

# The Pleckstrin Homology Domain of Diacylglycerol Kinase $\eta$ Strongly and Selectively Binds to Phosphatidylinositol 4,5-Bisphosphate\*

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Aiko Kume, Koki Kawase, Suguru Komenoi, Takako Usuki, Ena Takeshita, Hiromichi Sakai, and Fumio Sakane<sup>1</sup>

From the Department of Chemistry, Graduate School of Science, Chiba University, 1-33 Yayoi-cho, Inage-ku, Chiba 263-8522, Japan

Type II diacylglycerol kinase (DGK) isozymes ( $\delta$ ,  $\eta$ , and  $\kappa$ ) have a pleckstrin homology domain (PH) at their N termini. Here, we investigated the lipid binding properties of the PHs of type II DGK isozymes using protein-lipid overlay and liposome binding assays. The PH of DGK $\eta$  showed the most pronounced binding activity to phosphatidylinositol (PI) 4,5-bisphosphate (PI(4,5)P<sub>2</sub>) among the various glycerol- and sphingolipids including PI 3,4,5-trisphosphate, PI 3,4-bisphosphate, PI 3-phosphate, PI 4-phosphate, and PI 5-phosphate. Moreover, the PI(4,5)P<sub>2</sub> binding activity of the DGK $\eta$ -PH was significantly stronger than that of other type II DGK isozymes. Notably, compared with the PH of phospholipase C (PLC)  $\delta$ 1, which is generally utilized as a cellular PI(4,5)P<sub>2</sub> probe, the DGK $\eta$ -PH is equal to or superior than the PLC $\delta$ 1-PH in terms of affinity and selectivity for PI(4,5)P<sub>2</sub>. Furthermore, in COS-7 cells, GFP-fused wild-type DGK $\eta$ 1 and its PH partly translocated from the cytoplasm to the plasma membrane where the PLC $\delta$ 1-PH was co-localized in response to hyperosmotic stress in an inositol 5-phosphatase-sensitive manner, whereas a PH deletion mutant did not. Moreover, K74A and R85A mutants of DGK $\eta$ -PH, which lack the conserved basic amino acids thought to ligate PI(4,5)P<sub>2</sub>, were indeed unable to bind to PI(4,5)P<sub>2</sub> and co-localize with the PLC $\delta$ 1-PH even in osmotically shocked cells. Overexpression of wild-type DGK $\eta$ 1 enhanced EGF-dependent phosphorylation of ERK, whereas either K74A or R85A mutant did not. Taken together, these results indicate that the DGK $\eta$ -PH preferentially interacts with PI(4,5)P<sub>2</sub> and has crucial roles in regulating the subcellular localization and physiological function of DGK $\eta$ . Moreover, the DGK $\eta$ -PH could serve as an excellent cellular sensor for PI(4,5)P<sub>2</sub>.

Diacylglycerol kinase (DGK)<sup>2</sup> phosphorylates diacylglycerol to produce phosphatidic acid (1–5). Diacylglycerol (6–8) and phosphatidic acid (PA) (9, 10) are well recognized as lipid second messengers. DGK appears to participate in various physiological events by modulating the balance between the two bioactive lipids diacylglycerol and phosphatidic acid (1–5). Ten mammalian DGK isozymes ( $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$ ,  $\epsilon$ ,  $\zeta$ ,  $\eta$ ,  $\theta$ ,  $\iota$ , and  $\kappa$ ) containing two or three characteristic zinc finger-like C1 domains and a common catalytic region are divided into five groups (types I–V) according to their structural features (1–5).

Type II DGKs (11) consist of DGK $\delta$  (12),  $\eta$  (13), and  $\kappa$  (14). Moreover, alternative splicing products of DGK $\delta$  ( $\delta$ 1 and  $\delta$ 2) (15) and  $\eta$  ( $\eta$ 1 and  $\eta$ 2) (16) have been identified. Type II DGKs have a pleckstrin homology domain (PH) in common at their N termini and a catalytic domain that is divided into two subdomains (catalytic subdomains a and b). DGKs  $\delta$ 1,  $\delta$ 2, and  $\eta$ 2, but not DGKs  $\eta$ 1 and  $\kappa$ , contain a sterile  $\alpha$ -motif domain at their C termini. It has been demonstrated that DGKs  $\delta$ 1,  $\delta$ 2, and  $\eta$ 2 form oligomers through interactions between their sterile  $\alpha$ -motif domains and that this oligomer formation regulates the activities and subcellular localization of these DGK isoforms (15–19).

It has recently been demonstrated, using DGK $\delta$  knock-out mice and RNA interference, that DGK $\delta$  regulates the epidermal growth factor (EGF) receptor pathway in lung and skin epithelial cells (20) and insulin receptor signaling in skeletal muscle cells (21, 22) by modulating PKC activity. Moreover, a female patient with a disrupted DGK $\delta$  gene who exhibits seizures and a psychiatric disorder was found (23).

We recently reported that DGK $\eta$  is required for the Ras/B-Raf/C-Raf/MEK/ERK signaling cascade to be activated by EGF in HeLa cells, which are derived from cervical cancer (24). Importantly, DGK $\eta$  regulates the recruitment of B-Raf and C-Raf from the cytosol to membranes and controls their heterodimerization. Moreover, the study demonstrated that DGK $\eta$  activates C-Raf, but not B-Raf, in an EGF-dependent manner. The data show that DGK $\eta$  is a novel key regulator of the Ras/B-Raf/C-Raf/MEK/ERK signaling pathway. In addition,

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<sup>1</sup> To whom correspondence should be addressed. Tel./Fax: 81-43-290-3695; E-mail: sakane@faculty.chiba-u.jp.

<sup>2</sup> The abbreviations used are: DGK, diacylglycerol kinase; C1D, C1 domain; C1P, ceramide 1-phosphate; CL, cardiolipin; DG, diacylglycerol; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PH, pleckstrin homology domain; PI, phosphatidylinositol, PI(3)P, PI 3-phosphate; PI(4)P, PI 4-phosphate; PI(5)P, PI 5-phosphate; PI(3,4)P<sub>2</sub>, PI 3,4-bisphosphate; PI(4,5)P<sub>2</sub>, PI 4,5-bisphosphate; PI(3,4,5)P<sub>3</sub>, PI 3,4,5-trisphosphate; PLC, phospholipase C; SM, sphingomyelin; Sph, sphingosine; RFP, red fluorescent protein.

tion, Nakano *et al.* (25) reported that depleting DGK $\eta$  in lung cancer cell lines harboring a mutant EGF receptor reduced their growth on plastic and in soft agar and augmented the effects of afatinib, an EGF receptor inhibitor. In addition to cancer cells, DGK $\eta$  is also highly expressed in the brain (13, 16, 26). It is interesting to note that a genome-wide association study recently indicated that the gene encoding DGK $\eta$  is implicated in the etiology of bipolar disorder (27, 28). Moreover, it was reported that DGK $\eta$  was highly expressed in the brain of bipolar disorder patients (29).

DGK $\kappa$  is abundantly expressed in the testis (14, 30). A genome-wide association study indicated a potential relationship between DGK $\kappa$  and hypospadias (31).

As described above, type II DGKs are physiologically and pathologically important. However, the binding targets and functions of their PHs are still poorly understood. In this study, we investigated the lipid binding properties of the PHs of DGK $\delta$ ,  $\eta$ , and  $\kappa$  using protein-lipid overlay and liposome binding assays. We revealed that the PH of DGK $\eta$  strongly and highly selectively binds to phosphatidylinositol (PI) 4,5-bisphosphate (PI(4,5)P<sub>2</sub>). The DGK $\delta$ -PH also, but to a lesser extent, selectively associated with PI(4,5)P<sub>2</sub>. However, the PH of DGK $\kappa$  showed only weak binding activity to PI(4,5)P<sub>2</sub>.

## Experimental Procedures

**Materials**—Monoclonal anti-glutathione S-transferase (GST) (B-14) and anti-GFP antibodies were purchased from Santa Cruz Biotechnology. Polyclonal anti-RFP antibody was purchased from Medical and Biological Laboratories. Monoclonal anti-FLAG M2 antibody was purchased from Sigma-Aldrich. Anti-ERK monoclonal antibody and anti-phospho-ERK antibody were obtained from BD Transduction Laboratories and Cell Signaling Technology, respectively. Peroxidase-conjugated goat anti-mouse IgG antibody was acquired from Bethyl Laboratories. Peroxidase-conjugated goat anti-rabbit IgG antibody was obtained from Jackson ImmunoResearch Laboratories. Phosphatidylserine (PS) from bovine brain, dioleoyl-PI 3-phosphate (PI(3)P), dioleoyl-PI(4)P, dioleoyl-PI(5)P, dioleoyl-PI 3,4-bisphosphate (PI(3,4)P<sub>2</sub>), 1-stearoyl-2-arachidonoyl-PI(4,5)P<sub>2</sub> (18:0/20:4-PI(4,5)P<sub>2</sub>), dioleoyl-PI(4,5)P<sub>2</sub> (18:1/18:1-PI(4,5)P<sub>2</sub>), phosphatidylglycerol (PG) from chicken egg yolk, 1-stearoyl-2-arachidonoyl-dioleoyl-phosphatidic acid (18:0/20:4-PA), and 1,2-distearoyl-PA (18:0/18:0-PA) were purchased from Avanti Polar Lipids. 1-Stearoyl-2-arachidonoyl-phosphoinositol 3,4,5-trisphosphate (PI(3,4,5)P<sub>3</sub>) was purchased from Echelon Biosciences. All other lipids, dioleoyl-PA (18:1/18:1-PA), phosphatidylcholine (PC) from chicken egg yolk, phosphatidylethanolamine (PE) from chicken egg yolk, PI from bovine liver, cardiolipin (CL) from bovine heart, sphingomyelin (SM) from chicken egg yolk, sphingosine (Sph), ceramide 1-phosphate (C1P) from bovine brain, and dipalmitoyl-PI(4,5)P<sub>2</sub> (16:0/16:0-PI(4,5)P<sub>2</sub>) were purchased from Sigma-Aldrich. Myc/FLAG (DDK)-tagged human synaptotagmin 1 cDNA (pCMV6-Entry-human synaptotagmin 1) was purchased from OriGene.

**Plasmid Constructs**—pGEX-6P-1-DGK $\delta$ -PH, p3xFLAG-DGK $\eta$ 1 (splice variant 1), p3xFLAG-DGK $\eta$ 1- $\Delta$ PH, pAcGFP-DGK $\eta$ 1, pAcGFP-DGK $\eta$ -PH, pAcGFP-DGK $\eta$ -C1D, and pAcGFP-DGK $\eta$ -

PH+C1D were generated previously (32). To express GST-tagged DGK $\eta$ -PH and DGK $\kappa$ -PH, the cDNAs encoding these domains were generated from human DGK $\eta$ 1 (16) or DGK $\kappa$  (14) cDNA clones by PCR and subcloned into the pGEX-6P-1 vector (GE Healthcare). The DGK $\eta$ -PH cDNA was inserted into pDsRed monomer C1 (Takara Bio-Clontech). Human phospholipase C (PLC)  $\delta$ 1-PH cDNA (amino acids 21–130) (33) was amplified by reverse transcription-PCR with the following primers: forward, 5'-ATCTCGAGCGCTGCTG-AAGGGCAGCCAGCT-3'; reverse, 5'-ATGGATCCTGAGT-GGTGGATGATCTTGTGC-3'. It was then inserted into pDsRed-monomer C1.

DsRed monomer-DGK $\eta$ -PH(K74A) and -(R85A) mutants contain substitutions of Ala for Lys-74 and Arg-85, respectively. All mutants were made with the *in vitro* oligonucleotide mutagenesis system (Takara Bio-Clontech).

