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Molecular cloning and expression of a 90-kDa diacylglycerol kinase that predominantly localizes in neurons

(cloning/*in situ* hybridization)

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ABSTRACT **A diacylglycerol kinase cDNA was isolated from a rat brain cDNA library. This cDNA encoded an 801-amino acid protein of 90,287 Da. This 90-kDa diacylglycerol kinase showed 58% identity in deduced amino acid sequence with a previously isolated rat 80-kDa diacylglycerol kinase. EF-hand motifs, cysteine-rich zinc-finger-like sequences, and putative ATP-binding sites were all conserved between the two kinase species. However, mRNA encoding the 90-kDa kinase was confined to restricted neuronal populations such as the caudate–putamen, the accumbens nucleus, and the olfactory tubercle. Further, the 90-kDa kinase was found to exhibit high phosphorylation activity for long-chain diacylglycerols and was mainly associated with the membrane fraction when the cDNA was transfected into COS-7 cells.**

In the process of cell signal transduction, diacylglycerol (DG) kinase is thought to be involved in the resynthesis of phosphatidylinositol by converting the second messenger DG to phosphatidic acid (1, 2). DG kinase is thus regarded as an attenuator of the activity of protein kinase C (PKC) (3, 4). The importance of DG kinase in neurologic functions is indicated by the observation that the activity of this enzyme is lacking in the *Drosophila* retinal degeneration mutant (rdgA) (5).

In mammals, a soluble DG kinase has been purified from porcine brain cytosol (6), and cDNA encoding the porcine 80-kDa DG kinase has been revealed to specify EF-hand motifs, cysteine-rich zinc-finger-like sequences, and putative ATP-binding sites (7). Furthermore *in situ* hybridization followed by gene cloning of the rat homologue has demonstrated that mRNA for the 80-kDa DG kinase in brain is unexpectedly confined to oligodendrocytes, suggesting a regulatory involvement in myelin metabolism (8). To understand the functional significance of DG kinase in the process of neuronal signal transduction via the phosphatidylinositol cycle, it is crucial to identify neuronal species of DG kinase. Here we report the molecular cloning and expression of a DG kinase that localizes in neurons and appears to be associated with the membrane.*

MATERIALS AND METHODS

cDNA Cloning. A rat brain cDNA library was screened with a ^{32}P-labeled 1.0-kb *Xba* I fragment of rat 80-kDa DG kinase cDNA (8) under low-stringency conditions: 30% formamide/5× standard saline citrate (SSC)/1× Denhardt's solution/50 mM sodium phosphate, pH 7.2, with heat-denatured salmon sperm DNA (250 μg/ml) at 42°C. A single clone (4.7 kb, pNDGK1) showing weak signals was partially sequenced and found to be homologous to rat 80-kDa DG kinase. By rescreening a rat brain cDNA library with pNDGK1 as a probe under high-stringency conditions (8), three positive clones of 5.2, 5.9, and 2.7 kb (pNDGK2–4, respectively) were isolated. The cDNA inserts of the recombinant phages were subcloned into the *Eco*RI or *Not* I sites of plasmid pBluescript II SK(+) (Stratagene) and sequenced on both strands by the dideoxy chain-termination method (9) using Sequenase (United States Biochemical). Nucleotide and amino acid sequences were analyzed with GENETYX software (Software Development, Tokyo).

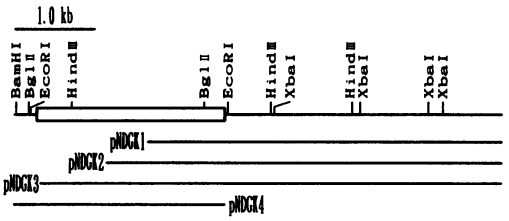

FIG. 1. Restriction map of cDNA encoding rat 90-kDa DG kinase. The sequences of the four cDNA clones pNDGK1–4 were combined to construct the composite cDNA, in which the open box and lines indicate the coding region and noncoding sequences, respectively.

