Cloning and Characterization of Diacylglycerol Kinase \( \tau \) Splice Variants in Rat Brain*

Received for publication, July 12, 2003, and in revised form, February 26, 2004
Published, JBC Papers in Press, March 15, 2004, DOI 10.1074/jbc.M312976200

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Diacylglycerol kinase (DGK) catalyzes phosphorylation of a second messenger diacylglycerol (DG) to phosphatidic acid in cellular signal transduction. Previous studies have revealed that DGK consists of a family of isozymes including our rat clones. In this study we isolated from rat brain cDNA library the cDNA clones for a rat homologue of DGK (\( r \)DGK\(-\tau \) ) that contains two zinc finger-like sequences, the highly conserved DGK catalytic domain, a bipartite nuclear localization signal, and four ankyrin repeats at the carboxyl terminus. In addition, we found novel splice variants, which contain either insertion 1 (71 bp) or insertion 2 (19 bp) or both in the carboxyl-terminal portion. Each of the insertions causes a frameshift, and the resultant premature stop codons produce two truncated forms (termed rDGK\(-\tau \)2 and \( \tau \)3), the former lacking the ankyrin repeats at the carboxyl terminus and the latter lacking a part of the catalytic domain and the ankyrin repeats. Truncation of the carboxyl-terminal portion clearly exerts effects on the detergent solubility and enzymatic activity of the splice variants, although all three variants showed similar cytoplasmic localization in cDNA-transfected cultured neurons despite the continued presence of the nuclear localization signal sequence. Immunoblot analysis using anti-rDGK antibody raised against the common amino-terminal portion clearly shows that these rDGK\(-\tau \) variants are indeed expressed in the brain. These results suggest that the carboxyl-terminal truncated forms of rDGK\(-\tau \) and \( \tau \)3 that exhibit reduced enzymatic activities might show a dominant negative effect against the intact rDGK\(-\tau \), and that the modulation of signal transduction by the splice variants may play some roles in the physiologic and/or pathologic conditions of neurons.

Extracellular signals, such as growth factors and neurotransmitters, trigger cascades of molecular changes at the plasma membrane, and many of them evoke a transient increase in levels of cellular diacylglycerol (DG)\(^1\) through hydrolysis of phosphatidylinositol 4,5-bisphosphate by phospholipase C (1). This rapid rise in cellular DG results in allosteric activation of protein kinase C (PKC), which mediates many cellular responses through phosphorylation of target proteins (2). Prolonged and excessive activation of PKC stimulated by proto-oncogene-related cascade leads to pathological conditions, which is commonly seen in transformed cells (2). This is supported by the fact that phorbol esters, which substitute for DG as signal mediators, serve as potent tumor promoters by persistently activating PKC (3). The functional significance of DG is not restricted to the PKC pathway; recently there has been evidence indicating that DG also activates several proteins including RasGRP (4), Unc-13 (5), the chimerins (6), and some mammalian homologues of transient receptor potential potential proteins (hTRPC3 and hTRPC6) (7), suggesting that DG is implicated in cellular events more widely than previously thought. Therefore, DG level must be strictly controlled to maintain the normal state.

Diacylglycerol kinase (DGK), which catalyzes phosphorylation of DG to phosphatidic acid (PA), is thought to be a key enzyme in the regulation of DG levels and, as a result, to be responsible for attenuating the activation of PKC (8). Furthermore, PA, the product of DGK, may also serve as a lipid second messenger, which has been reported to regulate a growing list of signaling proteins, including PKC-\( \tau \) (9), phosphatidylinositol-4-phosphate kinase (10), phospholipase C-\( \gamma \) (11), and many other molecules. Recently it was shown that PA directly interacts with the mammalian target of rapamycin, which governs cell growth and proliferation by mediating the mitogen- and nutrient-dependent signal transduction that regulates messenger RNA translation in a variety of cells (12). Thus, DGK is thought to be one of the key enzymes closely involved in the lipid-mediated cellular signaling events.

Because 80-kDa DGK was first purified and cloned from porcine brain and thymus cytosol (13, 14), many DGK isozymes have been isolated from various animal sources (reviewed in Refs. 15–17). These isozymes have been described to differ from each other with respect to the molecular masses, enzymological properties, activators, and substrate specificities. It is thus likely that these isozymes are operated under distinct regulatory mechanisms. Among these, DGK\( \tau \) was initially isolated

* This work was supported by grants-in-aid from the Ministry of Education, Science, Culture, and Sports of Japan and from Yamagata Health Supports and Taisho Pharmaceutical (to K. G.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AB058962, AB058963, and AB058964.

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1 The abbreviations used are: DG, diacylglycerol; NLS, nuclear localization signal; DGK, diacylglycerol kinase; r, rat; h, human; PA, phosphatidic acid; PKC, protein kinase C; RACE, rapid amplification of cDNA ends; MOPS, 4-morpholinoethanesulfonic acid; CDK6, cyclin-dependent kinase 6.
from the human cDNA library and revealed to belong to class IV of the DGK family because it contains four ankyrin repeats at its carboxyl terminus similar to DGKζ (18). This isoform was expressed only in the brain and retina in human tissues and was found to be in both the cytoplasm and nucleus by immunoblot analysis of the cDNA-transfected COS-7 cells (18).