**Expression and Purification of GST Fusion Proteins**—BL21 cells were transformed with pGEX-6P-1 constructs. GST alone and GST fusion proteins were expressed and purified according to the manufacturer's protocol (GE Healthcare). The expression of fusion proteins was induced with 0.1 mM isopropyl 1-thio- $\beta$ -D-galactopyranoside (Wako Pure Chemical Industries) at 37 °C for 3 h. The cells were then lysed by sonication in 50 mM Tris-HCl, pH 7.4, 0.25 M sucrose, 1% (v/v) Triton X-100 (Nacalai Tesque), 1 mM EDTA (Dojindo), 1 mM dithiothreitol, 20  $\mu$ g/ml aprotinin (Wako Pure Chemical Industries), 20  $\mu$ g/ml leupeptin (Nacalai Tesque), 20  $\mu$ g/ml pepstatin (Nacalai Tesque), 20  $\mu$ g/ml soybean trypsin inhibitor (Wako Pure Chemical Industries), and 1 mM phenylmethylsulfonyl fluoride (Wako Pure Chemical Industries). The insoluble material was removed by centrifugation. The supernatants were purified by affinity chromatography on a glutathione-Sepharose 4B column (GE Healthcare) at 4 °C. The purified proteins were dialyzed in 10 mM Tris-HCl, pH 7.4.

**Cell Culture and cDNA Transfection**—COS-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Wako Pure Chemical Industries) supplemented with 10% fetal bovine serum (Biological Industries), 100 units/ml penicillin, and 100  $\mu$ g/ml streptomycin (Wako Pure Chemical Industries) at 37 °C in an atmosphere containing 5% CO<sub>2</sub>. COS-7 cells were seeded in 60-mm dishes at a density of  $2.5 \times 10^5$  cells/dish. cDNA was transfected into COS-7 cells by electroporation with a Gene Pulser Xcell™ electroporation system (Bio-Rad) according to the manufacturer's instructions.

**Western Blotting Analysis**—COS-7 cells ( $\sim 1 \times 10^6$  cells/60-mm dish) expressing AcGFP-tagged proteins or DsRed monomer-tagged proteins were lysed in 150  $\mu$ l of 50 mM HEPES, pH 7.2, 150 mM NaCl, 5 mM MgCl<sub>2</sub>, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and Complete protease inhibitor mixture (Roche Applied Science). The mixture was centrifuged at 12,000  $\times g$  for 5 min at 4 °C. Cell lysates were separated using SDS-PAGE. The separated proteins were transferred to a polyvinylidene difluoride membrane (Bio-Rad) and blocked with 5% (w/w) skim milk. The membrane was incubated with polyclonal anti-RFP antibody (cross-reacts with DsRed monomer), monoclonal anti-FLAG M2 antibody, anti-ERK antibody, or anti-phospho-ERK antibody in 5% (w/v) skim milk for 1 h. The immunoreactive bands were visualized using peroxidase-

## PI(4,5)P<sub>2</sub> Binding of the DGK $\eta$ PH Domain

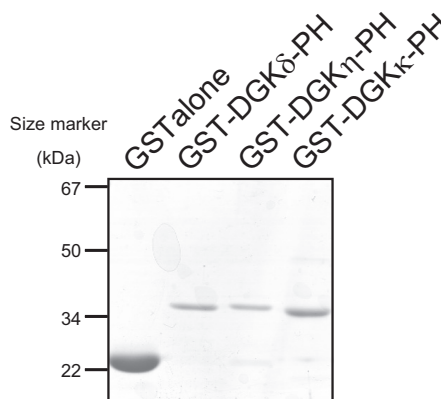
conjugated goat anti-mouse IgG antibody or goat anti-rabbit IgG antibody and the ECL Western blotting detection system (GE Healthcare).

**Protein-Lipid Overlay Assay**—One hundred picomoles of the indicated lipids were spotted onto a nitrocellulose membrane (Bio-Rad). The membranes were subjected to blocking with 1% skim milk in Tris-buffered saline, pH 7.4, for 1 h at room temperature. After the blocking, 10 ml of 3% fatty acid-free bovine serum albumin in Tris-buffered saline, pH 7.4, containing lysates from cells expressing GST fusion protein of interest (final concentration, 20 nM) or 80  $\mu$ l of the cell lysates containing DsRed monomer or 3xFLAG fusion protein were added to the membranes. The membranes were then incubated for 30 min at room temperature and then at 4 °C overnight. The membranes were incubated with an anti-GST antibody, anti-FLAG antibody, or anti-RFP antibody followed by incubation with peroxidase-conjugated goat anti-mouse IgG antibody or goat anti-rabbit IgG antibody. Finally, lipid-bound proteins were visualized using an ECL Western blotting detection system. Quantitative densitometry was performed using ImageJ software.

**Liposome Binding Assay**—The liposome preparation contained 1 mg/ml total lipid with the following composition: 100% (w/w) PC, 95% (w/w) PC and 5% (w/w) PS or 95% (w/w) PC and 5% (w/w) PA, and 95% (w/w) PC and 5% (w/w) PI(4,5)P<sub>2</sub>. The combined dried lipid mixture was resuspended in liposome buffer (100 mM NaCl, 1 mM dithiothreitol, and 20 mM HEPES, pH 7.4). Liposome formation was induced by 1-min sonication at 4 °C using a Branson Sonifier 450. For sedimentation assays, the cell lysates expressing GST alone and GST-DGK $\eta$ -PH were ultracentrifuged at 100,000  $\times$  *g* for 30 min. One hundred microliters of the cell lysates (100,000  $\times$  *g* supernatant) were mixed with 100  $\mu$ g of liposomes in 100  $\mu$ l of the liposome buffer, incubated for 60 min at 4 °C, and ultracentrifuged at 100,000  $\times$  *g* for 60 min at 4 °C. The supernatant and pellet were analyzed by SDS-PAGE followed by immunoblotting. Quantitative densitometry was performed using ImageJ software.

**DGK Activity Assay**—The octyl glucoside mixed micellar assay for DGK activity was performed as described previously (12). In brief, the assay mixture (50  $\mu$ l) contained 50 mM MOPS, pH 7.4, 50 mM octyl glucoside, 1 mM dithiothreitol, 100 mM NaCl, 20 mM NaF, 10 mM MgCl<sub>2</sub>, 1 mM EGTA, 5 mM phosphatidylserine, 1.5 mM diacylglycerol, and 1 mM [ $\gamma$ -<sup>32</sup>P]ATP (10,000 cpm/nmol; ICN Biomedicals). The reaction was initiated by adding cell lysates (5  $\mu$ g of protein) and continued for 5 min at 30 °C. Lipids were extracted from the mixture, and phosphatidic acid separated by thin layer chromatography was scraped and counted by a liquid scintillation spectrophotometer.

**Confocal Laser Scanning Microscopy**—Confocal laser scanning microscopy was carried out as described previously (34). Briefly, COS-7 cells were grown on poly-L-lysine-coated glass coverslips and transiently transfected with expression plasmids containing DGK $\eta$ 1 or its mutant cDNAs that were N-terminally fused with AcGFP and a DsRed monomer-tagged PLC $\delta$ 1-PH (35). After 48 h, the cells were serum-starved with DMEM (0.1% fetal bovine serum) for 3 h and incubated with sorbitol in DMEM (final concentration, 500 mM) for 30 min to induce hyperosmotic stress. The cells were then fixed in 3.7% formal-



**FIGURE 1. Expression and purification of GST fusion proteins.** GST alone and GST-tagged PHs of DGK $\delta$ ,  $\eta$ , and  $\kappa$  were expressed in BL21 *E. coli* cells and purified by affinity chromatography. These proteins were separated by SDS-PAGE and stained with Coomassie Brilliant Blue. A representative result of three independent experiments is shown.

dehyde. For immunofluorescence microscopy, COS-7 cells were permeabilized with 0.1% Triton X-100 in phosphate-buffered saline for 10 min at room temperature. Cells were incubated in phosphate-buffered saline containing 1% bovine serum albumin for 10 min at room temperature as a blocking step. The cells were then incubated in 1% bovine serum albumin in phosphate-buffered saline containing anti-FLAG monoclonal antibody for 30 min at room temperature. After being washed twice with phosphate-buffered saline, cells were incubated with the Alexa Fluor-conjugated secondary antibodies (Invitrogen). The coverslips were mounted using Vectashield (Vector Laboratories). Fluorescence images were acquired using an Olympus FV1000-D confocal laser scanning microscope.

**Cell Fractionation**—COS-7 cells ( $\sim 1 \times 10^6$  cells/60-mm dish) expressing AcGFP-tagged proteins were lysed in 150  $\mu$ l of 50 mM HEPES, pH 7.2, 150 mM NaCl, 5 mM MgCl<sub>2</sub>, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and Complete protease inhibitor mixture. The mixture was centrifuged at 12,000  $\times$  *g* for 5 min at 4 °C and further centrifuged at 100,000  $\times$  *g* for 60 min at 4 °C to separate supernatant and precipitate (membrane) fractions. AcGFP-tagged proteins were detected by Western blotting with anti-GFP antibody.

## Results

**Lipid Binding Activity of the PHs of DGK $\eta$ ,  $\delta$ , and  $\kappa$** —We expressed GST alone and the GST-fused PHs of DGK $\delta$ , DGK $\eta$ , and DGK $\kappa$  in *Escherichia coli* and highly purified them by affinity chromatography (Fig. 1). These proteins were soluble with expected molecular masses of 26, 38, 38, and 38 kDa, respectively (Fig. 1). We determined the lipid binding activities of the GST-fused PHs of DGK $\delta$ , DGK $\eta$ , and DGK $\kappa$  using a protein-lipid overlay assay. A nitrocellulose membrane was spotted with 100 pmol each of PI, PI(3)P, PI(4)P, PI(5)P, PI(3,4)P<sub>2</sub>, PI(4,5)P<sub>2</sub>, PI(3,4,5)P<sub>3</sub>, PC, PA, and PS as indicated (Fig. 2A). The PH of DGK $\eta$  strongly bound to PI(4,5)P<sub>2</sub> (Fig. 2, A and B). Compared with PI(4,5)P<sub>2</sub>, the PH was associated with PI(3,4)P<sub>2</sub> and PI(3,4,5)P<sub>3</sub> to a lesser extent. PI(3)P, PI(4)P, PI(5)P, and PA showed only weak binding activities. The binding activities of PI, PC, and PS were not detectable. The DGK $\delta$ -PH also strongly