RNA Extraction and Northern Blot Analysis. Total RNA was extracted from several tissues of adult rats by guanidine thiocyanate/phenol/chloroform extraction (10). Poly(A)$^+$ RNA was isolated by chromatography on an oligo(dT)-cellulose column and each poly(A)$^+$ RNA sample (5 μg per lane) was denatured with formaldehyde and size-separated by agarose gel electrophoresis (11). The RNA was transferred and fixed to a nylon membrane (Nytran, Schleicher & Schuell) and hybridized with a ^{32}P-labeled 1.2-kb *Eco*RI fragment of pNDGK1 cDNA for the 90-kDa DG kinase. Hybridization and washing were performed under high stringency (8). Autoradiography was at −80°C for 3 days.

Transfection and DG Kinase Activity. The cDNA for the presumed 90-kDa DG kinase and the cDNA for rat 80-kDa DG kinase (8) were subcloned into the simian virus 40-based expression vector pSRE (pcDL-SRα296 in ref. 12) as modified by Sakane *et al.* (13). The vector alone or the resulting constructs were transfected into COS-7 cells by use of DEAE-dextran (14). After 3 days the cells were harvested and lysed by sonication in lysis buffer (13). After removal of undisrupted cells by centrifugation (550 × *g*, 10 min), the supernatant, hereafter referred to as the lysate, was obtained and was further centrifuged at 100,000 × *g* for 30 min to separate soluble and membrane fractions. The membrane fraction was sonicated in 1 M KCl/20% (vol/vol) glycerol/10 mM Tris·HCl, pH 7.4/1 mM EDTA/1 mM EGTA/1 mM dithiothreitol and centrifuged at 100,000 × *g* for 30 min. DG kinase activity was measured by the octyl glucoside mixed-micelle assay (15). 1,2-Didecanoyl-*sn*-glycerol (diC_{10}) (Avanti Polar Lipids) was used as short-chain DG; 1,2-

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Abbreviations: DG, diacylglycerol; PLC, phospholipase C; PKC, protein kinase C; diC_{10}, 1,2-didecanoyl-*sn*-glycerol.

*The nucleotide sequence reported in this paper has been deposited in the GenBank database (accession no. D16100).

dioleoyl-*sn*-glycerol (18:1/18:1 DG) (Sigma), 1-stearoyl-2-linoleoyl-*sn*-glycerol (18:0/18:2 DG) (Biomol, Plymouth Meeting, PA), and 1-stearoyl-2-arachidonoyl-*sn*-glycerol (18:0/20:4 DG) (Biomol) were used as long-chain DGs. Before experimentation, we confirmed by TLC the DGs used had not isomerized to 1,3-DGs. Assays were performed in the presence of each substrate at a concentration of 1 mM. The reaction mixture (50 μl) contained 50 mM Mops (pH 7.2), 50 mM octyl glucoside (Calbiochem), 100 mM NaCl, 1 mM dithiothreitol, 20 mM NaF, 2.1 mM $CaCl_2$, 2.0 mM EGTA, 0.8 mM EDTA, 10 mM $MgCl_2$, 6.7 mM phosphatidylserine (Avanti Polar Lipids), and 1 mM [γ-^{32}P]ATP (10,000 cpm/nmol; ICN). In some experiments, the deoxycholate assay was performed using 1 mM deoxycholate and 0.5 mM substrate (6). The free Ca^{2+} concentration in the mixture of both assays was calculated to be 0.02 mM by using the computer programs described (16). The reaction was continued for 3 min at 30°C. Lipids were extracted (15) and separated on thin-layer plates of silica gel (Merck) (17). The band of phosphatidic acid detected by autoradiography was scraped with a sharp spatula and collected for liquid scintillation counting. Under the conditions described above, the rate of the reaction was linear with respect to protein concentrations and time up to 3 min.

***In Situ* Hybridization.** Cryostat sections of adult rat brain were hybridized with 0.5–1.0 × 10^6 cpm per slide of the same

