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Distinct expression and localization of the type II diacylglycerol kinase isozymes δ , η and κ in the mouse reproductive organs

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Abstract

Background: We have revealed that the type II diacylglycerol kinases (DGKs) δ , η and κ were expressed in the testis and ovary. However, these enzymes' functions in the reproductive organs remain unknown.

Results: In this study, we first identified the expression sites of type II DGKs in the mouse reproductive organs in detail. Reverse transcription-polymerase chain reaction and Western blotting confirmed that DGK δ 2 (splicing variant 2) but not DGK δ 1 (splicing variant 1) and DGK κ were expressed in the testis, ovary and uterus. DGK η 1 (splicing variant 1) but not DGK η 2 (splicing variant 2) was strongly detected in the ovary and uterus. Interestingly, we found that a new alternative splicing product of the DGK η gene, DGK η 3, which lacks exon 26 encoding 31 amino acid residues, was expressed only in the testis. Moreover, we investigated the distribution of type II DGKs in the testis, ovary and uterus through *in situ* hybridization. DGK δ 2 was distributed in the primary spermatocytes of the testis and ovarian follicles. DGK η 1 was distributed in the oviductal epithelium of the ovary and the luminal epithelium of the uterus. Intriguingly, DGK η 3 was strongly expressed in the secondary spermatocytes and round spermatids of the testis. DGK κ was distributed in the primary and secondary spermatocyte of the testis.

Conclusion: These results indicate that the expression patterns of the type II DGK isoforms δ 2, η 1, η 3 and κ differ from each other, suggesting that these DGK isoforms play specific roles in distinct compartments and developmental stages of the reproductive organs, especially in the processes of spermatogenesis and oocyte maturation.

Keywords: Diacylglycerol kinase, Alternative splicing, Spermatocyte, Ovarian follicle, Uterine luminal epithelium

Background

Diacylglycerol kinase (DGK) phosphorylates diacylglycerol (DG) to generate phosphatidic acid (PA) [1-4]. DG, which is liberated from phosphatidylinositol 4,5-bisphosphate and phosphatidylcholine upon cell stimulation, regulates a wide range of cellular functions. For instance, DG is an allosteric activator of conventional and novel protein kinase Cs (PKCs), Unc-13 and Ras guanyl nucleotide-releasing protein [5-7]. Therefore, DGK consumes DG and is thus responsible for attenuating DG-mediated signals. PA, the reaction product of DGK, has also been reported to regulate a number of signaling proteins such as phosphatidylinositol-4-phosphate 5-kinase, Ras GTPase-activating protein, C-Raf, mammalian target of rapamycin

and atypical PKC [8-10]. Therefore, DGK is thought to play roles not only in the downregulation of DG signaling but also in the production of another lipid mediator, PA.

It is well known that DGK represents a large enzyme family. Ten mammalian DGK isozymes, namely α , β , γ , δ , ϵ , ζ , η , θ , ι , and κ , which contain two or three characteristic cysteine-rich, zinc finger-like C1 domains and the catalytic region in common, are subdivided into five groups according to their structural features [1-4]. The type II DGK [11] comprises the δ [12], η [13] and κ [14] isozymes. The occurrence of alternative splicing was reported for DGK δ (δ 1 and δ 2) [15] and DGK η (η 1 and η 2) [16]. All of the type II DGK isoforms possess a pleckstrin homology domain at their N termini and a separated catalytic domain, and DGKs δ 1, δ 2 and η 2 but not DGKs η 1 and κ contain a sterile α -motif domain at their C termini. It has been reported that DGKs δ 1, δ 2 and η 2 formed oligomers through interactions among

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their sterile α -motif domains and that this oligomer formation regulates the activities and subcellular localizations of these DGK isoforms [15-19]. DGKs δ 2 and κ contain the Pro-rich 52 residues [15] and the 33 tandem repeats of Glu-Pro-Ala-Pro [14] extending from the N terminus, respectively.

