In order to clone novel diacylglycerol kinase (DGK) isozymes, we first obtained a DGK-related cDNA fragment by polymerase chain reaction using the human hepatoma cell line HepG2 mRNA and degenerated primers. The amplified fragment was subsequently used as a probe for screening the cDNA library from HepG2 cells. We obtained a cDNA clone coding for a novel DGK isozyme (designated DGKy) comprised of 791 amino acid residues. The amino acid sequence of DGKy was 52 and 62% identical to those of previously sequenced porcine 80-kDa and rat 90-kDa enzymes, respectively. DGKy, although initially cloned from the HepG2 cDNA libraries, was unexpectedly expressed in the human retina abundantly and to a much lesser extent in the brain. Other human tissues, including the liver and HepG2 cells, contained extremely low levels of DGKy mRNA. Furthermore, HepG2 cells and most of the human tissues except for the retina and brain expressed a truncated DGKy with an internal deletion of 25 amino acid residues (Ile641-Gly656). When transfected into COS-7 cells, the non-truncated cDNA gave phosphatidylyserine-dependent DGK activity with no apparent specificity with regard to the acyl compositions of diacylglycerol. In contrast the truncated cDNA failed to give DGK activity in spite of the expression of its mRNA and enzyme protein in COS cells, being undetectable in other cells such as neurons, hepatocytes, and platelets (12, 15, 17) (reviewed in Ref. 18). The cell-type specific mode of expression of DGK isozymes was further demonstrated recently by the cDNA cloning of the second 90-kDa enzyme (DGKβ) from rat brain (19). The expression of this DGK isozyme was strikingly limited to neurons of restricted brain areas (19). The knowledge on the two cloned DGK species suggests the presence of other DGK isozymes with different expression patterns depending on the cell types, since the activity of DGK has been detected in a wide range of animal tissues. Whether or not other DGKs with distinct properties are also structurally related to the two DGK isozymes so far cloned remains totally unknown.

In order to gain insight into the physiological significance of DGK isozymes, we have tried to clone novel DGK species. For this purpose we selected human hepatoma HepG2 cells, since the two DGKs so far sequenced have been known to be lacking in hepatocytes (10–12, 15–17). Furthermore, an earlier study (20) showed that the partially purified rat liver DGK had an apparent molecular mass of 120 kDa, which does not correspond to the molecular sizes so far described for DGKs from other tissues. In the present study the novel DGK cloned from HepG2 cells was unexpectedly found to be a retina-characteristic DGK species. We have also detected the expression of an internally truncated mRNA encoding inactive DGK. To date DGK isozymes have been designated according to their approximate molecular masses. However, the novel isozyme cloned in the present work has an approximate molecular mass of 90 kDa, indicating that a new naming system is required to distinguish the DGK isozymes of a similar molecular mass. In this report we tentatively designate the DGK isozymes following the order of their cDNA cloning. The novel DGK described here is the third to be sequenced to our knowledge and is thus designated DGKy.

Diacylglycerol kinase (DGK) reverses the normal flow of glycerolipid biosynthesis by phosphorylating diacylglycerol back to phosphatidic acid. The role of DGK in phospholipid metabolism remains to be clarified, but a number of experiments using the enzyme inhibitor R59022 (1) (reviewed in Ref. 2) have suggested that this enzyme may regulate the intracellular level of diacylglycerol, which is an established activator of protein kinase C (3).

Previous enzyme purification (4–9) and immunological (10–12) studies have suggested the occurrence of multiple DGK isozymes in animal cells. The isozymes have been described to differ from each other with respect to molecular masses (5–9), enzymological properties (5–9), antigenicity (10–12), and substrate specificity (8, 13, 14). DGK thus appears to form an enzyme family consisting of isozymes of considerably different properties. Indeed, the 80-kDa DGK (hereafter referred to as DGKα), first cloned from cDNA libraries from porcine thymus (15), human lymphocytes (16), and rat brain (17) has been shown to be expressed in lymphocytes and oligodendroglial cells, being undetectable in other cells such as neurons, hepatocytes, and platelets (12, 15, 17) (reviewed in Ref. 18). The amino acid sequence of the two cloned DGK species suggests the presence of other DGK isozymes with different expression patterns depending on the cell types, since the activity of DGK has been detected in a wide range of animal tissues. Whether or not other DGKs with distinct properties are also structurally related to the two DGK isozymes so far cloned remains totally unknown.

