Cloning and Characterization of a Glucocorticoid-induced Diacylglycerol Kinase*

(Received for publication, March 25, 1996, and in revised form, May 23, 1996)

Theresa M. Klauck, Xuequn Xu, Betty Mousseau, and Susan J. Jaken‡
From the W. Alton Jones Cell Science Center, Lake Placid, New York 12946

Diacylglycerol kinase (DGK) plays a key role in cellular processes by regulating the intracellular concentration of the second messenger diacylglycerol. We screened a hamster DDT1 smooth muscle cell library and isolated a unique, glucocorticoid-inducible cDNA with substantial homology to known DGKs. DGK activity was increased in lysates of insect cells infected with recombinant baculovirus containing this cDNA. Antibodies raised against expressed sequences recognized a glucocorticoid-inducible 130–140-kDa protein on immunoblots of DDT1 cell lysates. Thus, this sequence appears to be a new member of the DGK family that we refer to as DGKα. Homology to other DGKs was apparent in domains that are thought to be important for DGK function including the cysteine-rich motifs and potential catalytic domains. DGKα shares substantial homology with DGKβ including the N-terminal pleckstrin homology domain. The tissue distribution of DGKα message (determined by ribonuclease protection assays) and protein (determined by immunoblots) was more abundant than reported for other DGKs, indicating that DGKα may play a more general role in regulating cellular DG levels than other DGKs. Heterogeneity among DGK family members indicates that individual DGKs may have unique functions.

* This work was supported by National Institutes of Health Grants CA53841 and CA65874 (to S. J. J.). 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 abbreviations used are: DG, diacylglycerol; CRMs, cysteine-rich motifs; DGK, diacylglycerol kinase; PKC, protein kinase C; RACE, rapid amplification of cDNA ends; kb, kilobase pair(s); bp, base pair(s); RPA, ribonuclease protection analysis; MHCK, myosin heavy chain kinase.

**The nucleotide sequence(s) reported in this paper has been submitted to the GenBank®/EBI Data Bank with accession number(s) U94929.

‡ To whom correspondence should be addressed: W. Alton Jones Cell Science Center, 10 Old Barn Rd., Lake Placid, NY 12946. Tel.: 518-523-1260; Fax: 518-523-1849; E-mail: sjaken@northnet.org.

The abbreviations used are: DG, diacylglycerol; CRMs, cysteine-rich motifs; DGK, diacylglycerol kinase; PKC, protein kinase C; TAA, triamcinolone acetonide; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; kb, kilobase pair(s); bp, base pair(s); RPA, ribonuclease protection analysis; MHCK, myosin heavy chain kinase. Be second messengers as well (5–8). Thus, DGK has two important functions, first, to limit cellular levels of DG and second, to generate additional second messengers.

Several DGK activities have been purified or partially purified (4, 9), and a number of DGK and DGK-related clones have been isolated (10–18). DGKs appear to differ with respect to their molecular weight, cofactor regulation, tissue distribution, and substrate specificity; however, direct comparisons of DGK activities have been hampered by the lack of a standard assay technique and availability of specific antibodies. Alignment of DGK sequences has been used to identify motifs that may be important for DGK function and regulation. These include two calcium-binding EF hands that confer calcium-dependent activity (19), two cysteine-rich motifs (CRMs) that are similar to those found in PKC and Raf kinase (2), and a putative catalytic domain in the C-terminal half of the protein. The CRMs and the putative catalytic domain sequences are conserved in all DGKs including those from Drosophila (14) and Caenorhabditis elegans (13). Genetic analysis of Drosophila rdpA mutants has linked mutations in the DGK2 gene to retinal degeneration (20) indicating that this retinal specific form of DGK is essential for normal retinal function. Specific functions of other DGKs have not yet been identified.

In the course of studying PKCα regulation in hamster DDT1 smooth muscle cells, we identified a high molecular weight, glucocorticoid-inducible protein (referred to as DGKα) that cross-reacted with a PKCα-specific monoclonal antibody. Because of the possibility that this could have been a unique form of PKC, we immunoscreened a glucocorticoid-induced DDT1 cell cDNA library with the PKCα antibody. Several clones were isolated, none of which had appreciable homology to PKCs or other protein kinases. However, they were homologous to DGK sequences within CRMs and the putative catalytic domains. Expression of a partial cDNA confirmed that the sequence encodes a DGK activity. Our results demonstrate that DGKα is a unique form of DGK with broad tissue distribution. Differences from previously reported DGKs suggest that DGKα may have a specific role in cellular DG metabolism.

