The C-terminal part of diacylglycerol kinase α lacking zinc fingers serves as a catalytic domain

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All mammalian diacylglycerol kinase (DGK) isoenzymes so far cloned consist of four conserved regions, namely C1, C2 (tandem EF-hand structures), C3 (tandem cysteine-rich zinc finger sequences) and the C-terminal C4 domains. To determine the catalytic domain we expressed in COS-7 cells various truncation mutants of pig DGK α and assessed their enzyme activities. We found that the C4 domain lacking the whole N-terminal region including the zinc fingers possessed DGK activity that was dependent on the concentrations of diacylglycerol and ATP very similarly, as did the wild-type DGK α . Furthermore the DGK activity of the wild-type DGK and that expressed by the C4 domain were similarly activated by anionic amphiphiles such as phosphatidylserine, phosphatidylinositol and deoxycholate. It was also shown that a DGK mutant consisting of the zinc fingers and the C4 domain has enzymological properties very similar to those expressed by

the C4 domain alone. We also confirmed that the intact DGKs α , β and γ expressed in COS-7 cells displayed no detectable phorbol ester binding. These results show that the C4 domain of DGK is the catalytic region that is responsible for the enzyme activities sensitive to different activators. We cannot exclude the possibility that the N-terminal portion including the zinc fingers can still interact with diacylglycerol and activators without affecting the enzyme activity measured *in vitro*. However, it is quite likely that the DGK zinc fingers do not serve as diacylglycerol-binding sites, in contrast with those present in other proteins such as protein kinases C and *n*-chimaerin. Site-directed mutagenesis of all six putative ATP binding sites (Lys²⁴⁸, Lys³⁸³, Lys³⁹⁵, Lys⁴⁸³, Lys⁴⁹², and Lys⁵⁵⁴) did not significantly affect the enzyme activity. We therefore suggest that DGK does not contain a typical P-loop of ATP binding sites.

INTRODUCTION

On cell stimulation by various hormones, neurotransmitters, growth factors and other agonists, diacylglycerol (DG) is liberated from phosphatidylinositols and other phospholipids [1,2]. Because DG activates protein kinase Cs (PKCs), diacylglycerol kinase (DGK) is believed to act as an attenuator of PKC [3,4]. Moreover, phosphatidic acid (PA), the reaction product of DGK, is known to possess several biological activities as exemplified by its effects on a *ras*-GTPase-activating protein [5], PtdIns-4-phosphate kinase [6] and many other proteins involved in cellular signal transduction. DGK is thus thought to be one of the key enzymes involved in the regulation of signal transduction processes [3,4]; for example, DGK was reported to be involved in the production of interleukin 2 in T-lymphocytes [7] and retinal degeneration of *Drosophila melanogaster* [8]. In addition DGK might regulate the intracellular concentration of DG that is available for the synthesis of various glycerolipids in resting mammalian cells.

Three DGK isoenzymes, DGK α [9–11], β [12] and γ [13,14], have been cloned. The amino acid sequences of these DGK isoenzymes contain four conserved regions, namely C1, C2 (two sets of EF-hand), C3 (two sets of cysteine-rich zinc finger sequence) and C4 domains. The EF-hands are known to constitute Ca²⁺-binding sites in calmodulin [15] and calpain [16] for example. The cysteine-rich sequences with the motif His-Xaa_{11–12}-Cys-Xaa₂-Cys-Xaa_{9–14}-Cys-Xaa₂-Cys-Xaa₄-His-Xaa₂-Cys-Xaa_{6–7}-Cys are also found in PKC [17], Raf kinase [18], a *vav* proto-oncogene product [19], *n*-chimaerin [20] and a *Caenorhabditis elegans unc-13* gene product [21]. However, the C1 and C4 regions seem to be unique to DGK when searched for in the

sequences deposited in the protein database. Of the interesting functional domains of DGK, we have already reported that the two EF-hand structures are indeed high-affinity (K_d approx. 300 nM) Ca²⁺-binding sites [22]. Moreover, under Ca²⁺-free conditions, this region seemed to serve as an autoinhibitory domain by masking the putative phosphatidylserine (PtdSer)- and membrane-binding domain(s) of the enzyme [23].

The functions of the other domains (C1, C3 and C4) of DGK remain unclear. Because the C4 domain is the most highly conserved region of the DGK isoenzymes, it is presumed to be the catalytic domain. However, because the cysteine-rich sequences of PKC, *n*-chimaerin and the *unc-13* gene product have been shown to be the binding sites of DG or phorbol ester [17,21,24], the zinc finger sequences of DGK have also been speculated to be included in the catalytic action as the binding sites of DG. These vague assumptions have not been confirmed yet. In addition to Ca²⁺, several activators [PtdSer and deoxycholate (DOC)] were previously reported for DGK α [3,25]. However, the mechanism of the actions of these activators remains unknown.

To determine the catalytic region, in the present study we constructed a series of truncation mutants of DGK α and expressed them in COS-7 cells. By assessing the enzyme activities of these mutants, we attempted to define the catalytic region.

