Alternative Splicing of the Human Diacylglycerol Kinase δ Gene Generates Two Isoforms Differing in Their Expression Patterns and in Regulatory Functions*

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Fumio Sakane‡‡, Shin-ichi Imai‡, Keiko Yamada‡, Tomohiro Murakami‡, Shuichi Tsushima‡, and Hideo Kano‡

From the ‡Department of Biochemistry, School of Medicine, and ¶Department of Liberal Arts and Sciences, School of Health Sciences, Sapporo Medical University, South-1, West-17, Chuo-ku, Sapporo 060-8556, Japan

Diacylglycerol kinase (DGK) plays an important role in signal transduction through modulating the balance between two signaling lipids, diacylglycerol and phosphatidic acid. DGKδ (type II isozyme) contains a pleckstrin homology domain at the N terminus and a sterile α motif domain at the C terminus. We identified another DGKδ isoform (DGKδ2, 135 kDa) that shared the same sequence with DGKδ previously cloned (DGKδ1, 129 kDa) except for the 52 residues N-terminally extended. Analysis of panels of human normal and tumor tissue cDNAs revealed that DGKδ2 was ubiquitously expressed in all normal and tumor tissues examined, whereas the transcript of DGKδ1 was detected only in ovary and spleen, and in a limited set of tumor-derived cells. The expression of DGKδ2 was induced by treating cells with epidermal growth factor and tumor-promoting phorbol ester. In contrast, the levels of mRNA and protein of DGKδ1 were suppressed by phorbol ester treatment. Thus, it becomes clear that two DGKδ isoforms are expressed under distinct regulatory mechanisms. DGKδ1 was translocated through its pleckstrin homology domain from the cytoplasm to the plasma membrane in response to phorbol ester stimulation, whereas DGKδ2 remained in the cytoplasm even after stimulation. Further experiments showed that the δ2-specific N-terminal sequence blocks the phorbol ester-dependent translocation of this isoform. Co-immunoprecipitation analysis of differently tagged DGKδ1 and DGKδ2 proteins showed that they were able to form homo- as well as heterooligomers. Taken together, alternative splicing of the human DGKδ gene generates at least two isoforms, differing in their expressions and regulatory functions.

Diacylglycerol kinase (DGK)2 phosphorylates diacylglycerol to produce phosphatidic acid (PA) (1). The roles of diacylglycerol and PA as lipid second messengers have been attracting much attention. Diacylglycerol is known to be an activator of conventional and novel protein kinase Cs, chimerins, Unc-13, and Ras guanyl nucleotide-releasing protein (2–4), and PA has been reported in a number of studies to modulate phosphatidylinositol (PI)-4-phosphate kinase, Raf-1 kinase, atypical protein kinase C, and many other important enzymes (5, 6). PA is also known to have mitogenic effects in a variety of cells (5, 6). Because the cellular concentrations of these signaling lipids must be strictly regulated, DGK is thought to be one of the key enzymes involved in the cellular signal transduction.

It is now recognized that DGK represents a large gene family of isoforms differing remarkably in their structures, in the modes of tissue expression, and in the enzymological properties (7–10). To date, nine mammalian DGK isoforms (α, β, γ, δ, ε, η, θ, and ϵ) containing in common two or three characteristic zinc finger structures and the catalytic region are subdivided into five groups according to their structural features (7–10). Each subgroup is characterized by the subtype-specific functional domains such as calcium-binding EF-hand motifs and a recoverin homology domain (type I) (11–16), pleckstrin homology (PH) and sterile α motif domains (type II) (17, 18), no recognizable functional domains except for the zinc finger structures and the catalytic region (type III) (19), a myristoylated alanine-rich C kinase substrate (MARCKS) phosphorylation site domain and ankyrin repeats (type IV) (20, 21), and three (instead of two) zinc finger structures, a PH domain-like region and a Ras-associating domain (type V) (22). In contrast to mammals, only a few DGK isoforms have been identified in organisms such as Caenorhabditis elegans, Drosophila melanogaster, and Arabidopsis thaliana (8–10). Moreover, it is noteworthy that DGK gene has not yet been detected in yeast. It is therefore likely that DGK is needed to control functions unique to multicellular organisms, and that many mammalian DGK isoforms have essential roles in biological processes specific to higher vertebrates, such as development/differentiation, neural networking, the immune system, and tumorigenesis. However, our knowledge of specific functions of the individual DGK isoforms is still limited at present. Interestingly, the occurrence of alternative splicing was recently identified for three mammalian DGK genes, i.e. γ (14), ζ (23), and β (24) isoforms. Thus, the list of DGK isoform members is still growing. The

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The nucleotide sequence(s) reported in this paper has been submitted to the DDBJ/GenBank™/EBI Data Bank with accession number(s) AB078966.