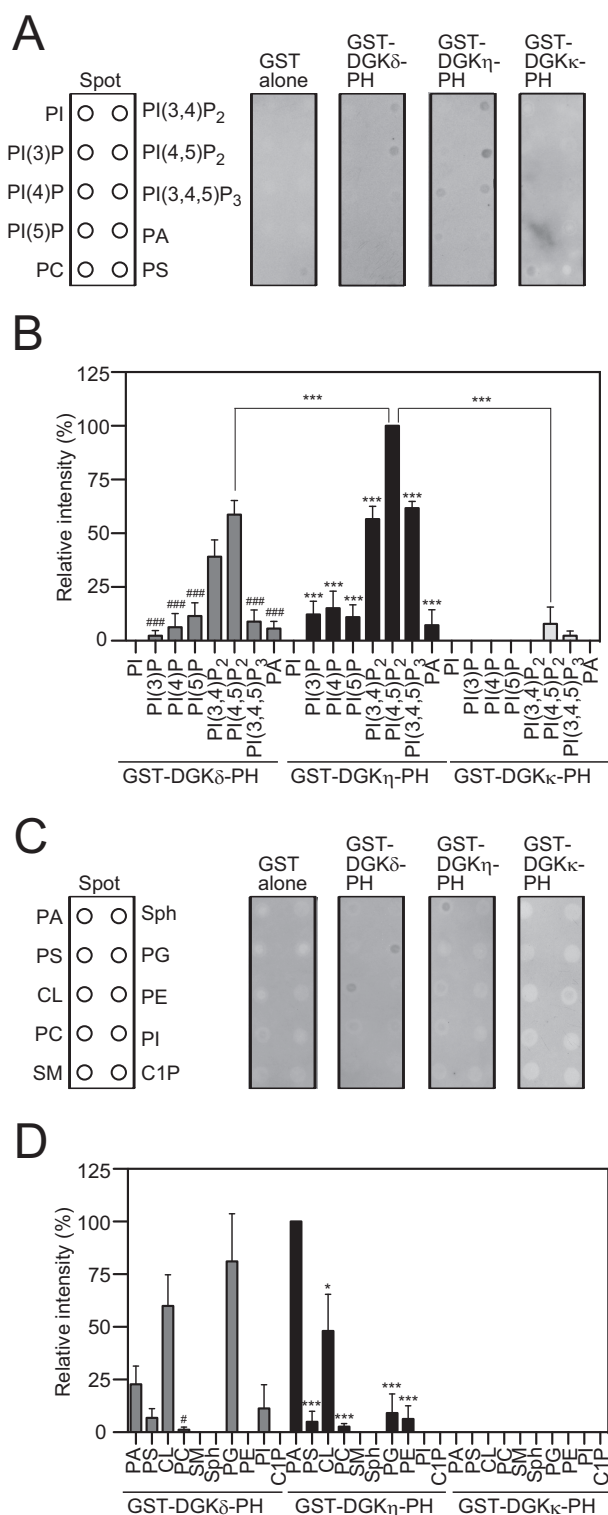


FIGURE 2. **Lipid binding activities of GST-tagged PHs of DGKδ, -η, and -κ.** A and C, equimolar amounts (100 pmol) of PI, PI(3)P, PI(4)P, PI(5)P, PC, PI(3,4)P<sub>2</sub>, PI(4,5)P<sub>2</sub>, PI(3,4,5)P<sub>3</sub>, PA, and PS (A) and PA, PS, CL, PC, SM, Sph, PG, PE, PI, and C1P (C) were spotted onto nitrocellulose membranes as indicated, respectively. The membranes were incubated with GST-tagged PHs of DGKδ, -η, and -κ as indicated. The lipid-bound PHs were detected by immunostaining with anti-GST antibody. B and D, the blots were scanned and quantified using ImageJ software. PI(4,5)P<sub>2</sub> (B) and PA (D) binding levels of DGKη-PH were set to 100%, respectively. The data are shown as the means ± S.D. of three independent experiments. Statistical significance compared with the PI(4,5)P<sub>2</sub> (B) and PA (D) binding activities of DGKη-PH (\* and \*\*\*) or DGKδ-PH (# and ###) was determined using Student's *t* test (\*, *p* < 0.05; \*\*\*, *p* < 0.005; #, *p* < 0.05; ###, *p* < 0.005). Error bars represent S.E.

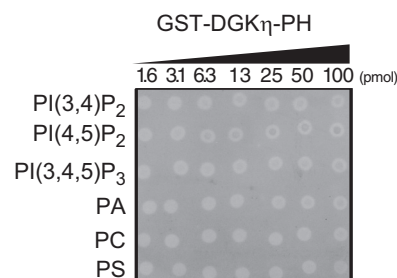


FIGURE 3. **Dose-dependent binding activities of DGKη-PH to PI(3,4)P<sub>2</sub>, PI(4,5)P<sub>2</sub>, PI(3,4,5)P<sub>3</sub>, PA, PC, and PS.** Indicated amounts (1.6, 3.1, 6.3, 13, 25, 50, and 100 pmol) of PI(3,4)P<sub>2</sub>, PI(4,5)P<sub>2</sub>, PI(3,4,5)P<sub>3</sub>, PA, PC, and PS were spotted onto a nitrocellulose membrane. The membrane was incubated with GST-tagged DGKη-PH. Lipid-bound DGKη-PH was detected by immunostaining with anti-GST antibody. A representative result of three independent experiments is shown. It was confirmed that lipid-bound GST alone was not detectable in this experiment (data not shown).

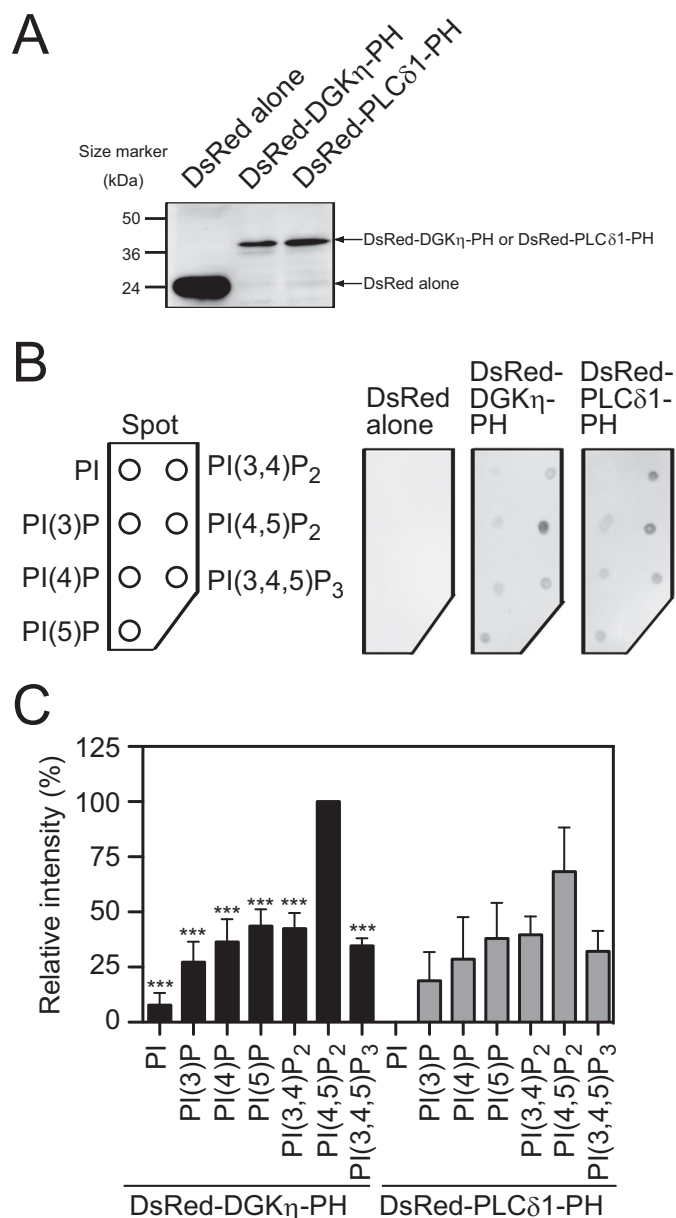
interacted with PI(4,5)P<sub>2</sub> (Fig. 2A). However, compared with the PH of DGKη, the PH of DGKδ bound less strongly to PI(4,5)P<sub>2</sub> (Fig. 2, A and B). The PH of DGKκ showed only very weak binding activity to PI(4,5)P<sub>2</sub> (Fig. 2, A and B). Therefore, among the PHs of type II DGKs, the PH of DGKη most strongly and selectively binds to PI(4,5)P<sub>2</sub>.

We examined the binding activities of the PHs of DGKη, -δ, and -κ compared with other glycerolipids and sphingolipids. In this experiment, their lipid binding activities were detected after longer exposure. Compared with PA, the DGKη-PH bound to CL to a lesser extent (Fig. 2, C and D). PG, PE, SM, Sph, and C1P exhibited either subtle or no detectable binding activities. In contrast, the PH of DGKδ more strongly bound to PG and CL than to PA and showed either subtle or no detectable binding activities to PE, SM, Sph, and C1P (Fig. 2, C and D). The binding activities of the PH of DGKκ to these lipids were not detectable (Fig. 2, C and D). Taken together, the PH of DGKη exhibited the most pronounced binding activity to PI(4,5)P<sub>2</sub> among the various glycerolipids and sphingolipids. Although the binding activity is weaker than that of the DGKη-PH, the PH of DGKδ also selectively interacts with PI(4,5)P<sub>2</sub>.

**Characterization of Selective Binding Activity of the DGKη-PH to PI(4,5)P<sub>2</sub>**—Next, we more quantitatively determined the affinity of the PH of DGKη for PI(4,5)P<sub>2</sub>. Various concentrations of PI(4,5)P<sub>2</sub>, PI(3,4)P<sub>2</sub>, and PI(3,4,5)P<sub>3</sub> were spotted on a nitrocellulose membrane. As shown in Fig. 3, the intensity of the spot of the DGKη-PH on 6.3 pmol of PI(4,5)P<sub>2</sub> was almost equivalent to those of 100 pmol of PI(3,4)P<sub>2</sub> and PI(3,4,5)P<sub>3</sub>. Therefore, the result indicates that the binding affinity of the DGKη-PH for PI(4,5)P<sub>2</sub> is markedly stronger than for PI(3,4)P<sub>2</sub> and PI(3,4,5)P<sub>3</sub>. Moreover, in the same experiment, the intensity of the spot of the DGKη-PH on 3.1 pmol of PI(4,5)P<sub>2</sub> was almost equivalent to that of 100 pmol of PI(4,5)P<sub>2</sub> of the DGKδ-PH (data not shown).

The effects of the tag and expression system were examined next. The DGKη-PH was tagged with DsRed monomer protein instead of GST and expressed in mammalian COS-7 cells instead of *E. coli* (Fig. 4A). It was confirmed that DsRed monomer-DGKη-PH was expressed with the expected molecular mass (38 kDa). As shown in Fig. 4, B and C, the DsRed monomer-DGKη-PH expressed in COS-7 cells strongly bound to PI(4,5)P<sub>2</sub>. Similar to the GST-DGKη-PH, the binding affinity

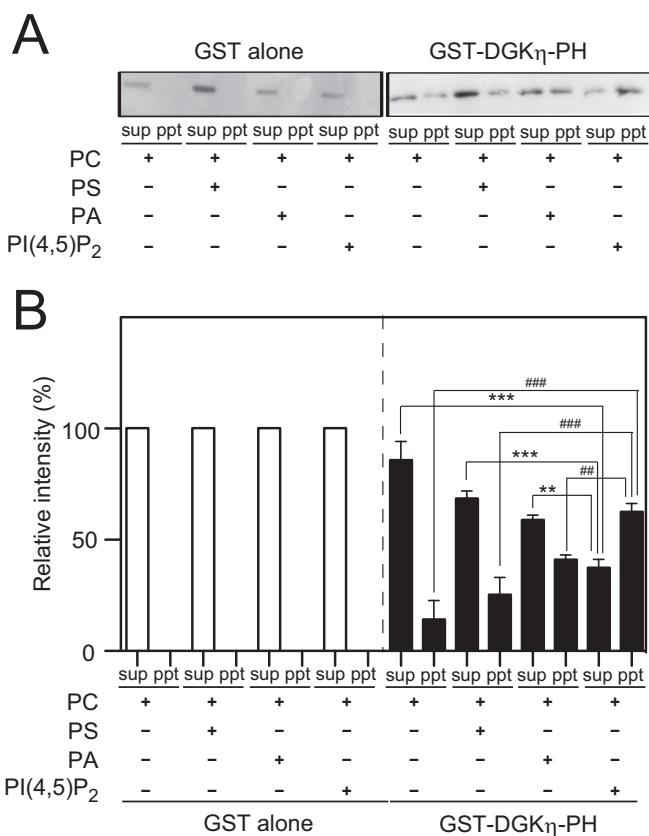
## PI(4,5)P<sub>2</sub> Binding of the DGK $\eta$ PH Domain



**FIGURE 4. Lipid binding activities of DsRed monomer-tagged DGK $\eta$ -PH and PLC $\delta$ 1-PH expressed in COS-7 cells.** *A*, expression of DsRed monomer alone, DsRed monomer-DGK $\eta$ -PH, and DsRed monomer-PLC $\delta$ 1-PH was confirmed by Western blotting. *B*, equimolar amounts (100 pmol) of PI, PI(3)P, PI(4)P, PI(5)P, PC, PI(3,4)P<sub>2</sub>, PI(4,5)P<sub>2</sub>, and PI(3,4,5)P<sub>3</sub> were spotted onto nitrocellulose membranes as indicated. The membranes were incubated with DsRed monomer-tagged DGK $\eta$ -PH and PLC $\delta$ 1-PH. Lipid-bound DsRed monomer-tagged proteins were detected by immunostaining with anti-RFP antibody, which cross-reacts with DsRed monomer. *C*, the blots were scanned and quantified using ImageJ software. The PI(4,5)P<sub>2</sub> binding level of DGK $\eta$ -PH was set to 100%. The data are shown as the means  $\pm$  S.D. of three independent experiments. Statistical significance compared with the PI(4,5)P<sub>2</sub> binding activity of DGK $\eta$ -PH was determined using Student's *t* test (\*\*\*,  $p < 0.005$ ). Error bars represent S.E.

for PI(4,5)P<sub>2</sub> was markedly stronger than those for PI(3,4,5)P<sub>3</sub>, PI(3,4)P<sub>2</sub>, PI(3)P, PI(4)P, PI(5)P, and PI.