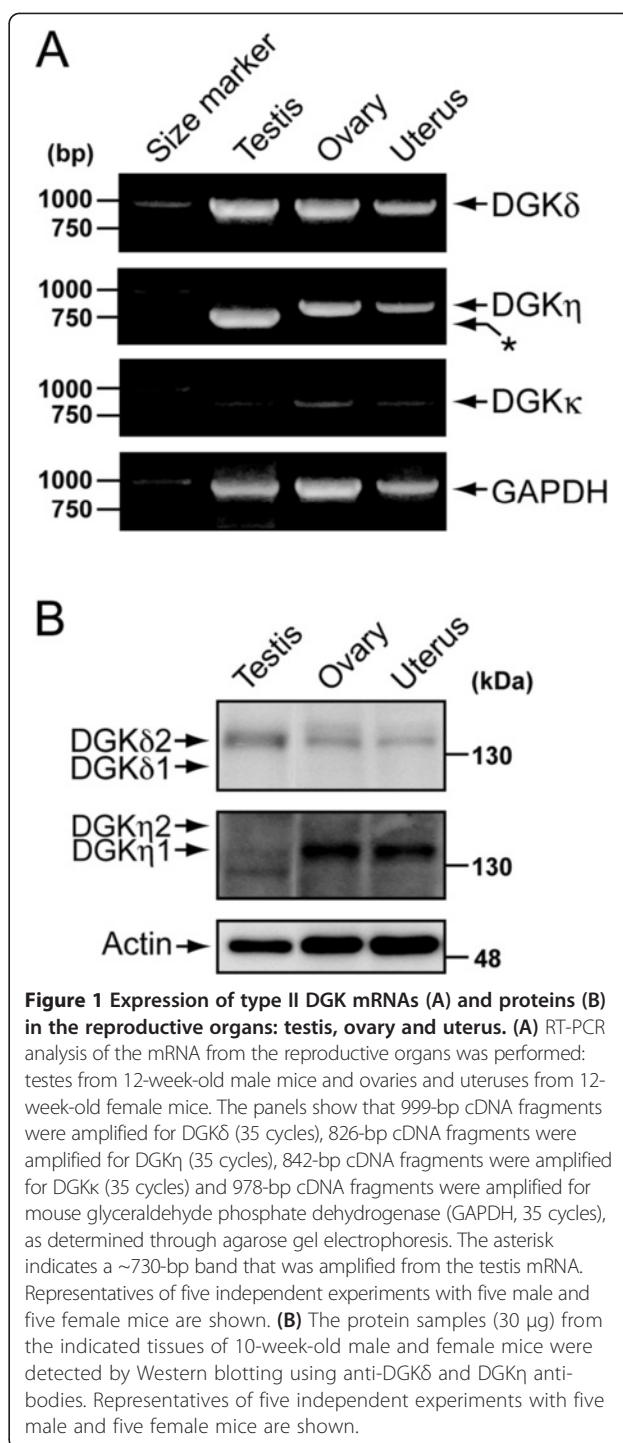
Based on the analysis of DGK δ -knockout mice, it was recently reported that DGK δ regulates the epidermal growth factor receptor pathway in epithelial cells of the lung and skin [20] and insulin receptor signaling in skeletal muscle [21,22] by modulating PKC activity. We recently reported that DGK η is expressed in stomach cancer and HeLa cervical cancer cells and that it is required for the Ras/B-Raf/C-Raf/MEK/ERK signaling cascade, which is activated by the epidermal growth factor [23]. Type II DGKs have been implicated in several diseases [24]. For example, DGK δ is a key enzyme that prevents insulin resistance and type 2 diabetes [21]. A female patient with a disrupted DGK δ gene who exhibits seizures and a psychiatric disorder was found [25]. DGK η was reported to be involved in lung cancer [26]. A genome-wide association study implicated the DGK η gene in the etiology of bipolar disorder [27,28]. A genome-wide association study also indicated a potential relationship between DGK κ and hypospadias [29].

Several reports have revealed that DGK δ , η and κ are abundantly expressed in the reproductive organs, testis and ovary [12-16]. However, the functions of DGKs δ , η and κ in the reproductive organs remain unclear, and not even their detailed distribution patterns in the organs have been revealed. Therefore, in this study, we examined the expression and spatial distribution of the DGKs δ 2, η 1 and κ proteins and mRNAs in the mouse reproductive organs. The results indicate distinct expression patterns, which were obviously different from each other. Moreover, we found a new splice variant of DGK η , DGK η 3, which was specifically expressed in the testis.

Results

Expression of DGK δ , η and κ in the mouse reproductive organs

We first confirmed the expression of DGK δ mRNA and protein in the mouse reproductive organs: the testis, ovary and uterus. The 999-bp cDNA fragment amplified from DGK δ mRNA was strongly detected in the testis and ovary and was clearly observed in the uterus by reverse transcription-polymerase chain reaction (RT-PCR) (Figure 1A). The Western blot analysis showed that the DGK δ 2 protein (the splice variant 2, calculated molecular mass: 135 kDa [15]) was substantially expressed in the testis and ovary and, to a lesser extent, in the uterus (Figure 1B). The DGK δ 1 protein (the splice variant 1, calculated molecular mass: 130 kDa [12]) was not detectable in the reproductive organs (Figure 1B).



The RT-PCR results demonstrated that DGK η mRNA was expressed strongly in the testis and ovary and moderately expressed in the uterus (Figure 1A). The RT-PCR product using DGK η mRNA from the testis (approximately 730 bp) was shorter than those obtained from the ovary and uterus (the 826-bp cDNA fragment) (Figure 1A). Therefore, the shorter product (~730 bp) is specifically generated in the testis. The Western blot analysis showed

that the DGK η 1 protein (the splice variant 1, calculated molecular mass: 128 kDa [13]) was strongly detected in the ovary and uterus (Figure 1B). The DGK η 1 protein was detected at a slightly higher position (132 kDa) in the SDS-polyacrylamide gel electrophoresis results than that corresponding to the calculated molecular mass (Figure 1B). The DGK η 2 protein (the splice variant 2, calculated molecular mass: 135 kDa (139 kDa in SDS-polyacrylamide gel electrophoresis) [16]) was not detected in the reproductive organs.

The sequence analysis of the RT-PCR product amplified from the testis mRNA revealed that the shorter mRNA (precisely 733 bp) is an alternative splicing product of the DGK η gene in which exon 26 is skipped (Figure 2). Therefore, we designate this product DGK η 3. Exon 26 of the DGK η gene contains 93 bp and encodes 31 amino acid residues. Therefore, no frame shift occurs due to alternative splicing. The exon/intron boundaries between exons 25 and 27 fulfill the GT/AG rule (Figure 2A). In addition to the ovary and uterus, the 733-bp band failed to be detected in the epididymis, sperm, vesicula seminalis, prostate gland, brain and Neuro 2a neuroblastoma cells (data not shown). These results further strongly suggest that

DGK η 3 is specifically expressed in the testis. The alternative splicing product is anticipated to produce a shorter DGK η protein with an expected molecular mass of 125 kDa (129 kDa as detected by SDS-polyacrylamide gel electrophoresis). We detected a 129-kDa band that reacted with anti-DGK η antibody in the testis (Figure 1B). However, we found that this band was also detected (data not shown) in the extracts from the testis of a recently created DGK η -knockout mouse (Isozaki, T. *et al.*, unpublished work). Therefore, although DGK η 3 mRNA was strongly expressed in the testis (Figure 1A), its protein band has not yet been identified (Figure 1B). The DGK η 3 protein may be unstable and quickly degraded.

