Nuclear Transportation of Diacylglycerol Kinase γ and Its Possible Function in the Nucleus*

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Diacetylglcerol kinases (DGKs) convert diacylglycerol (DG) to phosphatidic acid, and both lipids are known to play important roles in lipid signal transduction. Therefore, DGKs are considered to be one of the key players in lipid signaling, but its physiological function remains to be solved. In an effort to investigate one of nine subtypes, we found that DGKγ came to be localized in the nucleus with time in all cell lines tested while seen only in the cytoplasm at the early stage of culture, indicating that DGKγ is transported from the cytoplasm to the nucleus. Unlike DGKβ, γ didn’t necessarily need DGK activity, but its C1 domain was indispensable, suggesting that the C1 domain of DGKγ acts as a nuclear transport signal. Furthermore, to address the function of DGKγ in the nucleus, we produced stable cell lines of wild-type DGKγ and mutants, including kinase negative, and investigated their cell size, growth rate, and cell cycle. The cells expressing the kinase-negative mutant of DGKγ were larger in size and showed slower growth rate, and the S phase of the cells was extended. These findings implicate that nuclear DGKγ regulates cell cycle.

Diacetylglcerol (DG) is a second messenger regulating various cellular responses (1, 2). One of the important roles of DG is an activating protein kinase C (PKC) (1, 3, 4). DG is physiologically produced as a result of the signal-induced hydrolysis of phosphatidylinositol by phospholipase C. The generated DG is phosphorylated to phosphatidic acid by diacylglycerol kinase (DGK) or metabolized by DG lipase (2, 5, 6). Thus, DGK is an important enzyme to inactivate PKC by reducing the DG level, contributing to regulating of the cellular response. In addition, phosphatic acid itself activates ZPKC (7), phosphatidylinositol 4-phosphate 5-kinase (8, 9), and mammalian target of rapamycin (10), and modulates Ras GTPase-activating protein (11).

Molecular cloning studies revealed that mammalian DGK family consists of at least nine subtypes (2). Although all DGKs have cysteine-rich repeats similar to the C1A and C1B domains of PKCs in the N terminus and a catalytic domain in the C terminus, they are divided into five groups on the primary structure of these DGKs. Type I DGKs, including DGKα, -β, and -γ, have EF-hand motifs and two cysteine-rich regions (C1 domain) in the regulatory domain (12, 13), whereas Type II DGKs, DGKδ and -η, have a pleckstrin homology domain instead of the EF-hand motif in addition to the C1 domain (14, 15). The separated catalytic domains of DGKδ and -η are characteristic of Type II DGK. Type III, DGKε, has only one C1 domain in the regulatory domain (16). Myristoylated alanine-rich protein kinase C substrate phosphorylation site-like region and four ankyrin repeats are unique domains in Type IV DGK (17, 18). The final group, type V, includes DGKθ, which has three cysteine-rich regions and a pleckstrin homology domain with overlapping Ras-associating domain (19). The DGKs are thought to be involved in development, differentiation, construction of neural network and immunity, etc. However, subtype specific function and regulation mechanisms of DGKs are not clear.

Several groups have reported many different localization and translocation of DGKs, possibly contributing to their subtype-specific functions. In an effort to elucidate the function of DGK, we unexpectedly found that GFP-fused DGKγ (GFP-DGKγ) became localized in the nucleus as well as the cytoplasm a few days after transfection but was localized mainly in the cytoplasm just after expressed in CHO-K1 cells. Although nuclear transportation of DGKγ has never been reported, expression of DGKθ and DGKζ in the nucleus has been already described (20, 21). In addition, DGKζ is thought to be involved in the regulation of cell cycle (21). These findings, together with the facts that phosphatidylinositol turnover occurs within the nucleus and DG may be involved in the regulation of cell cycle (22–27), suggest that DGKγ has some physiological function in the nucleus. However, mechanism of the nuclear transportation and physiological functions of DGKγ are unknown. We, therefore, investigated molecular mechanism and physiological significance of nuclear transportation of DGKγ.

EXPERIMENTAL PROCEDURES

Materials—CHO-K1 cells were donated from Dr. M. Nishijima (National Institute of Health, Tokyo, Japan). COS-7 cells, SH-SYSY cells, and HeLa cells were purchased from the RIKEN Cell Bank. Fetal bovine serum, RNase A, and anti-FLAG M2 monoclonal antibody were obtained from Sigma. FuGENE 6 Transfection reagent was obtained from Roche Applied Science. Propidium iodide was obtained from Bio-Rad. FluoroLink Cy3-labeled goat anti-mouse IgG was purchased from Amersham Biosciences. We produced anti-GFP anti-

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body ourselves.\(^4\) \([\gamma-^{32}P]ATP\) was obtained from ICN Biomedicals (Irvine, CA). A TLC plate was obtained from Merck (Darmstadt, Germany). Geneticin was purchased from Invitrogen.

**Cell Culture**—CHO-K1 cells were cultured in Ham’s F-12 medium (Nacalaitesque, Japan). COS-7 and NIH3T3 cells were cultured in Dulbecco’s modified Eagles’ medium (Nacalaitesque, Japan). SH-SY5Y cells were cultured in Dulbecco’s modified Eagles’ medium/Ham’s F-12 medium (1:1) (Invitrogen). All cells were cultured at 37 °C in humidified atmosphere containing 5% CO\(_2\). All media contained 25 mM glucose, and all were buffered with 44 mM NaHCO\(_3\) and supplemented with 10% fetal bovine serum (Sigma), penicillin (100 units/ml), and streptomycin (100 \(\mu\)g/ml) (Invitrogen). The fetal bovine serum used was not heat-inactivated. The media for SH-SY5Y cells was added with 1% GlutaMAX-TM.1 Supplement (Invitrogen).