In this study we isolated cDNA clones for a rat homologue of DGKζ (rDGKζ-1) that contains two zinc-finger-like motifs, the highly conserved DGK catalytic domain, a bipartite nuclear localization signal (NLS), and four ankyrin repeats at the carboxyl terminus. Furthermore, we found novel splice variants, which either contain the insert 1 (71 bp) or the insertion 2 (19 bp) or both in the carboxyl-terminal portion. Each of the insertions causes a frameshift, and the resultant premature stop codons produce two truncated forms (termed rDGKζ-2 and rDGKζ-3), the former lacking the ankyrin repeats at the carboxyl terminus and the latter lacking a part of the catalytic domain in addition to the ankyrin repeats. Characterization of these variants with respect to the primary structure, detergent solubility, catalytic activity, and subcellular localization, together with the immunoblot data on the endogenous enzymes of the brain, suggests that the carboxyl-terminal truncated forms of rDGKζ-2 and rDGKζ-3 that exhibit reduced enzymatic activities might show a dominant negative effect against the intact rDGKζ-1.

**EXPERIMENTAL PROCEDURES**

**cDNA Cloning—** An oligo(dT)-primed agt10 adult rat brain cDNA library (6 × 10⁶ colonies) was screened with mixed cDNA probes encoding the highly homologous catalytic regions of rat DGKα, β, γ, and ζ under low stringency hybridization: 30% formamide, 5 × SSPE for 3 hr. One positive clone, pRMI, was obtained that was different from those of the previously cloned rat DGK isoforms but partly homologous to them. We obtained several other clones of different length by the second round screening using a fragment of pRMI as a probe under high stringency hybridization at 42 °C in the same solution described above, except 50% formamide was used instead of 30%. Sequencing was performed on both strands by the PerkinElmer Life Sciences 310 DNA autosequencer.

**Rat Brain cDNA Library Synthesis and 5′-RACE—** Rat brain mRNA (3 μg) was used to generate double-stranded cDNA using the Marathon cDNA amplification kit (Clontech Laboratories, Inc., Palo Alto, CA) according to the manufacturer’s instructions. The cDNA was ligated with the Marathon cDNA adaptor. 5′-RACE was performed using the cDNA as a template with rDGKζ gene-specific antisense primer RMI-1 (5′-TCTCTCACATGGATCGTCCAG-3′) and the adaptor primer AP1 or AP2 of the kit. Additional gene-specific antisense primers, RMI-2 (5′-GTCCCGTCTTACCTGTCGTACG-3′), RMI-3 (5′-GGCCGACATAACAGACGGATCG-3′), RMI-4 (5′-GTGTGATCGCGAAGACGATGTC-3′), RMI-5 (5′-GTCCTGCGGCAATGTGCTG-3′), RMI-7 (5′-GGATTAAGCTTACAGCTCCTCC-3′), RMI-8 (5′-GCGGAAAGAAGCCGAGCCG-3′), and RMI-9 (5′-GTCCTGCTTCACCTGCGACG-3′) were generated based on the sequences of progressively amplified 5′-RACE products.

**RNA Extraction and Northern Blot Analysis—** Total RNA was extracted from several tissues of adult Wistar rats as described previously (19–22). Each of the total RNA samples (20 μg per lane) was denatured with formaldehyde and size-separated by agarose gel electrophoresis. The RNA was transferred and fixed to a nylon membrane (Hybond-N, Amersham Biosciences) and hybridized with a 0.7-kb probe containing a 3′-noncoding sequence (nucleotides 3094–3579, GenBank™ accession number U12290) labeled with [α-32P]dATP. Sites of antigen-antibody reaction were visualized using the biotinylated secondary antibody and streptavidin-Alexa 488 Fluor conjugates (Molecular Probes). FLAG-tagged rDGKζ were photographed with a Leica fluorescence microscope and a confocal laser-scanning microscope (model TCS 4D, Leica Microsystems, Mannheim, Germany).

**Expression Ratio of the Splice Variants—** Two primer pairs, PS1 (5′-GAATCCGGAGAAGAATTCGAG-3′)-PS2 (5′-CAGAGCGATTGCCGACACATAA-3′) and PS3 (5′-GTTCGCCCAGGGTGGTCTCTGTCACA-3′)-PS4 (5′-GCGGAAAGAAGCCGAGCGG-3′), were designed to bracket insertion 1 and insertion 2, respectively. PCR amplification DNA for 2 h at room temperature. Hybridization was performed at 42 °C for 16 h in a moist chamber in the same solution (50 μl/mole), which contained an additional 10% dextran sulfate and 0.5–1.0 × 10⁶ cpm of the same cDNA probe as used for Northern analysis labeled with [α-32P]dATP by nick translation or the same amount of a control probe (pBR322, the plasmid vector). After hybridization the slides were sequentially rinsed in 2 × SSC, 0.1% sarcosyl at room temperature for 30 min, three times in 0.1 × SSC, 0.1% sarcosyl at 42 °C for 40 min each, and dehydrated in 70 and 100% ethanol. The sections were exposed to Hyperfilm-βmax (Amersham Biosciences) for 2–3 weeks, then dipped in Kodak NTB2 emulsion, and exposed for 2 months.

