Site-directed mutagenesis of the active site of diacylglycerol kinase α : calcium and phosphatidylserine stimulate enzyme activity via distinct mechanisms

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Diacylglycerol kinases (DAGKs) catalyse ATP-dependent phosphorylation of *sn*-1,2-diacylglycerol that arises during stimulated phosphatidylinositol turnover. DAGK α is activated *in vitro* by Ca²⁺ and by acidic phospholipids. The regulatory region of DAGK α includes an N-terminal RVH motif and EF hands that mediate Ca²⁺-dependent activation. DAGK α also contains tandem C1 protein kinase C homology domains. We utilized yeast, *Saccharomyces cerevisiae*, which lacks an endogenous DAGK, to express DAGK α and to determine the enzymic activities of different mutant forms of pig DAGK α *in vitro*. Six aspartate residues conserved in all DAGKs were individually examined by site-directed mutagenesis. Five of these aspartate residues reside in conserved blocks that correspond to sequences in the catalytic site of phosphofructokinases. Mutation of D434 (Asp⁴³⁴) or D650 abolished all DAGK α activity, whereas substitution of one among

INTRODUCTION

Diacylglycerol kinases (DAGKs) catalyse ATP-dependent phosphorylation of sn-1,2-diacylglycerol (DAG) that arises during stimulated phosphoinositide turnover [1,2]. Phosphoinositidederived DAG activates several signalling proteins, including PKC (protein kinases C) [3,4], β 2-chimaerin [5], Ras guanine nucleotide releasing proteins [6,7], the synaptic vesicle priming protein, UNC-13 [8,9] and transient receptor potential family calcium channels [10,11]. DAGKs attenuate all these DAGdependent signals. PA (phosphatidic acid) derived from DAG phosphorylation is also a lipid mediator [9-11]. All animal DAGKs share a homologous catalytic domain and two or three cysteine-rich, zinc finger-like regions commonly referred to as C1 domains [1,2]. They are classified into five families based on the presence of key regulatory motifs [1,2]. The C-terminal half of DAGK α has been subdivided into a catalytic domain (SMART ID: DAGKc) and an accessory domain (SMART ID: DAGKa). The DAGK catalytic domain is distantly related to the catalytic domains of PFKs (phosphofructokinases) and PP_i/ATP NAD kinases [12].

C1 domains are 50 residue structures comprising several short β -strands and an α -helix wound around two central Zn atoms via eight conserved cysteine and histidine residues [3]. In many proteins, C1 domains mediate reversible membrane association in response to extracellular signals. Direct interaction with membrane-associated DAG facilitates binding of some C1 domains

D465, D497, D529 and D697 decreased the activity to 6 % or less of that for wild-type DAGK α . Roles of homologous residues in phosphofructokinases suggested that the N-terminal half of the DAGK catalytic domain binds Mg-ATP and the C-terminal half binds diacylglycerol. A DAGK α mutant with its entire regulatory region deleted showed a much decreased activity that was not activated by Ca²⁺, but still exhibited PS (phosphatidylserine)dependent activation. Moreover, mutations of aspartate residues at the catalytic domain had differential effects on activation by Ca²⁺ and PS. These results indicate that Ca²⁺ and PS stimulate DAGK α via distinct mechanisms.

Key words: calcium, diacylglycerol, diacylglycerol kinase, phosphatidylserine, site-directed mutagenesis.

to membranes or artificial lipid vesicles [3]. Moreover, C1 domains are the targets of tumour-promoting phorbol esters, which are DAG analogues [3]. Acidic phospholipids, especially PS (phosphatidylserine), bind C1 domains synergistically with DAG and phorbol esters. This binding contributes to PS activation of PKC [13]. The role of the C1 domains in DAGKs is unknown. At least some DAGKs bind phorbol esters [14,15], and some are targeted to membranes in a PS and DAG-dependent manner [16,17]. A DAGK γ mutant, which disrupts one of the Zn-coordinating cysteine residues in its first C1 domain no longer translocates to membranes [18]. This suggests that the C1 domains of DAGKs also participate in membrane translocation. Some wellcharacterized plant DAGKs lack C1 domains, but still express catalytic activity in vitro [19]. Thus C1 domains may not be required for catalytic DAG binding. However, their role in regulating activity of animal DAGKs remains unknown.

