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Cloning and expression of a cytoskeleton-associated diacylglycerol kinase that is dominantly expressed in cerebellum

(88-kDa diacylglycerol kinase/*in situ* hybridization)

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ABSTRACT **A third species of diacylglycerol kinase (EC 2.7.1.107) cDNA was cloned from a rat brain cDNA library. The isolated cDNA encoded a 788-amino acid, 88-kDa polypeptide. This isozyme shared 58% identity with the previously isolated rat 80-kDa and 90-kDa diacylglycerol kinases. EF hand motifs, cysteine-rich zinc finger-like sequences, and putative ATP-binding site were all conserved among these isozymes. The 88-kDa diacylglycerol kinase was expressed specifically in brain and localized predominantly in cerebellar Purkinje cells. This isozyme was associated equally with particulate and supernatant fractions in cDNA-transfected COS-7 cells and dominantly with the particulate fraction in the brain. After Triton X-100 extraction, this isozyme remained in the detergent-insoluble cytoskeletal fraction of the brain and transfected COS-7 cells.**

Triggering of receptor complexes with hormones or neurotransmitters initiates signal transduction through phospholipase C (PLC)-mediated hydrolysis of phosphatidylinositols and subsequent production of second messengers, diacylglycerol (DG) and inositol 1,4,5-triphosphate (1, 2). DG kinase (EC 2.7.1.107) phosphorylates DG, an activator of protein kinase C, to produce phosphatidic acid, which is the first step for the resynthesis of phosphatidylinositols (3). Thus, DG kinase is thought to be a regulator of protein kinase C by attenuation of the levels of DG (3). In addition, there have been findings suggesting that phosphatidic acid, the product of DG kinase, might be a second messenger (4). Recent studies have shown that DG kinase can participate in signal transduction through not only G protein-coupled receptors but also receptor tyrosine kinases. In the latter case, DG kinase becomes associated with the cytoskeleton, presumably the actin filament system (5).

A variety of mammalian DG kinases have been purified from various tissues. The best characterized of these are an 80-kDa DG kinase from rat and porcine brain, a 90-kDa DG kinase from rat brain, and an 86-kDa human homologue of the 80-kDa DG kinase (6–9). Our previous *in situ* hybridization histochemical analyses have shown that the gene for 80-kDa DG kinase is predominantly expressed in T lymphocytes and oligodendrocytes (6) and the 90-kDa DG kinase is preferentially expressed in medium-sized neurons of the caudate-putamen of the brain (8). The primary structures deduced from all three molecules contain EF hand and zinc finger motifs (6–9).

As the third species of DG kinase, and the second expressed in neurons, we report here the molecular cloning of a brain-specific 88-kDa isozyme. The immunochemical and histochemical analyses demonstrated that this 88-kDa DG kinase is localized predominantly in cerebellum and is associated with the detergent-insoluble cytoskeletal fraction.*

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MATERIALS AND METHODS

cDNA Cloning. A rat brain cDNA library was screened with a mixed probe of a 1.0-kb *Xba* I fragment of rat 80-kDa DG kinase cDNA (6) and 1.2 kb of an *Eco*RI fragment of rat 90-kDa DG kinase cDNA (8) under low stringency conditions as described (8). Two positive clones, pDG1 and pDG3, were obtained. By another round of screening performed under high stringency conditions with a 5′ portion of the cloned cDNA as a probe, one positive clone, pG1, was isolated. The missing 5′ end was obtained by a rapid amplification of cDNA ends (RACE) polymerase chain reaction (PCR) (5′ AmpliFinder RACE kit, Clontech). The cDNA inserts were sequenced on both strands by the dideoxy chain-termination method using Sequenase (10). For the sequences obtained by RACE, two independent clones were completely sequenced.

RNA Extraction and Northern Blot Analysis. Total RNA was extracted from several tissues of adult Wister rats (3 months old) by guanidine thiocyanate/phenol/chloroform extraction (11). Poly(A) RNA was isolated by chromatography on an oligo(dT)-cellulose column (12). Conditions for hybridization and washing were performed as described (8).

