ARTICLE
Journal of Cellular Biochemistry 105:756–765 (2008)

Journal of Cellular Biochemistry

Diacylglycerol Kinase ζ Is Associated With Chromatin, but Dissociates From Condensed Chromatin During Mitotic Phase in NIH3T3 Cells

Hiroshi Hasegawa,[1,2] Tomoyuki Nakano,[1] Yasukazu Hozumi,[1] Michiaki Takagi,[2] Toshihiko Ogino,[2] Masashi Okada,[1] Ken Iseki,[3] Hisatake Kondo,[4] Masahiko Watanabe,[5] Alberto M. Martelli,[6] and Kaoru Goto[1*]

[1]*Department of Anatomy and Cell Biology, Yamagata University School of Medicine, Yamagata 990-9585, Japan*
[2]*Department of Orthopedics, Yamagata University School of Medicine, Yamagata 990-9585, Japan*
[3]*Department of Emergency Medicine, Yamagata University School of Medicine, Yamagata 990-9585, Japan*
[4]*Division of Histology, Department of Cell Biology, Tohoku University Graduate School of Medicine, Sendai 980-8575, Japan*
[5]*Department of Anatomy, Hokkaido University School of Medicine, Sapporo 060-8638, Japan*
[6]*Department of Human Anatomical Sciences, University of Bologna, 40126 Bologna, Italy*

ABSTRACT

Diacylglycerol kinase (DGK) converts diacylglycerol (DG) to phosphatidic acid, both of which act as second messengers to mediate a variety of cellular mechanisms. Therefore, DGK contributes to the regulation of these messengers in cellular signal transduction. Of DGK isozymes cloned, DGKζ is characterized by a nuclear localization signal that overlaps with a sequence similar to the myristoylated alanine-rich C-kinase substrate. Previous studies showed that nuclear DG is differentially regulated from plasma membrane DG and that the nuclear DG levels fluctuate in correlation with cell cycle progression, suggesting the importance of nuclear DG in cell cycle control. In this connection, DGKζ has been shown to localize to the nucleus in fully differentiated cells, such as neurons and lung cells, although it remains elusive how DGK behaves during the cell cycle in proliferating cells. Here we demonstrate that DGKζ localizes to the nucleus during interphase including G1, S, and G2 phases and is associated with chromatin although it dissociates from condensed chromatin during mitotic phase in NIH3T3 cells. Furthermore, this localization pattern is also observed in proliferating spermatogonia in the testis. These results suggest a reversible association of DGKζ with histone or its related proteins in cell cycle, plausibly dependent on their post-translational modifications. J. Cell. Biochem. 105: 756–765, 2008. © 2008 Wiley-Liss, Inc.

KEY WORDS: DIACYLGLYCEROL KINASE; CELL CYCLE; MITOTIC PHASE; CHROMATIN; SUBCELLULAR LOCALIZATION; PROTEIN KINASE C

Diacylglcerol kinase (DGK) phosphorylates diacylglycerol (DG) to produce phosphatidic acid (PA). DG acts as a key second messenger that mediates not only transmission of hormones and neurotransmitters, but also other cellular mechanisms, such as cell growth, differentiation, apoptosis, cytoskeletal organization, and cellular motility [Martelli et al., 2004]. In this regard, previous studies have shown that DG can be produced preferentially in various subcellular compartments including the plasma membrane, internal membranes, and nucleus [Nishizuka, 1992; Banfic et al., 1993; Martelli et al., 1995]. These studies clearly show that the second messenger DG may be generated locally in response to external stimuli or cellular conditions in order to operate different signal transduction pathways. DGK is known to consist of a family of isozymes that differ in terms of structural motifs, enzymological property, and tissue distribution [Topham, 2006; Goto et al., 2007; Sakane et al., 2007; Evangelisti et al., 2007a]. A recent study has

Hiroshi Hasegawa and Tomoyuki Nakano are contributed equally to this work.

Grant sponsor: The Ministry of Education, Science, Culture, Sports, Science and Technology (MEXT) of Japan; Grant number: 18590179; Grant sponsor: Taisho Pharmaceutical; Grant sponsor: CARISBO FOUNDATION; Grant sponsor: Progetti Strategici Università di Bologna EF2006.

*Correspondence to: Prof. Kaoru Goto, MD, PhD, Department of Anatomy and Cell Biology, Yamagata University School of Medicine, Iida-nishi 2-2-2, Yamagata 990-9585, Japan. E-mail: kgoto@med.id.yamagata-u.ac.jp

Received 15 April 2008; Accepted 1 July 2008 • DOI 10.1002/jcb.21873 • 2008 Wiley-Liss, Inc.
Published online 4 August 2008 in Wiley InterScience (www.interscience.wiley.com).

revealed that those isozymes are targeted differentially to subcellular sites including cytoskeleton, endoplasmic reticulum, Golgi complex, and nucleus, suggesting that each plays a unique role at distinct subcellular site [Kobayashi et al., 2007]. Of DGKs, DGKζ is characterized by ankyrin-repeats at the C-terminus and a nuclear localization signal (NLS) that overlaps with a sequence similar to the myristoylated alanine-rich C-kinase substrate (MARCKS) [Bunting et al., 1996; Goto and Kondo, 1996]. Previous studies have clearly shown that DGKζ is localized to the nucleus of several types of fully differentiated cells, including neurons in the brain [Hozumi et al., 2003] and dorsal root ganglion [Sasaki et al., 2006], and alveolar epithelial cells and macrophages in the lung [Katagiri et al., 2005]. Nuclear localization of DGKζ is also demonstrated in cultured cell lines and transfected cells [Goto and Kondo, 1996; Topham et al., 1998; Evangelisti et al., 2006; Kobayashi et al., 2007; Evangelisti et al., 2007a]. In this regard, MARCKS can be phosphorylated by conventional protein kinase C (PKC) α and γ isoforms, and it is suggested that the phosphorylation facilitates DGKζ export from the nucleus in transfected cells [Topham et al., 1998]. A previous study reported that nuclear DG levels fluctuate during the cell cycle and that changes in nuclear DG levels correlate with cell cycle progression through the G2/M phase [Sun et al., 1997]. With regard to the regulatory mechanisms for DG, that is, production of DG by phosphoinositol-specific phospholipase C (PI-PLC) and its attenuation by DGK, it is reported that a nuclear PI-PLC activity is activated during the G2 phase and that PI-PLC inhibitors lead to decreased nuclear PI-PLC activity and cell cycle blockade at the G2 phase [Sun et al., 1997]. DGKζ binds the retinoblastoma protein (pRB), a tumor suppressor and key regulator of the cell cycle, which depends on the phosphorylation status of pRB [Los et al., 2006]. Furthermore, overexpression of a wild-type DGKζ blocks the cell cycle at the G1 phase [Topham et al., 1998], which is accompanied by decreased levels of pRB phosphorylation on Ser-807/811 [Evangelisti et al., 2007b]. More importantly, it is shown that endogenous DGKζ acts as a repressor of DNA replication, as its down-regulation results in a higher percentage of cells being in both the S and G2/M phases of the cell cycle [Evangelisti et al., 2006, 2007b]. These results demonstrate that nuclear DGKζ is a key determinant of cell cycle progression and differentiation of C2C12 cells. However, it remains to be known how DGK behaves and changes its subcellular localization during the cell cycle in proliferating cells. To gain an insight into functional implications of DGKζ in cell cycle control, we investigated detailed subcellular localization of DGKζ at various phases of cell cycle in nontransformed mammalian cells. Here we show that DGKζ localizes to the nucleus during interphase including G1, S, and G2 phases and is associated with chromatin while it dissociates from condensed chromatin during mitotic phase. Furthermore, this localization pattern is also observed in proliferating spermatogonia in the testis. In addition, patterns of the subcellular localization of PKCs during the cell cycle are compared with that of DGKζ.

