

# 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

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DGK (diacylglycerol kinase) regulates the concentration of two bioactive lipids, diacylglycerol and phosphatidic acid. DGK $\delta 1$  or its PH (pleckstrin homology) domain alone has been shown to be translocated to the plasma membrane from the cytoplasm in PMA-treated cells. In the present study, we identified Ser-22 and Ser-26 within the PH domain as the PMA- and epidermal-growth-factor-dependent phosphorylation sites of DGK $\delta 1$ . Experiments *in vitro* and with intact cells suggested that the cPKC (conventional protein kinase C) phosphorylated these Ser residues directly. Puzzlingly, alanine/asparagine mutants at Ser-22 and Ser-26 of DGK $\delta 1$  and its PH domain are still persistently translocated by PMA treatment, suggesting that the PH domain phosphorylation is not responsible for the enzyme translocation and

that the translocation was caused by a PMA-dependent, but cPKC-independent, process yet to be identified. Interestingly, the aspartate mutation, which mimics phosphoserine, at Ser-22 or Ser-26, inhibited the translocation of full-length DGK $\delta 1$  and the PH domain markedly, suggesting that the phosphorylation regulates negatively the enzyme translocation. Our results provide evidence of the phosphorylation of the DGK $\delta 1$  PH domain by cPKC, and suggest that the phosphorylation is involved in the control of subcellular localization of DGK $\delta 1$ .

**Key words:** diacylglycerol kinase, pleckstrin homology domain, phorbol ester, protein kinase C, translocation.

## INTRODUCTION

Upon cell activation by various hormones, neurotransmitters, growth factors and other agonists, DG (diacylglycerol) is liberated from phosphatidylinositols and other phospholipids [1,2]. DGK (DG kinase) phosphorylates DG to produce phosphatidic acid [3]. The roles of DG and phosphatidic acid as lipid second messengers have been attracting much attention. DG is known to be an activator of cPKC and nPKC (conventional and novel protein kinase C respectively), Unc-13 and Ras guanine nucleotide-releasing protein [4–7]. Phosphatidic acid has been reported in a number of studies to modulate phosphatidylinositol-4-phosphate kinase, Raf-1 kinase, atypical PKC and many other important enzymes [8,9]. Because the cellular concentration of these signalling lipids must be strictly regulated, DGK is thought to be one of the key enzymes involved in the cellular signal transduction.

To date, nine mammalian DGK isoenzymes ( $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$ ,  $\epsilon$ ,  $\zeta$ ,  $\eta$ ,  $\theta$  and  $\iota$ ), containing in common the zinc-finger structures and the catalytic region, have been identified and classified into five subgroups according to their structural features [10–13]. Interestingly, the occurrence of alternative splicing, which supplements further the complexity of the DGK gene family members, has recently been detected for five mammalian DGK genes ( $\gamma$  [14],  $\zeta$  [15],  $\beta$  [16],  $\delta$  [17] and  $\eta$  [18] isoforms). The structural diversity and the tissue- and cell-dependent expression patterns observed distinctively for these isoforms [10–13] suggest that each member is regulated by a distinct mechanism, performing differentiated functions in particular types of cells.

We previously cloned a cDNA encoding DGK $\delta 1$  (type II isoenzyme), which contains a PH (pleckstrin homology) domain at the N-terminus and a SAM (sterile  $\alpha$  motif) domain at the C-terminus [19]. The alternative splicing product (DGK $\delta 2$ ) contains the 52 residues extended N-terminally [17]. The SAM domain, which is an evolutionally conserved protein–protein interaction motif, was found to occur in a wide range of proteins [20,21]. We demonstrated by gel filtration and co-immunoprecipitation analyses that DGK $\delta 1$  formed homo-oligomers [22] as well as hetero-oligomers with other type II DGK members, DGK $\delta 2$  and DGK $\eta 2$ , through their common SAM domains [17,18]. The PH domain is a small protein module of 100–120 amino acids that is found in many proteins involved in cell signalling, cytoskeletal rearrangement and other processes [23,24]. We reported that DGK $\delta 1$  was translocated from the cytoplasm to the plasma membrane in phorbol-ester-stimulated cells [22], and that the PH domain was critically involved in the translocation [17]. Interestingly, the subcellular localization of DGK $\delta 2$  was not changed even after phorbol ester stimulation [17].

We have found that, in addition to the translocation, endogenous or transiently expressed DGK $\delta 1$  is phosphorylated by endogenous protein kinase in response to phorbol ester stimulation in HepG2, COS-7 and HEK-293 (human embryonic kidney) cells [22]. However, the phosphorylation site(s) and the functional relationship between the enzyme phosphorylation and translocation remain to be explored. In the present study, we identified Ser-22 and Ser-26 within the PH domain as the phorbol-ester- and EGF (epidermal growth factor)-dependent phosphorylation

Abbreviations used: cPKC, conventional protein kinase C; DG, diacylglycerol; DGK, DG kinase; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; ERK, extracellular-signal-regulated kinase; GFP, green fluorescent protein; GST, glutathione S-transferase; HEK, human embryonic kidney; MEK, mitogen-activated protein kinase/ERK kinase; nPKC, novel protein kinase C; NTR, N-terminal region; PH, pleckstrin homology; PKC, protein kinase C; SAM, sterile  $\alpha$  motif; YFP, yellow fluorescent protein.

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sites. Several lines of evidence suggest that cPKC serves as a direct upstream kinase for these serine residues, and that, unexpectedly, the phosphorylation of one of these serine residues by cPKC regulates negatively, rather than positively, the translocation of DGK $\delta$ 1.

## EXPERIMENTAL

### Plasmid constructs

The cDNA fragments of DGK $\delta$ 1-NTR (amino acid residues 1–116), DGK $\delta$ 1- $\Delta$ NTR (amino acid residues 109–1170) and DGK $\delta$ 1-PH domain (amino acid residues 10–107) were generated from a human DGK $\delta$ 1 cDNA clone [19] by PCR. Mutagenesis was carried out using the QuikChange<sup>®</sup> site-directed mutagenesis kit (Stratagene, La Jolla, CA, U.S.A.). For subsequent subcloning into expression vectors, primers were designed such that the resulting DGK $\delta$ 1 cDNA fragments contained a 5'-EcoRI restriction site and a 3'-stop codon followed by a 3'-EcoRI site. The resulting DGK $\delta$ 1 fragments were fused in-frame with YFP (yellow fluorescent protein) in the pEYFP-C1 vector (Clontech, Tokyo, Japan) and with 3  $\times$  FLAG tag in the p3  $\times$  FLAG-CMV-7.1 (Sigma-Aldrich, Tokyo, Japan). Plasmids encoding the DGK $\delta$ 1-PH domain fused to GST (glutathione S-transferase) were obtained by inserting the PH domain cDNA fragments into the pGEX-6P-1 vector (Amersham Biosciences, Tokyo, Japan).

### Cell culture and transfection

HEK-293 and COS-7 cells were maintained in DMEM (Dulbecco's modified Eagle's medium; Sigma-Aldrich) containing 10% (v/v) foetal bovine serum at 37 °C in an atmosphere containing 5% CO<sub>2</sub>. Cells were transiently transfected with cDNAs using Effectene transfection reagent according to the manufacturer's instruction (Qiagen, Tokyo, Japan). After 24–48 h, cells were used for further analysis.