Transfection and DG Kinase Activity. The cDNA for 88-kDa DG kinase was subcloned in the expression vector, pSRE (8, 13). After incubating for 3 days, cDNA-transfected COS-7 cells were harvested and lysed by sonication in lysis buffer (13). Protein concentrations were determined by the method of Lowry *et al.*, with bovine serum albumin as a standard (14). DG kinase activity was measured by the octyl glucoside mixed-micelle assay and the deoxycholate assay as described (8, 15). 1,2-Didecanoyl-*sn*-glycerol (diC_{10}) was used as short-chain DG, whereas 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 long-chain DGs.

Antibody Preparation Against Bacterially Expressed 88-kDa DG Kinase Fusion Protein. A cDNA fragment encoding amino acids 219–449 of 88-kDa DG kinase was cloned into the expression vector, pMAL (protein fusion and purification system, New England Biolabs). The expressed fusion protein (66 kDa) was purified by preparative SDS/PAGE and injected into rabbits as described (6, 16).

Immunoprecipitation. Various amounts of the immune IgG were incubated for 1 hr on ice with the supernatant fraction of the transfected COS-7 cell lysate and the brain homogenate. Lysis buffer was used to adjust the final volume to 70 μl. Then, 50 μl of Pansorbin (Calbiochem) in 0.01 M phosphate-buffered saline (PBS) was added, and the mixture was

Abbreviations: DG, diacylglycerol; RACE, rapid amplification of cDNA ends; diC_{10}, 1,2-didecanoyl-*sn*-glycerol; PLC, phospholipase C; DAB, diaminobenzidine.

*The sequence reported in this paper has been deposited in the GenBank data base (accession no. D38448).

incubated for 1 hr on ice (17). The samples were centrifuged at 5000 × *g* for 5 min, and the enzyme activity remaining in the supernatant was determined in the deoxycholate assay. As a control, preimmune IgG was used instead of the immune IgG.

Cytoskeleton Extraction. COS-7 cells transfected with cDNA for 88-kDa DG kinase were grown for 3 days. The cells were extracted with 0.5% Triton X-100 in 20 mM Hepes, pH 7.4/50 mM NaCl/1 mM EGTA/1 mM phenylmethylsulfonyl fluoride/10 μg of leupeptin per ml/100 μM sodium orthovanadate/20 mM sodium fluoride (100 μl per 10^7 cells). Whole brain of an adult rat was homogenized in 3 volumes of the same buffer with 1% Triton X-100. After incubation for 15 min at 4°C, the cytoskeletons were pelleted at 12,000 × *g* for 3 min at 4°C (5, 18–20). Under the conditions described above, about 60% and 85% of total cellular protein was extracted from the transfected COS-7 cells and the brain, respectively.

Immunoblotting. COS-7 cells transfected with cDNA for either one of the three DG kinase isozymes and adult rat brains were homogenized separately with 10 mM Tris·HCl, pH 7.4/20 mM KCl/0.1 mM EDTA/0.25 M sucrose. The