MATERIALS AND METHODS

CELL CULTURE AND ANIMALS

NIH3T3 cells were grown in Dulbecco's modified Eagle's medium containing 25 mM glucose, 10% fetal bovine albumin, 100 U/ml penicillin, and 100 μg/ml streptomycin sulfate at 37°C and 5% CO_2. Wistar rats and C57BL/6 mice at 2–3 months of age were used throughout the experiments. They were treated in accordance with Guide for Animal Experimentation, Yamagata University School of Medicine.

RT-PCR ANALYSIS

Total RNAs were extracted from NIH3T3 cell lysate and whole mouse brain (positive control) by acid guanidinium thiocyanate/phenol/chloroform extraction (Trizol; Gibco BRL, Bethesda, MD). First-strand cDNA was synthesized from 2 μg of RNA using M-MLV reverse transcriptase (Promega, Madison, MI) following the manufacturer's instructions. Polymerase chain reaction (PCR) amplification was performed with KOD-plus-Taq polymerase (Toyobo, Tokyo, Japan) using gene specific oligonucleotide primers for mouse DGK isozymes and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as in Table I. PCR conditions were as follows: 95°C for 1 min; 30 cycles of 94°C for 30 s, 62°C for 30 s, and 68°C for 40 s; and 68°C for 2 min. PCR products amplified were separated by agarose gel electrophoresis and stained with ethidium bromide.

ANTIBODY PRODUCTION

Anti-PKCβII antibody was generated against an isoform-specific sequence encoding the C-terminus of mouse PKCβII (amino acid residues 664–673, Accession No. CAA37611) as described previously [Nomura et al., 2007]. The region was amplified by PCR using single-stranded cDNA library prepared from the adult mouse brain, subcloned into BamHI/EcoRI site of the pGEX4T-2 plasmid vector (Amersham Biosciences, Bucks, UK), and expressed as glutathione S-transferase (GST) fusion protein. The fusion protein was emulsified with Freund's complete adjuvant in the first immunization and incomplete adjuvant in the subsequent immunization (DIFCO, Detroit, MI), and injected subcutaneously into female rabbit at intervals of 2 weeks. Two weeks after the sixth injection, PKCβII-specific antibody was collected by affinity purification using GST-free polypeptides coupled to CNBr-activated Sepharose 4B (Amersham Biosciences). GST-free peptides were

TABLE I. Primers Used for Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Primer	Sequence (5′–3′)	Product (bp)
DGKα	FW: GTGACTGTGGGTTGCTCCGTG	401
DGKα	RV: ACAGCAACGGGAGGCACAGTG	
DGKβ	FW: GGACAGCATGTGTGGCGACTC	428
DGKβ	RV: GTTCCGGCAGTGGGCATAGTC	
DGKγ	FW: GTGGGATCCCACAGAGCTCAG	394
DGKγ	RV: GACGGAGGAGTTCCCTTCCAC	
DGKδ	FW: GACCAGCAGCTCAGGAAGCTG	398
DGKδ	RV: CTCCGGCTCAGCTCCTTGATC	
DGKε	FW: GCCACTTGGTCCTATGGACGC	435
DGKε	RV: CGTCGTGGACTGTCTTCTGGC	
DGKζ	FW: CTGCCCCAAGGTGAAGAGCTG	398
DGKζ	RV: GCTGTCTCCTGGTCCTCACGT	
DGKθ	FW: GTCCTGGAGCTTGGTGATGGG	413
DGKθ	RV: ACCTTGAGCCAGCCAGGGTAG	
GAPDH	FW: TTAGCACCCCTGGCCAAGG	541
GAPDH	RV: CCTACTCCTTGGAGGCCATG	

Forward (FW) and reverse (RV) primers for each diacylglycerol kinase (DGK) isozyme and GAPDH are listed together with the estimated product length (bp).

prepared by in-column thrombin digestion of GST fusion proteins bound to glutathione-Sepharose 4B media. Immunoblot analysis with the antibody detected a single band of the deduced molecular mass of PKCβII (~77 kDa).

IMMUNOBLOT

Rats and mice were anesthetized with pentobarbital (10 mg/kg body weight) and sacrificed by decapitation. Brains of rat and mouse were immediately removed and homogenized using a Potter homogenizer in a buffer containing 20 mM Tris–HCl (pH 7.4), 0.25 M sucrose, 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride (PMSF), and an appropriate amount of protease inhibitor cocktail (Sigma–Aldrich, St. Louis, MO). Cultured cells were harvested and lysed by sonication in the buffer. Homogenates were centrifuged for 10 min at 3,000*g* to remove debris, and the supernatant was used as a sample. In some cases, nuclear fraction was isolated from NIH3T3 cells as described previously with some modifications [Tabellini et al., 2003]. Briefly, cultured cells were washed with PBS and resuspended in 10 mM Tris–HCl (pH 7.4), 2 mM $MgCl_2$, 10 mM NaCl, 1mM PMSF, and protease inhibitor cocktail (Sigma–Aldrich). They were incubated on ice for 20 min, then 1% Triton X-100 was added. The cells were allowed to swell for 10 min, and sheared by 5 passages through a 26-gauge needle. Nuclei were recovered by centrifugation at 600*g* for 5 min, washed once in 10 mM Tris–HCl (pH 7.4), 5 mM $MgCl_2$, and protease inhibitor cocktail, and their purity was confirmed by phase-contrast microscope. Protein concentration was determined using BCA Protein Assay Reagent (Pierce Biotechnology, Rockford, IL). Proteins were separated on 10% sodium dodecyl sulphate (SDS)–polyacrylamide gel by electrophoresis, and the separated proteins were electrophoretically transferred onto polyvinylidene difluoride (PVDF) filters (Millipore Corp., Bedford, MA). Nonspecific binding sites on the PVDF filters were blocked by incubation with 5% skim milk for 12 h. The PVDF filters were then incubated with rabbit anti-DGKζ antibody (0.1 μg/ml) [Hozumi et al., 2003], rabbit anti-α-tubulin antibody (0.2 μg/ml, a cytoplasmic marker; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-PKCα antibody (1.0 μg/ml) [Nakano et al., 2006], and rabbit anti-PKCβII antibody (1.0 μg/ml) for 30 min at room temperature (RT). After washing with PBS containing 0.05% Triton X-100, the filters were incubated with goat anti-rabbit IgG-peroxidase (dilution 1:10,000) for 30 min. After three washes, immunoreactive bands were visualized with a chemiluminescence detection kit (ECL; Amersham Pharmacia Biotech, Buckinghamshire, UK).

CELL CYCLE ANALYSIS

The exact position of the analyzed cells during the cell cycle was assessed by combined analysis of synchronization by double thymidine block, DNA staining by propidium iodide (PI, 15 μg/ml, Sigma), 5′-bromo-2′-deoxyuridine (BrdU) incorporation, and phosphorylation of histone H3. First, to obtain a population of cells in G2 phase, NIH3T3 cells were arrested by double thymidine block according to the previous report with some modifications [Whitfield et al., 2000]. Cells were blocked for 17 h with 2 mM thymidine, released by washing out the thymidine, and then blocked again with 2 mM thymidine for 17 h to arrest all the cells at the beginning of S phase. The cells were released from the block by washing out the thymidine and fixed every 2 h with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 15 min for immunocytochemical staining. Cells in S phase were assessed by labeling cells with BrdU using the In Situ Cell Proliferation Kit (Roche Diagnostics, Basel, Switzerland) as described previously with some modifications [Citterio et al., 2004]. Briefly, cells were incubated with 10 μM BrdU for 10 min and fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 15 min. After permeabilization with 0.3% Triton X-100 in PBS for 15 min, cells were treated with 1 N HCl for 1 h at 37°C, followed by neutralization with 0.1 M boric acid and subjected to immunocytochemical detection of the incorporated BrdU.

