Diacylglycerol kinase (DGK) participates in regulating the intracellular concentrations of two bioactive lipids, diacylglycerol and phosphatidic acid. DGK\(\gamma\) (51, 128 kDa) is a type II isozyme containing a pleckstrin homology domain at the amino terminus. Here we identified another DGK\(\gamma\) isofrom (\(\gamma_2\), 135 kDa) that shared the same sequence with DGK\(\gamma_1\) except for a sterile \(\alpha\) motif (SAM) domain added at the carboxyl terminus. The DGK\(\gamma_1\) mRNA was ubiquitously distributed in various tissues, whereas the DGK\(\gamma_2\) mRNA was detected only in testis, kidney, and colon. The expression of DGK\(\gamma_2\) was suppressed by glucocorticoid in contrast to the marked induction of DGK\(\gamma_1\). DGK\(\gamma_2\) was shown to form through its SAM domain homo-oligomers as well as hetero-oligomers with other SAM-containing DGKs (\(\gamma_1\) and \(\gamma_2\)). Interestingly, DGK\(\gamma_2\) and DGK\(\gamma_1\) were rapidly translocated from the cytoplasm to endosomes in response to stress stimuli. In this case, DGK\(\gamma_1\) was rapidly relocated back to the cytoplasm upon removal of stress stimuli, whereas DGK\(\gamma_2\) exhibited sustained endosomal association. The experiments using DGK\(\gamma\) mutants suggested that the oligomerization of DGK\(\gamma_2\) mediated by its SAM domain was largely responsible for its sustained endosomal localization. Similarly, the oligomerization of DGK\(\gamma_2\) was suggested to result in negative regulation of its catalytic activity. Taken together, alternative splicing of the human DGK\(\gamma\) gene generates at least two isoforms with distinct biochemical and cell biological properties responding to different cellular metabolic requirements.

Upon cell stimulation by various hormones, growth factors, and other agonists, a variety of signaling lipids that regulate a wide range of biological output are liberated or synthesized. The cellular concentrations of such bioactive lipids must be strictly regulated by the action of metabolic enzymes. Diacylglycerol (DG)\(^1\) kinase (DGK) phosphorylates DG to yield phosphatidic acid (PA) (1). By numerous studies, DG and PA have been well recognized as lipid second messengers. DG is known to be an activator of conventional and novel protein kinase Cs (PKCs), chimaerins, Unc-13, and Ras guanyl nucleotide-releasing protein (2–4), and PA has been reported to modulate the activities of phosphatidylinositol-4-phosphate kinase, Ras GTPase-activating protein, Raf-1 kinase, atypical PKC, and many other important enzymes (5, 6). DGK thus appears to participate in various physiological events through modulating the balance between two bioactive lipids, DG and PA, in micro-environments within the cells.

It is now recognized that DGK represents a large enzyme family. The isoforms differ remarkably from each other with respect to their structures, the modes of tissue expression, and enzymological properties (7–10). To date, nine mammalian DGK isoforms (\(\alpha, \beta, \gamma, \delta, \epsilon, \zeta, \eta, \theta, \) and \(\iota\)) 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). Interestingly, the occurrence of alternative splicing was recently identified for four mammalian DGK genes (\(\gamma_1\) (11), \(\gamma_2\) (12), \(\beta_1\) (13), and \(\delta_1\) (14) isoforms). Thus, the list of DGK isoform members is still growing. The occurrence of alternative splicing in multiple DGK genes further adds to the complexity of the DGK gene family members and is probably essential for the action of DGKs regulating a wide range of cellular functions.

DGK\(\delta\) and DGK\(\gamma\) are closely related to each other and are thus classified together into the type II DGK subfamily (7–10). However, DGK\(\delta\) has a sterile \(\alpha\) motif (SAM) at the carboxyl terminus (15), whereas DGK\(\gamma\) contains no SAM domain (16). The SA domain, which is ~70 amino acids long, was first described as a module that is present in a small group of yeast sexual differentiation and Drosophila polyhomeotic proteins (17). The SA domain was subsequently found to occur in a wide range of proteins. Proteins containing SAM domains include the Eph family of receptor tyrosine kinases (18, 19), serine/threonine kinases (20), GTPase-activating protein, Raf-1 kinase, atypical PKC, and many other proteins. Such an extended occurrence suggests that SAM is an evolutionarily conserved protein-protein interaction domain that is involved in the regulation of numerous developmental processes among diverse eukaryotes. We recently demonstrated by gel filtration and co-immunoprecipitation analyses that DGK\(\delta\) formed homo-oligomeric structures in intact cells and that the SA domain was critically involved in the oligomerization (23). Moreover, the alternative splicing products of DGK\(\delta\) (DGK\(\delta_1\) and DGK\(\delta_2\)) were shown to binding protein; MAPK, mitogen-activated protein kinase; PA, phosphatidic acid; PKC, protein kinase C; RT-PCR, reverse transcriptase-polymerase chain reaction; SAM, sterile \(\alpha\) motif; TAA, tramcinolone acetonide; TPA, 12-O-tetradecanoylphorbol 13-acetate.
form hetero-oligomers via their SAM domains (14).