### Protein phosphorylation analysis in intact cells

COS-7 or HEK-293 cells expressing various 3  $\times$  FLAG-tagged DGK $\delta$ 1 constructs were used at 40 h post-transfection. The cells (35-mm-diameter dish) transfected were washed four times with Tris-buffered saline, incubated for 2 h in DMEM without sodium phosphate and sodium pyruvate (Invitrogen Life Technologies, Tokyo, Japan) containing 0.1% (w/v) BSA, and then labelled for 2 h with [<sup>32</sup>P]orthophosphate (0.1 mCi/ml; Amersham Biosciences) in phosphate-free DMEM/0.1% BSA. The labelled cells were subsequently incubated in phosphate-free DMEM/0.1% BSA containing 100 nM PMA or 0.1% DMSO for 30 min. When the effects of EGF stimulation were determined, cells were labelled for 4 h with 0.3 mCi/ml [<sup>32</sup>P]orthophosphate. Stimulated cells were then harvested in buffer 1 [50 mM Tris/HCl, pH 7.4, 1 mM EDTA, 1% (w/v) Nonidet P-40, 150 mM NaCl, 1 mM PMSF, Complete<sup>™</sup> protease inhibitor mixture (Roche Molecular Biochemicals, Tokyo, Japan) and phosphatase inhibitor cocktail II (Sigma-Aldrich)]. The suspension was sonicated and then centrifuged at 10 000 g for 20 min at 4 °C to give cell lysates. Cell lysates (300  $\mu$ l) were pre-cleared with 10  $\mu$ l of Protein A/G PLUS-agarose (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.). Anti-FLAG M2 monoclonal antibody (2  $\mu$ g; Sigma-Aldrich) was added to pre-cleared lysates to immunoprecipitate 3  $\times$  FLAG-tagged DGK $\delta$ 1 proteins. After 1 h, 5  $\mu$ l of Protein A/G PLUS-agarose was added, followed by a 1 h incubation at 4 °C. After washing the agarose beads five times with buffer 1, immunoprecipitated proteins were extracted with 50  $\mu$ l of SDS

sample buffer and then separated by SDS/PAGE. The radioactive signal in a dried gel was visualized by phosphorimaging using a BAS1800 Bio-Image Analyzer (Fuji Film, Tokyo, Japan).

### Western blot analysis

Pre-cleared cell lysates and immunoprecipitates were separated by SDS/PAGE. The separated proteins were transferred on to a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany) and blocked with 10% Block Ace (Dainippon Pharmaceutical, Tokyo, Japan) as described previously [22]. The membrane was incubated with anti-FLAG M2 monoclonal antibody in Block Ace for 1 h. The immunoreactive bands were visualized using horseradish-peroxidase-conjugated anti-mouse IgG antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, U.S.A.) and SuperSignal (Pierce, Rockford, IL, U.S.A.).

### Phosphoamino acid analysis

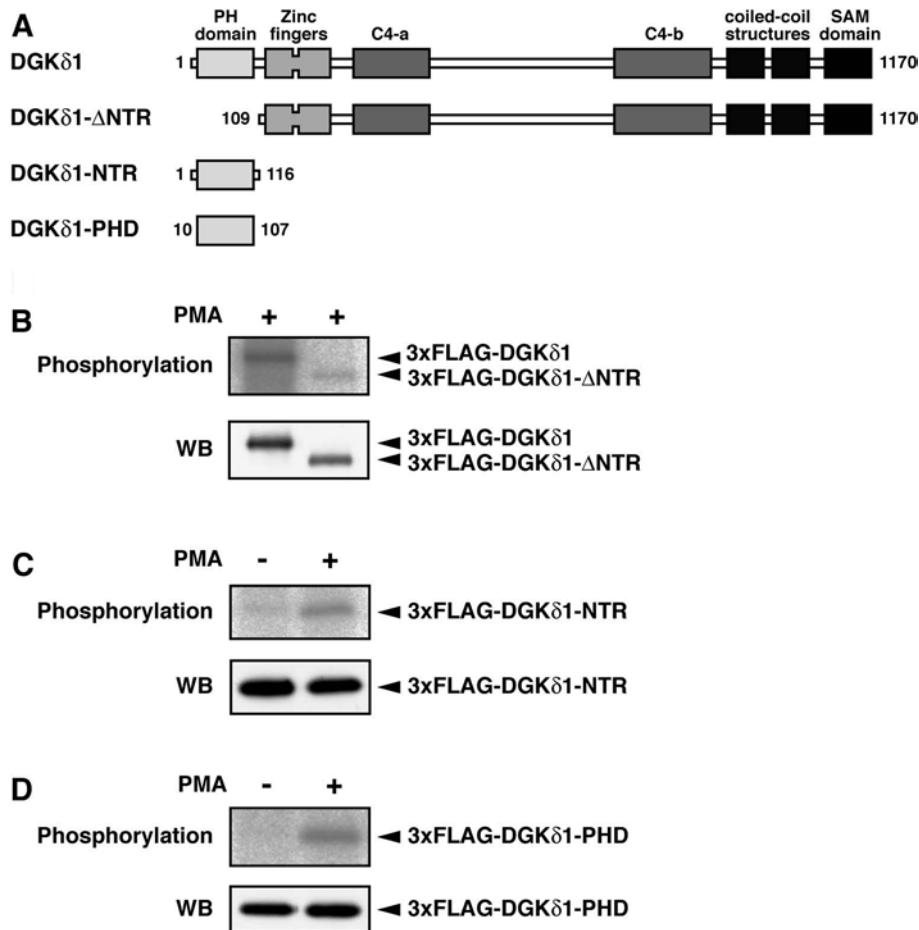
3  $\times$  FLAG-tagged DGK $\delta$ 1-PH domain labelled with <sup>32</sup>P was immunoprecipitated, separated by SDS/(16.5%) PAGE, and then transferred on to an Immobilon-P<sup>5Q</sup> membrane (Millipore, Tokyo, Japan). The transferred protein was visualized by autoradiography, excised from the membrane and hydrolysed in 6 M HCl at 110 °C for 90 min. The hydrolysate was dried under vacuum and redissolved in water containing unlabelled phosphoserine, phosphothreonine and phosphotyrosine standards. The hydrolysate was spotted on a cellulose TLC plate (Sigma-Aldrich). The electrophoresis was carried out in pH 3.5 buffer (5% ethanoic acid and 0.5% pyridine). After being dried, plates were sprayed with 0.25% (w/v) ninhydrin in acetone and heated at 65 °C to visualize the phosphoamino acid standards. The radioactive signal of phosphoamino acid was detected by phosphorimaging using a BAS1800 Bio-Image Analyzer.

### Expression and purification of GST-fusion proteins

XL1-Blue cells (Stratagene) were transformed by various pGEX-6P-1 constructs, and GST or GST-fusion proteins were expressed and purified according to the procedure recommended by the manufacturer (Amersham Biosciences). In this case, the expression of fusion proteins was induced by 1 mM isopropyl  $\beta$ -D-thiogalactoside at 37 °C for 3 h. Cells were then lysed by sonication in PBS, and insoluble material was removed by centrifugation at 10 000 g for 5 min. The supernatants were incubated in batches with glutathione-Sepharose 4B (Amersham Biosciences) for 2 h at 4 °C, and beads were then washed four times with PBS. The beads were finally washed once with the kinase buffer (see below) without ATP just before an *in vitro* protein kinase assay.

### *In vitro* protein kinase assay

An *in vitro* protein kinase assay was carried out at 30 °C for 30 min in the kinase buffer (20 mM Tris/HCl, pH 7.4, 1 mM CaCl<sub>2</sub>, 1 mM dithiothreitol, 10 mM MgCl<sub>2</sub>, 200  $\mu$ g/ml phosphatidylserine, 20  $\mu$ g/ml diolein, 1 mM ATP and 2.5  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP). Phosphatidylserine and diolein in chloroform were dried under nitrogen and dispersed in the buffer by sonication for 30 s at 4 °C, before the addition of enzyme and radioactive ATP. The beads that bound GST or GST-fusion proteins (5  $\mu$ g) were incubated with 15 m-units of purified rat PKC $\alpha$  (> 90% pure; Sigma-Aldrich). Reactions were terminated by centrifugation at 10 000 g for 5 min, and the beads were washed with the kinase buffer without ATP. GST or GST-fusion proteins were extracted with SDS sample buffer and then analysed by SDS/PAGE.