IMMUNOHISTOCHEMISTRY AND IMMUNOCYTOCHEMISTRY

Rats were anesthetized with ether and fixed with a transcardiac infusion of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The testis was removed, immersed in the same solution for further 2 h at 4°C, and kept in 30% sucrose in 0.1 M phosphate buffer (pH 7.0) until use. The tissue blocks were cut into 10 μm sections on a cryostat, placed on glass slides, and air-dried briefly. The sections were treated with 0.3% Triton X-100 in PBS for 15 min. Cultured cells were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 15 min and then permeabilized with 0.1% Triton X-100 in PBS for 5 min. Sections or cells were then treated with 5% normal goat serum in PBS (NGS/PBS) to block nonspecific binding sites and incubated with rabbit anti-DGKζ antibody (0.1 μg/ml) [Hozumi et al., 2003], rabbit anti-acetyl-histone H3 antibody (0.5 μg/ml; Upstate, Lake Placid, NY), mouse anti-phospho-histone H3 (Ser10) antibody (dilution 1:100; Upstate), rabbit anti-PKCα antibody (1.0 μg/ml) [Nakano et al., 2006], and rabbit anti-PKCβII antibody (1.0 μg/ml) overnight at 4°C in a moist chamber. Then, they were incubated with goat anti-rabbit IgG-Alexa 488 or goat anti-mouse IgG-Alexa 546 (dilution 1:400; Molecular Probes, Eugene, OR) in NGS/PBS for 30 min at RT. In some cases, sections or cells were also treated with PI to stain DNA, which gives red fluorescence similar to Rhodamine [Ali et al., 2004]. They were examined by fluorescent microscope or confocal laser-scanning microscope (LSM5 PASCAL; Carl Zeiss, Jena, Germany) at 543 nm helium excitation and 488 nm argon excitation, and fluorescent images were processed using Adobe Photoshop.

ChIP

Chromatin immunoprecipitation (ChIP) assays were performed essentially according to the protocol from Upstate Biotechnology with some modifications. Approximately 1×10^6 NIH3T3 cells in 10 cm dish were treated with 1% formaldehyde in PBS for 10 min at RT with gentle agitation. The cells were rinsed twice with PBS and scraped in ice-cold PBS containing 1 mM PMSF and protease inhibitor cocktail. After centrifugation at 3,000 rpm for 5 min at 4°C, the cells were resuspended in 200 μl of SDS lysis buffer containing 50 mM Tris–HCl (pH 8.0), 10 mM EDTA, 1% SDS, 1 mM PMSF, and protease inhibitor cocktail by vigorous pipetting and incubated for 10 min on ice. The lysates were sonicated to break chromatin into fragments with an average length of 0.5–1.0 kb and centrifuged at 15,000 rpm for 10 min at 4°C and the supernatant was used as a

sample for ChIP assays using anti-acetyl-histone H4 antibody (ChIP grade 06-866; Usptate Biotechnology).

RESULTS

We first performed RT-PCR analysis to examine the expression profile of the DGK isozymes in NIH3T3 cells, a mouse fibroblast cell line, using mouse brain as a control (Fig. 1A). Using the designed sets of primer (Table I), all the isozymes were successfully amplified with appropriate lengths in mouse brain where all of the isozymes examined were known to be expressed. In NIH3T3 cells, the expression signals were detected intensely for DGKζ, moderately for DGKδ and weakly for DGKα and -ε, while the signals for DGKθ were faint. The signals for DGKβ and -γ were below the detection levels. This showed that DGKζ is the major isozyme in NIH3T3 cells.

In immunoblot analysis, the antibody for DGKζ detected in total proteins of rat and mouse brains and NIH3T3 cells a major protein band of relative molecular mass of ~105 kDa, showing that the antibody solely recognizes DGKζ among the isozymes in both rat and mouse tissues and cells (Fig. 1B). However, a slight difference was observed between the immunoreactive band for rat brain and that of mouse brain and NIH3T3 cells, which might be attributed to different post-translational modifications between the animal species. In the nuclear fraction, the immunoreactive band was detected at the same molecular weight, confirming nuclear localization of DGKζ in NIH3T3 cells (Fig. 1C).

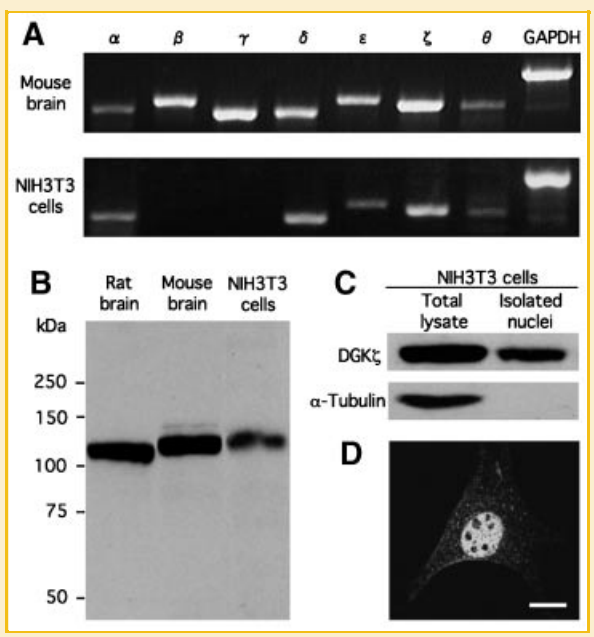

Fig. 1. Expression of DGKζ. In RT-PCR analysis of NIH3T3 cells and mouse brain, bands for DGK isozymes were amplified using specific primer for each isozyme listed in Table I and electrophoresed. GAPDH primer was also used as a control (A). Total proteins (25 μg) from rat brain, mouse brain, and NIH3T3 cells were analyzed by immunoblot using anti-DGKζ antibody (B). Molecular weight markers are indicated on the left in kDa. Nuclear fraction (10 μg) from NIH3T3 cell homogenate was also analyzed together with total protein (25 μg) using anti-DGKζ antibody and anti-α-tubulin antibody (cytoplasmic marker) (C). Immunocytochemical analysis of NIH3T3 cells (D). After fixation with 4% paraformaldehyde, NIH3T3 cells were immunolabeled with anti-DGKζ antibody followed by anti-rabbit IgG-Alexa 488 antibody and examined by fluorescent microscope. DGKζ-immunoreactivity appears in a speckled pattern in the nucleus, and nucleoli are negative. Bar = 10 μm.

In immunocytochemical analysis of asynchronized cells, presumably in interphase, DGKζ was mostly shown to be localized to the nucleus. The immunoreactivity was not homogeneous but appeared in a speckled pattern in the nucleus, and nucleoli were negative (Fig. 1D). This pattern of DGKζ-immunoreactivity was similar to that observed in neurons that do not proliferate after birth [Hozumi et al., 2003]. No labeling was observed when the primary antibody was omitted or absorbed with the antigen (data not shown).

We next examined changes in the subcellular localization of DGKζ during the cell cycle (Fig. 2A). For this purpose NIH3T3 cells were synchronized at the G1-S boundary by double thymidine-block protocol [Whitfield et al., 2000] and released into medium to allow synchronous progression through S phase, G2 phase, mitosis, and the subsequent G1 phase. We categorized the pattern of DNA staining of synchronized cells using PI in mitotic phase. During interphase DGKζ was essentially localized to the nucleus in a speckled pattern. During early prophase, coincident with visible chromatin condensation, DGKζ remained nuclear. However, at closer examination it was revealed that DGKζ was clearly segregated from condensed chromatin. During promethaphase, as nuclear envelope broke down, DGKζ was diffused in the cytoplasm. As the duplicated chromosomes moved apart in anaphase, DGKζ remained segregated from condensed chromatin, with a more intense staining around the area proximal to the chromosomes. Once anaphase was completed and the two daughter cells began to reform a nuclear envelope, DGKζ again became clearly localized to the nucleus.