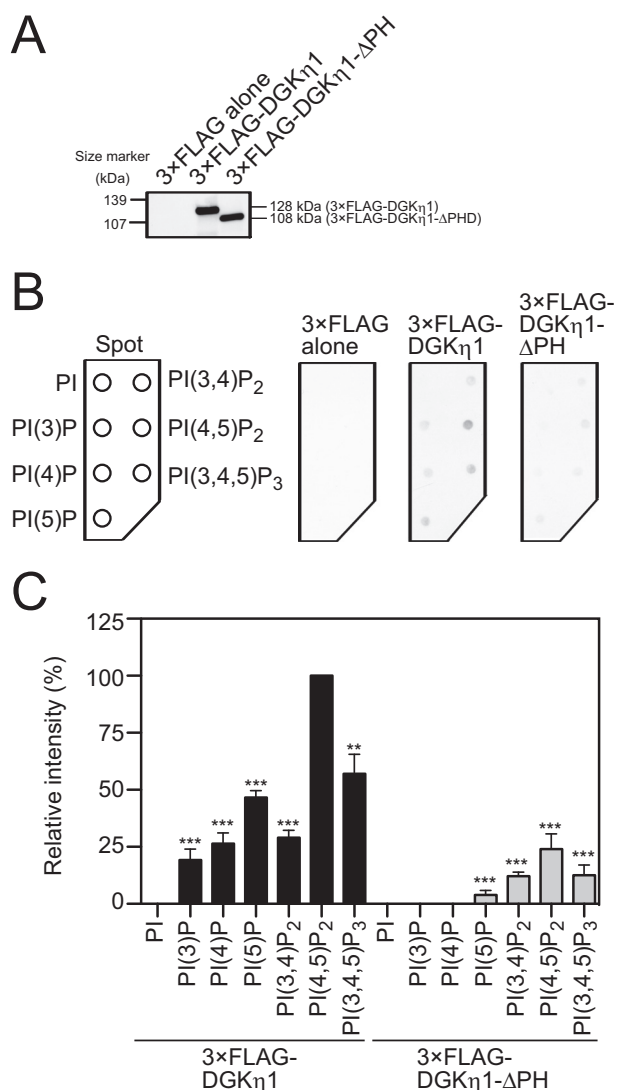
The PH of PLC $\delta$ 1 is a widely used PI(4,5)P<sub>2</sub>-binding probe (35, 36). The binding selectivity and affinity of the PLC $\delta$ 1-PH for PI(4,5)P<sub>2</sub> was compared with those of DGK $\eta$ . The DsRed monomer-tagged PLC $\delta$ 1-PH was also expressed with the expected molecular mass (40 kDa) in COS-7 cells (Fig. 4*A*). The



**FIGURE 5. Binding of DGK $\eta$ -PH to PI(4,5)P<sub>2</sub>-containing liposomes.** *A*, GST-tagged DGK $\eta$ -PH and GST alone were incubated with liposomes containing PC alone and a 9.5:0.5 molar ratio of PC to PS, PA, and PI(4,5)P<sub>2</sub> as indicated. Liposome-binding proteins were recovered by ultracentrifugation. Liposome binding (precipitate (ppt)) and lack of binding (supernatant (sup)) of GST-DGK $\eta$ -PH and GST alone were detected by Western blotting with anti-GST antibody. *B*, the blots were scanned and quantified using ImageJ software. Relative intensities of liposome binding (precipitate and lack of binding (supernatant)) of DGK $\eta$ -PH and GST alone are indicated, respectively. The data are shown as the means  $\pm$  S.D. of three independent experiments. Statistical significance was determined using Student's *t* test versus PI(4,5)P<sub>2</sub> liposome supernatant (\*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.005$ ) and versus PI(4,5)P<sub>2</sub> liposome precipitate (##,  $p < 0.01$ ; ###,  $p < 0.005$ ). Error bars represent S.E.

PLC $\delta$ 1-PH strongly bound to PI(4,5)P<sub>2</sub> and, to a lesser extent, to PI(3)P, PI(4)P, PI(5)P, PI(3,4)P<sub>2</sub>, and PI(3,4,5)P<sub>3</sub> (Fig. 4, *B* and *C*). The result is essentially the same as reported previously (37). Intriguingly, the intensity of the spot of the DGK $\eta$ -PH on PI(4,5)P<sub>2</sub> was equal to or greater than that of the PLC $\delta$ -PH (Fig. 4, *B* and *C*). Moreover, the selectivity of the DGK $\eta$ -PH for PI(4,5)P<sub>2</sub> compared with PI(3,4)P<sub>2</sub> and PI(3,4,5)P<sub>3</sub> was also equal to or better than that of the PLC $\delta$ 1-PH (Fig. 4, *B* and *C*).

In addition to the protein-lipid overlay assay, we confirmed the binding of the DGK $\eta$ -PH to PI(4,5)P<sub>2</sub> using a liposome binding assay. GST alone was not recovered in the precipitate fraction of PI(4,5)P<sub>2</sub> liposome, indicating that GST alone was not associated with PI(4,5)P<sub>2</sub> (Fig. 5, *A* and *B*). However, more than 60% of the GST-DGK $\eta$ -PH was recovered in the precipitate fraction of PI(4,5)P<sub>2</sub> liposome (Fig. 5, *A* and *B*). In contrast, only ~10, 20, and 40% of the DGK $\eta$ -PH was detected in PC, PS, and PA liposomes, respectively. Similar to the lipid-protein overlay assay, these results indicate that the DGK $\eta$ -PH more strongly binds to PI(4,5)P<sub>2</sub> than to PC, PS, and PA.



**FIGURE 6. PI(4,5)P<sub>2</sub> binding of full-length DGK $\eta$  and its PH-deleted mutant.** A, 3xFLAG-tagged full-length DGK $\eta$ 1 (splice variant 1) and DGK $\eta$ 1- $\Delta$ PH (amino acids 164–1164) were expressed in COS-7 cells. The Western blotting analysis was performed using anti-FLAG antibody. B, equimolar amounts (100 pmol) of PI, PI(3)P, PI(4)P, PI(5)P, PC, PI(3,4)P<sub>2</sub>, 18:0/20:4-PI(4,5)P<sub>2</sub>, PI(3,4,5)P<sub>3</sub>, PA, and PS were spotted onto nitrocellulose membranes as indicated. The membranes were incubated with COS-7 cell lysates containing 3xFLAG-tagged full-length DGK $\eta$  or its PH deletion mutant. C, the blots were scanned and quantified using ImageJ software. The PI(4,5)P<sub>2</sub> binding level of full-length DGK $\eta$  was set to 100%. The data are shown as the means  $\pm$  S.D. of three independent experiments. Statistical significance compared with the PI(4,5)P<sub>2</sub> binding activity of full-length DGK $\eta$  was determined using Student's *t* test (\*\*, *p* < 0.01; \*\*\*, *p* < 0.005). Error bars represent S.E.

**PI(4,5)P<sub>2</sub> Binding Activity of Full-length DGK $\eta$  via Its PH—** We next determined whether full-length DGK $\eta$  indeed binds to PI(4,5)P<sub>2</sub>. A lipid-protein overlay assay was performed using 3xFLAG-tagged full-length DGK $\eta$ 1 (splice variant 1) expressed in COS-7 cells (Fig. 6A). Similar to the PH alone (Figs. 2 and 4), 3xFLAG-full-length DGK $\eta$  most strongly interacted with PI(4,5)P<sub>2</sub> and, to a lesser extent, with PI(3)P, PI(4)P, PI(5)P, PI(3,4)P<sub>2</sub>, and PI(3,4,5)P<sub>3</sub> (Fig. 6, B and C). A DGK $\eta$ 1 mutant lacking a PH exhibited much less binding activity for PI(4,5)P<sub>2</sub> (Fig. 6, B and C). Therefore, these results indicate that the full-length DGK $\eta$ 1 strongly and selectively interacts with PI(4,5)P<sub>2</sub> through its PH.

We also examined whether PI(4,5)P<sub>2</sub> can activate DGK $\eta$ . Although 1.0 mol % PI(4,5)P<sub>2</sub> was added to the assay mixture, the activity of DGK $\eta$ 1 was not increased (data not shown). Thus, it seems likely that PI(4,5)P<sub>2</sub> is not directly involved in the activation mechanism of DGK $\eta$  *in vitro*.