**COS-7 Cell Transfection, Immunoblot, and DGK Activity Assay—** The cDNAs for the entire length of three rDGKζ variants were subcloned into the pASig vector (Invitrogen) and each rDGKζ-2 was composed of eight amino acids (FLAG marker peptide, Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys, by Eastman Kodak Co.) at the 5′ end of the coding region of the cDNAs. COS-7 transfection (4 μg of plasmid per 10-cm dish) was carried out using LipofectAMINE 2000 reagent (Invitrogen), and all steps were according to the protocol provided by the manufacturer. After incubating for 2–3 days, cDNA-transfected COS-7 cells were harvested and lysed by sonication in lysis buffer containing 20 mM Tris-HCl (pH 7.4), 0.25 M sucrose, 1 mM EDTA, 4 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 20 μg/ml leupeptin. After a low speed centrifugation (550 × g) for 5 min, protein concentration was determined using BCA Protein Assay Reagent (Pierce) according to the protocol provided by the manufacturer. Samples from transfected COS-7 cells (20 μg per lane) were subjected to SDS-10% PAGE. The proteins were then electrophoretically transferred to a polyvinylidene difluoride membrane. Immunoblotting was performed with anti-FLAG M2 antibody (Sigma) specific for the FLAG marker peptide. The immunoreactive band was detected using an enhanced chemiluminescence detection system (Amersham Biosciences).

For assaying DGK enzymatic activity, the amounts of the expressed proteins (rDGKζ-1, -2, and -3) were equalized by means of densitometric indication of the immunoreactive bands, and the protein concentrations were also adjusted. An equal amount of each lysate was used to measure DGK activity by the octyl glucoside mixed micelle assay as described previously (20, 23). The reaction mixture (20 μl) contained 50 mM MOPS (pH 7.2), 50 mM octyl glucoside, 100 μM NaCl, 1 mM dithiothreitol, 20 mM NaF, 5 mM phosphatidylserine (15.6 mol %) (Sigma), 2 mM 1,2-dioleoyl-sn-glycerol (6.25 mol %) (18:1/18:1 DG, Sigma), and 1 μM 6-[32P]PATP (10,000 cpm/nmol; ICN Biomedicals, Costa Mesa CA). In some experiments, 1-stearyl-2-oleoyl-sn-glycerol (18:0/18:2 DG, Biomol, Plymouth Meeting, PA) and 1-stearyl-2-arachidonyl-sn-glycerol (18:0/20:4 DG, Biomol) were also used as substrates. The reaction was initiated by adding lystate (10 μl) and continued for 3 min at 30 °C. Lipids were extracted from the mixture, and phosphatidic acid separated by thin layer chromatography was scraped and counted by liquid scintillation as described previously (20, 23).

**Expression Ratio of the Splice Variants—** To determine the expression ratio of the splice variants, transfected COS-7 cells were disrupted by sonication in lysis buffer as described above. Cell debris was removed by a centrifugation (550 × g, 10 min). The supernatant was centrifuged at 100,000 × g for 1 h to separate soluble (supernatant) from particulate (pellet) fractions consisting of membranes and cytoskeleton. The pellet was resuspended in an equal volume of Triton X-100 (1%) containing lysis buffer described above and centrifuged (100,000 × g, 30 min). Resultant pellet was resuspended in an equal volume of the same buffer and was used as Triton-insoluble particulate fraction. The supernatant contained dissolved membranes, and the pellet contained the cytoskeleton (24).

**Subcellular Localization in the cDNA-transfected Cultured Neurons—** Hippocampal neurons were isolated from newborn rats at postnatal day (P0) and cultured in B27-supplemented NEUROBASAL™ (Invitrogen) containing 0.5 mM glutamic acid (25). FLAG-tagged rDGKζ variants and rDGKζ were expressed in cultured hippocampal neuronal cells by calcium phosphate transfection (Clontech). After 24 h, the cells were fixed with 2% paraformaldehyde and incubated with an antibody against neuron antibody-reaction products, followed by using the biotinylated secondary antibody and streptavidin-Alexa 488 Fluor conjugates (Molecular Probes). FLAG-tagged rDGKζ were photographed with a Leica fluorescence microscope and a confocal laser-scanning microscope (model TCS 4D, Leica Microsystems, Mannheim, Germany).

**Interaction Ratio of the Splice Variants—** Two primer pairs, PS1 (5′-GAATCCGGAGAAGAATTCGAG-3′)-PS2 (5′-CAGAGCGATTGCCGACACATAA-3′) and PS3 (5′-GTTCGCCCAGGGTGGTCTCTGTCACA-3′)-PS4 (5′-GCGGAAAGAAGCCGAGCGG-3′), were designed to bracket insertion 1 and insertion 2, respectively. PCR amplification...
immunized by subcutaneous multiple injections of 400 µg of the protein provided by the manufacturer, and was used as an antigen. Rabbit sera were obtained after the fifth injection was used for immunization. Rabbits were injected with the supernatant fraction was purified by glutathione-Sepharose column chromatography (Amersham Biosciences), according to the protocol provided by the manufacturer, and was used as an antigen. Rabbits were immunized by subcutaneous multiple injections of 400 µg of the fusion protein emulsified with an equal volume of Freund’s complete adjuvant, and the sera obtained after the fifth injection was used for antibody. Rat brain was homogenized in a buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and Complete Protease Inhibitor Mixture (Roche Applied Science). After removal of undissrupted cells by a low speed centrifugation (550 x g, 10 min), the homogenate (30 µg) was subjected to immunoblot analysis using anti-rDGK antibody. The immunoreactive band was detected as described above.