Molecular Cloning of a Diacylglycerol Kinase Isozyme Predominantly Expressed in Human Retina with a Truncated and Inactive Enzyme Expression in Most Other Human Cells*

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Preparation and Screening of HepG2 cDNA Library—General manipulation of DNA was done following the standard protocol (21). Poly(A)+ RNA was isolated using a mRNA purification kit (Pharmacia Biotech Inc.) from total cellular RNA extracted from HepG2 cells by the guanidinium thiocyanate method (22). cDNA was constructed using a cDNA synthesis kit (Pharmacia). After addition of EcoRI adapters, the cDNA was inserted into λgt10 vector, and the recombinants were packaged into phage particles (Gigapack gold, Stratagene). Plaques of phages of Escherichia coli (NM514) lawn were screened using a 32P-labeled probe (275 bp), which was generated by RT/PCR from HepG2 mRNA as described below. The plaques were transferred to nitrocellulose membranes (Schleicher and Schuell) and screened by hybridization overnight at 60 °C in a solution containing 3 x SSC (1 x SSC = 0.15 M NaCl, 0.015 M sodium citrate), 10 x Denhardt’s solution, 0.5 mg/ml denatured salmon sperm DNA, and 0.1% SDS. The filters were washed twice at 37 °C in 3 x SSC, 0.1% SDS and finally once at 60 °C in 0.1 x SSC, 0.1% SDS. Approximately 2 x 106 plaques were screened. Positive clones were amplified by the BlueScript (Stratagene) and HindIII linker. The cDNA inserts were sequenced on both strands by the dideoxy chain termination method (23) using a Sequenase 2.0 kit (U. S. Biochemical Corp.). Sequences were analyzed using the GeneWorks software (IntelliGenetics, Inc.). The longest cDNA clone (3729 bp) obtained at this step was designated DGKG47 (Fig. 1).

RT/PCR Constructions—In order to obtain the probe for cDNA screening, RT/PCR was done following the protocol supplied with GeneAmp RNA PCR kit (Perkin-Elmer). In this case random hexamers were used as the primer of reverse transcriptase reaction with 100 ng of HepG2 mRNA. The reaction was continued for 30 min at 42 °C. The heat-inactivated (97 °C, 5 min) reverse transcription mixture (20 μl) was subjected to restriction mapping and sequence analysis. The fragment was 32P-labeled by a random prime reaction and subsequently hybridized to the corresponding site of DGKG4. The composite cDNA clone thus synthesized by an Applied Biosystems model 391 DNA synthesizer. In the case of DGK assay, phosphatidylinositol and 1,2-diacylglycerol (didecanoylglycerol, dioleoylglycerol, and stearoyl-arachidonoylglycerol) were bought from Sigma. n-Octyl β-D-glucoside was purchased from Dopindo Laboratories (Kumamoto, Japan).

Preparation and Screening of HepG2 cDNA Library—General manipulation of DNA was done following the standard protocol (21). Poly(A)+ RNA was isolated using a mRNA purification kit (Pharmacia Biotech Inc.) from total cellular RNA extracted from HepG2 cells by the guanidinium thiocyanate method (22). cDNA was constructed using a cDNA synthesis kit (Pharmacia). After addition of EcoRI adapters, the cDNA was inserted into λgt10 vector, and the recombinants were packaged into phage particles (Gigapack gold, Stratagene). Plaques of phages of Escherichia coli (NM514) lawn were screened using a 32P-labeled probe (275 bp), which was generated by RT/PCR from HepG2 mRNA as described below. The plaques were transferred to nitrocellulose membranes (Schleicher and Schuell) and screened by hybridization overnight at 60 °C in a solution containing 3 x SSC (1 x SSC = 0.15 M NaCl, 0.015 M sodium citrate), 10 x Denhardt’s solution, 0.5 mg/ml denatured salmon sperm DNA, and 0.1% SDS. The filters were washed twice at 37 °C in 3 x SSC, 0.1% SDS and finally once at 60 °C in 0.1 x SSC, 0.1% SDS. Approximately 2 x 106 plaques were screened. Positive clones were amplified by the BlueScript (Stratagene) and HindIII linker. The cDNA inserts were sequenced on both strands by the dideoxy chain termination method (23) using a Sequenase 2.0 kit (U. S. Biochemical Corp.). Sequences were analyzed using the GeneWorks software (IntelliGenetics, Inc.). The longest cDNA clone (3729 bp) obtained at this step was designated DGKG47 (Fig. 1).

