The cloning and developmental regulation of murine diacylglycerol kinase ζ

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Abstract Diacylglycerol kinases (DGKs) regulate the key signaling intermediates diacylglycerol (DAG) and phosphatidic acid (PA). We isolated cDNA clones of mouse diacylglycerol kinase ζ (mDGKζ) and found that it shares 88% identity at the nucleic acid level and 95.5% identity at the amino acid level with human DGKζ (hDGKζ). Murine DGKζ protein rose gradually during embryonic development, and was abundant in newborn and adult brains. By RNA whole-mount in situ hybridization, mDGKζ was shown to be expressed in spinal ganglia and limb buds at low level in E11.5 embryos and at higher level in E12.5 embryos. In E13.5 embryos, DGKζ mRNA was highly expressed in vibrissa follicles, in spinal ganglia, and in the interdigital regions of the developing limbs. Northern blotting showed that DGKζ expression was limited to specific anatomical regions of the brain. Thus, the expression of DGKζ is regulated temporally and spatially during mammalian development and correlates with the development of sensory neurons and regions undergoing apoptosis.

Key words: Diacylglycerol kinase; Embryonic development; Distribution

1. Introduction

Diacylglycerol (DAG) is a key intermediate in the synthesis of complex lipids and serves as an important second messenger. DAG exerts its signaling function by activating protein kinase C (PKC), which in turn regulates many cellular responses including growth, differentiation and apoptosis [1]. In addition, the level of DAG is essential for cellular events such as protein export from the Golgi complex in yeast [2]. The signals from DAG are terminated by its conversion to phosphatidic acid (PA), a reaction that is catalyzed by diacylglycerol kinase(s) (DGK). The role of DGK in regulating DAG levels was established by the observation that overexpression of DGKα decreased the elevated DAG level in *ras*-transformed fibroblasts [3]. In ending the signal(s) from DAG, the DGK reaction generates PA as the product which has been implicated in the regulation of DNA synthesis, in the induction of c-*myc*, c-*fos*, platelet-derived growth factor, in cAMP formation [4–6], and in modulating the activity of *n*-chimaerin [7] and NF1 [8]. Therefore, the DGK reaction occupies an interesting niche – it removes one lipid messenger but creates another. Besides its effect on DAG signaling, the DGK reaction is the first step in the recycling of phosphatidylinositol species following their hydrolysis. Disruption of this pathway can have severe consequences as demonstrated by the *Drosophila rdgA* (DGK2) mutant in which photoreceptor cells degenerate within a week after eclosion [9].

The known eight mammalian DGKs can be divided into five structural subgroups and differ in their expression patterns, activators, and substrate specificity. Type I includes DGKα [10,11], β [12], γ [13] and is defined by the presence of E-F hand motifs at the N-termini, which bind Ca^{2+} with different affinities [14]. DGKα was originally found in lymphocytes and oligodendrocytes, while DGKβ was cloned from brain and was found to be expressed in the olfactory tubercles, nucleus accumbens, caudate and putamen [12]. DGKγ, cloned from a HepG2 library, was found to be enriched in retina [13]. DGKδ [15] and DGKη [16] are type II DGKs, which have a pleckstrin domain (PH domain) at their N-termini. The PH domain has been found in a number of proteins involved in signal transduction and can serve as a site of protein-protein and protein-phospholipid interactions [17–19]. DGKδ was cloned from testis and HepG2 cells, and is primarily expressed in skeletal muscle and testis [15]. The expression of DGKη is found in a broad range of tissues and is regulated by glucocorticoids [16]. The third type of DGK, DGKε [20], has the simplest structure and shows substrate specificity for DAG with an arachidonoyl residue at the *sn*-2 position. DGKε mRNA has been found in retina, brain, testis, heart, spleen and lung [21]. Type IV is typified by DGKζ [22] which has two zinc fingers, four ankyrin repeats at its C-terminus and a unique region homologous to MARCKS phosphorylation site domain. DGKζ is highly expressed in the brain and skeletal muscle. RNA in situ hybridization by using rat brain section showed that DGKζ is expressed in cerebral cortex, hippocampus and olfactory bulb [23]. The *Drosophila* DGK2, *rdgA* gene also belongs to this type IV group and is expressed almost exclusively in the retina [9]. Proteins containing ankyrin repeats are involved in a variety of cellular processes such as gene regulation [24–26] and cell cycle control [27]. The type V DGK, DGKθ, is mostly expressed in brain and small intestine. It contains three cysteine-rich repeats – rather than two – and a ras-binding domain [28].