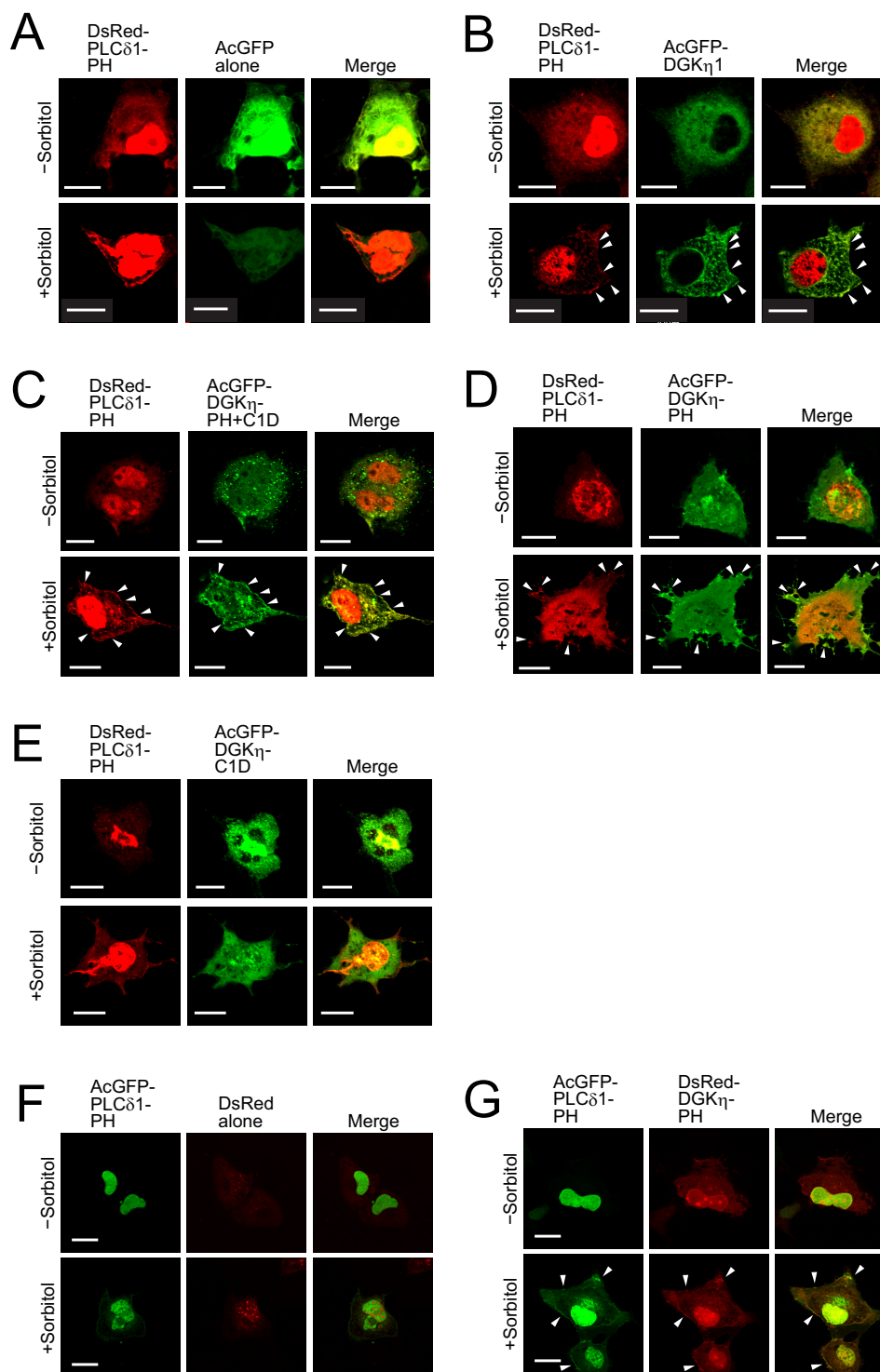
**Full-length DGK $\eta$ 1 and Its PH Co-localize with PLC $\delta$ 1-PH in Osmotically Shocked Cells—**Because the PH of PLC $\delta$ 1 recognizes and co-localizes with cellular PI(4,5)P<sub>2</sub> (35, 36), it is generally used as a cellular PI(4,5)P<sub>2</sub> probe. Moreover, osmotic shock increases the amount of PI(4,5)P<sub>2</sub> in the plasma membrane (38, 39). We next observed the co-localization of AcGFP-tagged full-length DGK $\eta$ 1 with the DsRed monomer-tagged PLC $\delta$ 1-PH in osmotically shocked cells. Indeed, the PLC $\delta$ 1-PH was partly localized to the plasma membrane in osmotically shocked cells (Fig. 7). As reported previously (16, 32), full-length DGK $\eta$ 1 was translocated to punctate vesicles in response to osmotic shock (Fig. 7B). Interestingly, full-length DGK $\eta$ 1 was partly co-localized with the PLC $\delta$ 1-PH at the punctate regions close to the plasma membrane (Fig. 7B). However, AcGFP alone did not exhibit such co-localization (Fig. 7A). These results indicate that in addition to punctate vesicles in the cytoplasm full-length DGK $\eta$ 1 was translocated to subcellular compartments enriched with PI(4,5)P<sub>2</sub> in the plasma membrane.

An AcGFP-tagged DGK $\eta$  mutant containing the PH and the C1 domains (C1Ds) was co-localized with the DsRed monomer-tagged PLC $\delta$ -PH at punctate vesicles close to the plasma membrane in osmotically shocked cells (Fig. 7C). However, the C1Ds alone lacking a PH were not co-localized with the PLC $\delta$ 1-PH (Fig. 7E). In contrast, the AcGFP-tagged DGK $\eta$ -PH alone was translocated to punctate regions in the plasma membrane and markedly co-localized with the DsRed monomer-tagged PLC $\delta$ 1-PH there in response to osmotic shock (Fig. 7D). We performed the same experiments using DsRed monomer-tagged DGK $\eta$ -PH and AcGFP-tagged PLC $\delta$ -PH and obtained essentially the same results (Fig. 7G). Taken together, these results indicate that the PH of DGK $\eta$  plays an important role in recognition and co-localization with PI(4,5)P<sub>2</sub>-containing subcellular compartments.

To further evaluate the co-localization of DGK $\eta$ -PH with PI(4,5)P<sub>2</sub>-containing subcellular compartments, we next examined the effect of overexpression of the PI(4,5)P<sub>2</sub> phosphatase synaptojanin. Synaptojanin dephosphorylates the D-5 position phosphate from PI(4,5)P<sub>2</sub> (40). As shown in Fig. 8, AcGFP-DGK $\eta$ -PH was markedly translocated from the cytoplasm to the plasma membrane in response to osmotic shock. However, synaptojanin significantly inhibited the osmotic shock-induced plasma membrane translocation of AcGFP-DGK $\eta$ -PH. The result further indicates that DGK $\eta$ -PH interacted and co-localized with PI(4,5)P<sub>2</sub> in an osmotic shock-dependent manner.

To confirm the subcellular translocation of AcGFP-DGK $\eta$ -PH, we performed cell fractionation. As shown in Fig. 9, the DGK $\eta$ -PH was translocated to the 100,000  $\times$  g precipitate (membrane) fraction in osmotically shocked cells, whereas AcGFP alone was not. It was also confirmed that the PLC $\delta$ 1-PH was translocated to the membrane fraction in response to hyperosmotic shock (data not shown).

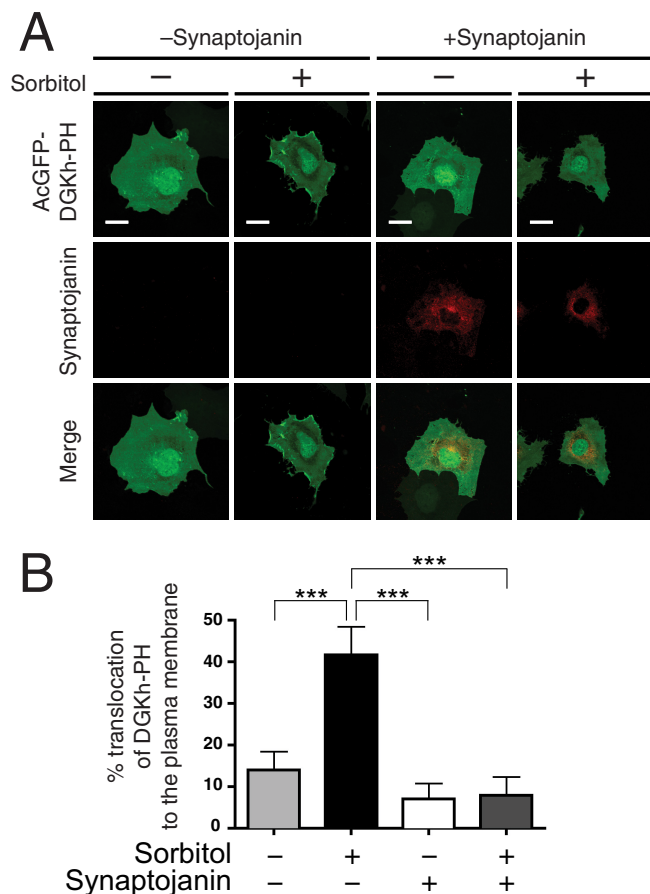
## PI(4,5)P<sub>2</sub> Binding of the DGK $\eta$ PH Domain



**FIGURE 7. Subcellular localization of full-length DGK $\eta$  and its mutants (DGK $\eta$ -PH+C1D, DGK $\eta$ -PH, and DGK $\eta$ -C1D) in COS-7 cells treated and untreated with hyperosmotic shock.** AcGFP alone (A), AcGFP-tagged full-length DGK $\eta$ 1 (splice variant 1) (B) and its mutants DGK $\eta$ -PH+C1D (C), DGK $\eta$ -PH (D), and DGK $\eta$ -C1D (E) were co-expressed with DsRed monomer-PLC $\delta$ 1-PH in COS-7 cells. DsRed monomer alone (F) and AcGFP-tagged DGK $\eta$ -PH (G) were co-expressed with AcGFP-PLC $\delta$ 1-PH in COS-7 cells. After 48 h of transfection, the cells were incubated with 500 mM sorbitol in DMEM for 30 min. The cells were fixed with 3.7% formaldehyde and imaged by inverted confocal laser microscopy (FV1000-D). Representative data of three independent experiments are shown. *Arrowheads* indicate co-localization of AcGFP-DGK $\eta$ 1, DGK $\eta$ -PH+C1D, or DGK $\eta$ -PH with DsRed monomer-PLC $\delta$ 1-PH and AcGFP-PLC $\delta$ 1-PH with DsRed monomer-DGK $\eta$ -PH. *Scale bars*, 20  $\mu$ m.

Ferguson *et al.* (41) reported that Lys-30, Lys-32, Arg-40, Ser-55, Arg-56, and Lys-57 in the PLC $\delta$ 1-PH play important roles in strong PI(4,5)P<sub>2</sub>-selective binding activity. The four amino acid residues Lys-30, Arg-40, Ser-55, and Arg-56 in the

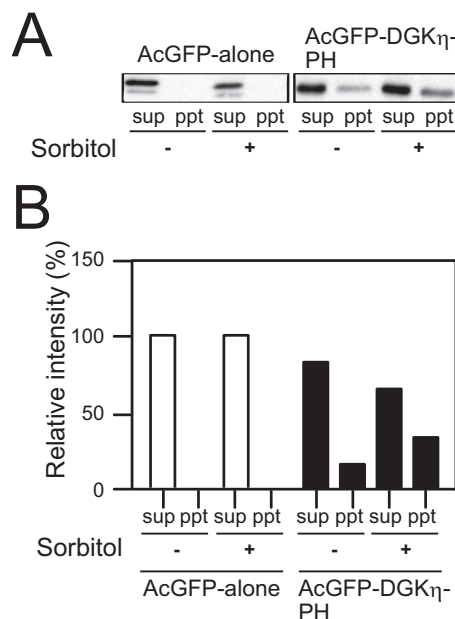
PLC $\delta$ 1-PH are conserved in the DGK $\eta$ -PH (Lys-74, Arg-85, Ser-100, and Lys-101, respectively) (Fig. 10). Therefore, we made DsRed monomer-DGK $\eta$ -PH(K74A) and -(R85A). It was confirmed that both DsRed monomer-DGK $\eta$ -PH(K74A) and



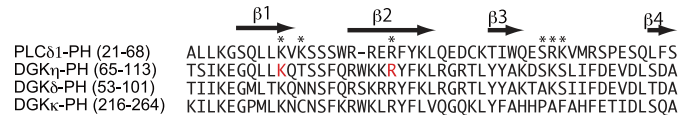
**FIGURE 8. Subcellular localization of DGK $\eta$ -PH in COS-7 cells treated with hyperosmotic shock in the presence of synaptojanin.** A, AcGFP-tagged DGK $\eta$ -PH was co-expressed with FLAG-tagged synaptojanin in COS-7 cells. After 48 h of transfection, the cells were incubated with 500 mM sorbitol in DMEM for 30 min. The cells were fixed with 3.7% formaldehyde, stained with anti-FLAG and Alexa Fluor 594-conjugated secondary antibodies, and observed using confocal laser scanning microscopy. Scale bars, 20  $\mu$ m. B, the percentages of cells exhibiting translocation of AcGFP-tagged DGK $\eta$ -PH to the plasma membrane in the presence or absence of synaptojanin were scored. More than 30 cells expressing AcGFP-tagged DGK $\eta$ -PH were counted in each experiment. The results are the means  $\pm$  S.D. of three independent experiments. The statistical significance (versus -synaptojanin +sorbitol) was determined using Student's *t* test (\*\*\*, *p* < 0.005). Error bars represent S.D.