MATERIALS AND METHODS

Materials

Restriction enzymes, DNA-modifying enzymes and linkers were purchased from Takara Shuzo (Kyoto, Japan), Toyobo (Osaka, Japan) or New England BioLabs. The *in vitro* mutagenesis

Abbreviations used: 6 × His, N-terminal short peptide containing six histidine residues and an enterokinase cleavage site; DG, diacylglycerol; DGK, diacylglycerol kinase; DOC, deoxycholate; PA, phosphatidic acid; PKC, protein kinase C; PDBu, phorbol 12,13-dibutyrate; Sph, sphingosine; PtdSer, phosphatidylserine.

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Figure 1 Structures of various DGK α mutant proteins

The mutations of the DGK α were made as described in the Materials and methods section. Stars indicate the positions of Lys residues replaced by Arg or Asn.

system (Version 2.0), the enhanced chemiluminescence Western blotting detection system and [α - 32 P]dCTP were bought from Amersham. [γ - 32 P]ATP was purchased from ICN Biochemicals. [3 H]phorbol 12,13-dibutyrate (PDBu) was from DuPont NEN. A sequencing kit (Sequenase version 2.0) based on the dideoxy chain-termination method [26] was bought from US Biochemical. 1,2-Dioleoylglycerol, liver phosphatidylcholine, liver phosphatidylethanolamine, liver PtdIns and brain sphingosine (Sph) were purchased from Sigma. Brain PtdSer was obtained from Avanti Polar Lipids. β -Octyl glucoside was obtained from Dojindo Laboratories (Kumamoto, Japan). PKC α cDNA (GeneBank/EMBL Data Bank accession No. X52479) was purchased from the American Type Culture Collection. Oligonucleotides were synthesized with an Applied Biosystems 380B DNA synthesizer. All other chemicals were of the highest purity commercially available.

Construction of expression plasmids

General manipulation of DNA was done with standard procedures [27]. All mutageneses were performed with pBluescript II SK+ (Stratagene) harbouring pig wild-type DGK α cDNA (pBluescript-DGK α) [23]. The preparation of expression vectors encoding wild-type DGK α (pSRE-DGK α) has been described previously [23].

pSRE-DGK α Δ1-196 and pSRE-DGK α Δ1-332 (Figure 1) were constructed by using PCR amplification. In this case the sense primers (S1, 5'-GCGAATTCCACCATGGCCTTGAAG-GACAATGG-3', and S2, 5'-GCGAATTCCACCATGGGATCC-CAGTGTCTTGGC-3') were derived from the peptide sequences Met-Thr-Leu-Lys-Asp-Asn-Gly (positions 197-203) and Ile-Tyr-Pro-Ser-Val-Leu-Ala (positions 333-339) [9] respectively. An anti-sense primer (A1, 5'-CTCACTATAGGGCGAATTGG-3') corresponds to positions 634-653 of the nucleotide sequence of the vector plasmid, pBluescript II SK+. To facilitate subcloning, *Eco*RI sites (underlined) were added to primers S1 and S2. As indicated by double underlines, nucleotide substitutions (A to G in S1 and CT to GG in S2) were introduced to create the initiation methionine codon and/or the Kozak consensus nucleotide sequence surrounding the ATG codon [28]. The peptide

sequences shown above were thereby changed to Met-Ala-Leu-Lys-Asp-Asn-Gly and Met-Asp-Pro-Ser-Val-Leu-Ala respectively. pBluescript-DGK α was used as template for PCR amplification with the primers S1 plus A1 (for amplification of a DGK α Δ1-196 cDNA fragment) and S2 plus A1 (for a DGK α Δ1-332 cDNA fragment) respectively. The reactions were performed with GeneAmp[®] PCR Reagent Kit (Perkin-Elmer Cetus) for 25 cycles on a thermoprocessor TR-100 (Taitec, Japan) under the following conditions: 94 °C for 1 min; 52 °C for 2 min and 72 °C for 3 min, followed by a final elongation step at 72 °C for 7 min. The amplified PCR products were digested with *Eco*RI restriction endonuclease and subsequently separated by a preparative-scale agarose gel electrophoresis. Bands at the predicted positions were recovered and cloned into the *Eco*RI site of pSRE plasmid, simian virus 40-based expression vector [23,29].

To construct an expression plasmid of a polyhistidine-tagged C-terminal half of DGK α , the insert encoding DGK α Δ1-357 was first excised from pBluescript-DGK α by *Dra*I and *Eco*RI restriction endonucleases, blunt-ended by Klenow fragment and ligated with *Bam*HI linker (5'-CCGGATCCGG-3'). After *Bam*HI digestion the cDNA fragment was inserted into the *Bam*HI site of pBlueBacHisA vector (Invitrogen), which encodes the N-terminal short peptide containing six histidine residues and an enterokinase cleavage site (6 $\times$ His tag). DGK α Δ1-357 cDNA containing an oligonucleotide encoding the 6 $\times$ His tag was excised from pBlueBacHisA by *Nde*I and *Eco*RI, blunt-ended by Klenow enzyme and ligated with *Eco*RI linker (5'-CCGGAATTCGG-3'). An *Eco*RI-digested fragment was subcloned into pSRE vector (pSRE-6 $\times$ His-DGK α Δ1-357). pBlueBacHisA vector containing no insert was used to construct a mock control plasmid (pSRE-6 $\times$ His).