Here, we report the cloning and characterization of murine DGKζ. We also studied the developmental expression of mDGKζ, and detected the protein as early as on day 10.5 of embryogenesis with a marked change at later stages of development. The spatial distribution of the mDGKζ message was determined by RNA whole-mount in situ hybridization in mouse embryos at various stages of development. We found that DGKζ expression is temporally and spatially restricted, and is highly expressed in tissues involved in sensory function.

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2. Materials and methods

2.1. Isolation and characterization of murine cDNAs

Hybridizations were performed in 5×SSPE, 5×Denhardt's, 0.2% SDS, and 0.1% $Na_2P_4O_7$ at 52.5°C for low stringency and 65°C for normal stringency. Primers for genomic PCR screening are: LD121-2, 5′ CAG CAT CTT AGC TAC ACA GTG CTG 3′ and LD121-3, 5′ ATC ACC AAG TCG GGC CTC CAG 3′.

2.2. Western immunoblots for murine DGKζ protein expression

Mouse embryos of different ages, newborn mouse brain and adult mouse brain were homogenized in 50 mM Tris-HCl pH 8.0, 2 μg/ml pepstatin A, 20 μg/ml leupeptin, 40 μg/ml TLCK, 20 μg/ml aprotinin, 20 μM BH_4, 3 mM DTT, 10 mM CHAPS and 1 mM PMSF. All homogenates were frozen and stored at −70°C until assayed. Samples with ∼100 μg protein were loaded on a 7.5% SDS-PAGE gel and Western blotting was performed as previously described [22]. The protein concentration of each sample was determined by standard BCA protein assay. The β-actin antibody was used as a control to ensure that an equal amount of protein was loaded in each lane. The Western blotting protocol with ECL detection is capable of detecting as little as 1 pg protein (Amersham). For the peptide competition experiment, the primary antibody was incubated with 4 volumes of peptide (0.1 mg/ml) at 4°C for 2 h prior to incubation with the membrane.

2.3. RNA whole-mount in situ hybridization

The RNA whole-mount in situ hybridization was performed as previously described [29]. The mouse strain used was Swiss Webster. The day that a copulation plug was observed was considered embryonic day 0.5 (E0.5). Embryos were collected from timed pregnant females. Embryos were dissected free of extraembryonic membranes in phosphate-buffered saline (PBS).

Two mDGKζ antisense RNA probes were used. The first probe was a 716-bp 3′ *Pst*I/*Eco*RI fragment. It was subcloned into pBluescript II and used to make digoxigenin-labeled RNA probe by in vitro transcription (Boehringer Mannheim). The second one was from the catalytic domain region of the mDGKζ gene. A pair of primers with T7 or T3 promoters attached was used to amplify a 558-bp mDGKζ cDNA fragment. Amplified PCR products were used as templates to make digoxigenin-labeled RNA probes by using an in vitro transcription kit (Boehringer Mannheim). The sequences of the primers were:

5' CAGAGATGCAATTAACCCTCACTAAAGGGAGAGTCTCGAGAAGCCAACCCA
T3
GAG 3',
5' CCAAGCTTCTAATACGACTCACTATAGGGAGACCCTGCTCACCTGGATCCTC
T7
AG 3'.

The probe concentration was 0.5 μg/ml.

2.4. Northern blotting

Human brain Northern blots I and II were purchased from Clontech. Two filters with 2 μg mRNA from different regions of the human brain were probed with a digoxigenin-labeled 761-bp fragment of hDGKζ as described previously [22]. The β-actin cDNA probe was used to ensure that an equal amount of mRNA was loaded in each lane. The detection limit for digoxigenin-labeled riboprobe is around 0.1 pg according to manufacturer estimation (Boehringer Mannheim).

