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Molecular cloning of a diacylglycerol kinase isozyme predominantly expressed in rat retina

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Abstract We have cloned and characterized a new diacylglycerol kinase (DGK) isozyme which is expressed in the retina and the brain of rat. The cDNA contains an open reading frame of 567 amino acid residues with a predicted protein of 64 kDa and shows very high homology to human DGKε. The new DGK isozyme contains two distinctive zinc-finger structures and a putative catalytic domain. This DGK expressed predominantly in the inner and outer nuclear layers of retina. This expression pattern is different from those of the previously cloned DGKs including the human DGKε, suggesting that this DGK isozyme has potential importance in visual functions as was the case in *Drosophila* retinal cells.

Key words: Diacylglycerol kinase (DGK); PI-signaling; Photo-transduction; Retina; (Rat)

1. Introduction

Diacylglycerol kinase (DGK) phosphorylates diacylglycerol (DAG) to produce phosphatidic acid (PA) [1]. DGK is thought to be one of the key enzymes which play an important role in signal transduction mediated by inositol-phospholipid turnover [2,3]. One of the roles of DGK must be the regulation of DAG concentration within the cell. In addition to serving as an important intermediate of inositol phospholipid biosynthesis, DAG has been shown to be a second messenger which activates protein kinase C (PKC). DAG is known to bind to and activate PKC, which, in turn, phosphorylates target proteins [4–6]. Recently, PA also has been attracting much attention as a lipid second messenger. PA has been reported in a number of studies to modulate activities of ras GTPase-activating proteins [7], PI-4-phosphate kinase [8] and many other important enzymes. Therefore the roles of DGK in the regulation of intracellular DAG and PA level must be critical for normal cellular functions.

Many DGK isozymes have been purified from various animal sources. These isozymes appear to differ with respect to their molecular weight, enzymological properties, activators, substrate specificity and tissue distribution. It is thus likely that each isozyme is operated under distinct regulatory mechanisms and has specific functions. For instance, DGK was found to be involved in interleukin-2 production in T-lymphocytes [9] and moreover in photoreceptor functions in *Drosophila melanogaster*. The critical importance of DGK in the visual function of *Drosophila* has been demonstrated in the rdgA mutant, which has a mutated eye-specific DGK (DGK2) gene [10,11]. The phosphoinositide cycle and the resulting molecules, inositol 1,4,5-trisphosphate (IP3) and DAG, are suggested to function as the primary second messenger in phototransduction in invertebrate photoreceptors [12]. On the other hand, cGMP has been shown to be a second messenger in rod photoreceptor cells in vertebrates [13]. Despite the common rhodopsin photoreceptive process, therefore, phototransduction in the vertebrate retina is apparently different from that in the invertebrate retina. However, it has been shown that PI synthesis and phosphorylation were enhanced by light in rat retinas [14,15], and IP3 receptors have been shown to localize to synaptic terminals of vertebrate retinal neurons, including rods and cones [16]. Thus, it is likely that PI-signal transduction also plays a role in the mammalian visual transduction process, directly or indirectly.

In the course of trying to isolate mammalian rdgA homologues, we identified a new DGK (rat DGKε) from rat retina. This DGK is expressed predominantly in a subset of neuronal cells in retina and brain, suggesting that rat DGKε may be important in retinal and brain function.

2. Materials and methods

2.1. Cloning and sequencing of rat DGKε cDNA

Degenerate primers were designed based on the amino acid sequences conserved among the sequenced catalytic domains of DGK isozymes. The forward primer P1 (5′-CTA/CAAC/TCCg/TCgA/CCAggTg/TTA/TC-3′) and the reverse primer P2 (5′-TgNATCCANggC/TTCNA/CCA/gTC-3′) were derived from the consensus amino acid sequences L–N–P–R–Q–V–F and D–G/N–E–P–W–M/I–Q found in the members of DGK family, i.e. *Drosophila* DGK1 [17], *Drosophila* DGK2 (rdgA) [11], porcine DGKα [18], human DGKγ [19], rat DGKI [20], rat DGKII [21], and rat DGKIII [22]. At this stage of investigation, the structure of human DGKδ [23], human DGKζ [24], human DGKε [25], hamster DGKη [26], and rat DGKIV [27] had not been revealed. Sufficient degeneracy was incorporated into the primer sequences to encompass divergence among the various isozymes at the nucleotide level. To facilitate subcloning, EcoRI and BamHI endonuclease sites were added to the 5′-ends of the P1 and P2 primers, respectively.

Rat adult (10–11 weeks) retina poly(A)$^+$RNA was purchased from Clontech. Single-strand cDNA was synthesized with these poly(A)$^-$RNA using SuperScriptII (BRL) and random hexamers. The reverse transcription mixture was then used as the template in the PCR amplification which was performed as follows: 94°C for 5 min, followed by 40 cycles of 94°C for 1 min, 52°C for 1 min, 72°C for 2 min, and concluding with a 7 min incubation at 72°C. The amplification products were gel-purified and digested with EcoRI and BamHI. They then were subcloned into the EcoRI/BamHI site of pBluescript SK(−) (Strategene). The cloned fragments were subjected to sequence analysis by the chain termination method using a BcaBest kit (TAKARA).

