We recently demonstrated that the activation of ceramide kinase (CERK) and the formation of its product, ceramide 1-phosphate (C1P), are necessary for the degranulation pathway in mast cells and that the kinase activity of this enzyme is completely dependent on the intracellular concentration of Ca\(^{2+}\) (Mitsutake, S., Kim, T.-J., Inagaki, Y., Kato, M., Yamashita, T., and Igarashi, Y. (2004) J. Biol. Chem. 279, 17570–17577). Despite the demonstrated importance of Ca\(^{2+}\) as a regulator of CERK activity, there are no apparent binding domains in the enzyme and the regulatory mechanism has not been well understood. In the present study, we found that calmodulin (CaM) is involved in the Ca\(^{2+}\)-dependent activation of CERK. The CaM antagonist W-7 decreased both CERK activity and intracellular C1P formation. Additionally, exogenously added CaM enhanced CERK activity even at low concentrations of Ca\(^{2+}\). The CERK protein was co-immunoprecipitated with an anti-CaM antibody, indicating formation of intracellular CaM-CERK complexes. 

An in vitro CaM binding assay also demonstrated Ca\(^{2+}\)-dependent binding of CaM to CERK. These results strongly suggest that CaM acts as a Ca\(^{2+}\) sensor for CERK. Furthermore, a CaM binding assay using various mutants of CERK revealed that the binding site of CERK is located within amino acids 422–435. This region appears to include a type 1-8-14B CaM binding motif and is predicted to form an amphipathic helical wheel, which is utilized in CaM recognition. The expression of a deletion mutant of CERK that contained the CaM binding domain but lost CERK activity inhibited the Ca\(^{2+}\)-dependent activation of CERK. Finally, we reveal that in mast cell degranulation CERK acts downstream of CaM, similar to CaM-dependent protein kinase II, which had been assumed to be the main target of CaM in mast cells.

Sphingolipids and their metabolites have emerged as a new class of lipid mediator of various cell functions (1–3). Ceramide (N-acylsphingosine; Cer), the precursor for all sphingolipids, functions as a lipid second messenger in a variety of cellular events (4). Many stimuli, such as tumor necrosis factor α, Fas ligand, γ-irradiation, anti-tumor reagents, and heat shock, cause an elevation in intracellular Cer content following the hydrolysis of sphingomyelin by endogenous sphingomyelinase. These changes can result in cell cycle arrest, cell differentiation, and apoptosis (5, 6). Cer is converted to sphingosine through the action of ceramidase, and in turn the sphingosine is metabolized to sphingosine 1-phosphate (S1P) by sphingosine kinase. S1P has been found to regulate cell growth (7) and motility (8, 9). Interestingly, S1P inhibits apoptosis induced by Cer and Fas ligand (10), indicating that the balance of Cer/sphingosine/S1P can affect cell phenotype. The Cer/sphingosine/S1P pathway had been considered to be the major metabolic pathway of Cer. However, in 2002, the enzyme ceramide kinase (CERK), which metabolizes Cer to ceramide 1-phosphate (C1P), was cloned (11), revealing a new pathway for Cer metabolism. CERK activity was initially described as a Ca\(^{2+}\)-stimulated lipid kinase activity that was co-purified with brain synaptic vesicles (12); it has since been reported in HL60 cells (13) and neutrophils (14). Additionally, CERK is thought to be involved in phagolysosome formation in polymorphonuclear leukocytes and to promote liposome fusion (15).

The product of CERK activity, C1P, has been reported to have mitogenic effects (16), although exogenously added C1P is rapidly hydrolyzed by a phosphatase (17). C1P was found to be a direct activator of cytosolic phospholipase A2 and to be involved in arachidonic acid release (18, 19). Additionally, our previous report revealed that CERK was a mediator of Ca\(^{2+}\)-dependent degranulation in mast cells (20). In both arachidonic acid release and mast cell degranulation, the intracellular elevation of Ca\(^{2+}\) is a crucial event that acts as a regulatory mechanism of CERK activity. However, there are no apparent Ca\(^{2+}\) binding domains in the primary structure of CERK (such as an EF-hand or C2 domain), and the Ca\(^{2+}\) regulatory mechanism has not been well understood. Calmodulin (CaM) has been recognized as a calcium sensor that interacts with and regulates multiple protein targets (21). When intracellular Ca\(^{2+}\) levels rise, four Ca\(^{2+}\) ions bind to CaM and the Ca\(^{2+}\)-CaM complex binds to the target protein, initiating various signaling cascades. In this manner, CaM is known to regulate ion channels, cell cycle, and cytoskeletal organization and to influence development (22, 23).