RESULTS

Isolation of cDNA Encoding Rat DGK—We first screened the rat brain cDNA library by low stringency hybridization with labeled cDNA fragments of rat DGK isoforms, and we obtained a cDNA clone encoding a putative DGK. Second round screening with the cloned fragment gave us the other cDNA clones of different lengths. Subsequently, the full-length sequence was obtained by using 5′-RACE. The composite cDNA contained a 3153-nucleotide open reading frame and an in-frame stop codon 150 bp upstream from the ATG site (Fig. 1). The deduced amino acid sequence encoded a protein of 1050 amino acids with a predicted molecular mass of 115 kDa, which was 80% identical in amino acid sequences to rDGK. Because data base search revealed it to be 91% identical to human DGK and 98% to mouse DGK, we regarded this to be a rat homologue of the human enzyme. Like rDGK (22) and hDGK (18), rat DGK (rDGK) contained the conserved cysteine-rich, zinc finger-like sequences near the amino terminus and four ankyrin repeats at the carboxyl terminus, in addition to the typical DGK catalytic domain, but lacked the EF-hand motifs. All of the structural features in rDGK were characteristic of the class IV enzyme. NLS of a bipartite type was also included in rDGK, as in rDGK, which contains two clusters of basic amino acids, residues 323–324 (KK) and residues 335–338 (KKKR), located next to the second zinc finger-like sequences (Fig. 1). Furthermore, the amino acid residues (-ETAV) at the carboxyl-terminal end of rDGK were consistent with the class I PDZ domain ligand consensus (S/T/XV) that is also found at the carboxyl-terminal end of DGK (26). A comparison of the primary structure between rDGK and rDGK showed high identity in catalytic domain (81%) but relatively low identities in the amino (28%) and carboxyl termini containing four ankyrin repeats (44%) (Fig. 2).

Tissue Distribution and mRNA Localization in the Brain—In Northern blot analysis, rDGK mRNA was detected exclusively in the brain and eye among the 11 tissues examined as a major band of 3.3 kb and a weak band of about 9.6 kb. The testis contained a short species of 1.2 kb, instead of those two bands, which was of unknown origin (Fig. 3A). Such a confined distribution pattern was quite different from that of rDGK, which is widely and abundantly expressed throughout the body (22). This pattern of expression suggests some neuron-specific function for rDGK.

We then examined mRNA localization in adult rat brain. By in situ hybridization analysis, intense hybridization signals for rDGK were detected in the hippocampus, cerebellar cortex, olfactory bulb, and olfactory tubercle. Moderate signals were detected in the cerebral cortex, caudate putamen, and thalamus (Fig. 3B). No hybridization signals were detected in the white matter, such as the corpus callosum and cerebellar medulla. In a control experiment, brain sections were hybridized with plasmid vectors of appropriate length or treated with RNase A before hybridization. In either case, no significant signals were detected in any brain regions (data not shown).

Alternative Splicing—In addition to the full-length rDGK, we found novel splice variants in rat brain, presumably produced by alternative splicing mechanism. A comparison of the sequences between several rat clones and the human full-length clone revealed two additional insertions, insertion 1 (71 bp) and insertion 2 (19 bp), in the rDGK cDNA (Fig. 4A). Because the nucleotide numbers of the insertions are not divisible by 3, the frameshift was presumed to occur in the coding sequences in association with premature stop codon in each case (Fig. 4B), resulting in generation of two putative alternatively spliced variants, termed rDGK and -2, in addition to the intact rDGK. The cDNA for rDGK-2 contained insertion 2 and that for rDGK-3 contained insertion 1, whereas the cDNA for rDGK-1 contained no insertions. Primary structures for rDGK-2 and -3 represent truncated forms of rDGK without ankyrin repeats, and in addition rDGK-3 contained an incomplete catalytic domain (Fig. 4C). A series of reverse transcriptase-PCR experiments using primer sets (PS1-PA1 or PS2-PA2) spanning each of the two putative alternatively spliced regions (insertions 1 and 2) confirmed the existence of these alternative splicing events. These experiments were carried out on different rat brain cDNA libraries prepared from discrete rat brains (Fig. 4A).

We next investigated the relative expression ratio of these variants in adult rat brain. For this purpose, PCR products using PS1 and PA2 primers spanning both insertions and cDNA library of normal adult rat brain as a template were subcloned and checked whether they include the insertion(s). Among 100 clones examined, the numbers of rDGK-1, -2, and -3 were 53, 5, and 42, respectively. These data suggest that rDGK-1 and -3 represent the major variants for rDGK in the adult rat brain (Table 1).

