The interaction between the pleckstrin homology domain of ceramide kinase and phosphatidylinositol 4,5-bisphosphate regulates the plasma membrane targeting and ceramide 1-phosphate levels

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Abstract

Ceramide kinase (CERK) converts ceramide (Cer) to ceramide-1-phosphate (C1P), which has recently emerged as a new bioactive molecule capable of regulating diverse cellular functions. The N-terminus of the CERK protein encompasses a sequence motif known as a pleckstrin homology (PH) domain. Although the PH domain was previously demonstrated to be an important domain for the subcellular localization of CERK, the precise properties of this domain remained unclear. In this study, we reveal that the PH domain of CERK exhibits high affinity for phosphatidylinositol (4,5) bisphosphate $(PI(4,5)P_2)_{[0]}$, among other lipids. Furthermore, in COS7 cells, GFP-fused CERK translocated rapidly from the cytoplasm to the plasma membrane in response to hyper-osmotic stress, which is known to increase the intracellular $PI(4,5)P_2$ levels, whereas a PH-domain deletion mutant did not. Additionally, in [^{32}P]orthophosphate-labeled COS7 cells, the translocation of CERK to the plasma membrane induced a 2.8 fold increase in C1P levels. The study presented here provides insight into the crucial role of the $CERK_{[0]}$-PH domain in plasma membrane targeting, through its binding to $PI(4,5)P_2$, and subsequent induction of C1P production in the vicinity of the membrane

Keywords: Ceramide kinase; Ceramide; Ceramide 1-phosphate; Pleckstrin homology domain; $PI(4,5)P_2$

Sphingolipids are ubiquitous constituents of eukaryotic cells that have essential roles in cell growth, survival and death [1, 2]. Their metabolites such as sphingosine (Sph), sphingosine 1-phosphate (S1P), ceramide (Cer), and ceramide 1-phosphate (C1P) are involved in signaling pathways that control various cell functions [3, 4, 5]. For instance Cer, the precursor for all sphingolipids, functions as a second messenger in a variety of cellular events including apoptosis and cell differentiation [6]. Cer can be also phosphorylated into C1P by Cer kinase (CERK) [7].

CERK activity was first identified in brain synaptic vesicles by Bajjalieh and co-workers [8], and was subsequently found in human leukemia (HL-60) cells [9]. A decade later, CERK was cloned based on its sequence homology to sphingosine kinase (SPHK) type 1 [10]. Both C1P and CERK have been implicated in the regulation of vital cellular processes, such as cell proliferation [11, 12], apoptosis [13], phagocytosis [14, 15] and inflammation [16, 17]. Recently, we demonstrated that CERK and C1P are required for the degranulation process in mast cells [18], and that this process is dependent on calmodulin [19].

Although it shares sequence homology with SPHKs, CERK differs significantly in its N-terminus. The N-terminus of CERK is myristoylated [20], which has little apparent effect on localization or enzyme activity [20], and contains a

pleckstrin homology (PH) domain [10]. The PH domain, a 120-amino acid residue stretch originally identified in pleckstrin but found in over 100 diverse proteins, has been the focus of extensive structural and functional studies [21-24]. Very recently, we demonstrated that the PH domain of CERK, especially the Leu10 residue, is not only indispensable for the enzyme's activity but also acts as a regulator of that activity [25]. To date, however, the precise target and roles of the PH domain in CERK have not been well characterized.

Most PH domain-containing proteins can bind with high specificity and affinity to certain phosphatidylinositol phosphate (PIP) lipids [26], a function generally attributed to the PH domain. Here, we show that the plasma membrane targeting of CERK is induced by the interaction of the PH domain and $PI(4,5)P_2$, and that the subsequent translocation of CERK enhances the formation of C1P in the vicinity of plasma membrane

Materials and methods

Cell culture and transfection

COS7 and HEK293 cells were obtained from ATCC. Cells were maintained on collagen-coated dishes in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS), 100 U/ml penicillin and 100 ng/ml streptomycin, at 37 °C in an atmosphere containing 5% CO_2. Cells were transiently transfected with plasmids using the Lipofectamine plus reagent, according to the instructions from the manufacturer (Invitrogen). Cells were then cultured for 24 h in the same DMEM medium and lysed in buffer containing 10 mM HEPES (pH 7.5), 2 mM EGTA, 1 mM dithiothreitol, 40 mM KCl, and Complete^{TM} protease inhibitor (Roche Diagnostics, Mannheim, Germany) as described previously [25]. Protein concentrations were measured using a BCA Protein Assay Reagent Kit (Pierce Biotechnology).

Construction of ceramide kinase mutants

A plasmid of the GST-fused PH domain of PLC-δ1 (GST-PH-PLC-δ1) was kindly provided by Dr. T. Takenawa (University of Tokyo, Tokyo, Japan). Constructs of wild-type CERK were generated in pcDNA3 (Invitrogen) vectors, as described

previously[0] [25]. The coding regions for the constructs were amplified by PCR from CERK cDNA, incorporating a *Bam*HI site at the end corresponding to the N-terminus and an *Eco*RI site at the opposite end. Appropriately digested PCR products were then subcloned into pcDNA3-FLAG and GST-tagged pGEX-4T3 (Amersham Biosciences) using the *Bam*HI/*Eco*RI sites. GFP-CERK and GFP-ΔPH-CERK constructs were cloned into N-terminally GFP-tagged EGFP-C1 (CLONTECH) at *Kpn*I/*Bam*HI restriction sites to generate the GFP-fusion constructs. The identity of each insert was confirmed by dyedeoxynucleotide sequencing.