In the work presented here, we investigated the mechanism of the Ca\(^{2+}\)-dependent activation of CERK and found that CaM was in fact involved. Utilizing point mutation analysis, we also identified the CaM binding site in CERK.

**EXPERIMENTAL PROCEDURES**

**Materials**—Ceramide (C18:0, d18:1), cardiolipin, p-nitrophenyl N-acetyl β-D-glucosaminide, A23187, and an anti-FLAG monoclonal antibody (M2) were all purchased from Sigma. \(^{32}\)P]ATP and \(^{3}H\)sphingosine were from PerkinElmer and American Radiolabeled Chemicals (St. Louis, MO), respectively. The calmodulin antagonist W-7 and the calmodulin-dependent kinase II inhibitor KN-93 were from Seikagaku Corp. (Tokyo, Japan). An imidooester cross-linker, dimethyl 3,3-dithiobispropionimidate/2HCl, and an anti-CaM antibody were from Pierce and Zymed Laboratories Inc. (South San Francisco, CA), respectively. All reagents were of the highest purity available.

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CERK Assays—The kinase activity of CERK was assayed as described previously (20), with some modifications. Briefly, cells were lysed in a buffer comprising 10 mM HEPES, 1 mM diithiothreitol, 40 mM KCl, and Complete™ protease inhibitor mixture (Roche Applied Science). Each lysate was incubated for 30 min at 30 °C in a reaction mixture containing 20 mM HEPES, 80 mM KCl, 1 mM cardioliopin, 1.5% β-octylglucoside, 0.2 mM diethylenetriaminepentaacetic acid, 20 mM [γ-32P]ATP, and 40 μM Cer (C18:0, d18:1). As indicated, varying amounts of CaCl2, EGTA, and bovine CaM were added to this mixture. In certain experiments, lysates were preincubated with an inhibitor, W-7 or KN-93, at the indicated concentration for 10 min, and then the enzyme activity was assayed. Lipids were extracted and separated on Silica Gel 60 high performance TLC (HPTLC) plates (Merck) using chloroform/acetone/methanol/acetic acid/water (10:4:3:2:1, v/v) as the solvent system. Bands corresponding to C1P were quantified using an imaging analyzer BAS2500 (Fuji Film).

Cell Culture and Metabolic Labeling—Rat basophilic leukemia (RBL-2H3) cells and the previously established CERK transfectants RBL-CK3 and CK4 (20) were cultured as monolayers in Eagle’s minimum essential medium (Sigma) supplemented with 10% fetal bovine serum, penicillin, and streptomycin. Chinese hamster ovary (CHO-K1) cells were maintained in Ham’s nutrient mixture F12 medium (Sigma), supplemented with 10% fetal bovine serum, penicillin, and streptomycin. Human embryonic kidney 293FT cells were purchased from Invitrogen and cultured according to the manufacturer’s directions. Cells were transfected with the indicated DNA using Lipofectamine Plus (Invitrogen) for CHO-K1 cells and Lipofectamine 2000 (Invitrogen) for human embryonic kidney 293FT cells, respectively.

For metabolic labeling, RBL-CK3 cells (106) were incubated with 2 μCi (2 μl) of carrier-free [32P]orthophosphoric acid (PerkinElmer) in 5 ml of Eagle’s minimal essential medium for 90 min at 37 °C. After the incubation, the cells were harvested and washed twice with Tyrode’s buffer (25 mM PIPES (pH 7.2), 119 mM NaCl, 5 mM KCl, 0.4 mM MgSO4, 5.6 mM glucose, 1 mM CaCl2, and 0.1% bovine serum albumin). As indicated, W-7 was added to the Tyrode’s buffer, and cells were preincubated for 10 min. Subsequently, the cells were stimulated at 37 °C with 0.1 Ci (2 μl) of carrier-free [32P]orthophosphoric acid (PerkinElmer) in 5 ml of Eagle’s minimal essential medium for 90 min at 37 °C. After the lysates were diluted with 4 volumes of dilution buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM MgCl2, 1 mM dithiothreitol, and 0.5% Triton X-100), they were centrifuged (20,000 × g, 10 min at 4 °C). The supernatants were preincubated with an inhibitor, W-7 or KN-93, at the indicated concentration for 10 min, and then the enzyme activity was assayed. Lipids were extracted and separated on Silica Gel 60 high performance TLC (HPTLC) plates (Merck) using chloroform/acetone/methanol/acetic acid/water (10:4:3:2:1, v/v) as the solvent system. Bands corresponding to C1P were quantified using an imaging analyzer BAS2500 (Fuji Film).