‡‡ To whom correspondence should be addressed. Tel.: 81-11-611-2111; Fax: 81-11-622-1918; E-mail: sakane@spmed.ac.jp.

§ The abbreviations used are: DGK, diacylglycerol kinase; EGF, epidermal growth factor; GFP, green fluorescent protein; HEK, human embryonic kidney; PI, phosphatidylinositol; PH, pleckstrin homology; TAA, triaminocinolone acetonide; TPA, 12-O-tetradecanoylphorbol 13-acetate; BSA, bovine serum albumin; DMEM, Dulbecco’s modified Eagle’s medium; RT, reverse transcriptase; PBS, phosphate-buffered saline; PA, phosphatidic acid; MARCKS, myristoylated alanine-rich C kinase substrate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; aa, amino acid(s).
occurrence of alternative splicing in multiple DGK genes further supplements the complexity of the DGK gene family members, and apparently contributes to fine-tuning the action of DGKs regulating a wide range of cellular functions. Indeed, in the case of DGKδ, the control of the alternative splicing of this gene is hypothesized to be involved in the pathogenesis of human mood disorders (24).

We previously cloned a cDNA encoding DGKδ (type II isozyme) having a calculated molecular mass of 130 kDa (17). When the cloned DGKδ cDNA was expressed in COS-7 cells, indeed, a 130-kDa protein band was observed by Western blot analysis using anti-DGKδ antibody (17). However, we noted in subsequent experiments using HepG2 cell homogenates that, in addition to the 130-kDa band expected, an endogenous 140-kDa band strongly reacted with the anti-DGKδ antibody (see Fig. 2A). This observation has suggested the existence of a splice variant of DGKδ gene with a larger molecular size. In the present study, we identified the occurrence of alternative splicing yielding a novel DGKδ isoform with distinct structural and functional properties.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—COS-7 and human embryonic kidney (HEK) 293 cells were maintained in DMEM (Sigma-Aldrich, Tokyo, Japan) containing 10% fetal bovine serum at 37 °C in an atmosphere containing 5% CO2. Cells were transiently transfected with cDNAs using Effectene transfection reagent according to the instructions from the manufacturer (Qiagen, Tokyo, Japan). After 2 days, cells were used for further analysis.

Rapid Amplification of cDNA Ends (RACE)—RACE was performed to extend the 5′ end of human DGK δ cDNA using Marathon-Ready cDNA of human brain (Clontech, Tokyo, Japan) as templates. cDNA was synthesized with an adaptor primer 1 provided by the manufacturer and an antisense gene-specific primer (nucleotide positions 743–770, 5′-TGAAACATGGCCCTTGACCAAGAGCG-3′ (Ref. 17)). The longest cDNA clone amplified (DKD2-10) was selected for sequence analysis.

Reverse Transcriptase (RT)-PCR—Total RNA was isolated from HepG2, HL-60, or HEK293 cells using Isogen (Nippon Gene, Tokyo, Japan) according to the instructions from the manufacturer. Reverse transcription into cDNAs was achieved using the SuperScript preamplification system (Invitrogen, Tokyo, Japan) according to the protocol from the manufacturer. cDNAs from human normal tissues and tumor-derived cells were purchased from Clontech. PCR amplification was performed with Takara Ex Taq (Takara Biomedicals, Tokyo, Japan) using gene-specific oligonucleotide primers as follows: a DGKδ forward primer (nucleotide positions −22 to +3, 5′-GGGCACCTTGAAAACGCAAGTTTATG-3′), a DGKδ forward primer (nucleotide positions 132–156, 5′-CACCTGGGGGATCCAGCGAGAAGG-3′), and a common reverse primer (nucleotide positions 411–435 and 543–567 in DGKδ1 and DGKδ2, respectively; 5′-CATCCCTGAGAAGTCTGCTGATCTG-3′). PCR conditions were as follows: 94 °C for 4 min; 35 cycles of 94 °C for 30 s, 68 °C for 2 min; and 68 °C for 7 min. For normalization, human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was simultaneously amplified (25 cycles). PCR products amplified were separated by agarose gel electrophoresis, stained with ethidium bromide, and subjected to densitometric analysis.

COS-7 Cell Transfection and Determination of DGK Activity—One μg each of pSR-e-DGKδ1, pSR-e-DGKδ2, or pSR-e vector alone was transfected into COS-7 cells (60-mm dish) using Effectene transfection reagent (Qiagen, Tokyo, Japan). After 2 days, the cells were harvested and lysed by sonication in lysis buffer (0.5 ml/60-mm dish) containing 20 μM Tris-HCl (pH 7.4), 0.25 μM sucrose, 1 μM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, Complete Protease Inhibitor Mixture (Roche Molecular Biochemicals, Tokyo, Japan) and Phosphatase Inhibitor Mixture II (Sigma-Aldrich). After a low speed centrifugation (15,000 × g for 10 min), the resultant supernatant was used as enzyme source. The octyl glucoside mixed micellar assay using diolein as substrate was done as described (17).