Marathon RACE, an anchored PCR procedure, was performed to

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Abbreviations: DAG, diacylglycerol; PA, phosphatidic acid; DGK, diacylglycerol kinase; PI, phosphatidyl inositol; IP3, inositol 1,4,5-trisphosphate; PLC, phospholipase C; PKC, protein kinase C; RT/PCR, reverse transcription/polymerase chain reaction

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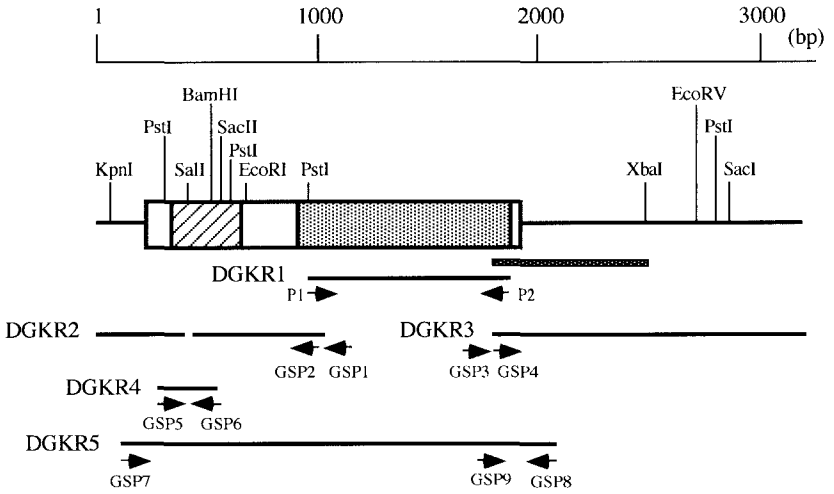

Fig. 1. Schematic map of rat DGKε clones. A composite of rat DGKε (3240 bp) was constructed by combining sequences from the overlapping clones (DGKR2, DGKR3, and DGKR5). DGKR5 includes an open reading frame. The box and line indicate the coding region and noncoding sequences, respectively; ▩, zinc-finger structures; ▩, catalytic domain; solid bar, the probe used for Northern blot analysis.

extend the 5′- and 3′-end of the novel DGK-related cDNA (DGKR1) using the Marathon cDNA Amplification Kit (Clontech) and rat adult (10–11 weeks) retina poly(A)$^+$RNA (Clontech). PCR amplification was performed as follows with gene-specific primers (GSP1–8): GSP1(985 to 1019), GSP2(762 to 793), GSP3(1379 to 1413), GSP4(1505 to 1531), GSP5(104 to 131), GSP6(283 to 309), GSP7(−104 to −75), GSP8(1801 to 1827). And the reaction was performed as described in the protocol provided by the manufacturer. The amplification products were cloned directly into the pCR vector with the TA cloning kit (Invitologen). To generate a cDNA containing an open reading frame, we again performed Marathon RACE. We design GSP7 and GSP8 primers based on the sequence obtained from DGKR2 and DGKR3. Purified plasmid DNAs were sequenced by the by the dideoxy chain termination method using a BcaBest kit (TAKARA) and ALF express DNA sequencers (Pharmacia Biotech). Independent two clones of each DGKR2, 3, 4, and 5 were sequenced.

2.2. RNA blot hybridization analysis

Poly(A)$^+$RNAs (4.5 mg each) of rat adult retina and brain were separated on 1.2% agarose formaldehyde gel and transferred to a nylon membrane (Hybond N$^+$, Amersham). Rat multiple tissue Northern (MTN) blot (Clontech) was also used. Partial fragments of DGKI [20], DGKII [21], and DGKIII [22] cDNA were used as the probes (nucleotide 1505–2329 of DGKε, nucleotide 1639–2239 of DGKI, nucleotide 1803–2492 of DGKII, and nucleotide 1827–2454 of DGKIII). Those fragments of DGK I, II and III were made using RT/PCR. Hybridization was carried out in ExpressHyb.Hybridization solution (Clontech). The conditions of hybridization and washing were as described in the protocol provided by the manufacturer.