CERK Mutants—For micropreparation, harvested cells were incubated for 10 min at 4 °C in IP buffer (50 mM Tris-HCl (pH 7.2), 150 mM NaCl, 5 mM EDTA, 0.5% Triton X-100, 10% glycerol, and Complete™ protease inhibitor mixture). After centrifugation at 15 × 10³ rpm for 5 min, primary antibodies were added to the supernatants, and samples were incubated overnight at 4 °C. Antibody complexes were precipitated by incubating for 3 h at 4 °C with 20 μl of 50% protein G-Sepharose FF (Amersham Biosciences) in IP buffer. Prior to their use in CERK assay, the beads were pelleted and washed three times in IP buffer. For CERK assays the beads were repeatedly washed with phosphate-buffered saline to remove detergent and other reagents.

For use in Western blotting, the beads were taken up in 20 μl of SDS loading buffer containing 2% 2-mercaptoethanol, and the proteins were separated by SDS-PAGE according to the method of Laemmli (26). Proteins were transferred onto a polyvinylidifluoride membrane according to the method described by Towbin et al. (27). After a 1-h incubation with 3% skim milk in Tris-buffered saline containing 0.2% Tween 20 (T-TBS), the membrane was incubated with antibodies at 4 °C overnight. After a wash with T-TBS, the membrane was incubated for 2 h with horseradish peroxidase-conjugated anti-rabbit IgG (Amersham Biosciences). After another wash with T-TBS, the membranes were developed using an ECL kit (Amersham Biosciences) as recommended by the manufacturer and were then visualized using x-ray film.

CaM Affinity Binding Assays—CHO cells overexpressing CERK or CERK mutants were harvested and lysed with lysis buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM MgCl2, 1 mM dithiothreitol, 0.5% Triton X-100, and Complete™ protease inhibitor mixture). After the lysates were diluted with 4 volumes of dilution buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM MgCl2, and 1 mM dithiothreitol), they were centrifuged for 1 h at 4 °C to remove debris. The supernatants (50 μl) were added to 50 μl of CaM-Sepharose 4B beads that had been equilibrated with binding buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM MgCl2, and 0.1% Triton X-100) containing 2 mM CaCl2 or 4 mM EGTA, and the mixture was incubated for 1 h at 4 °C. Unbound proteins were removed by washing three times with binding buffer. Proteins bound to the CaM-Sepharose beads were then analyzed by Western blotting as described above. The blot was probed with an anti-CERK antibody that had been previously prepared (21).

Degranulation Assays—RBL-2H3, -CK3, or -CK4 cells were harvested and washed with Tyrode’s buffer. After a 10-min preincubation in the presence or absence of the indicated concentration of W-7 or KN-93, the cells were stimulated at 37 °C with 0.1 μM A23187 for 30 min. Cells were then centrifuged at 800 × g for 5 min, and the β-hexosaminidase activity was measured in both the supernatant and cell pellet, using p-nitrophenyl N-acetyl β-D-glucosaminide (Sigma) as a chromogenic substrate (28). Degranulation was expressed as a percentage of the β-hexosaminidase activity released into the supernatant.
CaM Involved in Ca\(^{2+}\) Activation of CERK