COS-7 Cell Transfection—COS-7 cells (1–107 cells, 60-mm dish) co-expressing 3′-FLAG-tagged DGKδ and GFP-DGKδ were harvested in lysis buffer containing 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1% Nonidet P-40, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, Complete Protease Inhibitor Mixture, and Phosphatase Inhibitor Mixture II. The mixture was centrifuged at 100,000 × g for 30 min at 4 °C to give cell lysates. Cell lysates (400 μl) were precleared with Protein A/G PLUS-agarose (10 μl, Santa Cruz Biotechnology, Santa Cruz, CA). Anti-GFP (2 μl, Living Colors full-length A.v. polyclonal antibody, Clontech) was added to the lysates to immunoprecipitate GFP-fusion proteins. After 1 h at 4 °C, Protein A/G PLUS-agarose beads (5 μl) were added and further incubated at 4 °C for 1 h. After the agarose beads were washed five times with wash buffer (50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 0.1% Triton X-100, 1.6 M KC1), immunoprecipitated proteins were extracted with 50 μl of SDS sample buffer and then analyzed by SDS-PAGE.

Cell Fractionation—GFP alone, DGKδ-PH-GFP, or DGKδ-S2-PH-GFP (see Fig. 6 for DGKδ constructs) was transfected into HEK293 cells (60-mm dish) using Effectene transfection reagent. After 24 h, the cells were washed four times with PBS and then cultured in DMEM plus 0.1% BSA. After 3 h, the medium was exchanged with DMEM plus 0.1% BSA containing 1 μM TPA, and cells were cultured for another 1 h. The cells were then harvested and lysed by sonication in lysis buffer (0.3 ml) containing 50 mM Tris-HCl (pH 7.4), 5 mM MgCl2, 150 mM NaCl, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, Complete Protease Inhibitor Mixture, and Phosphatase Inhibitor Mixture II. After a low speed centrifugation (550 × g for 10 min), the homogenate was separated into the supernatant fractions by a centrifugation at 100,000 × g for 30 min. The particulate fraction was suspended in the original volume (0.5 ml) of the lysis buffer. The aliquots (20 μl) of these fractions were subjected to immunoblot analysis using anti-GFP antibody.

Western Blot Analysis—Cell lysates and immunoprecipitates were separated on SDS-PAGE (7.5 or 15%). The separated proteins were transferred to a polyvinylidene difluoride membrane (Bio-Rad, Tokyo, Japan) and blocked with Block Ace (Dainippon Pharmaceutical, Tokyo, Japan). The membrane was incubated with anti-FLAG M2 monoclonal antibody or anti-GFP monoclonal antibody (B-2, Santa Cruz Biotechnology) in Block Ace for 1 h. The immunoreactive bands were visualized by peroxidase-conjugated anti-rabbit or mouse IgG antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) and ECL (Amersham Biosciences, Tokyo, Japan).

Fluorescence Microscopy—HEK293 cells were grown on poly-l-lysine-coated glass coverslips and transiently transfected with expression plasmids containing DGKδ cDNAs N- or C-terminally fused with GFP. After 24 h, HEK293 cells were washed four times with PBS and then cultured in DMEM plus 0.1% BSA. After 3 h, the medium was exchanged with DMEM plus 0.1% BSA containing 1 μM TPA, and cells were cultured for another 1 h. The cells were then fixed with 3.7% formaldehyde in PBS for 10 min and washed five times with PBS at room temperature. The coverslips were mounted using Vectashield (Vector Laboratories, Burlingame, CA). Cells were examined using an inverted confocal laser scanning microscope (Zeiss LSM 510). Images were processed using Adobe Photoshop 6.0 (Adobe Systems, San Jose, CA).