2.3. In situ hybridization

Two non-overlapping antisense oligonucleotides were complementary to nucleotide residues 101–145 (DGK-5′) and 1700–1745 (DGK-3′) of the rat DGKε cDNA. These oligonucleotides were labeled with ^{33}P-dATP using terminal deoxyribonucleotidyl transferase (BRL) to a specific activity of 0.5×10^9 dpm mg^{-1} DNA. Under deep pentobarbital anesthesia, brains and eyes were obtained from rats at 10–11 weeks. Fresh frozen sections through the brain and retina (20 mm in thickness) were mounted on the same glass slides precoated with 3-aminopropyltriethoxysilane, and were processed for in situ hybridization histochemistry, as described previously [28]. Briefly, following fixation with 4% paraformaldehyde and prehybridization, hybridization was performed at 42°C for 10 h in buffer containing 50% formamide, 30 mM Tris-HCl (pH 7.5), 0.6 M NaCl, 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin, 0.25% SDS, 200 mg/ml of yeast tRNA, 10% dextran sulfate, 20 mM dithiothreitol and 1×10^4 dpm/ml of ^{33}P-labeled oligonucleotide probe. The slides were washed at room temperature for 40 min in 2× SSC containing 0.1% salcosyl and further washed twice at 55°C for 40 min in 0.1× SSC containing 0.1% salcosyl. The sections were exposed for 1–2 months to Hyperfilm-β max (Amersham) for macro-autoradiography, and dipped in nuclear track emulsion (NTB2, Kodak) for 2 months.

3. Results

3.1. Isolation and identification of rat DGKε cDNA

To identify DGK isozymes in rat retina, we carried out RT/PCR using poly(A)$^+$RNA from rat adult retina and degenerate primers (P1 and P2 in Fig. 1) that corresponded to amino acid sequences L–N–P–R–Q–V–F and D–G/V–E–P–W–M/I–Q. The amino acid sequences are highly conserved among all mammalian and *Drosophila* DGK isozymes [11,17–26]. The PCR products had the expected size of ~760 bp. We subcloned and sequenced these products, and found a novel DGK-related fragment (DGKR1) (Fig. 1). The DGKR1 contained the conserved sequences that the PCR strategy was designed to detect, but otherwise had a unique primary structure. Besides DGKR1, partial sequences of the other DGKs (DGKI, II, III) that were already isolated from rat brain [20–22] were also obtained. The DGKR1 sequence was used as primers to perform the Marathon RACE, and we isolated DGKR2 and DGKR3 (5′ and 3′ regions) and DGKR5 containing an open reading frame. However, the sequence comparison showed that there was an apparent gap of 25 amino acids in DGKR2 that corresponds to the zinc-finger structure which is highly conserved among other DGKs [11,17–26]. We suspected an internal deletion in this area. Therefore, we next performed RT/PCR using the retina poly(A)$^+$RNA and the primers flanking the site of possible deletion (GSP5 and GSP6). When the PCR products were gel-electrophoresed, we noted a trace amount of a 209-bp product but not of a 131-bp product as expected from the sequence of DGKR2.

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Fig. 2. The nucleotide and deduced amino acid sequences of rat DGKε. The nucleotide and deduced amino acid sequence of rat DGKε cDNA. The open reading frame consists of 1701 nucleotides and encodes 567 amino acids. Single-letter symbols for the amino acids are below the second nucleotide of each codon. The two conserved zinc-finger structures are underlined. The catalytic domain is doubly underlined. * = termination codon.

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-209                                                                  CGGCCAGAGGCTTGGCTGGAGGTGGTACC
-180 CGAGACCGCGAGAGCGAACTGGGGTCTCCAGAGACCTCGCGATAGGGGCGCGGGCGAGGCCCAGCGCGACGGCCGTTGTGTGAGCACGCG
 -90 CAGAAGGACGAGCTGCAACCCCACGCTGTGAGTCCTAGAGCAAGTCCCCTGGACCCGGCCTCTCCGTAGGCCACGCCTGGGCCTGGGAAG

   1 ATGGAGGGAGACCAGCGGTCCGGCCCATCGGCGCAGGGCCTGCTCCCCGATGGCCACTTGATCCTATGGACGCTGTTCTCCGTGCTGTTG
   1 M  E  G  D  Q  R  S  G  P  S  A  Q  G  L  L  P  D  G  H  L  I  L  W  T  L  F  S  V  L  L

  91 CCGGTGTTCATCACCTTATGGTGTAGTCTGCAGCGTTCGCGCCGGCAGCTGCACCGCAGGGACATCTTCCGCAAGAGCAAGCACTGCTGG
  31 P  V  F  I  T  L  W  C  S  L  Q  R  S  R  R  Q  L  H  R  R  D  I  F  R  K  S  K  H  C  W

 181 CGTGACACCGACCTGTTCAGCCACCCCACCTACTGCTGCGTGTGCGCCCAGCACATCCTGCAAGGAGCCTTCTGCGACTGCTGCGGGCTC
  61 R  D  T  D  L  F  S  H  P  T  Y  C  C  V  C  A  Q  H  I  L  Q  G  A  F  C  D  C  C  G  L

 271 CGCGTGGACGAGGGCTGCCTCAAGAAGGTCGACAAGCGCTTCCCGTGCAAGGAGATCATGCTCAAGAACGACAGCAGGGCCGCAGATGCG
  91 R  V  D  E  G  C  L  K  K  V  D  K  R  F  P  C  K  E  I  M  L  K  N  D  S  R  A  A  D  A

 361 ATGCCCCACCACTGGATCCGCGGCAACGTCCCCCTGTGCACTTACTGTGTAATCTGCAGGCAGCAGTGTGGCAGCCAGCCTAAACTCTGC
 121 M  P  H  H  W  I  R  G  N  V  P  L  C  T  Y  C  V  I  C  R  Q  Q  C  G  S  Q  P  K  L  C