**Constructs of Plasmids Encoding DGK\(\gamma\)-fused GFP and Mutants**—The constructs encoding DGK\(\gamma\) having GFP at its N terminus (GFP-DGK\(\gamma\)) or at C terminus (DGK\(\gamma\)-GFP) and mutants having substitution of Cys-285 to Gly in the C1A domain (GFP-DGK\(\gamma\)C1Am) or Cys-348 to Gly in the C1B domain (DGK\(\gamma\)-GFP-DGK\(\gamma\)C1Bm) were previously described (28). The constructs encoding the mutants lacking the C1A or C1B domain (DGK\(\gamma\)-GFP-DGK\(\gamma\)\(\Delta\)C1A or DGK\(\gamma\)-DGK\(\gamma\)\(\Delta\)C1B) were also described previously (29). Kinase negative mutant of DGK\(\gamma\) (GFP-DGK\(\gamma\)KN) was produced by substitution of Gly-491 to Asp. Briefly, using the plasmid encoding GFP-DGK\(\gamma\), site-directed mutagenesis was performed according to the manufacturer’s recommended protocol with an ExSite PCR-based site-directed mutagenesis kit (Stratagene). The primers were 5’-GCTGAGATCCTTGAGATATGTTGGACAAGG-3’ and 5’-CAACTGTTGCATCTCAGCAGGCAA-3’. The mutation was confirmed by verifying its sequence. Furthermore, to make kinase-negative mutants having substitution of Cys-285 or Cys-348 to Gly (GFP-DGK\(\gamma\)KNC1Am and GGFP-DGK\(\gamma\)KNC1Bm), the cDNA of DGK\(\gamma\) KN was digested with Sall and Smal and then subcloned into the Sall and Smal site in the plasmid encoding GFP-DGK\(\gamma\)C1Am and GFP-DGK\(\gamma\)C1Bm, respectively.

In addition, to construct DGK\(\gamma\) KN having a nuclear localization signal (NLS), cDNA fragments of DGK\(\gamma\) KN with BspEI site at the N terminus and Xhol site at the C terminus were produced by a PCR using cDNA for kinase-negative mutant of DGK\(\gamma\) constructed as described above. The PCR products were first subcloned into a pGEM T-Easy vector (Promega, Madison, WI). After digestion with BspEI and Xhol, the cDNA encoding DGK\(\gamma\) KN was subcloned into the BspEI and Xhol site in the pECPF-Nuc vector (Clontech). The primers were 5’-AATCCGGGATGATGACTGGGCAAATTCG-3’ and 5’-GCTGAGAGCTCTTGAAGGCTTTTACT-3’.

**Confocal Microscopy**—The fluorescence of Cy3 and GFP were observed under confocal laser scanning fluorescent microscopy (Carl Zeiss, Jena, Germany). The GFP fluorescence was monitored at 488 nm argon laser excitation with 515 nm low pass barrier filter. Cy3 fluorescence was monitored at 543 nm HeNe 1 excitation with a 560–615 nm band pass filter.

Upon quantitating, the subcellular distribution of the proteins fused GFP, which we described below. The cells in which the ratio of GFP fluorescence intensity of cytoplasm and nucleus was under 0.3 were defined as “only in the cytoplasm,” and the cells with the ratio of 0.3–0.8 were defined as “abundantly in the cytoplasm.” In the case the ratio was over 0.8, the cells were defined as “equally in the cytoplasm and the nucleus.”

**Immunoblotting and Kinase Assay**—Plasmids (\(\sim\)32 \(\mu\)g) were electroporated into COS-7 cells using a GenePulser (Bio-Rad, 975 microfarads, 220 \(\text{V} \cdot \text{cm}\)\(^{-1}\)) or transfected into NIH3T3 cells using FuGENETM6 transfection reagent (Roche Applied Science) and 1 \(\mu\)g of DNA according to the manufacturer’s protocol. Transfected cells were cultured at 37 °C for \(\sim\)24 h before use.

**Immunostaining**—CHO-K1 cells transfected cDNA coding FLAG fusion proteins as described above, and those without transfection were fixed for 1 h at room temp with 4% paraformaldehyde, 0.2% picric acid in 0.1 M phosphate buffer and permeabilized with 0.3% Triton X-100 in 0.01 M PBS for 15 min at room temp. Cells were sequentially incubated with 10% normal goat serum, mouse anti-FLAG antibody (Sigma), or anti-DGK\(\gamma\) antibody, and then Fluorolink Cy3-labeled goat anti-mouse antibody. At each step, transfected cells were washed three times with 0.01 M PBS containing 0.03% Triton X-100 (PBS-T) for 5 min.

For preparation of anti-DGK\(\gamma\) antibody, an oligopeptide corresponding to amino acids 778–791 of human DGK\(\gamma\) (31) was used for antigen. This antibody was purified by antigen-immobilized affinity column. We finally confirmed that the purified DGK\(\gamma\) antibody has no cross-reactions with pig DGK\(\alpha\) or rat DGK\(\beta\).