Characterization of rDGK Variants—We characterized features of rDGK variants in terms of the enzymatic activity and detergent solubility by using the mammalian expression system with the FLAG epitope tag. The cDNA expression constructs for rDGK-1, -2, or -3 with the epitope tag were transfected into COS-7 cells, and individual homogenates were prepared for both enzymatic assay and immunoassay. Immunoreactive bands with molecular masses corresponding to those for rDGK-1, -2, or -3 were recognized by the anti-FLAG antibody in homogenates of the transfected cells (Fig. 5A), confirming that the insertions 1 and 2 are meaningful sequences in living cells. No immunoreactive bands were recognized in homogenates of cells transfected with the vector alone (data not shown). In assays for DGK enzymatic activity, homogenates from cells transfected with rDGK-1 showed 20-fold greater activity than those transfected with vector alone, indicating that rDGK-1 represents a functionally active entity for DGK. The enzymatic activity of homogenates transfected with cDNA for rDGK-2 was about half that of rDGK-1, whereas that of homogenates for rDGK-3 was at almost background levels (Fig. 6). When incubated with the three different substrates containing distinct acyl chains, both rDGK-1 and -2 displayed almost equal activity for either of 1,2-dioleoyl-sn-
FIG. 1. Nucleotide sequence and deduced primary structure of the composite cDNA for rDGK/H9259-1. Nucleotides and amino acids are numbered starting from A of the translation initiation codon ATG and the initiator methionine, respectively. An in-frame stop codon that precedes the translation initiation is underlined. The cysteine-rich zinc finger-like sequences are underlined, and conserved cysteine and histidine residues are marked with solid boxes. The bipartite nuclear localization signal, in which two clusters of basic amino acids are displayed by boxes, is indicated by a dotted line. Glycine residues characteristic of ATP-binding motif are marked by solid triangles. Four ankyrin repeats at the carboxyl terminus are heavily underlined. Positions of the insertions 1 and 2 are indicated by open triangles. Glycine residues characteristic of ATP-binding motif are marked by solid triangles. Four ankyrin repeats at the carboxyl terminus are heavily underlined. Positions of the insertions 1 and 2 are indicated by open triangles.
glycerol, 1-stearoyl-2-linoleoyl-sn-glycerol, and 1-stearoyl-2-arachidonoyl-sn-glycerol. Therefore enzymatic activity of rDGK\textsubscript{z} has no preference for any of the DGs in terms of the acyl chain composition, which is similar to DGK\textsubscript{z} as reported previously (22, 27).

It was puzzling to note that rDGK\textsubscript{z}-2 gave a markedly reduced enzymatic activity despite the presence of intact catalytic domain. We therefore performed enzyme kinetics experiments to assess the different catalytic properties. As shown in Fig. 7A, we first confirmed that the enzyme reaction proceeded linearly for at least 5 min, indicating that the reduced activity of rDGK\textsubscript{z}-2 is not due to its instability. We then tested the effects of various concentrations of ATP and DG on the enzymatic activities of the transfected COS-7 cell lysates (Fig. 7B).
and C). The carboxy-terminal truncation resulted in the increased $K_{\text{m}}$ value for ATP (−3-fold) in the case of rDGK-2 compared with the intact rDGK-1 (Fig. 7B). On the other hand, the apparent $K_{\text{m}}$ value for DG (−3 mol %) remained unaffected (Fig. 7C). We also confirmed (data not shown) that rDGK-1 and -2 were activated similarly by phosphatidylserine (EC50, 11–14 mol %). Throughout these experiments, rDGK-3 failed to give detectable enzymatic activity, thus confirming its lack of catalytic activity.

In ultra-centrifugal fractionation of the transfected COS-7 cell lysate, the immunoreactive band for rDGK-1 was detected in both the soluble and particulate fractions, whereas those for rDGK-2 and -3 were recovered solely in the particulate fraction (Fig. 5B). When the particulate fraction was solubilized in a buffer containing 1% Triton X-100 and then centrifuged, rDGK-2 and -3 were recovered in the Triton-insoluble fraction. Furthermore, a considerable part (−50%) of the particulate rDGK-1 was also found to be Triton-insoluble. Similar results were obtained by the experiment using lysates of COS-7 cells transfected under milder conditions (−4–5-fold increase of DGK activity in cell lysates using a reduced amount of plasmid (2 μg per 10-cm dish) and a shorter incubation time (−24 h)), suggesting that the distribution of rDGK variants is not an artifact due to the overexpression system. This suggests that the intact rDGK-1 may shuttle between the soluble and Triton-insoluble fractions by the carboxy-terminal mediated process.

We then examined the subcellular localization of rDGK variants in the hippocampal neurons that express the mRNA endogenously. When individual cDNAs for the three rDGK variants were transfected into primary neuronal cells in vitro, all of the rDGK variants were localized in the cytoplasm of the transfected neurons despite the sequence of an NLS included. The immunoreactivity was detected diffusely in the somata although less intensely in the periphery (Fig. 5C). This is in sharp contrast to the nuclear localization of rDGKα that also contains an NLS of the same type (22, 28).