In the course of genome data base search for the human DGK\(\eta\) gene, we identified in silico a potential splice variant of DGK\(\eta\) with a SAM domain at the carboxyl terminus. Here we cloned the novel DGK\(\eta\) isoform generated through the alternative splicing. Therefore, the splice products of DGK\(\eta\) were revealed to possess biochemical and cell biological properties distinct from each other. Moreover, we found that the new DGK\(\eta\)2 isoform formed hetero-oligomers with other type II members, DGK\(\xi\)1 and DGK\(\xi\)2.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfection—**COS-7, human embryonic kidney (HEK) 293, and HepG2 cells were maintained in DMEM (Sigma) containing 10% fetal bovine serum at 37 °C in an atmosphere containing 5% CO\(_2\). Cells were transiently transfected with cDNAs using Effectene transfection reagent according to the instructions from the manufacturer (QIAGEN, Tokyo, Japan). 24 or 48 h after transfection, cells were used for further analysis.

**Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)—**Total RNA was isolated from HepG2 or HEK293 cells using Isogcen (Nippon Gene, Tokyo, Japan) according to the protocol from the manufacturer. Reverse transcription into cDNA was achieved using the SuperScript preamplification system (Invitrogen) according to the instructions from the manufacturer. cDNAs from human normal tissues and tumor-derived cells were purchased from BD Biosciences, Tokyo, Japan. PCR amplification was performed with Takara Ex Taq (Takara Biomedicals, Tokyo, Japan) using gene-specific oligonucleotide primers as follows: a DGK\(\xi\)3 common forward primer (nucleotide positions 2992–3116), 5'-TGAATAAAGCCAACCCAAGGTGCC-3', and a common reverse primer (nucleotide positions 3601–3627, 5'-TAAAATCCCTCGAGAATTCCGCTTTAC-3'), and a common reverse primer (nucleotide positions 3601–3627, 5'-TTAAATCCCTCGAGAATTCCGCTTTAC-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 5 min. For normalization, human glyceraldehyde phosphate dehydrogenase (GAPDH) mRNA was simultaneously amplified (25 cycles). PCR products were separated by agarose gel electrophoresis and stained with ethidium bromide.

**Plasmid Constructs—**cDNAs coding for DGK\(\xi\)-AS1L2 (amino acid residues 1–1147), DGK\(\xi\)-3PDZ (amino acid residues 1–1217) and DGK\(\xi\)-SAM (amino acid residues 1144–1220) were generated by PCR from a human DGK\(\xi\)2 cDNA clone. DGK\(\xi\)-2W1151G cDNA clone was generated by replacing the Trp-1151 in the SAM domain with Gly using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). For subsequent subcloning into expression vectors, primers were designed such that the resulting DGK\(\xi\)2 cDNA fragments contained a 5'-BamHI restriction site and a 3'-stop codon followed by a 3'-SalI site. The resulting DGK\(\xi\)2 fragments were fused in frame (the BglII/SalI site) with cDNA encoding a maltose binding protein (MBP, 43 kDa) obtained by inserting the MBP cDNA into the pMAL-c2X vector (New England Biolabs, Beverly, MA).

**Determination of DGK Activity—**One \(\mu\)g each of p3×FLAG-CMV-DGK\(\xi\)1, p3×FLAG-CMV-DGK\(\xi\)2, or p3×FLAG-CMV vector alone was transfected into COS-7 cells (60-mm dish). After 48 h, the cells were harvested and lysed by sonication in buffer A (0.5 ml/tray 60-mm dish) containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, complete protease inhibitor mixture (Roche Applied Science), and phosphate inhibitor mixture II (Sigma). After a low-speed centrifugation (550 g for 5 min), the resultant supernatant was used as enzyme source. The octyl glucoside mixed micellar assay using diolein as substrate was done as described (15).

**Western Blot Analysis—**COS-7 cells were grown on poly-L-lysine-coated glass coverslips and serum-starved for 3 h and then incubated in DMEM with or without 1 % Nonidet P-40. The cells were then washed with Tris buffer (pH 7.4), 1 mM EDTA, 1 mM DTT, 0.2% Triton X-100, 0.6 M KCl, and sodium pyruvate (Invitrogen) containing 0.1% bovine serum albumin. For subsequent subcloning into expression vectors, primers were designed such that the resulting DGK\(\xi\)2 cDNA fragments contained a 5'-BamHI restriction site and a 3'-stop codon followed by a 3'-SalI site. The resulting DGK\(\xi\)2 fragments were fused in frame (the BglII/SalI site) with cDNA encoding a maltose binding protein (MBP, 43 kDa) obtained by inserting the MBP cDNA into the pMAL-c2X vector (New England Biolabs, Beverly, MA).