DGK α K248R, DGK α K492R, DGK α K383N, DGK α K395N, DGK α K483N and DGK α K554N mutants contained substitutions of Arg or Asn for Lys²⁴⁸, Lys⁴⁹², Lys³⁸³, Lys³⁹⁵, Lys⁴⁸³ and Lys⁵⁵⁴ respectively. All mutants were made with the *in vitro* oligonucleotide mutagenesis system (Amersham) in accordance with the protocol supplied and subcloned into pSRE. The oligonucleotide primers used were as follows: for K248R, 5'-GCAGGGCCCTCATGGCAC-3'; K492R, 5'-GTACCACCC TACTTGCT-3'; K383N, 5'-CCCGCCACTGTTGGGGTTGAC-3'; K395N, 5'-ATACTGGAAGTTCCAAAGC-3'; DGK α K483N, 5'-CTCTAAATCGTTGAGGATC-3'; K554N, 5'-AGTACCACAGGTTGTTCTTCA-3' (substituted nucleotides underlined).

Rat pSRE-DGK β [12] was donated by Dr. K. Goto and Dr. H. Kondo (Tohoku University, Sendai, Japan). Human pSRE-DGK γ was constructed as described [13]. To construct pSRE-PKC α , used as a positive control for phorbol ester binding experiments, the human PKC α cDNA [30] was excised from pBluescript II KS+ by *Eco*RI and inserted into pSRE.

Expression in COS-7 cells

The cDNA transfection of COS-7 cells was performed with the DEAE-dextran method as described [31]. After 3 days the cells were washed twice with PBS, harvested and lysed by sonication in ice-cold lysis buffer (0.5 ml per 100 mm-dish) containing 25 mM Tris/HCl, pH 7.4, 0.25 M sucrose, 5 mM EGTA, 1 mM EDTA, 1 mM dithiothreitol, 20 μ g/ml each of leupeptin, aprotinin and pepstatin, 50 μ g/ml soybean trypsin inhibitor and 1 mM PMSF. After removal of undisrupted cells by centrifugation (550 *g* for 10 min at 4 °C), the supernatant (cell extract) was used for the phorbol ester binding assay. For the assay for DGK activity the cell extract was subsequently centrifuged at

100 000 *g* (Beckmann TL-100 Ultracentrifuge) for 30 min at 4 °C to remove membranes and aggregated protein products. The resultant supernatant was used for kinetic and immunoblot analyses. Approx. 50 % and 90 % of the DGK activities of DGK α Δ1–196 and DGK α Δ1–357 respectively were recovered in the soluble fraction. More than 90 % of the activities of DGK α K248R, DGK α K492R, DGK α K383N, DGK α K395N, DGK α K483N and DGK α K554N mutants were recovered in the soluble fraction. The extract obtained from COS-7 cells transfected with the pSRE vector without DGK inserts was used to determine control values (background).

Purification of the polyhistidine-tagged DGK mutant

Purification under native conditions of 6 × His-DGK α Δ1–357 expressed in COS-7 cells was done with a ProBond (a Ni²⁺ charged Sepharose resin) column (1.2 ml) in accordance with the instructions provided (Invitrogen). The bound protein was eluted by loading Native-Imidazole Elution Buffer; six fractions (0.5 ml each) were collected. After assay of DGK activity, 0.2 ml each of the fractions was precipitated by adding 80 μ l of trichloroacetic acid (25 %, w/v) and 2 μ l of 100 g/ml BSA. The resultant pellet was subjected to immunoblot analysis with anti-DGK α antibody.

Assay of DGK activity

The octyl glucoside mixed-micellar assay of DGK activity was done as described [22]. Briefly, the assay mixture (50 μ l) contained 50 mM Mops, pH 7.4, containing 50 mM octyl glucoside, 1 mM dithiothreitol, 100 mM NaCl, 20 mM NaF, 10 mM MgCl₂, 0.2 mM CaCl₂, 2 mM (6.1 mol %) dioleoylglycerol, 6 mM (18.2 mol %) PtdSer and 1 mM [γ -³²P]ATP (approx. 100 000 c.p.m./nmol). The 100 000 *g* supernatants (15 μ g of protein) of the COS cell extracts were used as enzymes.

Phorbol ester binding assay

The [³H]PDBu binding assay was performed as described [32]. The binding was determined in 250 μ l incubation mixtures containing [³H]PDBu (40 nM), 550 *g* supernatant of COS-7 cells (50 μ g of protein), 50 mM Tris/HCl, pH 7.4, 0.1 mM CaCl₂, 100 μ g/ml PtdSer and 4 mg/ml bovine γ -globulin. The conditions provided saturable binding for PKC [1,32]. The incubation was performed in 1.5 ml polypropylene Eppendorf microcentrifuge tubes for 30 min at 37 °C. The samples were then chilled for 5 min at 0 °C and 187 μ l of 35 % (w/w) poly(ethylene glycol) 6000 in 50 mM Tris/HCl, pH 7.4, was added. The mixtures were then incubated for 15 min at 0 °C to permit precipitation of proteins. The mixtures were centrifuged at 11 000 *g* in a Kubota 1700 centrifuge at 4 °C for 20 min. The supernatant was removed by aspiration and blotted with Kimwipe. After the tip of the centrifuge tube had been cut off, the radioactivity in the pellet was measured to determine bound [³H]PDBu. Non-specific binding determined in the presence of 40 μ M unlabelled PDBu was subtracted from the data.