RESULTS

Identification and Characterization of a Novel DGKδ Isoform Generated by Alternative Splicing—To clone an alternative splicing product of the DGKδ gene, we performed 5′-RACE using a human brain cDNA as template. Sequencing of 14 clones amplified showed that all of them shared a 5′ sequence that was lacking in the DGKδ cDNA previously isolated from HepG2 cell cDNA library (17). The longest clone (DKD2-10) had a 168-bp-long (open reading frame, 156 bp) 5′ sequence that was different from the 104-bp-long (open reading frame, 24 bp) 5′ sequence of DGKδ cDNA previously cloned (17) (Fig. 1A). We tentatively designated the original (from HepG2 cells) and new (from human brain) DGKδ clones as DGKδ1 and DGKδ2, respectively. DGKδ2 had a predicted translation initiation site (nucleotide positions 1–3) different from that of DGKδ1 (Fig. 1, A and B), whose flanking regions were confirmed well to the Kozak consensus sequence (CC(A/G)CCATGG/AG). Analysis of a genomic clone (accession no. AC013726) deposited in DNA data base showed that the exon/intron boundaries fulfill the GT/AG rule and that DGKδ1 uses the second exon of DGKδ2 as the first exon (Fig. 1, A and B). The open reading frames of DGKδ1 and DGKδ2 encode 1170- and 1214-aa pro...
The translation initiation codon, ATG, of DGKα1 is underlined. B, the exon-intron structure of the DGKα gene (exons 1–3) and the alternative splicing that yields the two different forms are shown. Nucleotide and amino acid numbers are indicated at left in italic and plain text, respectively. The 5′ end of the exon encoding DGKα2 is set at nucleotide 1. The translation initiation codon, ATG, of DGKα1 is underlined. B, the exon-intron structure of the DGKα gene (exons 1–3) and the alternative splicing that yields the two different forms are shown. C, the domain architectures of DGKα isoforms. The conserved functional domains are indicated, as well as the isoform-specific regions. D, alignment of the specific sequences of human DGKα isoforms with the N-terminal sequence of hamster DGKα. Identical amino acids are indicated at left in italic, and similar amino acids are shaded. Dashes indicate gaps inserted to maximize alignment.

In an attempt to characterize the properties of the two DGKα isoforms, we cloned each of the cDNAs into an expression vector and transfected them into COS-7 cells. Proteins with apparent molecular masses of 130 and 140 kDa were recognized by antibody against the C-terminal part of DGKα (17) in the cells transfected with DGKα1 and DGKα2 cDNAs, respectively (Fig. 2A). The apparent molecular masses of DGKα1 and DGKα2 expressed from the cDNAs corresponded with the 130- and 140-kDa bands of DGKα endogenously present in HepG2 cells, respectively (Fig. 2A). We found that HepG2 cells contained 2–5-fold more DGKα2 than DGKα1 isozyme. To address whether the novel DGKα2 cDNA encodes a functional enzyme, homogenates from cells transfected with the DGKα2 cDNA or vector alone were assayed for DGK activity. In this case, Western blot analysis showed that the proteins of DGKα1 and DGKα2 were expressed to a comparable extent in COS-7 cells (Fig. 2A). As shown in Fig. 2B, DGKα1 and DGKα2 displayed comparable DGK activities when assayed in vitro, confirming that both of the splice variants are active enzymes.

Differential Modes of Expression and Induction of DGKα Isoforms—To investigate the expression patterns of DGKα isoforms, panels of cDNAs synthesized from human normal and tumor tissues and cell lines were analyzed by RT-PCR. As shown in Fig. 3 (A–C), the DGKα2 transcript was detectable in all normal and tumor tissues and cell lines examined and was expressed predominantly in testis, peripheral blood leukocytes, lung carcinomas (GI-117 and LX-1), colon adenocarcinomas (CX-1 and GI-112), a prostatic adenocarcinoma (PC3), HepG2, HL-60, and HEK293 cells. On the other hand, the DGKα1 transcript was detected only in ovary, and to a lesser extent in spleen. The expression of DGKα1 in human tumor tissues and cell lines was also much more limited and was detected in HepG2, HL-60 cells, LX-1, CX-1, and several other tumor tissues. Thus, DGKα2 showed a much broader tissue distribution, whereas the expression of DGKα1 was highly tissue-specific. Although it is difficult to compare the relative amounts of PCR products generated using different primers, we noted that the expression level of DGKα2 was generally higher than that of DGKα1 except for ovary. To confirm these results obtained using RT-PCR, we examined the protein levels of the DGKα isoforms in several human normal tissues (kidney, testis, and ovary) and cell lines (HepG2, HL-60, and HEK293 cells) by Western blotting. As shown in Fig. 3D, the protein levels of the DGKα isoforms were consistent with the corresponding mRNA levels. Although further work is required, it seems likely that the expression of DGKα1 is relatively tissue-specific, and that DGKα2 is the major product of DGKα gene in most tissues and cells except for ovary.