**Figure 1** Phosphorylation of DGK $\delta 1$ -PH domain in response to PMA stimulation

(A) A schematic representation of the DGK $\delta 1$  constructs used for the phosphorylation assay. (B–D) COS-7 cells were transfected with a plasmid encoding 3  $\times$  FLAG–DGK $\delta 1$  (B), 3  $\times$  FLAG–DGK $\delta 1$ - $\Delta$ NTR (C) or 3  $\times$  FLAG–DGK $\delta 1$ -NTR (D). After serum-starvation and  $^{32}$ P-labelling, cells were incubated for 30 min in the presence of 100 nM PMA (+) or 0.1 % DMSO (–). FLAG-tagged proteins were immunoprecipitated with anti-FLAG antibody and separated by SDS/PAGE. The radioactive signal was visualized by a BAS1800 Bio-Image Analyzer (upper panels), and the immunoprecipitated 3  $\times$  FLAG-tagged proteins were analysed by Western blotting using anti-FLAG antibody (lower panels). Representatives of three repeated experiments are shown. PHD, PH domain.

## Fluorescence microscopy

HEK-293 cells were grown on poly(L-lysine)-coated glass coverslips and transiently transfected with cDNAs encoding various DGK $\delta 1$  constructs N-terminally fused with YFP. After 24 h, HEK-293 cells were washed four times with PBS and then cultured in DMEM/0.1 % BSA. After 3 h, the medium was exchanged with DMEM/0.1 % BSA containing 100 nM PMA or 0.1 % DMSO. After 30 min, cells were fixed with 3.7 % (w/v) formaldehyde in PBS for 10 min and then washed five times with PBS at room temperature (25 °C). The coverslips were mounted using Vectashield (Vector Laboratories, Burlingame, CA, U.S.A.). Cells were examined using an inverted confocal laser scanning microscope (Zeiss LSM 510).

## RESULTS

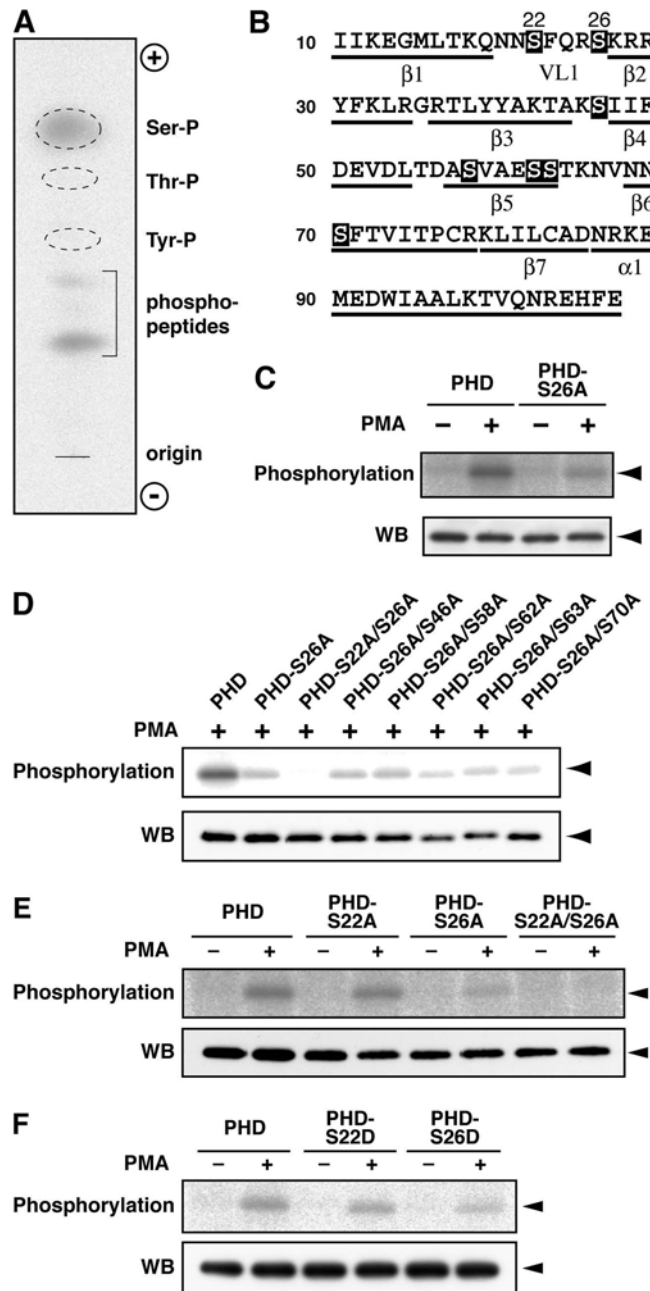
### Phosphorylation of the DGK $\delta 1$ -PH domain by PMA stimulation

We previously described the phosphorylation of DGK $\delta 1$  in PMA-stimulated cells [22]. To identify the phosphorylation sites, we first

assessed phosphorylation of wild-type DGK $\delta 1$  and its truncation mutant lacking the N-terminal region (DGK $\delta 1$ - $\Delta$ NTR; Figure 1A). As shown in Figure 1(B), when compared with the wild-type enzyme, the phosphorylation of DGK $\delta 1$ - $\Delta$ NTR was markedly reduced, but still persisted in PMA-stimulated COS-7 cells. On the other hand, DGK $\delta 1$  N-terminal region (DGK $\delta 1$ -NTR) alone was also phosphorylated in a PMA-dependent manner (Figure 1C). Because DGK $\delta 1$ -NTR contains the DGK $\delta 1$ -specific sequences in addition to the PH domain, we next tested whether or not the PH domain alone was phosphorylated. As shown in Figure 1(D), the DGK $\delta 1$ -PH domain alone was phosphorylated by PMA stimulation. These results indicate that DGK $\delta 1$  has PMA-dependent phosphorylation sites in both the PH domain (amino acid residues 10–107) and the remaining C-terminal region (amino acid residues 109–1170).

### Identification of phosphorylation sites within the DGK $\delta 1$ -PH domain

We next attempted to identify the phosphorylation site(s) within the DGK $\delta 1$ -PH domain. The domain contains seven serine, seven



**Figure 2** Phosphorylation at Ser-22 and Ser-26 in the DGK $\delta$ 1-PH domain

(A) Phosphoamino acid analysis of the DGK $\delta$ 1-PH domain. The analysis was performed as described in the Experimental section. Ser-P, phosphoserine; Thr-P, phosphothreonine; Tyr-P, phosphotyrosine; phosphopeptides, undigested phosphopeptides. (B) Primary and predicted secondary structure of the DGK $\delta$ 1-PH domain. Serine residues are highlighted.  $\alpha$ ,  $\alpha$ -helix;  $\beta$ ,  $\beta$ -sheet; VL1, variable loop 1. (C–F) Effects of point mutations on phosphorylation of the DGK $\delta$ 1-PH domain. COS-7 cells were transfected with expression plasmids as indicated. After starvation and  $^{32}$ P-labelling, cells were incubated for 30 min in the presence of 100 nM PMA (+) or 0.1% DMSO (–). FLAG-tagged proteins were immunoprecipitated with anti-FLAG antibody and analysed by SDS/PAGE. The radioactive signal was visualized by a BAS1800 Bio-Image Analyzer (upper panels), and the immunoprecipitated 3  $\times$  FLAG-tagged proteins were analysed by Western blotting using anti-FLAG antibody (lower panels). The position of the DGK $\delta$ 1-PH domain and its mutants is indicated by arrowheads. Representatives of three repeated experiments are shown. PHD, PH domain.

threonine and three tyrosine residues (Figure 2B). To determine which amino acid residues are phosphorylated in PMA-stimulated cells, we first carried out a phosphoamino acid analysis of the

DGK $\delta$ 1-PH domain. As shown in Figure 2(A), we detected radioactive phosphoserine. However, no phosphorylation signal of threonine and tyrosine residues was detected.