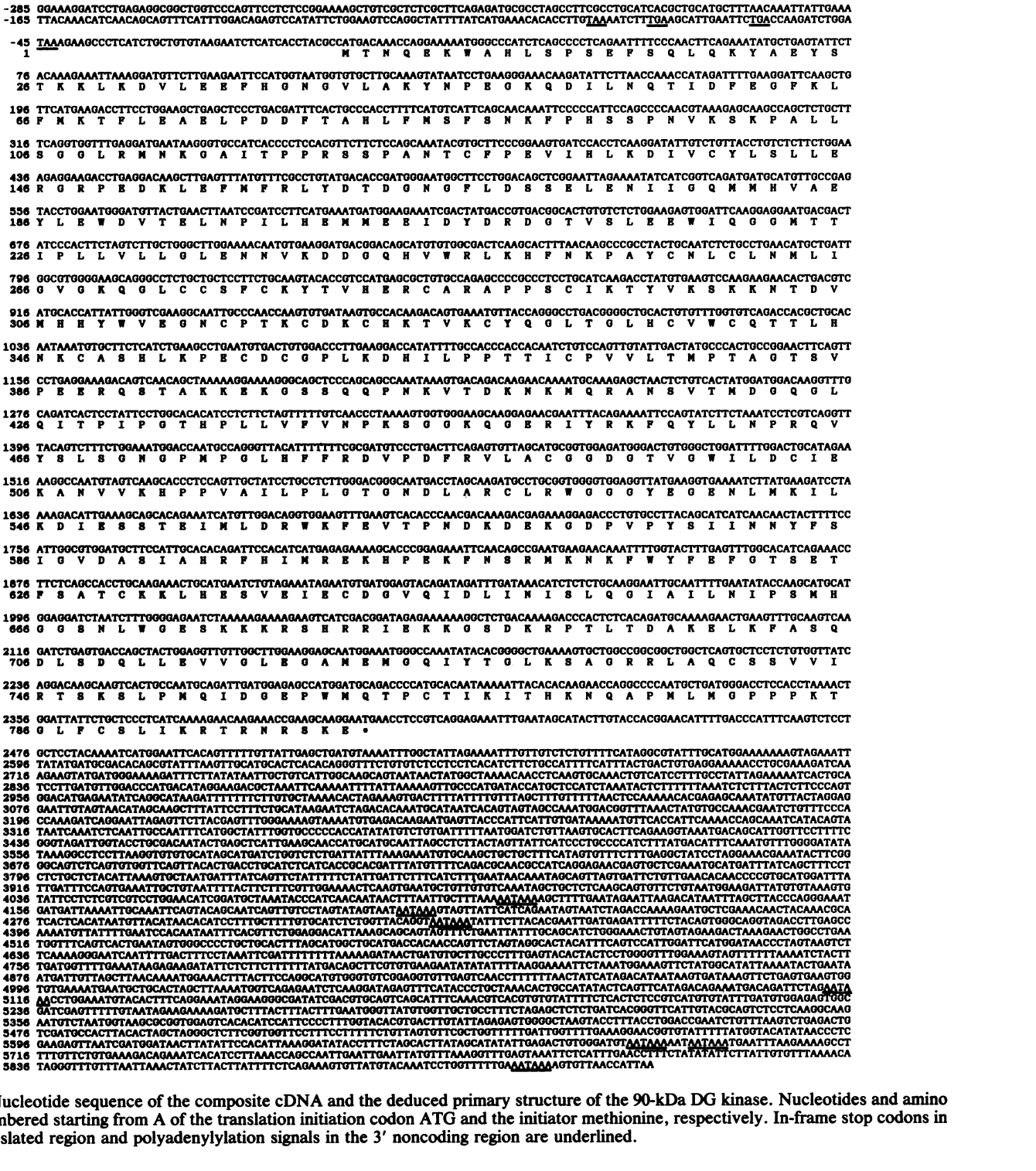

FIG. 2. Nucleotide sequence of the composite cDNA and the deduced primary structure of the 90-kDa DG kinase. Nucleotides and amino acids are numbered starting from A of the translation initiation codon ATG and the initiator methionine, respectively. In-frame stop codons in the 5′ untranslated region and polyadenylylation signals in the 3′ noncoding region are underlined.

cDNA probe labeled with [α-[35S]thio]dATP as used for Northern analysis and washed as described (8). After exposure to Hyperfilm-βmax (Amersham) for 2–3 weeks, the sections were dipped in Kodak NTB2 emulsion and exposed for 3 months.