DGK κ mRNA was modestly expressed in the testis, ovary and uterus (Figure 1A). DGK κ protein was not detected in these mouse reproductive organs by Western blotting using anti-human DGK κ antibody (data not shown). Anti-DGK κ antibody against human DGK κ [14] may not effectively recognize the murine DGK κ protein.

Distribution of DGK δ , η and κ mRNAs in the testis

We then performed *in situ* hybridization using the anti-sense probe to examine the spatial distribution of DGK δ 2

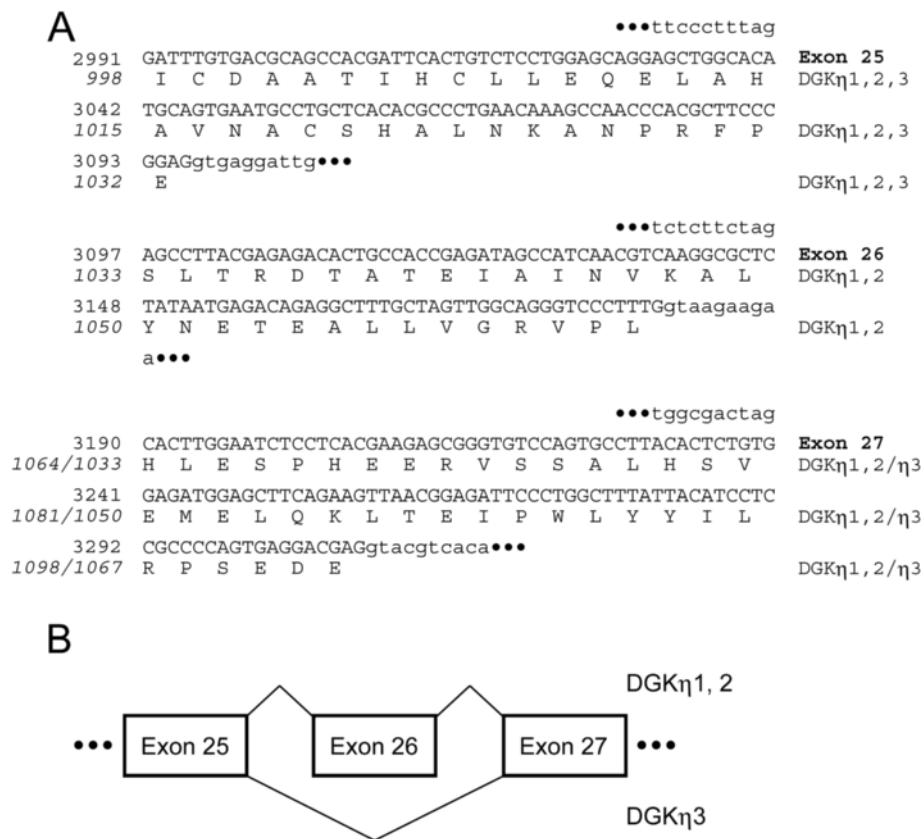


Figure 2 Alternative splicing products of DGK η . **(A)** The nucleotide sequences and deduced amino acid sequences of DGK η 1, DGK η 2 and DGK η 3 are shown. The nucleotide and amino acid numbers are indicated in the left in italic and plain text, respectively. **(B)** The exon-intron structure of the DGK η gene (exons 25–27) and the alternative splicing that yields the two different forms (DGK η 1/2 and DGK η 3) are shown.

mRNA in the mouse testis (Figure 3). Although the testis sections were also hybridized with the sense probe as a control, no distinct staining was detected (Figure 3). As shown in Figure 3, DGK δ mRNA was mainly expressed in primary spermatocytes. The mRNA was moderately detected in the spermatogonia and, to a lesser extent, in the secondary spermatocytes. However, no obvious staining of DGK δ mRNA was detected in the round and elongated spermatids and the Leydig cells. The expression intensities of DGK δ in these testicular cells are summarized in Table 1.

The RT-PCR results demonstrated that DGK η 3 mRNA but not DGK η 1/2 mRNA was mainly detected in the testis (Figure 1). Compared with DGK δ 2 mRNA, DGK η 3 mRNA was detected in the inner area of the testis. DGK η 3 mRNA was strongly expressed in the secondary spermatocytes and the round spermatids and was weakly detected in the primary spermatocytes (Figure 3 and Table 1). In the spermatogonia, elongated spermatids and the Leydig cells, DGK η 3 mRNA was not detectable. No distinct staining was detected with the sense probe as a control (Figure 3).

Compared with the DGK δ and DGK η mRNAs, DGK κ mRNA was expressed in the intermediate region between these isozymes in the testis. DGK κ mRNA was substantially detected in the primary and secondary spermatocytes and weakly distributed in the spermatogonia and round spermatids (Figure 3 and Table 1). However, no obvious hybridization with DGK κ mRNA was detected in the round and elongated spermatids and the Leydig cells. No detectable staining was observed with the sense probe as a control (Figure 3).