**Fluorescence Microscopy—**For immunofluorescence microscopy, COS-7 cells were grown on poly-l-lysine-coated glass coverslips and transiently transfected with expression plasmids containing DGK\(\eta\) cDNAs amino-terminal fused with GFP. After 24 h, cells were serum-starved for 3 h and then incubated in DMEM with or without 500 mM sorbitol for 30 min. The cells were then fixed in 3.7% formaldehyde. When non-GFP fusion proteins were needed to be detected, cells were fixed as above and then reacted with an antiserum to GFP (Santa Cruz Biotechnology) and ECL (Amersham Biosciences) in phosphate-free DMEM 0.1% bovine serum albumin. After washing, the coverslips were incubated successively with the primary antibodies and the appropriate rhodamine- or mouse IgG antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) and ECL (Amersham Biosciences).
Fig. 1. Sequence of DGKα1 and DGKα2 cDNAs and organization of the human DGKα gene. A, the nucleotide sequences and deduced amino acid sequences of DGKα1 and DGKα2 are shown. Amino acid and nucleotide numbers are indicated at left in italic and plain text, respectively. A potential target sequence for class I PDZ domains is underlined. B, the exon-intron structure of the DGKα gene (exons 27–30) 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 SAM domain of human DGKα with that of human DGKβ. Identical amino acids are indicated in reverse type. Similar amino acids are shaded.

RESULTS

Identification and Characterization of a Novel DGKα Isoform—In the course of performing a genome data base search (accession numbers of human genomic clones: AL157932, AL139328, and AC018379) using as a probe the nucleotide sequence encoding the human DGKα SAM domain (15), we found a potential splice variant of human DGKα (Fig. 1A). We tentatively designated the human homolog of hamster DGKα cloned previously by Klauck et al. (16) and the newly identified clone as DGKα1 and DGKα2, respectively. As shown in Fig. 1, A and B, DGKα2 utilizes all of the 30 exons, whereas DGKα1 skips exon 29. The open reading frames of DGKα1 and DGKα2 encode 1164- and 1220-amino acid proteins with calculated molecular masses of 128 and 135 kDa, respectively (Fig. 1C). Interestingly, DGKα2 has a SAM domain encoded by exons 29 and 30 at the carboxyl terminus (Figs. 1, A–C). Because the phase of open reading frame used in the exon 30 of DGKα1 is different from that used by DGKα2 (Fig. 1A), the carboxy-terminal portions (amino acids 1148–1164 for DGKα1 and 1148–1220 for DGKα2) have no similarity with each other. The sequence of DGKα2 SAM domain (amino acids 1148–1220) shows high homology (78.1% identity and 87.7% similarity) to that of DGKβ (15) (Fig. 1D). Moreover, we noted that the last three residues (Ser-Glu-Val) of DGKα2 are a potential target sequence, S/TXΦ (Φ, a hydrophobic amino acid, usually Val, Ile, or Leu; X, unspecified amino acid), for class I PDZ domains (24–26).

To confirm that the two DGKα1 clones encode active enzymes, we subcloned each of the cDNAs into an expression vector, p3 × FLAG-CMV-7.1, which was subsequently transfected into COS-7 cells. We detected in the transfected cells proteins corresponding to the predicted molecular masses of 128 kDa (DGKα1) and 135 kDa (DGKα2) upon Western blotting using anti-FLAG antibody (not shown). Both enzymes were confirmed to possess DGK catalytic activities when the cell homogenates were assayed in vitro (not shown). Interestingly, DGKα1 showed an apparently 4-fold higher activity than that of DGKα2, suggesting the possibility that the SAM domain exerts inhibitory effects on the catalytic activity (see Fig. 7).

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 were analyzed by RT-PCR. As shown in Fig. 2, the DGKα1 transcript was detectable in most of the normal tissues and all of tumor-derived cells examined, with exceptionally low expression in the lung and skeletal muscle. On the other hand, the DGKα2 transcript was detected only in testis, kidney, and colon. Compared with DGKα1, the expression of DGKα2 in tumor-derived cells appeared to be relatively low, although the lack of antibody against human DGKα hindered us from confirming the protein levels of isoforms in the tissues.

Differential tissue distribution patterns of DGKα1 and DGKα2 mRNAs led us to hypothesize that the expressions of these isoforms are regulated under distinct mechanisms. Because Klauck et al. (16) reported that glucocorticoid, triamcinolone acetonide (TAA), increased the expression level of the hamster DGKα (a counterpart of human DGKα1), we first tested the effect of TAA. When HepG2 cells, which are known to express glucocorticoid receptors (27), were treated with 10 nm TAA for 72 h, the level of DGKα1 mRNA was markedly increased (Fig. 3A). In contrast, the DGKα2 mRNA was moderately decreased. We recently found that expression of DGKα2, another type II DGK isoform, was induced by treating...
cells with epidermal growth factor (EGF) and TPA, whereas the levels of mRNA and protein of DGK1 were suppressed by phorbol ester treatment (14). Thus, we next examined effects of TPA (100 nM, 24 h) and EGF (10 ng/ml, 48 h) on the expression levels of DGK1 isoforms. As shown in Fig. 3B, the mRNA level of DGK1 was not much affected by TPA whereas that of DGK12 was significantly increased. We next examined the effect of EGF on the expression of cellular DGK1 enzymes. For this purpose, we used HEK293 cells because HepG2 cells failed to respond to EGF. EGF had no detectable effects on the mRNA levels of DGK1 and DGK12 (Fig. 3C). The results collectively demonstrated that the induction patterns of mRNA levels of DGK1 and DGK12 were clearly different from each other. Moreover, the expression patterns of DGK1 are distinct from those of the closely related isoforms (DGK11 and DGK12).