Differential tissue distribution patterns of DGKα1 and DGKα2 mRNAs suggested that the expression of the isoforms is regulated under distinct mechanisms. Because Klauck et al. (18) reported that glucocorticoid, triamcinolone acetonide (TAA), increased the expression level of other type II isozyme,
DGK$_\gamma$, we first tested the effect of TAA. When HepG2 cells, known to express glucocorticoid receptors (25), were treated with 10 nM TAA for 72 h, the protein levels of DGK$_1$ and DGK$_2$ remained almost unchanged (Fig. 4A), although we confirmed in the same experiment that the level of DGK$_\gamma$ mRNA was markedly increased as described by Klauck et al. (18). Because the DGK$_2$ transcript was expressed in a wide range of tumor-derived cells, we next tested the effects of a tumor-promoting phorbol ester (TPA) and epidermal growth factor (EGF) on the expression levels of DGK$_\delta$ isoforms. When HepG2 cells were cultured for 24 h in the presence of TPA (100 nM), the DGK$_\delta_2$ protein level detected as a 140-kDa band was markedly (2.8-fold) increased (Fig. 4B). In contrast, the DGK$_\delta_1$ protein was decreased almost to an undetectable level. To ask whether these changes of protein levels were caused by transcriptional regulation, we measured the levels of the transcripts of the isoforms by RT-PCR. Consistent to the changes of enzyme proteins, the DGK$_\delta_2$ transcript was increased (2.0-fold) by TPA stimulation, whereas that of DGK$_\delta_1$ was decreased to an undetectable level (Fig. 4C). We next examined the effect of EGF on the expression of cellular DGK$_\delta$ enzymes. For this purpose, we used HEK293 cells because HepG2 cells failed to respond to EGF. When HEK293 cells were incubated for 48 h in the presence of EGF (10 ng/ml), the DGK$_\delta_2$ protein was substantially (2.3-fold) increased (Fig. 4D). However, DGK$_\delta_1$ protein was not detectable both in the absence and presence of EGF. We confirmed that the DGK$_\delta_2$ transcript was increased (2.3-fold) by EGF stimulation, whereas that of DGK$_\delta_1$ remained undetectable (Fig. 4E). These results indicate that the protein levels of DGK$_1$ and DGK$_2$ are regulated, at least in part, at the transcriptional stage and that, although derived from a single gene, the transcription of the two forms of DGK$_\delta$ is regulated under distinct regulatory mechanisms.

**Intracellular Localizations of DGK$_\delta$ Isoforms**—We have recently found TPA-induced translocation of DGK$_\delta_1$ from cytoplasmic vesicles to the plasma membrane in HEK293 cells (26).
Therefore, we intended to see the intracellular localization of DGKδ2 with a unique N-terminal sequence and also to see whether DGKδ2 was translocated by TPA stimulation similarly as observed for DGKδ1. Although cytoplasmic/nuclear localization of GFP alone was not affected by TPA stimulation, DGKδ1 was confirmed to be translocated from cytoplasmic vesicles to the plasma membrane in TPA-stimulated cells (Fig. 5A) as similarly observed for DGKδ1. In contrast, DGKδ2 localized at cytoplasmic vesicles in resting cells failed to be significantly translocated in TPA-stimulated cells. The results indicate that, in TPA-stimulated cells, the two DGK isoforms can be spatially segregated, reflecting their distinct roles in the regulation of cellular functions

As shown in Fig. 5A, DGKδ1 and DGKδ2 share most of the C-terminal sequence (as many as 1162 aa residues), differing from each other only in the short stretches of the N-terminal sequences. It is therefore reasonable to assume that the N-terminal sequence unique to each of the two enzymes is responsible for their discrepant responses to TPA stimulation.

In the experiments using HEK293 cells, we first examined the intracellular localization of an N-terminally truncated mutant, DGKδ lacking both the PH domain and the DGKδ1-specific sequence (GFP-DGKδ-ΔS1,ΔPH). As shown in Fig. 6B, this mutant lost the ability of the TPA-dependent translocation. The result indicates that the PH domain and/or the DGKδ1-specific sequence is necessary for the translocation of DGKδ1. Thus, we next asked whether the DGKδ PH domain alone (DGKδ-PH-GFP) is sufficient for the translocation. As observed with wild-type DGKδ1, DGKδ-PH-GFP showed the TPA-dependent translocation from the cytoplasm to the plasma membrane (Fig. 6B). To determine the functions of the DGKδ1- and DGKδ2-specific sequences, the localization of the PH domain containing one of these sequences (DGKδ1-S1-PH-GFP or DGKδ2-S2-PH-GFP) was analyzed. DGKδ1-S1-PH-GFP showed the TPA-dependent translocation like DGKδ-PH-GFP, whereas DGKδ2-S2-PH-GFP did not (Fig. 6B). To confirm the translocation detected by microscopic observation, we performed an alternative approach: cell fractionation by a centrifugation at 100,000 g. The extract from HEK293 cells expressing DGKδ-PH-GFP or DGKδ2-S2-PH-GFP was fractionated, and the contents of the GFP fusion proteins in each fraction were determined by Western blot analysis using anti-GFP antibody (Fig. 6C). The amount of DGKδ-PH-GFP in the particulate (membrane) fraction increased markedly after TPA stimulation, although a predominant amount of DGKδ-PH-GFP remained still cytosolic. However, DGKδ2-S2-PH-GFP and GFP alone were completely recovered in the cytosolic fraction without detectable membrane association. Taken together, the DGKδ PH domain is necessary and sufficient for the TPA-dependent enzyme translocation and the DGKδ2-specific sequence is likely to have inhibitory effect(s) on the translocation. We previously reported that TPA-dependent phosphorylation of DGKδ1 is closely linked to its translocation to the plasma membrane (26). The role of protein phosphorylation in the cellular localization of DGKδ remains unclear, because we observed that, in TPA-stimulated HepG2 cells, both of the endogenous DGKδ1 and DGKδ2 were similarly phosphorylated.4