Distribution of DGK δ , η and κ mRNAs in the ovary and oviduct

DGK δ mRNA was broadly and modestly expressed in the primary, secondary and mature follicles and the corpus lutea (Figure 4 and Table 2). In the medulla, weak staining was observed. DGK δ mRNA was weakly detected in the oviductal epithelium (Figure 5). Although the ovary and oviduct sections were also hybridized with the sense probe as a control, no distinct staining was detected (Figures 4 and 5).

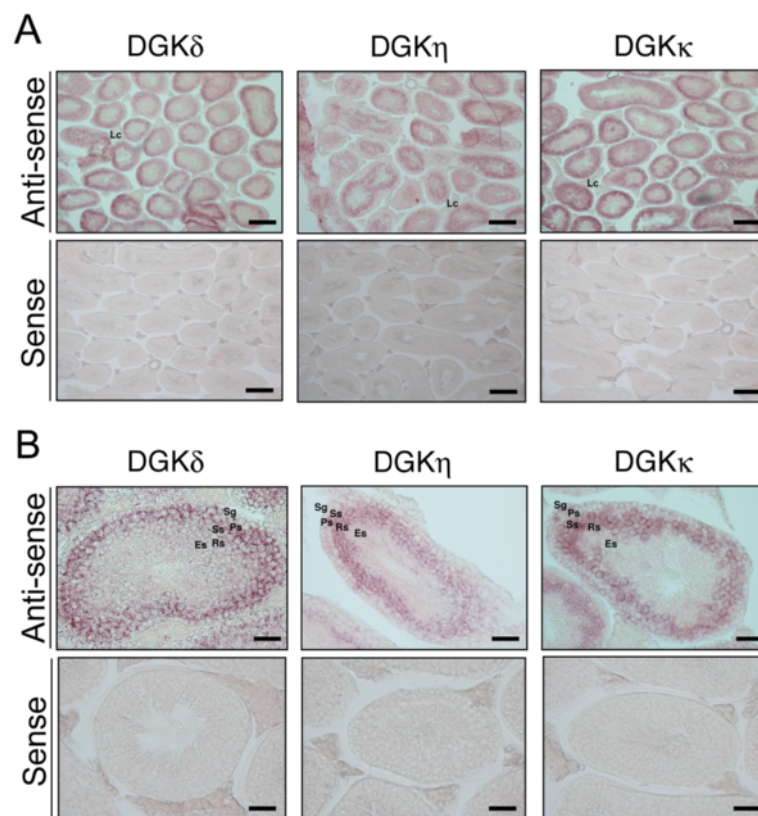


Figure 3 *In situ* hybridization of the type II DGK mRNAs in the testis of a 12-week-old male mouse. **(A)** The type II DGK mRNAs were hybridized and detected with the antisense probe of the type II DGK mRNAs. The testis sections were also hybridized with the sense probes as controls. **(B)** High-magnification image of the seminiferous tubules. Representatives of three independent experiments with three male mice are shown. Sg, spermatogonium; Ps, primary spermatocyte; Ss, secondary spermatocyte; Rs, round spermatid; Es, elongated spermatid; Lc, Leydig cell. The scale bars in **(A)** represent 200 μ m, and the scale bars in **(B)** represent 40 μ m.

Table 1 Expression of type II DGKs in the testis

Testis	Spermatogonium	Primary spermatocyte	Secondary spermatocyte	Round spermatid	Elongated spermatid	Leydig cell
DGK δ	++	+++	+	-	-	-
DGK η	-	+	++	+++	-	-
DGK κ	+	++	+++	+	-	-

- : not detected; + : weakly detected; ++: moderately detected; +++: strongly detected.

Strong staining of DGK η mRNA was observed in the granulosa cells (follicular epithelium) of the primary and secondary follicles (Figure 4 and Table 2). Moreover, the mature follicles and corpus lutea, which are matured from secondary follicles, only modestly expressed DGK η mRNA. In the medulla, only weak staining was observed. DGK η mRNA was strongly expressed in the oviductal epithelium (Figure 5 and Table 2). With the sense probe as a control, no distinct staining was detected (Figures 4 and 5).

DGK κ mRNA was slightly expressed in the medulla of the ovary (Figure 4 and Table 2). However, DGK κ mRNA

failed to be detected in the ovarian follicles and corpus lutea. This mRNA was not detectable in the oviductal epithelium (Figure 5 and Table 2). No obvious staining was detected with the sense probe as a control (Figures 4 and 5).

Distribution of DGK δ , η and κ mRNAs in the uterus

DGK η mRNA was strongly expressed in the luminal epithelium but not in the endometrium (Figure 6 and Table 3). DGK η mRNA was clearly detected in the uterine glands. However, the mRNA was not detectable in the endometrium or myometrium (data not shown). No