Preparation of Anti-DGK Antibodies and Immunoblot Analysis—The cDNA fragment (407 bp) encoding amino acids 1–134 of DGKα was obtained by digestion of DGKα with Dral and PvuII. The fragment was cloned into the T7 RNA polymerase-dependent pBluescript II vector. The expression was done in Escherichia coli strain BL21(DE3)/pLysS and induced in the presence of 1 mM isopropyl-β-D-thiogalactopyranoside as described (31). The expressed fusion protein recovered in insoluble fractions was solubilized with 8 M urea and dialyzed against 30 mM Tris-HCl (pH 7.4) containing 30 mM NaCl, 20 mM NαF, 2.06 mM CaCl2, 10 mM MgCl2, 2.0 mM EDTA, 1.5 mM diacylglycerol, 2.9 mM phosphatidylinositol, and 1 mM (10,000 cpm)32P-γATP. Both fractions (soluble and insoluble) were subjected to SDS/PAGE and transferred to nitrocellulose membranes (Schleicher and Schuell). Blotted DNA was cross-linked to the membrane by ultraviolet light and hybridized for 18 h at 60 °C to a 32P-labeled cDNA probe obtained by KpnI-BglII treatment of DGKα (nt 1812–2669). The hybridized membrane was washed as described for the cDNA screening and exposed to Fuji x-ray film at −80 °C with the help of an intensifying screen. A parallel amplification of glyceraldehyde-3-phosphate dehydrogenase cDNA fragment (316 bp) (25) was done using the human glyceraldehyde-3-phosphate dehydrogenase control amplifier set (Clontech) to assess the integrity and concentrations of mRNAs used.

cDNA Transfection and Assay of DGK Activity—The two cDNA clones, DGK4 and DGK47 with and without the internal deletion, were digested with Dral and ApaLI, blunted with T4 DNA polymerase, and ligated with E.coli linker. The cDNAs were then subcloned into the simian virus 40-based expression vector pSRE (26), which was derived by modification of the original pcDL-SR-α-296 vector (27). The resulting constructs (approximately 10 μg) and the expression vector DGK12 harboring porcine DGKα cDNA (26) were transfected into COS-7 cells using DEAE-dextran (28) as described previously (26). After 3 days, the cells were harvested and lysed by sonication in the lysis buffer (0.5 M/100-mm dish) containing 20 mM Tris-HCl (pH 7.4), 0.25 M sucrose, 1 mM EDTA, 4 mM EGTA, 1 mM diithiothreitol, 20 μg/ml each of leupeptin, pepstatin, aprotinin, and soybean trypsin inhibitor, and 1 mM phenylmethylsulfonyl fluoride. After a low speed centrifugation (500 × g for 10 min), the supernatant was used for DGK activity assay.

The octyl glucoside mixed micellar assay of DGK activity was done as described (29). In brief, the assay mixture (50 μl) contained 50 mM MOPS (pH 7.4), 50 mM octyl glucoside, 1 mM diithiothreitol, 100 mM NaCl, 20 mM NaF, 2.06 mM CaCl2, 10 mM MgCl2, 2.0 mM EDTA, 1.5 mM diacylglycerol, 2.9 mM phosphatidylinositol, and 1 mM (10,000 cpm)32P-γATP. The mixture was calculated to be 50 μl using the computer program based on the data described (30). The reaction was initiated by adding enzyme (less than 5 μg of protein) and continued for 3 min at 30 °C. Lipids were extracted from the mixture, and phosphatidic acid was separated by thin layer chromatography (13) was scraped and counted by a liquid scintillation spectrophotometer.
cDNA Cloning of Diacylglycerol Kinase Isozyme

