Molecular Cloning of a Novel Human Diacylglycerol Kinase Highly Selective for Arachidonate-containing Substrates*

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Diacylglycerol (DAG) is a second messenger that activates protein kinase C and also occupies a central role in phospholipid biosynthesis. Conversion of DAG to phosphatidic acid by DAG kinase regulates the amount of DAG and the rate it takes. We used degenerate primers to amplify polymerase chain reaction products from cDNA derived from human endothelial cells. A product with a novel sequence was identified and used to clone a 2.6-kilobase cDNA from an endothelial cell library. When transfected with a truncated version of this cDNA, COS-7 cells had a marked increase in DAG kinase activity, which demonstrated clear selectivity for arachidonoyl-containing species of diacylglycerol. The open reading frame of this clone has 567 residues with a predicted molecular mass of about 64 kDa. This enzyme, which we designated DGKε, has two distinctive zinc finger-like structures in its N-terminal region, but does not contain the E-F hand motifs found in some other mammalian DGKs. The catalytic domain of DGKε, which is related to other DGKs, contains two ATP-binding motifs. Northern blotting demonstrated that DGKε is expressed predominantly in tests. This unique diacylglycerol kinase may terminate signals transmitted through arachidonoyl-DAG or may contribute to the synthesis of phospholipids with defined fatty acid composition.

Diacylglycerol occupies a central position in the biosynthesis of phospholipids and triglycerides. It also is an important intracellular messenger because it can bind to and activate protein kinase C, which, in turn, phosphorylates target proteins (1). This pathway has been implicated in many cellular responses including growth, differentiation, and other events such as secretion. The mechanisms by which the signaling pathway and the synthesis of complex lipids are differentially regulated is not clear, but the concentration of DAG within the cell is almost certainly one important component. In response to a variety of signals, the DAG level rises by the activation of one or more phospholipases C and, in some cases, a phospholipase D followed by phosphatidic acid phosphohydrolase. Either pathway causes a rise in the amount of diacylglycerol by degrading phospholipids. The level of DAG also is influenced by the rate at which it is converted into other products. One pathway for decreasing DAG is its conversion to phosphatidic acid, a reaction catalyzed by DAG kinases (EC 2.7.1.107).

The stimulated rise in DAG levels is an integral component of the response of cells to a variety of stimuli that lead to growth or differentiation, and the effects of phorbol esters, which are tumor promoters, are through activation of protein kinase C. Thus, the level of DAG may be an important determinant of growth. In support of this, we found that rapidly growing endothelial cells have severalfold higher levels of DAG than quiescent cells, and others observed that transformation of cells by several oncogenes results in an increased content of DAG even in the absence of an additional stimulus (2–4). The conversion of DAG to phosphatidic acid may dampen such signals, but the precise effects on cellular behavior are hard to predict because phosphatidic acid also may influence the growth response (5–7). Another role of the DAG kinase reaction may be to resynthesize phosphatidylinositol, which, unlike most phospholipids, has a characteristic fatty acid composition: 1-stearoyl-2-arachidonoyl (8). The mechanism for achieving this composition has never been elucidated although one possibility is that an enzyme(s) in the synthetic, or a salvage, pathway are specific for precursors with the appropriate molecular composition. Following the stimulated turnover of phosphatidylinositol, there is a later rise in phosphatidic acid, which has been thought to be the result of a DGK-catalyzed reaction. If this enzyme were specific for arachidonate-containing species of DAG, then multiple cycles might progressively enrich phosphatidylinositol with arachidonate.

The first isoform of DAG kinase characterized at a molecular level, DGKα, has a molecular mass of about 80 kDa (9, 10) and is found predominantly in lymphocytes and oligodendrocytes (11). A second form, DGKβ,was cloned from brain where it is mainly expressed (12). Kai et al. (13) isolated a cDNA for DGKγ from a human liver library, but subsequently found it to be expressed mostly in retina (13). A homologous rat cDNA is highly expressed in cerebellar cells (14). We recently identified DGKζ from human endothelial cells and showed that it has broad distribution, with highest levels in brain and muscle (15). None of these isoforms exhibits a strong preference for substrates with specific fatty acids. However, MacDonald et al. (15, 16) described an activity that had marked preference for DAG species that contain an arachidonoyl residue. Walsh et al. (17) reported the purification of DAG kinase from bovine testis and