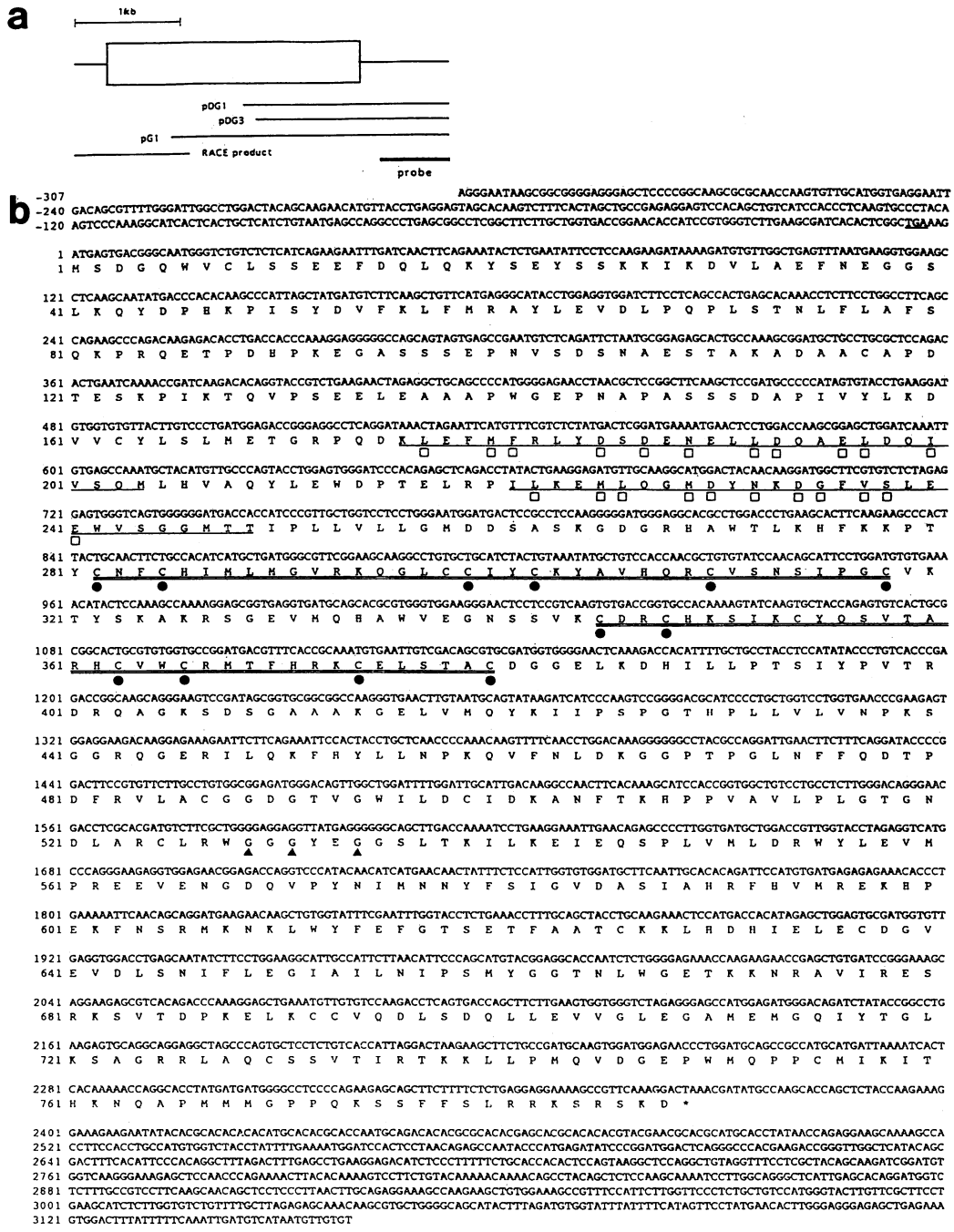

FIG. 1. (*a*) Map of cDNA encoding rat 88-kDa DG kinase. Isolated clones and RACE product were combined to construct the composite cDNA, in which the open box and lines indicate the coding region and untranslated sequences, respectively. The probe used for Northern blot analysis and *in situ* hybridization histochemistry is also indicated. (*b*) Nucleotide sequence of the composite cDNA and the deduced primary structure of the 88-kDa DG kinase. 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 in the 5′ untranslated region is underlined. Ca^{2+}-coordinating residues in EF hand motifs (underline) are indicated (□). Cysteine residues making up zinc finger-like sequences (double underline) are also shown (●). Residues characteristic of ATP-binding sites found in various protein kinases are marked (▲).

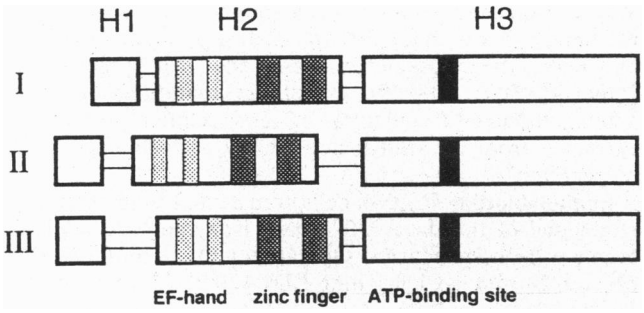

FIG. 2. Linear representation of 80-kDa DGK-I (I), 90-kDa DGK-II (II), and 88-kDa DGK-III (III). The regions displaying sequence similarity are indicated by large boxes (H1, H2, H3). EF hand motifs, cysteine-rich zinc finger-like sequences, and putative ATP-binding site are shown.

lysates were boiled for 4 min in Laemmli's sample buffer and subjected to SDS/7.5% PAGE (21). The proteins were then electrophoretically transferred to a nitrocellulose membrane (0.45-μm pore size). After blocking the nonspecific binding sites in 5% skim milk (wt/vol) in PBS, the membrane was incubated for 2 hr at room temperature with the anti-rat 88-kDa DG kinase antibody and then treated with peroxidase-conjugated anti-rabbit IgG antibody for 1 hr.