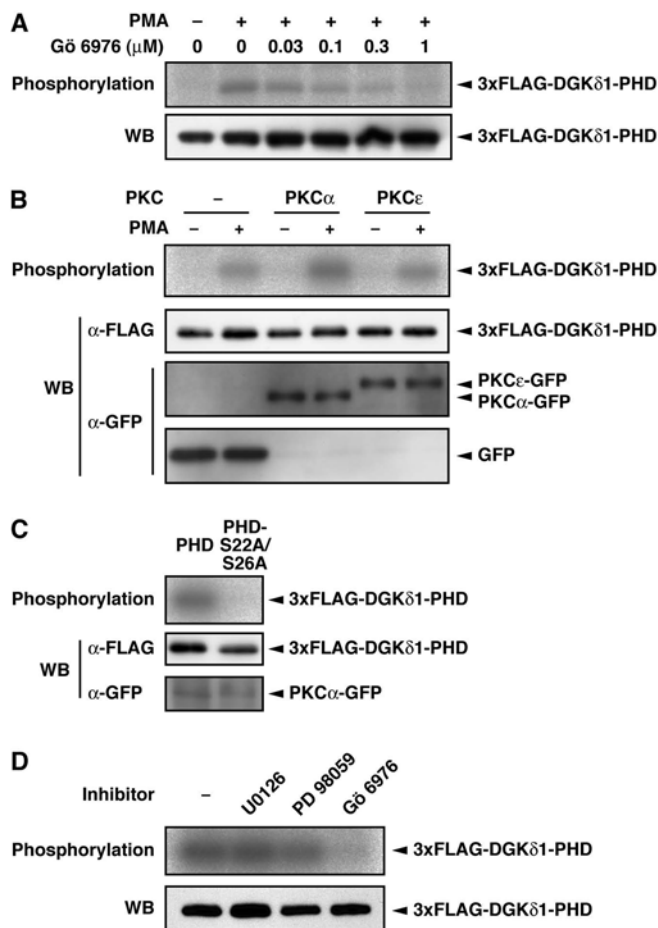
We next used alanine-scanning mutagenesis to determine which serine residues within the DGK $\delta$ 1-PH domain are targets of endogenous protein kinase. The PMA-dependency of phosphorylation implies that cPKC and/or nPKC may phosphorylate the serine residue(s). It is known that PKC preferentially phosphorylates serine and threonine flanked with arginine and lysine at –3 to –1 and +1 to +3 positions [25]. In this context, Ser-26 is flanked with four arginine/lysine residues at the appropriate positions (Figure 2B). This serine residue was thus replaced with alanine (S26A). As shown in Figure 2(C), the DGK $\delta$ 1-PH domain-S26A mutant showed a considerable loss of phosphorylation signal compared with the wild-type domain. However, because some phosphorylation signal still persisted with this mutant, we next attempted to identify the other serine residues that were phosphorylated. We further substituted alanine for one of six serine residues (Ser-22, Ser-46, Ser-58, Ser-62, Ser-63 and Ser-70) of the S26A mutant (Figure 2B). Each of these double mutants was expressed in COS-7 cells, and the phosphorylation levels were assessed. Only the S22A/S26A mutant showed a complete loss of phosphorylation signal (Figure 2D).

Although the phosphorylation level of the S26A mutant was markedly reduced, an alanine mutation at Ser-22 alone reduced only slightly the PH domain phosphorylation (Figure 2E). We further constructed S22D and S26D mutants, in which a negative charge (aspartic acid) was introduced to mimic phosphoserine. Reduction patterns of phosphorylation of the aspartate mutants were essentially the same as those of the corresponding alanine mutants (Figures 2E and 2F), suggesting that the phosphorylation of one of the two serine residues occurs independently of each other. These results indicate that Ser-26 is the major phosphorylation site within the PH domain in response to PMA stimulation. Essentially the same results were obtained using HEK-293 cells (results not shown).

#### cPKC is involved in the phosphorylation of Ser-22 and Ser-26 within the DGK $\delta$ 1-PH domain

In the next set of experiments, we explored the endogenous protein kinase involved in the phosphorylation of the DGK $\delta$ 1-PH domain. We showed previously that staurosporine, a non-selective PKC inhibitor, inhibits PMA-dependent phosphorylation of DGK $\delta$ 1 [22]. In the present study, we tested the effects of a cPKC-selective inhibitor, Gö 6976, on phosphorylation. As shown in Figure 3(A), Gö 6976 clearly reduced the phosphorylation signal in a dose-dependent manner. Gö 6976 at a concentration of 1  $\mu$ M achieved a complete loss of the signal. The result indicates that the DGK $\delta$ 1-PH domain was phosphorylated through the cPKC-dependent pathway in PMA-stimulated cells.

To substantiate the cPKC-dependent phosphorylation, we examined the effects of cPKC expression. We first investigated the expression patterns of endogenous PKC isoenzymes in COS-7 and HEK-293 cells by Western blotting. Among cPKC isoenzymes, only PKC $\alpha$  was highly expressed, whereas PKCs  $\beta$  and  $\gamma$  were not detected in the two cell lines (results not shown). Thus, in subsequent experiments, we focused on PKC $\alpha$ . The phosphorylation of the PH domain was appreciably enhanced by the overexpression of PKC $\alpha$  (Figure 3B). In contrast, nPKC ( $\epsilon$ -isoform), which was an nPKC isoform solely detectable in COS-7 and HEK-293 cells (results not shown), did not markedly affect the phosphorylation level. The double alanine mutant (DGK $\delta$ 1-PH domain-S22A/S26A) was not phosphorylated in the PKC $\alpha$ -overexpressing cells (Figure 3C), indicating that at least one of Ser-22 and

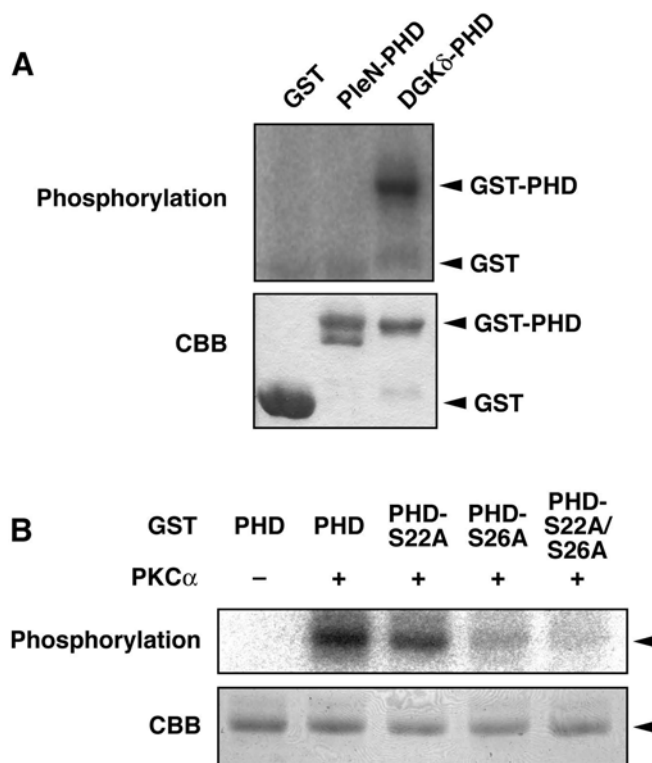


**Figure 3** Effects of a cPKC-selective inhibitor, Gö 6976, and PKC $\alpha$  expression on the DGK $\delta 1$ -PH domain phosphorylation