EXPERIMENTAL PROCEDURES

Materials—Male Syrian hamsters weighing 100–150 g were purchased from Charles River Breeding Lab Inc., Wilmington, MA. Fetal bovine serum, Dulbecco’s modified Eagle’s medium, and Ham’s F12 were from Life Technologies, Inc. Insulin and transferrin were from Sigma. Vitrogen 100 was from Collagen Corp., Palo Alto, CA. Escherichia coli diacylglycerol kinase was from Lipidex, Inc., Westfield, NJ. 1,2-Dioleoyl-sn-glycero-diolein (diolein) was from Avanti Polar Lipids, Inc., Alabaster, AL. [γ-32P]ATP (3000 Ci/mmol) and [α-32P]UTP (3000 Ci/mmol) were from DuPONT NEN. All restriction enzymes, Taq polymerase, Prime-a-Gene Kit, and alkaline phosphatase-conjugated goat anti-rabbit or anti-mouse immunoglobulins were from Promega, Madison, WI. The MAXIscript T7/RFI in vitro transcription and RPA II Kits were from Ambion, Austin, TX. The TA Cloning Kit was from Invitrogen Corp., San Diego, CA. ECL Western blot reagents were from Amersham Corp. Affi-Gel 10 and protein assay reagents were from Bio-Rad. Nitro-PLus was from Micron Separations Inc., Westborough, MA. Recombi-
nant baculovirus expressing PKCα was a kind gift from Dr. R. Bell, Duke University Medical Center, Durham, NC. All other reagents not listed were of the highest quality and from the best source possible.

Cloning and Sequencing—A Lambda Zap II expression library (kindly provided by Dr. Steve Harris, University of Texas, San Antonio) prepared from glucocorticoid-induced DDT1 cells was immunoscreened with a PKCα-specific monoclonal antibody (M6) (21) according to standard protocols. Three positive clones were isolated out of two million plaques screened. Bluescript® SK phagemid (pSK) containing the positive inserts was excised from lambda phage and used for sequencing. Additional clones were isolated by rescreening the library with digoxigenin-labeled cDNA probes prepared from pSK4 (see Fig. 2) according to standard protocols. To obtain additional 5′ sequences, a second DDT1 library (kindly provided by Dr. J. Im, Norris, Medical University of South Carolina, Charleston, SC) was screened with a cDNA probe prepared from a 5′ fragment of pSKA21, (pSKA21a) and two identical overlapping fragments. The full product was gel-purified, blunt-ended, and subcloned into pSK for further sequencing.

Both 5′ RACE and inverse PCR strategies were used to obtain the remaining 5′ end. The 5′ Amplifier RACE kit (Clontech Laboratories, Inc., Palo Alto, CA) was used with GP45 (5′-CTCATGGGTTCGGCTGACTAC-3′) for reverse transcription and the nested primer GP23 (5′-GCTGATCAGCTAGCTCCTAC-3′) and an anchor primer for amplification. For inverse PCR, cDNA was reverse-transcribed from GP43 (5′-CTCTCTGCTGCACATGCTACGAATGGGCG-3′) to obtain unknown 5′ sequence (23). The PCR products from 5′ Amplifier and inverse PCR were cloned directly into pCR1 with the TA Cloning kit.

Inserts of positive clones were sequenced on both strands using a combination of manual and automated dyeoxy-chain termination reactions. A USB Sequenase Version 2.0 kit was used for the manual sequencing. Automated sequencing was done using an Applied Biosystems 370A Automated DNA Sequencer (Applied Biosystems, San Francisco, CA) using either dye-primer or dye-terminator protocols. Double-stranded DNA for sequencing was prepared by Promega Magic Miniprep columns. Sequencing data were analyzed using GeneWorks ( IntelliGenetics, Inc., Mountain View, CA) and DNASIS (Hitachi Software Engineering America, Ltd., Brisbane, CA).

Source of Primary Antibodies—The M6 monoclonal antibody used in this study was prepared to purified rabbit PKCα and recognizes the catalytic domain of PKCα (21). The antipeptide antibody A10 was prepared to a synthetic peptide consisting of the 15 C-terminal amino acids of the DGKα predicted amino acid sequence. A10 antiserum was purified to homogeneity by affinity purification with Affi-Gel (BioRad). The digoxigenin-labeled antibodies A12 and A21 were raised in rabbits against purified fusion proteins produced in bacteria expressing the pSK4 or the pSKA21b insert, respectively. The pSK4 insert was expressed as a glutathione S-transferase fusion protein from a pGEX3X vector (Phar- macia Biotech Inc.) and purified by electrophoresis. Subclone A21b was expressed as a histidine-tagged fusion protein from pQE31 (Qiagen, Inc., Chatsworth, CA) and purified under nonnaturating conditions with a nickel affinity column according to the Qiagen protocol. Both antisera were affinity purified on Affi-Gel-10 columns containing the cognate fusion protein.

Western Blots—Proteins were separated by SDS-polyacrylamide gel electrophoresis (7.5% unless indicated otherwise) (24). The proteins were transferred to Nitro-Plus, and blots were immunostained as described previously (25) and developed with either alkaline phosphatase substrates or enhanced chemiluminescence reagents.