The nuclear DNA of eukaryotic cells is complexed with histone proteins to form the nucleoprotein complex termed chromatin [Wolffe, 1999]. The N-terminal tails of each of the core histones is subject to several types of covalent modifications, including acetylation and methylation of lysine residue, and phosphorylation of serine residue [Downs et al., 2007]. It has been suggested that these modifications on histones can provide an epigenetic language, that is, histone code that modulates the genetic information transmitted in DNA [Strahl and Allis, 2000; Jenuwein and Allis, 2001]. For example, acetylation of specific lysine residues in the amino termini of the core histones plays a fundamental role in transcriptional regulation. In most species, histone H3 is primarily acetylated at lysines 9, 14, 18, and 23. Therefore, staining with antibody against acetyl-histone H3, a good marker for both dispersed and condensed chromatin, produced an image compatible with PI-stained DNA (data not shown). On the other hand, phosphorylation of histone H3 on Ser10 is a specific marker for condensed chromatin during mitotic phase [Hendzel et al., 1997]. Consistent with this, phospho-histone H3 staining was not detected in the nucleus in interphase but was observed solely onto condensed chromatin during mitotic phase (Fig. 2B). It should be noted that DGKζ was clearly segregated from phospho-histone H3-positive condensed chromatin. During mitotic phase, condensed chromatin and DGKζ were never overlapped with each other.

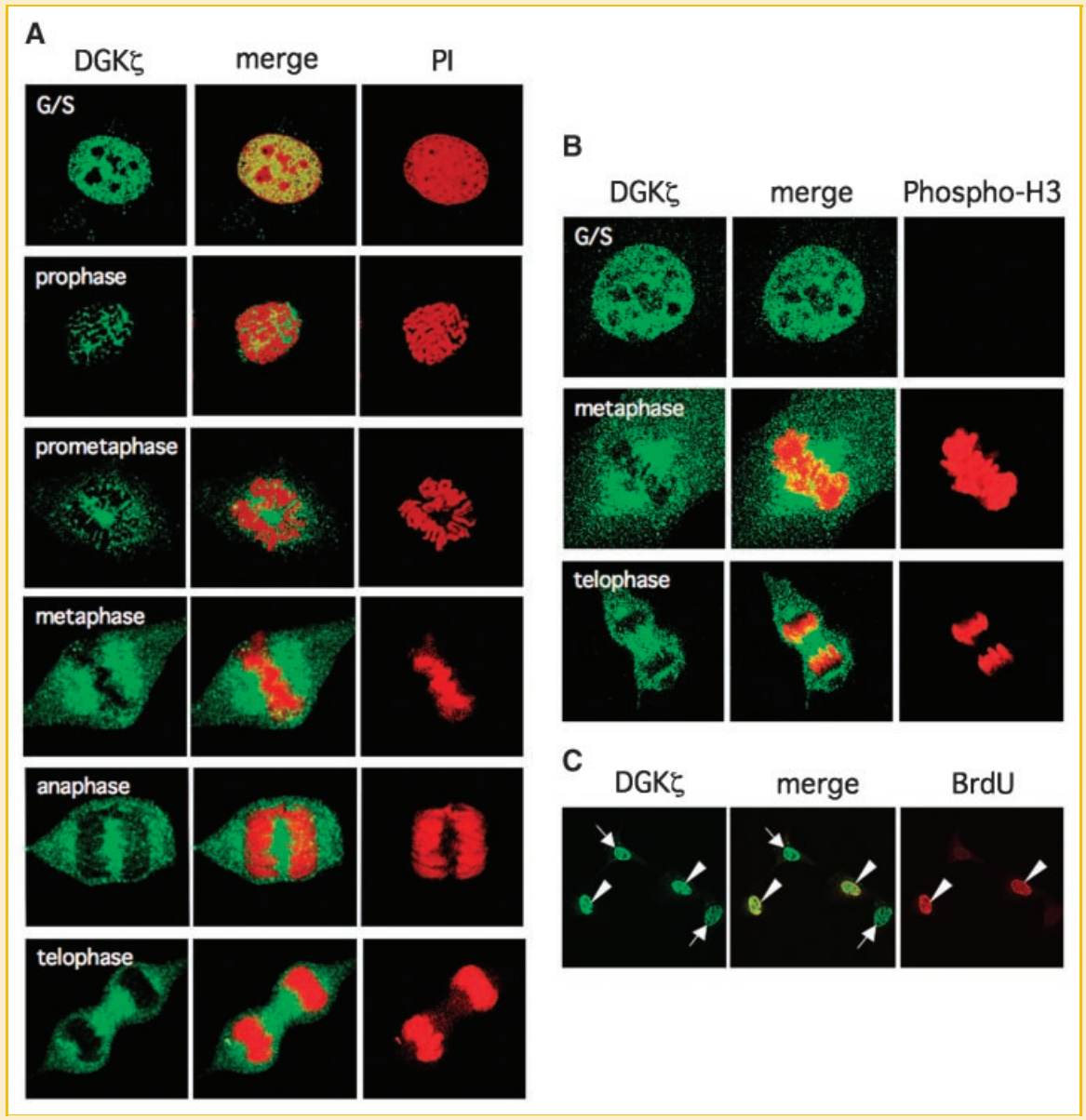

Fig. 2. Subcellular localization of DGKζ during the cell cycle in NIH3T3 cells. The position of the analyzed cells during mitotic phase was assessed by combined analysis of synchronization by double thymidine block, DNA staining with PI, phosphorylation of histone H3, and BrdU incorporation. After fixation with 4% paraformaldehyde, NIH3T3 cells were immunolabeled with anti-DGKζ antibody (green), where DNA was stained by PI (red, in A) or condensed chromatin was stained by anti-phospho-histone H3 (red, in B). To identify cells in S phase, NIH3T3 cells were incubated with 10 μM BrdU for 10 min. After fixation, incorporated BrdU was detected using anti-BrdU antibody as described in Materials and Methods Section (C). Fluorescent labeling was examined by confocal laser-scanning microscope. During interphase including G/S phase, DGKζ is essentially localized to the nucleus in a speckled pattern as shown in Figure 1, while it is segregated from condensed chromatin during mitotic phase (A). More specifically, DGKζ never overlapped with condensed chromatin labeled by anti-phospho-histone H3 antibody that does not stain dispersed chromatin during interphase (B). DGKζ is localized in the nucleus of both BrdU-positive (in S phase) and BrdU-negative (in G1 or G2 phase) cells in a similar fashion with each other, and no significant differences in the expression and localization are observed (C).

As described above, DGKζ was shown to colocalize with dispersed chromatin region during interphase but dissociate from condensed chromatin during mitotic phase. However, it remained undetermined whether the subcellular localization of DGKζ would change during S phase. For this purpose, we used BrdU to label cells in S phase and performed double immunostaining for DGKζ and BrdU (Fig. 2C). After incubation of cells in the presence of BrdU for 10 min, 10–20% of cells were labeled by BrdU, which are considered as S phase cells, whereas almost all of the cells showed immunoreactivity for DGKζ. When we compared the staining pattern of DGKζ between BrdU-positive (in S phase) and BrdU-negative (in G1 or G2 phase) cells, no significant difference was observed. This suggests that DGKζ displays no changes in its expression and subcellular distribution during S phase. We also performed the experiments described above using different cultured cell lines including Swiss 3T3 and HepG2 cells and obtained essentially similar data (data not

shown), suggesting that this pattern of subcellular localization of DGKζ during the cell cycle is a feature common to most, if not all, proliferating cells.

We further examined whether the characteristic pattern of subcellular localization of DGKζ is observed during the cell cycle in native cells proliferating in organisms. Therefore, we performed immunohistochemical investigation of testis in which sperms are continuously generated. As shown in Figure 3, DGKζ immunoreactivity was observed in the nucleus of spermatogonia during interphase as in NIH3T3 cells, demonstrating nuclear localization of DGKζ in proliferating cells in situ. Furthermore, it was also observed that DGKζ was segregated from condensed chromatin in spermatogonia during mitotic phase, as seen in proliferating cultured cells. However, it is noteworthy that DGKζ was not detected anymore in spermatocytes containing tightly packed, transcriptionally inactive chromatin. This suggests that DGKζ expression is down-regulated in transcriptionally inactive cells.