RESULTS

Sequence and restriction analyses of four positive clones obtained (pNDGK1–4) indicated that all four were replicas of a single clone (Fig. 1). The composite nucleotide sequence contained an open reading frame encoding 801 aa residues (including the initiating methionine), from which the molecular mass was calculated to be 90,287 Da. The nucleotide sequence around the initiation codon fulfilled Kozak's criteria for eukaryotic initiation sites (18). In-frame stop codons were found at nt −45, −60, −75, and −84 in the 5′ untranslated region. Polyadenylylation signals appeared at nt 4104, 4209, 4334, 5112, 5683, 5692, and 5906 (Fig. 2).

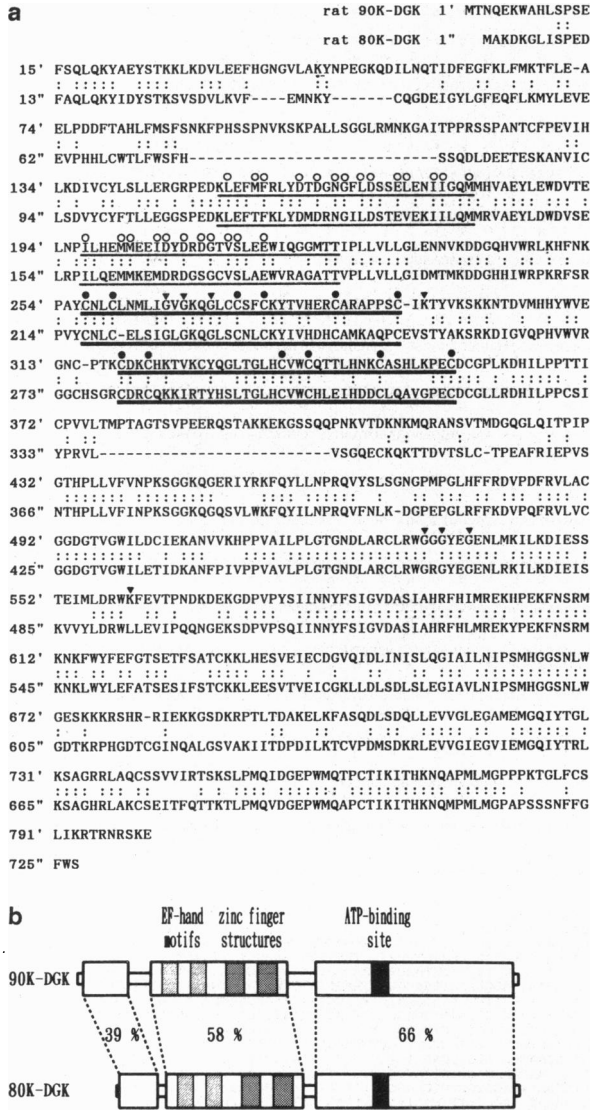

FIG. 3. Comparison of the 90-kDa DG kinase (90K-DGK) and the 80-kDa DG kinase (80K-DGK). (*a*) Alignment of the deduced amino acid sequences. Identical amino acid residues (:) and deleted residues (−) are shown. Ca^{2+}-coordinating residues in EF-hand motifs (thin underline) are indicated (○). Cysteine residues making up zinc-finger-like sequences (thick underline) are also shown (●). Residues characteristic of ATP-binding sites found in protein kinases are marked (▲). (*b*) A linear representation of 90K-DGK and the 80K-DGK. The regions displaying sequence similarity are indicated by large boxes. EF-hand motifs, cysteine-rich zinc-finger-like sequences, and putative ATP-binding sites are shown. Amino acid identities of the regions are indicated as percentages.

This 90-kDa DG kinase and the previously isolated rat 80-kDa DG kinase (8) shared 58% amino acid sequence identity overall. In particular, Ca^{2+}-coordinating residues of two EF-hand motifs (aa 152–180 and 197–225), cysteine residues of two cysteine-rich zinc-finger-like sequences (aa 257–292 and 319–356), and putative ATP-binding sites (aa 266–294 within the first cysteine-rich sequence and aa 533–560) were well conserved (Fig. 3).

By Northern blot analysis, a band of 6.2-kb mRNA, consistent with nearly the full length of the 90-kDa DG kinase cDNA, was detected predominantly in brain and less abundantly in adrenal gland and small intestine (Fig. 4). A faint band of mRNA was detected in heart. No hybridization bands were recognized in RNAs from thymus or spleen, which had been shown to express the 80-kDa DG kinase mRNA abundantly (8).