Homo- and Hetero-oligomer Formation of DGKδ Isoforms—We have recently found that DGKδ1 forms oligomeric

4 S. Imai, F. Sakane, and H. Kanoh, unpublished observation.
(at least tetrameric) structures in vivo and that the sterile α motif domain plays a critical role for the oligomer formation (26). To confirm homo-oligomer formation of DGKδ in vivo, we carried out co-immunoprecipitation analysis using the lysates of COS-7 cells co-expressing GFP-tagged and 3×FLAG-tagged DGKδ proteins. When GFP-tagged DGKδ was immunoprecipitated with anti-GFP antibody, 3×FLAG-tagged DGKδ was co-immunoprecipitated (Fig. 7A). To examine whether the two DGKδ isoforms form hetero-oligomer structures in vivo, we performed the same experiment using the lysates of COS-7

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**Fig. 6. Roles of the PH domain and the N-terminal sequences in the subcellular localization of DGKδ isoforms.** A, schematic representation of DGKδ mutants used. B, HEK293 cells were transfected with expression plasmids encoding DGKδ-ΔS1,2ΔPH (DGKδ lacking the DGKδ1- or DGKδ2-specific sequence and the PH domain), DGKδ-S1+PH (the PH domain with DGKδ1-specific sequence), DGKδ-S2+PH (the PH domain with DGKδ2-specific sequence), and DGKδ-PH (the PH domain without the DGKδ1- or DGKδ2-specific sequences), which are N- or C-terminally fused with GFP. After starvation, cells were incubated for 1 h in the presence of 1 μM TPA or 0.1% Me2SO. After stimulation, HEK293 cells were fixed with 3.7% formaldehyde and then mounted onto glass slides. Cells were examined using an inverted confocal laser scanning microscopy (Zeiss LSM 510). The arrowheads indicate the plasma membrane localization of DGKδ-PH and DGKδ-S1+PH. A representative of three repeated experiments is shown. Bar = 10 μm. C, Homogenate (H), particulate (P), and soluble (S) fractions (20 μl each) from HEK293 cells expressing GFP alone, DGKδ-PH-GFP, or DGKδ-S2+PH-GFP were separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The GFP fusion proteins were detected with anti-GFP antibody. A representative of twice repeated experiments is shown.
cells co-expressing GFP-tagged DGK\textsubscript{1} and 3×FLAG-tagged DGK\textsubscript{2}. When GFP-tagged DGK\textsubscript{1} was immunoprecipitated with anti-GFP antibody, 3×FLAG-tagged DGK\textsubscript{2} was co-immunoprecipitated (Fig. 7B). The result shows that DGK\textsubscript{1} and DGK\textsubscript{2} are able to form homo- as well as hetero-oligomer structures \textit{in vivo}.

**DISCUSSION**

The present study added a new DGK isoform into the growing list of mammalian DGK gene family (altogether nine independent genes and four alternative splicing products at present). The results further point to the potential importance of DGK\textsubscript{5} as a signaling molecule by disclosing the existence of two DGK\textsubscript{5} isoforms differing with respect to TPA-dependent translocation and transcriptional regulation, although the two enzymes can interact with each other to form hetero-oligomers.

Alternative splicing is an important means for the generation of protein diversity in eukaryotes. The alternative selection of exon sequences during splicing is often regulated in different tissues and cells. We thus speculate the possible existence of additional isoforms of other DGKs through alternative splicing. The marked divergence of DGK isoforms achieved through alternative splicing may reflect their physiological importance and their needs to respond to a variety of signaling pathways operating under distinct regulatory mechanisms.

The mRNA levels of DGK\textsubscript{2} were significantly augmented in several tumor-derived cells, whereas that of DGK\textsubscript{1} was only moderately increased. This suggests that the alternative selection of DGK\textsubscript{5} exon sequences during splicing is regulated in cell growth- and tumorigenesis-dependent manners. Indeed, both of a tumor promoter, TPA, and a tumor-related growth factor, EGF, enhanced the DGK\textsubscript{2} expression. In contrast, DGK\textsubscript{1} expression did not respond to or was negatively regulated by these factors. Thus, highly expressed DGK\textsubscript{5}, especially DGK\textsubscript{2}, may be involved in cell growth and tumorigenesis. Because DGK\textsubscript{1} and DGK\textsubscript{2} genes have different transcription initiation sites, their transcription must be regulated by different transcription factors. Activation protein-1 is known to be important for TPA- and growth factor-induced gene expression in many cases (28, 29). However, our preliminary search failed to detect typical activation protein-1-binding sequences in the 5′-flanking region (1 kb long) of the translation initiation site of DGK\textsubscript{2} gene. Further studies are needed to reveal mechanisms of the transcriptional regulation of DGK\textsubscript{5} gene.