 451 GACTACCGGTGTGTTTGGTGCCAGCAGACAGTCCACGATGAGTGCATGAGAGGCAGCTTAAAGAGTGAGAAGTGTGATTTTGGAGAATTC
 151 D  Y  R  C  V  W  C  Q  Q  T  V  H  D  E  C  M  R  G  S  L  K  S  E  K  C  D  F  G  E  F

 541 AAAAATCTCATCATCCCTCCAAGTTATTTAACGTCCATTAACCAGATGCGTAAAGACAAAAACACAAATTATGAAGTGCTTGCTTCTAAG
 181 K  N  L  I  I  P  P  S  Y  L  T  S  I  N  Q  M  R  K  D  K  N  T  N  Y  E  V  L  A  S  K

 631 TTTGGAAAGCAGTGGACCCCATTAATAATCCTGGCCAACTCTCGTAGTGGAACTAACATGGGAGAAGGACTGTTGGGAGAGTTTAAGATG
 211 F  G  K  Q  W  T  P  L  I  I  L  A  N  S  R  S  G  T  N  M  G  E  G  L  L  G  E  F  K  M

 721 CTGTTAAATCCCGTCCAGGTGTTCGATGTCACTAAAACTCCTCCGATCAAAGCTCTGCAGCTGTGCACTCTTCTTCCTTATTACTCCGTT
 241 L  L  N  P  V  Q  V  F  D  V  T  K  T  P  P  I  K  A  L  Q  L  C  T  L  L  P  Y  Y  S  V

 811 CGAGTCCTTGTTTGTGGAGGGGATGGGACTGTGGGCTGGGTCCTCGATGCAATTGATGAAATGGAGATTAAGGGACAAGAAAAATACATT
 271 R  V  L  V  C  G  G  D  G  T  V  G  W  V  L  D  A  I  D  E  M  E  I  K  G  Q  E  K  Y  I

 901 CCAGAGGTTGCAGTCTTACCTCTGGGAACAGGCAACGACCTATCCAACACCCTGGGCTGGGGTACTGGCTATGCTGGAGAAATCCCGGTT
 301 P  E  V  A  V  L  P  L  G  T  G  N  D  L  S  N  T  L  G  W  G  T  G  Y  A  G  E  I  P  V

 991 GCACAGGTCTTAAGGAATGTAATGGAAGCGGATGGAATGAAACGAGACAGATGGAAAGTGCAGGTAACAAATAAAGGCTACTACAGTTTA
 331 A  Q  V  L  R  N  V  M  E  A  D  G  M  K  R  D  R  W  K  V  Q  V  T  N  K  G  Y  Y  S  L

1081 AGGAAACCCAAGGAGTTCACAATGAACAACTACTTCTCTATTGGACCCGATGCCCTCATGGCTCTCAATTTTCATGCTCATCGTGAGAAG
 361 R  K  P  K  E  F  T  M  N  N  Y  F  S  I  G  P  D  A  L  M  A  L  N  F  H  A  H  R  E  K

1171 GCGCCGTCTCTGTTTTCCAGCCGAAACCTTAACAAGGCTGTTTATTTATTCTATGGAACTAGAGATTGCTTGGTGCAAGAGTGTAAAGAT
 391 A  P  S  L  F  S  S  R  N  L  N  K  A  V  Y  L  F  Y  G  T  R  D  C  L  V  Q  E  C  K  D

1261 TTGAATAAAAAAATTGAGCTAGAGCTGGATGGTGAGCGAGTTGAACTGCCTAACTTGGAGGGAGTTATTGTTCTGAACATTGGCTACTGG
 421 L  N  K  K  I  E  L  E  L  D  G  E  R  V  E  L  P  N  L  E  G  V  I  V  L  N  I  G  Y  W

1351 GGCGGTGGCTGCCGACTGTGGGAAGGAGTGGGAGATGAGACGTATCCTCTAGCCAGACATGATGATGGCTTACTGGAAGTTGTTGGAGTA
 451 G  G  G  C  R  L  W  E  G  V  G  D  E  T  Y  P  L  A  R  H  D  D  G  L  L  E  V  V  G  V

1441 TATGGGTCTTTCCACTGTGCTCAAATCCAGGTGAAACTGGCAAATCCTTTTCGAATCGGACAGGCCCATACCGTGCGGCTGACTTTGAAG
 481 Y  G  S  F  H  C  A  Q  I  Q  V  K  L  A  N  P  F  R  I  G  Q  A  H  T  V  R  L  T  L  K

1531 TGCTCCAGGATGCCGATGCCGATGCAGGTGGATGGGGAGCCCTGGGCCCAAGGGCCTGGTACTGTCACCATAACGCACAAGACACATGCA
 511 C  S  R  M  P  M  P  M  Q  V  D  G  E  P  W  A  Q  G  P  G  T  V  T  I  T  H  K  T  H  A