Cell Culture—Hamster DDT1-MF2 cells (kindly provided by Dr. Steve Harris, University of Texas, San Antonio) were plated at a density of 1 × 10⁶ cells/150-mm tissue culture dishes coated with 10 μg/ml Vitrogen. Cells were maintained in 11 Dulbecco's Modified Eagles medium/12 supplemented with 5 μg/ml insulin, 5 μg/ml transferrin, 3 × 10⁻⁶ M selenium (DFITS), 100 units/ml penicillin, 0.1 mg/ml streptomycin, and 1% charcoal-stripped fetal bovine serum (CSFSB). For glucocorticoid-induction experiments the cells were plated at 1 × 10⁶ cells/cm² on 100-mm tissue culture dishes coated with Vitrogen and grown in DFITS + 1% 2 × CSFSB. Triamcinolone acetonide (TTA, 1 × 10⁻⁶ M) was added after 3 days where indicated. Cell lysates were collected in lysis buffer (0.25 M sucrose, 25 mM Tris, pH 7.4, 0.25 M magnesium acetate, 1 mM diithiothreitol, 2.5 mM EDTA, 10 μM leupeptin, 1 mM phenylmethylsulfonyl fluoride, 10 μM aprotinin) and prepared for electrophoresis.

Baculovirus Expression—A partial DGKα construct (pSKDGKα) was prepared in pSK by ligating together pSK5 R1 and pSKB24 using convenient restriction sites (see Fig. 2 for a schematic map of DGKα clone). The total size of the insert was 4,112 kb, and the predicted protein size was 116 kb corresponding to an open reading frame of 31 kb. This insert was dosed into the blueBaclastHis transfer vector (Invitrogen Corp., San Diego, CA) for preparation of recombinant baculovirus (BdGKα-p). The full-length DGKα baculovirus (BdGKα) was constructed by addition of a pCR1154/5 fragment to the 5′ end of pSKα-a. Subsequently, the BamHI/HindIII fragment containing the full-length DGKα coding region was dosed into pBlueBacHisA for preparation of recombinant baculovirus.

Recombinant baculoviruses were prepared by cotransfecting the transfer vector (0.4 μg) with linearized baculovirus DNA (0.1 μg) (Baculogold from Pharmingen, San Diego, CA) in Sf9 insect cells with Lipofectin (Life Technologies, Inc.). For expression studies, Sf9 cells were infected with the viral stock and collected 3 days post-transfection in sucrose/ATP buffer containing 0.25 M sucrose, 25 mM Tris, pH 7.4, 0.005 M EDTA, 0.5 mM dithiothreitol, 2.5 mM EDTA, 10 μM leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 10 μM aprotinin. Protein values were determined by the method of Bradford (26).

DGK Assay—The DGK assay was carried out as described previously with some minor changes (27). Samples were collected in sucrose/ATP buffer described above. The assay was performed in a 50-μl reaction volume containing 100 mM Tris, pH 7.4, 1 mM sodium deoxycholate, 0.5 mM diethiothreitol, 1 mM diol, 1.6 mM (γ-32P)-ATP (5000 cpm/nmol), 5 μM magnesium chloride, and 50 μg of sample protein. The stock solution (0.25 mCi) (Avanti Polar Lipids, Alabaster, AL) was freshly prepared by sonicating on ice in 100 mM Tris, pH 7.4, containing 25 mM sodium deoxycholate and 1.25 mM diethiothreitol. An aliquot (20 μl) of this solution was added to 20 μl of the protein in 20 μl of 50 mM Tris buffer (pH 7.4). All reactions were made at 30°C and initiated by adding 10 μl of a 5 × solution containing 8 μM [γ-32P]ATP and 25 mM magnesium chloride. Samples were incubated at 30°C for 10 min. Concentrated hydrochloric acid (50 μl) was added to stop the reaction. The lipids were extracted by adding 0.5 ml of water and 0.33 ml of butanol. After vortexing, the tubes were centrifuged for 3 min at 2000 rpm. The upper layer was transferred to a new tube and washed with an equal volume of butanol-saturated water. An aliquot of the upper layer (50 μl) was assayed on a scintillation counter.

Tissue Distribution Analysis—Male Syrian hamsters (100-150 g) were anesthetized with 90 mg/kg pentobarbital by injection into the lower abdominal cavity. For immunoblotting, the appropriate tissues were removed, rinsed in ice-cold homogenization buffer (20 mM Tris, pH 7.4, 5 mM EDTA, 2 μg/ml diethiothreitol, 0.25 mM sucrose, weighed, and homogenized in a 1:5 ratio of tissue homogenization buffer (3 volumes/g tissue) containing 10 μg/ml leupeptin, 1 mM phenylmethylsulfon fluoride, and 10 μM aprotinin) was added before homogenization in a Dounce homogenizer and sonicated 5 × 10 s. Protein values were determined by the method of Bradford (26). Samples (100 μg protein/lane) were separated on 7.5% SDS-polyacrylamide gels and blotted to NitroPlus.