To confirm that 90-kDa DG kinase really possesses DG kinase activity, COS-7 cells were transfected with psreN-DGK, now termed psre90K-DGK, and then lysed. pSRE vector and psre80K-DGK (80-kDa DG kinase cDNA) were used as controls. In the octyl glucoside mixed-micelle assay, the lysate derived from cells transfected with psre90K-DGK had >15-fold higher DG kinase activity for each long-chain DG species compared with that from cells transfected with pSRE alone (Fig. 5*a*). It further showed 3- to 5-fold preference for long-chain DGs over diC_{10}. The 80-kDa DG kinase showed no phosphorylation selectivity among the DG species used. Similar results were obtained in the deoxycholate assay (Fig. 5*b*). The 90-kDa DG kinase activity was undetectable in the Triton X-100 assay (19) (data not shown). In addition, the 90-kDa DG kinase activity with 18:0/20:4 DG showed a 3-fold stimulation by Ca^{2+} [6.1 nmol/(min·mg) in the presence of 2 mM EGTA, 16.4 nmol/(min·mg) in the presence of 0.02 mM Ca^{2+} (values represent the means from two experiments)]. The 90-kDa DG kinase activity was recovered exclusively in the membrane fraction, whereas the 80-kDa DG kinase activity remained soluble (Fig. 6). This 90-kDa DG kinase activity was largely (82%) extracted from membranes by 1 M KCl buffer. The recovery of the enzyme activity was about 60% in the extraction procedure.

By *in situ* hybridization analysis of adult rat brain, the most intense expression of the gene was detected in the caudate–putamen, the accumbens nucleus, the olfactory tubercle, and the hippocampal pyramidal-cell layer (Fig. 7*a*). In the caudate–putamen, the hybridization signals were densely deposited throughout the entire region except for scattered small structures corresponding to the striate fiber bundles, indicating that glial cells were negative. At higher magnification, the hybridization signals were concentrated on many neu-

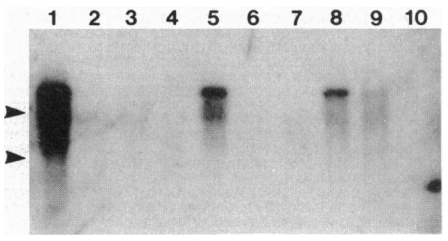

FIG. 4. Northern blot analysis of the 90-kDa DG kinase mRNA in various rat tissues. Poly(A)+ RNAs (5 μg per lane) from various tissues were electrophoresed and transferred to a nylon membrane. The filter was hybridized with 32P-labeled 1.2-kb *Eco*RI fragment of pNDGK1. Lanes: 1, brain; 2, spleen; 3, thymus; 4, testis; 5, adrenal gland; 6, liver; 7, kidney; 8, small intestine; 9, heart; 10, salivary gland. Size markers (arrowheads) are 28S and 18S rRNAs.

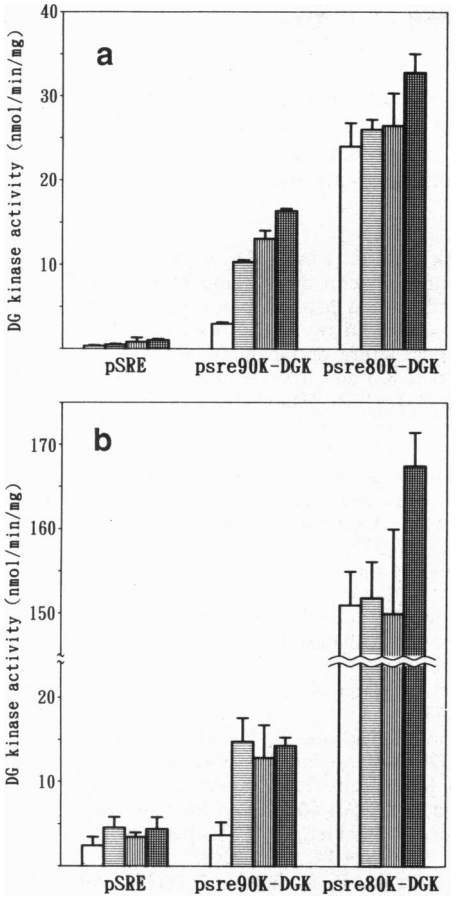

FIG. 5. DG kinase activities in COS-7 cells transfected with pSRE vector alone or the constructs designated psre90K-DGK (90-kDa DG kinase) and psre80K-DGK (80-kDa DG kinase). Lysates were assayed for kinase activity toward single DG species in the octyl glucoside mixed-micelle assay (*a*) and the deoxycholate assay (*b*). Values shown are means ± SD (n = 3). From left to right in each quartet: diC_{10}, 18:1/18:1 DG, 18:0/18:2 DG, and 18:0/20:4 DG.