Immunoblot analysis

Immunoblot analysis with anti-DGK α antibody [33] was performed as previously described, but with the enhanced chemiluminescence detection system instead of ¹²⁵I-labelled Protein A. Epitope mapping of the anti-DGK α antibodies by using several bacterially expressed DGK α fragments showed that the antibody recognized only the C4 domain (results not shown).

RESULTS

Determination of the catalytic region

To determine the catalytic region, we first constructed two truncation mutants, DGK α Δ1–196 and DGK α Δ1–332 (Figure 1) lacking the C1–C2 and the C1–C3 regions respectively, and expressed them in COS-7 cells. The expression of the protein products with expected molecular masses (wild-type DGK α , 83 kDa; DGK α Δ1–196, 61 kDa; DGK α Δ1–332, 45 kDa) was confirmed by immunoblot analysis (Figure 2). A smaller band of the wild-type protein might be a proteolytic product. Densitometric quantification showed that the expression level of DGK α Δ1–196 was about 1.5- and 2.2-fold those of wild-type enzyme and DGK α Δ1–332 respectively. As shown in Table 1, the 100 000 *g* supernatant from COS-7 cells expressing the wild-type enzyme as positive control showed a marked increase in activity (approx. 11-fold) when compared with mock-transfected cells. The supernatants from DGK α Δ1–196- and DGK α Δ1–332-transfected cells also gave markedly high DGK activities (approx. 30-fold and 5-fold respectively). The activity of the DGK α Δ1–332-transfected cells was low compared with DGK α Δ1–196. However, the relative amount of the expressed DGK α Δ1–332 suggests that the specific activity of this mutant is not greatly different from that of DGK α Δ1–196 (Table 1).

The possibility still existed that the expression product (DGK α Δ1–332) having no DGK activity might have some

Figure 2 Immunoblot analysis of wild-type DGK α , DGK α Δ1–196 and DGK α Δ1–332

The 100 000 *g* supernatants (15 μ g of protein) of extracts of COS-7 cells transfected with the constructs were subjected to SDS/PAGE and transferred on to a nitrocellulose membrane. The DGK proteins were detected with anti-DGK α antibodies as described in the Materials and methods section. Abbreviation: KD, kDa.

Table 1 DGK activities of wild-type DGK α , DGK α Δ1–196 and DGK α Δ1–332

DGK activities of the 100 000 *g* supernatants (15 μ g of protein) were measured as described in the Materials and methods section; the results are means $\pm$ S.D. for three to five independent experiments. The DGK activities normalized for levels of expression are also shown; the results are expressed as a percentage of the values of DGKΔ1–196.

Construct	DGK activity (nmol/min per mg)	Normalized DGK activity (%)
Mock	2.0 $\pm$ 0.2	—
Wild-type	22.2 $\pm$ 4.4	51.1 $\pm$ 10.4
Δ1–196	63.2 $\pm$ 8.5	100
Δ1–332	10.4 $\pm$ 1.2	32.4 $\pm$ 4.1

Figure 3 Purification of 6 × His-DGKαΔ1–357

(a) The 100 000 *g* supernatant (4 ml) of the COS-7 cell extract containing the 6 × His-DGKαΔ1–357 (●) or the 6 × His peptide alone (△) was purified on a ProBond column (1.2 ml). The bound protein was eluted by loading Native-Imidazole Elution Buffer; six 0.5 ml fractions were collected. DGK activity was measured by using 20 µl of each fraction. The recovery of the increased DGK activity in the COS-7 cell extract containing the 6 × His-DGKαΔ1–357 was 31%. (b) Aliquots (0.2 ml) of the eluates (6 × His-DGKαΔ1–357) were concentrated and subjected to immunoblot analysis as described in the Materials and methods section. Abbreviation: KD, kDa.

stimulatory effects on endogenous DGK of COS-7 cells, resulting in the apparently increased DGK activity. To confirm that DGKαΔ1–332 itself had intrinsic DGK activity, we expressed a polyhistidine-tagged C-terminal half of DGKα (6 × His-DGKαΔ1–357; Figure 1) in COS-7 cells and subsequently purified it from the crude extract by using metal-affinity resin. As shown in Figure 3(a), the eluate from the column applied with the cell extract containing 6 × His-DGKαΔ1–357 contained sig-

Table 2 *K_m* values for DG and ATP and EC₅₀ values for PtdSer of wild-type DGKα, DGKαΔ1–196 and DGKαΔ1–332

Values (means ± S.D.) were obtained from three independent experiments including those given in Figure 4.