Lipid overlay assay

To assess the PIP-binding properties of CERK, a protein-lipid overlay assay was performed using commercially available PIP membranes (Echelon Bioscience Inc.). HEK293 cells were transiently transfected with either FLAG or FLAG-tagged CERK. The cells were lysed for 10 min at 4 ºC in IP buffer (50 mM Tris-HCl (pH 7.2), 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1% Tween 20 and Complete^{TM} protease inhibitor (Roche Diagnostics)). After a 5 min centrifugation at 16,000 X g, the cell lysates (100 μg) were incubated with anti-FLAG antibodies (1:200; Sigma) overnight at 4 ºC with gentle rocking. Immunocomplexes were precipitated by 3 h incubation at 4 ºC with

protein A/G-agarose (Amersham Biosciences) in IP buffer. The beads were washed three times in IP buffer. The PIP strip membranes were blocked for 1 h in 3% fatty acid-free bovine serum albumin (BSA) in TBS/T (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.1% Tween 20), then incubated overnight at 4 ºC with one of the FLAG-tagged proteins (1 μg/ml) diluted in TBS/T. The membranes were washed six times over 30 min in TBS/T, then incubated for 1 h with a 1:1000 dilution of anti-CERK polyclonal antibody [18]. The blots were washed with TBS/T, then incubated with a 1:5000 dilution of anti-rabbit IgG F(ab_)$_2$ fragment (Amersham Biosciences). The proteins were detected using an enhanced chemiluminescence (ECL) western blotting detection kit (Amersham Biosciences).

In vitro lipid binding assay

In vitro lipid binding assay was performed as previously described [27]. Briefly, lipid vesicles (phosphatidylethanolamine (PE)/ phosphatidylcholine (PC) = 1/1; total 2 μg) containing 10% synthetic phosphoinositides with C16 palmitic acids (Sigma) and resuspended in ethanol, were coated on a 96-well microtiter plate and dried at room temperature. The wells were blocked with phosphate-buffered saline (PBS) containing 5% BSA. GST fusion proteins, expressed and purified as described previously [27],

were diluted to 1.0 mg/ml in PBS containing 5% BSA. The proteins were added to the wells, and incubated for 45 min. After washing with PBS containing 0.05% Tween 20, the wells were incubated for 30 min with 200 μl glutathione horseradish peroxidase conjugate (Sigma) diluted to 1 μg/ml in PBS containing 5% BSA. The plate was washed with PBS containing 0.05% Tween 20, and the bound proteins were detected using the substrate o-phenylenediamine dihydrochloride (OPD) (Sigma). The colorimetric reaction was measured at 492 nm in an ELISA plate reader (Bio-Rad).

Fluorescence microscopy

Cells were transfected with the GFP constructs and after 24 h, were replated into sterile glass dishes (Iwaki). COS7 cells were allowed to adhere and grow another 24 h, then the cells were serum-starved for 6 h , to induce hyper-osmotic stress, and incubated with sorbitol in DMEM (final concentration: 1 M). Cells were examined using an inverted LSM510 confocal laser scanning microscope (Carl Zeiss).

Ceramide kinase assay

COS7 cells were transfected with an empty vector or a pcDNA3 expression vector encoding wild-type CERK. After 24 h, the cells were serum-starved for 6 h

then incubated for 20 min with sorbitol in DMEM (final concentration 1 M) and lysed in buffer (10 mM HEPES (pH 7.5), 2 mM EGTA, 1 mM dithiothreitol, 40 mM KCl, and Complete^{TM} protease inhibitor). The protein concentrations of the lysates were determined with a BCA Kit. The CERK activity of each cell lysate was assayed as described previously [25], using ceramide (C18:0, d18:1; Avanti Polar Lipids) and [γ-^{32}P] ATP (Perkin-Elmer Life Sciences) as substrates. The lipids were 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.

To determine the effect of ceramide-PI or ceramide-PI(4,5)P2 micelles on the enzyme activity, 40 μM of ceramide, in lysis buffer to final concentrations of 0, 1, 10 and 100 nM of PI or PI(4,5)P_2, was added to lysates prepared from COS7 cells expressing wild-type CERK, and assayed as above. The labeled C1P was quantified using a BAS 2500 imaging analyzer (Fuji Photo Film). A unit of CERK activity was defined as the amount of enzyme required to produce 1 pmol of C1P per min.

Metabolic labeling of cells

COS7 cells were incubated at 37 °C for 90 min with 2 μCi of carrier-free [^{32}P]orthophosphoric acid (PerkinElmer Life Sciences) in Eagle's minimal essential

medium without free phosphate. After incubation, the cells were washed twice with serum-free medium then were stimulated at 37 °C with 1 M sorbitol for 20 min. Reactions were terminated by adding 7 volumes of chloroform/methanol (1:1, v/v). Two phases were generated by adding 1.6 volumes of 1 M KCl. The organic phase was dried and subjected to a mild alkaline treatment to remove glycerophospholipids as described [9]. Lipids were re-extracted by the method of Bligh and Dyer [28]. [^{32}P]C1P was detected by HPTLC using the same method as described above.