3. Results

3.1. Isolation and characterization of murine cDNA and genomic clones

We screened a mouse brain cDNA library with a *Sma*I-*Hin*dIII probe derived from the hDGKζ cDNA by low stringency hybridization. The first identified clone (3-2) differed from any known mouse DGKs and showed a high percentage of sequence identity with hDGKζ, suggesting it was the murine homolog of hDGKζ. The translation initiation site was not found either in this clone or others (7-1 and 8-1) isolated from another two rounds of screening. A 500-bp mouse genomic fragment was amplified by polymerase chain reaction (PCR) and used to isolate four P1 genomic clones. Splicing patterns were conserved between hDGKζ [30] and mDGKζ as demonstrated by reverse transcriptase-PCR. As summarized in Fig. 1A, the composite mDGKζ sequence was obtained from mouse cDNA clone 3-2, 7-1, 8-1 and the mouse P1 genomic clones. The initiation ATG of the open reading frame was identified as the first ATG sequence following with an in-frame stop codon 9-bp upstream. The first zinc finger of the mDGKζ protein includes the sequence $HX_{11}CX_6CX_{12}CX_2CX_4HX_2CX_{10}C$ and the second includes $HX_{11}CX_2CX_{19}CX_2CX_4HX_4CX_9C$. The cysteine and histidine residues of the two zinc fingers are completely conserved between hDGKζ and mDGKζ proteins. The MARCKS phosphorylation site is conserved between hDGKζ and mDGKζ proteins except for a (m)Arg^{270} to (h)Lys^{269} replacement in the mDGKζ protein. In addition, the catalytic domain of the mDGKζ protein is similar to all known DGKs. The main feature that distinguishes DGKζ is the ankyrin repeats, and the deduced mDGKζ protein, like the human protein, has four such repeats (Fig. 1B). Mouse DGKζ shares 88% and 95.5% identity with the human sequence at the nucleotide and amino acid level, respectively.

3.2. Immunodetection of the murine DGKζ protein: the DGKζ protein accumulates during late embryogenesis and post-natal development

Human DGKζ and murine DGKζ proteins have an identical peptide sequences at their C-termini, thus, we were able to use a previously described antibody [22]. This antibody recognized a ∼120-kDa protein in homogenates of the mouse brain, and pre-incubation with the peptide antigen block the recognition indicating specific binding (Fig. 2A). We next examined the expression of mDGKζ during embryonic development by performing Western immunoblots of homogenates from whole embryos and from brains of newborn and adult mice. The mDGKζ protein was detected in the homogenates of E10.5 embryos, and the protein level increased gradually during development (Fig. 2B). A band (indicated by an ar-

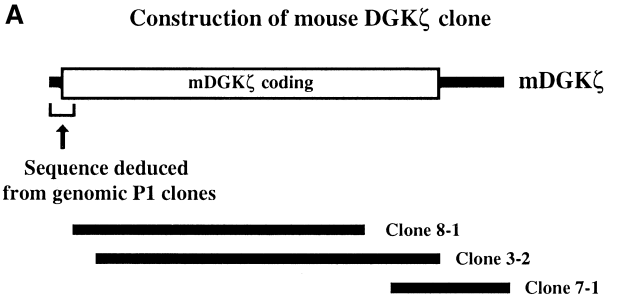

Fig. 1. Sequence of the murine DGKζ cDNA. A: The overlapping map of representative clones. The cDNA and genomic clones were isolated as described (Section 3). The clones were sequenced in an automated ABI system. B: The nucleic acid sequence and deduced amino acid sequence of mDGKζ. The zinc fingers are underlined, and conserved cysteine and histidine residues are marked with *. Serine residues within the MARCKS homology region are marked by +. The residues within the ATP binding motif are double underlined. The ankyrin motifs are displayed within boxes.

B

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CGGTGCGGAGCCGGCGTGCTGAGCCCCGGCCGCCGGCCCGGCATGGGCGTCTCCCGCGGG 60
CCCGCCGCCGGCCGGGGCTAGGGCCTGATGGAGCCGCGGGACCCCAGCCCCGAGGGCCGG 120
                          M  E  P  R  D  P  S  P  E  G  R

AGCAGCGACTCGGAGTCGGCCTCCGCCTCGTCCAGCGGCTCCGAGCGCGACGCGGGTCCG 180
 S  S  D  S  E  S  A  S  A  S  S  S  G  S  E  R  D  A  G  P

GAGCCCGACAAGGCGCCGCGGCGCCTGACCAAGCGGCGCTTCCCGGGGCTGCGGCTCTTT 240
 E  P  D  K  A  P  R  R  L  T  K  R  R  F  P  G  L  R  L  F