(A) COS-7 cells were transfected with a plasmid encoding the 3  $\times$  FLAG-DGK $\delta 1$ -PH domain. Cells were starved,  $^{32}$ P-labelled and incubated with Gö 6976 at the indicated concentrations for 30 min before PMA stimulation. Cells were stimulated with 100 nM PMA (+) or 0.1 % DMSO (–) for 30 min in the presence of Gö 6976. (B) COS-7 cells were co-transfected with p3  $\times$  FLAG-CMV-DGK $\delta 1$ -PH domain and either pEGFP alone, pEGFP-PKC $\alpha$  or pEGFP-PKC $\epsilon$ . After starvation and  $^{32}$ P-labelling, cells were incubated for 30 min in the presence of 100 nM PMA (+) or 0.1 % DMSO (–). (C) COS-7 cells were co-transfected with pEGFP-PKC $\alpha$  and either p3  $\times$  FLAG-CMV-DGK $\delta 1$ -PH domain or -PH domain-S22A/S26A. After starvation and  $^{32}$ P-labelling, cells were incubated for 30 min in the presence of 100 nM PMA. (D) COS-7 cells were transfected with a plasmid encoding the 3  $\times$  FLAG-DGK $\delta 1$ -PH domain. Cells were starved,  $^{32}$ P-labelled and incubated with U0126 (50  $\mu$ M), PD098059 (100  $\mu$ M) or Gö 6976 (1  $\mu$ M) for 30 min before PMA stimulation. Cells were treated with 100 nM PMA for 30 min in the presence of the inhibitors. FLAG-tagged proteins were immunoprecipitated with anti-FLAG antibody and analysed by SDS/PAGE. The radioactive signal was visualized by a BAS1800 Bio-Image Analyzer (A–D, top panels), and the 3  $\times$  FLAG-tagged proteins immunoprecipitated (A–D) and the GFP-fusion proteins in cell lysates (B and C) were analysed by Western blotting using anti-FLAG and anti-GFP antibodies (bottom panels, WB).

Ser-26 was indeed phosphorylated through the cPKC-dependent pathway.

PKC is known to activate the MEK [mitogen-activated protein kinase/ERK (extracellular-signal-regulated kinase) kinase]/ERK pathway [26,27]. However, MEK inhibitors, U0126 (50  $\mu$ M) and PD098059 (100  $\mu$ M) [28], failed to inhibit the phosphorylation (Figure 3D). In this experiment, we confirmed that U0126 (50  $\mu$ M) and PD098059 (100  $\mu$ M) inhibited phosphorylation of ERK by 95 % and 70 % respectively (results not shown). The result suggests that PKC-activated protein kinases including MEK, ERK and further downstream kinases of the ERK pathway



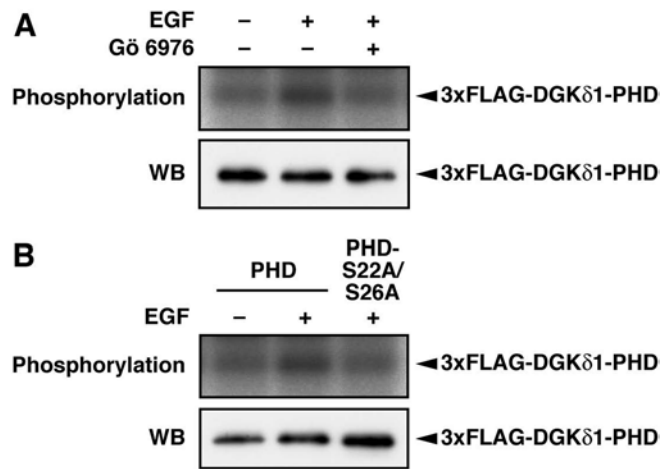
**Figure 4** *In vitro* phosphorylation of the DGK $\delta 1$ -PH domain by PKC $\alpha$

(A) GST, GST-pleckstrin N-terminal PH domain (PleN-PHD) and GST-DGK $\delta 1$ -PH domain (DGK $\delta$ -PHD) proteins were incubated with (+) or without (–) purified rat PKC $\alpha$  (> 90 % purity). (B) GST-DGK $\delta 1$ -PH domain (PHD), GST-DGK $\delta 1$ -PH domain-S22A (PHD-S22A), GST-DGK $\delta 1$ -PH domain-S26A (PHD-S26A) or GST-DGK $\delta 1$ -PH domain-S22A/S26A (PHD-S22A/S26A) proteins were incubated with (+) or without (–) PKC $\alpha$ . Subsequently, the phosphorylated products were separated by SDS/PAGE followed by Coomassie Brilliant Blue staining (CBB, lower panels). The radioactive signal was visualized by a BAS1800 Bio-Image Analyzer (upper panels). The position of the GST-DGK $\delta 1$ -PH domain and its mutants is indicated by an arrowhead.

are not involved in the phosphorylation of the DGK $\delta 1$ -PH domain.

#### cPKC directly phosphorylates Ser-22 and Ser-26 of the DGK $\delta 1$ -PH domain *in vitro*

We next asked whether cPKC directly phosphorylates the DGK $\delta 1$ -PH domain. The DGK $\delta 1$ -PH domain fused with GST was purified and then incubated with purified rat PKC $\alpha$  in the presence of [ $\gamma$ - $^{32}$ P]ATP. As shown in Figure 4(A), the GST-DGK $\delta 1$ -PH domain was phosphorylated by PKC $\alpha$  *in vitro*. On the other hand, the N-terminal PH domain of pleckstrin was not phosphorylated by PKC $\alpha$ , suggesting that the phosphorylation is specific for the DGK $\delta 1$ -PH domain. We confirmed that the double alanine mutant (GST-DGK $\delta 1$ -PH domain-S22A/S26A) failed to be phosphorylated by cPKC (Figure 4B). Moreover, the single alanine mutants (GST-DGK $\delta 1$ -PH domain-S22A and -S26A) exhibited approx. 30 % and 80 % losses of phosphorylation signal respectively, compared with the wild-type PH domain. These results indicate that PKC $\alpha$  is capable of phosphorylating both Ser-22 and Ser-26 directly within the DGK $\delta 1$ -PH domain, and that, consistent with the phosphorylation patterns obtained using intact cells (Figure 2E), the phosphorylation of Ser-26 *in vitro* by PKC $\alpha$  is dominant over that of Ser-22 *in vitro*.



**Figure 5** Phosphorylation of the DGK $\delta$ 1-PH domain by EGF stimulation

(A) COS-7 cells were transfected with a plasmid encoding 3  $\times$  FLAG-DGK $\delta$ 1-PH domain (PHD). Cells were starved,  $^{32}$ P-labelled, and incubated with Gö 6976 (1  $\mu$ M) for 30 min before EGF stimulation. Cells were incubated with (+) or without (-) 100 ng/ml EGF for 10 min in the presence of Gö 6976. (B) COS-7 cells were transfected with either p3  $\times$  FLAG-CMV-DGK $\delta$ 1-PH domain (PHD) or -PH domain-S22A/S26A (PHD-S22A/S26A). After starvation and  $^{32}$ P-labelling, cells were incubated with (+) or without (-) 100 ng/ml EGF for 10 min. FLAG-tagged proteins were immunoprecipitated with anti-FLAG antibody and aliquots were analysed by SDS/PAGE. The radioactive signal was visualized by a BAS1800 Bio-Image Analyzer (upper panels), and the immunoprecipitated 3  $\times$  FLAG-tagged proteins were analysed by Western blotting using anti-FLAG antibody (lower panels).