1621 CTGATGCTGTACTTCTCTGGGGAGCAGTCGGATGATGACCTCTCAAGCCCTTCAGATCACGAGGACTTAAAGGAGGCCGAGTAGAAGGCT
 541 L  M  L  Y  F  S  G  E  Q  S  D  D  D  L  S  S  P  S  D  H  E  D  L  K  E  A  E  *

1711 GAGATGAAGCGTATCTGCCCAGATGGGTGACATGTCGCCCACAGGACACTGCTGGCCGTCAGCTGTCATTTCCTTCCTAGCATTGTGTGT
1801 GAGCATGTGAACAGCATTTCCACAGCTAGGCTTCAGTATCACAGACACACTCTCCTGTCAGCAACACACTCTGATCATTATCACTAACTG
1891 AAAAGAAAAACCCACCACAGAGAGGTCCAGGAAGATGAAACACTTCTCCCGGCTGGACATTGTAAGACTGGCCTCACGCCGCATTGTGCA
1981 CTGCCTTGGTGGACAGGATGTGGACGGAGAGATCATTCCCTCCCTCAGTTCTTGGTGTGTATTAACGCGTTAAACGTTAGCTTGCTTTTG
2071 AACTGCCCTGGTTAACATGGGCTTCCTCCCGCACTGTGCCAAGGAAGATGCCCCACACCTCAAACCAAATGGACCCTGCATTCTGATTCC
2161 TAGGAGCACCTGAGGAGAAAAGTGAAAGAAAGATGGCGCTTACCATTTTCACAGTAGATATTTCCTATCAGGCTTTCAGATTTTCACAAC
2251 CATATTGAACTGAAGTGCAAAATCTTTTTTTTTTAATTGTTTTTGTTTGGGCAGTACTAAGGATTGAACCTCTGGAAGTCTAGATGAATG
2341 CTCTATCATTGAACTACACTCCAGCACTGGTGTGTATGCACACACAAACACACACACACACACACACACACACGTATATATGTATA
2431 TACAACTAGATAGGTAGATGGGGCTAAAGTAAGATTTAGAAAACTTTGGAATGGAAGCTCTACAGTTCACCAGCTTTTCTTTAGACTAAA
2421 AGATATCATTGACTGGAAAAAATAGACACATAAGGGAAGCCTCCAAGTCAGATTAGTTACTCAAAAAATAGTCCAGGCTGACTGCCTTCA
2611 AGTTGAGTGGTCTTGTGTAGTGAAACACTGCAGGACTCTTGACAGCATTGCCCTCAGAGAGGGAGCTCTGCTAAGATGTTTGTATCCTAT
2701 TTATTTCAAAGTATGAAAGATATATTTTTAACTTATTGTTTACCTGTGGCGTGATGCTTCTGTTAAGCGTTTACACTGTATTGTATAGCT
2791 TTATTTAGTAAAGTGGTCACATAAATCTGCTGGTCACAGTCACATAGCACTCTCTCTCTCTGTTCCATGGCATATATACTCACTGTGCTC
2881 ATTCCTCAGACCTTAAAAGGGAGGGCACATGAATAAGAAAGAACTTGAGGGGTTGGGGATTTAGCTCAGTGGTAGAGCACTTGCCTAGGA
2971 AGCGCAAGGCCCTGGGTTCGGTCCCCAGCTCCGGGAAAAAAAAAAAAAAAAAAAAAAAAA
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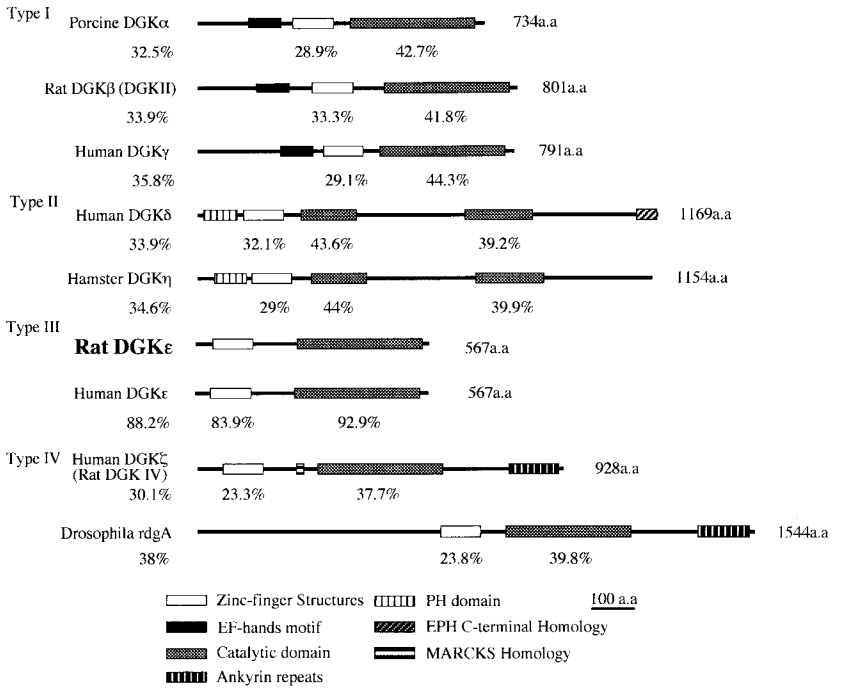

Fig. 3. The schematic structure of DGKs. The structure of rat DGKε is shown with reference to the previously sequenced DGK gene family.