-(R85A) were expressed with the expected molecular mass (38 kDa) (Fig. 11A) and that these mutants failed to bind to PI(4,5)P<sub>2</sub> *in vitro* (Fig. 11B). These results indicate that Lys-74 and Arg-85 in DGK $\eta$ -PH are also critical residues for PI(4,5)P<sub>2</sub> binding activity. We confirmed that DsRed monomer-DGK $\eta$ -PH(K74A) and -(R85A) were unable to translocate to the plasma membrane and co-localize with the PLC $\delta$ 1-PH even in osmotically shocked cells (Fig. 11, C and D), suggesting that Lys-74 and Arg-85 are important to detect cellular PI(4,5)P<sub>2</sub> and further indicating that the PH of DGK $\eta$  plays an important role in recognition and co-localization with PI(4,5)P<sub>2</sub>-containing subcellular compartments.

**Effects of DGK $\eta$ 1(K74A) and DGK $\eta$ 1(R85A) on EGF-dependent Activation of ERK**—DGK $\eta$ 1 is a positive regulator of the EGF receptor/Ras/B-Raf/C-Raf/MEK/ERK signaling pathway (24). To address physiological significance of PI(4,5)P<sub>2</sub> binding activity of DGK $\eta$ 1, we tested the effects of overexpression of wild-type DGK $\eta$ 1 and the PI(4,5)P<sub>2</sub> binding-negative (full-



**FIGURE 9. Subcellular fractionation of DGK $\eta$ -PH in COS-7 cells treated and untreated with hyperosmotic shock.** COS-7 cells expressing AcGFP alone and AcGFP-tagged DGK $\eta$ -PH were lysed. The mixture was centrifuged at 12,000  $\times$  *g* for 5 min at 4  $^{\circ}$ C and further centrifuged at 100,000  $\times$  *g* for 60 min to separate supernatant (*sup*) and precipitate (*ppt*) fractions. A, AcGFP alone and AcGFP-tagged DGK $\eta$ -PH were detected by Western blotting with anti-GFP antibody. B, the blots were scanned and quantified using ImageJ software. Relative intensities of precipitate and supernatant fractions of AcGFP alone and AcGFP-tagged DGK $\eta$ -PH are indicated, respectively. Representative data of three independent experiments are shown.



**FIGURE 10. Alignment of the PHs of PLC $\delta$ 1, DGK $\eta$ , DGK $\delta$ , and DGK $\kappa$ .** Lys-30, Lys-32, Arg-40, Ser-55, Arg-56, and Lys-57 in the PLC $\delta$ 1-PH indicated by asterisks are important for strong PI(4,5)P<sub>2</sub>-selective binding activity. Lys-74 and Arg-85 in the DGK $\eta$ -PH are indicated by red font.

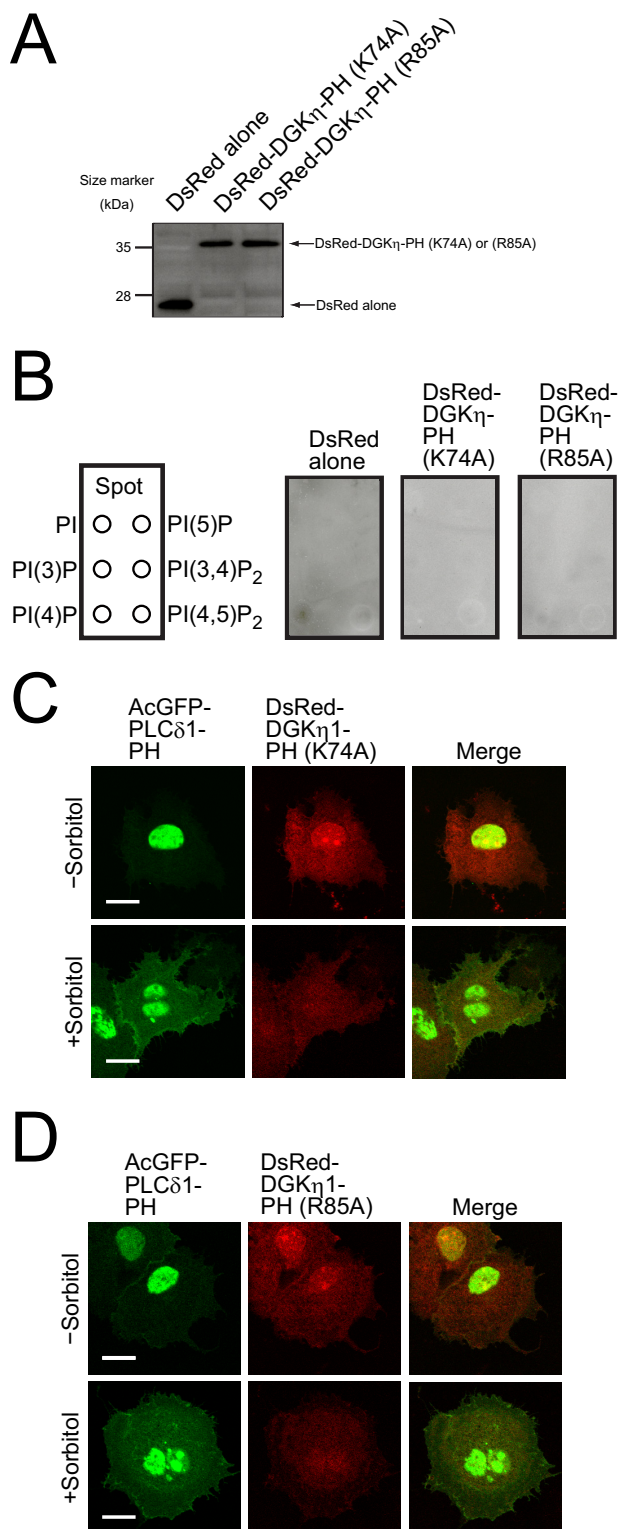
length) mutants DGK $\eta$ 1(K74A) and DGK $\eta$ 1(R85A) on EGF-induced ERK phosphorylation. As shown in Fig. 12, wild-type DGK $\eta$ 1 markedly increased EGF-dependent phosphorylation levels of ERK. However, neither DGK $\eta$ 1(K74A) nor DGK $\eta$ 1-(R85A) enhanced the ERK phosphorylation even though their protein expression levels were similar to that of wild-type DGK $\eta$ 1. These results suggest that PI(4,5)P<sub>2</sub> binding activity of DGK $\eta$ 1 plays an important role in regulating the EGF receptor/ERK signal transduction pathway.

**Discussion**

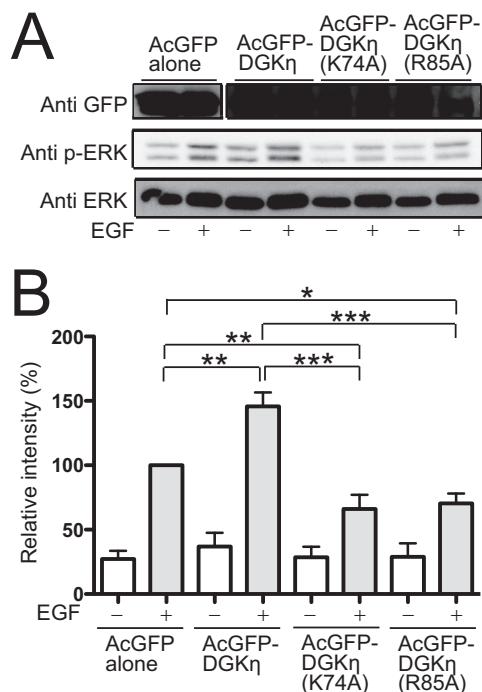
The lipid binding properties of the PH of DGK $\eta$  have not been clear. In this study, we demonstrated that the DGK $\eta$ -PH is a PI(4,5)P<sub>2</sub>-selective binding domain with high affinity (Figs. 2 and 4). It was also confirmed that the full-length DGK $\eta$ 1 strongly and selectively interacts with PI(4,5)P<sub>2</sub> through its PH (Fig. 6). Affinities for various phosphoinositides and phospholipids were as follows: PI(4,5)P<sub>2</sub>  $\gg$  PI(3,4)P<sub>2</sub>  $\approx$  PI(3,4,5)P<sub>3</sub> > PA  $\approx$  PI(3)P  $\approx$  PI(4)P  $\approx$  PI(5)P  $\gg$  CL  $\gg$  PG  $\approx$  PE  $\approx$  PS  $\approx$  PC > PI  $\approx$  SM  $\approx$  Sph  $\approx$  C1P (Figs. 2 and 3). Therefore, these results indicate that the DGK $\eta$ -PH is highly selective for PI(4,5)P<sub>2</sub>.



## PI(4,5)P<sub>2</sub> Binding of the DGK $\eta$ PH Domain



**FIGURE 11. Lipid binding activities and subcellular localization of DsRed monomer-tagged DGK $\eta$ -PH(K74A) and DGK $\eta$ -PH(R85A) expressed in COS-7 cells.** *A*, expression of DsRed monomer alone, DsRed monomer-DGK $\eta$ -PH(K74A), and DsRed monomer-DGK $\eta$ -PH(R85A) was confirmed by Western blotting. *B*, equimolar amounts (100 pmol) of PI, PI(3)P, PI(4)P, PI(5)P, PC, PI(3,4)P<sub>2</sub>, and PI(4,5)P<sub>2</sub> were spotted onto nitrocellulose membranes as indicated. The membranes were incubated with DsRed monomer alone, DsRed monomer-DGK $\eta$ -PH(K74A), and DsRed monomer-DGK $\eta$ -PH(R85A). Lipid-bound DsRed monomer-tagged proteins were detected by immunostaining with anti-RFP antibody. Even after much longer exposure than that in Fig. 4, spots of PI(4,5)P<sub>2</sub> were not detected. DsRed monomer-DGK $\eta$ -PH(K74A)



**FIGURE 12. Effects of DGK $\eta$ 1(K74A) and DGK $\eta$ 1(R85A) on EGF-dependent activation of ERK.** COS-7 cells were transfected with pAcGFP vector, pAcGFP-DGK $\eta$ 1, pAcGFP-DGK $\eta$ 1(K74A), or pAcGFP-DGK $\eta$ 1(R85A). After 24 h, the cells were serum-starved for 5 h and then stimulated with 100 ng/ml EGF for 5 min. ERK1/2 phosphorylation (p-ERK) and total ERK2 in the cell lysates were analyzed by Western blotting. *A*, representative results of Western blotting analysis are shown. *B*, the phospho-ERK levels (phosphorylated ERK/total ERK) were quantified by densitometry. Phospho-ERK levels in cells that were transfected with pAcGFP vector alone and stimulated with EGF for 5 min were set to 100. The data are shown as means  $\pm$  S.D. of four independent experiments. The statistical significance was determined using analysis of variance followed by Tukey's post hoc test (\*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.005$ ). Error bars represent S.E.

Compared with the PHs of DGK $\delta$  and DGK $\kappa$ , the affinity of the PH of DGK $\eta$  for PI(4,5)P<sub>2</sub> is markedly strong (DGK $\eta$ -PH  $\gg$  DGK $\delta$ -PH  $\gg$  DGK $\kappa$ -PH) (Fig. 2). Partial formation of the proper conformation may cause low affinity for PI(4,5)P<sub>2</sub>. However, high concentrations of the PHs of DGK $\delta$  and DGK $\kappa$ , which probably increase the number of the domains that have the proper conformation, showed no effect on their binding affinities for PI(4,5)P<sub>2</sub>, indicating that the different affinities are intrinsic properties.