Construct	<i>K_m</i> (DG) (mol %)	<i>K_m</i> (ATP) (mM)	EC ₅₀ (PtdSer) (mol %)
Wild-type	3.4 ± 0.3	0.13 ± 0.02	14.0 ± 1.4
Δ1–196	4.1 ± 1.3	0.19 ± 0.02	7.9 ± 0.6
Δ1–332	5.9 ± 1.6	0.57 ± 0.24	11.0 ± 2.2

nificant DGK activity, whereas the extract from the mock transfection (pSRE-6 × His) in the parallel experiment showed no detectable enzyme activity. A single band of molecular mass 47 kDa (the mutant protein) was found to account for the enzyme activity (Figure 3b). Although the result strongly suggests that the C4 domain alone could serve as the catalytic domain, we could not exclude the possibility that the other regions (C1–C3) were still partly involved in the catalytic function. We thus attempted to compare the enzymic properties of the wild-type enzyme and the truncation mutants.

To analyse the kinetic properties of the truncation mutants, we determined the effects of various concentrations of DG (Figure 4a) and ATP (Figure 4b) on the activities of the 100 000 *g* supernatants from COS-7 cells transfected with wild-type DGK, DGKαΔ1–196 and DGKαΔ1–332 cDNA species. Because the *K_m* values obtained for DG and ATP of wild-type DGKα expressed in COS-7 cells agreed well with those (DG, 3.3 mol %; ATP, 0.10 mM) [22] of DGKα purified from pig thymus (see Table 2), the activity measured with the crude fraction did not seem to be significantly affected by contaminating factors such as ATP and PA phosphatase. All of the DGK activities of the wild-type enzyme and the truncation mutants were similarly increased up to a concentration of approx. 1 mol % DG or 0.125 mM ATP, and nearly saturated at a concentration of about 7 mol % DG or 1 mM ATP (Figures 4a and 4b). As summarized in Table 2, the *K_m* values for DG and ATP of DGKαΔ1–332 were not significantly different from those of the wild-type DGKα and DGKαΔ1–196. DGKα is known to have no marked selectivities

Figure 4 Effects of DG (a), ATP (b) and PtdSer (c) on DGK activities of wild-type DGKα, DGKαΔ1–196 and DGKαΔ1–332

The 100 000 *g* supernatants (15 µg) of extracts from COS-7 cells transfected with pSRE-DGKα (■), pSRE-DGKαΔ1–196 (●) and pSRE-DGKαΔ1–332 (▲) were incubated with various concentrations of DG, ATP or PtdSer as indicated. The concentrations of the non-varied assay components in each panel were 1 mM ATP and 18.2 mol % PtdSer in (a), 6.1 mol % DG and 18.2 mol % PtdSer in (b) and 6.1 mol % DG and 1 mM ATP in (c). To facilitate comparison, background activities (the control cells transfected with the vector alone) were subtracted. The background activities (▽) are also shown. The values are the averages of duplicate determinations, which differed by less than 5% of the mean. The results presented are representative of three separate experiments. Abbreviation: PS, PtdSer.

Table 3 DGK activities of wild-type DGK α , DGK α Δ1–196, DGK α Δ1–332 and 6 × His-DGK α Δ1–357 in the presence of various lipid activators

The 100 000 *g* supernatants (15 μ g of protein) from COS-7 cells transfected with pSRE-DGK α (wild-type), pSRE-DGK α Δ1–196 and pSRE-DGK α Δ1–332 were incubated in the presence (18.2 mol %) of phosphatidylcholine (PtdChol), phosphatidylethanolamine (PtdE), PtdSer, PtdIns or Sph as an activator. The sample (20 μ l) of purified 6 × His-DGK α Δ1–357 was also incubated in the presence of PtdSer. The results are expressed as fold increase over the value obtained without lipid activators. Abbreviation: n.d., not determined.

Construct	Relative activity (fold)					
	None	PtdChol*	PtdE*	PtdSer†	PtdIns*	Sph†
Wild-type	1.0	1.0 ± 0.1	4.2 ± 0.3	15.2 ± 1.4	10.3 ± 0.7	8.0 ± 2.7
DGK α Δ1–196	1.0	1.0 ± 0.0	4.5 ± 0.1	16.3 ± 2.0	12.3 ± 0.4	1.0 ± 0.0
DGK α Δ1–332	1.0	1.0 ± 0.1	5.1 ± 0.1	17.6 ± 0.6	14.1 ± 0.8	1.1 ± 0.2
Purified 6 × His-Δ1–357	1.0	n.d.	n.d.	16.4 ± 1.2	n.d.	n.d.

* Means ± range for two independent experiments.

† Means ± S.D. for three independent experiments.

with regard to the acyl compositions of DG [13]. When several DG species, such as 1,2-didecanoylglycerol, 1,2-dioleoylglycerol and 1-stearoyl-2-arachidonoylglycerol, were used as substrates, the truncation mutants did not show significant preferences to any DG species examined (results not shown). Taken together the results indicate that the intact enzyme, DGK α Δ1–196 and DGK α Δ1–332 share very similar enzymic properties. Thus the C4 domain alone is most likely to contain the necessary information to confer DGK activity. In contrast the C1–C3 domains seem to be the regulatory domain. We could not deny the possibility that the N-terminal portion including the zinc fingers can interact with DG without affecting the enzyme activity measured *in vitro*. Unexpectedly, however, it is most likely that the DGK zinc fingers do not serve as DG-binding sites in contrast with those present in other proteins such as PKCs and *n*-chimaerin.