Total RNA for ribonuclease protection analysis (RPA) was prepared from male Syrian hamster tissues. Tissues were placed in RNA extraction buffer (REB, 4 M guanidine isothiocyanate, 25 mM sodium citrate, 0.1 M β-mercaptoethanol) at 10 μg/tissue and homogenized for 30 s in a Brinkman Polytron. Total RNA was prepared by cesium chloride centrifugation using standard procedures. The total RNA was resuspended in ribonuclease-free water to a concentration of 2 μg/ml. The ribonuclease protection assay is described below.

Ribonuclease Protection Assay—An antisense RNA probe was prepared from a linearized pSK vector containing a 197-bp HindIII/EcoRI fragment (nucleotides 3255-3451) of partial DGKα cDNA with T3 RNA polymerase and [32P]UTP according to the Ambion MAXIscript T7/T3 In Vitro Transcription kit protocol (Ambion, Inc., Austin, TX). Total RNA (20 μl of tissue sample) was processed according to standard protocol or with the Ambion RPA II Kit. Samples were separated on a 5% sequencing gel that was dried and autoradiographed overnight. Hamster probes for a suitable standard (such as glyceraldehyde phosphate dehydrogenase) were not available for these studies. Therefore, results are normalized to total RNA per tissue sample. The relative distributions of DGKα

140-kDa Diacylglycerol Kinase

5.25

3.8

3.2

2.9

2.6

2.3

2.0

1.7

1.4

1.1

0.8

0.5

0.2

0.0
140-kDa Diacylglycerol Kinase

Fig. 1. Glucocorticoid induction of DGK\textsubscript{1} in DDT1 cells. DDT1 cells were treated with 10\textsuperscript{-8} M TAA for the indicated times. Total cell lysates were collected, and aliquots (100 \textmu g of protein/lane) were separated by SDS-polyacrylamide gel electrophoresis and blotted to Nitro-Plus. The blot was probed with M6, a monoclonal antibody directed toward the catalytic domain of PKC\textsubscript{α} (21).

message were similar with two independent preparations of tissue RNAs.

RESULTS

Isolation of DGK\textsubscript{1} Clones—In studies designed to study the hormonal regulation of PKC\textsubscript{α} in the steroid-responsive hamster smooth muscle DDT1 cell line, we determined that glucocorticoids such as triamcinolone acetonide (TAA) did not influence the level of immunoreactive 80-kDa PKC\textsubscript{α}. However, TAA treatment did increase the level of a 140-kDa protein (DGK\textsubscript{1}) that cross-reacted with the PKC\textsubscript{α} monoclonal antibody M6 (Fig. 1). In order to isolate the cDNA for this potential PKC\textsubscript{α}-related protein, the M6 antibody was used to immunoscreen a glucocorticoid-induced DDT1 Lambda Zap II expression library. Three overlapping clones (pSK4, pSK14, and pSK31) were isolated (Fig. 2). The pSK4 insert (2.6 kb) was selected for further study. Sequence analysis indicated that it contained a 1.3-kb open reading frame and 1.3 kb of 3’ noncoding sequence. The bacterially expressed sequence was 47 kDa, which correlated with the predicted size of the translated cDNA, and was recognized by M6 (data not shown). However, the deduced amino acid sequence did not have significant homology to PKC\textsubscript{α} or other protein kinases. Thus, the reason for M6 recognition of this expressed sequence is not due to primary sequence homology and, instead, may be due to some secondary structure common to both proteins.

To obtain additional 5’ sequence, the library was rescreened with the pSK4 insert. Three larger overlapping clones (pSKA5, A20, and A21, 4.0 kb) were isolated. Since these still appeared to be only partial cDNAs, a 5’ fragment of pSKA21 (pSKA21a) was used to screen a second DDT1 cDNA library from which clone pSKB24 (4.3 kb) was isolated. 5’ RACE and inverse PCR were used to generate additional 5’ sequence. The first 5’ RACE product (pSK5R1, 0.679 kb) extended 198 bp beyond the 5’ end of pSKB24. The second 5’ RACE product (pCRII15R4, 0.452 kb) extended 322 bp further from the 5’ end of 5’R1. The sequence obtained by inverse PCR (pCRII62/26) was identical to 5’R4, except that it was 8 base pairs longer. Fig. 2 indicates the spatial relationships of the various DGK\textsubscript{1} clones. The composite sequence is 4,793 kb with an open reading frame of 3,462 kb and a predicted protein size of 127 kDa (Fig. 3). A putative start methionine with a consensus Kozak sequence was identified 25 base pairs after the beginning of the cDNA.