We have now examined the requirements for catalytic activity of DAGK α expressed in *Saccharomyces cerevisiae*. This yeast does not express an endogenous DAGK activity, allowing for detection of very low activities of heterologously expressed mutant DAGKs. A mutant lacking both C1 domains expressed readily measurable, albeit much decreased activity in this yeast, demonstrating that the C1 domains are not absolutely required for catalytic DAG phosphorylation. The roles of six aspartate residues were examined by site-directed mutagenesis. These aspartate residues span the entire C-terminal half of DAGK α and are conserved in all DAGK sequences. Five of these aspartate residues

Abbreviations used: DAG, diacylglycerol; DAGK, DAG kinase; HRP, horseradish peroxidase; OG, octylglucoside; PA, phosphatidic acid; PFK, phosphofructokinase; PKC, protein kinase C; PS, phosphatidylserine; for brevity, the one-letter system for amino acids has been used: D434, Asp⁴³⁴. ¹ To whom correspondence should be addressed, at the Section of Endocrinology and Metabolism (e-mail jpwalsh@iupui.edu).

are in conserved sequence blocks that correspond to the catalytic site of PFKs. Mutants of all six aspartate residues exhibited no or markedly decreased DAGK activities. These findings indicate that the entire C-terminal region of DAGK α comprises its catalytic domain. Effects of these mutations on Ca²⁺ and PS stimulation of activity suggested that these effectors activate DAGK α via distinct mechanisms.

EXPERIMENTAL

Materials

Restriction and DNA-modifying enzymes were from Promega or Gibco BRL (Rockville, MD, U.S.A.). Oligonucleotides were from Gibco BRL. $[\gamma^{-32}P]$ ATP was from DuPont-New England Nuclear (Boston, MA, U.S.A.). sn-1-Palmitoyl 2-oleoyl phosphatidylserine and sn-1-palmitoyl 2-oleoyl phosphatidic acid were from Avanti Polar Lipids (Birmingham, AL, U.S.A.). sn-1-Palmitoyl 2-oleoyldiacylglycerol (16:0,18:1-DAG) was prepared by digestion of the corresponding phosphatidylcholine (Avanti) with Bacillus cereus phospholipase C [20]. Octyl- β -D-glucopyranoside (octylglucoside, OG), sodium deoxycholate, Triton X-100, ATP, dihexadecylphosphate, diethylenetriaminepenta-acetic acid, EDTA, EGTA, leupeptin, aprotinin, PMSF, pepstatin A and sorbitan trioleate were from Sigma. A chemiluminescent Westernblotting detection system was from Pierce. Anti-Flag M2 antibody was from Eastman Kodak (Rochester, NY, U.S.A.). Reagents for protein assay and electrophoresis were from Bio-Rad. DEAE-cellulose (DE52) ion-exchange resin was from Whatman. Silica gel 60 plates were from Merck.

Expression of DAGKs in COS-1 cells

DAGK α -pCDNA3 and DAGK α Δ 196-pCDNA3, which express DAGK α and DAGK α Δ 196 in COS-1 cells, have been described previously [21]. A cDNA construct expressing DAGK α Δ 332, pSRE-DAGK α Δ 332, was provided by Dr F. Sakane and Dr H. Kanoh (Department of Biochemistry, Sapporo Medical University School of Medicine, Sapporo, Japan) [22]. To express DAGK α and the Δ 332 mutant in COS-1 cells, fragments containing DAGK α and the mutant sequences were subcloned into pCDNA3 (Invitrogen). To facilitate quantification of expression, a FLAG epitope was introduced at the C-termini as described previously [21]. Growth and transfection of COS-1 cells have also been described [21]. After 48 h, cells were harvested and lysed by sonication in ice-cold 20 mM Tris/HCl (pH 7.4), 0.25 M sucrose, 100 mM NaCl, 2.5 mM EGTA, 1 mM MgCl₂, 1 mM dithiothreitol, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, 1 mM PMSF, 50 μ M ATP and 0.02 % Triton X-100. After removal of undisrupted cells by brief centrifugation, the extracts were centrifuged at 100 000 g (Beckmann TL-100) for 20 min at 4 °C to pellet membranes. The resultant supernatants were rapidly frozen in a solid CO₂/ethanol bath and stored at -70 °C until assayed.

