoplasm to the Golgi apparatus in accordance with its dephosphorylation (21). We, therefore, examined the effect of expression of PP2Ce on subcellular localization of

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**FIGURE 4. VAPA interacts with PP2Ce in vivo.** a, GST-VAPA was co-expressed with either PP2Ce-FLAG, FLAG-ΔN-PP2Ce, or FLAG-PP2Ceβ in HEK293 cells. GST-VAPA was pulled down using glutathione-agarose beads 48 h after the transfection and immunoblotted using an anti-FLAG antibody, to determine the VAPA binding region of PP2Ce. GST fused with amino-terminal region of PP2Ce (Fig. 3a, N1-, N2-, and N3-GFP) as well as GST fused with full-length PP2Ce (FL-GFP) were expressed in HEK293 cells, and the cell extracts were submitted to GST pulldown analysis using an anti-GFP antibody (upper panel). Whole cell extracts (WCL) were also immunoblotted with anti-GFP and anti-GST antibodies (lower panel). c and d, to determine the PP2Ce binding region of VAPA, expression plasmids for GST fused with full-length VAPA (GST-VAPA) and two deletion mutants of VAPA (GST-VAPA-1 and GST-VAPA-2) were prepared (c). GST-VAPA, GST-VAPA-1, or GST-VAPA-2 was expressed in HEK293 cells, and the cell extracts were subjected to GST pulldown analysis using an anti-GFP antibody (d, top panel). The whole cell extracts were also immunoblotted with anti-GST (d, middle panel) or an anti-PP2Ce antibody (d, bottom panel).

**FIGURE 5. PP2Ce dephosphorylates CERT in a VAPA-dependent manner.** HEK293 cells were transfected with the expression plasmid for HA-CERT with or without the expression plasmid for GST-VAPA in the presence or absence of the expression plasmid for PP2Ce-FLAG, PP2Ce(D302A)-FLAG (a phosphatase activity-deficient mutant of PP2Ce), orFLAG-PP2Ceβ. The cells were harvested after culturing for 48 h, and the cell extracts were subjected to immunoblot analysis using an anti-HA antibody (upper panel) or anti-FLAG and anti-GST antibodies (lower panel).
CERT. HA-CERT and GST-VAPA were transiently expressed in HeLa cells, and the intracellular distribution of HA-CERT was analyzed by indirect immunostaining with an anti-HA antibody and an antibody against GM130, a Golgi marker protein. Although HA-CERT was distributed throughout the cytoplasm when expressed only with GST-VAPA (Fig. 6d), co-expression of PP2Ce greatly redistributed HA-CERT to the Golgi apparatus (Fig. 6d). CERT (S132A), which was previously shown to act as a mimic of the dephosphorylated form of CERT, was localized to the Golgi region even in the absence of PP2Ce, as we demonstrated previously (Fig. 6g) (21). These results suggest that PP2Ce enhances phosphatidylinositol 4-monophosphate binding activity of CERT through its dephosphorylation.

PP2Ce Enhanced the Interaction between CERT and VAPA—We next examined whether dephosphorylation of CERT by PP2Ce affected the interaction between CERT and VAPA. When HEK293 cells were transfected with expression plasmids of GST-VAPA and HA-CERT, no interaction between these proteins was observed (Fig. 7a, lanes 1 and 2 in the top panel). In contrast, expression of PP2Ce-FLAG in combination with GST-VAPA and HA-CERT caused an increase in the interaction between GST-VAPA and HA-CERT, in accordance with dephosphorylation of CERT (Fig. 7a, lanes 3 and 4 in the top and middle panels). Neither interaction between CERT and VAPA nor dephosphorylation of CERT was observed when PP2Ce(D302A) (Fig. 7a, lanes 5 and 6 in the top and middle panels) or PP2Ce (Fig. 7b, lanes 7 and 8 in the top and middle panels) were co-expressed. These results suggest that PP2Ce enhances the interaction between CERT and VAPA through dephosphorylation of CERT. Interestingly, although expression of AN-PP2Ce caused a mobility shift of CERT protein to a similar extent as full-length PP2Ce (Fig. 7b, lanes 5 and 6 in the

![Image](https://example.com/image1.png)

**FIGURE 6. Expression of PP2Ce redistributes CERT to the Golgi apparatus.** HeLa cells transiently expressing HA-CERT and GST-VAPA (a–c), HA-CERT, GST-VAPA, and PP2Ce (d–f), or CERT(S132A) and GST-VAPA (g–i) were double-labeled with anti-HA antibody (green) and anti-GM130 antibody (red) by indirect immunostaining. DIC indicates differential interference images.