***In Situ* Hybridization Histochemistry.** Fresh frozen blocks of brains from adult male rats were sectioned at 30-μm thickness on a cryostat. The sections were mounted on silane-coated glass slides, fixed with paraformaldehyde, pretreated, and hybridized with dATP[^{35}S]-labeled probe as described (6). After exposure to Hyperfilm-β max (Amersham) for 2–3 weeks, the sections were dipped in Kodak NTB2 emulsion and exposed for 2 months.

Immunocytochemistry. Adult male rats were perfused through the heart under pentobarbital anesthesia (40 mg/kg) with 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.4 (PB). Cryostat sections were incubated with anti-rat 88-kDa DG kinase antibody overnight at 4°C in a moist chamber. Sites of antigen–antibody reaction were visualized using the avidin-biotinylated peroxidase complex (ABC) system (Vector Laboratories) with DAB as a substrate (6).

RESULTS

We isolated two cDNA clones that are different from cDNAs for 80-kDa and 90-kDa DG kinases but homologous to them in nucleotide sequence. The nucleotide and deduced amino acid sequences of the composite cDNA are presented in Fig. 1*a*. The putative initiation codon was preceded by an in-frame stop codon at nt −6 and occurred in a Kozak consensus sequence for the initiation of translation (Fig. 1*b*). The deduced amino acid sequence encoded a protein of 788 amino

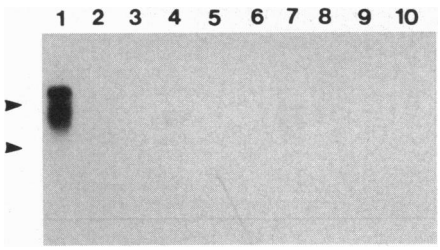

FIG. 3. Northern blot analysis of the 88-kDa-DGK mRNA in various rat tissues. Poly(A)$^+$RNAs (5 μg per lane) were electrophoresed and transferred to a nylon membrane. The blot was hybridized with ^{32}P-labeled probe. Lanes: 1, brain; 2, spleen; 3, thymus; 4, testis; 5, adrenal gland; 6, liver; 7, kidney; 8, small intestine; 9, heart; 10, salivary gland. Size markers (arrowheads) represent 28S and 18S rRNAs.

Table 1. DG kinase activities in COS-7 cells transfected with pSRE vector alone or psreDGK-III

	DG kinase activity, nmol/min per mg			
	Octyl glucoside		Deoxycholate	
DG species	pSRE	psreDGK-III	pSRE	psreDGK-III
diC$_{10}$	1.3	25.5	3.1	106.2
18:1/18:1 DG	1.4	35.9	5.5	158.0
18:0/18:2 DG	1.8	39.0	3.6	130.0
18:0/20:4 DG	2.1	43.2	3.9	135.6

Lysates were assayed for kinase activity toward single DG species in the octyl glucoside mixed-micelle assay and deoxycholate assay. Values represent the means of triplicate determinations. Similar results were obtained in two separate experiments.

acids with a calculated molecular weight of 88,520. The primary structure of this molecule was closely related to, but clearly distinct from, the sequences of previously characterized DG kinases (6, 8). Thus this cDNA encodes a further DG kinase. We termed this newly identified 88-kDa DG kinase DGK-III and, in addition, termed previously identified 80-kDa DG kinase and 90-kDa DG kinase DGK-I and DGK-II, respectively.