**Nuclear Transportation and Function of DGK\(\gamma\)**

For preparation of anti-DGK\(\gamma\) antibody, an oligopeptide corresponding to amino acids 778–791 of human DGK\(\gamma\) (31) was used for antigen. This antibody was purified by antigen-immobilized affinity column. We finally confirmed that the purified DGK\(\gamma\) antibody has no cross-reactions with pig DGK\(\alpha\) or rat DGK\(\beta\).

For determination of the kinase activity of DGK\(\gamma\) in transfected cells, the cells were harvested and centrifuged at 5500 \(\times\) g for 3 min. The cells were resuspended in homogenizing buffer (250 \(\text{mM}\) sucrose, 10 \(\text{mM}\) EGTA, 2 \(\text{mM}\) EDTA, 50 \(\text{mM}\) Tris-HCl, 200 \(\mu\)g/ml leupeptin, 1 \(\mu\)g/ml benzamidine (Sigma), 1 \(\mu\)g/ml pepstatin, 1 \(\mu\)g/ml apotinin, 5 \(\mu\)g/ml aprotinin, 0.01 \(\mu\)g/ml trypsin inhibitor, 2 \(\mu\)g/ml pepsin inhibitor, 5 \(\mu\)g/ml soybean trypsin inhibitor, 0.5 \(\mu\)g/ml antipain, 0.5 \(\mu\)g/ml apronin, 0.5 \(\mu\)g/ml pepstatin A, 0.5 \(\mu\)g/ml E-64, 0.5 \(\mu\)g/ml leupeptin A, 1% Triton-X-100, pH 7.4) and sonicated (UD-210 Tomy, Japan, output 3, 15 s, 2 times).

For immunoblotting, the samples were subjected to 7.5% SDS-polyacrylamide gel electrophoresis, followed by blotting onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA). After being cultured, the cells were harvested and centrifuged at 5500 \(\times\) g for 3 min. The cells were resuspended in homogenizing buffer (250 \(\text{mM}\) sucrose, 10 \(\text{mM}\) EGTA, 2 \(\text{mM}\) EDTA, 50 \(\text{mM}\) Tris-HCl, 200 \(\mu\)g/ml leupeptin, 1 \(\mu\)g/ml benzamidine, 1% Triton-X-100, pH 7.4) and sonicated (UD-210 Tomy, Japan, output 3, 15 s, 2 times).

For immunoblotting, the samples were subjected to 7.5% SDS-polyacrylamide gel electrophoresis, followed by blotting onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA). Nonspecific binding sites were blocked by incubation with 5% skim milk in PBS-T at 4 °C overnight. The membrane was incubated with anti-GFP antibody for 1 h at room temperature. After washing with PBS-T, the membrane was incubated with peroxidase-labeled anti-rabbit IgG (Jackson, ImmunoResearch Laboratories, West Grove, PA) for 30 min. After three rinses with PBS-T, the immunoreactive bands were visualized using a chemiluminescence detection kit (ECL, Amersham Biosciences).

To determine the kinase activity of DGK\(\gamma\) and mutants, appropriate volumes of the homogenate samples, which contain comparative

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\(^4\) Y. Yamaguchi, Y. Shirai, T. Matsubara, N. Ohshiro, K. Yoshino, K. Yonezawa, Y. Ono, and N. Salto, manuscript in preparation.
amounts of the fusion protein of DGKγ or mutants assessed by immunoblotting, were subjected to octyl glucoside mixed-micelle assay (14) by subtle modification. 1-Steroyl-2-arachidonoyl-sn-glycerol (Biomol, Plymouth Meeting, PA) was used as substrate. The radioactivity of phosphatidic acid was separated on 20-cm Silica gel 60 TLC plates (Merck) using a chloroform:methanol:acetic acid (65:15:5) solution and detected by using a BAS2500 (Fujix, Tokyo, Japan).

**Measurement of the Diameter and Thickness of the Cells Transiently Expressing Fluorescent Proteins**—Plasmids (~5.5 μg) encoding GFP-DGKγ, GFP-DGKγ KN, GFP-DGKγ ΔC1B, GFP-DGKγ KNC1Am, and GFP alone were transfected into CHO-K1 cells by lipofection as described above. Diameters of the respective cells were measured by LSM510 software under confocal microscopy. The thickness of the cells was measured using re-constructed image of three-dimensional sections.
FIGURE 2. Comparison of properties of GFP-DGKγ and its mutants. A, constructs of fusion proteins of DGKγ with GFP and mutants. GFP was fused at the N terminus of DGKγ (GFP-DGKγ). GFP-DGKγ ΔC1A and GFP-DGKγ ΔC1B lacked the C1A domain (from 269 to 318 aa) and C1B domain (from 334 to 380 aa), respectively. Cys-285 in the C1A domain or Cys-348 on the C1B domain was replaced to Gly in the GFP-DGKγ C1Am and GFP-DGKγ C1Bm, respectively. In GFP-DGKγ KN, Gly-491 in the ATP binding site was substituted to Asp. GFP-DGKγ KNC1Am and GFP-DGKγ KNC1Bm were made by replacing Cys-285 or Cys-348 to Gly in the C1A domain or C1B domain of GFP-DGKγ KN, respectively. DGKγ-GFP has GFP at the C terminus of DGKγ. B, immunoblot analysis of GFP-DGKγ, DGKγ-GFP, and mutants by anti-GFP antibody. The plasmids were transfected to COS-7 cells by electroporation. The lysates of the transfected cells were subjected to SDS-PAGE and Western blotting as described under "Experimental Procedures." The immunoreactive bands were detected by using anti-GFP antibody. The molecular mass of marker protein is indicated on the left. C, kinase activity of GFP-DGKγ, DGKγ-GFP, and mutants. Comparative amount of the GFP-DGKγ, DGKγ-GFP, and mutants (shown in B) were subjected to the octyl-glucoside method using 1-steroyl-2-arachidonoyl-sn-glycerol as substrate. Reaction products were separated on TLC plate and detected by BAS2500.
Production of Stable Cell Lines—Plasmids (~5.5 µg) encoding GFP-DGKγ KN, GFP-DGKγ KN, GFP-DGKγ KNC1Am, and GFP alone were transfected by lipofection using FuGENE™6 transfection reagent according to the manufacturer’s protocol. Geneticin (0.5 mg/ml) was added to the medium 24 h after the transfection. After being cultured for more than 24 h, the transfected cells were transferred to 96-well
plates at 0.5 cell/well for cloning. The positive clone was identified by the fluorescence under confocal microscopy.