This fragment (DGKR4) contained an additional 78 bp encoding 25 amino acids, which were highly conserved in other DGKs. These results indicate that the deletion in DGKR2 was a cloning artifact, or an alternative form but expressed at a very low level. The clones of DGKR1 ~ 5 are summarized in Fig. 1.

3.2. Analysis of the deduced amino acid sequence for rat DGKε

As summarized in Fig. 2, the composite rat DGKε cDNA sequence was obtained from clones DGKR2, 3 and 5. The initiation ATG of the open reading frame was identified as the first ATG sequence following an in-frame termination codon at nucleotides −23 to −21 from the initiating methionine. This new DGK has 88.2% identity with human DGKε [25]; the zinc finger motifs and catalytic domain showed 83.9 and 92.9% identity, respectively (Fig. 3). We thus believe that it should be a rat counterpart of human DGKε, so that we named this new DGK as rat DGKε. The rat DGKε encodes a putative protein of 567 amino acid residues having a calculated M_r of 64 000. The primary structure displays several characteristic features shared by other DGK isozymes cloned to date (Fig. 3).

3.3. Tissue distribution of rat DGKε mRNA

The expression pattern of the corresponding transcripts was analyzed in a variety of rat tissues by Northern blot analysis. The ~3.3 kb mRNA expression was most abundant in the retina. The mRNA could also be detected in the brain and to a lesser extent in the heart, spleen, lung, kidney and testis (Fig. 4A). On the other hand, its expression in the liver and skeletal muscle was undetectable. The size of the hybridized RNA was consistent with the size of rat DGKε cDNA which we cloned (Fig. 2). In addition to this band, larger species of ~6.5 and ~8 kb were also detected. All three bands were detectable with various probes, including 3′ non-coding region (1501–2329 bp), 5′ coding region (DGKR4), and catalytic domain (DGKR1) (data not shown). Therefore, the ~6.5 and ~8 kb bands, which are much larger than the cDNA we cloned (3204 bp), may be alternatively spliced forms or have a very long untranslated region.

Four other DGK isozymes (DGKI, II, III, IV) have been isolated from rat brain [20–22,27]. DGKIV was expressed

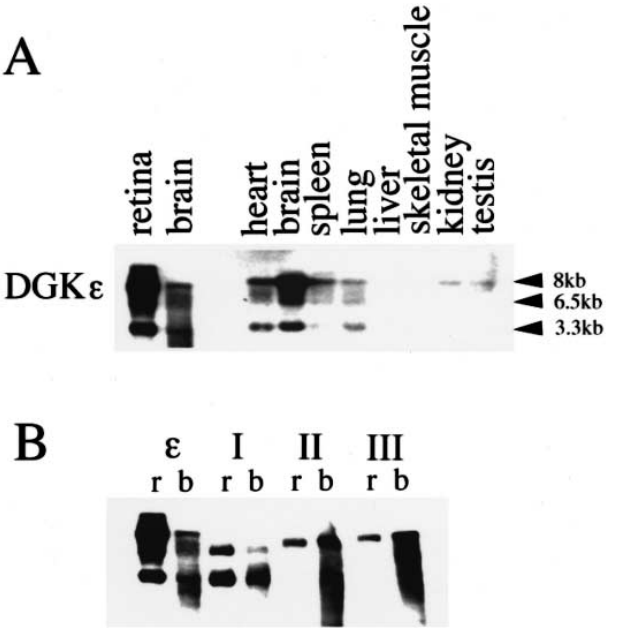

Fig. 4. Tissue-specific expression of rat DGKε. Poly(A)$^+$RNA (4.5 μg each) from retina and brain was analyzed as described under in Section 2. Except for these tissues, commercially available multiple tissue Northern Blot (Clonthech) was employed. A: The filters were hybridized with the 3′ non-coding region (solid bar in Fig. 1). B: The filters were hybridized with rat DGKε (ε), DGKI (I), DGKII (II) and DGKIII (III) specific probes, respectively. r = retina; b = brain.