The DGK-III amino acid sequence was identical to those of DGK-I and DGK-II at 58% of residues. Ca^{2+}-coordinating EF hand motifs and cysteine-rich zinc finger-like sequences were tandemly repeated, as seen in the other DG kinases. An ATP-binding consensus sequence Gly-Xaa-Gly-Xaa-Xaa-Gly was also conserved (Fig. 1). Comparison of the structures of the DGK-I, -II, and -III revealed three homologous regions (tentatively termed H1, H2, and H3) and hinged regions of variable length among them (Fig. 2). The H2 region (51–63% identity) containing two EF hand motifs and two zinc finger-like sequences is thought to regulate kinase activity through Ca^{2+} ions and phospholipids. The H3 region showing high identity (66–75%) is thought to be the catalytic domain, given the presence of the ATP-binding consensus sequence (22). The role of the relatively low homologous H1 region (33–39% identity) remains unclear.

In Northern blot analysis, DGK-III mRNA was detected exclusively in brain as a major band of 6.4 kb and a lesser band of 4 kb with a noncoding probe (Fig. 3). Since the same

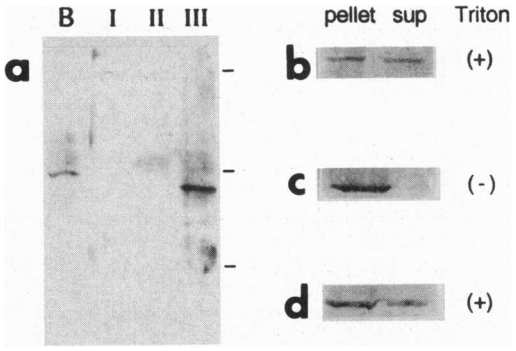

FIG. 4. Immunoblot analysis of transfected COS-7 cells with antibody against bacterially expressed DGK-III fusion protein. (*a*) Total lysates of DGK-I, -II, or -III cDNA-transfected COS-7 cells and the brain (B) were analyzed by SDS/7.5% PAGE. Bound antibody was visualized by the chemiluminescent system. (*b*) COS-7 cells transfected with DGK-III cDNA were extracted with 0.5% Triton X-100 and centrifuged at 12,000 × *g* for 3 min. (*c* and *d*) Brain was homogenized in a buffer with (*d*) or without (*c*) 1% Triton X-100 and centrifuged as described in the text. Resulting pellet and supernatant (sup) were analyzed as well. Bound antibody was visualized by DAB as a substrate for peroxidase. Size markers indicated on the right represent 200, 97.4, and 68 kDa from top to bottom. Applied volume: *a*, 100 μg per lane; *b*, 25 μg per lane; *c*, 75 μg per lane; *d*, 48 μg per lane.

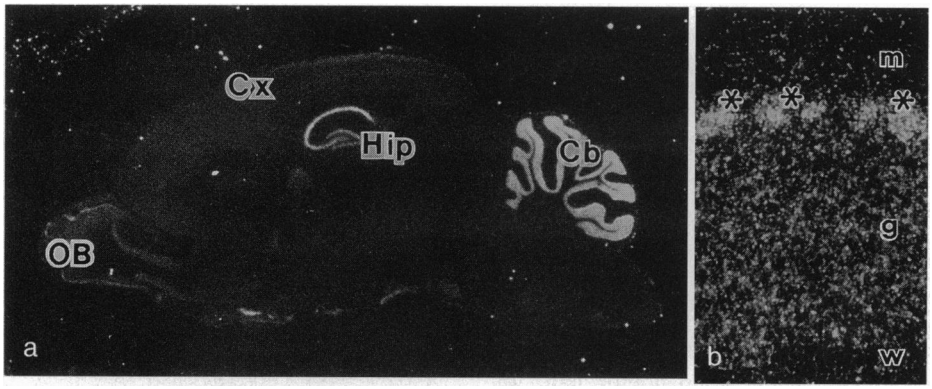

FIG. 5. *In situ* hybridization of DGK-III mRNA in the adult rat brain. (*a*) Film autoradiographic image of parasagittal section through the caudate-putamen. Note intense expression signals in the cerebellar cortex (Cb), moderate signals in the hippocampus (Hip), and weak signals in neuronal layers of the olfactory bulb (OB). Cx, cerebral cortex. (×4.0.) (*b*) Dark-field photomicrograph of the cerebellum on the emulsion-dipped section at higher magnification. Note intense signals in the Purkinje cells (*) and moderate signals in the granule cells (g). m, Molecular layer; w, white matter. (×175.)

pattern of hybridization bands was also observed when two other coding regions of this cDNA were employed as a probe (data not shown), these two mRNAs were suggested to be derived from an alternative use of poly(A) tail. This confined distribution was quite different from those of DGK-I and -II (6, 8), suggesting some neuron-specific function for this molecule.