Expression of DAGKs in yeast

Point mutations of full-length DAGK α were prepared using an Altered Sites II mutagenesis kit (Promega) according to the instructions provided by the supplier. All mutant sequences were confirmed by automated DNA sequencing. To express DAGK α constructs in *S. cerevisiae*, DNA fragments containing the desired sequences were excised with *Eco*RI and subcloned into pYCDE2. The plasmid constructs were introduced into *S. cerevisiae* strain

WY294 and transformed cells were grown in 50 ml of SD medium, supplemented with 20 mg/l uracil, 30 mg/l leucine, 30 mg/l isoleucine and 150 mg/l valine as described previously [23]. Subsequent steps were conducted at 4 °C or on ice. Cells were collected by centrifugation and washed in 3 ml of 25 mM Tris/HCl (pH 7.4), 25 mM NaCl, 0.25 M sucrose, 1 mM EGTA, 1 mM MgCl₂, 1 mM dithiothreitol, 0.02 % Triton X-100, 50 μ M ATP, 2 μ g/ml aprotinin, 2 μ g/ml leupeptin, 3 μ g/ml pepstatin, 1 mM benzamidine and 1 mM PMSF (lysis buffer). The cells were then disrupted by vortex-mixing with glass beads in 0.75 ml of lysis buffer. The lysate was clarified by centrifugation. The pellet was then extracted with a second 0.75 ml volume of lysis buffer. The extracts were combined and applied to a 0.7 ml (0.6 cm \times 2.5 cm) DEAE-cellulose column equilibrated with lysis buffer. The column was washed with 2 ml of lysis buffer containing 50 mM NaCl, and the DAGK activity was then eluted with 1.5 ml of lysis buffer containing 125 mM NaCl. All DAGK activity measurements were linear with time and protein concentration after the DEAE step, presumably owing to the removal of interfering phosphatase or lipase activities. Aliquots of this preparation were rapidly frozen in solid CO₂/ethanol for later analysis.

Immunoblot analysis

Equal amounts (5–10 μ g) of lysate from COS-1 cells transiently expressing DAGK α or the truncation mutants were separated by SDS/PAGE. Proteins were electrophoretically transferred to nitrocellulose membranes (Schleicher and Schuell). After blocking in TBS-T (Tris-buffered saline/Tween) buffer containing 5% (w/v) non-fat dry milk for 1 h, the membrane was incubated with anti-Flag M2 antibody (1:2000 in TBS-T) for 1 h. Membranes were then washed with TBS-T and incubated with HRP (horseradish peroxidase)-conjugated sheep anti-mouse IgG (1:5000 dilution in TBS-T) for 30 min. After washing four times in TBS-T buffer, the HRP conjugates were detected by chemiluminescence. Truncated DAGKs expressed in S. cerevisiae were similarly applied to SDS/polyacrylamide gel and transferred to nitrocellulose. After blocking in PBS-T containing 5 % nonfat dry milk, the membrane was incubated with anti-DAGK α antibody (a gift from Dr F. Sakane and Dr H. Kanoh) in PBS-T (1:2000) for 1 h. The membrane was then incubated with HRPconjugated goat anti-rabbit IgG (1:10000 in PBS-T) for 1 h. After washing four times in TBS-T buffer, the HRP conjugates were detected by chemiluminescence. DAGK α catalytic domain point mutants were detected similarly, except that a polyclonal antibody against the DAGK α regulatory region was used [24]. Densities of immunoblot bands were determined from scanned images using Scion Image 3b. Scion Image is a Windows-based version of the public domain NIH Image program developed at the NIH. This software is freely available at the Scion Corporation website (http://www.scioncorp.com/).

Other methods

DAGK activity was assayed in OG/PS mixed micelles or with 0.1 mM DAG dispersed in 1 mM deoxycholate as described previously [21,23]. Note that activities determined in the presence of detergents may not reflect those in membranes or lipid vesicles. Analytic methods for measurement of the concentrations of protein, DAG and phospholipids were also as described previously [21]. Protein sequences homologous with the DAGK α catalytic domain were identified by PSI BLAST [25] and Meta-MEME [26] searches. Lipid kinases were aligned using ClustalX [27].

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Figure 1 Structures of DAGK α (DGK α) truncation mutants used in the present study

Diagram of the DAGK α mutants used in the present study are shown. RVH, EF hand, C1 and catalytic (CAT) domains are indicated. All of the constructs also included an N-terminal FLAG epitope to facilitate detection and quantification on immunoblots. The wild-type and Δ 196 constructs have been described previously [21]. Construction and expression of DAGK α Δ 332 are described in the Experimental section. In all Figures, DGK α is used to represent DAGK α .