Proliferation of Stable Cell Lines—0.6 × 10⁶ cells of each stable cell lines were split on three 10-cm dishes (0.2 × 10⁶ each). After 24 (day 1), 48 (day 2), and 72 h (day 3), cells were treated with trypsin-EDTA after washed PBS(−) and collected by centrifugation (1250 × g for 10 min, at 4°C). The harvested cells were resuspended in 1 ml of PBS(−), stained with 0.4% Trypan blue in PBS(−), and counted. The doubling time of the lines were calculated from simple regression based on the number of the cells. Difference of correlation coefficients of the regression lines was determined by testing the t value.

Live Imaging of Cell Division of HeLa Cells Expressing CFP-NLS and CFP-DGKγKN-NLS—Plasmids encoding CFP-NLS and CFP-DGKγ KN-NLS were transfected into HeLa cells. Images of both phase contrast and CFP fluorescence were taken every 10 min from 24 to 72 h after transfection using fluorescent microscopy, BZ-8000 (Keyence, Osaka, Japan) equipped with cultivation system, INU-KI-F1 (Tokai Hit, Shizuoka, Japan). Based on the images, the doubling time of respective cells, the time period between the first and the second division, was measured.

Flow Cytometry—0.2 × 10⁶ cells of each stable cell lines were spread on 10-cm dishes. The cells were synchronized at the beginning of S phase by double thymidine block and release protocol. Briefly, the cells were treated with 10 mM (for GFP stable cells) or 5 mM (for GFP-DGKγ KN stable cells) thymidine for the first 18 h, followed by an interval of thymidine-free incubation for 10 h, and the second thymidine incubation for 8 h (32). To release the cell cycle, the cells were washed well and cultured in normal medium containing serum. Every hour after the release, cells were treated with trypsin-EDTA and collected in a 1.5-ml tube. Washed by PBS(−), the cells were fixed by 70% ethanol for 1 h on ice, and treated sequentially by 0.25 mg/ml RNase A and 0.05 mg/ml propidium iodide. Cell cycle analysis was performed by using a FACS-Calibur (BD Biosciences).

RESULTS
Changes in Subcellular Localization of DGKγ—During the cultivation of CHO-K1 cells expressing GFP-DGKγ, we observed different localization of GFP-DGKγ; some cells had GFP-DGKγ in the nucleus and some did not. Specifically, just after the transfection many of the CHO-K1 cells express GFP-DGKγ only in the cytoplasm (Fig. 1A, CHO-K1, type A) but 2–3 days later, this enzyme was localized equally in the cytoplasm and nucleus (Fig. 1A, CHO-K1, type B). Here, we considered the possibility that the nuclear localization of GFP-DGKγ was due to the degradation, because GFP itself is localized in the nucleus and the cytoplasm as shown in below in Fig. 4C. However, no degraded product of GFP-DGKγ was found even at day 3 by immunoblotting using GFP antibody (Fig. 1B), indicating that the enzyme is transported from the cytoplasm to the nucleus during cultivation. Therefore, we investigated time-dependent changes of GFP-DGKγ localization. Fig. 1C shows that the number of the CHO-K1 cells expressing GFP-DGKγ only in the cytoplasm (Type A) decreased, while the number of the cells expressing the fusion protein both in the cytoplasm and the nucleus (Type B) increased with time. Percentage of the type B CHO-K1 cells was 34% at day 1, but it increased to 53% at day 2. The ratio of type B to type A cells changed from 0.826 at day 1 to 2.790 at day 2. This ratio increased further to 3.110 at day 3. We also carried out the same experiment using NIH3T3 cells. In NIH3T3 cells, the percentage of the type A cells and that of the type B cells are similar at day 1, but the type B cells became the major population with time; it increased to 72% at day 2 and 86% at day 3 (Fig. 1C, NIH3T3). Furthermore, we performed this experiment using the neuroblastoma cell line, SH-SY5Y, because DGKγ are abundantly expressed in the brain. In SH-SY5Y cells, GFP-DGKγ showed different types of the localization (Fig. 1A, SH-SY5Y, type A and type B). Percentage of the type B SH-SY5Y cells was 47% at day 1, and it was 52.2% at day 2 with the change in ratio from 1.395 to 2.373 (Fig. 1C, SH-SY5Y). Finally, the ratio reached 4.183 at day 3. These results indicate that DGKγ is transported to the nucleus depending on cultivation periods in all cell lines tested.