DG kinase activity of the protein encoded by this cDNA was measured by expression of DGK-III cDNA (psreDGK-III) in the COS-7 cells. As controls, cDNAs for rat DGK-I and -II (psreDGK-I and -II, respectively) as well as pSRE vector alone were also expressed. In the octyl glucoside mixed-micelle assay for DG kinase activity, a lysate from COS-7 cells transfected with psreDGK-III showed >20-fold higher activity with each DG species than the control lysate (Table 1). DGK-III showed slightly lower phosphorylation activity for diC_{10} among the DG species used. Similar results were obtained in the deoxycholate assay. The DGK-III activity decreased to a control level by deprivation of Ca^{2+} (2 mM EGTA). After separation of the lysate into supernatant and particulate fractions, DGK-III activity was recovered equally in both fractions (31.5 and 25.8 nmol/min per mg of activity toward 18:0/20:4 DG in the octyl glucoside assay, respectively).

In immunoblot analysis using the antibody against bacterially expressed DGK-III of COS-7 cells, a single band of 88 kDa, which was consistent with the deduced molecular mass, was detected in the psreDGK-III lysate but not in the psreDGK-I and psreDGK-II lysates, showing that this antibody specifically recognized DGK-III among these isozymes (Fig. 4*a*). After extraction with Triton X-100, the DGK-III-immunoreactivity was detected equally in Triton-soluble and -insoluble cytoskeletal fractions (Fig. 4*b*). On the other hand, a larger band of about 97 kDa instead of 88 kDa was detected in homogenate of the whole brain in the immunoblot analysis (Fig. 4*a*). This 97-kDa band in the brain disappeared when using the antibody preabsorbed with bacterially expressed DGK-III, showing the 97-kDa band shared the same antigenicity with DGK-III. After separation of the brain homogenate by centrifugation, the immunoreactive band was dominantly recognized in the particulate fraction (Fig. 4*c*). When the brain homogenate was extracted with Triton X-100, the immunoreactive band for DGK-III appeared more densely in the detergent-insoluble cytoskeletal fraction than in the supernatant (Fig. 4*d*). This antibody immunoprecipitated a considerable portion (40%) of the soluble DG kinase activity in the COS-7 cells transfected with DGK-III cDNA. The DG kinase activity of cerebellar supernatant fraction (4.23 nmol/min per mg) was also immunoprecipitated by this antibody, though to a lesser extent (15%), suggesting that DGK-III is the minority among the DG kinase isozymes in the cerebellar supernatant fraction.

By *in situ* hybridization analysis, the gene for DGK-III was expressed most intensely in the cerebellum (Fig. 5*a*). At higher magnification, the hybridization signals were deposited intensely in the Purkinje cell somata and moderately in the granule cell layer (Fig. 5*b*). Outside the cerebellum, the septum, the hippocampal pyramidal, and dentate granule cell layer expressed the mRNA moderately to intensely, whereas the olfactory mitral cell and inner granular layer and the cerebral cortex expressed it weakly and faintly. No hybridization signals were discerned in white matter, such as the corpus callosum and cerebellar medulla. In control experiments, brain sections were hybridized with plasmid vector of appropriate length or treated with RNase A before hybridization. In either case, no significant hybridization signals were detected in any brain regions (data not shown).

By immunocytochemical analysis for DGK-III in the cerebellum, which expressed the mRNA predominantly, the Purkinje cell somata were most intensely immunoreactive, in accord with the *in situ* hybridization results (Fig. 6). The granule cells were weakly immunoreactive. Weak immunoreactivity was also seen diffusely in the molecular layer. In contrast, the cerebellar medulla was immunonegative. When cerebellar sections were incubated with antibody that had been preabsorbed with the bacterially expressed DGK-III fusion protein, no immunoreactivity was detected in any portions of the sections.