Collectively, these results suggest that truncation of the carboxy-terminal portion clearly exerts effects on the enzymatic activity and detergent solubility of the splice variants, although all three variants show similar cytoplasmic localization in cDNA-transfected cultured neurons.

**Immunoblot Analysis of Rat Brain**—To confirm whether proteins of these rDGK variants are endogenously expressed in the brain, we performed immunoblot analysis using anti-rDGK antibody raised against the amino-terminal portion of the enzyme. As shown in Fig. 8A, this antibody clearly recognized all of the three rDGK variants, but not rDGKα, in the transfected COS-7 cell lysates, thus confirming the specificity of the antibody. In rat brain homogenate, the antibody detected intense bands corresponding to the major variants, rDGK-1 and -2, and a faint band corresponding to -2 (Fig. 8B). Failure to detect a clear band for rDGK-2 may be explained by the fact that the mRNA for this variant was estimated to be expressed at a very low level compared with those for rDGK-1 and -2 as shown in Table 1. The immunoreactive band for rDGK-1 was rather intense compared with that for rDGK-3, which is also consistent with our data on the estimation of the expression ratio (Table 1). These data strongly suggest that the messages for the rDGK splice variants are indeed translated in the brain.

**DISCUSSION**

We isolated cDNA for a rat homologue of human DGKα (18). Sequences of both species share a 91% identity in the amino acid and include two zinc finger-like motifs and four ankyrin repeats in addition to the highly conserved DGK catalytic domain, all of which are characteristic of class IV enzyme. Rat DGKα also contains an NLS of bipartite type similar to that of rDGK. The NLS sequence overlaps with a motif similar to the phosphorylation site domain of the myristoylated alanine-rich protein kinase C substrate proteins found in rat and human DGKα (22, 27) and hDGKα (18). The present in situ hybridization histochemistry of the brain reveals unique localization pattern of mRNA for rDGKα. Strong signals are detected in the hippocampus, cerebellar cortex, olfactory bulb, and olfactory tubercle and moderate signals in cerebral cortex, caudate-putamen, and thalamus, which is roughly consistent with the expression pattern in the human brain detected previously by regional Northern blot analysis (18). This localization pattern of gene expression in the brain is in sharp contrast to those of previous reports for other rat isozymes (rDGKα, -β, -γ, -ζ, and -ε) (16). Briefly, the signals for rDGKα are detected in glial cells (oligodendrocytes) and those for rDGKβ and rDGKγ predominantly in neurons of the caudate-putamen and the cerebellum, respectively. On the other hand, rDGKα and rDGKβ show relatively ubiquitous expression in most of the gray matter regions. These comparative data suggest that a specific type of neurons expresses specific sets of isozymes and that more than one isozyme is co-expressed in a single neuron in most of the brain regions. In this regard we are currently investigating subcellular localization of these isozymes by using cDNA transfection method, which suggests that they are localized to distinct subcellular sites.

In this study we also found novel splice variants of rDGKα in rat brain and characterized their features. First, the different solubility for the variants by cell fractionation analysis should be noted. The two truncated variants, rDGK-2 and -3, are solely recognized in the Triton-insoluble particulate fraction, whereas rDGK-1 is detected both in the soluble and particulate fractions. By comparison of the amino acid sequences among three variants, it is possible to speculate that this difference in the solubility may be due to the presence or absence of the ankyrin repeats in their sequences. Most ankyrin repeat-containing proteins, such as a transcriptional factor NFκB (29, 30), GA-binding protein (31), and a tumor suppressor p16 (32, 33) consist of four or more complete forms of the repeats, which

| Insertion 1 | Insertion 2 | Splice variant | Amino acids | Mass | No. clones/100 colonies |
|------------|------------|----------------|-------------|------|------------------------|
|            | -          | rDGK-1         | 1050        | 115  | 53                     |
| -          | +          | rDGK-2         | 840         | 93   | 5                      |
| +          | -          | rDGK-3         | 591         | 64   | 5/42                   |
| +          | +          | rDGK-3         | 591         | 64   | 37/42                  |

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provide stabilizing interactions between adjacent modules. In the case of GA-binding protein, the subunit contains a series of ankyrin repeats, whereas the subunit contains a DNA-binding domain that is a member of the ETS family. These dissimilar subunits form a tight heterodimer through ankyrin repeats and bind to DNA (31). The INK4 family containing p16 functions as cell cycle regulators through interaction and inhibition of cyclin-dependent kinase 6 (CDK6) (34). The ankyrin groove of p16 clamps the amino-terminal lobe of CDK6 that results in preventing CDK6 activation.