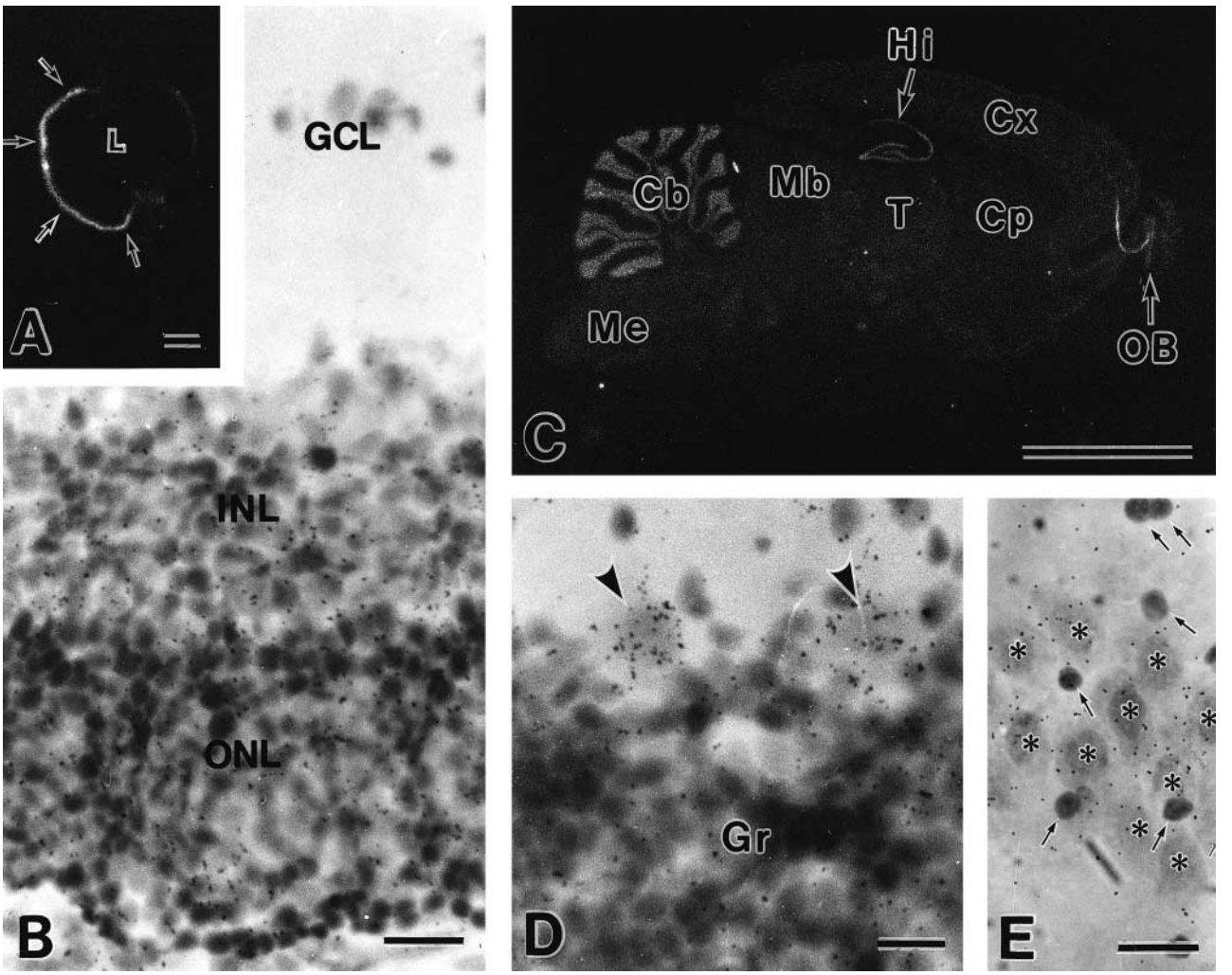

Fig. 5. Expression of rat DGKε in the adult rat eye and brain. A: Eye; arrowheads indicate strong hybridization signals in the retina. B: Retina; hybridization signals were detected in the INL and the ONL. C: Brain. D: Cerebellar cortex. Arrowheads in panel C indicate hybridization signals in the Purkinje cells. E: Hippocampus; arrows indicate hybridization-negative putative glia cells and asterisks indicate pyramidal cells in the CA3. Cb = cerebellum; Cx = cerebral cortex; Cp = caudate putamen; GCL = ganglion cell layer; Gr = granular layer; Hi = hippocampus; INL = inner nuclear layer; L = lens; Mb = midbrain; Me = medulla oblongata; Ob = olfactory bulb; ONL = outer nuclear layer; T = thalamus. Scale bars: A, 2 mm; B,D,E, 20 μm; C, 5 mm.

primarily in thymus and brain and was relatively low in retina [27]. To confirm that rat DGKε is predominantly expressed in retina, the expression of other three DGKs (DGKI, II, III) in retina was also examined by Northern blotting (Fig. 4B). Only rat DGKε was abundantly expressed in retina, and the other three DGKs were not. This feature makes rat DGKε unique among the known DGKs.