Homo- and Hetero-oligomer Formation of DGK1 Isoforms—We next examined biochemical and cell biological properties of DGK11 and DGK12. We have recently found that DGK11 and DGK12 form oligomeric (at least tetrameric) structures in vitro and in vivo and that the SAM domain plays a critical role in the oligomer formation (14, 23). To study the multimeric nature of the DGK12 SAM domain in vitro, MBP-DGK12-SAM purified was analyzed using a Superose 12 HR column. As already observed for DGK5 (23), MBP-DGK12-SAM (40 μM) was eluted at the tetramer position (~240 kDa) (Fig. 4A). In the case of EphB2 receptor (28), an aromatic residue, Tyr-912, at 8th position in the SAM domain (corresponding to Trp-1151 in DGK12) was reported to be critical for dimer formation. On the other hand, the SAM domain of the EphA2 receptor, in which the critical Tyr is replaced with Gly, did not form dimer structure (29). Moreover, when the corresponding residue (Trp-1101) of DGK1 was mutated to Gly, the mutant did not form the multimeric structures (23). Trp-1151 in the DGK12 SAM domain was thus substituted by Gly to see whether this Trp is critical for the interaction. In contrast to the wild-type SAM, MBP-DGK12-SAM-W1151G (40 μM) was eluted at the monomer position (~60 kDa) (Fig. 4A). These data, essen-

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**Fig. 3. Regulation of expression of DGK11 and DGK12 by TAA, TPA, and EGF.** A, HepG2 cells were treated with 10 nM TAA for 72 h. B, HepG2 cells were treated with 100 nM TPA for 24 h. C, HEK293 cells were treated with 10 ng/ml EGF for 48 h. Total RNA was isolated, and the aliquots (500 ng) were subjected to RT-PCR. An upper panel in each set shows 536- and 405-bp cDNA fragments amplified for DGK11 (39 cycles) and DGK12 (39 cycles), respectively, in agarose gel electrophoresis. Lower panels in each set exhibit 983-bp cDNA fragments amplified for human glyceraldehyde phosphate dehydrogenase (GAPDH, 25 cycles). A representative of three repeated experiments is shown.

**Fig. 4. Homo-oligomerization of DGK12 through its SAM domain.** A, gel filtration analysis of oligomer formation of MBP fusion proteins in vitro. The purified MBP-DGK12-SAM or MBP-DGK12-SAM-W1151G (40 μM, 100 μl) was eluted from a Superose 12 HR column. The monomer (~60 kDa), dimer (~120 kDa), and tetramer (~240 kDa) positions estimated by a calibration curve of molecular mass standards (23) are indicated. Vo, void volume. B–E, co-immunoprecipitation analysis of oligomer formation of DGK12 in vitro. B, COS-7 cells were co-transfected with pEGFP or pEGFP-DGK12 and p3-FLAG-CMV-DGK12-SAM or MBP-DGK12-SAM-W1151G (40 μM) was eluted from a Superose 12 HR column. The monomer (~60 kDa), dimer (~120 kDa), and tetramer (~240 kDa) positions estimated by a calibration curve of molecular mass standards (23) are indicated. Vo, void volume. C, COS-7 cells were co-transfected with pEGFP or pEGFP-DGK12 and p3-FLAG-CMV-DGK12-SAM or MBP-DGK12-SAM-W1151G and p3-FLAG-CMV-DGK12-W1151G or pEGFP-DGK12-S12 and p3FLAG-CMV-DGK12-S12 as indicated. D, COS-7 cells were co-transfected with pEGFP-DGK12 and p3-FLAG-CMV-DGK12-W1151G or pEGFP-DGK12-S12 and p3FLAG-CMV-DGK12-S12 as indicated. E, COS-7 cells were co-transfected with pEGFP-DGK12 and p3-FLAG-CMV-DGK12-W1151G. After 48 h, the cells were serum-starved and subsequently stimulated by 1 μM TPA for 1 h. Anti-FLAG or anti-FLAG antibody was used for immunoprecipitation (IP) and Western blotting (WB) as indicated. Lysates and IP fractions were analyzed by Western blotting. Representatives of three repeated experiments are shown.
homo-oligomer formation of DGK\textsuperscript{\eta}1 was not observed (Fig. 4C).

To assess further the contribution of the SAM domain to the oligomer formation, we performed co-immunoprecipitation experiments using DGK\textsuperscript{\eta} mutants DGK\textsuperscript{\eta}2-W1151G and DGK\textsuperscript{\eta}-\textdelta S1,2 lacking the SAM domain. As shown in Fig. 4D, in comparison with wild-type DGK\textsuperscript{\eta}, significantly less 3×FLAG-DGK\textsuperscript{\eta}-\textdelta S1,2 and 3×FLAG-DGK\textsuperscript{\eta}2-W1151G were found in the anti-GFP immunoprecipitated complexes. These results indicate that the SAM domain contributes critically to the oligomer formation of DGK\textsuperscript{\eta}2. We noted, however, that a faint but detectable band of 3×FLAG-tagged DGK\textsuperscript{\eta}2-W1151G was still co-immunoprecipitated with the GFP-tagged counterpart (Fig. 4D), suggesting that, in addition to Trp-1151, the other residues of the domain weakly contribute to the formation of oligomer. It was previously reported that DGK\textsuperscript{\delta}1-oligomer was dissociated to a monomer by TPA stimulation. However, such disassembly of DGK\textsuperscript{\delta}1-oligomer was not detected (Fig. 4E).