Verification That Clones Are DGK\textsubscript{1}—To verify that the isolated sequences coded for the original 130–140-kDa protein recognized by the PKC\textsubscript{α} M6 antibody, polyclonal antibodies were prepared to three sequences from different regions of the composite partial DGK\textsubscript{1} cDNA clone. Sequences used for antibody production are shown in Fig. 4A and include: 1) a synthetic 15-amino acid peptide corresponding to the C-terminal deduced amino acid sequence of pSK4 (AJ10), 2) a PSK4-glu- tathione S-transferase fusion protein (AJ12), and 3) a pSKA21b-histidine-tagged fusion protein (AJ21). All antisera were affinity purified against the cognate protein or peptide. Each of the antibodies recognized a TAA-inducible protein of 130–140 kDa on immunoblots of DDT1 cell lysates, thus confirming the relationship between the clones and DGK\textsubscript{1} (Fig. 4B). Small differences in DGK\textsubscript{1} recognition among the antibodies were also apparent. Whereas M6, AJ 12, and AJ 21 clearly recognized a doublet at 140 kDa, AJ 10 (the C-terminal antibody) recognized only one band. Careful comparison of adjacent lanes stained with M6 and AJ 10 indicated that AJ 10 recognized the lower (major) band of the doublet. The TAA-mediated increase in DGK\textsubscript{1} protein was accompanied by a TAA-mediated increase in specific mRNA species detected either by Northern blot or ribonucleotide protection analysis.

Partial and full-length DGK\textsubscript{1} constructs were expressed from baculovirus (BvDGK\textsubscript{1}-p and BvDGK\textsubscript{1}, respectively) in Sf9 cells. The expressed proteins were approximately 120 and 130 kDa, respectively (Fig. 4C). The molecular weights of endogenous hamster brain DGK\textsubscript{1} and recombinant full-length DGK\textsubscript{1} were nearly identical indicating that the putative start methionine indicated in Fig. 3 is at or very near the beginning of the open reading frame.

Sequence Analysis—The composite DGK\textsubscript{1} sequence contains motifs found in other DGK family members including cysteine-rich motifs (CRM’s) and the putative catalytic subdomains (Fig. 3). DGK\textsubscript{1} is closely related (>57% homology) to DGK\textsubscript{δ} (16) in each of these functional motifs. Unlike other DGK family members, DGK\textsubscript{δ} and DGK\textsubscript{1} do not contain identifiable EF hands but do contain an N-terminal pleckstrin homology domain (77% homology). A long intervening sequence between the putative catalytic subdomains is also unique to these DGK’s; however, they differ substantially in this intervening sequence (<38% homology). The C terminus of DGK\textsubscript{δ} has homology to the C terminus of the EPH receptor tyrosine kinases. This domain, which is not present in PKC\textsubscript{α}, is thought to be a regulatory domain (16). Thus, despite their similarities in functional motifs, DGK\textsubscript{δ} and DGK\textsubscript{1} are distinct sequences that comprise a new subfamily of DGKs.

The spacing of the cysteines in the N terminus of the DGK\textsubscript{1} sequence is characteristic of the general motif HX\textsubscript{12}, CX\textsubscript{5–7}, C found in a variety of signaling molecules including PKCs, Raf kinases, and DGKs (2).

2 T. Klauck, manuscript in preparation.
Alignment of the PKCα, DGKη, and c-Raf CRMs shows that the spacing of the cysteines and histidines is highly conserved; however, there is no significant homology outside of these residues (Fig. 5A). In contrast, alignment of the DGK CRMs demonstrates several potentially significant conserved residues (see Consensus in Fig. 5B). In particular, each of the DGK CRM I motifs (except DGKε) begins with the sequence GXHXW. An invariant P (or G) occurs two residues before the first cysteine which suggests the importance of secondary structure in this region. CRM 2 motifs also have a conserved W two residues from the beginning and an invariant W between the third and fourth cysteines. The end of CRM 2 is defined by a GXGXXK sequence that is unique to the DGK family. As noted previously (17), spacing of residues in the CRMs of DGKε is somewhat unique and could potentially be linked to the unique substrate specificity of this isozyme. Thus, the DGK CRMs share sequences that are likely to distinguish their functions from CRMs found in other signaling molecules such as c-Raf, PKCs, unc 13, vav, and n-chimaerin.

All DGK family members contain a second domain of conserved sequence homology that is likely to be important for DGK function and possibly for catalytic activity. In DGKδ and DGK γ, this putative catalytic domain is separated into two subdomains (Fig. 6, A and B). These subdomains are also separated in Drosophila DGK1 (28). Subdomain sequences from each of the DGKs were >50% identical to the porcine DGKα corresponding sequences. Subdomain 1 contains a GXGXXG12–14K motif (at G473-K492 in DGKα) that is known to participate in ATP binding to protein kinases (10). Although the GXGXXG box is conserved among DGKs, the downstream K, which is essential for ATP binding in protein kinases, is not. Furthermore, recent studies demonstrated that mutations in this region do not affect DGKα activity (29). Therefore, this sequence does not appear to be the functional ATP-binding site for DGK phosphotransferase activity. Significant homology between subdomain 1 (but not subdomain 2) and MHCK (30) was also apparent. It should be noted that the GXGXXG motif found in this portion of the MHCK sequence (321–326) does not appear to be the functional ATP-binding domain (which has been mapped to residues 467–473). The homology of MHCK to DGK subdomain 1 may indicate a more general rather than a DGK-specific function.