Results

CERK interacts directly with PI(4,5)P_2 through the PH domain

In a previous study, we reported the cytosolic distribution of CERK in mast cells, as well as some membrane expression in RBL-2H3 cells [18]. Additionally, Carre et al. found that deleting the N-terminal pleckstrin homology (PH) domain in CERK altered its location from the perinuclear region to the homogeneous cytoplasmic region of COS1 and HUVEC cells [20]. We obtained similar results using wild-type CERK transfected COS7 cells, but localization at the plasma membrane was also observed (Fig.1, *arrow*). These observations promoted us to investigate in detail the mechanism behind the altered subcellular distribution of CERK.

PH domains are known to regulate the membrane association or activity of proteins through high affinity binding to PIPs [24, 29, 30]. We therefore examined the affinity of CERK to PIPs of varying sizes, by performing a protein-lipid overlay assay. CERK bound to most of the PIPs tested, whereas this enzyme did not bind to lysophosphatidic acid (LPA), lysophosphocholine (LPC), sphingosine 1-phosphate (S1P), phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidic acid (PA), or phosphatidylserin (PS) (Fig. 2A). To investigate this interaction in more detail, *in vitro* lipid binding assays were performed with purified GST-fusion proteins of the

full length CERK (GST-CERK) and its PH domain (GST-PH-CERK) [27]. GST alone (GST) and a GST fusion protein of the PH domain of PLC-δ1 (GST-PH-PLC-δ1) were successfully used as a negative (Fig. 2B, *a*) and positive control (Fig. 2B, *b*), respectively. GST-CERK again bound to all of the PIPs tested (Fig. 2B, *c*). GST-PH-CERK was found to bind with the highest affinity to $PI(4,5)P_2$ (Fig. 2B, *d*), implicating that this interaction may be very important for the intracellular localization of CERK.

Hyper-osmotic stress induces the translocation of CERK to the plasma membrane

The intracellular content of PIPs is known to be altered by osmotic swelling [31, 32]. To investigate whether the localization of CERK changes in response to $PI(4,5)P_2$ elevation, we examined GFP-tagged CERK (GFP-CERK) or ΔPH-CERK (GFP-ΔPH-CERK) in transfected COS7 cells under hyper-osmotic conditions. Treatment of the GFP-CERK-transfected COS7 cells with 1 M sorbitol induced the translocation of GFP-CERK to the plasma membrane, especially at the perinuclear region, within 15 min (Fig. 3A, *arrow*). However, no similar change was observed in the distribution of the GFP-ΔPH-CERK (Fig. 3B). These results indicate that the interaction between the CERK PH domain and $PI(4,5)P_2$ could mediate CERK translocation.

Intracellular C1P levels are increased by hyper-osmotic stress

Next, we tested whether the translocation of CERK leads to elevated C1P levels in cells. Hyper-osmotic stress induced a 2.8 fold increase in the [^{32}P]C1P levels in the CERK-transfected COS7 cells after 20 min (Fig. 4), yet no increase was observed in the ΔPH-CERK-transfected cells (data not shown). These results indicate that the localization of CERK to the plasma membrane is crucial for its C1P formation.

To evaluate whether the increases in C1P observed during hyper-osmotic stress were due to enhanced kinase activity, an *in vitro* kinase assay was performed utilizing transiently wild-type CERK-transfected COS7 cells. The enzyme activity exhibited a weak and insignificant increase (from 100% to 118%) in response to the hyper-osmotic challenge (Fig. 5A). Additionally, we examined whether a direct interaction between the PH domain and PI(4,5)P_2 enhances the activity of CERK. COS7 cells transiently expressing CERK were lysed and analyzed for CERK activity using Cer-PI or Cer-PI(4,5)P_2 liposomes as the substrate. As shown in Fig. 5, the content of the substrate complex had almost no affect on the enzyme activity. These results suggest that the interaction of the PH domain of CERK with PI(4,5)P_2 may not directly contribute to the increase in C1P formation observed with hyper-osmotic stress. The translocation might facilitate CERK to interact with their substrates.

Discussion

Since Bajjalieh *et al.* first reported that CERK activity was co-purified with synaptic vesicles [7], the CERK protein has been considered to be membrane-associated. However, in a recent study, we suggested that CERK is principally a cytosolic protein, mediating Ca^{2+}-dependent degranulation in RBL2H3 cells [18]. In another report, Carre *et al.* suggested that the CERK is associated with the Golgi complex in COS1 and HUVEC cells [20]. Together, these studies indicate that CERK localization varies in different cell types or under different conditions. The structure of CERK, which is not shared by its SPHK homologues, includes a PH domain (amino acids 8-126) with short N-terminal extensions [10]. Carre *et al.* further demonstrated that deletion of the PH domain alters the localization of CERK [20]. This suggests the possibility that the subcellular distribution of CERK is determined by the action of this domain.