To examine whether DGK\textsuperscript{\eta}2 and the DGK\textsuperscript{\delta} isoforms, possessing in common a SAM domain, form hetero-oligomer structures in vivo, we performed experiments using COS-7 cells co-expressing GFP-tagged DGK\textsuperscript{\eta}2 and 3×FLAG-tagged DGK\textsuperscript{\delta}1 or DGK\textsuperscript{\delta}2. When GFP-tagged DGK\textsuperscript{\eta}2 was immunoprecipitated with anti-GFP antibody, 3×FLAG-tagged DGK\textsuperscript{\eta}2 was co-immunoprecipitated (Fig. 4D). Reciprocally, when GFP-tagged DGK\textsuperscript{\eta}2 was immunoprecipitated with anti-GFP antibody, 3×FLAG-tagged DGK\textsuperscript{\delta}2 was co-immunoprecipitated. On the other hand, apparently due to lack of the SAM domain, homo-oligomer formation of DGK\textsuperscript{\eta}1 was not observed (Fig. 4C).

T. Murakami, F. Sakane, and H. Kanoh, unpublished observation.
Phosphorylation of DGK\(\eta\) Isoforms by TPA Stimulation—Because we have recently demonstrated that DGK\(\eta\)1 was phosphorylated upon TPA cell stimulation (23), protein phosphorylation of DGK\(\eta\)1 and DGK\(\eta\)2 was investigated. As shown in Fig. 6A, DGK\(\eta\)1 was clearly phosphorylated in TPA-stimulated cells, whereas DGK\(\eta\)2 was not. This result suggested that because the DGK\(\eta\)1-specific region at the carboxyl terminus contains four serine residues and one threonine (Fig. 1A), this region might have served as a phosphorylation site. Alternatively, the DGK\(\eta\)2-specific region (the SAM domain) might be involved in the suppression of phosphorylation. To address these possibilities, we constructed a DGK\(\eta\)1 mutant lacking both of the DGK\(\eta\)1- and DGK\(\eta\)2-specific sequences (DGK\(\eta\)1-ΔS1,2). Subsequent phosphorylation experiments showed that wild-type DGK\(\eta\)1 and DGK\(\eta\)1-ΔS1,2 were phosphorylated to a similar extent (Fig. 6B). The result indicates that the DGK\(\eta\)1-specific region does not contain the phosphorylation site and that the SAM domain quite likely exerts inhibitory effect(s) on the phosphorylation.

Catalytic Activity of DGK\(\eta\) Isoforms—When expressed in COS-7 cells, DGK\(\eta\)1 was already noted to give ~4-fold higher activity than that of DGK\(\eta\)2 (Fig. 7B). We therefore attempted to delineate the mechanism underlying the different catalytic efficiencies of the two DGK\(\eta\) isozymes. Because DGK\(\eta\)1 was phosphorylated in TPA-stimulated cells (Fig. 6A), we first examined the effects of phosphorylation on the catalytic activities of the DGK\(\eta\) isoforms. When COS-7 cells expressing DGK\(\eta\)1 or DGK\(\eta\)2 were treated with TPA, the DGK activity measured in vitro was not affected (Fig. 7A). We therefore concluded that the catalytic activity of DGK\(\eta\) isoforms was not significantly regulated by protein phosphorylation.

It is possible that the DGK activity is positively affected by the DGK\(\eta\)1-specific sequence (amino acids 1148–1164). Conversely, the activity may be negatively regulated by the DGK\(\eta\)2-specific sequence (the SAM domain, amino acids 1148–1220). To assess these possibilities, we determined catalytic activity of a mutant lacking both the DGK\(\eta\)1- and DGK\(\eta\)2-specific sequences, DGK\(\eta\)1-ΔS1,2. As shown in Fig. 7B, DGK\(\eta\)1-ΔS1,2 exhibited almost the same activity with that of wild type DGK\(\eta\)1, thus suggesting that the SAM domain negatively regulates the activity.

We next attempted to see whether the SAM domain inhibits the DGK\(\eta\)2 activity directly or indirectly through mediating enzyme oligomerization. The point mutant (DGK\(\eta\)2-W1151G) with a considerably impaired ability to form homo-oligomer (see Fig. 4D) gave DGK activity more than 2-fold higher than that of the wild-type enzyme (Fig. 7B). Although the activity of DGK\(\eta\)2-W1151G was lower than that of wild-type DGK\(\eta\)1 and DGK\(\eta\)1-ΔS1,2, this is probably because of the weak interaction still occurring between the mutant proteins (see Fig. 4D). On the other hand, the addition of excess amounts of MBP-DGK\(\eta\)2-SAM or MBP-DGK\(\eta\)2-SAM-W1151G invariably failed to affect the catalytic activity of the mutant (DGK\(\eta\)1-ΔS1,2) lacking the SAM domain (Fig. 7C). The result strongly suggests that the SAM domain does not directly inhibit the activity. Taken together, we infer that oligomerization through the SAM domain is, at least in part, responsible for the suppressed catalytic activity of DGK\(\eta\)2.