DGKα, -β, and -γ contain two EF hand motifs that are known to participate in calcium binding and regulation of several proteins, including DGKα (19). The absence of EF hand motifs in DGKδ and -γ indicate that they belong to a distinct subfamily of calcium-independent DGKs. These results indicate differences in calcium regulation of various DGK activities.

Tissue Distribution of DGKη—The distribution of DGKη in hamster tissues was characterized at the message level by ribonuclease protection analysis (RPA) and at the protein level by immunoblot analysis. To study the expression of DGKη message, a 197-bp antisense RNA probe was made to the 3' end of the coding sequence (3.255–3.452 kb) and hybridized to total

---

**Fig. 3.** Nucleotide and deduced amino acid sequences of the composite DGKη cDNA. The nucleotide and deduced amino acid sequences for the composite DGKη cDNA described in Fig. 2 are shown. The open reading frame consists of 3462 nucleotides and encodes 1154 amino acids. Single letter symbols for the amino acids are below the second nucleotide of each codon. The two conserved cysteine-rich domains are boxed. The two (putative) catalytic subdomains are doubly underlined. The N-terminal pleckstrin homology domain is singly underlined. *, termination codon.
RNA samples from various tissues (Fig. 7). This probe corresponds to the final 66 amino acids of hamster DGK \( \eta \) that were present in the antigen used to prepare antibody AJ 21 used in the immunoblot analysis. Message was detected in every tissue examined with testes being the most abundant. Message levels in brain, lung, spleen, and prostate were also relatively abundant (prostate data not shown).

Immunoblots of total lysates from various hamster tissue were probed with antibody AJ 12 (prepared to the final 433 amino acids of DGK \( \eta \)) (Fig. 8). These results also demonstrated that DGK \( \eta \) is expressed to some degree in most tissues, although abundant message levels (i.e. in the testes) did not always correlate with abundant protein levels. This is potentially due to tissue-specific differences in protein stability or message processing. Of the tissues examined, highest levels were found in brain. Tissue distribution in rats was similar to that described in the testes being the most abundant. Message levels in brain, lung, spleen, and prostate were also relatively abundant (prostate data not shown).

DGK Activity—The substantial sequence and domain homologies clearly demonstrate that DGK \( \eta \) is related to the DGK family of proteins. To determine if the DGK \( \eta \) protein actually had DGK activity, a partial DGK \( \eta \) protein (Fig. 9) were distributed approximately equally between the soluble and particulate fractions. The partial cDNA used in these expression studies does not contain the pH domain that may influence membrane association. However, we have also noted roughly equal partitioning of DGK between soluble and particulate fractions of cultured cells including fibroblasts and mammary and pituitary epithelial cells. These data show that expression of DGK \( \eta \) is associated with increased DGK activity. The specific activity and fold increase are similar to that reported for extracts of COS cells expressing human DGK \( \alpha \) or -\( \delta \) (12, 16). It should be noted that DGK activity is dependent upon the type of detergent or lipid included in the assay and on the substrate used (13, 31, 32). We have not yet explored the optimal assay requirements for DGK \( \eta \) activity. The catalytic activity of the partial DGK \( \eta \) was extremely unstable as over 50% of the activity was lost after overnight storage at 4°C or at –70°C in the presence of 10% or 20% glycerol.

**DISCUSSION**

In this study we report the isolation of a unique cDNA (DGK \( \eta \)) with considerable homology to known DGKs in the CRMs and the two putative catalytic subdomains. Extracts from cells expressing recombinant partial DGK \( \eta \) contained significantly more DGK activity than control cells or cells expressing recombinant PKC\( \alpha \) or -\( \delta \), thus confirming that DGK \( \eta \) protein has DGK activity. The positions of the functional domains in DGK family members are summarized in Fig. 10. In general, CRMs are located within the N-terminal half of the proteins, whereas the putative catalytic subdomains are found in the C-terminal regions. Type I DGKs (\( \alpha \), \( \beta \), \( \gamma \)) contain EF hands that are associated with the calcium-dependent activities of this type of DGK. Whereas wild type DGK\( \alpha \) is a calcium-dependent enzyme, EF hand deletion mutants are calcium-inde-
pendent(19). Type II DGKs (\(d\) and \(h\)) are not calcium-sensitive enzymes (16). The pleckstrin homology and/or the EPH C-terminal tail homology domains found in these DGKs are likely to play an important role in regulating their activities. DGK \(e\) represents a third type of DGK that is distinguished according to its selective hydrolysis of diglycerides containing arachidonate (17). To date, structural motifs that are responsible for restricting the substrate specificity of DGK \(e\) have not been identified. Finally, DGK \(z\) represents a fourth type of DGK that contains two identifiable motifs not found in other mammalian DGKs (18). DGK \(z\) contains four tandem ankyrin repeats that are also found in \(Drosophila\) DGK2 (14). Ankyrin repeats are known to be a general protein recognition motif (33) and could function similarly in DGKs. DGK \(z\) also contains a sequence homologous to the PKC phosphorylation site on the major PKC...
of individual CRM have been described for some proteins. For
the intervening residues is unique for each CRM. Functions
ily, zinc-binding CRMs that have been identified in the PKC fam-
and other DGK is very similar to the conserved 50-amino acid
sequences and regulation of this sequence in DGK 
phosphatidylserine, actin, and calmodulin (34, 35). The func-
has been reported to be involved in PKC-regulated binding of
myristoylated alanine-rich C-kinase substrate, this sequence
substrate myristoylated alanine-rich C-kinase substrate. In
that the porcine DGK 
denctosuggestthattheDGKCRMsbindDGorthattheyare
does not bind phorbol esters. To date, there is no direct evi-
dence that expression of DGK 
activities and DGK
probably that other DGKs also have specific roles in DG me-
spatially and, consequently, in the regulation of DG-dependent
biochemical processes. Further studies will be necessary to
determine how expression of individual DGKs influences ago-
with other proteins and/or phospholipids. It is not yet known if the DGK CRMs mediate DGK interactions
interactions with other proteins (50, 51); however, the role of
individual CRMs in these interactions has not yet been studied. It is not yet known if the DGK CRMs mediate DGK interactions with other proteins and/or phospholipids.