Morphological analysis showed that DGKζ staining pattern closely resembled chromatin pattern in interphase. This raised a question whether DGKζ might be physically associated with chromatin. To examine this possibility we performed chromatin immunoprecipitation (ChIP) assays on NIH3T3 cells. As DGKζ staining was segregated from condensed chromatin during mitotic phase, it was suggested that DGKζ might reversibly bind to chromatin depending on the modification of histones or histone-binding proteins. Therefore, to detect weak or reversible association with chromatin we briefly crosslinked chromatin using 1% formaldehyde and processed samples for ChIP assays as described in detail in Materials and Methods Section. As shown in Figure 4, ChIP assays demonstrated that the immunoprecipitate by acetyl histone H4 antibody contained DGKζ, suggesting physical association of DGKζ with chromatin. Considering the morphological data together, it is suggested that DGKζ is bound to dispersed chromatin during interphase and dissociates from condensed chromatin during mitotic phase.

Previous studies have suggested that the subcellular localization of DGKζ might be controlled by phosphorylation by PKCα and βII [Topham et al., 1998; Luo et al., 2003]. Therefore, we next examined how PKCs behave during the cell cycle. Immunoblot analysis using the specific antibodies confirmed the expression of PKCα and βII in NIH3T3 cells, although the expression level of α was much higher than that of PKCβII (Fig. 5D). Following the same protocol as for the experiments on DGKζ, we synchronized NIH3T3 cells at the G1-S boundary by double thymidine-block and then monitored the immunocytochemical distribution of PKCα and βII. As shown in Figure 5A, during interphase PKCα was essentially localized to the cytoplasm. During prophase, as chromatin was visibly condensed, PKCα was also detected in the region between condensed chromatin. At this time point we could not discern whether the nuclear envelope was intact or broken down, that is, whether PKCα was translocated from the cytoplasm through the nuclear pore to the nucleus or rather diffused to the region between condensed chromatin. During metaphase and thereafter, PKCα remained diffusely dispersed in the cytoplasm and segregated from condensed chromatin.

On the other hand, PKCβII was essentially localized to the nucleus and was observed as scattered coarse dots during interphase (Fig. 5B). During prophase, coarse dotted pattern of PKCβII was mainly distributed in the region between condensed chromatin in

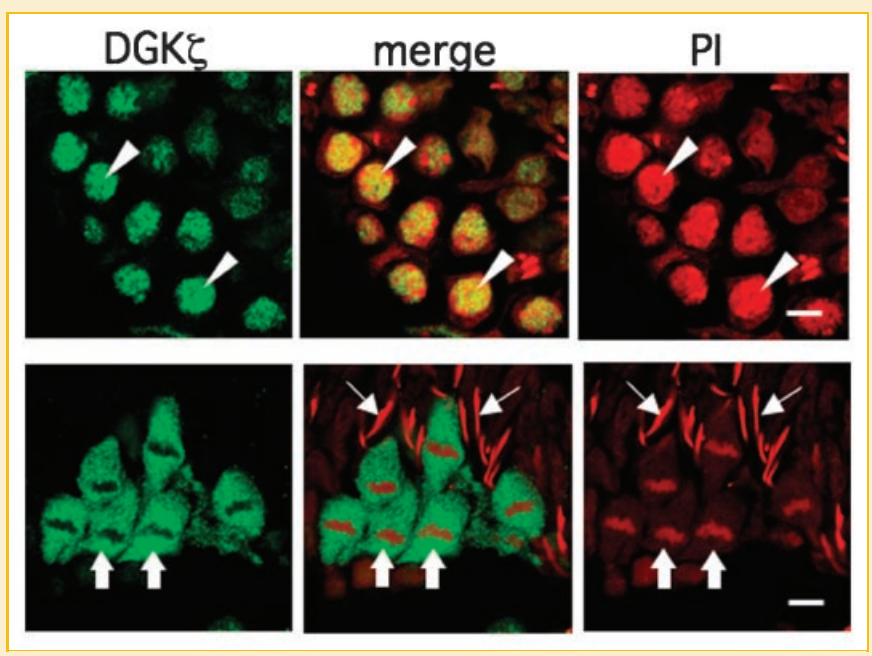

Fig. 3. Immunohistochemical analysis for DGKζ in the testis. Sections from rat testis were immunolabeled by anti-DGKζ antibody (green). DNA was visualized by staining with PI (red). DGKζ is observed in the nucleus of immature spermatogonia (arrow heads) during interphase while it is segregated from condensed chromatin during mitotic phase (thick arrows). Note that DGKζ is not detected anymore in spermatocytes containing tightly packed chromatin (thin arrows). Bar = 10 μm.

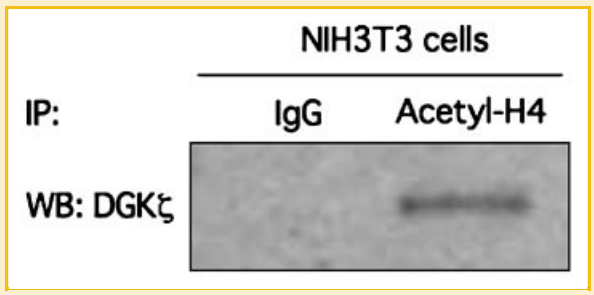

Fig. 4. Chromatin immunoprecipitation (ChIP) for DGKζ. Approximately 1×10^6 NIH3T3 cells were treated with 1% formaldehyde, and the chromatin fraction was isolated and sonicated to be broken into fragments. ChIP assays were performed using anti-acetyl-histone H4 antibody (ChIP grade) and unimmunized IgG as a control according to the protocol from Upstate Biotechnology. Each sample loaded represents 5% of total.

the nucleus. During metaphase and thereafter, PKCβII was rather dispersed throughout the cytoplasm and segregated from condensed chromatin similarly to PKCα. Taken together, it is concluded that the subcellular localization pattern of DGKζ is different from those of PKCα and βII although during mitotic phase all of them are dispersed throughout the cytoplasm and segregated from condensed chromatin.

We next asked whether PKC activation might lead to changes in the subcellular localization of PKCα and DGKζ in NIH3T3 cells. Consistent with the previous findings [Leach et al., 1989; Wagner et al., 2000], when stimulated by phorbol ester (PMA), PKCα was shown to be translocated mainly to the nuclear envelope and also to the nucleoplasm and the plasma membrane to some extent (Fig. 5C). On the other hand, DGKζ remained in the nucleus as in unstimulated cells and none of the cells showed cytoplasmic localization of DGKζ, suggesting no effect of PKC activation on the subcellular localization of DGKζ. However, it remained unclear whether DGKζ was phosphorylated by PKCs under these experimental conditions although PKCα and βII might have a chance to interact with DGKζ in the nucleus.

DISCUSSION

In the present study we have revealed the detailed subcellular localization of DGKζ during the cell cycle in non-transformed proliferating cells: DGKζ localizes to the nucleus during interphase including G1, S, and G2 phases and is associated, to some extent, with chromatin while it dissociates from condensed chromatin in mitotic phase in NIH3T3 cells, showing reversible association of DGKζ with chromatin. Furthermore this main localization pattern is also observed in proliferating spermatogonia in the testis.