In this study, we demonstrated that the PH domain of DGK\textsubscript{5} is essential and sufficient for the TPA-dependent translocation. To date several reports are available concerning intracellular translocation of DGK isozymes, \textit{i.e.} DGK\textsubscript{\textgamma} (from the nucleus to the cytoplasm by TPA stimulation) (30), DGK\textsubscript{\textgamma} (from the cytoplasm to the plasma membrane by TPA stimulation) (31), and DGK\textalpha (from the cytoplasm to the plasma membrane by T cell receptor stimulation) (32). In these cases various regulatory domains of DGKs were noted to be involved in the enzyme translocation, such as a basic amino acid-rich, nuclear localization signal (MARCKS phosphorylation site-like region) in DGK\textsubscript{\textgamma}, a zinc finger structure in DGK\textgamma, and zinc fingers and/or the catalytic domain in DGK\textalpha. These results collectively indicate that DGK isozymes are translocated through distinct mechanisms using different functional domains. A subset of PH domains in different proteins was reported to translocate from the cytoplasm to the plasma membrane by a variety of agonist stimulations (33, 34). However, the TPA-dependent translocation of a PH domain is the first to be reported to our knowledge. The translocation mechanisms through the DGK\textsubscript{5} PH domain are not clear at present. Takeuchi et al. (35) demonstrated that the DGK\textsubscript{5} PH domain showed an affinity for a PI 3,4,5-trisphosphate analog 12 times higher than that of PI 4,5-bisphosphate. By analyzing the ability of different PH domains to rescue cdc25\textsuperscript{\textalpha} yeast in a PI 3-kinase-dependent manner, however, Isakoff et al. (36) showed that the DGK\textsubscript{5} PH domain was not recruited to the plasma membrane by PI 3-kinase products. Thus, physiological lipid target(s) of the PH domain, if any, is still unclear at present. It is possible that the DGK\textsubscript{5} PH domain binds to protein target(s) instead of phosphoinositides. Interestingly, we found that the DGK\textsubscript{5} PH domain was phosphorylated in TPA-stimulated cells. Instead of increased amounts of target lipids or proteins, the conformational change of the PH domain by phosphorylation may be essential for the translocation. Analyses of the translocation mechanisms through the DGK\textsubscript{5} PH domain and of its phosphorylation site(s) are the targets of future investigation.
The DGKδ2-specific sequence but not the DGKδ1-specific one inhibited the PH domain-mediated enzyme translocation. One of the possible mechanisms is that the DGKδ2-specific sequence binds to unknown anchoring protein(s) in the cytoplasm and that this binding occurs with an affinity stronger than that operating between the DGKδ PH domain and its binding factor(s) in the plasma membrane. Alternatively, it is possible that the DGKδ2-specific sequence masks a certain sequence that is required for the recognition of plasma membrane-targeting factor(s) and/or for phosphorylation occurring in TPA-stimulated cells (26). In this respect, the C-terminal half of the DGKδ2-specific sequence, which contains many acidic amino acid residues, shows significant homology to the N-terminal part of hamster DGKγ (18). DGKγ expressed in HEK293 cells also failed to be translocated in the presence of TPA, suggesting that the homologous sequences are important to block the translocation of DGKδ2 and DGKγ. Because basic amino acid residues in PH domains are known to play important roles in the membrane translocation (33, 34), the acidic regions of DGKδ2 and DGKγ may exert negative effects on the enzyme translocation.

It is interesting to note that DGKδ1 and DGKδ2 can form hetero-oligomer structures. Because DGKδ is known to form at least tetramer structures (26), permutations and combinations of the heterotetramer yield 24 (= 16) variations. If the DGKδ isoforms form oligomeric structures larger than the tetramer, the diversity increases significantly. Because both DGKδ1 and DGKδ2 are co-expressed in ovary, a lung carcinoma (LX-1), and a colon adenocarcinoma (CX-1), the isoforms can form hetero-oligomer structures at least in these tissues and cells. The marked heterogeneity of the hetero-oligomer structures of DGKδ isoforms, again, may reflect their physiological importance and diverse functions.