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1 The abbreviations used are: DAG, diacylglycerol; DGK, diacylglycerol kinase; HUVEC, human umbilical vein endothelial cells; PIP2, phosphatidylserine; PCR, polymerase chain reaction; bp, base pair(s); MOPS, 4-morpholinopropanesulfonic acid.
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EXPERIMENTAL PROCEDURES

Materials—[γ-32P]ATP (6000 Ci/mmole), [α-35S]ATP (1000 Ci/mmole), [α-32P]dCTP (6000 Ci/mmole), and Escherichia coli DGK were bought from Amersham. Phosphatidylinerse (PS) and phosphatic acid were bought from Avanti Polar Lipids (Alabaster, AL). All 1,2-diacylglycerols were purchased from Serdary (Engwood Cliffs, NJ). OCTyl-β-glucopyranosidase, RNase, and all steps were followed by the protocol provided by the manufacturer.

Reverse Transcription-PCR—Degenerate primers containing insns were designed based on the amino acid sequences conserved among the sequenced catalytic domains of DGK isozymes. The forward primer, 5'-TG/CTTGGGGGAGT/CAGTAGAATG/CCTGAGCAT-3' (GP-1) where I is in- nisine, was based on the amino acid sequence, CGCGDGTG, and corresponded to the amino acids Cys432-Gly439 of human DGK (10) and the corresponding sequences, following optimal alignment, of other DGKs. The reverse primer, 5'-CAAGGCTCTACG/ACGCTTCATG/CCTGAGCAT-3' (GP-2) was based on the amino acid sequence, MOVBDGVEIPW, which corresponds to amino acids Met695-Trp702 of human DGK (10) and the aligned sequences of other DGKs except Drosophila DGK2 (19). Total RNA from confluent HUVEC monolayers was isolated by the guanidinium thiocyanate method (20). Single-strand cDNA was synthesized with 1 μg of total RNA from HUVEC at 37°C for 1 h using oligo(dT)12-18 as the primer and M-MLV reverse transcriptase (Life Technologies, Inc.). The reverse transcription mixture was then used as the template in the PCR amplification which was performed as follows: 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 2 min and 30 s for 5 cycles, followed by 94°C for 30 s, 65°C for 30 s, and 72°C for 2 min for 30 cycles. The PCR amplification was performed as follows: 94°C for 30 s, 50°C for 30 s, and 72°C for 2 min. To detect the presence of DGK, 1.0 μl of the PCR products was analyzed by 0.7% agarose gel and stained with ethidium bromide.

RESULTS AND DISCUSSION

CDNA Cloning of DGKe—In previous experiments we had observed increased DGK activity as endothelial cells became quiescent (22). To identify the enzymes responsible for the increased activity, we carried out reverse transcription-PCR using degenerate primers and total RNA from confluent cultures of human endothelial cells. We cloned and sequenced 29 PCR products; 20 were identical with human DGKe (10). However, we also found two novel DGKs that encoded novel sequences that the PCR strategy was designed to detect, but otherwise had unique primary structure. One of the PCR products, which had a length of 761 bp, was named DGKz and will be reported separately (17). The other novel product had a length of 746 bp, and we designated it DGKx. This PCR fragment was used as a probe to screen a CDNA library from human endothelial cells. Of two million plaques screened, there was one positive, DGKx (1 Fig. 1A). The 5′-portion of DGKx was subsequently used as the probe in another round of screening, which yielded two more positive clones (out of 2 × 106), DGK2 and DGK3 (1 Fig. 1A), all of which were sequenced.

The cDNA of DGKx has an open reading frame encoding 567 amino acids including the initiator methionine (calculated Mr, 63,884) (Fig. 1B). The translation initiation codon corresponds well with the Kozak sequence (23). However, in the clones shown, we did not detect in-frame stop codons in the 5′-untranslated region, nor were there typical polyadenylation signals in the 3′-untranslated region. Thus, the full-length messenger RNA for this enzyme is likely to be larger than the clone we isolated (see below). In a subsequent experiment, we screened a library from human testis (Clontech) and isolated another DGKe clone with a longer 5′ region, and an in-frame
stop codon was found at position 2129 from the initiating methionine (data not shown). DGK has 34%, 36%, 36%, and 32% identity with human DGK (10), rat DGK (12), human DGK (13), and human DGK, respectively. However, DGK clearly differs from the other cloned DGKs as it does not contain the N-terminal conserved region and E-F hand sequences found in other mammalian DGKs (Fig. 2). Moreover, the two zinc finger-like cysteine-rich sequences (residues His60-Cys108 and His125-Cys177, Fig. 1B) found in DGK have distinctive patterns; the sequence of the first is Cys-X2-Cys-X9-Cys-X2-Cys-X7-Cys-X9-Cys, while the second has the sequence: Cys-X2-Cys-X14-Cys-X2-Cys-X2-Cys-X2-Cys. These precise zinc finger motifs are not found in any other DGKs, or in protein kinases C. In particular, the number of amino acids separating the last two cysteines in both the first and the second zinc finger-like motifs is 9 in DGK instead of 5–8 in most other mammalian DGKs or protein kinases C. These features make DGK unique among the known DGKs.