Mechanism for Nuclear Transportation of DGKγ—To analyze whether enzymatic activity is required for the nuclear transportation of DGKγ, several mutants were generated as shown in Fig. 2A, and correlation between their activities and nuclear transportation was investigated. Immunoblotting using anti-GFP antibody revealed that each mutant had appropriate molecular size (Fig. 2B) and that no significant degraded products were detected. Fig. 2C shows that GFP-DGKγ had kinase activity, whereas DGKγ fused GFP at the N terminus (DGKγ-GFP) had no activity as previously reported (28). The mutants having mutations in the C1A or C1B domains (GFP-DGKγ C1Am and C1Bm) and lacking the C1A or C1B domain (GFP-DGKγ ΔC1A and ΔC1B) showed lower but significant activity than wild-type GFP-DGKγ. Interestingly, mutation in the C1A domain rather than in the C1B domain affected the kinase activity. All the kinase-negative mutants, GFP-DGKγ KN, KNC1Am, and KNC1Bm, whose Gly-491 in the ATP binding site was replaced to Asp, showed no kinase activity.

Despite no kinase activities, both GFP-DGKγ KN and DGKγ-GFP were localized in the nucleus as well as the cytoplasm of CHO-K1 cells, but the transportation of GFP-DGKγ KN into the nucleus was slower than wild type (Fig. 3, GFP-DGKγ KN and GFP-DGKγ KN). On the other hand, GFP-DGKγ C1Am and C1Bm did not show the increase in the number of cells expressing the mutants equally in the cytoplasm and the nucleus, although they possessed significant kinase activity (Fig. 3, GFP-DGKγ C1Am and GFP-DGKγ C1Bm). Similarly, GFP-DGKγ ΔC1A, ΔC1B, KNC1Am, and KNC1Bm did not change their localization (Fig. 3, GFP-DGKγ ΔC1A, GFP-DGKγ ΔC1B, GFP-DGKγ KNC1Am, and GFP-DGKγ KNC1Bm). Table 1 summarizes the kinase activities of the mutants and their transportation to the nucleus. These results show that there is no significant correlation between the kinase activity and the nuclear transportation, suggesting that nuclear transportation of DGKγ does not require its kinase activity.

On the other hand, as seen in the cases of GFP-DGKγ C1Am, C1Bm, ΔC1A, and ΔC1B and GFP-DGKγ KNC1Am and KNC1Bm, mutation in the C1A or C1B domains eliminated the nuclear transportation of DGKγ, suggesting that the C1 domain is important for the nuclear transportation of DGKγ and contains a nuclear transport signal. To confirm the function of C1 domain as a nuclear transport signal and

| TABLE 1 | The activity of GFP-DGKγ, DGKγ -GFP, and mutants and their nuclear localization in CHO-K1 cells |
|----------------|-------------------------------------------------------------|
| The activity of DGKγ | Localization in the nucleus |
| GFP-DGKγ | ++ + + |
| GFP-DGKγ KN | - + |
| DGKγ-GFP | - - |
| GFP-DGKγ C1Am | ++ + |
| GFP-DGKγ C1Bm | ++ + |
| GFP-DGKγ ΔC1A | - + |
| GFP-DGKγ ΔC1B | ++ + + |
| GFP-DGKγ KNC1Am | - - |
| GFP-DGKγ KNC1Bm | - - |
identify which part of the C1 domain is important for the function, we generated six fragments from the C1 domain fused with FLAG; entire C1 domain, C1A domain plus Hinge, Hinge plus C1B domain, C1A domain, Hinge and C1B domain (Fig. 4A) and investigated their localization using FLAG antibody. The entire C1 domain was dominantly localized in the nucleus but the other fragments did not show nuclear localization (Fig. 4B). Similarly, GFP-tagged C1 domain was localized specifically in the nucleus, whereas GFP alone was seen throughout the cells (Fig. 4C). These results represent that the entire C1 domain is necessary to act as a nuclear localization signal at least in DGKγ.

FIGURE 4. Localization of the C1 domain and its fragments of DGKγ in CHO-K1 cells. A, schematic illustration of the C1 domain and its fragments used. C1 domain is from 269 to 380 aa, C1A domain plus Hinge is from 269 to 333 aa, Hinge plus C1B domain is from 319 to 380 aa, C1A domain is from 269 to 318 aa, Hinge is from 319 to 333 aa and C1B domain is from 334 to 380 aa of rat DGKγ. B, localization of FLAG-tagged C1 domain and its fragments in CHO-K1 cells. The transfected cells were cultured for 24 h and fixed. The FLAG fusion proteins were detected by mouse anti-FLAG antibody and Cy3-labeled anti-mouse IgG. C, localization of GFP-tagged C1 domain in CHO-K1 cells. Plasmids of GFP-C1 domain of DGKγ and GFP was transfected into CHO-K1 cells and fixed 24 h after the transfection. The fluorescence was detected by confocal microscopy. The micrographs are the images of GFP fluorescence, and the merged images represent GFP fluorescence and Nomarski images.
FIGURE 5. Differences in size of the CHO-K1 cells expressing GFP-DGKγ, GFP-DGKγ KN, GFP-DGKγ ΔC1B, and GFP-DGKγ KNC1Am. Diameters of CHO-K1 cells expressing GFP, GFP-DGKγ, and mutants were measured 72 h after the transfection. The number of cells of different diameter was counted (total number of each cell type was 100). In the case of GFP, GFP-DGKγ, and GFP-DGKγ KN, the cells expressing both in the cytoplasm and the nucleus were measured, whereas the cells expressing them only in the cytoplasm were selected in the case of GFP-DGKγ ΔC1B and GFP-DGKγ KNC1Am. Typical images of GFP, GFP-DGKγ, and mutants in CHO-K1 cells are also shown to represent their localization and cell size. Furthermore, typical cross-section images and average thickness are shown on the right side.
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TABLE 2