RESULTS

cDNA Cloning of DGKY—In an attempt to identify novel DGKs, we performed RT/PCR using degenerated primers and mRNA from HepG2 cells, since previously sequenced DGKs are known to be lacking in hepatocytes. The amino acid sequence encoded by the 275-bp PCR fragment (DGKGl, Fig. 1) was 64 and 80% identical to but still clearly distinct from those of porcine (15) or human (16) DGKs and rat DGKβ (19), respectively. We thus initially considered that this fragment represented a novel DGK isozyme expressed in hepatocytes. DGKGl was subsequently used as a probe for screening cDNA libraries constructed from HepG2 mRNA. Of the 2 × 106 plaques screened, we obtained six positive clones, which were found to represent a single clone when restriction mapped and sequenced. As shown in Fig. 2, the longest 3729-bp cDNA (DGKG4) contained the 5'- and 3'-untranslated sequences with an open reading frame encoding 766 amino acids including the initiator methionine (calculated M, = 86,077). There were several in-frame stop codons in the 5'-untranslated region, but we could not detect typical polyadenylation signals in the 3' sequence. The nucleotide sequence around the initiation codon fulfilled criteria for eucaryotic initiation sites (33).

The basic structure of this putative DGK was very similar to those of previously sequenced DGKs. In particular, the sequence contained the structural characteristics previously noted in other DGKs: the two Ca2+-coordinating EF-hand sequences (residues Lys179–Met207 and Ile224–Thr252) and the two zinc finger-like cysteine-rich sequences (residues His372–Cys380 and His387–Cys398). Furthermore, there were an N-terminal conserved region (C1, Leu55–Gln58) and a highly identical region (C4, Ile199–Pro775, numbered as in the composite DGKG47, see later) at the C-terminal portion, as summarized in Fig. 3. It was noted, however, that the putative ATP binding motif, GXGXXG—K (X, any amino acid), conserved in the first zinc finger and the C-terminal portion of both DGKs α and β was only partially retained (see the sequence starting from Gly304 and Gly309, Fig. 2). It was also noted that the identity of amino acid sequences of the two zinc fingers was not particularly high among different DGKs sequenced (Fig. 3), although the relative locations of critical residues like His and Cys were well conserved. In addition, Gly309 of the first zinc finger of DGKG4, which has been always retained at the corresponding sites of all sequenced DGKs and other proteins having a similar structure, was substituted for Ala in the second finger (Ala316) (Fig. 2). These structural features strongly suggested that the DGKG4 clone encoded a novel DGK, and we designated this enzyme DGKG4. However, the sequence comparison showed that there was an apparent gap of 25 amino acids in the C4 region (Fig. 2). Since the C4 region is most highly conserved among DGKs, we suspected an internal deletion in this area. Furthermore, the lysate of COS-7 cells transfected with DGKG4 failed to give an increased DGK activity, suggesting that the encoded protein may be catalytically inactive as will be described later.

In order to confirm the suspected deletion, we next performed the second round of RT/PCR of the HepG2 mRNA using the amplimers flanking the site of possible deletion. When the PCR products were gel electrophoresed, we noted a trace amount of a 708-bp fragment in addition to the relatively abundant 633-bp product as expected from the sequence of DGKG4 (not shown). Both cDNAs were subcloned, and three independent clones originated from each fragment were subjected to sequence analysis. We confirmed that the 633-bp fragment contained the same sequence as determined for DGKG4. However, the 708-bp product (DGKG7, Fig. 1) contained an additional 75 bp encoding 25 amino acids, which were highly conserved in other DGKs (Fig. 2). These results confirmed that HepG2 cells contained two species of DGK mRNA, i.e., truncated and full-length species, and that the internal deletion we detected was not due to cloning artifacts. The composite cDNA thus constructed (Fig. 1, DGKG47) encoded the full-length DGK comprised of 791 amino acid residues of the calculated M, = 88,895 (Fig. 2). As summarized in Fig. 3, the full-length DGKy shared 52 and 62% identical amino acid sequences overall with porcine DGKα (15) and rat DGKβ (19), respectively. It is apparent that these DGKs shared common basic structures.