The results we have obtained for rDGK variants from cell fractionation experiments of the transfected COS-7 cells raise the possibility that the ankyrin repeats in the rDGK-1 may contribute to the mechanism that allows this molecule to stay soluble and/or to stabilize itself to Triton-insoluble components, such as cytoskeleton, depending on different molecule(s) interacting with this motif. It is reported that the carboxyl terminus including ankyrin repeats and PDZ-binding motif in rDGK may interact with the PDZ domain of 1-syntrophin and Ras-

FIG. 5. Expression of rDGK variant proteins and the subcellular localization in cDNA-transfected cultured neurons. A, expression of proteins for rDGK variants was confirmed in cDNA-transfected COS-7 cells. COS-7 cells were transfected with cDNAs for the FLAG-tagged rDGK variants. After 48 h, the transfected cell lysates (20 μg each) were examined by immunoblot analysis by using anti-FLAG antibody M2 with the chemiluminescence. The amounts of the expressed proteins (rDGK-1, -2, and -3) were equalized by means of densitometric indication of the immunoreactive bands, and the protein concentrations were also adjusted. These normalized proteins were also used for assessing the DGK enzymatic activity shown in Fig. 6. B, cell fractionation analysis. The transfected COS-7 cell lysates were separated into soluble and particulate fractions by a centrifugation (100,000 × g, 1 h) after removing cell debris. The pellet was resuspended in an equal volume of the buffer and was used as particulate fraction. 10 μg of protein from each fraction was loaded in each lane. The particulate fraction was separated into Triton-soluble and Triton-insoluble fractions in a buffer containing 1% Triton X-100 by further centrifugation (100,000 × g, 30 min). Each fraction was adjusted to an equal volume, and the aliquots (10 µl each) of these fractions were subjected to immunoblot analysis as described above. A representative of triplicate experiments is shown. C, primary cultured hippocampal neurons were transfected with FLAG-tagged rDGK variants or vector alone. After 24 h, the cells were fixed with 2% paraformaldehyde, and FLAG-tagged rDGKs were visualized using mouse anti-FLAG antibody, the biotinylated anti-mouse IgG antibody, and streptavidin-Alexa 485 Fluor conjugates. Cells were examined using a confocal laser-scanning microscope. The immunoreactivities for the full-length rDGK-1 and the truncated forms of rDGK-2 and -3 are detected primarily in the cytoplasm in a similar fashion with each other. They are detected diffusely in the somata although less intensely in the periphery. Note the sharp contrast of the immunoreactivities for the rDGK variants to that for rDGKζ, which is detected predominantly in the nucleus.

FIG. 6. DGK activities in COS-7 cells transfected with cDNAs for rDGK variants. COS-7 cells were transfected with cDNAs for the FLAG-tagged rDGK variants or vector alone. After adjusting the amounts of the expressed proteins (rDGK-1, -2, and -3) on the immunoblot by means of densitometric indication of the corresponding bands as in Fig. 5A, equal amounts of each lysate was assayed for DGK activity by the octyl glucose mixed micelle assay. 1,2-Dioleoyl-sn-glycerol (18:1/18:1 DG), 1-stearoyl-2-linoleoyl-sn-glycerol (18:0/18:2 DG), and 1-stearoyl-2-arachidonoyl-sn-glycerol (18:0/20:4 DG) were used as substrates. Values represent the means of triplicate determinations. Similar results were obtained in three separate experiments.
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Fig. 7. Time course and enzyme kinetics of rDGK variants. COS-7 cell lysates transfected with cDNAs for rDGK-1 (●) and -2 (▲) were incubated as described under “Experimental Procedures” for different periods (A) or with varied concentrations of ATP (B) and DG (C) as indicated. B and C, a double-reciprocal plot of the data is presented. The result is a representative of duplicate experiments, which gave quite reproducible results. Data points are the average of two determinations, which differed by less than 5% of the mean.

GRP, a guanine nucleotide exchange factor that is specific for Ras (26, 35). It is also reported that DGKz may interact via its ankyrin repeats with the receptor for leptin, a peptide involved in energy balance (36). Ankyrin repeat motif is included in a number of proteins and has been detected in organisms ranging from viruses to human (37). It is thought that ankyrin repeat motif-containing proteins do not bind selectively to a single class of protein target. Rather, the diversity of biological roles of these proteins is paralleled by the diversity of unrelated proteins with which they interact. Therefore, molecules associated with DGKz may not always interact with DGKs. However, with regard to the PDZ-binding motif (-ETAV) at the carboxyl-terminal end of rDGK-1, it is highly possible that rDGK-1 may also interact with PDZ domain of y1-syntrophin because the sequence of the motif is completely consistent with that of rDGKz (-ETAV) (26). Further study is needed to elucidate molecules associated with the carboxyl terminus of rDGK-1 together with the distribution of rDGKz variants in neurons that express different subset of proteins from COS-7 cells.

Fig. 8. Immunoblot analysis of rat brain with anti-rDGK antibody. A, specificity of the antibody raised against the amino terminus (amino acids 1–157) of rDGK. COS-7 cells were transfected with cDNAs for the FLAG-tagged rDGK variants or rDGKz. After 48 h, the cells were harvested and examined by immunoblot analysis with the polyclonal rabbit antibody against rDGK. Transfected COS-7 cell homogenate (20 μg of protein) was loaded in each lane. The expressed proteins were confirmed by using anti-FLAG antibody (data not shown). Note that the anti-rDGK antibody clearly detects all of the rDGK variants but not rDGKz. B, rat brain homogenate (total protein, 30 μg) was analyzed by immunoblot using anti-rDGK antibody. Note that major variants, rDGK-1 (arrowhead) and -2 (arrow), are detected in rat brain homogenate and that a minor variant, rDGK-2 (asterisk), is also detected very faintly below rDGK-1. A representative of triplicate experiments is shown. Size markers are indicated on the left in kDa.