**RESULTS**

CaM Is Involved in the Ca\(^{2+}\)-dependent Activation of CERK and the Ca\(^{2+}\)-dependent Formation of C1P—We previously reported that CERK is involved in Ca\(^{2+}\)-dependent degranulation in mast cells and that C1P elevation following CERK activation is required for the elevation of intracellular Ca\(^{2+}\) (20). This suggests that the activation of CERK by Ca\(^{2+}\) is required for degranulation. Sugiura et al. (11) previously reported that nanomolar-order concentrations of Ca\(^{2+}\) were sufficient to activate CERK in vitro. However, in our previous studies, immunoprecipitated CERK required micromolar-order concentrations of Ca\(^{2+}\) for activation. The major difference between these two experiments was the source of the enzyme. Sugiura used whole cell lysates from CERK-overexpressing cells, whereas we used the immunoprecipitated enzyme. These facts suggested the presence of a cofactor for CERK that enhances its Ca\(^{2+}\) sensitivity, a cofactor that is lost during the immunoprecipitation.

CaM is a well known Ca\(^{2+}\) sensor that modulates the activity of a number of metabolic enzymes (21). We investigated whether CaM might be involved in the Ca\(^{2+}\) activation of CERK, initially by examining the effects of the CaM antagonist W-7 on CERK activity in vitro. We used a previously established cell line, RBL-CK3, that stably expresses CERK and displays an activity ~250 times greater than that of its parent RBL-2H3 cells (20). Enzymes were prepared from the RBL-CK3 cells as whole cell lysates (lysate-CERK) or immunoprecipitated CERK. As shown in Fig. 1, W-7 had almost no effect on the activity of the immunoprecipitated CERK, whereas it decreased the CERK activity in the lysate in a dose-dependent manner. W-7 is known to bind to CaM and to inhibit Ca\(^{2+}\)/CaM-regulated enzyme activity. It is possible that the immunoprecipitation process might cause the displacement of CaM associated with the CERK, in which case the W-7 would have no effect on the activity. However, in the enzyme assays of the cell lysate, W-7 increasingly excluded the binding of Ca\(^{2+}\) from Ca\(^{2+}\)/CaM-CERK complexes, resulting in a dose-dependent decrease in the Ca\(^{2+}\)-dependent activation of CERK. Even in excess Ca\(^{2+}\), the presence of W-7-CaM complexes might interfere with the association of CERK and Ca\(^{2+}\). The baseline activity (determined in the absence of Ca\(^{2+}\)) of the lysate CERK was actually greater than that of the immunoprecipitated CERK, but it exhibited only moderate activation by exogenous Ca\(^{2+}\) compared with the immunoprecipitated CERK, suggesting that endogenous Ca\(^{2+}\)/CaM complexes had already activated the CERK in the lysate to some extent.

To further evaluate the possible involvement of CaM in the activation of CERK, purified CaM from bovine brain was added to reaction mixtures of the immunoprecipitated CERK, and CERK assays were performed. Free Ca\(^{2+}\) concentrations were adjusted using a CaCl\(_2\)/EGTA mixture as calculated by the MAXChelator program and adjusted by adding CaCl\(_2\) and EGTA. The CERK activity was measured in the presence of 4 \(\mu\)M CaM (black bars) or 0.4 \(\mu\)M CaM and 50 \(\mu\)M W-7 (gray bars) or the absence of both (white bars). CERK activity is expressed as a percentage compared with the absence of Ca\(^{2+}\), CaM, and W-7. B, RBL-CK3 cells were labeled by incubation with \([^{32}\text{P}]\)orthophosphoric acid and then stimulated with the calcium ionophore A23187 to activate the enzyme. The amount of C1P elevation induced by 0.1 \(\mu\)M A23187 is expressed as 100%.

Next, we examined whether the intracellular C1P formation would be affected by W-7. In a previous report, we showed that C1P formation in RBL-CK3 cells could be induced by the calcium ionophore A23187 (20). Therefore, RBL-CK3 cells were preincubated with \([^{32}\text{P}]\)orthophosphoric acid for 90 min and then stimulated for 30 min with 0.5 \(\mu\)M A23187. W-7 treatment decreased the observed Ca\(^{2+}\)-dependent formation of \([^{32}\text{P}]\)C1P in a dose-dependent manner (Fig. 2B). This is consistent with the hypothesis that CaM acts as a calcium sensor for CERK.