How is this pattern of the subcellular localization of DGKζ implicated in its functional roles? First, its association with chromatin suggests a possible role for DGKζ in chromatin functions. Chromatin is the physiological template of all eukaryotic genetic information and is subject to a diverse array of posttranslational modifications largely exerted on histone tails, thereby regulating access to the underlying DNA [Jenuwein and Allis, 2001]. According to a recent perspective, chromatin is regarded as a highly dynamic structure, in which binding sites are continuously being scanned by nuclear proteins in a random, undirected fashion [van Holde and Zlatanova, 2006]. In relation to the cell cycle control, previous studies have shown that overexpression of DGKζ in the nucleus leads to cell cycle arrest at G1 phase in the transfected COS cells [Topham et al., 1998] and C2C12 cells [Evangelisti et al., 2007b], while down-regulation of endogenous DGKζ by short interfering RNA increases the number of cells in both S and G2/M phases of the cell cycle [Evangelisti et al., 2007b]. In this regard, DGKζ may bind to the retinoblastoma protein (pRB), a tumor suppressor and key regulator of the cell cycle, whose activity depends on the phosphorylation status of pRB [Los et al., 2006]. Furthermore, the enhanced activity of DGKζ in the nucleus down-regulates phosphorylation of Ser808/811 of pRB [Evangelisti et al., 2007b]. It has been shown that pRB mutant with alanine substitutions at Ser-807/811 enhances growth suppressing activity [Driscoll et al., 1999]. Moreover, phosphorylation of Ser-807/811 led to an inactivation of pRB tumor suppressor activity in uveal melanoma [Brantley and Harbour, 2000], suggesting that these two residues are key determinants of pRB activity. Since pRB plays a major role in cell cycle control [De Falco et al., 2006], it is plausible that DGKζ is involved in the control of cell cycle progression through the phosphorylation status of pRB on critical serine residues, even though the precise molecular mechanism underlying this phenomenon still requires investigation. Because this event requires DGK activity in the nucleus, it is conceivable that either attenuated levels of DG or enhanced levels of PA might change the balance of the status, leading to down-regulated levels of Ser-807/811 pRB. In this regard, attenuated levels of DG down-regulates the activity of the PKC family, a well-known DG-dependent protein kinase [Nishizuka, 1984, 1992]. It is reported that one of the classical PKC family, PKCβII, directly phosphorylates pRB although the phosphorylative events do not occur on Ser-807/811 [Suzuma et al., 2002]. Therefore other DG-dependent protein kinase(s) including different PKC isoforms might be involved in this event.

Second, the present study reveals reversible association of DGKζ with chromatin, that is, DGKζ is bound to dispersed chromatin during interphase and dissociates from condensed chromatin during mitotic phase. Similar localization pattern during the cell cycle has been reported for TIF1α, which is a non-histone chromosomal protein tightly associated with highly accessible euchromatic regions of the genome and exhibits a finely granular distribution in euchromatin during interphase nuclei, whereas it is mostly excluded from condensed chromatin in mitotic nuclei [Remboutsika et al., 1999]. Considering that TIF1α, one of the transcriptional intermediary factors, mediates ligand-dependent transcriptional regulation by nuclear receptors and acts on transcription initiation through chromatin remodeling and/or modulation of the activity of the transcription machinery [Glass et al., 1997], it is plausible that DGKζ might share some role(s) with those factors. Further studies need to be done.

With respect to the association with chromatin, bromodomain represents an extensive family of evolutionarily conserved protein modules found in many chromatin-associated proteins and in nearly all known nuclear histone acetyltransferases [Jeanmougin et al.,

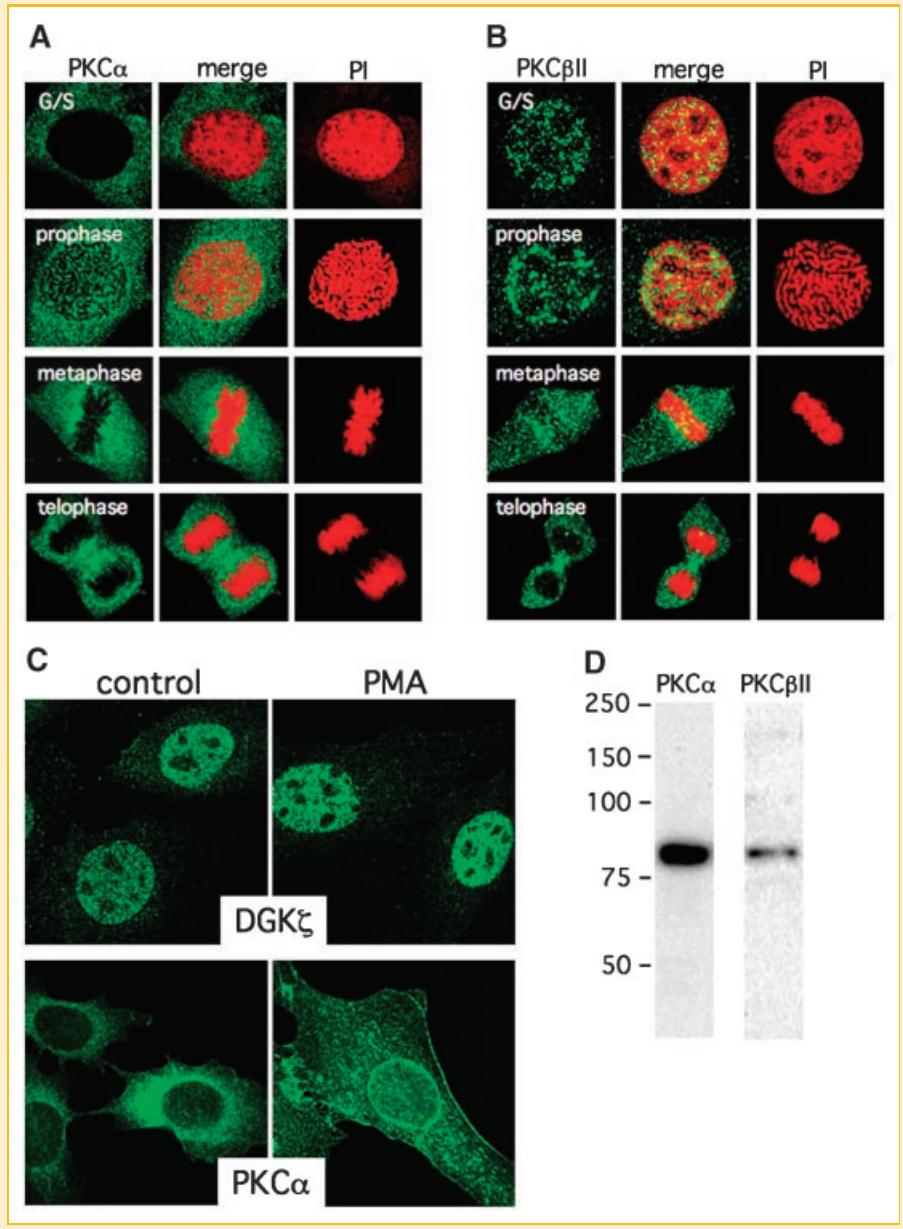

Fig. 5. Subcellular localization of PKCα and PKCβII during the cell cycle in NIH3T3 cells and the effect of PMA on the localization of DGKζ. The position of the analyzed cells during mitotic phase was assessed by combined analysis of synchronization by double thymidine block and DNA staining. After fixation with 4% paraformaldehyde, NIH3T3 cells were immunolabeled with anti-PKCα antibody (green in A) and anti-PKCβII (green in B). DNA was visualized by staining with PI (red). To examine the effect of PKC activation, some cells were treated with 5 μM PMA for 60 min. After fixation, NIH3T3 cells were immunolabeled with anti-DGKζ antibody and anti-PKCα antibody (C). During interphase including G/S phase, PKCα is essentially localized to the cytoplasm, while it is diffused into the region between condensed chromatin during mitotic phase (A). On the other hand, PKCβII is essentially localized to the nucleus in a scattered, coarse-dotted pattern during interphase, while it is diffused throughout the cytoplasm and excluded from condensed chromatin during mitotic phase (B). Upon PMA stimulation, PKCα is translocated from the cytoplasm to the nuclear envelope and nucleoplasm and, to some extent, to the plasma membrane, although DGKζ remains in the nucleus as in unstimulated conditions (C). Total protein (25 μg) from NIH3T3 cells was analyzed by immunoblot using anti-PKCα and PKCβII antibodies (D). Molecular weight markers are indicated on the left in kDa.