REFERENCES
1. Kanoh, H., Yamada, K., and Sakane, F. (1990) Trends Biochem. Sci. 15, 47–50
2. Nishizuka, Y. (1992) Science 258, 607–614
3. Hurley, J. H., Newton, A. C., Parker, P. J., Blumberg, P. M., and Nishizuka, Y. (1997) Protein Sci. 6, 477–480
4. Roh, D., and Kazanietz, M. G. (1999) FASEB J. 13, 1658–1676
5. Exton, J. H. (1994) Biochim. Biophys. Acta 1212, 26–42
6. English, D. (1996) Cell. Signal. 8, 341–347
7. Sakane, F., and Kanoh, H. (1997) Int. J. Biochem. Cell Biol. 29, 1139–1143
8. Topham, M. K., and Prescott, S. M. (1999) J. Biol. Chem. 274, 11447–11450
9. van Blitterswijk, W. J., and Houssa, B. (2000) Cell. Signal. 12, 585–605
10. Kanoh, H., Yamada, K., and Sakane, F. (2002) J. Biochem. (Tokyo) 131, 629–633
11. Sakane, F., Yamada, K., Kanoh, H., Yokoyama, C., and Tanabe, T. (1990) Nature 344, 345–348
12. Schaap, D., de Wit, J., van der Wal, J., Vandekerckhove, J., van Damme, J., Gussov, D., Ploegh, H. L., van Blitterswijk, W. J., and van der Bend, R. L. (1990) FEBS Lett. 275, 151–158
13. Goto, K., and Kondo, H. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 7588–7602
14. Bai, M., Sakane, F., Imai, S., Wada, I., and Kanoh, H. (1994) J. Biol. Chem. 269, 18492–18498
15. Goto, K., Funayama, M., and Kondo, H. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 13042–13046
16. Jiang, Y., Qian, W., Hawes, J. W., and Walsh, J. P. (2000) J. Biol. Chem. 275, 34092–34099
17. Sakane, F., Imai, S., Kai, M., Wada, I., and Kanoh, H. (1996) J. Biol. Chem. 271, 8394–8401
18. Klauck, T. M., Xu, X., Mousseau, B., and Jaken, S. (1996) J. Biol. Chem. 271, 15761–15768
19. Tang, W., Bunting, M., Zimmerman, G. A., McIntyre, T. M., and Prescott, S. M. (1996) J. Biol. Chem. 271, 10237–10241
20. Bunting, M., Tang, W., Zimmerman, G. A., McIntyre, T. M., and Prescott, S. M. (1996) J. Biol. Chem. 271, 10230–10236
21. Ding, L., Traer, E., McIntyre, T. M., Zimmerman, G. A., and Prescott, S. M. (1998) J. Biol. Chem. 273, 32746–32752
22. Houssa, B., Schaap, D., van der Val, J., Goto, K., Kondo, H., Yamakawa, K., Shibata, M., Takenawa, T., and van Blitterswijk, W. J. (1997) J. Biol. Chem. 272, 10422–10428
23. Ding, L., Bunting, M., Topham, M. K., McIntyre, T. M., Zimmerman, G. A., and Prescott, S. M. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 5519–5524
24. Caricasole, A., Bettini, E., Sala, C., Roncarati, R., Kobayashi, N., Caldara, F., Goto, K., and Terstappen, G. C. (2002) J. Biol. Chem. 277, 4780–4786
25. Raddatz, D., Henneken, M., Armbrust, T., and Ramadori, G. (1996) Hepatology 24, 928–933
26. Imai, S., Sakane, F., and Kanoh, H. (2002) J. Biol. Chem. 277, 35323–35332
27. Topham, M. K., and Prescott, S. M. (2001) J. Cell Biol. 152, 1135–1144
28. Angel, P., Imagawa, M., Chiu, K., Stein, B., Iimura, R. J., Rahmsdorf, H. J., Jonat, C., Herrlich, P., and Karin, M. (1987) Cell 49, 729–739
29. Lee, W., Mitchell, P., and Tjian, R. (1997) Cell 90, 741–752
30. Topham, M. K., Bunting, M., Zimmerman, G. A., McIntyre, T. M., Blackshear, P. J., and Prescott, S. M. (1998) Nature 394, 697–700
31. Shirai, Y., Segawa, S., Kuriyama, M., Goto, K., Sakai, N., and Saito, N. (2000) J. Biol. Chem. 275, 24760–24766
32. Sanjana, M. A., Jones, D. R., Izquierdo, M., and Merida, I. (2001) J. Cell Biol. 153, 207–220
33. Lemmon, M. A., and Ferguson, K. M. (2000) Biochem. J. 350, 1–18
34. Maffucci, T., and Falasca, M. (2001) FEBS Lett. 506, 173–179
35. Takeuchi, H., Kanematsu, T., Misumi, Y., Sakane, F., Konishi, H., Kikkawa, U., Watanabe, Y., Katan, M., and Hirata, M. (1997) Biochim. Biophys. Acta 1339, 275–285
36. Isakov, S. J., Cardozo, T., Andreev, J., Li, Z., Ferguson, K. M., Abagyan, R., Lemmon, M. A., Aronheim, A., and Skolnik, E. Y. (1998) EMBO J. 17, 5374–5387