**Fig. 7.** Catalytic activities of DGK\(\eta\)1 and DGK\(\eta\)2. A, effects of cell treatment with TPA. COS-7 cells were transfected with plasmids encoding either 3×FLAG-DGK\(\eta\)1 or 3×FLAG-DGK\(\eta\)2. After starvation for 3 h, cells were incubated for 1 h in the presence of 1 μM TPA (+) or 0.1% Me\(\text{SO}\) (−). The cell lysates (15 μg of protein/sample) were assayed for DGK activity. To facilitate comparison of enzyme activities, background activities obtained for the control cells transfected with the vector alone were subtracted. The results are presented as the percentage of the value of wild type DGK\(\eta\)2 and the mean ± S.D. of the values obtained in three separate experiments. C, effects of the addition of the SAM domain. COS-7 cells were transfected with plasmids encoding 3×FLAG-DGK\(\eta\)-ΔS1,2. The cell lysates (10 μg of protein/sample) were preincubated with 5 μl each of purified MBP, MBP-DGK\(\eta\)2-SAM, or MBP-DGK\(\eta\)2-SAM-W1151G at 4°C for 1 h and then assayed for DGK activity. To facilitate comparison, background activities (the control cells transfected with the vector alone) were subtracted. The results are presented as the percentage of the value of DGK\(\eta\)-ΔS1,2 with no addition. A representative of twice-repeated experiments is shown.
FIG. 8. Subcellular localization of DGKν1 and DGKν2. A, COS-7 cells were plated in glass-bottom chambers and transfected with expression plasmids encoding GFP alone, GFP-DGKν1, or GFP-DGKν2 as indicated. After 24 h, cells were incubated for 5 min in the presence of 500 mM sorbitol. After replacement of the medium by sorbitol-free medium, cells were further incubated for 5 min. A p38 MAPK inhibitor (SB203580) or a mitogen-activated ERK activating kinase inhibitor (U0126) was added 30 min prior to the stimulation. Cells were examined using inverted confocal laser scanning microscopy (Zeiss LSM 510). A representative of three repeated experiments is shown. Bar = 10 μm. B, COS-7 cells were plated in glass-bottom chambers and transfected with expression plasmids encoding GFP-DGKν-ΔS1,2, GFP-DGKν2-ΔPDZ, GFP-DGKν2-W1151G, GFP-DGKν2-SAM, or GFP-DGKν2-SAM-W1151G as indicated. After 24 h, cells were incubated for 5 min in the presence of 500 mM sorbitol. After replacement of the medium by sorbitol-free medium, cells were further incubated for 5 min. A p38 MAPK inhibitor (SB203580) or a mitogen-activated ERK activating kinase inhibitor (U0126) was added 30 min prior to the stimulation. Cells were examined using inverted confocal laser scanning microscopy (Zeiss LSM 510). A representative of three repeated experiments is shown. Bar = 10 μm.
Intracellular Localizations of DGK\textsubscript{\eta} Isoform Proteins—We have recently found in HEK293 cells TPA-induced translocation of DGK\textsubscript{1} from cytoplasmic vesicles to the plasma membrane (23). However, no translocation of DGK\textsubscript{\eta} isoforms was detected when HEK293 and COS-7 cells were treated with TPA.\textsuperscript{2} We next examined effects of stress stimuli on the intracellular localization of DGK\textsubscript{\eta}. As shown in Fig. 8A, both DGK\textsubscript{\eta1} and DGK\textsubscript{\eta2} expressed in COS-7 cells were diffusely distributed in the cytoplasm. In response to osmotic shock (500 mM sorbitol), both isoforms were rapidly (within less than 5 min) translocated from the cytoplasm to punctate vesicles in more than 95% of COS-7 cells expressing the enzymes. Essentially the same results were obtained in cells exposed to oxidative stress (0.5 mM \ce{H2O2}).\textsuperscript{2} A similar translocation pattern was also observed in the cDNA-transfected HEK293 cells.\textsuperscript{2} Because more than 90% of DGK\textsubscript{\eta2} became associated with the Triton X-100 insoluble cytoskeletal fraction in the presence of 500 mM sorbitol, we could not assess the relationship between oligomer formation and enzyme translocation. In the presence of sorbitol, DGK\textsubscript{\eta2} (and DGK\textsubscript{\eta1})\textsuperscript{2} markedly co-localized with an endosome marker protein, RhoB (30) (Fig. 8C).

Osmotic shock and oxidative stress are known to activate p38 mitogen-activated protein kinase (MAPK), c-Jun-NH\textsubscript{2}-terminal kinase/stress-activated protein kinase, and extracellular signal-regulated kinase (ERK) (31, 32). When p38 MAPK inhibitor, SB203580, was added, the translocation induced by sorbitol was significantly inhibited (Fig. 8A). On the other hand, an inhibitor of mitogen-activated ERK activating kinase, U0126, did not exert such effects. Essentially the same results were obtained with DGK\textsubscript{\eta2}.\textsuperscript{2} The results strongly suggest that translocation was mainly regulated through the p38 MAPK pathway.