Transient Expression of DGKY—In order to investigate the catalytic properties of DGKY, DGKG4 and DGKG47, encoding the truncated and full-length enzymes, respectively, were subcloned into the expression vector, pSRE, and transfected into COS-7 cells. The expression vector, DKG212, encoding porcine DGKα (26), was also used in parallel as a control. As shown in Fig. 4A, the cell lysate transfected with DGKG47 gave much higher DGK activity when compared with that obtained from cells transfected with the vector alone. The activities toward didecanoylglycerol, dioleoylglycerol, and stearoylarachidonoyl-glycerol were not much different, indicating that DGKY possessed no marked specificity with regard to the acyl compositions of diacylglycerol. In contrast to DGKG47, DGKG4 encoding the truncated DGK failed to increase the DGK activity of the transfected cells in repeated experiments, suggesting that the truncated DGK was catalytically inactive. To confirm this, total RNA and the lysates from transfected cells were subjected to Northern (not shown) and Western blot analysis, respectively. The DGKy mRNA was detected for both DGKG4 and DGKG47 transfections to a comparable extent (not shown). The expression of the truncated and full-length DGK enzyme proteins could be confirmed by immunoblotting with antibodies raised against the N-terminal portion of the enzyme (Fig. 4B). Immunoreactive proteins corresponding to the molecular masses of the two species of DGK were detected to a similar level in cells transfected with the two cDNAs, whereas no bands were detected in the control cells. It was also shown that the antibodies did not cross-react with DGKα (Fig. 4B, lane 4). The results demonstrated that the truncated DGKy mRNA detected in HepG2 cells encoded inactive enzyme and strongly suggested that the internal truncation occurred in a catalytically essential region.

In contrast to DGKα expressed in COS cells, which was exclusively present in soluble fraction as previously reported (26), the DGK activity was recovered in both soluble (40%) and membrane (60%) fractions (average of two transfections). Most of the membrane-associated activity (more than 80%) was released when treated with 1 M KCl. This may suggest that a part
tifs and zinc fingers are underlined with composite cDNA encoding single primers used. The rows (GPI-GPS) sequence, in-frame stop codons are indicated in boldface letters.

**FIG. 2.** Nucleotide sequence of the composite cDNA encoding human DGKγ and the deduced amino acid sequence. Two sets each of EF-hand motifs and zinc fingers are underlined with a single and double lines, respectively. Arrows (GP1-GP5) correspond to the PCR primers used. The boxed sequence was deleted in DGKγ4. In the 5'-flanking region, in-frame stop codons are indicated in boldface letters.
DGKγ can be loosely bound to the membranes as has been noted for rat DGKβ (19). We next studied the effects of phosphatidylserine and Ca\(^{2+}\) on the enzyme activity, since the two compounds are known to be required for the maximal activities of both DGKs α (26, 29) and β (19). Different from DGKα, the activity of which was dependent on both phosphatidylserine and Ca\(^{2+}\) as reported previously (26, 29), the DGKγ activity became maximal with phosphatidylserine alone (Fig. 5). The Ca\(^{2+}\) independence of the DGKγ activity was confirmed in the experiments in which the Ca\(^{2+}\) concentrations were varied from 0.1 to 100 μM in the presence and absence of phosphatidylserine (not shown). We therefore do not know the function of EF-hands of DGKγ, although the sequence adequately conforms to the criteria proposed to be required for the Ca\(^{2+}\) binding (34).