It was reported that Lys-30, Lys-32, Arg-40, Ser-55, Arg-56, and Lys-57 in the PLC $\delta$ 1-PH are important for strong PI(4,5)P<sub>2</sub>-selective binding activity (41). The four amino acid residues Lys-30, Arg-40, Ser-55, and Arg-56 in the PLC $\delta$ 1-PH are conserved in the DGK $\eta$ -PH (Lys-74, Arg-85, Ser-100, and Lys-101, respectively) (Fig. 10). We confirmed that K74A and R85A in DGK $\eta$ -PH are indeed critical residues for PI(4,5)P<sub>2</sub> binding activity (Fig. 11B). In the DGK $\delta$ -PH, the three amino acid residues Lys-62, Arg-73, and Lys-89, corresponding to Lys-30, Arg-40, and Arg-56 in the PLC $\delta$ 1-PH, are conserved. How-

(C) and DsRed monomer-DGK $\eta$ -PH(R85A) (D) were co-expressed with AcGFP-PLC $\delta$ 1-PH in COS-7 cells. After 48 h of transfection, the cells were incubated with 500 mM sorbitol in DMEM for 30 min. The cells were fixed with 3.7% formaldehyde and imaged by inverted confocal laser microscopy (FV1000-D). Representative data of three independent experiments are shown. Scale bars, 20  $\mu$ m.

ever, in the DGK $\kappa$ -PH, only two amino acid residues, Lys-225 and Arg-236, are conserved. Therefore, it is possible that the differences in the sequences of these PHs cause the distinct affinities for PI(4,5)P<sub>2</sub> among them (DGK $\eta$ -PH > DGK $\delta$ -PH > DGK $\kappa$ -PH; see Fig. 2). The presence of Gly at the end of the  $\beta$ 1 strand of PH is important to confer PI(3,4,5)P<sub>3</sub> binding activity (42, 43). Because the amino acid residue at the end of the  $\beta$ 1 strand of the DGK $\eta$ -PH is Asn, it is reasonable that it does not preferably bind to PI(3,4,5)P<sub>3</sub> (Fig. 2). However, the determination of the three-dimensional structure of the PHs of DGK $\eta$ , - $\delta$ , and - $\kappa$  is required to analyze their different PI(4,5)P<sub>2</sub> binding properties in more detail.

A previous study demonstrated that the PH of DGK $\delta$  non-selectively interacted with PI(4,5)P<sub>2</sub> because it also strongly bound to PS (37). However, high amounts (~20 times higher) of lipids were used in the overlay assay in the previous report (37). Therefore, it is likely that the selectivity of the PH of DGK $\delta$  for PI(4,5)P<sub>2</sub> was detected in the present study (Fig. 2), which used relatively low amounts of phospholipids. The PH of DGK $\delta$  is important for its phorbol ester-dependent plasma membrane localization (15, 44). However, it is still unclear whether PI(4,5)P<sub>2</sub> is involved in the translocation.

The EGF receptor signaling pathway, which is regulated by DGK $\eta$  (24), augments PI(4,5)P<sub>2</sub> (45). Osmotic shock also increases the amount of PI(4,5)P<sub>2</sub> at the plasma membrane (38, 39). In this study, we also found that the PLC $\delta$ 1-PH, which is known to be a PI(4,5)P<sub>2</sub> sensor, was localized at the plasma membrane in response to osmotic shock (Fig. 7). DGK $\eta$ -PH(K74A) and -(R85A), which lacked PI(4,5)P<sub>2</sub> binding activities (Fig. 11B), failed to translocate to the plasma membrane and to co-localize with the PLC $\delta$ 1-PH even in osmotically shocked cells (Fig. 11, C and D). These results strongly suggest that PI(4,5)P<sub>2</sub> was increased in the plasma membrane. The PH of DGK $\eta$  was also co-distributed with the PLC $\delta$ 1-PH at the plasma membrane. Moreover, depletion of PI(4,5)P<sub>2</sub> by the PI(4,5)P<sub>2</sub> phosphatase synaptojanin significantly reduced the osmotic shock-dependent plasma membrane localization of DGK $\eta$ -PH (Fig. 8). The result further indicates that DGK $\eta$ -PH binds to and is co-localized with PI(4,5)P<sub>2</sub> in cells. Therefore, the PH of DGK $\eta$  could also function as a cellular PI(4,5)P<sub>2</sub> sensor. Compared with the PH of PLC $\delta$ 1, the PH of DGK $\eta$  is equal to or superior to the PLC $\delta$ 1-PH in terms of affinity and selectivity for PI(4,5)P<sub>2</sub> (Fig. 4). The PH of PLC $\delta$ 1 is widely used as a cellular PI(4,5)P<sub>2</sub> detector. Therefore, the PH of DGK $\eta$  could serve as an excellent PI(4,5)P<sub>2</sub>-selective probe with high affinity and selectivity like the PLC $\delta$ 1-PH.

We have reported that DGK $\eta$ 1 was osmotic shock-dependently translocated to punctate vesicles and that the PH and C1Ds are important for the redistribution (16, 32). In this study, we observed that the PH alone was translocated to the punctate regions of the plasma membrane where the PLC $\delta$ 1-PH was co-localized in response to osmotic shock (Fig. 7, D and G), whereas the C1 domains alone, which were distributed to punctate vesicles in the cytoplasm, were not co-localized with the PLC $\delta$ 1-PH (Fig. 7E). In osmotically shocked cells, full-length DGK $\eta$  and a DGK $\eta$  mutant containing both the PH and the C1Ds were distributed to both PLC $\delta$ 1-PH-co-localized punctate regions at the plasma membrane and punctate vesicles in

the cytoplasm, which do not co-localize with the PLC $\delta$ 1-PH (Fig. 7, B and C). These results suggest that the PH and C1Ds competitively recruit DGK $\eta$  to subcellular compartments with and without PI(4,5)P<sub>2</sub>, respectively.

We did not detect obvious competition between the PLC $\delta$ 1-PH and the DGK $\eta$ -PH in both confocal microscopy and cell fractionation. Whether the PI(4,5)P<sub>2</sub> binding competition between these PHs occurs or not is probably dependent on the amount of PI(4,5)P<sub>2</sub> in the plasma membrane. The amount of PI(4,5)P<sub>2</sub> produced in osmotically shocked cells may be greater than the sum of the amounts of the PLC $\delta$ 1-PH and DGK $\eta$ -PH that are able to access PI(4,5)P<sub>2</sub> in the plasma membrane.

We reported previously that DGK $\eta$ 1 positively regulates the EGF receptor/Ras/B-Raf/C-Raf/MEK/ERK signaling pathway (24). Wild-type DGK $\eta$ 1 markedly increased EGF-dependent phosphorylation levels of ERK, whereas both DGK $\eta$ 1(K74A) and DGK $\eta$ 1(R85A) failed to show such effect (Fig. 12). Therefore, it is possible that PI(4,5)P<sub>2</sub> binding activity of DGK $\eta$ 1 regulates its stimulation-dependent subcellular localization and plays an important role in regulating the signal transduction pathway.

DGK $\eta$  was reported to be implicated in the etiology of bipolar disorder (27–29). In the brain, DGK $\eta$  is enriched in the dentate gyrus of the hippocampus and the Purkinje cells of the cerebellum (26), which are known to be associated with bipolar disorder (46–48). A common treatment for bipolar disorder is a mood stabilizer, lithium, which attenuates PI turnover (49). Therefore, DGK $\eta$  may regulate the pathogenesis of bipolar disorder through the PH-dependent binding to PI(4,5)P<sub>2</sub> generated by PI turnover in the dentate gyrus and the Purkinje cells.

Among more than 300 PHs, only the PH of PLC $\delta$ 1 is an established PI(4,5)P<sub>2</sub>-selective binding domain (50, 51). In this study, we added the DGK $\eta$ -PH to the list as a new member. Although the affinity of the DGK $\delta$ -PH for PI(4,5)P<sub>2</sub> is relatively lower than that of the DGK $\eta$ -PH, the PH of DGK $\delta$  is an additional PI(4,5)P<sub>2</sub>-selective PH. The PH of DGK $\eta$  could be an excellent cellular sensor for PI(4,5)P<sub>2</sub> that is equal or superior to the PLC $\delta$ 1-PH. DGK $\eta$ 1 has been reported to be involved in EGF-dependent cell proliferation (24) and in the pathogenesis of lung cancer (25) and bipolar disorder (27–29). It will be interesting to determine what role the DGK $\eta$  isozyme, which has the highly PI(4,5)P<sub>2</sub>-selective binding motif, plays in modulating these physiologically and pathologically important events.

*Author Contributions*—F. S. conceived and coordinated the study and wrote the paper. A. K. designed, performed, and analyzed the experiments shown in Figs. 1–7 and 10. T. U. designed, performed, and analyzed the experiments shown in Figs. 2, 7–9, 11, and 12. S. K. designed, performed, and analyzed the experiments shown in Figs. 7–9, 11, and 12. K. K. designed, performed, and analyzed the experiments shown in Figs. 8, 11, and 12. E. T. designed, performed, and analyzed the experiments shown in Fig. 2. H. S. designed the experiments shown in Figs. 1–7 and 10, provided technical assistance, and contributed to the preparation of the figures. All authors reviewed the results and approved the final version of the manuscript.

## References

- Goto, K., Hozumi, Y., and Kondo, H. (2006) Diacylglycerol, phosphatidic acid, and the converting enzyme, diacylglycerol kinase, in the nucleus.