| Sizes of the stable cell lines of GFP, GFP-DGKγ, GFP-DGKγ KN, and GFP-DGKγ KNC1Am |
|-------------------------------------------------|
| The cells were spread onto glass bottom dishes. After being cultured for 72 h, the cells were fixed, and the size of each cell was measured using LSM510 software. Averages of sizes of the 100 cells are shown. In the case of GFP, GFP-DGKγ, and GFP-DGKγ KN, the cells expressed both in the cytoplasm and the nucleus were chosen. On the other hand, the cells expressed only in the cytoplasm were selected in the case of GFP-DGKγ KNC1Am. |

Average of cell size

|            | μm² |
|------------|-----|
| GFP        | 333.84 |
| GFP-DGKγ (localizes in cytoplasm and nucleus) | 391.67 |
| GFP-DGKγ KN (localizes in cytoplasm and nucleus) | 1332.5 |
| GFP-DGKγ KNC1Am (localizes in cytoplasm) | 412.67 |

Physiological Functions of Nuclear DGKγ—To investigate physiological function of DGKγ in the nucleus, we first measured the size of CHO-K1 cells transiently expressing GFP, GFP-DGKγ, or GFP-DGKγ KN, because we had noticed that the size of cells expressing DGKγ KN were bigger than that of cells expressing wild-type DGKγ. Apparently, the diameter of the typical CHO-K1 cells expressing GFP and GFP-DGKγ was 20–30 μm, whereas that of GFP-DGKγ KN was 30–40 μm (Fig. 5, left side), suggesting the importance of DGKγ activity in cell shape. However, it is not clear whether the difference in the cell size was due to the lack of DGKγ activity in the nucleus or in the cytoplasm because GFP-DGKγ KN expressed both in the cytoplasm and the nucleus. We, therefore, further examined the size of cells expressing DGKγ mutants only in the cytoplasm to examine an effect of cytoplasmic DGK activity on the cell size. We used the cells expressing GFP-DGKγ KNC1Am, because the mutants have no kinase activity and localizes only in the cytoplasm. On the other hand, the cells expressing GFP-DGKγ AC1B were used as the cells with cytoplasmic kinase activity (Figs. 2C and 3). No differences were found in the size of cells expressing GFP-DGKγ KNC1Am and GFP-DGKγ ΔC1B; the size of cells expressing GFP-DGKγ ΔC1B and KNC1Am were similar to that of GFP- or GFP-DGKγ-expressing cells (Fig. 5, left side). Furthermore, we measured the thickness of the CHO-K1 cells transiently expressing these proteins to investigate whether overexpression of DGKγ KN flattened the cells and/or resulted in increase in cell volume. As shown in Fig. 5, the cells expressing GFP-DGKγ KN were slightly flatter than those of the cells expressing other proteins. In addition, volume of the GFP-DGKγ KN cells was ~1.36-fold; the average of the diameter and thickness of GFP-DGKγ KN cells were 34.7 μm and 7.98 μm, whereas those of the other cells were 28.86 μm and 8.46 μm, respectively. These results indicated that the overexpression of DGKγ KN affected the cell shape and volume. To confirm this, we further made stable CHO-K1 cell lines of GFP, GFP-DGKγ, GFP-DGKγ KN, and GFP-DGKγ KNC1Am and compared the size of the cells. The stable cells expressing GFP-DGKγ KN in the nucleus and the cytoplasm were remarkably bigger than the others (Table 2, Importantly). The size of cells expressing GFP-DGKγ, and GFP-DGKγ KNC1Am were almost same. These results indicate that dominant negative effect on the nuclear DGKγ, but not cytoplasmic DGKγ, influences the size and volume of cells.

Next, to elucidate the mechanism of DGKγ KN-induced enlargement of cells, we compared proliferation of the stable cell lines (Fig. 6). Fig. 6 shows that the DGKγ KN cells stated the division more slowly than the others and that doubling time of GFP-DGKγ KN stable cells was 19.1 h, which was calculated from its regression line. On the other hand, those of GFP, GFP-DGKγ, and GFP-DGKγ KNC1Am cells were ~15.4, 15.8, and 15.8 h. These results indicated that the proliferation rate of GFP-DGKγ KN was slightly slower than others. To directly investigate inhibitory effect of nuclear DGKγ KN on the proliferation, we constructed CFP-DGKγ KN having nuclear localization signal (CFP-DGKγ KN-NLS) and CFP-NLS as control and compared their proliferation rate. The cell expressing CFP-DGKγ KN-NLS had a tendency to die, compared with control cells. Doubling time of the cells transiently expressing CFP-DGKγ KN-NLS was 17.36 ± 1.99 h (n = 25), whereas that of control cells was 15.58 ± 1.69 h (n = 50). These results demonstrated that nuclear GFP-DGKγ KN causes the disorder of cell cycle.