The actual site of the catalytic domain in DGK, a lipid kine-
has not yet been defined. Conservation of two homologous
subdomains among all known DGKs provides strong evidence
that these sequences are essential for DGK activity. These
substrates are contiguous in most mammalian DGKs and in
Drosophila DGK2 but are separated in DGK 
, - , and Drosoph-
ila DGK1. Surprisingly, a sequence homologous to subdomain 1
is also found in the protein kinase, myosin heavy chain kinase.

Therefore, subdomain 1 is not unique to DGKs and may have a
more general function. Although the protein kinase
GXGXGXG12–14K ATP-binding motif was found in the C termi-
us of DGK , recent studies demonstrate that this sequence is
not necessary for DGK activity (29).

Previous work demonstrated that expression of DGK , - , -
, and -e are highly tissue-specific. For example, expression of
porcine DGK was limited to the thymus, lymphocytes, and
specific regions of the porcine brain (52, 53). In contrast, DGK 
showed a much broader tissue distribution and was highly
expressed in brain and testes. In particular, the abundance
of message in brain, lung, and spleen distinguish PKC from the
more narrowly expressed PKC. The broad tissue and cellular
distributions suggest that DGK may play a general role in
cellular DG homeostasis.

In conclusion, we have identified a novel DGK that is
significantly different from previously cloned DGKs based upon
its sequence and tissue distribution. Furthermore, glucocorticoid
induction of other DGKs has not been noted to date. It is clear
that, similar to other signaling molecules such as PKC and
phospholipase C, DGK is a heterogeneous family of proteins.

There is evidence that individual DGKs may have unique
properties and, consequently, unique functions. For example, a
number of studies indicate that membrane-associated DGK
activities and DGK (17) preferentially phosphorylate 1-stear-
yl-2-arachidonyl DG, a molecular species that is primarily
derived from phosphatidylinositol turnover (32, 54–56). It is
probable that other DGKs also have specific roles in DG me-
tabolism and, consequently, in the regulation of DG-dependent
biochemical processes. Further studies will be necessary to
determine how expression of individual DGKs influences ago-
nist-stimulated and steady state DG levels. Such studies will
begin to address the relative importance of different DG pools
in DG-dependent cellular processes, such as activation of spe-
cific PKC isoforms.

REFERENCES

1. Jaken, S. (1996) Curr. Opin. Cell Biol. 8, 168–173
2. Stabel, S., and Parker, P. J. (1991) Pharmacol. & Ther. 51, 71–95
3. Shariff, A., and Luna, E. J. (1992) Science 256, 245–247
4. Kanoh, H., Yamada, K., and Sakane, F. (1990) Trends Biochem. Sci. 15, 47–50
5. Van Corven, E. J., Van Rijsijkwijk, A., Alink, K., van der Bend, R. L., van
Blitterswijk, W. J., and Moolenaar, W. H. (1992) Biochem. J. 281, 163–169
6. Durieux, M. E., and Lynch, K. K. (1993) Trends Pharmacol. Sci. 14, 249–254
7. Knauss, T. C., Jaffer, F. E., and Abboud, H. E. (1990) J. Biol. Chem. 265, 14457–14463
8. Moolenaar, W. H., Kruijer, W., Tilly, B. C., Verlaan, L., Bierman, A. J., and
Laat, S. W. (1986) Nature 323, 171–173
9. Walsh, J. P., Suen, R., Lemaître, R. N., and Glomset, J. A. (1994) J. Biol. Chem. 269, 21135–21164
10. Sakane, F., Yamada, K., Kanoh, H., Yokoyama, C., and Tanabe, T. (1990)
Nature 344, 345–348
11. Goto, K., Watanabe, M., Kondo, H., Yuasa, H., Sakane, F., and Kanoh, H.
(1992) Mol. Brain Res. 15, 75–87
12. Schap, D., de Witt, J., van der Wal, J., Vandenbossche, J., van Damme, J.,
Gussow, D., Ploegh, H. L., van Blitterswijk, W. J., and van der Bend, R. L.
(1990) FEBS Lett. 275, 151–158

![Graph](https://via.placeholder.com/150)

**Fig. 9. DGK- expression in Sf9 cells.** Partial DGK (BvDGK ) was expressed from recombinant baculovirus BvDGK-p in Sf9 cells. Aliquots of total lysates (Total), soluble (Sol) and particulate (Part) fractions (50 μg of protein/lane) from cells with (+) or without (−) BvDGK-p infection were blotted and probed with AJ 12.