## PI(4,5)P<sub>2</sub> Binding of the DGK $\eta$ PH Domain

- Biochim. Biophys. Acta* **1761**, 535–541
- Mérida, I., Avila-Flores, A., and Merino, E. (2008) Diacylglycerol kinases: at the hub of cell signalling. *Biochem. J.* **409**, 1–18
  - Sakane, F., Imai, S., Kai, M., Yasuda, S., and Kanoh, H. (2007) Diacylglycerol kinases: why so many of them? *Biochim. Biophys. Acta* **1771**, 793–806
  - Shulga, Y. V., Topham, M. K., and Epand, R. M. (2011) Regulation and functions of diacylglycerol kinases. *Chem. Rev.* **111**, 6186–6208
  - van Blitterswijk, W. J., and Houssa, B. (2000) Properties and functions of diacylglycerol kinases. *Cell. Signal.* **12**, 595–605
  - Nishizuka, Y. (1992) Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. *Science* **258**, 607–614
  - Hurley, J. H., Newton, A. C., Parker, P. J., Blumberg, P. M., and Nishizuka, Y. (1997) Taxonomy and function of C1 protein kinase C homology domains. *Protein Sci.* **6**, 477–480
  - Ron, D., and Kazanietz, M. G. (1999) New insights into the regulation of protein kinase C and novel phorbol ester receptors. *FASEB J.* **13**, 1658–1676
  - Exton, J. H. (1994) Phosphatidylcholine breakdown and signal transduction. *Biochim. Biophys. Acta* **1212**, 26–42
  - English, D. (1996) Phosphatidic acid: a lipid messenger involved in intracellular and extracellular signalling. *Cell. Signal.* **8**, 341–347
  - Sakai, H., and Sakane, F. (2012) Recent progress on type II diacylglycerol kinases: the physiological functions of diacylglycerol kinase  $\delta$ ,  $\eta$  and  $\kappa$  and their involvement in disease. *J. Biochem.* **152**, 397–406
  - Sakane, F., Imai, S., Kai, M., Wada, I., and Kanoh, H. (1996) Molecular cloning of a novel diacylglycerol kinase isozyme with a pleckstrin homology domain and a C-terminal tail similar to those of the EPH family of protein tyrosine kinase. *J. Biol. Chem.* **271**, 8394–8401
  - Klauck, T. M., Xu, X., Mousseau, B., and Jaken, S. (1996) Cloning and characterization of a glucocorticoid-induced diacylglycerol kinase. *J. Biol. Chem.* **271**, 19781–19788
  - Imai, S., Kai, M., Yasuda, S., Kanoh, H., and Sakane, F. (2005) Identification and characterization of a novel human type II diacylglycerol kinase, DGK $\kappa$ . *J. Biol. Chem.* **280**, 39870–39881
  - Sakane, F., Imai, S., Yamada, K., Murakami, T., Tsushima, S., and Kanoh, H. (2002) Alternative splicing of the human diacylglycerol kinase  $\delta$  gene generates two isoforms differing in their expression patterns and in regulatory functions. *J. Biol. Chem.* **277**, 43519–43526
  - Murakami, T., Sakane, F., Imai, S., Houkin, K., and Kanoh, H. (2003) Identification and characterization of two splice variants of human diacylglycerol kinase  $\eta$ . *J. Biol. Chem.* **278**, 34364–34372
  - Harada, B. T., Knight, M. J., Imai, S., Qiao, F., Ramachander, R., Sawaya, M. R., Gingery, M., Sakane, F., and Bowie, J. U. (2008) Regulation of enzyme localization by polymerization: polymer formation by the SAM domain of diacylglycerol kinase  $\delta$ 1. *Structure* **16**, 380–387
  - Imai, S., Sakane, F., and Kanoh, H. (2002) Phorbol ester-regulated oligomerization of diacylglycerol kinase  $\delta$  linked to its phosphorylation and translocation. *J. Biol. Chem.* **277**, 35323–35332
  - Knight, M. J., Joubert, M. K., Plotkowski, M. L., Kropat, J., Gingery, M., Sakane, F., Merchant, S. S., and Bowie, J. U. (2010) Zinc binding drives Sheet formation by the SAM domain of diacylglycerol kinase  $\delta$ . *Biochemistry* **49**, 9667–9676
  - Crotty, T., Cai, J., Sakane, F., Taketomi, A., Prescott, S. M., and Topham, M. K. (2006) Diacylglycerol kinase  $\delta$  regulates protein kinase C and epidermal growth factor receptor signaling. *Proc. Natl. Acad. Sci. U.S.A.* **103**, 15485–15490
  - Chibalin, A. V., Leng, Y., Vieira, E., Krook, A., Björnholm, M., Long, Y. C., Kotova, O., Zhong, Z., Sakane, F., Steiler, T., Nylén, C., Wang, J., Laakso, M., Topham, M. K., Gilbert, M., et al. (2008) Downregulation of diacylglycerol kinase  $\delta$  contributes to hyperglycemia-induced insulin resistance. *Cell* **132**, 375–386
  - Miele, C., Paturzo, F., Teperino, R., Sakane, F., Fiory, F., Oriente, F., Ungaro, P., Valentino, R., Beguinot, F., and Formisano, P. (2007) Glucose regulates diacylglycerol intracellular levels and protein kinase C activity by modulating diacylglycerol-kinase subcellular localization. *J. Biol. Chem.* **282**, 31835–31843
  - Leach, N. T., Sun, Y., Michaud, S., Zheng, Y., Ligon, K. L., Ligon, A. H., Sander, T., Korf, B. R., Lu, W., Harris, D. J., Gusella, J. F., Maas, R. L., Quade, B. J., Cole, A. J., Kelz, M. B., and Morton, C. C. (2007) Disruption of diacylglycerol kinase  $\delta$  (DGKD) associated with seizures in humans and mice. *Am. J. Hum. Genet.* **80**, 792–799
  - Yasuda, S., Kai, M., Imai, S., Takeishi, K., Taketomi, A., Toyota, M., Kanoh, H., and Sakane, F. (2009) Diacylglycerol kinase  $\eta$  augments C-Raf activity and B-Raf/C-Raf heterodimerization. *J. Biol. Chem.* **284**, 29559–29570
  - Nakano, T., Iravani, A., Kim, M., Hozumi, Y., Lohse, M., Reichert, E., Crotty, T. M., Stafforini, D. M., and Topham, M. K. (2014) Diacylglycerol kinase  $\eta$  modulates oncogenic properties of lung cancer cells. *Clin. Transl. Oncol.* **16**, 29–35
  - Usuki, T., Sakai, H., Shionoya, T., Sato, N., and Sakane, F. (2015) Expression and localization of type II diacylglycerol kinase isozymes  $\delta$  and  $\eta$  in the developing mouse brain. *J. Histochem. Cytochem.* **63**, 57–68
  - Baum, A. E., Akula, N., Cabanero, M., Cardona, I., Corona, W., Klemens, B., Schulze, T. G., Cichon, S., Rietschel, M., Nöthen, M. M., Georgi, A., Schumacher, J., Schwarz, M., Abou Jamra, R., Höfels, S., et al. (2008) A genome-wide association study implicates diacylglycerol kinase  $\eta$  (DGKH) and several other genes in the etiology of bipolar disorder. *Mol. Psychiatry* **13**, 197–207
  - Weber, H., Kittel-Schneider, S., Gessner, A., Domschke, K., Neuner, M., Jacob, C. P., Buttenschon, H. N., Boreatti-Hümmer, A., Volkert, J., Herterich, S., Baune, B. T., Gross-Lesch, S., Kopf, J., Kreiker, S., Nguyen, T. T., et al. (2011) Cross-disorder analysis of bipolar risk genes: further evidence of DGKH as a risk gene for bipolar disorder, but also unipolar depression and adult ADHD. *Neuropsychopharmacology* **36**, 2076–2085
  - Moya, P. R., Murphy, D. L., McMahon, F. J., and Wendland, J. R. (2010) Increased gene expression of diacylglycerol kinase  $\eta$  in bipolar disorder. *Int. J. Neuropsychopharmacol.* **13**, 1127–1128
  - Shionoya, T., Usuki, T., Komenoi, S., Isozaki, T., Sakai, H., and Sakane, F. (2015) Distinct expression and localization of the type II diacylglycerol kinase isozymes  $\delta$ ,  $\eta$  and  $\kappa$  in the mouse reproductive organs. *BMC Dev. Biol.* **15**, 6
  - van der Zanden, L. F., van Rooij, I. A., Feitz, W. F., Knight, J., Donders, A. R., Renkema, K. Y., Bongers, E. M., Vermeulen, S. H., Kiemeny, L. A., Veltman, J. A., Arias-Vásquez, A., Zhang, X., Markljung, E., Qiao, L., Baskin, L. S., et al. (2011) Common variants in DGK $\eta$  are strongly associated with risk of hypospadias. *Nat. Genet.* **43**, 48–50
  - Matsutomo, D., Isozaki, T., Sakai, H., and Sakane, F. (2013) Osmotic shock-dependent redistribution of diacylglycerol kinase  $\eta$ 1 to non-ionic detergent-resistant membrane via pleckstrin homology and C1 domains. *J. Biochem.* **153**, 179–190
  - Guo, Y., Philip, F., and Scarlata, S. (2003) The pleckstrin homology domains of phospholipases C- $\beta$  and - $\delta$  confer activation through a common site. *J. Biol. Chem.* **278**, 29995–30004
  - Tsushima, S., Kai, M., Yamada, K., Imai, S., Houkin, K., Kanoh, H., and Sakane, F. (2004) Diacylglycerol kinase  $\gamma$  serves as an upstream suppressor of Rac1 and lamellipodium formation. *J. Biol. Chem.* **279**, 28603–28613
  - Lemmon, M. A., Ferguson, K. M., O'Brien, R., Sigler, P. B., and Schlesinger, J. (1995) Specific and high-affinity binding of inositol phosphates to an isolated pleckstrin homology domain. *Proc. Natl. Acad. Sci. U.S.A.* **92**, 10472–10476
  - Watt, S. A., Kular, G., Fleming, I. N., Downes, C. P., and Lucocq, J. M. (2002) Subcellular localization of phosphatidylinositol 4,5-bisphosphate using the pleckstrin homology domain of phospholipase C  $\delta$ 1. *Biochem. J.* **363**, 657–666
  - Kavran, J. M., Klein, D. E., Lee, A., Falasca, M., Isakoff, S. J., Skolnik, E. Y., and Lemmon, M. A. (1998) Specificity and promiscuity in phosphoinositide binding by pleckstrin homology domains. *J. Biol. Chem.* **273**, 30497–30508
  - Hayes, M. J., Merrifield, C. J., Shao, D., Ayala-Sanmartin, J., Schorey, C. D., Levine, T. P., Proust, J., Curran, J., Bailly, M., and Moss, S. E. (2004) Annexin 2 binding to phosphatidylinositol 4,5-bisphosphate on endocytic vesicles is regulated by the stress response pathway. *J. Biol. Chem.* **279**, 14157–14164
  - Yamamoto, M., Chen, M. Z., Wang, Y. J., Sun, H. Q., Wei, Y., Martinez, M., and Yin, H. L. (2006) Hypertonic stress increases phosphatidylinositol 4,5-bisphosphate levels by activating PIP5K1 $\beta$ . *J. Biol. Chem.* **281**, 32630–32638

40. McPherson, P. S., Garcia, E. P., Slepnev, V. I., David, C., Zhang, X., Grabs, D., Sossin, W. S., Bauerfeind, R., Nemoto, Y., and De Camilli, P. (1996) A presynaptic inositol-5-phosphatase. *Nature* **379**, 353–357
41. Ferguson, K. M., Lemmon, M. A., Schlessinger, J., and Sigler, P. B. (1995) Structure of the high affinity complex of inositol trisphosphate with a phospholipase C pleckstrin homology domain. *Cell* **83**, 1037–1046
42. Isakoff, S. J., Cardozo, T., Andreev, J., Li, Z., Ferguson, K. M., Abagyan, R., Lemmon, M. A., Aronheim, A., and Skolnik, E. Y. (1998) Identification and analysis of PH domain-containing targets of phosphatidylinositol 3-kinase using a novel *in vivo* assay in yeast. *EMBO J.* **17**, 5374–5387
43. Lemmon, M. A., and Ferguson, K. M. (2000) Signal-dependent membrane targeting by pleckstrin homology (PH) domains. *Biochem. J.* **350**, 1–18
44. Imai, S., Kai, M., Yamada, K., Kanoh, H., and Sakane, F. (2004) The plasma membrane translocation of diacylglycerol kinase  $\delta 1$  is negatively regulated by conventional protein kinase C-dependent phosphorylation at Ser-22 and Ser-26 within the pleckstrin homology domain. *Biochem. J.* **382**, 957–966
45. Noh, D. Y., Shin, S. H., and Rhee, S. G. (1995) Phosphoinositide-specific phospholipase C and mitogenic signaling. *Biochim. Biophys. Acta* **1242**, 99–113
46. Hagihara, H., Takao, K., Walton, N. M., Matsumoto, M., and Miyakawa, T. (2013) Immature dentate gyrus: an endophenotype of neuropsychiatric disorders. *Neural Plast.* **2013**, 318596
47. Maloku, E., Covelo, I. R., Hanbauer, I., Guidotti, A., Kadriu, B., Hu, Q., Davis, J. M., and Costa, E. (2010) Lower number of cerebellar Purkinje neurons in psychosis is associated with reduced reelin expression. *Proc. Natl. Acad. Sci. U.S.A.* **107**, 4407–4411
48. Walton, N. M., Zhou, Y., Kogan, J. H., Shin, R., Webster, M., Gross, A. K., Heusner, C. L., Chen, Q., Miyake, S., Tajinda, K., Tamura, K., Miyakawa, T., and Matsumoto, M. (2012) Detection of an immature dentate gyrus feature in human schizophrenia/bipolar patients. *Transl. Psychiatry* **2**, e135
49. Belmaker, R. H., Agam, G., van Calker, D., Richards, M. H., and Kofman, O. (1998) Behavioral reversal of lithium effects by four inositol isomers correlates perfectly with biochemical effects on the PI cycle: depletion by chronic lithium of brain inositol is specific to hypothalamus, and inositol levels may be abnormal in postmortem brain from bipolar patients. *Neuropsychopharmacology* **19**, 220–232
50. Lemmon, M. A. (2008) Membrane recognition by phospholipid-binding domains. *Nat. Rev. Mol. Cell Biol.* **9**, 99–111
51. Maffucci, T., and Falasca, M. (2001) Specificity in pleckstrin homology (PH) domain membrane targeting: a role for a phosphoinositide-protein co-operative mechanism. *FEBS Lett.* **506**, 173–179