![Image](https://example.com/image2.png)

**FIGURE 7. PP2Ce enhances the interaction between CERT and VAPA.** a, HEK293 cells were transiently transfected with the expression plasmids for HA-CERT and GST-VAPA with or without the expression vector for PP2Ce-FLAG or PP2Ce(D302A)-FLAG. The cells were harvested after culturing for 48 h, and the GST pulldown assay was performed with the cell extracts using anti-HA and -GST antibodies (top panel). The whole cell extracts (WCL) were also immunoblotted with anti-HA and anti-GST antibodies (middle panel) or anti-FLAG antibody (bottom panel). b, HEK293 cells were transiently transfected with expression plasmids for HA-CERT and GST-VAPA with or without the expression vector for PP2Ce-FLAG, FLAG-AN-PP2Ce, or FLAG-PP2Ceβ. The cells were harvested after culturing for 48 h, and a GST pulldown assay was performed using anti-HA and anti-GST antibodies (top panel). The whole cell extracts were also immunoblotted with anti-HA and anti-GST antibodies (middle panel) or anti-FLAG antibody (bottom panel). c, HEK293 cells were transfected with the expression plasmids for HA-CERT, GST-VAPA, and PP2Ce (lane 1, 0 μg; lane 2, 0.005 μg; lane 3, 0.01 μg; lane 4, 0.03 μg; lane 5, 0.15 μg; lane 6, 0.3 μg). The cells were harvested after culturing for 48 h, and a GST pulldown assay was performed with the cell extracts using an anti-HA antibody (top panel). The whole cell extracts were also immunoblotted with anti-HA (second panel), anti-GST (third panel), or anti-PP2Ce antibody (bottom panel). d, as in c, except for using expression plasmid for CERT(S132A) instead of that for HA-CERT.
Absence of PP2Cε could only marginally promote the interaction of VAPA and CERT (Fig. 7b, lanes 5 and 6 in top panel) (see “Discussion”).

To examine whether the enhanced interaction between CERT and VAPA by PP2Cε is mediated through dephosphorylation of the SR motif, we conducted binding assays with the S132A mutant of CERT. As shown in Fig. 7d, the interaction between CERT(S132A) and VAPA was observed even in the absence of PP2Cε. Co-expression of PP2Cε did not further increase CERT(S132A)-VAPA interaction (Fig. 7d), whereas the interaction between wild-type CERT and VAPA was dependent on PP2Cε (Fig. 7c). These results suggest that PP2Cε regulates the interaction between CERT and VAPA through dephosphorylation of the SR motif in CERT.

Knockdown of Expression of PP2Cε Attenuates the Interaction between VAPA and CERT—We were interested in determining whether endogenous PP2Cε is indeed responsible for dephosphorylation of CERT. To this end we used RNA interference to knock down the expression of PP2Cε in vivo. Although the interaction of CERT and VAPA is low in HEK293 cells unless PP2Cε is co-expressed, a significant interaction between these proteins is observed in HeLa cells even in the absence of co-expression of PP2Cε. We, therefore, used HeLa cells in knockdown experiments. Transfection of HeLa cells with two distinct siRNAs directed against PP2Cε mRNA (ε-3 and ε-4) resulted in a 70 and 75% reduction in PP2Cε expression 24 h after transfection, respectively (Fig. 8a). At the same time point, interaction between HA-CERT and GST-VAPA was decreased by 35 and 47% in the cells transfected with ε-3 and ε-4, respectively (Fig. 8b, lane 3–6).

We finally examined whether knockdown of PP2Cε affects SM synthesis in intact cells. We have previously shown that the treatment of cells with cholesterol adsorbent methyl-β-cyclodextrin stimulated dephosphorylation of CERT (21). We, therefore, employed this experimental condition for the knockdown study. The amount of radioactivity incorporated into SM during 2 h of labeling in HeLa cells were reduced by 20 and 40% by transfection with two siRNAs, ε-3 and ε-4, respectively, without affecting the rate of ceramide synthesis (Fig. 8c). These results support the conclusion that PP2Cε is involved in regulation of ceramide trafficking in the cells.

**DISCUSSION**

**PP2Cε Is a Transmembrane Protein**—In the present study we provide evidence that PP2Cε is an ER resident type I integral membrane protein, with a carboxyl-terminal catalytic domain facing the cytoplasm and a short amino-terminal hydrophobic domain localizing inside the membrane. Genomic analysis revealed that the amino acid sequence surrounding the transmembrane domain of mammalian PP2Cε is conserved in distantly related organisms, such as insects and sea urchins (Fig. 3d), supporting the notion that membrane localization is important for the in vivo function of PP2Cε.