Ca\(^{2+}\)-dependent Association of CERK with CaM—To examine whether the CaM is bound directly to CERK, co-precipitation assays were performed using CaM-Sepharose 4B. Lysates from CHO cells expressing CERK were incubated with CaM-Sepharose 4B in the presence of 1 mM CaCl\(_2\) or 2 mM EGTA. CERK was co-precipitated with CaM-Sepharose 4B only in the presence of Ca\(^{2+}\) (Fig. 3A). This result supports the contention that CaM binds to CERK and that this binding is Ca\(^{2+}\)-dependent.

Next, we examined whether endogenous CaM is also bound to CERK in situ. CERK was overexpressed in CHO cells, and the cells were treated with the cleavable protein-protein cross-linker, dimethyl 3,3-dithiobispropionimidate. CaM was immunoprecipitated using an anti-CaM antibody, the cross-linker was cleaved with 2-mercaptoethanol, and the sample was processed for Western blotting. CERK was co-immunoprecipitated with CaM only when the anti-CaM antibody was added (Fig.
as well as endogenous CaM binds to CERK in a Ca\(^{2+}\) dependent manner.

**Identification of the CaM Binding Site in CERK**—To determine the CaM binding domain in CERK, we prepared a series of truncation mutants of CERK as shown in Fig. 4. Each mutant was expressed in CHO cells, and cell lysates were incubated with CaM-Sepharose in the presence of CaCl\(_2\) or EGTA. Full-length CERK (FL) and the dC450 mutant both bound CaM in the presence of Ca\(^{2+}\), whereas dC330 did not. The C-terminal fragment (dN330) was also able to bind CaM. Together, these results indicate that CaM binding to CERK is restricted to the C-terminal region between residues 330–450.

Most CaM targets contain one of two types of recognition motifs, a Ca\(^{2+}\)-independent IQ motif or one of the Ca\(^{2+}\)-dependent motifs 1-5-10 or 1-8-14 or its variants (21). Types 1-5-10 and 1-8-14 motifs are defined by amphipathic helices with a net positive charge, in which hydrophobic residues occur preferentially at positions 1-8-14 or its variants (21). Types 1-5-10 and 1-8-14 motifs are preferred CaM binding motifs. CaM binding activity of CERK, indicating that although these amino acids might not be important for CaM recognition, they are important for maintaining the helical structure of the CaM binding site.

We also generated the C429R mutation, which had the most significant impact on the CaM binding of the full-length CERK (Fig. 5A). The CaM binding activity of this mutant was greatly reduced compared with that of wild type CERK (Fig. 5C). Furthermore, the activities of C431R and L435R (not shown) were greatly reduced in comparison to that of the wild type CERK (Fig. 5D). Although diminished, weak binding of CaM to C431R remained detectable (Fig. 5C, lane C). This may indicate the presence of yet another CaM binding site, although the binding was much weaker compared with the full-length construct, suggesting that most of the binding occurs in the region from amino acids 422 to 435. Thus, this region can be considered the CaM binding site in CERK.

To evaluate the role of this CaM binding domain in vivo, we examined whether the CaM binding domain could inhibit Ca\(^{2+}\)-dependent formation of C1P. As shown in Fig. 5E, when the cells were transfected with FL and empty vector, calcium ionophore A23187 increased intracellular C1P contents efficiently (p >0.005 by analysis of variance). These results agreed with our previous report (20) and suggested that CERK was activated by intracellular Ca\(^{2+}\). However, when the cells were transfected with FL and dN330, which contained the CaM binding domain
CaM Involved in Ca²⁺ Activation of CERK

**FIGURE 5. Analysis of the CaM binding site of CERK.** A, identification of the CaM binding site in CERK. The point mutants dN330L422R, dN330F429R, dN330F431R, and dN330L435R, and dN330L422R/F429R were constructed, and CaM-Sepharose co-sedimentation assays were performed as in Fig. 3A, B, helical wheel projection of amino acids 420–437 in mouse CERK. Amino acids and their position numbers are presented with hydrophobic amino acids shown as white circles, basic amino acids as gray circles, and hydrophilic amino acids as black circles. The arrow indicates the hydrophobic moment. C, CaM binding experiment comparing full-length CERK and a point mutant, FL-F431R. The gene construction and CaM-Sepharose co-sedimentation assays were performed as under “Experimental Procedures” and Fig. 3A, D, the CERK activities of FL, FL-F431R, and vector-transfected CHO cells. E, the expression of the CaM binding peptide dN330 interrupted Ca²⁺-dependent C1P formation. The CERK (FL) were co-transfected with empty vector or dN330 to human embryonic kidney 293FT cells. The cells were labeled by incubation with [⁴⁰⁰P]orthophosphoric acid for 90 min and then incubated with or without the calcium ionophore A23187 for 30 min. [⁴⁰⁰P]C1P was then extracted and quantified as detailed under “Experimental Procedures.” The amount of C1P obtained from the cells transfected with both FL and empty vector is expressed as 100%. The results are expressed as mean ± S.D. (analysis of variance; *, p < 0.005 for treatment of A23187 versus no treatment of A23187 in FL and empty vector transfected cells).