GGGCACAGGAAAGCCATCACCAAGTCGGGCCTCCAGCACCTGGCACCCCCTCTTCCCACG 300
 G  H  R  K  A  I  T  K  S  G  L  Q  H  L  A  P  P  L  P  T

CCCGGGGCCCCGTGCGGTGAATCTGAGGAGCAGATCCAGAGCACTGTGGACTGGAGTGAG 360
 P  G  A  P  C  G  E  S  E  E  Q  I  Q  S  T  V  D  W  S  E

TCAGCAGTGTATGGGGAGCACATCTGGTTTGAGACCAACGTGTCCGGTGACTTCTGCTAT 420
 S  A  V  Y  G  E  H  I  W  F  E  T  N  V  S  G  D  F  C  Y
                   *                       *

GTCGGGGAGCAGCACTGTGTAGCTAAGATGCTGCCGAAGTCAGCGCCCAGAAAAAAGTGT 480
 V  G  E  Q  H  C  V  A  K  M  L  P  K  S  A  P  R  K  K  C
                   *                       *

GCAGCCTGTAAGATCGTGGTGCATACCCAATGCATTAAGCAGCTGGAAAAGATCAATTTC 540
 A  A  C  K  I  V  V  H  T  Q  C  I  K  Q  L  E  K  I  N  F
       *           *        *

CGCTGTAAGCCGTCCTTCCGCGAATCAGGCTCCAGGAATGTCCGTGAGCCAACCTTCGTA 600
 R  C  K  P  S  F  R  E  S  G  S  R  N  V  R  E  P  T  F  V
    *

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 R  H  H  W  V  H  R  R  R  Q  D  G  K  C  R  H  C  G  K  G
    *                             *        *

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 F  Q  Q  K  F  T  F  H  S  K  E  I  V  A  I  S  C  S  W  C
                                                *        *

AAACAGGCATACCACAGCAAGGTGTCGTGCTTCATGATGCAACAGATTGAGGAACCCTGC 780
 K  Q  A  Y  H  S  K  V  S  C  F  M  M  Q  Q  I  E  E  P  C
          *           *                          *

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 S  L  G  V  H  A  A  V  V  I  P  P  T  W  I  L  R  A  R  R

CCCCAGAACACCCTCAAGGCCAGCAAGAAGAAAAAGAGAGCGTCCTTCAAGAGGAGGTCC 900
 P  Q  N  T  L  K  A  S  K  K  K  K  R  A  S  F  K  R  R  S
                                           +           +

AGCAAGAAAGGACCTGAGGAAGGCCGCTGGAGACCCTTCATCATCAGACCCACCCCATCC 960
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 +

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 P  L  M  K  P  L  L  V  F  V  N  P  K  S  G  G  N  Q  G  A

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Fig. 1 (*continued*).

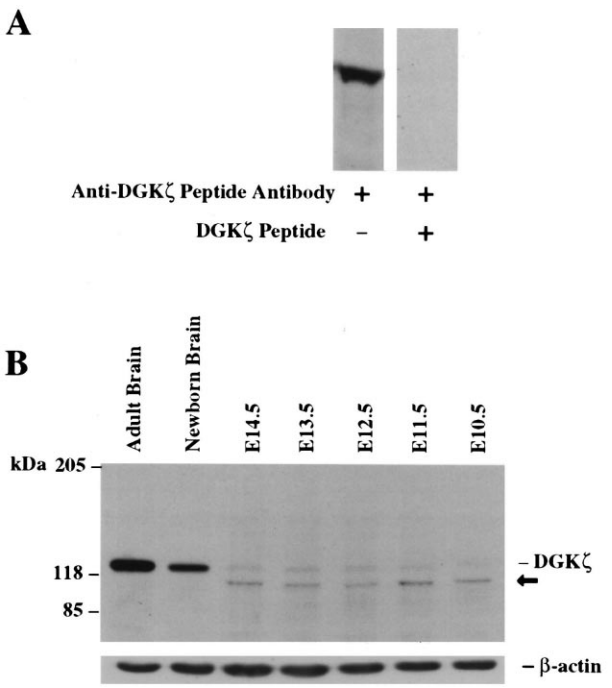

Fig. 2. The mDGKζ protein level changes during mouse embryogenesis. A: 100 μg of protein of mouse brain homogenate was loaded in each lane and probed with the affinity purified anti-DGKζ antibody at a concentration of 1 μg/ml. In the control experiment, pre-incubation with the immunogen-peptide (4 volumes 0.1 mg/ml peptide+1 volume 1 mg/ml antibody) significantly blocked the recognition of the DGKζ protein. B: Embryos at different stages were dissected and homogenized. 100 μg of protein from embryo homogenates, newborn brain and adult brain homogenates was loaded in each lane. They were probed with the affinity purified anti-DGKζ antibody. The β-actin antibody was used as a control to show that an equal amount of protein was loaded in each lane. This is a representative result from five independent experiments. ←