In the current study we have focused on the functional role of the PH domain of CERK. PH domains have been detected in over 100 protein sequences [36]. Despite low sequence conservation, the general structures of characterized PH domains are extremely well conserved [37]. This domain can also mediate protein-protein interactions, for example that which occurs between the β adrenergic receptor protein kinase and the β/γ subunit of heterotrimeric G-proteins [38]. PH domains also share an

ability to associate with the plasma membrane and/or intracellular membranes through high affinity binding to particular PIP lipids [29, 39]. Our results here verify that the PH domain of CERK is also capable of interacting with PIP lipids. The GST fusion protein comprising the PH domain of CERK bound with the highest affinity to $PI(4,5)P_2$ (Fig. 2B). Considering the higher quantity of $PI(4,5)P_2$ in cells, compared to other PIPs, our results suggest that the binding of this lipid to the PH domain of CERK is important for membrane targeting. To examine this possibility further, we took advantage of the susceptibility of COS7 cells to hyper-osmotic challenge, which is known to induce $PI(4,5)P_2$ formation [33-35]. Hyper-osmotic stress did induce a 2.8 fold increase in the level of C1P in COS7 cells (Fig. 4), however, the CERK-$PI(4,5)P_2$ micelle failed to adversely affect CERK activity (Fig. 5B). Therefore, we concluded that the plasma membrane targeting of CERK is induced by the interaction of the PH domain and $PI(4,5)P_2$, and, consequently, the translocation of CERK enhances the formation of C1P in the vicinity of the plasma membrane. The translocation might facilitate CERK to interact with their substrates.

Shayman and co-workers reported that C1P is formed during phagocytosis and that it promotes the fusion of complex liposomes [14, 15]. We recently demonstrated that the calcium-dependent activation of CERK is involved in the degranulation

pathway, possibly through the fusion of the plasma membrane with the secretory granules in the mast cells [18]. The translocation of CERK and the subsequent C1P elevation in the plasma membrane might be important for the membrane fusion process. However, the extent of the involvement and any physiological roles of membrane-targeted CERK in such membrane fusion processes, including mast cell degranulation and phagocytosis will need to be examined in future studies.

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Figure legends

Fig. 1. ΔPH-CERK fails to localize in plasma membrane.

COS7 cells expressing either GFP-CERK or GFP-ΔPH-CERK were grown for 24 h on sterile glass dishes. Live cells expressing GFP fusion proteins were observed using an LSM510 confocal microscope (Zeiss).

Fig. 2. The PH domain of CERK interacts with PIPs.

(A) CERK binds to most PIPs. HEK293 cells were transiently transfected with a FLAG vector or a plasmid encoding Flag-tagged CERK, then lysed. Proteins were immunoprecipitated with an anti-FLAG monoclonal antibody, then incubated with PVDF membranes containing the indicated phospholipids (100 pmol per spot). The membranes were washed, and the PIP-bound CERKs were detected using an anti-CERK polyclonal antibody and an enhanced chemiluminescence (ECL) kit. (B) The PH domain of CERK binds with high affinity to $PI(4,5)P_2$. (a-d) Lipid vesicles (PE/PC = 1/1; total 2 μg) containing 10% synthetic phosphoinositides with C16 palmitic acids were coated on a 96-well microtiter plate. Plates were then overlaid with 1.0 μg/ml GST (a, *negative control*), GST-PH-PLC-δ1 (b, *positive control*), GST-CERK (c), or GST-PH-CERK (d). The bound proteins were detected by incubating with 200 μl glutathione-

HRP (1 μg/ml in PBS containing 5% BSA) and developing with o-phenylenediamine dihydrochloride (OPD). The plates were measured using an ELISA plate reader (492 nm). Results from three independent experiments are represented as mean values ± S.D.

Fig. 3. Hyper-osmotic stress induces the translocation of CERK to the plasma membrane.

COS7 cells were transiently transfected with plasmids encoding either GFP-CERK (A) or GFP-ΔPH-CERK (B) and grown for 24 h on a sterile glass dish. The cells were serum-starved for 6 h and then incubated with sorbitol in DMEM (final concentration 1 M). Live cells expressing GFP fusion proteins were observed using an LSM510 confocal microscope (Zeiss). The *arrows* indicate the plasma membrane region of the cell.

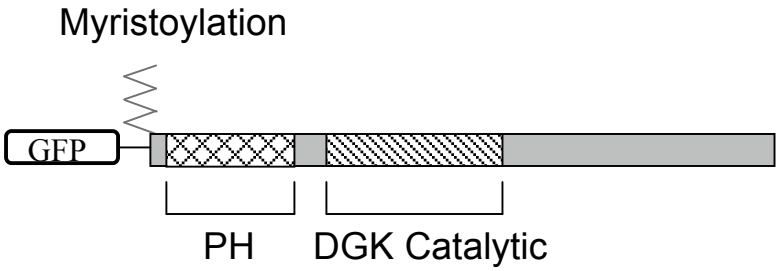
Fig. 4. Hyper-osmotic stress induces the increase of intracellular C1P content.

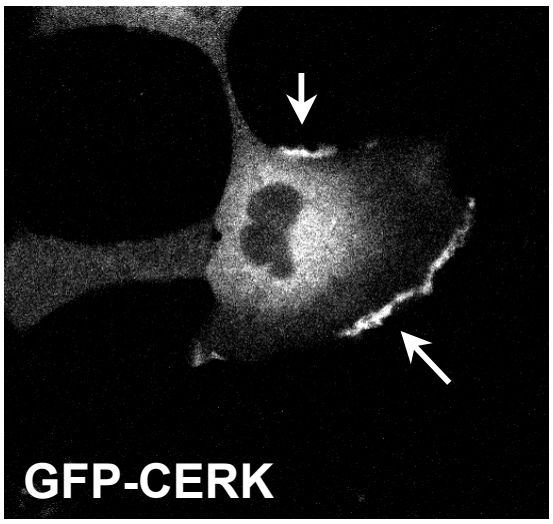
COS7 cells were transiently transfected with a plasmid encoding CERK. Twenty-four hours after transfection, cells expressing CERK were labeled for 90 min with 2 μCi of carrier-free [^{32}P]orthophosphoric acid and washed twice with serum-free medium. Labeled cells were stimulated with 1 M sorbitol for 20 min at 37 ºC. Lipids were