Characterization of DGK—We subcloned the full-length cDNA of DGK into the XhoI site of pcDNAI/Neo and transformed it into COS-7 cells. In our initial experiments, we could not detect DGK activity after transient expression (data not shown). We considered the possibility that this result was caused by rapid degradation of the mRNA since the 3'-untrans-
**Fig. 3.** Diacylglycerol kinase ε has marked specificity for arachidonate-containing diacylglycerols. COS-7 cells were transfected with vector alone (pcDNAI) or with a vector containing the coding sequence of DGKε (pcDNAI/DGKε). Total lysates of the cells were assayed for diacylglycerol kinase activity as described under “Experimental Procedures.” A, DGK activity was assayed with the following substrates (indicated on the right): 1,2-dihexanoyl-sn-glycerol (10/0/10:0), 1-palmitoyl-2-oleoyl-sn-glycerol (16/0/18:1), 1-oleoyl-2-palmitoyl-sn-glycerol (18/1/16:0), 1,2-dioleoyl-sn-glycerol (18/1/18:1), 1-stearoyl-2-arachidonoyl-sn-glycerol (18/0/18:1), 1-stearoyl-2-linoleoyl-sn-glycerol (18/0/18:2), and 1-stearoyl-2-araachidonoyl-sn-glycerol (18/0/20:4). The data were collected from two independent transfections, which are shown as individual experiments. B, comparison of the substrate specificity of different diacylglycerol kinases. We carried out transfections as above, but, as a control, we also transfected cells with DGKα (pcDNAI/DGKα). Additionally, assays were performed with recombinant DGK from E. coli (1 μl of enzyme from the Amersham diacylglycerol assay kit). The substrates used are indicated using the abbreviations as above. For ease of comparison, the values obtained with each DGK using 1-stearoyl-2-arachidonoyl-sn-glycerol (18/0/20:4) as the substrate are shown as 100%. The activities measured using the other substrates are expressed as relative to the arachidonate-containing diacylglycerol. The values shown are the averages of two separate experiments.

The enzyme encoded by the cDNA that we isolated, DGKε, is distinctive compared to other known DGKs as it is very selective for arachidonoyl-DAG. The basis for this property is not clear. The distinctive zinc finger-like structures, which likely are the sites for DAG binding, may contribute to the substrate specificity, but DGKε, which does not have the arachidonoyl-DAG specificity, has similar sequences. A DAG kinase with such specificity might play an important role in phospholipid metabolism by producing a precursor of phosphatidylinositol, phosphatidic acid, that is enriched in arachidonic acid. Multi-
ple cycles of phosphatidylinositol hydrolysis and resynthesis could lead to progressive enrichment in arachidonic acid. However, DGKε seems unlikely to serve this function generally as it has a very restricted pattern of expression. However, it is possible that other tissues have very low level expression, below what we could detect by Northern blotting, since the arachidonate-specific DGK activity has been reported previously in aortic endothelium (25). Alternatively, there could be another isoform with similar substrate preference.

Walsh et al. (17) recently reported purification of an arachidonoyl-specific DGK from bovine testis, and it is likely that DGKε is its homolog (17). For example, the bovine enzyme has an apparent Mr of 58,000 which is comparable to the calculated Mr of DGKε, they share the substrate specificity, and both are highest in testis. However, unlike the bovine enzyme, which is inhibited by PS and insensitive to DGK inhibitors, DGKε is insensitive to PS and moderately inhibited by R59949. These differences may reflect the assay conditions used or may indicate that there are two isoforms of DGK with specificity for diacylglycerol substrates containing arachidonic acid.

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Note Added in Proof—During the publication of this manuscript, the identification of another diacylglycerol kinase (δ) was reported. The nomenclature of this manuscript was chosen to include this new member of the diacylglycerol kinase family.

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