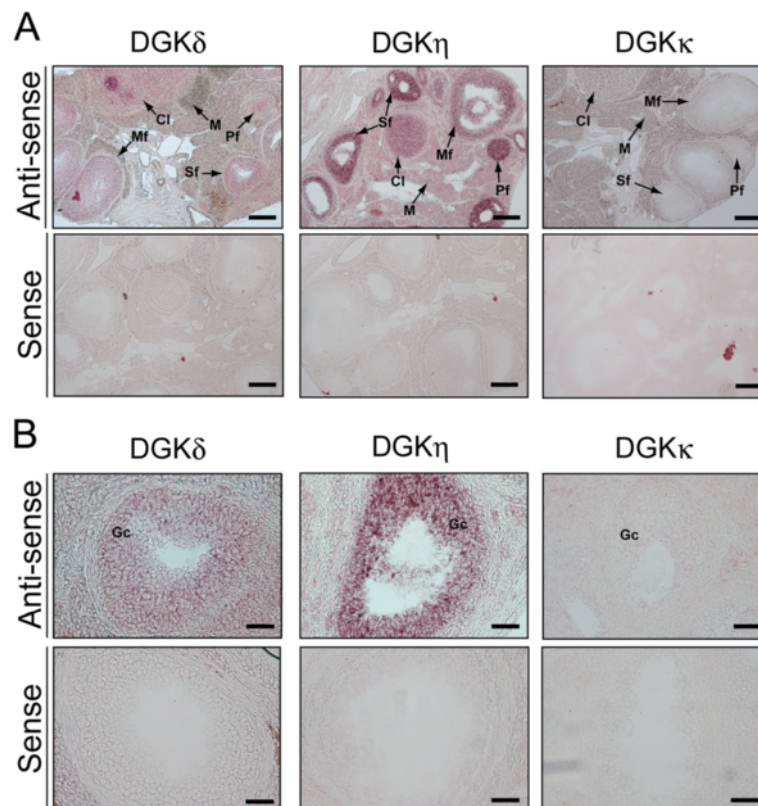


Figure 4 *In situ* hybridization of the type II DGK mRNAs in the ovary of a 12-week-old female mouse. **(A)** The type II DGK mRNAs were hybridized and detected with the antisense probes of the type II DGK mRNAs. The ovary sections were also hybridized with the sense probes as controls. **(B)** High-magnification image of the ovarian follicles (secondary follicles). Although we observed histological specimens from five female mice in different estrous cycles, essentially the same results were obtained. Representatives of five independent experiments with five female mice are shown. Pf, primary follicle; Sf, secondary follicle; Mf, mature follicle; Cl, corpus luteum; Gc, granulosa cell; M, medulla. The scale bars in **(A)** represent 200 μ m, and the scale bars in **(B)** represent 40 μ m.

Table 2 Expression of type II DGKs in the ovary and oviduct

Ovary						
	Primary follicle	Secondary follicle	Mature follicle	Corpus luteum	Medulla	Oviductal epithelium
DGK δ	++	++	++	++	+	+
DGK η	++++	++++	++	++	+	++
DGK κ	-	-	-	-	+	-

- : not detected; +: weakly detected; ++: strongly detected; ++++: very strongly detected.

distinct staining was detected with the sense probe as a control (Figure 6).

DGK δ and DGK κ were broadly distributed and only weakly detected in the luminal epithelium of the uterus (Figure 6 and Table 3).

Discussion

This study provides the first detailed demonstration of the expression and distribution of the type II DGK isozymes DGK δ , η and κ in the mouse reproductive organs. DGK δ , η and κ were expressed in the mouse reproductive organs, namely the testis, ovary and uterus. However, their expression patterns were obviously different from each other.

Exon 26, which encodes 31 amino acid residues, is skipped in the new alternative splicing product of the DGK η gene, which is denoted DGK η 3 (Figures 1 and 2). DGK η 3 was strongly expressed in the secondary spermatocytes and the round spermatids and was not detected in other tissues, such as the ovary, uterus, epididymis, sperm, vesicula seminalis, prostate gland, brain and Neuro 2a neuroblastoma cells (data not shown), implying that DGK η 3 is specifically expressed in the testis, particularly in the secondary spermatocytes and the round spermatids. Therefore, this isoform plays a specialized role in these testicular cells. It is interesting to investigate the functional difference that is generated by the absence of exon 26. Although a motif search was

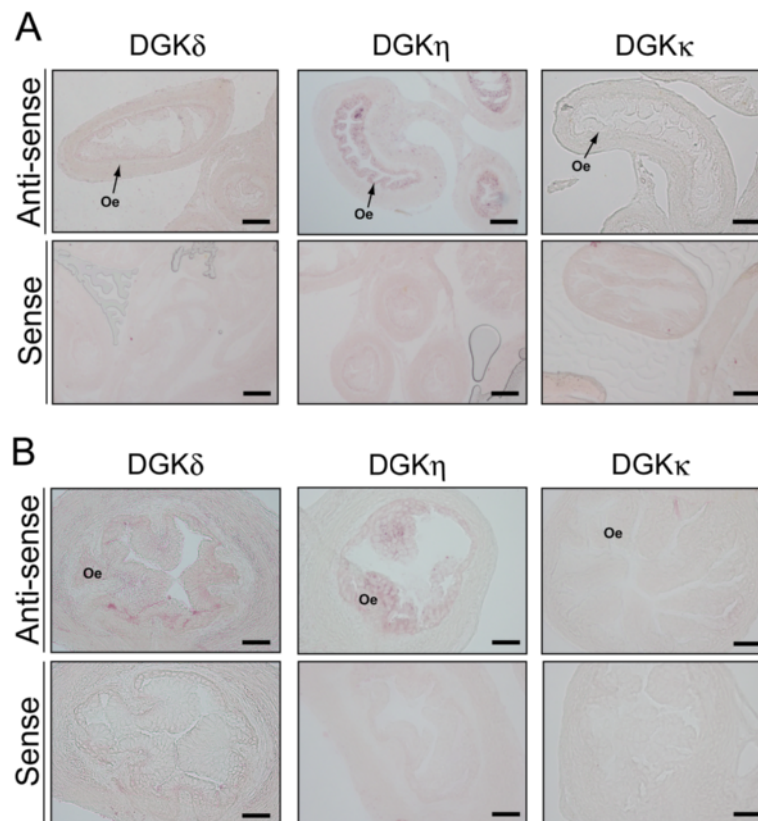


Figure 5 *In situ* hybridization of the type II DGK mRNAs in the oviduct of a 12-week-old female mouse. **(A)** The type II DGK mRNAs were hybridized and detected with the antisense probes of the type II DGK mRNAs. The oviduct sections were also hybridized with the sense probes as controls. **(B)** High-magnification image of the ovarian oviduct. Representatives of four independent experiments with four female mice are shown. Oe, oviductal epithelium. The scale bars in **(A)** represent 100 μ m, and the scale bars in **(B)** represent 40 μ m.