DISCUSSION

The DGK isozyme termed DGK-III was 58% identical in its amino acid sequence to the previously cloned isozymes, now

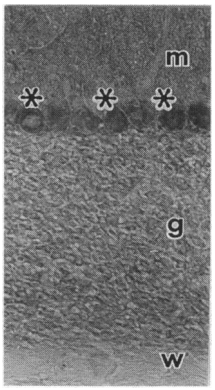

FIG. 6. Immunolight micrograph of DGK-III in the adult rat cerebellum. Purkinje cell somata (*) are most intensely immunoreactive. Weak immunoreactivity is seen in the granule cells (g) and molecular layer (m). Note the absence of immunoreactivity in the cerebellar medulla (w). (×160.)

termed DGK-I and DGK-II. DGK-III contained EF hand motifs, zinc finger-like sequences, and an ATP-binding site in common with the other two isozymes. In accord with the occurrence of EF hand motifs, the phosphorylation activity of DGK-III was regulated by Ca^{2+}, similar to DGK-I and -II. However, DGK-III had several characteristics markedly different from DGK-I and -II isozymes.

The major difference was the subcellular distribution. The activity of DGK-III was detected in supernatant and particulate fractions of COS-7 cells transfected with cDNA specific to DGK-III. This feature is in contrast to the dominant appearance of DGK-I activity in the supernatant fraction and that of DGK-II in the particulate fraction as reported previously (8). The appearance of DGK-III in the particulate fraction was much dominant in the brain homogenate. In addition, the immunoblotting analysis indicated the slightly higher molecular weight of DGK-III in the brain homogenate. Different posttranslational modification of DGK-III, such as phosphorylation, methylation, or some other, might explain the difference in the subcellular appearance of DGK-III as well as that in the apparent molecular weight of DGK-III between the native enzyme in the brain and that expressed in the COS-7 cells, although the precise reason remains to be elucidated. Since DGK-III remained in the detergent-insoluble fraction after Triton X-100 extraction of the transfected COS-7 cells and the brain, DGK-III in the particulate fraction is considered to represent its association with the cytoskeleton.

The appearance of DGK-III in supernatant and particulate fractions suggests that translocation of this isozyme molecule between the two intracellular compartments is constantly active. A Ca^{2+}- or DG-induced translocation of DG kinase activity from supernatant to particulate fraction has been demonstrated to occur in homogenates of thymus, brain, and liver (15, 23). The phosphorylation of DG kinase by protein kinase C (PKC) or cAMP-dependent protein kinase (PKA) has also been shown to induce such a translocation (24). Potential phosphorylation sites for PKC and PKA, according to Pearson and Kemp (25), are found in DGK-III at amino acids 21, 328, 680, 683, 776, and 784 for PKC and 784 for PKA.

Furthermore, evidence has recently been presented for involvement of tyrosine phosphorylation in the translocation of DG kinase from cytosol to cytoskeleton fraction (5). Evidence has also been presented suggesting that DG kinase may be a direct substrate for the activated epidermal growth factor (EGF) receptor (4). In this regard, a tyrosine residue at amino acid 210 in the primary structure of DGK-III may serve as a candidate for the phosphorylation site by the EGF receptor based on similarity to protein kinase phosphorylation site motifs (26). Neuron-specific expression and association with the cytoskeleton of this molecule suggest that DGK-III is involved in the reorganization of the neuronal cytoskeleton—for example, synaptic plasticity.

In addition to the subcellular distribution, DGK-III shows a pattern of gene expression in the brain different from those of DGK-I and -II. This expression pattern closely resembles that of PLCγ-1 already reported by Ross *et al.* (27). The intense expression of both molecules is confined to the olfactory mitral cells, the hippocampal pyramidal cells and dentate granule cells, and the cerebellar Purkinje cells and granule cells. Our previous study has pointed out the resemblance in gene expression between DGK-II and PLCβ-1 in the brain. On the other hand, no clear correlations between DGK-III or -II and any subtypes of protein kinase C, targets for DG, are found in their gene expression patterns, although a correlation has been noted between DGK-I and the subtype III/α of protein kinase C in terms of gene expression in the oligodendrocytes. These similarities in gene expression between subtypes of DG kinase and PLC strongly suggest that DG kinase and PLC, in a subtype-specific paired fashion, at least in the brain, participate in inositol phospholipid-related signal transduction. Since a total of eight distinct cDNAs for PLC isozymes has so far been isolated from mammalian cells (2), it is possible that additional isozymes other than the three DG kinases might be expressed in a region- or cell-specific manner in the brain.

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