3.4. In situ hybridization

Expression of rat DGKε was examined in the retina and brain of adult rats, by in situ hybridization with ^{33}P-labeled antisense oligonucleotide probes (Fig. 5). Higher expression of the rat DGKε mRNA was detected in the retina (Fig. 5A). By bright-field microscopy, inner and outer nuclear layers were labeled, while the ganglion cell layer lacks the signals within the retina (Fig. 5B). The bipolar cells are distributed in the inner nuclear layers (INL) and the photoreceptors are distributed in the outer nuclear layers (ONL). In addition to retinal expression, rat DGKε expression was distributed in various regions of the brain, with higher signals in the cerebellar cortex and hippocampus (Fig. 5C). By bright-field microscopy, the Purkinje cells and granular cells were labeled in the cerebellum (Fig. 5D). In the hippocampus, signals were found over the pyramidal cell layer of Ammon's horn and the granule cell layer of the dentate gyrus (Fig. 5E). The specificity of these signals was verified by identical expression patterns with use of another non-overlapping oligonucleotide probe, and by disappearance of the characteristic signals when hybridization was carried out in the presence of a 20-fold excess amount of unlabeled oligonucleotides (data not shown).

4. Discussion

In this study we have reported the isolation of a novel DGK cDNA (rat DGKε) with a considerable homology to known DGKs in the zinc-finger structures and the catalytic domain. Recently, a human DGKε was isolated and was shown to have a clear selectivity for arachidonoyl-containing species of diacylglycerol [25]. The rat DGKε shared 88.2% identical amino acid sequence with the human DGKε, while

homology is as low as 30.1 ~ 38% with other DGKs (Fig. 3). We thus believe that the rat DGKε is a rat counterpart of the human DGKε. So the rat DGKε may also have selectivity for arachidonoyl-containing species of DAG like human DGKε. However, the expression pattern of the rat and human DGKε was significantly different. Although the rat DGKε was expressed predominantly in retina and was low in testis, the human DGKε was predominantly expressed in testis (retina was not examined) [25].

Four DGK isozymes (I, II, III, IV) have been cloned from rat brain [20–22,27]; DGK I was predominantly expressed in T-lymphocytes and oligodendrocytes, DGK II was preferentially expressed in medium-sized neurons of the caudate putamen of the brain, DGK III was predominantly expressed in cerebellar Purkinje cells, and DGKIV was primarily expressed in thymus and brain but was faint in intestine and retina. By Northern blotting (Fig. 4A,B), only rat DGKε was expressed predominantly in retina. This feature makes the newly isolated rat DGKε unique among the known DGKs, suggesting that rat DGKε may have important functions in retina. Although retinal expression of human DGKε was not examined, it may be expressed predominantly in retina like rat DGKε.

In vertebrate retina, there are two photoreceptor cell types—rods and cones—which are concerned with night and daytime vision, respectively. In rods, it has been shown that cGMP cascade is used for phototransduction. The role of the phosphoinositide cycle and the resulting bifurcating signaling molecules, DAG and IP3, has not been clearly demonstrated in the function of vertebrate retina. The DGK activity was not detected in the bovine rod outer segments [29], and phosphorylation of rhodopsin by PKC and PLC activity in the bovine retina was not light-dependent [30,31]. On the other hand, the light-stimulated turnover of phosphoinositides was suggested to occur in rat retina [14,15], and the activation of PLC by arrestin in the bovine rod outer segment was also suggested to be light-dependent [32]. In contrast, PI signal transduction has been shown to be directly involved in the function of *Drosophila* photoreceptor cells, and many visual mutants were found to harbor abnormal phosphoinositide metabolism. For example, a norpA mutant which has a genetic defect in a retina-specific PLC showed the loss of light-evoked photoreceptor responses (receptor potential) [33,34]. Recently a rat and bovine norpA homologue has been isolated [35,36]. This vertebrate norpA homologue was expressed in cone photoreceptor cells and other retinal neurons [37], which suggests that PI signal transduction may be used in the phototransduction cascade in cones. Furthermore, the critical importance of DGK in the visual function of *Drosophila* has been demonstrated in the rdgA mutant which has a mutated eye-specific DGK gene leading to photoreceptor cell degeneration [11]. It is likely that DGK also participates in the renewal and degradation process of membrane phospholipids in the vertebrate retina, which is directly or indirectly linked to phototransduction. Within the retina, rat DGKε was almost exclusively expressed in inner and outer nuclear layers (Fig. 5B), suggesting the potential importance of DGK in visual functions. Retinal PI responses to light may be associated with photoreceptor functions and/or with neurons which make synapses with these cells.

Recent studies demonstrated that the mGluR family may play a key role in synaptic modulation by specializing their synaptic localization [38]. mGluR1, one of eight subtypes, is linked to PI turnover and subsequent PKCγ activation [39,40]. Activation of mGluR1 is indicated to play an important role in cerebellar long-term depression (LTD) and hippocampal long-term potentiation (LTP) [41,42]. LTD and LTP are thought to be a molecular mechanism of learning and memory. By the electron-microscopic immunogold method, mGluR1 is preferentially located at a perisynaptic position surrounding the postsynaptic density [43]. From in situ hybridization, rat DGKε and mGluR1 mRNAs are expressed in the same region in cerebellar and hippocampus (Fig. 5C,D,E). Therefore, there is a possibility that rat DGKε is in the same cascade as mGluR1 and PKCγ.

To elucidate the rat DGKε functions in the retina and brain, we shall have to perform further investigations into the intracellular localization of DGKε protein.

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