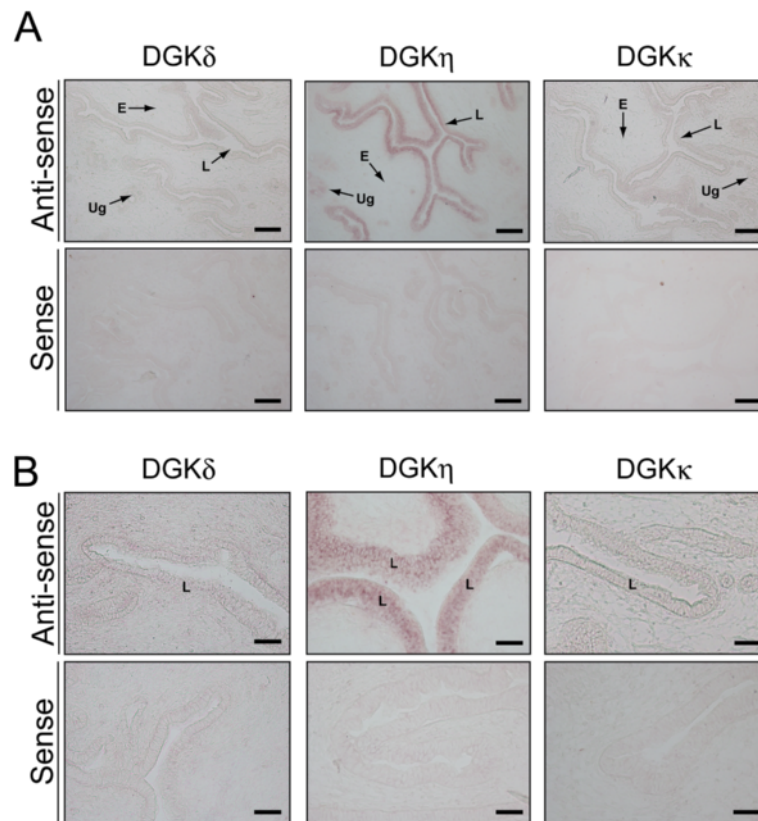


Figure 6 *In situ* hybridization of the type II DGK mRNAs in the uterus of a 12-week-old female mouse. **(A)** The type II DGK mRNAs were hybridized and detected with the antisense probes of the type II DGK mRNAs. The uterus sections were also hybridized with the sense probes as controls. **(B)** High-magnification image of the endometrial epithelium. Representatives of four independent experiments with four female mice are shown. L, luminal epithelium; E, endometrium; Ug, uterine gland. The scale bars in **(A)** represent 200 μ m, and the scale bars in **(B)** represent 40 μ m.

carried out, no obvious functional motifs were not found in the 31-amino acid sequence encoded by exon 26. A protein database search indicated that the 31-amino acid sequence showed high similarity (34.5% identity (79.3% similar) in 29-aa overlap) with the C-terminal region (aa 797–825) of promyelocytic leukemia protein isoform 1, which is a tumor suppressor of acute promyelocytic leukemia [30,31]. However, the function of the region of promyelocytic leukemia protein isoform 1 has not been revealed. Therefore, the function of the 31-amino acid sequence lacking in DGK η 3 remains unclear at present.

In the testis, different expression patterns of type II DGKs were observed. During the process of spermatogenesis, the

primary spermatocytes are developed from the spermatogonia through mitosis [32,33]. The secondary spermatocytes are then derived from the primary spermatocytes via the first meiotic division. Moreover, the round spermatids are generated from the secondary spermatocytes through the second meiotic division. DGK δ 2 mRNA was mainly expressed in the spermatogonia and primary spermatocytes (Figure 3 and Table 1). Strong staining of DGK κ was detected in the primary and secondary spermatocytes (Figure 3 and Table 1). DGK η 3 mRNA was strongly expressed in the secondary spermatocytes and the round spermatids (Figure 3 and Table 1). These results imply that DGKs δ 2, η 3 and κ are involved in mitosis and the first and second meiotic divisions, respectively. The sperm fertilization-related protein equatorin, which is involved in fusion with the oolemma, was reported to be strongly expressed in secondary spermatocytes and round spermatids and was weakly detected in the primary spermatocytes [34]. This expression pattern of equatorin is very similar to that of DGK η 3 (Figure 3). Therefore, equatorin and DGK η 3 may be functionally linked to each other.

A genome-wide association study indicated that DGK κ is involved in hypospadias [29]. Hypospadias is thought

Table 3 Expression of type II DGKs in the uterus

Uterus				
	Luminal epithelium	Endometrium	Uterine gland	Myometrium
DGK δ	+	-	-	-
DGK η	+++	-	+	-
DGK κ	+	-	-	-

--: not detected; +: weakly detected; +++: strongly detected.

to relate to androgen production [35]. Androgen is mainly produced in Leydig cells [35]. However, DGK κ was not detected in Leydig cells. Moreover, the expression pattern of DGK κ was not similar to that of androgen receptor, which was mainly expressed in spermatogonia and primary spermatocytes [36]. Therefore, a relationship between the distribution pattern of DGK κ and hypospadias was not found in this study.