Interestingly, after removal of sorbitol, DGK\textsubscript{\eta1} was rapidly (within less than 5 min) relocated back to the cytoplasm in about 95% of cells expressing the isoform (Fig. 8A). On the other hand, DGK\textsubscript{\eta2} remained associated with the vesicles in more than 90% of DGK\textsubscript{\eta2}-transfected cells. Even 15 min after the wash out, DGK\textsubscript{\eta2} still associated with the vesicles in about 70% of transfectants. The DGK\textsubscript{\eta1} mutant lacking the DGK\textsubscript{\eta1} specific sequence (DGK\textsubscript{\eta1}-ASL12) showed the same translocation pattern as observed for wild-type DGK\textsubscript{\eta1} (Fig. 8B), indicating that the region does not act as a repulsive element in endosomes in the absence of sorbitol. Moreover, because the DGK\textsubscript{\eta2} mutant lacking the PDZ binding sequence (DGK\textsubscript{\eta2}-\text{PDZ}) showed the same translocation pattern as observed for wild-type DGK\textsubscript{\eta2}, the SAM domain, but not the PDZ binding sequence, is considered to be essential for sustained endosomal localization.

It is possible that the oligomer formation through the SAM domain is responsible for the sustained localization. Alternatively, the domain itself may directly stabilize the membrane association. As shown in Fig. 8B, DGK\textsubscript{\eta2}-W1151G failed to exhibit sustained endosomal association, indicating that the enzyme oligomerization is involved at least in part in the stabilization of membrane association. The SAM domain alone (DGK\textsubscript{\eta2}-SAM) was translocated from the cytoplasm to endosomes upon sorbitol treatment, but different from the intact enzyme, DGK\textsubscript{\eta2}-SAM was rapidly dissociated from the membranes by removal of sorbitol. Moreover, the W1151G mutation in the SAM domain (DGK\textsubscript{\eta2}-SAM-W1151G) did not affect the transient nature of the enzyme translocation. Because the same point mutation introduced into full-length DGK\textsubscript{\eta2} markedly reduced the sustained endosomal localization, the SAM domain alone cannot directly stabilize the membrane association. Taken together, the oligomer formation is indicated to mainly contribute to the sustained endosomal localization of DGK\textsubscript{\eta2}.

**DISCUSSION**

The present study added a new DGK isoform to the growing list of the mammalian DGK gene family (altogether nine independent genes and five alternative splicing products at present). Interestingly, although alternative splicing of the DGK\textsubscript{\eta} gene replaces the most amino-terminal sequence (14), that of the DGK\textsubscript{\eta} gene generates a DGK\textsubscript{\eta} isoform having the carboxy-terminal addition, including the SAM domain and a PDZ-binding motif. Exchanges of distinct functional domains at separate sites give a great diversity of structure and function to the type II DGKs. As expected from the alternative splicing of the DGK\textsubscript{\eta} gene (14), DGK\textsubscript{\eta1} and DGK\textsubscript{\eta2} were expressed in different manners. Moreover, we revealed that skipping only a single exon (exon 29) of the DGK\textsubscript{\eta} gene resulted in generating two splice variants having many different biochemical and cell biological properties, such as catalytic activity, protein phosphorylation, and subcellular localization.

Klauck et al. (16) reported that the hamster DGK\textsubscript{\eta1} was glucocorticoid (TAA)-inducible. In this report, we confirmed that TAA significantly increased the DGK\textsubscript{\eta1} mRNA level in HepG2 cells. Interestingly, the mRNA level of the newly identified DGK\textsubscript{\eta2} was, if anything, decreased by the TAA treatment. Although TPA also enhanced the DGK\textsubscript{\eta1} expression, but not DGK\textsubscript{\eta2}, EGFP failed to affect the expressions of both isoforms. These results suggest that choices of the splice sites occur in highly inducer-specific manners. However, because of the general lack of understanding of how cells choose and change particular splice sites (33), regulatory mechanisms of alternative splicing of the DGK\textsubscript{\eta} gene are still unclear. DGK\textsubscript{\eta1} showed a much broader tissue distribution, whereas the expression of DGK\textsubscript{\eta2} was relatively tissue-specific. The results suggest that DGK\textsubscript{\eta1} plays a more general role in regulating cellular DG and PA levels when compared with DGK\textsubscript{\eta2}.

DGK\textsubscript{\eta1} showed a molecular activity in vitro ~4-fold higher than that of DGK\textsubscript{\eta2}. A DGK\textsubscript{\eta1} mutant lacking the SAM domain also had about 4-fold higher activity in vitro than that of wild-type DGK\textsubscript{\eta1}.\textsuperscript{3} Thus, it is likely that the molecular activities of type II DGK isoforms lacking the SAM domain are generally higher than those of the isoforms equipped with this domain. In this study, we inferred that oligomer formation through the SAM domain rather than the direct effects of this domain resulted in the inhibition of the catalytic activity. Thus, the amino-terminal part of DGK\textsubscript{\eta2} oligomerized through the SAM domain may interfere with the catalytic action. Alternatively, it remains possible that the amino-terminal portion of monomeric DGK\textsubscript{\eta} interacts with an unknown activating factor for the DGK activity.