1997]. Recently, this domain has been discovered to function as acetyl-lysine binding domain, which prompted us to survey this domain, although no apparent sequences have been currently found in the primary structure of DGKζ.

Third, we reveal that fully differentiated spermatozoa, that is, elongated spermatids, with highly condensed chromatin representing transcriptionally inactive state exhibit no DGKζ-immunoreactivity, suggesting that DGKζ expression is closely associated with the activity in the nuclear events. Within the sperm heads the haploid genome is compacted to a volume of about 5% of that of a somatic cell nucleus. This remarkable repackaging event is achieved by replacing histones with protamines [Mills et al., 1977;

Sassone-Corsi, 2002; Meistrich et al., 2003]. The replacement of histones and deposition of protamines involves major remodeling of the chromatin. This remodeling is presumably responsible for the genome-wide cessation of transcription of RNA during this stage [Kierszenbaum and Tres, 1975]. Absence of DGKζ expression in sperms might be related to the replacement of histones with protamines.

With regard to the relationship between nuclear activity and DGKζ expression, previous study shows that transcriptionally active neurons show intense DGKζ-immunoreactivity in the nucleus while non-neuronal cells in the brain show no DGKζ-immunoreactivity under normal conditions [Hozumi et al., 2003]. However, under pathological conditions such as ischemia and infarction, DGKζ quickly disappears from the damaged neurons but appears in non-neuronal cells, such as glial cells and endothelial cells [Ali et al., 2004; Nakano et al., 2006]. From these studies, it may be that DGKζ is upregulated in cells under active nuclear events, the reason for which remains unveiled. One possibility might be suggested from the present and previous findings that show that DGKζ is associated with chromatin and that overexpression of DGKζ on cardiomyocytes is clearly protective under diseased conditions, such as high pressure overload, ischemic stress, and infarction [Takahashi et al., 2005; Arimoto et al., 2006; Harada et al., 2007; Niizeki et al., 2007]. For example, cardiac-specific overexpression of DGKζ attenuates left ventricular remodeling and improves survival after myocardial infarction [Niizeki et al., 2007]. From these findings, it is hypothesized that DGKζ might be involved in the maintenance of chromatin machinery, in which ''system errors'' might be brought during active transcription or replication. The next challenge will be to elucidate the molecular details of how DGKζ exerts such protective support in the nucleus.

Fourth, we found PKC stimulation by PMA does not affect the subcellular localization of DGKζ although the treatment induces translocation of PKCα. Previous studies have suggested that phosphorylation of a sequence similar to MARCKS facilitates DGKζ export from the nucleus in transfected cells [Topham et al., 1998]. However, the present finding that DGKζ stays in the nucleus under both unstimulated and PMA-stimulated conditions is contradictory to the previous one. One possibility is that the difference is ascribed to the experimental conditions, that is, we employed untransfected NIH3T3 cells while the previous study was performed by using transfected cells, where proteins are presumed to be overexpressed beyond the physiological range. Considering the previous study that shows that DGKζ is quickly translocated from the nucleus to the cytoplasm in ischemic hippocampal neurons [Ali et al., 2004], the nucleo-cytoplasmic translocation of DGKζ may be a phenomenon under pathological conditions in response to critical insults, mediated through undetermined mechanism. Further studies are needed to examine a nucleocytoplasmic shuttling mechanism of DGKζ in more detail.

From the present study using non-transformed cultured cells and proliferating cells in the testis we have learned that DGKζ is reversibly associated with chromatin during the cell cycle, which gives us an important clue to address functional roles of DGKζ. Further studies are warranted to address the hypothesis we have raised.

ACKNOWLEDGMENTS

The work was supported by Grant-in Aid and the 21st Century of Excellence Program from the Ministry of Education, Science, Culture, Sports, Science and Technology (MEXT) of Japan and from Taisho Pharmaceutical (K.G.) and CARISBO FOUNDATION and Progetti Strategici Università di Bologna EF2006 (A.M.M.).

REFERENCES

Ali H, Nakano T, Saino-Saito S, Hozumi Y, Katagiri Y, Kamii H, Sato S, Kayama T, Kondo H, Goto K. 2004. Selective translocation of diacylglycerol kinase zeta in hippocampal neurons under transient forebrain ischemia. Neurosci Lett 372:190–195.

Arimoto T, Takeishi Y, Takahashi H, Shishido T, Niizeki T, Koyama Y, Shiga R, Nozaki N, Nakajima O, Nishimaru K, Abe J, Endoh M, Walsh RA, Goto K, Kubota I. 2006. Cardiac-specific overexpression of diacylglycerol kinase zeta prevents Gq protein-coupled receptor agonist-induced cardiac hypertrophy in transgenic mice. Circulation 113:60–66.

Banfic H, Zizak M, Divecha N, Irvine RF. 1993. Nuclear diacylglycerol is increased during cell proliferation in vivo. Biochem J 290(Pt 3):633–636.

Brantley MA Jr, Harbour JW. 2000. Inactivation of retinoblastoma protein in uveal melanoma by phosphorylation of sites in the COOH-terminal region. Cancer Res 60:4320–4323.

Bunting M, Tang W, Zimmerman GA, McIntyre TM, Prescott SM. 1996. Molecular cloning and characterization of a novel human diacylglycerol kinase zeta. J Biol Chem 271:10230–10236.

Citterio E, Papait R, Nicassio F, Vecchi M, Gomiero P, Mantovani R, Di Fiore PP, Bonapace IM. 2004. Np95 is a histone-binding protein endowed with ubiquitin ligase activity. Mol Cell Biol 24:2526–2535.

De Falco G, Comes F, Simone C. 2006. Prb: Master of differentiation. Coupling irreversible cell cycle withdrawal with induction of muscle-specific transcription. Oncogene 25:5244–5249.

Downs JA, Nussenzweig MC, Nussenzweig A. 2007. Chromatin dynamics and the preservation of genetic information. Nature 447:951–958.

Driscoll B, T'Ang A, Hu YH, Yan CL, Fu Y, Luo Y, Wu KJ, Wen S, Shi XH, Barsky L, Weinberg K, Murphree AL, Fung YK. 1999. Discovery of a regulatory motif that controls the exposure of specific upstream cyclin-dependent kinase sites that determine both conformation and growth suppressing activity of pRB. J Biol Chem 274:9463–9471.

Evangelisti C, Riccio M, Faenza I, Zini N, Hozumi Y, Goto K, Cocco L, Martelli AM. 2006. Subnuclear localization and differentiation-dependent increased expression of DGK-zeta in C2C12 mouse myoblasts. J Cell Physiol 209:370–378.

Evangelisti C, Bortul R, Fala F, Tabellini G, Goto K, Martelli AM. 2007a. Nuclear diacylglycerol kinases: Emerging downstream regulators in cell signaling networks. Histol Histopathol 22:573–579.

Evangelisti C, Tazzari PL, Riccio M, Fiume R, Hozumi Y, Fala F, Goto K, Manzoli L, Cocco L, Martelli AM. 2007b. Nuclear diacylglycerol kinase-zeta is a negative regulator of cell cycle progression in c2c12 mouse myoblasts. FASEB J 21:3297–3307.

Glass CK, Rose DW, Rosenfeld MG. 1997. Nuclear receptor coactivators. Curr Opin Cell Biol 9:222–232.

Goto K, Kondo H. 1996. A 104-kDa diacylglycerol kinase containing ankyrin-like repeats localizes in the cell nucleus. Proc Natl Acad Sci USA 93:11196–11201.

Goto K, Hozumi Y, Nakano T, Saino SS, Kondo H. 2007. Cell biology and pathophysiology of the diacylglycerol kinase family: Morphological aspects in tissues and organs. In: Jeon KW, editor. International review of cytology. San Diego: Academic Press. pp 25–63.