row), ~5–10 kDa smaller than the full-length protein, was detected in embryo homogenates and its recognition was also blocked by pre-incubation with the peptide antigen. We speculate that it is a proteolytic product since its intensity was variable and a PEST sequence was found in the N-terminus of DGKζ [22]. In five independent experiments, we found a high level of DGKζ protein in the brain of newborn mice, and a further two-fold increase at the DGKζ protein level in adult mouse brain. The DGKζ signal was normalized to β-actin (Fig. 2B).

3.3. *Patterns of DGKζ expression: examination of DGKζ by RNA whole-mount in situ hybridization indicates that DGKζ is expressed predominantly in sensory structures*

After finding the change in DGKζ expression during development, we examined its tissue distribution by in situ hybridization for mRNA on whole-mount preparations of mouse embryos. Antisense and sense probes to mDGKζ were synthe-

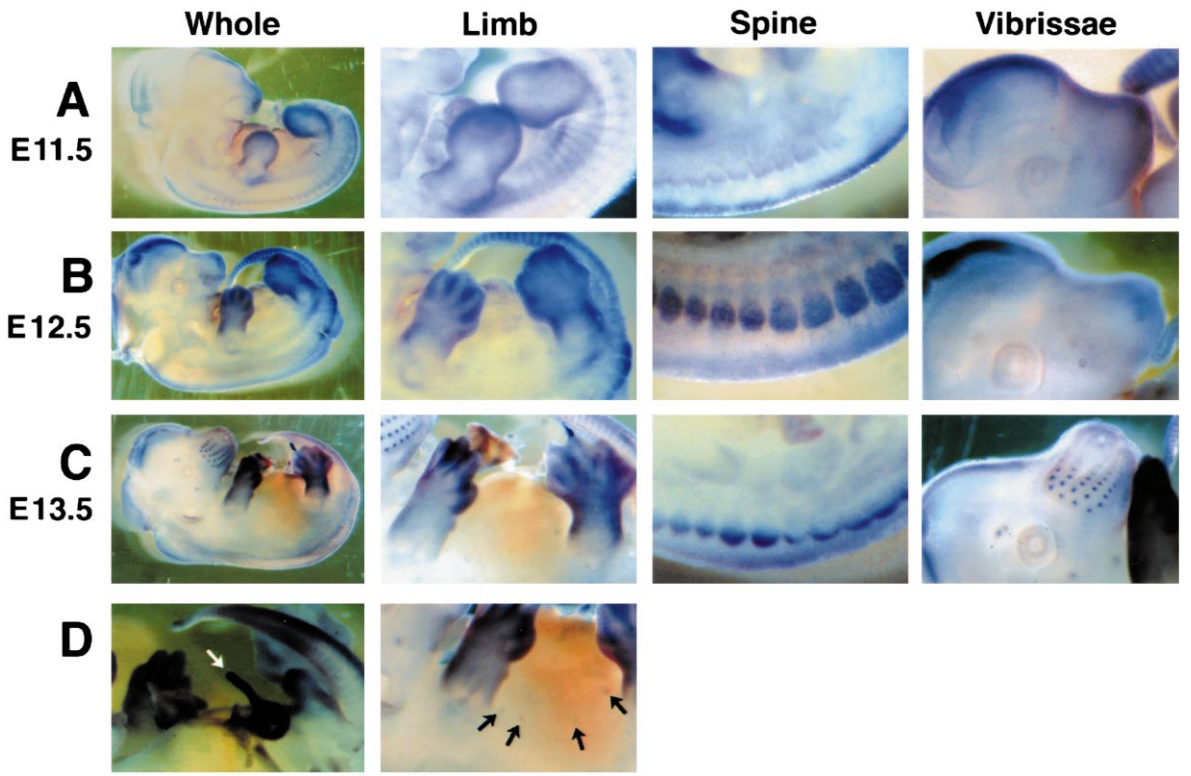

Fig. 3. Murine DGKζ is highly expressed in the sensory nerve system during mouse embryogenesis. Mouse embryos at different stages were hybridized with DGKζ antisense and sense digoxigenin-labeled probes as described in Section 2.3 (blue-purple staining represents the positive signals). The DGKζ sense probe gave no specific staining. A: Low level expression of DGKζ in somites, limb buds and spinal ganglia in E11.5 day embryos. B: Strong expression of DGKζ in spinal ganglia, limb buds and low level expression in the follicles of vibrissae in E12.5 day embryos. C: Constitutively high level expression in limbs and spinal ganglia, and the follicles of vibrissa in E13.5 day embryos. D: Expression of DGKζ in umbilical vessels and nipple primordia.

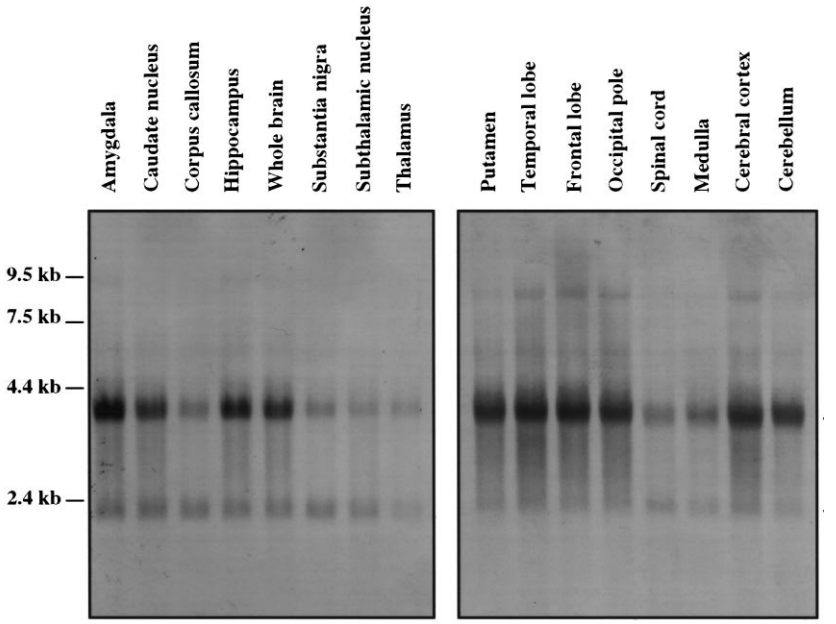

Fig. 4. Analysis of DGKζ mRNA from different regions of the human brain. Two filters with 2 μg mRNA from different brain regions were probed with a fragment of the hDGKζ. A sample from whole brain shows a band at 3.7 kb. The expression level of DGKζ varies among different regions of brain, high in cerebral cortex, cerebellum, hippocampus, occipital pole, frontal lobe, temporal lobe, putamen, amygdala and caudate nucleus, and low in spinal cord, medulla, corpus callosum, substantia nigra, subthalamic nucleus and thalamus.