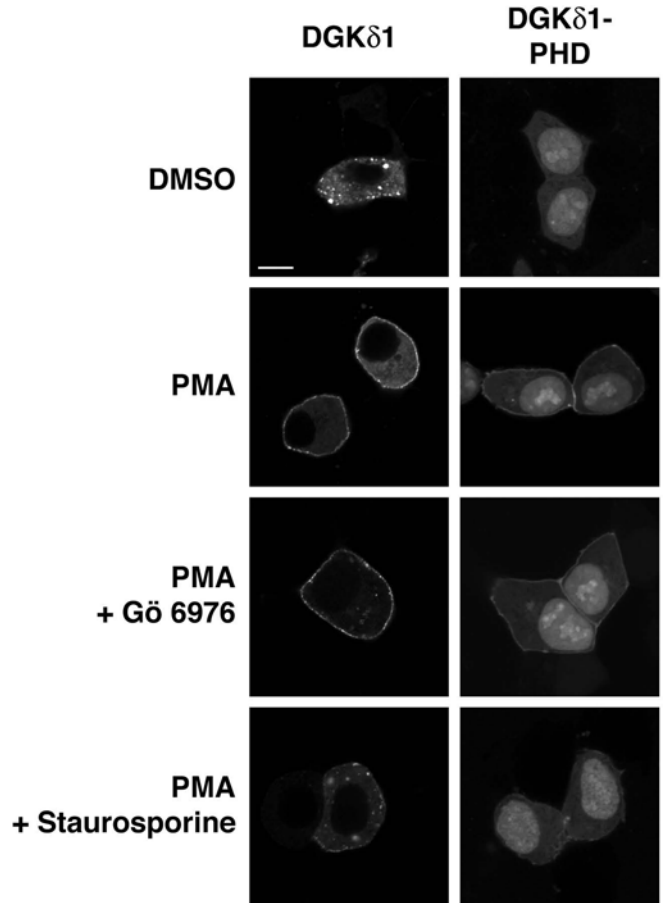
#### EGF-dependent phosphorylation at Ser-22 and Ser-26 within the DGK $\delta$ 1-PH domain

We examined whether, in addition to phorbol ester stimulation, Ser-22 and Ser-26 were phosphorylated by physiological stimulation such as growth factors. When COS-7 cells were stimulated with EGF, the PH domain expressed was phosphorylated (Figure 5A). This phosphorylation was markedly inhibited by Gö 6976, indicating that cPKC plays a critical role in the signalling pathway from the EGF receptor stimulation to the DGK $\delta$ 1-PH domain phosphorylation. The double alanine mutant (DGK $\delta$ 1-PH domain-S22A/S26A) exhibited a markedly decreased phosphorylation level (Figure 5B). These results indicate that at least one of Ser-22 and Ser-26 was phosphorylated via cPKC in EGF-stimulated cells.

#### Effects of PKC inhibitors on the translocation of the DGK $\delta$ 1-PH domain

We next attempted to reveal the physiological relevance of the phosphorylation of DGK $\delta$ 1 at Ser-22 and Ser-26. We reported previously that in response to PMA stimulation, DGK $\delta$ 1 is translocated from the cytoplasm to the plasma membrane in HEK-293 cells [22], and that the DGK $\delta$ 1-PH domain is necessary and sufficient for the DGK $\delta$ 1 translocation [17]. Thus we examined the effects of PKC inhibitors on intracellular localization of the DGK $\delta$ 1-PH domain in HEK-293 cells.

The YFP-tagged DGK $\delta$ 1-PH domain exhibited cytoplasmic/nucleoplasmic localization in the resting cells (Figure 6). However, as reported previously [17], the DGK $\delta$ 1-PH domain was partly translocated from the cytoplasm to the plasma membrane upon PMA stimulation. The subcellular localization of YFP alone was not affected by PMA stimulation (see Fig. 7). As reported previously for full-length DGK $\delta$ 1 [22], the cell treatment with a non-selective PKC inhibitor, staurosporine, markedly inhibited the translocation of the PH domain (Figure 6). In contrast,



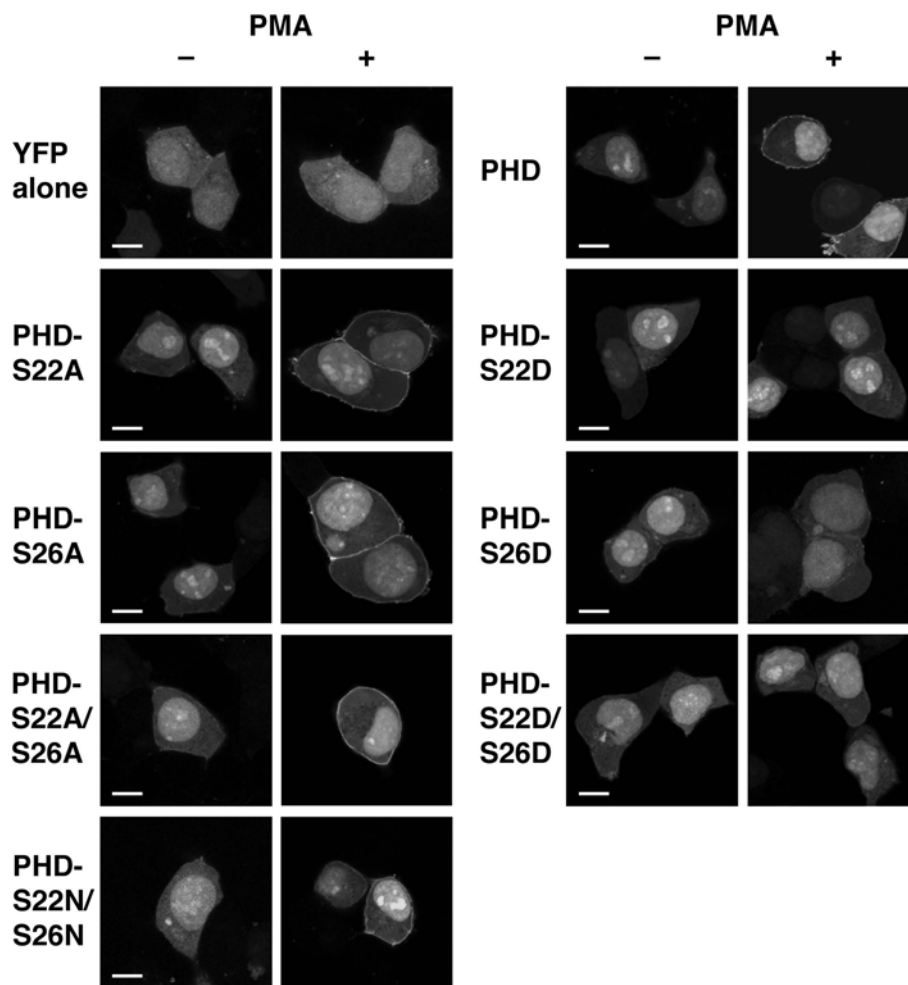
**Figure 6** Effects of PKC inhibitors on the subcellular distribution of DGK $\delta$ 1 and its PH domain

HEK-293 cells were transfected with a plasmid encoding YFP-DGK $\delta$ 1 or YFP-DGK $\delta$ 1-PH domain (PHD). After starvation, cells were pre-incubated with 1  $\mu$ M Gö 6976 or 300 nM staurosporine for 30 min. Cells were stimulated with 100 nM PMA for 30 min in the presence of Gö 6976 or staurosporine. After stimulation, HEK-293 cells were fixed with 3.7% (w/v) formaldehyde and then mounted on to glass slides. Cells were examined using an inverted confocal laser scanning microscopy (Zeiss LSM 510). Scale bar, 10  $\mu$ m. Representatives of three repeated experiments are shown.

Gö 6976 unexpectedly failed to block the PMA-dependent translocation. Essentially the same inhibitory pattern was obtained using full-length DGK $\delta$ 1. These results strongly suggest that cPKC-dependent phosphorylation is not involved in the positive regulation of the translocation, and that unknown mechanisms, which are dependent on PMA and independent of cPKC, operate in this event.

#### Effects of mutations at Ser-22 and Ser-26 on the translocation of DGK $\delta$ 1-PH domain

To analyse further the functional relationship between the phosphorylation of the PH domain and the PMA-dependent translocation, we examined subcellular distribution of the alanine mutants (YFP-DGK $\delta$ 1-PHD-S22A, -S26A and -S22A/S26A), in which Ser-22 and/or Ser-26 were replaced with alanine to prevent phosphorylation. As observed for the experiment using Gö 6976, the alanine mutants were translocated markedly from the cytoplasm to the plasma membrane by PMA stimulation (Figure 7), suggesting further that the cPKC-dependent phosphorylation does not participate in the enhancement of translocation.