Conserved motifs in DAGKs, PFKs, NAD kinases and other protein families were identified and aligned using MEME [28] and Block Maker [29].

RESULTS

Expression of the DAGK $\!\alpha$ catalytic domain in COS-1 cells and yeast

Animal DAGKs include a conserved C-terminal catalytic region and two or three cysteine-rich motifs, which are commonly referred to as C1 domains. Diagrams of DAGK α constructs used in the present study are shown in Figure 1. To examine whether the C1 domains of DAGK α are required for catalytic activity, a $\Delta 332$ truncation mutant was expressed in COS-1 cells. Densitometric scanning of immunoblots against the C-terminal FLAG epitope indicated that the DAGK $\alpha \Delta 332$ truncated protein was present in these COS-1 lysates at approx. 50 % of the level of wild-type DAGK α . This mutant expressed a DAGK activity in the deoxycholate assay which, after subtraction of the COS-1 background and correction for the decreased level of expression, was approx. 10 % of the activity measured for the wild-type enzyme.

To exclude interference rigorously from endogenous COS-1 DAGKs, the $\triangle 332$ mutant was also expressed in S. cerevisiae. This yeast does not express a DAGK. Searches of the S. cerevisiae genome revealed only its two long-chain base kinases, which are only distantly related to animal DAGKs (see below). These enzymes phosphorylate only dihydrosphingosine and related sphingoid bases [30]. Similar searches identified no Saccharomyces sequences homologous with bacterial DAGKs. Extracts of yeast transformed with the pYCDE2 vector were examined for DAGK activity using the deoxycholate and OG/PS assays, as well as assays employing Triton and hexadecyl sulphobetaine or Triton and octadecyltrimethylammonium chloride [23]. Radioactive product co-migrating with the PA standard was not observed with any of these assays. Figure 2(B) (left lane) shows an autoradiogram of the products of DAG phosphorylation by pYCDE2 yeast extract in the OG/PS assay. The DAGK α Δ 332 construct expressed a protein product with the expected molecular mass on immunoblots (results not shown). The activity of S. cerevisiae lysates expressing the DAGK α Δ 332 protein was 0.7% of that with wild-type DAGK α . The lipid product of the $\Delta 332$ reaction co-migrated with authentic PA on TLC (Figure 2B). After DEAEcellulose chromatography, this activity was completely dependent on exogenous DAG (Figure 2B). These findings demonstrate that DAGK α lacking its C1 domains is still capable of catalysing DAG



Figure 2 Stimulation of DAGK $\alpha \Delta 332$ activity by PS

DAGK α Δ 332 was expressed in COS-1 cells or yeast and assayed in OG/PS as described in the Experimental section. (**A**) Total DAGK activities of COS-1 lysates transfected with DAGK α Δ 332 or the pCDNA3 vector. Activities are shown as means \pm S.D. and are not corrected for vector background or expression level. Activity of the Δ 332 mutant in 10 mol % PS was not significantly greater than the vector background. However, in 20 mol % PS, the difference was significant (P = 0.004). (**B**) Autoradiogram of a TLC of the reaction products of DAGK α Δ 332 expressed in yeast. [³²P]Product co-migrating with the PA standard (arrow) was not detected with lysates of yeast transformed with the pYCDE2 vector, or in assays from which DAG was excluded. (**C**) Effect of PS on the activity of DAGK α Δ 332 expressed in yeast. Activities are shown as means \pm S.D. Increasing the PS concentration significantly stimulated the Δ 332 activity (P = 0.001). Unpaired Student's *t* tests comparing enzyme activities were performed using Sigma Plot 2001.

phosphorylation, albeit at much decreased levels compared with full-length DAGK α .