ronal somata possessing chromatin-poor nuclei (Fig. 7*b*). Moderate levels of gene expression were observed in neuronal layers of the olfactory bulb. Cerebral neocortex, cerebellar cortex, and the dentate granular-cell layer expressed

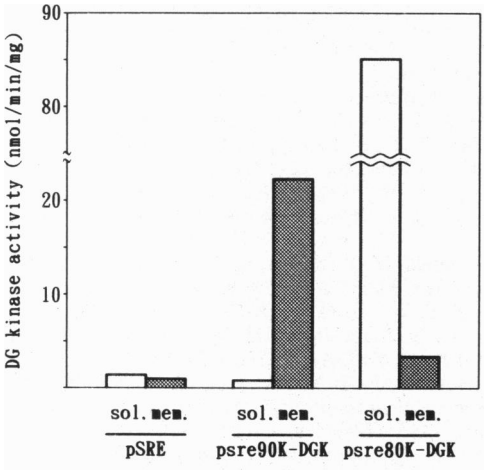

FIG. 6. Partition of DG kinase activities in transfected COS-7 cells. Lysates were centrifuged at 100,000 × *g* for 30 min to separate the soluble (sol.) and membrane (mem.) fractions. Both of them were examined in the octyl glucoside mixed-micelle assay with 18:0/20:4 DG. Values are the averages of duplicate determinations. Similar results were obtained in two repeated experiments.

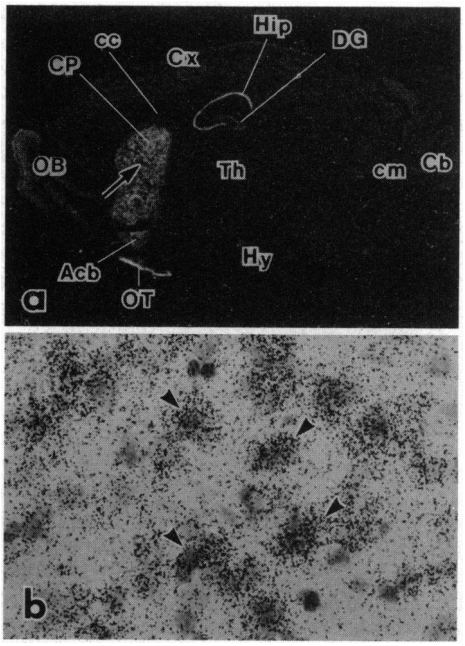

FIG. 7. *In situ* hybridization of the 90-kDa DG kinase mRNA in parasagittal section through the caudate–putamen of adult rat brain. (*a*) Note intense expression signals in the caudate–putamen (CP), the accumbens nucleus (Acb), the olfactory tubercle (OT), and the hippocampal pyramidal-cell layer (Hip) and moderate signals in neuronal layers of the olfactory bulb (OB). Also note the absence of hybridization in the white matter, such as scattered small structures within the caudate–putamen corresponding to the fiber bundles (arrow), the corpus callosum (cc), and the cerebellar medulla (cm). (×3.) Cx, cerebral cortex; Cb, cerebellar cortex; DG, dentate granular-cell layer; Th, thalamus; Hy, hypothalamus. (*b*) Bright-field microscopy of higher magnification of the caudate–putamen. Note specific concentration of the hybridization signals on many neuronal somata possessing chromatin-poor nuclei (arrowheads). (×335.)

the gene weakly. No significant hybridization signals were detected in the thalamus, hypothalamus, or most brainstem nuclei. Further, no hybridization signals were discerned in white matter such as that of the corpus callosum, the anterior commissure, and the cerebellar medulla, in contrast to the gene expression for the 80-kDa DG kinase (8). In control experiments brain sections were hybridized with the plasmid vector of an appropriate length, or some were pretreated with RNase A before hybridization. In either case, no significant hybridization signals were detected in any brain regions (data not shown).