Truncation of the carboxyl-terminal portion clearly exerts effects on the enzymatic activity of rDGKs (Fig. 6). It is not surprising that rDGKs contains no enzymatic activity because it has incomplete catalytic domain. However, we found that rDGK-2 also gives about 50% reduced activity of the intact rDGK-1, although it has an intact catalytic domain. The enzyme kinetics showed that the carboxyl-terminal truncation affected the apparent enzyme affinity for ATP (Fig. 7B). This may be explained by the hypothesis that protein-protein or protein-lipid interaction mediated through the carboxyl-terminal ankyrin repeats may affect the catalytic properties of the enzyme. Further study is needed to clarify this point. In any case, it is clear that by the alternative splicing mechanism the signal propagation involved with rDGKs may be modulated in three grades according to the enzymatic activity. In the immunocytochemical analysis by transfection of epitope-tagged cDNAs for three variants into primary hippocampal neurons (Fig. 5C), the predominant localization of all three variants in the cytoplasm, but not in the nucleus, is unexpected because the NLS is contained in all of them. This is somewhat different from the previous finding of hDGKz by Ding et al. (18) in which the protein was found to be in both the cytoplasm and nucleus by Western blot of cDNA-transfected COS cell lysates, although no photographic data are available in their report. This discrepancy may be due to the difference in the cell types used. Regardless of the reason for this discrepancy, it is highly plausible that rDGK-2 and -3 may act in a dominant negative manner against the intact rDGK-1, because the carboxyl-terminal truncated forms of rDGK-2 and -3 show 50 and 0% enzymatic activities compared with the full-length rDGK-1, respectively. They may compete with one another for the “specified signaling position,” and the extent of the enzymatic activity in the signal transduction may be determined by the molecule that occupies the signaling position. On the whole, the total activity would then be dependent upon the expression ratio of the splice variants. We roughly estimated this ratio in adult rat brain, showing that rDGK-1 and -3 are the major splice variants and that rDGK-2 is a minor one under normal conditions (Table I). In support of our estimation, immunoblot analysis of endogenous enzymes using the anti-rDGK antibody, which recognizes all of the three rDGKz variants, detects major bands for rDGK-1 and -3 and a faint band for rDGK-2 in the brain homogenate (Fig. 8). It is plausible that the ex-
pression ratio would be altered by physiologic and/or pathologic stimuli. Given the hypothesis that rDGK may also play some role in the nucleus as suggested by the presence of NLS, it is also plausible that translocation of rDGK between the cytoplasm and nucleus could occur under some conditions, which might function as a regulatory mechanism for nuclear enzymes and also as a means of signal propagation between the two subcellular compartments.

It remains to be elucidated whether a similar alternative splicing mechanism exists for human enzyme. However, human genome data (National Center for Biotechnology Information, www.ncbi.nlm.nih.gov/) enable us to compare hDGK and rDGK cDNA sequences, which reveal that the insertions 1 and 2 of rDGK are located in introns 18 and 27 of the hDGK genome, respectively. We found two possible 3′-splicing acceptor sites (invariant AG nucleotides) in introns 18 and 27 of the human genome (Fig. 9). We speculate that alternative use of either of the two putative 3′-splicing acceptor sites in these introns might determine the presence or absence of the insertions 1 and 2. A similar mechanism is reported for rat PKCζ splice variants, which are produced by the different usage of 5′-splicing donor sites, instead of 3′ acceptor sites (38). In this case 83-bp insertion at the V3 region of PKCζ results in the truncation of the full-length PKCζ protein. It should be noted here that the existence of inactive and truncated form of hDGK has also been detected, although the exact mechanism of the alternative splicing remains unknown (39).

A splicing variant of cAMP-response element-binding protein has also been reported to be up-regulated in cAMP-response element-binding protein transgenic mice (40). Moreover, in the thyroid carcinoma cells, Knauf et al. (41) have identified a rearrangement and an amplification of truncated PKCε mRNA encoding amino-terminal residues 1–116 of the subtype fused to an unrelated sequence. This naturally occurring truncated PKCε, by a dominant negative effect, has been demonstrated to block cell death triggered by a variety of stimuli. These previous reports support the idea that the alternative splicing mechanism of rDGK plays some roles in neuronal function. It would be intriguing to examine the expression ratio of rDGK variants and their localization in the brain area in developmental stages and under pathologic conditions, such as ischemia and psychiatric disorders.

In summary, our study reports novel splice variants of DGKs in rat brain that contain truncated carboxyl-terminal portions, and this truncation clearly exerts effects on the enzymatic activity and detergent solubility of the splice variants, although all three variants show similar cytoplasmic localization in cDNA-transfected cultured neurons. Our data raise a possibility that the modulation of signal transduction by the splice variants may play some roles in the physiologic and/or pathologic conditions of neurons. Further investigation is required to clarify these questions.

Acknowledgments—We are grateful to Dr. John A. Glomset, Howard Hughes Medical Institute Research Laboratories, University of Washington, for critical comments and suggestions. We also thank Dr. Hayato Sasaki for help to generate the antibody and Dr. Cindy Reed-Reynolds for reading the manuscript.

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