In the ovary, DGK η 1 mRNA was strongly expressed in the granulosa cells (follicular epithelium) of the primary and secondary follicles and modestly detected in the mature follicles and corpus luteum (Figure 4). Because granulosa cells are vigorously proliferated in the primary and secondary follicles during the maturation of ovarian follicles [37], it is possible that DGK η 1 is involved in the proliferation of granulosa cells. We reported that DGK η 1 enhances cell proliferation through the activation of C-Raf [23]. McPhillips, F. *et al.* reported that C-Raf (Raf-1), which is expressed in granulosa cells, mediates growth factor-stimulated growth in ovarian cancer [38]. Therefore, these results allow us to speculate that DGK η 1 is involved in ovarian carcinogenesis through the activation of C-Raf (Raf-1). DGK δ 2 mRNA was also expressed in the granulosa cells (follicular epithelium) of the primary and secondary follicles and modestly detected in the mature follicles and corpus luteum (Figure 4). However, its expression levels were not changed during the follicle development. Therefore, the function of DGK δ 2 is likely different from that of DGK η 1.

DGK η 1 mRNA was strongly expressed in the luminal epithelium of the uterus (Figure 6), and DGK δ 2 and κ mRNAs were weakly detected in these cells. Similar to the granulosa cells in the ovary, the luminal epithelium proliferates during the estrus cycle [37]. Epidermal growth factor receptor is known to be highly expressed in the luminal epithelium [39]. Among type II DGKs, DGK η 1 enhances cell proliferation downstream of epidermal growth factor receptor [23], and DGK δ 2 activates epidermal growth factor receptor through inactivation of protein kinase C [20]. Therefore, these DGK isoforms may positively regulate proliferation through the receptor in the luminal epithelium.

Conclusions

Our data demonstrate that the spatial expression patterns of DGKs δ 2, η 1, η 3 and κ in the murine reproductive organs are different from each other. These results support the hypothesis that DGKs δ 2, η 1, η 3 and κ can play specific roles in distinct compartments and developmental stages of the reproductive organs, especially in the processes of spermatogenesis and oocyte maturation. In addition, we revealed the existence of a new alternative splicing product of DGK η , which we denoted DGK η 3. We recently established DGK η -knockout mice (Isozaki, T. *et al.* unpublished work). To analyze the

reproductive organ-related phenotypes of the knockout mice, it is important to understand the expression patterns of DGK η in the reproductive organs. Studies are underway to examine the physiological functions of DGK η 1 and η 3 in the reproductive organs using the newly established DGK η -knockout mice.

Methods

Animals and tissue preparation

C57BL/6 N mice were obtained from SLC Japan Inc. (Shizuoka, Japan). Mouse tissues were removed immediately after decapitation. All of the procedures using experimental animals were approved by the Ethics Committee of Chiba University (No. 26–96) and were performed according to the guidelines for the Care and Use of Laboratory Animals of Chiba University.

RT-PCR

The testis, ovary and uterus from 10 to 12-week-old male and female mice were homogenized in QIAzol lysis reagent (Qiagen, Venlo, Netherlands), and the total RNA was isolated with a Direct-zol RNA MiniPrep kit (Zymo Research, Irvine, CA, USA). cDNA synthesis was performed with the Transcriptor First-Strand cDNA Synthesis Kit (Roche Diagnostics, Mannheim, Germany), using 0.5 μ g of total RNA and random hexamer primers. PCR amplification was performed using rTaq polymerase (Toyobo, Osaka, Japan) and the following mouse DGK δ , η and κ -specific oligonucleotide primers. The DGK δ primers were the following: forward primer (nucleotide positions 4506–4527, 5'-CGGGATCCGGAAGTGACATATGCCATGAGA-3') and reverse primer (nucleotide positions 5484–5505, 5'-GGGGTACCTCCTTCATTCATCCCTCTCCA-3'). The PCR conditions were as follows: 94°C for 3 min, 35 cycles of 94°C for 30 sec, 56°C for 30 sec, and 72°C for 1.5 min, and 72°C for 5 min. The DGK η primers were the following: forward primer (nucleotide positions 2417–2436, 5'-GGGAATTCCGGGAGCTACTACAGAGATC-3') and reverse primer (nucleotide positions 3224–3243, 5'-GGGGGTCGACCTCACAGAGTGTAAGGCAC-3'). The PCR conditions were as follows: 94°C for 3 min, 35 cycles of 94°C for 30 sec, 58°C for 30 sec, and 72°C for 2 min, and 72°C for 5 min. The DGK κ primers were the following: forward primer (nucleotide positions 3758–3778, 5'-GGGAATTCACCTTGGGCAACTACAGTGTT-3') and reverse primer (nucleotide positions 4581–4600, 5'-GGGGGTCGACGGGTCAGTTCAGGTCGAGT-3'). The PCR conditions were as follows: 94°C for 3 min, 35 cycles of 94°C for 30 sec, 50°C for 30 sec, and 72°C for 2 min, and 72°C for 5 min. For normalization, the mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was simultaneously amplified (35 cycles) using the following GAPDH-specific oligonucleotide primers: forward primer

(nucleotide positions 103–128, 5'-TCGGTGTGAACG-GATTTGGCCGTATT-3') and reverse primer (nucleotide positions 1056–1079, 5'-CATGTAGGCCATGAGGTC-CACCAC-3'). The PCR conditions were as follows: 94°C for 3 min, 35 cycles of 94°C for 30 sec, 45°C for 30 sec, and 72°C for 1 min, and 72°C for 5 min. The amplified PCR products were separated by agarose gel electrophoresis and stained with ethidium bromide (Wako Pure Chemical, Osaka, Japan).