(Fig. 5A), calcium ionophore A23187 failed to increase intracellular C1P contents, suggesting that dN330 could saturate the CaM and hence block Ca²⁺-induced activation of CERK. These results indicate that the CaM binding domain of CERK indeed interacts with Ca²⁺/CaM and that this interaction is important for the Ca²⁺-dependent regulation of CERK.

**DISCUSSION**

CaM can bind with high affinity to a relatively small α-helical region of amino acids, called an amphipathic helix, in many proteins. The binding mechanism between CaM and its target proteins is roughly classified as hydrophobic interaction. To determine the CaM binding site in the CERK protein, we introduced mutations to disrupt the helical structure and the hydrophobicity of its amphipathic helix. These mutants lost their CaM binding ability, allowing us to determine the CaM binding site. The disruption of the CaM binding also affected enzymatic activity, so we were unable to establish the precise connection between the CaM binding and the CERK activity in vitro. However, when the cells were pretreated with the calcium ionophore A23187 for 30 min. [⁴⁰⁰P]C1P was then extracted and quantified as detailed under “Experimental Procedures.” The amount of C1P obtained from the cells transfected with both FL and empty vector is expressed as 100%. The results are expressed as mean ± S.D. (analysis of variance; *, p < 0.005 for treatment of A23187 versus no treatment of A23187 in FL and empty vector transfected cells).
transfected with dN330 that contained the CaM binding domain, Ca\(^{2+}\)-dependent formation of C1P was inhibited, suggesting that dN330 could saturate the CaM and hence block Ca\(^{2+}\)-induced activation of CERK. Our results firmly establish that CaM is involved in Ca\(^{2+}\)-dependent CERK activation.

In the presence of Ca\(^{2+}\), the activity of the immunoprecipitated CERK was not affected by W-7 treatment (Fig. 1), indicating that activation independent of CaM can also occur. Regardless of the stimuli though, the range of intracellular concentrations of Ca\(^{2+}\) rises to several micromoles. Thus, under restricted intracellular conditions, CaM can facilitate CEM-dependent Ca\(^{2+}\) ionophore-induced C1P formation was greatly decreased with W-7 treatment (Fig. 2B), indicating that the contribution of CaM is more critical than was previously perceived from the in vitro experiment.

CERK was originally cloned based on sequence homology to sphingosine kinase 1 (SPHK1) (11). Recently, translocation of SPHK1 was greater than was previously perceived from the topology has been determined (35–41). Of the Cer metabolic enzymes, ceramidase, glucosyltransferase, and sphingomyelin synthase have long been known to be regulated by Cer (4, 32–34). Thus, the control of intracellular Cer levels is very important in many signaling pathways. Ceramidase, glucosyltransferase, and sphingomyelin synthase have long been known to metabolize Cer. These enzymes have been cloned, and their catalytic topology has been determined (35–41). Of the Cer metabolic enzymes, only CERK seems to be able to metabolize Cer in the cytosolic region. The above-mentioned potential targets of Cer (protein kinase C, phosphatidylinositol 3-kinase, phospholipase D, ceramide-activated protein kinase, and ceramide-activated phosphatase) are all cytosolic proteins. Considering these facts, it is plausible that CERK acts as a key enzyme in the regulation of intracellular Cer content. Unlocking the mechanism of its activation through Ca\(^{2+}\)/CaM, as reported here, advances our understanding of this enzyme and will facilitate future study into the function of intracellular Cer and C1P as second messengers.

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