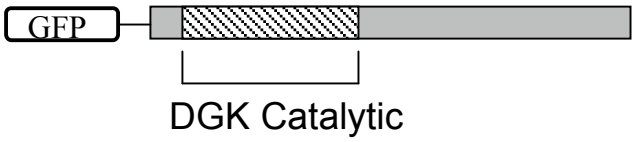
extracted and separated by thin-layer chromatography. [^{32}P]C1P was quantified using an image analyzer (BAS2500, Fuji Film). Results are each expressed as a percentage of the control and represent the mean ± S.D. of three experiments.

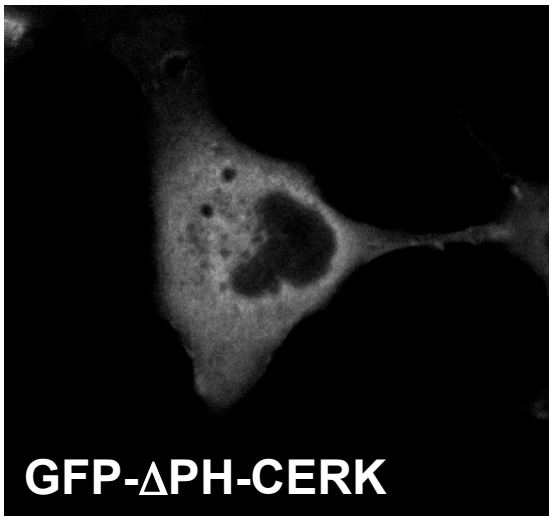
Fig. 5. Hyper-osmotic stress and PI(4,5)P_2 have no direct effect on the activity of CERK.

(A) COS7 cells were transiently transfected with an empty vector or a plasmid encoding CERK. Twenty-four hours after transfection, the cells were stimulated with 1 M sorbitol for 20 min, then harvested and lysed. *In vitro* CERK activities were determined as described in Materials and Methods. To analyze the enzyme activities, C18-Cer was used as a substrate with [γ-^{32}P] ATP. (B) COS7 cells were transiently transfected as in (A). Twenty-four hours after transfection, the cells were lysed and the enzyme activities were analyzed using [γ-^{32}P] ATP together with Cer-PI or Cer-PI(4,5)P_2 as the substrates. Results are each expressed as a percentage of the control and represent the mean ± S.D. of three experiments.