Interaction sites of activators

In addition to Ca²⁺, negatively charged amphiphiles such as PtdSer, PtdIns and DOC have also been reported to activate DGK [3,25]. We next determined the dependencies of the truncation mutants on the known DGK activators. Figure 4(c) and Table 3 demonstrate that both DGK α Δ1–196 and DGK α Δ1–332 (and purified 6 × His-DGK α Δ1–357) were activated (approx. 17-fold) in the presence of 18 mol % PtdSer, as observed with the wild-type enzyme. Moreover the EC₅₀ values for PtdSer estimated for various forms of DGK α were not significantly different from each other (Figure 4c and Table 2). Another acidic phospholipid, PtdIns, also activated the wild-type enzyme and both of the mutants to almost the same extents (10–14-fold). In contrast, the non-charged phospholipids, phosphatidylethanolamine and phosphatidylcholine, had only weak or no detectable effects on their activities. When the suspension assay was employed, DOC was also found to be a strong activator for both mutants (results not shown). We could not exclude the possibility that the N-terminal portion including the zinc fingers could interact with the lipids without affecting the enzyme activity. However, the results indicate that the C4 domain recognizes negatively charged amphiphiles such as PtdSer, PtdIns and DOC as the enzyme activators. Because PKC is known to be activated by PtdSer through the zinc finger sequences [34], the

Table 4 PDBu binding activity of DGK isoenzymes and PKC

PDBu binding activities of the extracts (50–100 μ g of protein) from COS-7 cells transfected with pSRE (mock), pSRE-DGK α , pSRE-DGK β , pSRE-DGK γ and pSRE-PKC α were determined as described in the Materials and methods section. Each value shown represents data after subtraction of non-specific binding activity from an actual value. The non-specific [³H]PDBu binding activities were less than 10% of the total [³H]PDBu binding activity in all cellular extracts. DGK activities of the extracts (10 μ g of protein) are also shown. The results are means ± S.D. for three independent assays.

Construct	Bound [³ H]PDBu (pmol/mg)	DGK activity (nmol/min per mg)
Mock	1.39 ± 0.13	1.0 ± 0.1
pSRE-DGK α	1.50 ± 0.15	17.9 ± 2.2
pSRE-DGK β	1.44 ± 0.14	8.2 ± 1.1
pSRE-DGK γ	1.46 ± 0.16	40.0 ± 0.4
pSRE-PKC α	4.72 ± 0.84	1.0 ± 0.1

activation mechanisms of DGK by PtdSer seem to be different from those of PKC.

We have demonstrated that a positively charged lipid, Sph, also activated DGK α [25]. Table 3 shows that Sph activated only the wild-type enzyme. In contrast, both of the truncation mutants failed to be activated by Sph. The result indicates clearly that the activation mechanisms of Sph are different from those of the anionic lipids; it also suggests that Sph may interact at least in part with the C1 and/or C2 regions.

Phorbol ester binding activity of zinc fingers of intact DGK isoenzymes

The above results suggested that the properties of the zinc fingers of DGK α were different from those of PKCs and *n*-chimaerin with respect to DG binding. To analyse further the properties of zinc fingers, we next tested the phorbol ester binding activity of DGK. So far, only the bacterially expressed zinc finger region of DGK α has been reported to show no detectable phorbol ester binding activity [24]. We also obtained essentially the same negative finding by using the same DGK region expressed in *Escherichia coli* (F. Sakane and H. Kanoh, unpublished work). However, it is still possible that the zinc fingers expressed in prokaryotic cells (*E. coli*) are not properly folded. Furthermore the two new DGK isoenzymes (β and γ), which also contain zinc finger structures, have recently been cloned. It is thus necessary to test their phorbol ester binding activities. In the present study we thus attempted to see whether the intact DGK α and the newly cloned DGK isoenzymes expressed in mammalian cells could bind phorbol ester or not. The extracts from COS-7 cells transfected with DGK α , β and γ cDNA species showed DGK activities 16.9, 7.2 and 39.0 nmol of PA/min per mg of protein higher than the basal activity respectively (Table 4). Because the specific activity of DGK α purified from pig thymus was approx. 10 μ mol of PA/min per mg of protein [25,35], the increased activity represented about 21 pmol of the DGK protein per mg of cellular protein. However, Table 4 shows that the extract from COS-7 cells transfected with DGK α exhibited no detectable PDBu binding activity. Moreover the COS-7 cell extract containing DGKs β and γ also had no detectable binding activity. In contrast, the extract from PKC transfectant cells as a positive control showed markedly increased PDBu binding activity. The result further supports the notion that the zinc finger sequences of DGK isoenzymes belong to a subgroup functionally different from those of PKCs and *n*-chimaerin.