Harada M, Takeishi Y, Arimoto T, Niizeki T, Kitahara T, Goto K, Walsh RA, Kubota I. 2007. Diacylglycerol kinase zeta attenuates pressure overload-induced cardiac hypertrophy. Circ J 71:276–282.

Hendzel MJ, Wei Y, Mancini MA, Van Hooser A, Ranalli T, Brinkley BR, Bazett-Jones DP, Allis CD. 1997. Mitosis-specific phosphorylation of histone H3 initiates primarily within pericentromeric heterochromatin during G2 and spreads in an ordered fashion coincident with mitotic chromosome condensation. Chromosoma 106:348–360.

Hozumi Y, Ito T, Nakano T, Nakagawa T, Aoyagi M, Kondo H, Goto K. 2003. Nuclear localization of diacylglycerol kinase zeta in neurons. Eur J Neurosci 18:1448–1457.

Jeanmougin F, Wurtz JM, Le Douarin B, Chambon P, Losson R. 1997. The bromodomain revisited. Trends Biochem Sci 22:151–153.

Jenuwein T, Allis CD. 2001. Translating the histone code. Science 293:1074–1080.

Katagiri Y, Ito T, Saino-Saito S, Hozumi Y, Suwabe A, Otake K, Sata M, Kondo H, Sakane F, Kanoh H, Kubota I, Goto K. 2005. Expression and localization of diacylglycerol kinase isozymes and enzymatic features in rat lung. Am J Physiol Lung Cell Mol Physiol 288:L1171–L1178.

Kierszenbaum AL, Tres LL. 1975. Structural and transcriptional features of the mouse spermatid genome. J Cell Biol 65:258–270.

Kobayashi N, Hozumi Y, Ito T, Hosoya T, Kondo H, Goto K. 2007. Differential subcellular targeting and activity-dependent subcellular localization of diacylglycerol kinase isozymes in transfected cells. Eur J Cell Biol 86: 433–444.

Leach KL, Powers EA, Ruff VA, Jaken S, Kaufmann S. 1989. Type 3 protein kinase C localization to the nuclear envelope of phorbol ester-treated NIH 3T3 cells. J Cell Biol 109:685–695.

Los AP, Vinke FP, de Widt J, Topham MK, van Blitterswijk WJ, Divecha N. 2006. The retinoblastoma family proteins bind to and activate diacylglycerol kinase zeta. J Biol Chem 281:858–866.

Luo B, Prescott SM, Topham MK. 2003. Association of diacylglycerol kinase zeta with protein kinase C alpha: Spatial regulation of diacylglycerol signaling. J Cell Biol 160:929–937.

Martelli AM, Cataldi A, Manzoli L, Billi AM, Rubbini S, Gilmour RS, Cocco L. 1995. Inositides in nuclei of Friend cells: Changes of polyphosphoinositide and diacylglycerol levels accompany cell differentiation. Cell Signal 7:53–56.

Martelli AM, Fala F, Faenza I, Billi AM, Cappellini A, Manzoli L, Cocco L. 2004. Metabolism and signaling activities of nuclear lipids. Cell Mol Life Sci 61:1143–1156.

Meistrich ML, Mohapatra B, Shirley CR, Zhao M. 2003. Roles of transition nuclear proteins in spermiogenesis. Chromosoma 111:483–488.

Mills NC, Van NT, Means AR. 1977. Histones of rat testis chromatin during early postnatal development and their interactions with DNA. Biol Reprod 17:760–768.

Nakano T, Hozumi Y, Ali H, Saino-Saito S, Kamii H, Sato S, Kayama T, Watanabe M, Kondo H, Goto K. 2006. Diacylglycerol kinase zeta is involved in the process of cerebral infarction. Eur J Neurosci 23:1427–1435.

Niizeki T, Takeishi Y, Arimoto T, Takahashi H, Shishido T, Koyama Y, Goto K, Walsh RA, Kubota I. 2007. Cardiac-specific overexpression of diacylglycerol kinase zeta attenuates left ventricular remodeling and improves survival after myocardial infarction. Am J Physiol Heart Circ Physiol 292:H1105–H1112.

Nishizuka Y. 1984. The role of protein kinase C in cell surface signal transduction and tumour promotion. Nature 308:693–698.

Nishizuka Y. 1992. Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. Science 258:607–614.

Nomura S, Fukaya M, Tsujioka T, Wu D, Watanabe M. 2007. Phospholipase cbeta3 is distributed in both somatodendritic and axonal compartments and localized around perisynapse and smooth endoplasmic reticulum in mouse Purkinje cell subsets. Eur J Neurosci 25:659–672.

Remboutsika E, Lutz Y, Gansmuller A, Vonesch JL, Losson R, Chambon P. 1999. The putative nuclear receptor mediator TIF1alpha is tightly associated with euchromatin. J Cell Sci 112:1671–1683.

Sakane F, Imai S, Kai M, Yasuda S, Kanoh H. 2007. Diacylglycerol kinases: Why so many of them? Biochim Biophys Acta 1771:793–806.

Sasaki H, Hozumi Y, Hasegawa H, Ito T, Takagi M, Ogino T, Watanabe M, Goto K. 2006. Gene expression and localization of diacylglycerol kinase isozymes in the rat spinal cord and dorsal root ganglia. Cell Tissue Res 326:35–42.

Sassone-Corsi P. 2002. Unique chromatin remodeling and transcriptional regulation in spermatogenesis. Science 296:2176–2178.

Strahl BD, Allis CD. 2000. The language of covalent histone modifications. Nature 403:41–45.

Sun B, Murray NR, Fields AP. 1997. A role for nuclear phosphatidylinositol-specific phospholipase C in the G2/M phase transition. J Biol Chem 272: 26313–26317.

Suzuma K, Takahara N, Suzuma I, Isshiki K, Ueki K, Leitges M, Aiello LP, King GL. 2002. Characterization of protein kinase C beta isoform's action on retinoblastoma protein phosphorylation, vascular endothelial growth factor-induced endothelial cell proliferation, and retinal neovascularization. Proc Natl Acad Sci USA 99:721–726.

Tabellini G, Bortul R, Santi S, Riccio M, Baldini G, Cappellini A, Billi AM, Berezney R, Ruggeri A, Cocco L, Martelli AM. 2003. Diacylglycerol kinase-theta is localized in the speckle domains of the nucleus. Exp Cell Res 287: 143–154.

Takahashi H, Takeishi Y, Seidler T, Arimoto T, Akiyama H, Hozumi Y, Koyama Y, Shishido T, Tsunoda Y, Niizeki T, Nozaki N, Abe J, Hasenfuss G, Goto K, Kubota I. 2005. Adenovirus-mediated overexpression of diacylglycerol kinase-zeta inhibits endothelin-1-induced cardiomyocyte hypertrophy. Circulation 111:1510–1516.

Topham MK. 2006. Signaling roles of diacylglycerol kinases. J Cell Biochem 97:474–484.

Topham MK, Bunting M, Zimmerman GA, McIntyre TM, Blackshear PJ, Prescott SM. 1998. Protein kinase C regulates the nuclear localization of diacylglycerol kinase-zeta. Nature 394:697–700.

van Holde K, Zlatanova J. 2006. Scanning chromatin: A new paradigm? J Biol Chem 281:12197–12200.

Wagner S, Harteneck C, Hucho F, Buchner K. 2000. Analysis of the subcellular distribution of protein kinase C alpha using PKC-GFP fusion proteins. Exp Cell Res 258:204–214.

Whitfield ML, Zheng LX, Baldwin A, Ohta T, Hurt MM, Marzluff WF. 2000. Stem-loop binding protein, the protein that binds the 3′ end of histone mRNA, is cell cycle regulated by both translational and posttranslational mechanisms. Mol Cell Biol 20:4188–4198.

Wolffe AP. 1999. Chromatin: Structure and function, 3rd edition. San Diego: Academic Press.