Therefore, we measured cell cycle of GFP-DGKγ KN by flow cytometry and compared it with control cell lines expressing GFP alone and GFP-DGKγ KNC1Am, the latter of which is inactive and cytostatic. At 24 h after the release from serum starvation, the profile of GFP-DGKγ KN stable cells was different from those of GFP and GFP-DGKγ KNC1Am stable cells, indicating that nuclear, but not cytosolic DGKγ KN affected the cell cycle. Then, we analyzed the cell cycle more precisely using GFP and GFP-DGKγ KN stable cells synchronized at the beginning of S phase by the double thymidine block method (32). Prior to the experiment, we determined proper concentration of thymidine for synchronizing the cells (supplemental Fig. S1). In the case of GFP-DGKγ KN stable cell lines, treatment with 2.5 mM thymidine resulted in partial G1 arrest, and 5 mM thymidine exerted a much clearer effect. For GFP stable cell lines, G1 arrest was not induced at 5 mM, and 10 mM was needed for complete G1 arrest. From these results, we decided to perform double thymidine block at 10 mM for GFP stable cells and 5 mM for GFP-DGKγ KN cells for cell-cycle analysis.

At 4 h after washing out thymidine, ~37% of the control cells transited to G2/M phase, whereas the cells expressing GFP-DGKγ KN in G2/M phase were only 17% (Fig. 7). ~70% of the control cells were in G2/M phase at 5 h, but it took 6–7 h for the population of GFP-DGKγ KN in G2/M phase to reach maximum. The GFP stable cells almost returned to G1 phase at 7 h, and the size of population of them in G1 phase reached 60% at 8 h, the biggest value through this experiment. On the other hand, 7 h after the release, ~51% of GFP-DGKγ KN stable cells still remained in G2/M phase, and only 14% cells were in G1 phase. Finally, a major population of GFP-DGKγ KN transited to G1 phase at 9 h; it was delayed by 2 h compared with the GFP stable cells. These results clearly reveal that the S phase of GFP-DGKγ KN was extended, suggesting that the extension causes enlargement of the cell. The disorder of cell cycle by nuclear expression of DGKγ KN suggested that alteration of the cell cycle affects the intracellular localization of DGKγ. Therefore, we observed the localization of GFP-DGKγ in the stable cells after serum starvation for 24 h. Under control conditions, the percentage of the stable cells expressing GFP-DGKγ both in the cytoplasm and the nucleus was 34% and that expressing only in the cytoplasm was 51% of all cells (Fig. 8A, control). The number of the cells expressing GFP-DGKγ both in the cytoplasm and the nucleus (type B) increased from 35% to 54% by serum starvation. In contrast, the number of the cells expressing the fusion protein only in the cytoplasm (type A) decreased from 51% to 34%. The ratio of type B to type A increased from 0.692 to 1.557. These results indicate that GFP-DGKγ is transported from the cytoplasm to the nucleus under serum-starved conditions.

However, these experiments were carried out under artificial conditions using exogenously expressed GFP-DGKγ. Therefore, to confirm nuclear transportation of endogenous DGKγ, we performed immunostaining using anti-DGKγ antibody. In many control cells, endogenous DGKγ was localized in the cytoplasm but not in the nucleus (Fig. 8B, control). After 24-h serum starvation, DGKγ localized in the nucleus as much as in the cytoplasm (Fig. 8, serum starvation), indicating that, as well as GFP-DGKγ, endogenous DGKγ can be transported to the nucleus, and the nuclear transportation is induced by serum starvation. These results, together with the inhibitory effect of DGKγ KN on cell cycle, strongly suggest an important physiological function of nuclear DGKγ in the cell cycle regulation.
FIGURE 6. Effect of nuclear expression of DGKγ KN on the proliferation. A, proliferation of the CHO-K1 cells stably expressing GFP, GFP-DGKγ, GFP-DGKγ KN, and GFP-DGKγ KN1Am. Each stable cell line was split into dishes at $0.2 \times 10^6$ cells/dish. Cells were harvested after 24-, 48-, and 72-h incubations and stained by 0.4% Trypan blue in PBS(−). Then the numbers of living cells were counted. Each point and vertical bar indicates the mean ± S.D. of six independent experiments. The regression coefficient of GFP-DGKγ KN was significantly different from those of the other three ($p < 0.001$). B, live imaging of cell division of HeLa cells expressing CFP-NLS and CFP-DGKγKN-NLS. The cell division of HeLa cells transiently expressing CFP-DGKγ KN-NLS and CFP-NLS were observed as described under “Experimental Procedures.” Typical images of CFP-DGKγKN-NLS and CFP-NLS cells are shown. Numbers on the right side show average of doubling time ($n = 25$ for CFP-DGKγKN-NLS, $n = 50$ for CFP-NLS), which is from the first division to the second one. *, difference between the average of doubling time was significant ($p < 0.001$).
DISCUSSION

In this report, we showed for the first time that DGKγ is transported from the cytoplasm to the nucleus (Fig. 1). The nuclear transportation of DGKγ was independent of kinase activity of DGKγ (Fig. 3 and Table 1), but the mutant lacking kinase activity (DGKγ KN) showed much slower nuclear transportation than that of wild type. The mutation eliminating kinase activity of GFP-DGKγ C1Am or GFP-DGKγ C1Bm made a remarkable decrease in the number of the cells expressing equally in the cytoplasm and the nucleus (GFP-DGKγ KN C1Am versus GFP-DGKγ C1Am or GFP-DGKγ KN C1Bm versus GFP-DGKγ C1Bm in Fig. 3). These results suggest that the kinase activity, although not essential, may have some roles in the nuclear transportation of DGKγ.