**Fig. 10. Conserved domains of cloned DGKs.** Positions of conserved DGK domains (C1–C4) and functional motifs are illustrated.
13. Goto, K., and Kondo, H. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 7598–7602
14. Masai, I., Okazaki, A., Hosoya, T., and Hotta, Y. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 1137-1136
15. Kai, M., Sakane, F., Imai, S., Wada, I., and Kanoh, H. (1994) J. Biol. Chem. 269, 18492–18498
16. Sakane, F., Imai, S., Kai, M., Wada, I., and Kanoh, H. (1996) J. Biol. Chem. 271, 8394–8401
17. Tang, W., Bunting, M., Zimmerman, G. A., McIntyre, T. M., and Prescott, S. M. (1996) J. Biol. Chem. 271, 10237–10241
18. Bunting, M., Tang, W., Zimmerman, G. A., McIntyre, T. M., and Prescott, S. M. (1996) J. Biol. Chem. 271, 10230–10236
19. Sakane, F., Imai, S., Yamada, K., and Kanoh, H. (1991) Biochem. Biophys. Res. Commun. 181, 1015–1021
20. Inoue, H., Yoshioka, T., and Hotta, Y. (1989) J. Biol. Chem. 264, 5996–6000
21. Leach, K. L., Powers, E. A., McGuire, J. C., Dong, L., Kiley, S. C., and Jaken, S. (1988) J. Biol. Chem. 263, 13223–13230
22. Frohman, M. A., Dush, M. K., and Martin, G. R. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 8998–9002
23. Zeiner, M., and Gehring, U. (1995) BioTechniques 17, 1050–1054
24. Laemmli, U. K. (1970) Nature 227, 680–685
25. Kiley, S., Schaap, D., Parker, P., Hsieh, L. L., and Jaken, S. (1990) J. Biol. Chem. 265, 15704–15712
26. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
27. Kanoh, H., Kondoh, H., and Ono, T. (1983) J. Biol. Chem. 258, 1767–1774
28. Masai, I., Hosoya, T., Kojima, S. I., and Hotta, Y. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 6030–6034
29. Schaap, D., van der Wal, J., and van Blitterswijk, W. J. (1995) Biochem. J. 304, 661–664
30. Ravid, S., and Spudich, J. A. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 5877–5881
31. Ishitoya, J., Yamakawa, A., and Takenawa, T. (1987) Biochem. Biophys. Res. Commun. 144, 1025–1030
32. Besterman, J. M., Pollenz, R. S., Booker, E. L., Jr., and Cuatrecasas, P. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 9378–9382
33. Yamada, K., and Kanoh, H. (1988) Biochim. Biophys. Acta 1169, 211–216
34. Walsh, J. P., Suen, R., and Glomset, J. A. (1995) J. Biol. Chem. 270, 28647–28653
35. Bunting, M., Tang, W., Zimmerman, G. A., McIntyre, T. M., and Prescott, S. M. (1996) J. Biol. Chem. 271, 10237–10241
36. Bunting, M., Tang, W., Zimmerman, G. A., McIntyre, T. M., and Prescott, S. M. (1996) J. Biol. Chem. 271, 10230–10236
37. Inoue, H., Yoshioka, T., and Hotta, Y. (1989) J. Biol. Chem. 264, 5996–6000
38. Leach, K. L., Powers, E. A., McGuire, J. C., Dong, L., Kiley, S. C., and Jaken, S. (1988) J. Biol. Chem. 263, 13223–13230
39. Kiley, S., Schaap, D., Parker, P., Hsieh, L. L., and Jaken, S. (1990) J. Biol. Chem. 265, 15704–15712
40. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
41. McCaffery, J. M., and Pollenz, R. S., Jr., and Cuatrecasas, P. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 9378–9382
42. Besterman, J. M., Pollenz, R. S., Booker, E. L., Jr., and Cuatrecasas, P. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 9378–9382
43. Yamada, K., and Kanoh, H. (1988) Biochim. Biophys. Acta 1169, 211–216
44. Walsh, J. P., Suen, R., and Glomset, J. A. (1995) J. Biol. Chem. 270, 28647–28653
45. Yamada, K., and Kanoh, H. (1988) Biochim. Biophys. Acta 1169, 211–216
46. Walsh, J. P., Suen, R., and Glomset, J. A. (1995) J. Biol. Chem. 270, 28647–28653