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\textsuperscript{2} S. Imai, F. Sakane, and H. Kanoh, unpublished observation.
Translocation of DGKδ1 from cytoplasmic vesicles to the plasma membrane was dependent on TPA stimulation (23). In addition to DGKδ1, translocations of many DGKs to the plasma membrane or to the nucleus have been reported so far in TPA-treated cells or in activated T-lymphocytes (7–10). In this report, we demonstrated for the first time that DGKδ1 and DGKδ2 were significantly translocated from the cytoplasm to endosomes by stress stimuli. TPA is known to stimulate PKC (2) and subsequently the Raf-1/ERK pathway (34). ERKs are predominantly activated by mitogenic stimuli, and other p38 MAPKs and c-Jun-NH2-terminal kinases/stress-activated protein kinases are preferentially activated by non-mitogenic stimuli, including environmental stresses. However, osmotic shock and oxidative stress are known to activate all of p38 MAPKs, c-Jun-NH2-terminal kinases/stress-activated protein kinases, and ERKs. Because a p38 MAPK inhibitor (SB203580), but not an inhibitor of mitogen-activated ERK activating kinase (U0126), significantly inhibited the translocation of DGKδ1, this event is considered to be mainly regulated by the p38 MAPK pathway. This concept is supported by the fact that TPA, which activates the ERK pathway, did not markedly enhance the translocation. At present, we cannot deny the possibility that c-Jun-NH2-terminal kinases/stress-activated protein kinases also partly contribute to the enzyme translocation. Interestingly, the endosomal association of DGKδ1 induced by sorbitol was quite transient, whereas that of DGKδ2 was rather sustained. Using DGKδ1 mutants we found that the sustained association of DGKδ1 with endosomes was largely caused by its oligomer formation through its SAM domain. Such distinct modes of membrane association should reflect their different physiological functions. Many signaling proteins such as phospholipase Cγ1, p120 Ras GTPase-activating protein, and c-Raf were found associated with endosomal compartments (35). Because it is known that phospholipase Cγ1 liberates DG and that the activities of p120 Ras GTPase-activating protein and c-Raf are regulated by PA (7–10), DGKδ1 and DGKδ2 may be involved in these signaling pathways on transient or continuous demands, respectively.

DGKδ1, but not DGKδ2, was phosphorylated by phorbol ester stimulation. Obvious phosphorylation of the DGKδ1 isoform was not detected by sorbitol stimulation, suggesting that the phosphorylation is probably dependent on PKC. Because the DGKδ1-specific sequence was not considered to serve as a phosphorylation site, phosphorylation occurs in the region common to the two isoforms. To explore physiological consequence of the isoform-selective protein phosphorylation, identification of phosphorylation site(s) and protein kinase(s) involved is needed.

One of the most remarkable features of the SAM domain of DGKδ2 is its ability to interact with DGKδ itself and with the closely related isoforms, DGKδ1 and DGKδ2. Because DGKδ2, DGKδ1, and DGKδ2 (14) are all co-expressed in lung carcinomas (LK-1 and GI-117), a colon adenocarcinoma (CX-1), a prostatic adenocarcinoma (PC3), pancreatic adenocarcinoma (GI-103), and HepG2 cells, these isoforms can potentially form hetero-oligomer structures at least in these tissues and cells. Because DGKδ1 is known to form at least tetramer structures (23), permutations and combinations of the hetero-tetramer yield 3^4 (=81) variations. What is the physiological implication of the DGK hetero-oligomers being greatly diversified? This question is the target of future investigation.

In addition to the DGKδ gene, alternative splicing was found in the DGKβ (13), DGKγ (11), DGKα (14), and DGKζ (12) genes to date. DGKβ isoforms differ at their carboxyl termini through alternative splicing and one variant was associated with the plasma membrane, whereas the other isoform was predominantly localized in the cytoplasm (13). Active DGKγ is predominantly expressed in human retina, but an inactive form lacking 25 amino acids within the catalytic domain is detected in most human tissues and cells, such as kidney, testis, HepG2 cells, and HL-60 cells (11). The expression of DGKδ2 was induced by treating cells with EGF and TPA, whereas the levels of mRNA and protein of DGKδ1 were suppressed by the phorbol ester treatment (14). Moreover, 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. In the case of DGKδ1, the initial 54 amino acids are replaced with a 262-amino acid fragment (12). DGKζ1 is a more widely expressed form, whereas a DGKζ2 protein was predominantly detected in muscle tissues. Inhibitory effects on Ras guanyl nucleotide-releasing protein and subsequent localization of the DGKζ isoforms were different from each other (36).

In plants, alternative splicing of the tomato DGK gene generates an isoform having the calmodulin-binding domain near the carboxyl terminus (37). Calcium recruited only the calmodulin-binding DGK from soluble to membrane fractions. Therefore, alternative splicing may be a common mechanism in regulating DGK functions in different tissues and cells. The marked divergence of DGK isoforms elicited by alternative splicing may reflect their physiological importance and the needs to respond to a variety of signaling pathways operating under distinct regulatory mechanisms.

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