**Figure 7** Effects of alanine/asparagine and aspartate mutations of Ser-22 and Ser-26 on subcellular distribution of the DGK $\delta 1$ -PH domain

HEK-293 cells were transfected with plasmids encoding YFP alone, YFP-DGK $\delta 1$ -PH domain (PHD), YFP-DGK $\delta 1$ -PH domain-S22A (PHD-S22A), YFP-DGK $\delta 1$ -PH domain-S26A (PHD-S26A), YFP-DGK $\delta 1$ -PH domain-S22A/S26A (PHD-S22A/S26A), YFP-DGK $\delta 1$ -PH domain-S22D (PHD-S22D), YFP-DGK $\delta 1$ -PH domain-S26D (PHD-S26D), YFP-DGK $\delta 1$ -PH domain-S22D/S26D (PHD-S22D/S26D) or YFP-DGK $\delta 1$ -PH domain-S22N/S26N (PHD-S22N/S26N) as indicated. After starvation, cells were incubated for 30 min in the presence of 100 nM PMA (+) or 0.1% DMSO (-). After stimulation, HEK-293 cells were fixed with 3.7% (w/v) formaldehyde and then mounted on to glass slides. Cells were examined using an inverted confocal laser scanning microscopy (Zeiss LSM 510). Scale bar, 10  $\mu$ m. Representatives of three repeated experiments are shown.

We next examined subcellular distribution of the aspartate mutants (YFP-DGK $\delta 1$ -PHD-S22D, -S26D and -S22D/S26D), in which one or two aspartate residues were introduced to mimic phosphoserine. In contrast with the alanine mutants, the aspartate mutants localized at the cytoplasm in resting cells and failed to translocate even in PMA-stimulated cells. In contrast, YFP-DGK $\delta 1$ -PHD-S22N/S26N, in which asparagine was substituted for Ser-22 and Ser-26, was translocated markedly to the plasma membrane. These results suggest that the phosphorylation of one of Ser-22 and Ser-26 prevents the translocation and/or enhances dissociation from the plasma membrane following the enzyme translocation. Western blot analysis showed that the expression levels of the mutants were almost the same (Figure 2; and results not shown). Therefore we consider that the differences of localization are not caused by variable expression levels of the enzyme constructs.

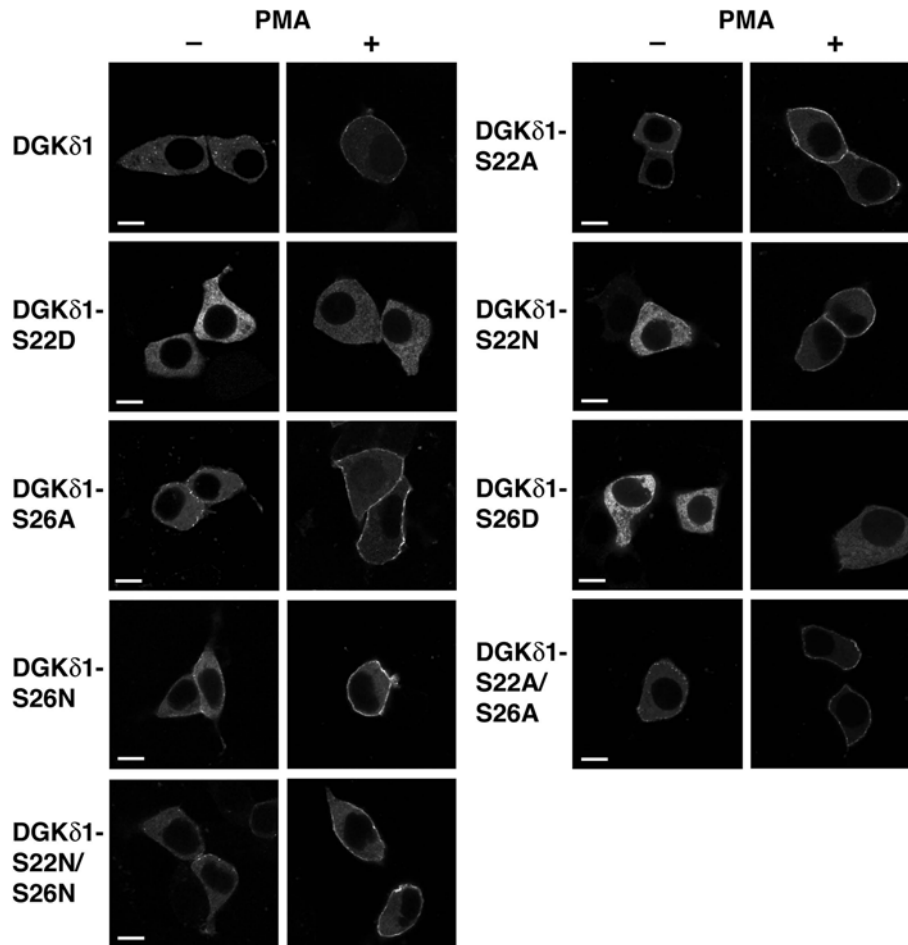
#### Effects of mutations at Ser-22 and Ser-26 on translocation of full-length DGK $\delta 1$

We next examined whether the phosphorylation of Ser-22 or Ser-26 indeed affected the subcellular localization of full-length

DGK $\delta 1$ . As reported previously [22], wild-type DGK $\delta 1$  was translocated from the cytoplasm to the plasma membrane by PMA stimulation (Figure 8). However, the aspartate mutants, YFP-DGK $\delta 1$ -S22D and -S26D, remained at the cytoplasm even in PMA-stimulated cells. In contrast with the aspartate mutants, alanine mutants, YFP-DGK $\delta 1$ -S22A, -S26A and -S22A/S26A, failed to block the PMA-dependent translocation. Moreover, all of the asparagine mutants, YFP-DGK $\delta 1$ -S22N, -S26N and -S22N/S26N, clearly underwent the PMA-induced translocation, indicating that the negative charge on the aspartate residue is essential to prevent the translocation. These results suggest that the phosphorylation of either Ser-22 or Ser-26 negatively regulates the translocation of full-length DGK $\delta 1$  via its PH domain in agreement with the results of the expression of the PH domain alone (Figure 7).

#### DISCUSSION

In the present study, we report that Ser-22 and Ser-26 of DGK $\delta 1$  within the PH domain are phosphorylated in PMA- and EGF-stimulated cells. The results presented here indicate that cPKC is



**Figure 8** Effects of alanine/asparagine and aspartate mutations of Ser-22 and Ser-26 on the subcellular distribution of full-length DGK $\delta$ 1

HEK-293 cells were transfected with plasmids encoding YFP-DGK $\delta$ 1 (DGK $\delta$ 1), YFP-DGK $\delta$ 1-S22A (DGK $\delta$ 1-S22A), YFP-DGK $\delta$ 1-S22D (DGK $\delta$ 1-S22D), YFP-DGK $\delta$ 1-S22N (DGK $\delta$ 1-S22N), YFP-DGK $\delta$ 1-S26A (DGK $\delta$ 1-S26A), YFP-DGK $\delta$ 1-S26D (DGK $\delta$ 1-S26D), YFP-DGK $\delta$ 1-S26N (DGK $\delta$ 1-S26N), YFP-DGK $\delta$ 1-S22A/S26A (DGK $\delta$ 1-S22A/S26A) or YFP-DGK $\delta$ 1-S22N/S26N (DGK $\delta$ 1-S22N/S26N) as indicated. After starvation, cells were incubated for 30 min in the presence of 100 nM PMA (+) or 0.1% DMSO (-). After stimulation, HEK-293 cells were fixed with 3.7% (w/v) formaldehyde and then mounted on to glass slides. Cells were examined using an inverted confocal laser scanning microscopy (Zeiss LSM 510). Scale bar, 10  $\mu$ m. Representatives of three repeated experiments are shown.