Figure 5 Effects of site-directed mutageneses at six putative ATP binding sites on DGK activity

(a) The 100 000 g supernatants (15 µg of protein) from COS-7 cells transfected with each construct were subjected to SDS/PAGE. The DGK proteins were detected by immunoblotting with anti-DGKα antibodies as described in the Materials and methods section. (b) DGK activities of the 100 000 g supernatants (15 µg) from COS-7 cells transfected with the constructs were measured as described in the Materials and methods section. To facilitate comparison, background activities (the control cells transfected with the vector alone) were subtracted. The results are expressed as a percentage of the control value (the wild-type enzyme). The results were means ± S.D. for three independent experiments.

Analysis of putative ATP binding sites by site-directed mutagenesis

We next attempted to identify the ATP binding site. Our earlier study [9] noted that DGKα contained within the C3 and C4 regions two putative ATP binding sites with the motif Gly-Xaa-Gly-Xaa₂-Gly-Xaa₁₃₋₁₆-Lys, which has been found in protein kinases [36]. However, substitution of the lysine residue (Lys²⁴⁸ or Lys⁴⁹²) in each motif with an arginine residue, which is known to inactivate PKC [37], did not affect the DGK activity (Figure 5b). Immunoblotting showed that the mutant proteins and the wild-type protein were similarly expressed (Figure 5a). Schaap et al. [38] also reported essentially the same result. Thus it is confirmed that the putative sequences do not serve as the ATP binding site. This conclusion is supported by the facts that the first sequence exists in the C3 region, which is not included in the catalytic domain as shown in the present study, and that the second sequence is not conserved in rat DGKβ and human DGKγ isoenzymes recently cloned [12,13].

A lysine residue surrounded by glycine-rich sequences is known to be essential for nucleotide binding in several ATP- and GTP-binding proteins such as adenylate kinases, Ras proteins and phosphoglycerate kinases [39]. We therefore next searched the lysine residues conserved among all mammalian DGK isoenzymes [9–14] and *Drosophila* DGK homologues [8,40] so far cloned. In this way we found four conserved lysine residues (Lys³⁸³, Lys³⁹⁵, Lys⁴⁸³ and Lys⁵⁵⁴ in pig DGKα). They are also surrounded by Gly (or amino acids with a small side chain such as Ala, Pro and Ser)-rich sequences as follows: Pro-Lys³⁸³-Ser-Gly-Gly-Xaa₂-Gly-Xaa₅-Lys³⁹⁵, Gly-Gly-Gly-Xaa₂-Gly-Xaa₄-Lys⁴⁸³ and Ser-Xaa-Gly-Xaa₂-Ala-Xaa₂₃-Lys⁵⁵⁴. We constructed DGKαK383N, DGKαK395N, DGKαK483N and

DGKαK554N mutants containing substitutions of Asn for Lys³⁸³, Lys³⁹⁵, Lys⁴⁸³ and Lys⁵⁵⁴ respectively. Immunoblotting showed that DGKαK383N, DGKαK483N, DGKαK554N and wild-type proteins were expressed to similar levels (Figure 5a). Exceptionally, the expression level of DGKαK395N was approx. 50 % of that of the wild-type enzyme. The activities of DGKαK383N, DGKαK395N, DGKαK483N, DGKαK554N were always more than 65 % of the control activity obtained for the wild-type DGK (Figure 5b). This result suggests that the Lys residues are not critical for ATP binding and that DGK does not have a typical P-loop at the ATP binding site [39].

DISCUSSION

On the basis of the amino acid sequences deduced from the DGK cDNA species, four conserved regions (C1–C4) have been recognized in DGKs α, β and γ [9–14]. Because the C4 domain is the most highly conserved region among the DGK isoenzymes, it was thought to be the catalytic domain. However, the Cys₆His₂ zinc finger sequences of PKCs [1,17], *n*-chimaerin [20] and the protein product of *unc-13* [21] have been revealed to be responsible for the binding of DG and/or phorbol ester. Therefore the zinc finger sequences (the C3 region) of DGK were also speculated to be the DG-binding site and are inevitably included in the catalytic domain. However, these vague assumptions had not been confirmed. In the present study we have unexpectedly revealed that the C4 region of the enzyme alone can serve as a catalytic domain. At present, we cannot exclude the possibility that the N-terminal portion including the zinc finger structures could still interact with DG without affecting the enzyme's activity. However, the zinc fingers of DGKα would not be included in the catalytic domain as the DG binding site. This notion was supported by the presence of a *Drosophila* DGK homologue, DGK1, that contains no cysteine-rich sequence [40]. The finding has suggested that the zinc fingers might not be essential for the catalytic action of DGKs. In addition, the phorbol ester binding activities of intact DGKs α, β and γ expressed in mammalian cells (Table 4) and bacterially expressed zinc finger sequences (F. Sakane and H. Kanoh, unpublished work, and [24]) were not detected, in contrast with those of PKCs and *n*-chimaerin. The C1–C3 regions are thus likely to serve as the regulatory domains, and their functions remain to be explored.