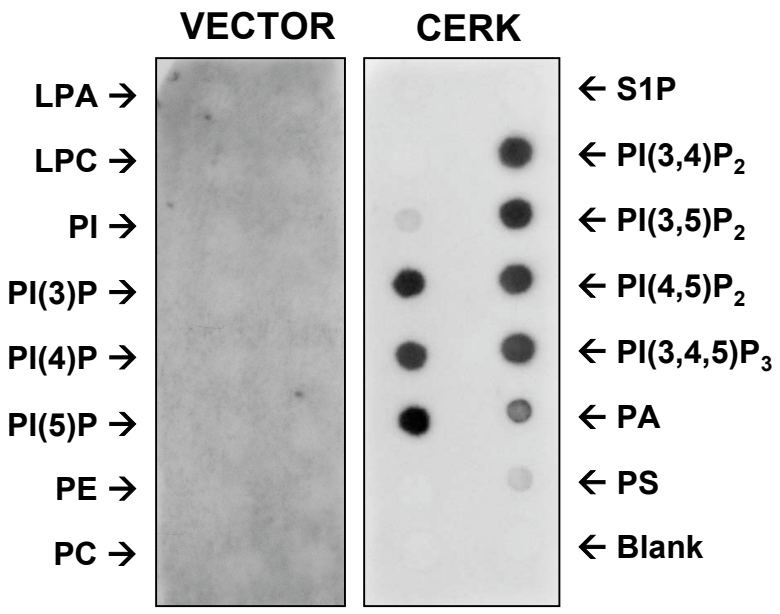






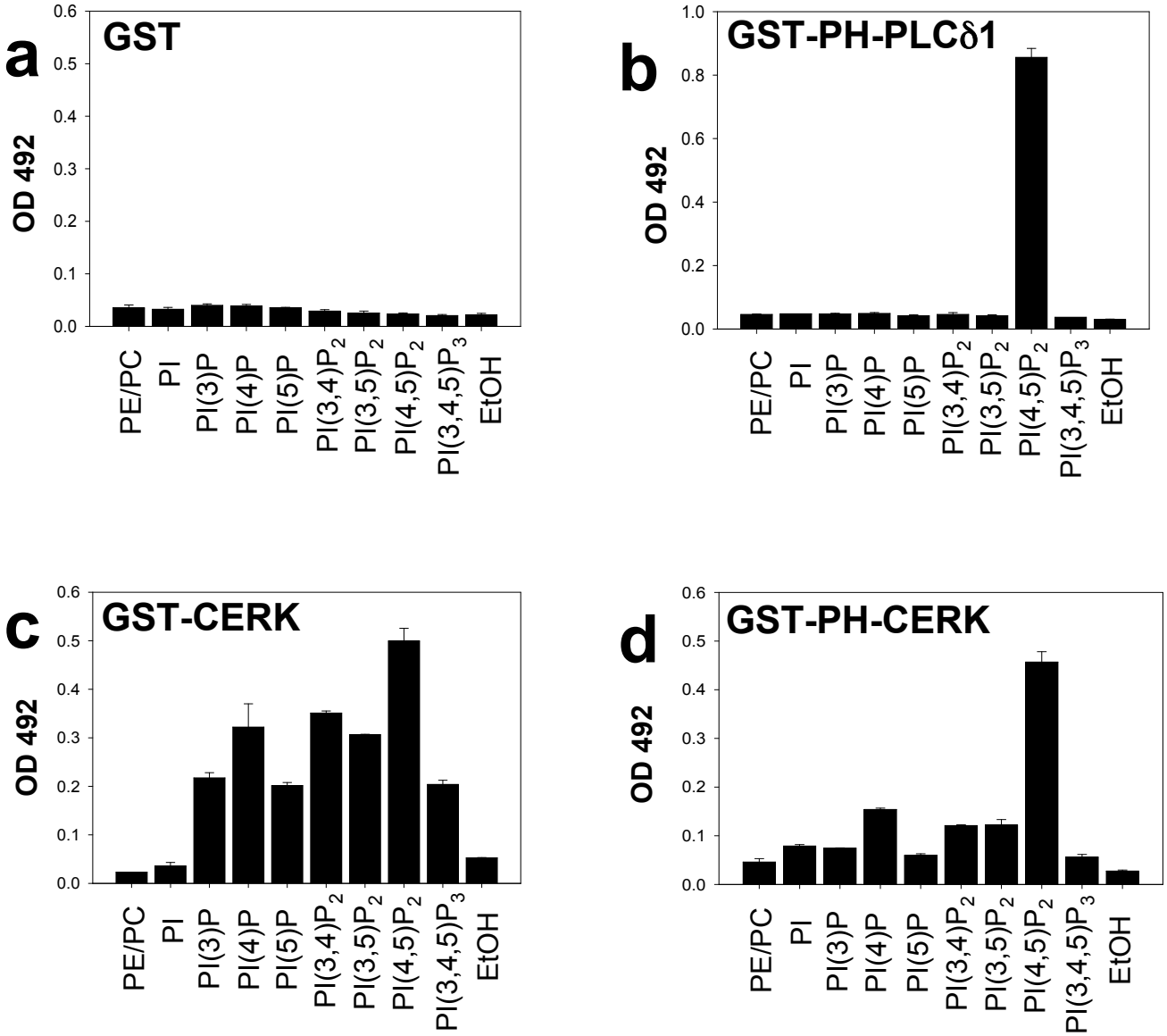
Kim et al. Fig. 1

A

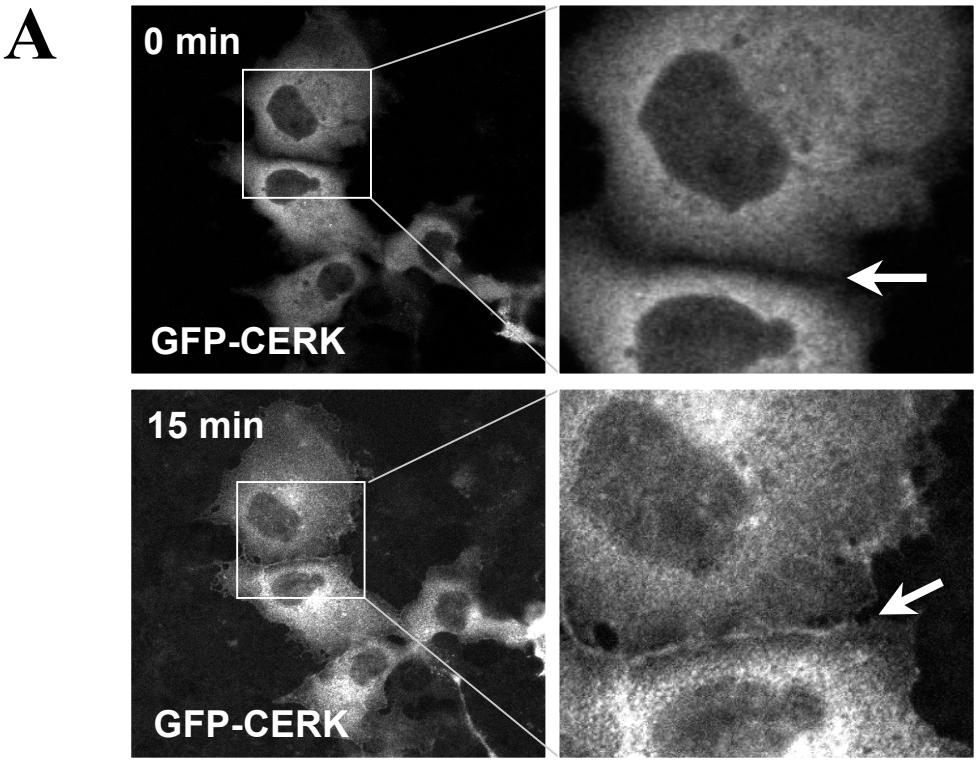