**DISCUSSION**

DGKγ cDNA cloned from HepG2 cells turned out to encode an isozyme characteristically expressed in the human retina. However, the use of HepG2 cells as the source of cDNA led us to detect the expression of an internally truncated mRNA, which was shown to encode a catalytically inactive enzyme.
Fig. 5. Effects of phosphatidylserine and Ca\(^{2+}\) on the activity of DGK\(\gamma\) transiently expressed in COS-7 cells. The lysates were prepared from COS cells transfected with the full-length DGK\(\gamma\) cDNA (DGKG47) and with porcine DGK\(\alpha\) (DGK212). The DGK activity was measured by the octyl glucoside mixed micellar assay in the presence and absence of 2.9 mM phosphatidylserine and 50 \(\mu\)M Ca\(^{2+}\) as indicated in the figure. The results are means \(\pm\) S.D. from three independent assays.

Fig. 6. Southern blot analysis of the RT-PCR products from different human cells. A, following 20 cycles of PCR amplification using mRNAs (100 ng each) of different human tissues and cells, the products (5 \(\mu\)l of 100-\(\mu\)l reaction mixture) were gel electrophoresed and transferred to a nylon membrane. The amplification products were detected using \(^{32}\)P-labeled DGK\(\gamma\) cDNA as a probe. Lane 1, retina; lane 2, liver; lane 3, brain; lane 4, Jurkat; lane 5, HL60; and lane 6, HepG2 cells. In lanes 7 and 8, PCR amplification using the human retina mRNA (100 ng) was done for 18 and 22 cycles, respectively, and the linearity of the amplification signals was confirmed by a densitometric scanning. The figure is a representative of twice repeated experiments with a similar result. In this experiment each mRNA gave a comparable level of glyceraldehyde-3-phosphate dehydrogenase cDNA fragment (983 bp) in a parallel amplification experiment (not shown). B, the PCR amplification using 100 ng each of different mRNAs was increased to 30 cycles, and 3 \(\mu\)l of the mixture was analyzed as described in A except for brain (lane 1) and retina (lane 9) in which samples were diluted 10-fold before electrophoresis. Lane 2, Jurkat; lane 3, HL60; lane 4, HepG2 cells; lane 5, liver; lane 6, kidney; lane 7, spleen; and lane 8, testis. Furthermore, the critical importance of DGK in the visual function of the fruit fly has been demonstrated in the rdgA mutant, which has a mutated eye-specific DGK gene (44, 45). It is likely that also in the mammalian retina DGK participates in the renewal and degradation process of membrane phospholipids, which is directly or indirectly linked to the visual functions. The existence of DGK\(\gamma\) almost exclusively expressed as an active form in the human retina strongly suggests the potential importance of DGK in the visual functions.

The basic structure of DGK\(\gamma\) was shown to be very similar to those of DGKs \(\alpha\) and \(\beta\). DGK\(\alpha\) is known to be expressed in oligodendrocytes of rat brain (17), while the expression of DGK\(\beta\) is confined to neurons of the striatum (19). Thus, all of the three cloned DGKs happen to be typically expressed in the central nervous system. It is interesting to see whether other DGKs with distinct properties and cellular distributions also possess similar basic structures. It is also almost certain that DGK\(\gamma\) only weakly expressed in the brain is located in a very limited cell population within the brain.

The function of the conserved regions of DGKs remains largely unknown. Although the EF-hands of DGK\(\alpha\) have been shown to be high affinity Ca\(^{2+}\) binding sites (26, 29), DGK\(\gamma\) with a similar structure failed to be activated by Ca\(^{2+}\) in contrast to DGK\(\alpha\). We therefore do not know the function of EF-hands of DGK\(\gamma\), although this region fulfills the conditions required for Ca\(^{2+}\) binding (34) including the distribution of seven oxygens in the putative Ca\(^{2+}\) binding loops. The deletion of 25 amino acid residues at the C-terminal portion resulted in a complete loss of DGK activity. This deletion occurred in the C4 region most highly-conserved not only in the mammalian DGKs but also in...
the two Drosophila DGK homologs (24, 45). However, the deleted amino acid sequence did not contain apparent ATP binding consensus sequences so far noted for protein kinases (46) and other ATP binding proteins (47). The putative ATP binding sequences previously noted for both DGKs (15, 16) and β (19) were retained only incompletely and did not reside in the deleted region. DGK may have unique ATP binding sites(s), or the deletion caused a conformational change leading to the inactivation. These possibilities and the function of the conserved regions of DGKs are the targets of further investigation.

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