The RT-PCR product amplified from the DGK η mRNA in the testis was purified and then sequenced using the DGK η -specific forward and reverse primers described above. The sequencing was carried out by Eurofins Genomics (Tokyo, Japan).

Western blotting

The testis, ovary and uterus from 10- to 12-week-old male and female mice were homogenized in lysis buffer (50 mM HEPES, pH7.2, 150 mM NaCl, and 5 mM MgCl₂) containing complete EDTA-free protease inhibitor cocktail (Roche Diagnostics) and centrifuged at 1,000 × *g* for 5 min. The protein concentration in the supernatants was determined using a bicinchoninic acid protein assay kit (Thermo Scientific, Hudson, NH, USA). The tissue lysates (30 μg of protein) were separated on SDS-PAGE, and the separated proteins were transferred to a polyvinylidene difluoride membrane (Pall Life Sciences, Port Washington, NY, USA). The membrane was blocked with 5% skim milk and incubated with an anti-DGK δ polyclonal antibody [15] and an anti-DGK η polyclonal antibody (ProteinTech Group, Chicago, IL, USA) overnight at 4°C. The immunoreactive bands were visualized using a peroxidase-conjugated anti-rabbit IgG antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) and the ECL Western Blotting Detection System (GE Healthcare Bio-Sciences, Piscataway, NJ, USA).

Preparation of plasmids and digoxigenin-labeled Riboprobes for in situ hybridization

The PCR products of DGKs from the mouse brain were subcloned into the BamHI/KpnI and EcoRI/SalI sites of the pBluescript SK (+) vector (Stratagene-Agilent Technologies, Santa Clara, CA, USA). The plasmids were cut with either KpnI (Roche Diagnostics) or BamHI for DGK δ mRNA, and with either SalI or EcoRI for DGK η and κ mRNA to produce the sense or anti-sense probes. The digoxigenin (DIG)-labeled probes were synthesized by transcribing 3 μg of template linear DNA with T3 and T7 RNA polymerase (Promega, Madison, WI, USA) according to the DIG RNA labeling kit protocol (Roche Diagnostics).

In situ hybridization

The testis, ovary and uterus isolated from 10- to 12-week-old male and female mice were rapidly frozen in

100% OCT compound (Tissue-Tek, Sakura Finetek USA, Inc., Torrance, CA, USA) in a dry ice-hexane bath. The frozen tissues were sectioned at 25-μm thickness (Leica, Solms, Germany) and mounted on APS-coated slides (S8441, Matsunami, Tokyo, Japan). The sections were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) (137 mM NaCl, 2.67 mM KCl, 8.09 mM Na₂HPO₄ · 12H₂O, and 1.47 mM KH₂PO₄) (Wako Pure Chemical) for 20 min. The sections were treated with proteinase K (1 μg/μl, Qiagen) for 30 min at 37°C and fixed with 4% paraformaldehyde in PBS for 5 min. The basic proteins were acetylated with 0.25% acetic anhydride in 0.1 M triethylamine (pH 8.0), and both before and after this process, the slides were rinsed with PBS. Pre-hybridization was carried out with hybridization buffer (40% deionized formamide, 10% dextran sulfate, 1× Denhard's solution, 4× SSC (1× SSC: 0.15 M NaCl, and 15 mM sodium citrate), 10 mM dithiothreitol, 1 mg/ml tRNA from baker's yeast, 1 mg/ml boiled deoxyribonucleic acid from salmon sperm, and 0.25 mg/ml DIG-labeled riboprobe denatured at 65°C for 30 min) without riboprobes for 30 min at 60°C. The hybridization was performed overnight with hybridization buffer at 60°C. At the end of the incubation period, the sections were rinsed stepwise at 60°C with 4× SSC, 2× SSC in 50% deionized formamide, and 0.1× SSC and blocked through a 30 min incubation with blocking reagent solution (Roche Diagnostic). The sections were incubated for 30 min at room temperature in the presence of anti-DIG Fab fragments conjugated with alkaline phosphatase (1/5,000 dilution) in blocking reagent solution. The staining was eventually developed using nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate staining solution according to the DIG Nucleic Acid Detection Kit protocol (Roche Diagnostic). We then performed light microscopic observation of serial sections of histologic specimens.

Availability of supporting data

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank/EMBL Data Bank with accession number(s) KP329574.

Abbreviations

DGK: Diacylglycerol kinase; DG: Diacylglycerol; PA: Phosphatidic acid; PKC: Protein kinase C; RT-PCR: Reverse transcription-polymerase chain reaction; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; DIG: Digoxigenin; PBS: Phosphate buffered saline; Sg: Spermatogonium; Ps: Primary spermatocyte; Ss: Secondary spermatocyte; Rs: Round spermatid; Es: Elongated spermatid; Lc: Leydig cell; Pf: Primary follicle; Sf: Secondary follicle; Mf: Mature follicle; Cl: Corpus luteum; Gc: Granulosa cell; M: Medulla; Oe: Oviductal epithelium; L: Luminal epithelium; E: Endometrium; Ug: Uterine gland.

Competing interests

The authors declare that they have no competing interests.

Authors' contribution

TS designed and performed the experiments, analyzed the data and wrote the manuscript. TU, SK and TI carried out the experiments. HS designed the

experiments. FS conceived, supervised and designed the study and drafted the manuscript. All authors read and approved the final manuscript.

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