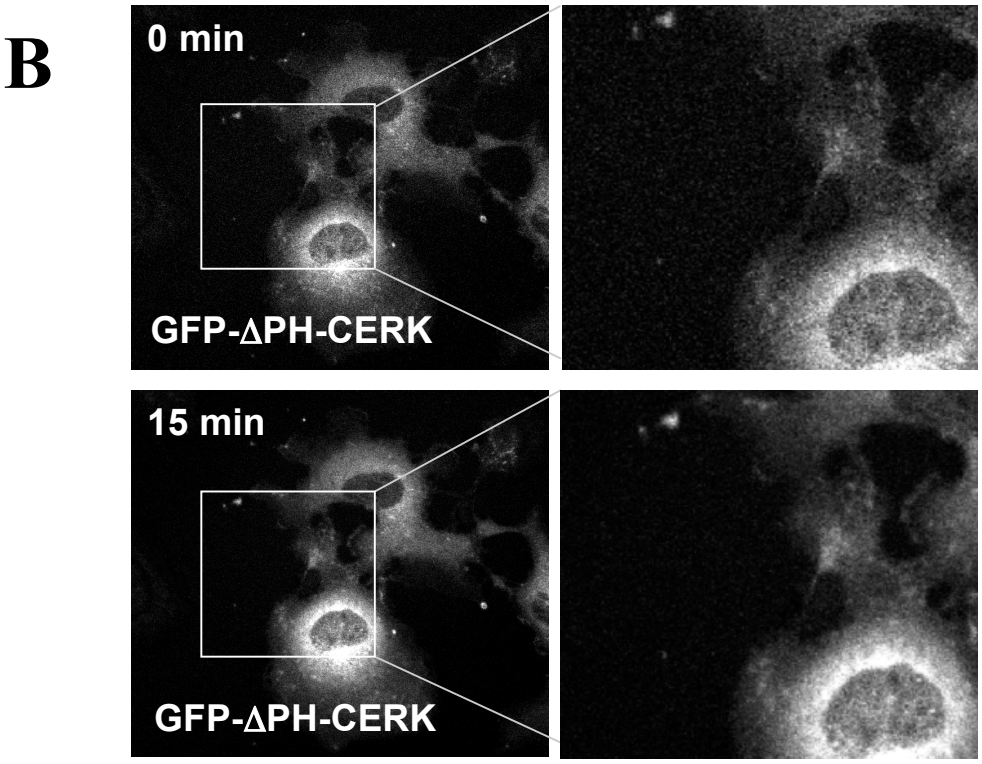
Kim et al. Fig. 2A

B

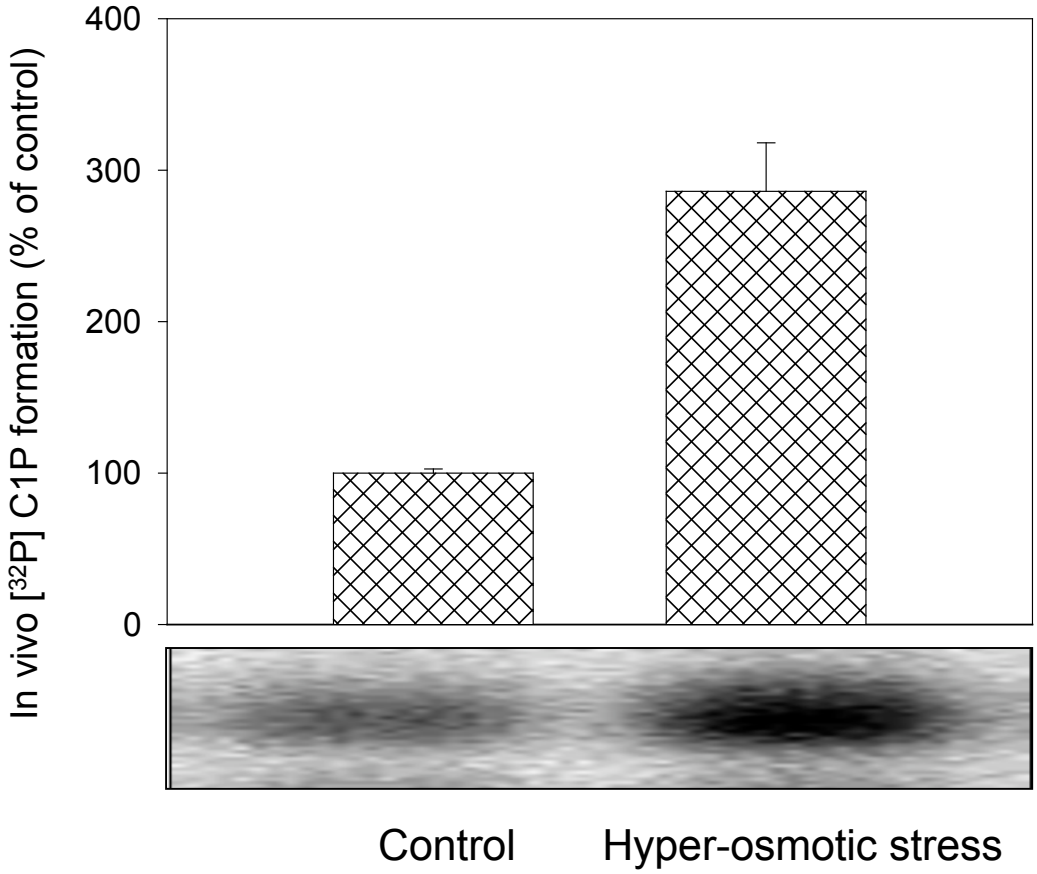


Kim et al. Fig. 2B



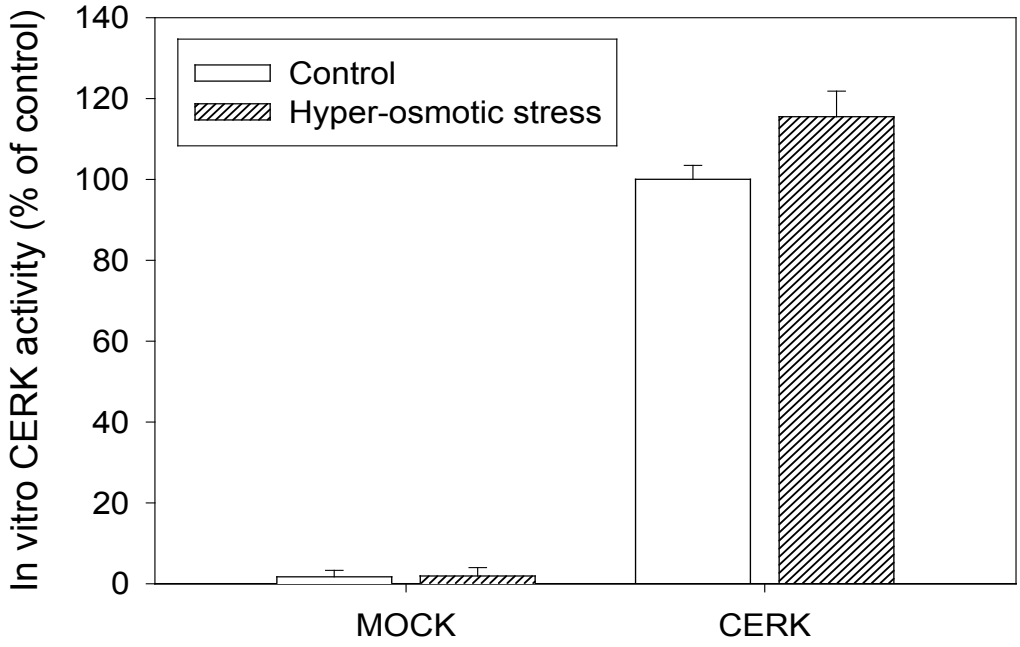