involved in the phosphorylation of DGK $\delta$ 1. Although we cannot rule out the possibility that an unknown protein kinase that functions downstream of cPKC phosphorylates the DGK $\delta$ 1 PH domain in cells, it is likely that cPKC is a direct upstream kinase for Ser-22 and Ser-26. This conclusion is based on the observation that: (i) purified PKC $\alpha$  phosphorylated the purified DGK $\delta$ 1-PH domain directly *in vitro* (Figure 4), (ii) the inhibition of MEK, which is the major downstream effector of PKC [26,27], did not affect the phosphorylation of the DGK $\delta$ 1-PH domain (Figure 3D), (iii) Ser-22 and Ser-26 appreciably fulfil the canonical phosphorylation site motif for PKC $\alpha$  as discussed in the next paragraph, and (iv) the preference of the phosphorylation site (Ser-26 > Ser-22) detected for the endogenous protein kinase was essentially the same as that exhibited by PKC $\alpha$  *in vitro* (Figures 2E and 4B). Because PKC $\alpha$  was the solely detectable cPKC in HepG2, HEK-293 and COS-7 cells (results not shown) where cellular or transfected DGK $\delta$ 1 was phosphorylated by endogenous protein kinase [22], it is likely that PKC $\alpha$  mainly participates in the DGK $\delta$ 1-PH domain phosphorylation in these cells. Although co-immunoprecipitation experiments were performed, we failed to detect any appreciable association of PKC $\alpha$  with DGK $\delta$ 1 (results not shown). This suggests that the phosphorylation of DGK $\delta$ 1

at Ser-22 and Ser-26 by PKC $\alpha$  occurs transiently, and that the complex between the two proteins, if any, cannot be recovered under detergent lysis conditions.

The flanking sequence of Ser-22 (QNNS<sup>22</sup>FQR, where underlines indicate residues identical with the corresponding residues in the canonical phosphorylation site motif for PKC $\alpha$ ) and that of Ser-26 (FQRS<sup>26</sup>KRR) partly fulfil the canonical phosphorylation site motif for PKC $\alpha$  {[RXXS\*F(R/K)(R/K)], where the asterisk indicates the phosphorylation site} [29]. It is known that each PKC isoform has an isoform-specific recognition motif [29]. Because the flanking sequences of Ser-22 and Ser-26 show no agreement with the canonical phosphorylation site motif for PKC $\epsilon$  [R(K/E/R)XS\*XXX], these Ser residues are predicted to be poor substrates of PKC $\epsilon$ . Indeed, in contrast with PKC $\alpha$ , overexpressed PKC $\epsilon$  did not significantly affect the phosphorylation level of the DGK $\delta$ 1-PH domain (Figure 3B).

DGK $\delta$ 1 lacking the PH domain was considerably phosphorylated (Figure 1B). Moreover, full-length DGK $\delta$ 1 having double alanine or aspartate mutations of Ser-22 and Ser-26 was also phosphorylated (50–80% of control) (results not shown). The results indicate that DGK $\delta$ 1 has at least one additional phosphorylation site outside of the PH domain. Staurosporine, a non-selective



PKC inhibitor, completely inhibited PMA-dependent phosphorylation of full-length DGK $\delta$ 1 [22], whereas Gö 6976, the cPKC-selective inhibitor, only partly inhibited the phosphorylation (approx. 30% inhibition) (results not shown). Thus PMA-dependent, staurosporine-sensitive and Gö 6976-insensitive protein kinase, for example nPKC, may participate in the phosphorylation of the additional site(s) present in the C-terminal portion of DGK $\delta$ 1. In this context, the action of this DGK isoform would be controlled by multiple protein kinases in a complex manner. The physiological importance of the phosphorylation outside of the PH domain is unclear. Since any mutations at Ser-22 and Ser-26 did not affect the oligomer formation of DGK $\delta$ 1 (results not shown), the additional phosphorylation site(s) may be involved in the dissociation of oligomer structure [22].

NMR and crystal structures have now been reported for eight different PH domains [23,24]. By alignment of the amino acid sequence of the DGK $\delta$ 1-PH domain with those of the eight PH domains, Ser-22 and Ser-26 are predicted to locate in the first variable loop between the first and second  $\beta$ -sheets (see Figure 2B). Binding targets of most of the PH domains are negatively charged molecules such as PtdIns(4,5) $P_2$  and PtdIns(3,4,5) $P_3$ . NMR studies showed that the interaction between the phosphoinositide and the PH domain is mediated by the positively charged surface of the domain that contains variable loops 1, 2 and 3 [23,24]. Although target molecule(s) of the DGK $\delta$ 1-PH domain have not yet been identified, it is easily imagined that phosphorylation, introduction of negative charges, within the variable loop 1 would directly affect affinity for putative target molecule(s) of the enzyme. Alternatively, conformational changes caused by the phosphorylation may be more important for target molecule recognition by the PH domain.

We have provided results suggesting that the phosphorylation of one of Ser-22 and Ser-26 negatively regulates the translocation of DGK $\delta$ 1 to the plasma membrane (Figures 6–8). It is puzzling to see that PMA, while causing the membrane translocation of DGK $\delta$ 1, also suppresses the translocation through the enzyme phosphorylation by cPKC. In this context, it can be assumed that these discrepant actions of PMA operate in a segregated manner. The existence of negative regulation of the translocation implies that the intracellular localization of DGK $\delta$ 1 is timely and strictly regulated by the balance between enhancement and suppression of the translocation. Because the PMA-dependent translocation of DGK $\delta$ 1 was clearly detected (Figures 6–8), it is likely that only a part of the enzyme is phosphorylated by cPKC at the PH domain.

Unfortunately, the present study did not reveal positive regulatory mechanisms of the DGK $\delta$ 1-translocation. However, we can conclude that the PH domain alone is sufficient for the PMA-induced translocation, and that the positive regulation of translocation is independent of phosphorylation in the DGK $\delta$  PH domain because the double alanine mutations at Ser-22 and Ser-26 and a Gö 6976 treatment, which gave a complete loss of phosphorylation signal (Figures 2 and 3), failed to inhibit the translocation of the PH domain (Figures 6 and 7). Moreover, the phosphorylation analysis using PKC inhibitors (Figure 6) allows us to speculate that a PMA-dependent, staurosporine-sensitive and Gö 6976-insensitive factor modifies a certain targeting molecule other than DGK $\delta$ 1 itself that recruits the enzyme to the plasma membrane. Although nPKC fulfils these three criteria, it is unclear whether this enzyme is indeed involved in the positive regulation of DGK $\delta$ 1-translocation. Further studies searching for the molecule(s) responsible for recruiting the DGK $\delta$ 1-PH domain by using, for example, the yeast two-hybrid system will address the positive-regulatory mechanisms of the translocation.

Recently, Luo et al. [30,31] reported that PKC $\alpha$  interacted with and phosphorylated DGK $\zeta$  (type IV isoform). These proteins

co-localized in the cytoplasm in resting cells. However, after addition of PMA, PKC $\alpha$  translocated from the cytoplasm to the plasma membrane, whereas DGK $\zeta$  remained in the cytoplasm. In contrast, our results demonstrated that DGK $\delta$ 1 is translocated to the plasma membrane in response to PMA stimulation. These results suggest that although both DGK $\delta$ 1 and DGK $\zeta$  are commonly phosphorylated by PKC $\alpha$ , they are spatiotemporally segregated from each other in PMA-stimulated cells.

In conclusion, our results provide evidence of the phosphorylation of the DGK $\delta$ 1 PH domain by cPKC in PMA- and EGF-stimulated cells and suggest that the translocation of DGK $\delta$ 1 to the plasma membrane is negatively and critically regulated by the phosphorylation. These results imply that the action of DGK $\delta$ 1 is adeptly regulated in a complex manner.

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