sized and used to examine the expression of DGKζ in E10.5, E11.5, E12.5, and E13.5 embryos. In E10.5 embryos, we could not define a tissue-specific pattern of expression because the signals were not sufficiently above the background. In E11.5 embryos, we observed low level expression in somites, spinal ganglia, and limb buds (Fig. 3A). We detected much stronger expression of DGKζ in spinal ganglia and limb buds in E12.5 embryos (Fig. 3B). Additionally, very strong expression was found in the interdigital regions of the limb where cells are undergoing apoptosis to create the fully developed digits. DGKζ staining was very strong in spinal ganglia and highlighted the segmentation of the embryo. Interestingly, the spinal cord was not highly labeled (Fig. 3B). The dorsal root ganglia are comprised of the cell bodies of sensory neurons and our results demonstrate that DGKζ is highly expressed in them. When we examined E13.5 day embryos, we found that DGKζ was highly expressed in the follicles of the vibrissae, in spinal ganglia, and in limb buds (Fig. 3C). The vibrissa follicle contains venous sinuses and sensory nerve fibers within a connective tissue sheath and is first observable during the 13th embryonic day. The strong DGKζ staining in the follicles of the vibrissae of E13.5 embryos suggests that DGKζ is expressed from the initiation of vibrissae. We also observed staining in the umbilical vessels of E13.5 embryos (Fig. 3D), which is noteworthy since hDGKζ was originally cloned from an umbilical vein endothelial cell library [22]. Interestingly, expression of DGKζ also was detected in the nipple primordia of E13.5 embryos (Fig. 3D). We observed DGKζ staining in the brain of the embryos, but the precise anatomical localization of the signal was not easily revealed by this technique. Both probes mentioned in Section 2.3 gave us identical staining patterns. In all cases hybridization with the sense probe was performed to confirm that the pattern observed with the antisense probe was specific.