Kim et al. Fig. 3



Kim et al. Fig. 4

A



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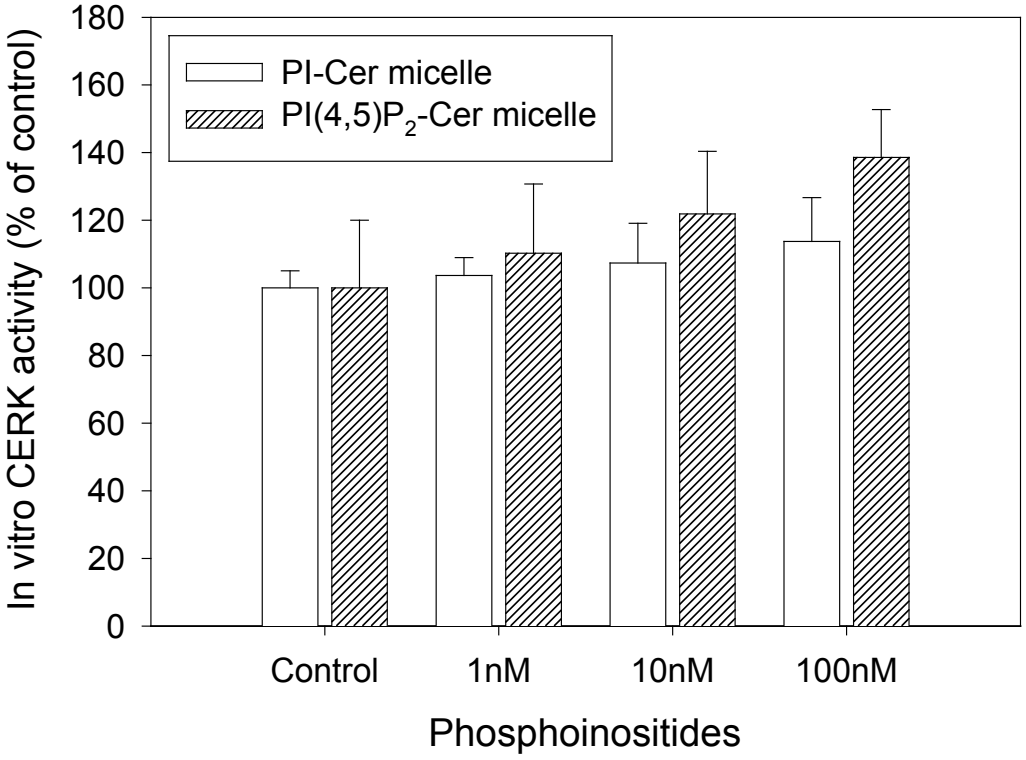

Kim et al. Fig. 5