In addition to the conserved Cys and His residues, several amino acid residues such as Phe, Tyr, Pro, Leu, Trp and Gln at certain positions within the phorbol ester binding domain of PKCs were reported to be critical for interaction with the ligand [41]. However, all of the important residues are also conserved or replaced by similar residues in the zinc finger structures of DGKs α, β and γ [9–14]. Therefore the critical residues by which the lack of phorbol ester binding in the DGK isoenzymes is rationalized are still unknown. Interestingly, Redman et al. [42] recently reported that DGK purified from pig testis membranes was inhibited by the anti-tumour agent calphostin C, which is known to interact with the phorbol ester binding site in PKCs [43]. We also confirmed that calphostin C inhibited the activity of the DGKαΔ1–332 (F. Sakane and H. Kanoh, unpublished work). Because the inhibition was competitive with respect to DG, it was suggested that there are similarities between the DG binding site of DGK and the phorbol ester and/or DG binding site of PKC [42]. Kazanietz et al. [41] reported that the loop comprising nine amino acids, (Leu/Phe)-(Leu/Ile/Val/Met)-(Trp/Tyr)-Gly-(Leu/Phe/Ile)-(Gly/Val/Ile/Leu/Met/Ala/Asn)-(Lys/Arg/His/Ala)-Gln-Gly, in the zinc fingers of PKCs

was directly implicated in the phorbol ester binding activity. We thus searched for similar amino acid sequences in the C4 domain of DGK α . It was found that there are two such sequences (residues 470–478 and 609–617), Leu-Arg-Trp-Gly-Gly-Gly-Tyr-Glu-Gly and Asn-Leu-Trp-Gly-Asp-Thr-Lys-Arg-Pro (double and single underlines indicate identical and similar amino acids respectively). Moreover the sequences were conserved in the other DGK isoenzymes. It is therefore puzzling that DGK fails to bind phorbol esters.

The zinc finger sequences of DGK are thus apparently functionally distinct from those of PKCs, *n*-chimaerin and the protein product of *unc 13* with respect to phorbol ester binding. In this view, the Cys₆His₂ zinc finger structure can be at least divided into two subgroups. The zinc finger sequences of PKCs ζ , λ and μ and the proto-oncogene products of *vav* and *c-ras* were recently found to have no detectable phorbol ester binding activity [2,44–46]. Therefore the zinc finger sequences of these proteins and DGKs belong to a subgroup without the phorbol ester binding ability. Besides phorbol ester/DG binding, diverse functions of the Cys₆His₂ zinc finger structure have recently been found. For example, PKC ϵ was localized to the Golgi via its zinc finger domain [47]. The zinc fingers of *n*-chimaerin were demonstrated to interact with several lipids such as PA and lysoPA [48]. This structure of Raf-1 kinase is known to bind p21^{ras} [49,50]. Thus the zinc fingers of DGK might also possess some new and unexpected functions. Interestingly, the overall identity of amino acid sequences of the zinc fingers is not particularly high among different DGK isoenzymes, suggesting that they might have functions different from each other. To analyse the functions of DGK zinc fingers in more detail, further work is required with several additional mutants (for example a mutant with amino acid substitutions in the zinc fingers by site-directed mutagenesis and a deletion mutant containing only zinc finger sequences). However, we could not perform further experiments employing them because the expression levels and solubilities of these mutants were found to be extremely low.

All negatively charged lipids, PtdSer, PtdIns and DOC, activated the enzyme activity of the mutant containing the catalytic domain (C4) alone, suggesting that their activation mechanisms are the same. Although PKC is known to be activated with PtdSer at least in part through the zinc finger sequences [34], the activation mechanisms of DGK by PtdSer would be different from those of PKC. We have previously reported that PtdSer increased the affinity of DGK α for ATP [22]. We therefore suggest that the ATP binding site and the PtdSer-interaction region might be functionally linked. In contrast, Sph could not activate the deletion mutants lacking the C1 and C2 regions. This indicates that the activation mechanisms of Sph are different from those of the anionic lipids (Table 3). Because the EF-hand region contains many negatively charged amino acid residues, these structures might be involved in the activation process of Sph.

In this study we could not identify the ATP binding site of DGK. A lysine residue surrounded by a Gly-rich sequence is known to be essential for nucleotide binding in many ATP- and GTP-binding proteins such as adenylate kinases, Ras proteins and phosphoglycerate kinases [39]. However, the results shown in Figure 5 demonstrate that mutants containing the substitution of lysine for asparagine (DGK α K383N, DGK α K395N, DGK α K483N and DGK α K554N) possessed almost the same enzyme activity as the wild-type enzyme. It is therefore most likely that DGK does not have a canonical P-loop of nucleotide-binding site. The C4 domain did not reveal significant similarities to the sequences of nucleotide-binding proteins deposited in the protein database. This suggests that DGK has a new ATP

binding sequence. Arg residues conserved in all DGK isoenzymes and homologues, such as Arg⁴²⁷, Arg⁴⁶⁸, Arg⁴⁹⁸, Arg⁵⁴⁰ and Arg⁵⁵⁰ in DGK α , might have an important role in binding the negatively charged substrate ATP. Further analysis such as photoaffinity labelling is required to determine the ATP binding site.

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