DISCUSSION

We have cloned a mammalian DG kinase species that localizes in neurons. The conservation of EF-hand motifs in the regulatory domain of both the 90-kDa DG kinase and the 80-kDa DG kinase suggests that both isozymes may be regulated by Ca^{2+}. Indeed, the 90-kDa DG kinase was activated by Ca^{2+}, like the 80-kDa DG kinase (15). However, the 90-kDa kinase is distinct from the 80-kDa one in terms of subcellular distribution and expression cell types.

The membrane association of the 90-kDa DG kinase observed in the COS expression system is an intrinsic property of this molecule but was not due to the expression system itself, because the 80-kDa DG kinase was recovered exclusively in the soluble fraction in concurrent experiments (Fig. 6). However, by hydropathy analysis with GENETYX software (20), no amino acid sequences sharing significantly extended hydrophobicity were found in the primary structure of the 90-kDa kinase (data not shown). This, together with the

observation that the membrane-bound 90-kDa DG kinase can be solubilized with high salt (1 M KCl) in the absence of detergents, indicates that this enzyme is not an intrinsic membrane protein but may be loosely associated with the membrane. Amphipathic α-helices, which have been proposed to constitute a domain involved in protein–lipid interaction in some proteins (21, 22), have also been found in the structure of the 80-kDa DG kinase (15) and the 90-kDa DG kinase (data not shown). Although the α-helix was originally hypothesized to be involved in interaction between the 80-kDa DG kinase and membrane phospholipids (15), this possibility was denied later by the same authors using the EF-hand-deleted mutant (13). Thus this lack of interaction may also be the case for the α-helix in the 90-kDa kinase.

Glomset and coworkers (17, 23) have reported the existence and biological characteristics of a membrane-bound DG kinase in Swiss 3T3 cells that selectively phosphorylates arachidonoyl DGs at a rate 2- to 8-fold higher than that for other naturally occurring long-chain DGs. Different from their DG kinase species, the present 90-kDa DG kinase has no marked substrate selectivity among long-chain DGs, including arachidonoyl DG, although it has much lower kinase activity for short-chain DGs such as diC_{10} (Fig. 5). Since the present assays were performed at low temperature (30°C) without preincubation, the absence of this substrate selectivity in this species of DG kinase was not caused by the experimental inactivation of this enzyme, which has been described to occur with higher temperature and longer preincubation (17). In Northern blot analysis no hybridization bands corresponding to the 90-kDa DG kinase were detected in testis (Fig. 4), which has been shown to contain the membrane-bound, arachidonoyl DG kinase in baboon (19). Further, *in situ* hybridization analysis shows that the gene for the 90-kDa DG kinase is expressed intensely in restricted brain regions such as the caudate–putamen and olfactory tubercle (Fig. 7). This expression pattern is in sharp contrast to that for the 80-kDa DG kinase (8), whose mRNA is predominant in oligodendrocytes in the white matter, including the corpus callosum; in the caudate–putamen the message for the 80-kDa DG kinase is confined to the glial cells in the striate fiber bundles, which are negative for the 90-kDa DG kinase mRNA. All these findings suggest that DG kinase may be composed of multiple isozymes, each of which has a characteristic regional pattern of expression in different tissues and even in the brain. Actually, we have already obtained from the brain cDNA library two additional species of cDNA clones distinct from that of this 90-kDa DG kinase; details will be described elsewhere.

As noted in the Introduction, DG kinase represents an "off" signal for PKC-mediated response through the attenuation of DG, whereas phospholipase C (PLC) represents its "on" signal through the synthesis of DG from phosphatidylinositol. Both PKC and PLC consist of multiple isoforms, and each of them exhibits differential expression patterns of genes and proteins in the brain (24, 25). Among those various isoforms, the genes for PKC-β and PLC-β are expressed intensely in the caudate–putamen, a major representative of the brain regions showing high expression of the 90-kDa DG kinase mRNA. This parallel expression suggests that the 90-kDa kinase may preferentially attenuate the presumed system of PLC-β-mediated activation of PKC-β, at least in the caudate–putamen. Since the caudate–putamen is a major dopaminergic projection field (26) and because the dopamine receptor may be coupled to activation of PLC and phosphatidylinositol phosphate metabolism (27), detailed investigations of the relation between the function of the 90-kDa DG kinase and dopaminergic transmission are warranted. Further, the moderate expression of the 90-kDa DG kinase mRNA in the adrenal gland and intestine makes it necessary to examine whether this DG kinase species is involved in the secretion of adrenal chromaffin cells or cortical cells or in some functions of intestinal epithelial cells or smooth muscle cells.