Instead of kinase activity, the C1 domain of DGKγ was essential for the nuclear transportation of DGKγ. Namely, none of the C1 domain mutants...
(GFP-DGK γC1Am and C1Bm or GFP-DGK γΔC1A and ΔC1B) showed transportation from the cytoplasm to the nucleus (Fig. 3 and Table 1). In addition, FLAG and GFP tagging the entire C1 domain were dominantly localized only in the nucleus, and the entire C1 domain was necessary for the nuclear localization (Fig. 4). These results suggest that, at least in DGK γ, the entire C1 domain acts as a nuclear localization signal.

However, the C1 domain of DGK γ doesn’t possess a well known NLS, and it is not clear how it acts as a nuclear transport signal. It has been already reported that C1 domains of PKC, DGK, and chimaerin can bind to some lipids (29, 33, 34). Thus, lipids including DG may be involved in the nuclear transportation of DGK γ. In fact, the mutations in the C1 domain, which are predicted to weaken or abolish their lipid binding based on the fact that corresponding mutations in the PKC C1 domain abrogate the phorbol 12,13-dibutyrate binding (35), inhibited the nuclear transportation. Alternatively, carrier proteins such as importin (36, 37) may participate in the nuclear transportation by associating with C1 domain, because the C1 domain of DGK can interact with some proteins (38). The identification of lipid(s) or protein(s) binding to DGK γC1 domain would be helpful to understand the mechanism of C1 domain as NLS. Furthermore, it is interesting to study whether other C1 domains in other DGKs and PKCs are important for nuclear localization or not.

In addition to the nuclear transportation mechanism, DGK γ seems to have export mechanism. Although the C1 domain of DGK γ was localized dominantly in the nucleus (Fig. 4, B and C), full-length DGK γ was expressed equally in the cytoplasm and the nucleus (Figs. 1 and 3). We could not find the cells that expressed DGK γ in the nucleus dominant over the cytoplasm. In addition, the photobleaching of cytoplasmic fluorescence of the cells expressing GFP-DGK γ caused a rapid decrease in the nuclear fluorescence within 5–10 min, and then the cytoplasmic fluorescence reached a level equal to that of the nucleus (data not shown). These results suggest that DGK has not only nuclear transport mechanism but also export mechanism and that the DGK level in the nucleus is maintained as much as that in the cytoplasm. Probably, the shuttling of DGK γ between the cytoplasm and the nucleus is regulated through the C1 domain and other region(s).

We also investigated physiological function of nuclear DGK γ by expressing kinase negative DGK γ in the nucleus, which is expected to inhibit the endogenous nuclear DGK γ by dominant-negative effects. The cells expressing GFP-DGK γ KN were larger in the size (Fig. 5 and Table 2), proliferated slowly (Fig. 6), and their S phase was extended (Fig. 7), suggesting that enlargement of cell size is induced by protein synthesis during extended S phase. In addition, serum starvation induced nuclear transportation of endogenous DGK γ and GFP-DGK γ stably expressing in the CHO-K1 cells (Fig. 8). These results strongly implicate that DGK γ in the nucleus is involved in regulation of cell cycle.

Involvement of DGK in cell-cycle regulation is supported by several reports as follows. First, inhibition of DGK activity prevents transition from G1 to S phase (39). Second, the involvement of other DGK subtypes such as DGK ɛ in the cell cycle has also been reported, although there are some differences; COS-7 cells expressing DGK ɛ showed the increase in the size of G1 cell population and decrease in that of G2/M cell population but no effects of DGK ɛ KN on the cell cycle were detected (21). The differences may be due to subtype specificity and/or different cell types used.

Both DGK γ and DGK ɛ are abundantly expressed in the brain (40), although it is unclear about their functions as a regulator of the cell cycle in the central nervous system. However, expression of DGK γ has been recently reported in HL-60, U937, and NIH3T3 cells (41–43). We also confirmed the expression of DGK γ in various tissues, including kidney, muscle, and other tissues, in addition to brain (supplemental Fig. S2). Therefore, at least in these cells and tissues, it is expected that nuclear DGK γ is involved in cell-cycle regulation. In fact, regulation of differentiation by DGK γ in HL-60 cells has been suggested (41, 43).

However, it still remains to be solved how DGK can regulate the cell cycle. The amount of nuclear phosphatidylinositol, including phosphatidylinositol 4,5-bisphosphate, fluctuates during the cell cycle (22), and the DG mass in the nucleus increases in G2/M phase (27). Furthermore, βIIIPKC translocates into the nucleus and phosphorylates lamin B.
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in the G2/M phase (44). These reports suggest that the DG derived from phosphatidylinositol 4,5-bisphosphate recruits PKC to regulate G1/M phase. In other words, regulation of nuclear DG mass is important for cell-cycle regulation, and DGK might be involved in the control of nuclear DG. Alternatively, PA produced by DGK may have some function in cell-cycle regulation. In fact, the amount of PA in the nucleus also changes by the stimulation of α-thrombin, which has a mitogenic effect (45). Accordingly, the mechanism of cell-cycle regulation by DGKγ is an important issue to be solved next.

In conclusion, the present results indicate that DGKγ is transported from the cytoplasm to the nucleus via its C1 domain and suggest that DGKγ is involved in cell-cycle regulation.

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