We previously proposed that PP2Cε was translated beginning at the fourth Met codon of the open reading frame to produce a 303-amino acid polypeptide based on the results of the experiments using in vitro translation system (28). To examine whether PP2Cε was translated from fourth Met codon in vivo, we examined the size of the endogenous protein. Endogenous PP2Cε in mouse brain co-migrated with recombinant protein translated from the first Met codon, which consists of 360 amino acids (result not shown). Furthermore, the fact that amino acid sequence surrounding the transmembrane domain of mouse PP2Cε is conserved in distantly related organisms supports the idea that the amino-terminal non-catalytic region is translated in these animals. In contrast, the sequence surrounding fourth Met codon of mouse PP2Cε is not conserved among other organisms (data not shown). Collectively, these lines of evidence suggest that endogenous PP2Cε is actually longer than was previously thought, although we cannot rule out the possibility that the shorter polypeptide, which is composed of 303 amino acids, also exists in vivo.
Physiological Significance of Dephosphorylation of CERT by PP2C—In this study we provide evidence that PP2C dephosphorylates CERT in vivo. Our conclusion that CERT is a physiological substrate of PP2C is based on the following observations. First, PP2C dephosphorylated CERT in a manner depending on VAPA expression, whereas PP2Cβ did not dephosphorylate CERT in vivo, even in the presence of co-expressed VAPA. Second, dephosphorylation of CERT by PP2C caused redistribution of CERT from the cytoplasm to the Golgi apparatus. Third, dephosphorylation of CERT by PP2C, but not PP2Cβ, also caused the enhanced interaction between CERT and VAPA. Fourth, knockdown of PP2C mRNA by siRNA reduced the interaction between CERT and VAPA. These lines of evidence together with the observation that PP2C specifically associated with VAPA both in the yeast two-hybrid system and in mammalian cells strongly suggest that VAPA is a physiological binding partner of PP2C in the ER. Considering the fact that dephosphorylation of CERT by PP2C occurred in a VAPA-dependent manner and that PP2C and CERT were found to bind to different regions of VAPA, we propose that VAPA may act as a scaffold protein that interacts with PP2C and CERT simultaneously and stimulates dephosphorylation of CERT by PP2C.

Previously, we showed that CERT is phosphorylated at multiple sites in the SR motif and that the phosphorylation of these sites results in down-regulation of ceramide trafficking activity of CERT through inhibition of the START and PH domains (21). Our finding that PP2C dephosphorylates CERT and that dephosphorylation enhanced the interaction of CERT with both ER and Golgi membranes suggest that PP2C functions as an activator of ceramide transport (Fig. 9). This conclusion is supported by the fact that knockdown of PP2C expression reduced the level of ceramide-trafficking SM synthesis. In this context we previously suggested that dephosphorylation of the SR motif was required for the association of CERT with phosphatidylinositol 4-monophosphate in the Golgi apparatus but not with VAPA in the ER. Our conclusion was based on the observation that CERT(S132A) and CERT(10E) in which all the Ser/Thr residues of the SR motif were replaced by Glu to mimic the phosphorylated state had similar affinity for VAPA as wild-type CERT (21). Although the reason for the discrepancy between our previous and present studies is not known, the difference in experimental conditions for the pulldown assay may account for it. Although the cells were treated with chemical cross-linker before the pulldown assay in the previous study, no cross-linking reagent was used in this study. A pulldown assay without cross-linking pretreatment may detect only strong and stable interaction between CERT and VAPA. Alternatively, the conformational change induced by replacement of Ser/Thr residues with Glu only partially mimicked that induced by phosphorylation of these sites. This partial conformational change may not have been enough to inhibit the association between the mutant CERT and VAPA, although it was sufficient to suppress the interaction between the mutant CERT and Golgi. Further studies are required to answer this question.

One unexpected observation was that the expression of ΔN-PP2C caused a mobility shift of CERT protein to the same extent as wild-type PP2C, although in contrast to the expression of wild-type PP2C, ΔN-PP2C expression had only a modest stimulatory effect on the association of CERT with VAPA (Fig. 7). These results suggest that the phosphorylation site(s) in SR motif of CERT responsible for the regulation of interaction with VAPA differs from that responsible for the mobility shift on SDS-PAGE and that localization to ER membrane is required for PP2C to dephosphorylate the former site(s) precisely. Alternatively, association with PP2C may be required for VAPA to keep an appropriate conformation for binding to the dephosphorylated CERT.

CERT-mediated ceramide trafficking is thought to occur at the narrow cytoplasmic gaps termed the ER-Golgi membrane contact site. Two models have been proposed to depict this process (19, 32). In the “neck-swinging model,” CERT might simultaneously associate with both ER and Golgi, and the ceramide is transferred by the START domain by its oscillating motion. In contrast, the “short-distance shuttle model” proposes that CERT might quickly shuttle between the two organelle membranes. Our observation that dephosphorylation of CERT by PP2C enhances its affinity for both the ER membrane and the Golgi apparatus would support the neck-swinging model (Fig. 9).

It has been reported that VAPA interacts with hepatitis C virus nonstructural protein 5A (NS5A) and that the interaction of NSSA with VAPA is required for efficient replication of the hepatitis C virus genome (17). Interestingly, the ability of NSSA to bind VAPA is enhanced by dephosphorylation of NSSA, whereas hyperphosphorylation of NSSA disrupts viral RNA replication (17, 33). Whether PP2C and VAPA also regulate the hepatitis C virus life cycle in human liver through dephosphorylation of NSSA remains to be elucidated.

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