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1. Bishop, W. R., Ganong, B. R. & Bell, R. M. (1986) *J. Biol. Chem.* **261**, 6993–7000.
2. Kanoh, H., Yamada, K. & Sakane, F. (1990) *Trends Biochem. Sci.* **15**, 47–50.
3. de Chaffoy de Courcelles, D., Roevens, P. & Van Belle, H. (1985) *J. Biol. Chem.* **260**, 15762–15770.
4. Nishizuka, Y. (1984) *Nature (London)* **308**, 693–698.
5. Inoue, H., Yoshioka, T. & Hotta, Y. (1989) *J. Biol. Chem.* **264**, 5996–6000.
6. Kanoh, H., Hondoh, H. & Ono, T. (1983) *J. Biol. Chem.* **258**, 1767–1774.
7. Sakane, F., Yamada, K., Kanoh, H., Yokoyama, C. & Tanabe, T. (1990) *Nature (London)* **344**, 345–348.
8. Goto, K., Watanabe, M., Kondo, H., Yuasa, H., Sakane, F. & Kanoh, H. (1992) *Mol. Brain Res.* **16**, 75–87.
9. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 5463–5468.
10. Chomczynski, P. & Sacchi, N. (1987) *Anal. Biochem.* **162**, 156–159.
11. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY).
12. Takebe, Y., Seiki, M., Fujisawa, J., Hoy, P., Yokota, K., Arai, K., Yosida, M. & Arai, N. (1988) *Mol. Cell. Biol.* **8**, 466–472.
13. Sakane, F., Imai, S., Yamada, K. & Kanoh, H. (1991) *Biochem. Biophys. Res. Commun.* **181**, 1015–1021.
14. Okayama, H., Kawauchi, M., Brownstein, M., Lee, F., Yokota, T. & Arai, K. (1987) *Methods Enzymol.* **154**, 3–28.
15. Sakane, F., Yamada, K., Imai, S. & Kanoh, H. (1991) *J. Biol. Chem.* **266**, 7096–7100.
16. Fabiato, A. & Fabiato, F. (1979) *J. Physiol. (London)* **75**, 463–505.
17. MacDonald, M. L., Mack, K. F., Williams, B. W., King, W. C. & Glomset, J. A. (1988) *J. Biol. Chem.* **263**, 1584–1592.
18. Kozak, M. (1986) *Cell* **44**, 283–292.
19. Lemaitre, R. N., King, W. C., MacDonald, M. L. & Glomset, J. A. (1990) *Biochem. J.* **266**, 291–299.
20. Kyte, J. & Doolittle, R. F. (1982) *J. Mol. Biol.* **157**, 105–132.
21. Kaiser, E. T. & Kezdy, F. J. (1987) *Annu. Rev. Biophys. Biophys. Chem.* **16**, 561–581.
22. Kalmer, G. B., Kay, R. J., Lachance, A., Aebersold, R. & Cornell, R. B. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 6029–6033.
23. MacDonald, M. L., Mack, K. F., Richardson, C. N. & Glomset, J. A. (1988) *J. Biol. Chem.* **263**, 1575–1583.
24. Young, S. W., III (1988) *J. Chem. Neuroanat.* **1**, 177–194.
25. Ross, C. A., MacCumber, M. W., Glatt, C. E. & Snyder, S. H. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 2923–2927.
26. Palacios, J. M. & Wamsley, J. K. (1984) in *Handbook of Chemical Neuroanatomy*, eds. Björklund, A., Hökfelt, T. & Kuhar, M. J. (Elsevier, Amsterdam), Vol. 3, pp. 325–334.
27. Mahn, L. C., Burch, R. M., Monsma, F. J., Jr., & Sibley, D. R. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 2196–2200.