3.4. Northern blotting of isolated regions of brain defines specific patterns of expression

To refine the localization of DGKζ expression in the brain, we used Northern blotting to examine mRNA samples from specific regions of human brain. The membranes were hybridized with a 761-bp digoxigenin-labeled hDGKζ probe. The hybridization was performed as previously described [22]. We detected a band at 3.7-kb position in the whole brain sample, as expected. Interestingly, the expression level varied among the different regions with strong expression in cerebellum, cerebral cortex, and hippocampus. In contrast, the expression of DGKζ in the spinal cord, medulla of the brain, and thalamus was low (Fig. 4). A β-actin probe was used as a control to confirm the same amount of the mRNA loaded and the integrity of the mRNA in each lane demonstrating the same amount of mRNA from different regions of brain was loaded. These results are consistent with the in situ hybridization experiment in which we did not detect significant staining in the spinal cord of embryos of different ages. Thus, DGKζ expression is limited to specific regions of the human brain.

4. Discussion

To study the developmental regulation and functions of DGKζ, we cloned the mDGKζ and analyzed the expression of this gene during mouse embryogenesis. Murine DGKζ has highest sequence similarity with hDGKζ (97%) and *Drosophila* DGK2, *rdgA* (49%) at the amino acid level. The deduced mDGKζ protein has the domain motifs that define the type IV DGKs: two cysteine-rich repeats, a conserved catalytic domain, and four ankyrin repeats at the C-terminus. Like the other members of the DGK family, mDGKζ shares the greatest homology with other DGKs in the catalytic domain. Within that domain DGKζ has the conserved putative ATP

binding site GXGXXG, of which a mutation in the second glycine in *Drosophila* DGK2 causes the retinal degeneration phenotype [9].

We studied mDGKζ expression in embryos from E10.5 to E14.5 and found that the expression increases gradually during embryogenesis. We observed that brains of both newborn and adult mice have abundant DGKζ protein suggesting that DGKζ may be involved in neuronal development and function. We determined the location of the DGKζ mRNA in developing embryos and found that it is highly expressed in the sensory nerve system including the dorsal root ganglia and vibrissa follicles. The expression pattern of DGKζ shows similarity to the expression pattern of the *patched* regulatory gene at some stages [31]. *Patched* is a part of the hedgehog signaling pathway, which is down-regulated by protein kinase A. There is no known relationship between this pathway and the DGK-mediated signaling, but our findings show they have overlapping expression during development. This suggests a potential interaction between them but more experimental evidence is needed to distinguish whether this indicates functional relationship. Our whole-mount in situ hybridization method was not sufficiently sensitive to define subregions of the mouse embryonic brain, so we used samples from different anatomical regions of the human brain to test whether the DGKζ mRNA is limited to specific regions of the brain. DGKζ is highly expressed in the sensory nervous system, while expression is low in other areas, particularly the spinal cord.

Our data demonstrate that the expression of DGKζ is subject to strict temporal and spatial regulation during mammalian development. Its tissue-specific expression pattern is characterized by selective expression in many sensory components of the nerve system. At the same time, the expression of DGKζ overlaps significantly with the pattern observed with other DGK isoforms [12,21,28]. Although DGK isoforms differ in substrate specificity and cofactor usage, when two or more DGK isoforms are expressed in the same cells, they may have complementary functions, or act in concert to regulate the level of DAG and/or other important lipid messengers. They may also be functionally redundant in these cells, meaning one can compensate for the functional loss of the other. On the other hand, DGKζ may have unique and indispensable functions especially where it is expressed alone or at a much higher level than other DGK isoforms. Taken together, it is likely that different DGK isoforms have unique functions, but also may act cooperatively to control cellular events such as growth and differentiation.

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