Many C1 domains mediate DAG and phospholipid-dependent targeting of proteins to membranes [3]. Thus it is possible that the very low activity of the DAGK α Δ 332 protein reflects loss of C1mediated PS binding, which might target the enzyme to mixed micelles and thereby increase its activity in the assay in vitro. To test this, we examined the activation of DAGK α and several deletion mutants by PS. Increasing the surface concentration of PS in the assay from 10 to 20 mol % stimulated wild-type DAGK α 7.8-fold and DAGK α Δ 196 3.8-fold [21]. Stimulation by acidic lipids is not a general property of DAGKs, e.g. DAGK δ and DAGK ε are inhibited by PS [31,32]. Note also that the COS-1 background DAGK activity was stimulated < 2-fold by PS (Figure 2A). At 10 mol % PS, the activity of COS-1 lysates expressing the $\triangle 332$ activity was only slightly higher than that of lysates transfected with the pCDNA3 vector (Figure 2A). Increasing the PS to 20 mol % stimulated the $\triangle 332$ activity markedly more than the vector control, indicating that PS is stimulating the $\Delta 332$ activity (Figure 2A). To quantify this stimulation, we examined the activities expressed in DEAE-purified yeast lysates. Increasing the PS from 10 to 20 mol% stimulated wild-type DAGK α 7.5-fold, which is similar to the stimulation of COS-1-expressed enzyme [21]. The $\triangle 332$ activity was stimulated 6.3-fold, which is similar to the stimulation seen with wild-type enzyme

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DGKa, pig DGK, human DGKq, human DGKe, D. melanogaster DGKe, human DGK8, human DGK1, A. thaliana	572 K L E 499 D L J 752 N L E 407 H I E 422 D L M 792 S L F 560 D F	ESLTVE KHTRV QRVQLE EKLTLV IKKVELE KQTRLQ WQVRVE	IC GG (10) C DI GG (11) C DI GG (7) L DI N (7) L DI GG (7) V ELR (7) V DI GG (9)	E G I A V G C V Y F G G I A V G A L V F E G I I V E G L I F E G F L V	L L L E J A	N I P N I P N I D N I G N I G N I G	STHG RYCA SYAG SWGA YWGG SWG3 SYMG	G (38) G (15) G (15) G (16) G (16) G (15) G (7) G (16)	PD QR PS NS AR PR QS	L S D I H D D C F D D I I S D I H D D C N D D C N H D I	<pre>< R L I A Y L I A Y L I A Y L I A I L I A L L I A L L I </pre>	E V V E V J E V V E V F E V V E V V E V V	G L E G F T G I F G V T G V T S I S	(23) E (20) E (22) T (22) Q (22) T (22) Y (22) Y (22) A	T F V V L V K I I R L V R L F R V V K I	H T T T T S T I F Q V K I L K T L L Q L C	- K 1 - K / (3) (- E 1 C S M - K / - K /	Г L P I А I P G V P I V P I И M P I А Т Р Р L P	N Q I V Q V N Q V N Q V N Q V N Q V V Q I		PWMC PCKL AWVC PWMC PWAC PWVC	A A S P P G S P A G P G G P G D A P G D A P G	Т К Я Я Т К Т В	I T H I A L I V H L S S I T H I S A I S H	R N Q R N Q K N R R S Q K T H A G P H G C	- M P - A T - A Q - A R A M M K V H - A F	M L V G M V Q K M L T R V L K L L Y F S M L R K M L K R	721 624 877 531 546 905 685
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Figure 3 Alignments of DAGKs and related lipid kinases

DAGKs, the prokaryotic DAGK homologues and sphingosine/ceramide kinases were separately aligned using ClustalX. The DAGK and sphingosine/ceramide kinase alignments were then each aligned with the prokaryotic kinases using the profile alignment utility of ClustalX. Representative sequences from the alignment are shown. Numbering of amino acids is according to the DAGKα sequence. Groups of conserved amino acids are shaded light grey. Asparate residues conserved in DAGKs are shown by white letters on black background. GenBank[®] accession numbers for these sequences are as follows: DAGKα, *Sus scrufa* (pig), 125323; DAGKζ, *Homo sapiens*, 4503317; DAGKη, *Homo sapiens*, 13559036; DAGKε, *Drosophila melanogaster*, 2801431; DAGKε, *Homo sapiens*, 4503313; DAGKθ, *Homo sapiens*, 4557519; DAGK1, *Arabidopsis thaliana*, 2494034; yerQ, *B. subtilis*, 7474554; *Listeria innocua* protein, 16415238; *Clostridium perfringens* protein, 18144990; *Streptococcus pneumoniae* protein, 14972520; bmrU, *B. subtilis*, 728972; yegS, *E. coli*, 2495625; *Bacillus halodurans* protein, 10174571; *Streptomyces coelicolor* protein, 6434737; Iong-chain base kinase 1 (LCBK1), *S. cerevisiae*, 6324745; sphingosine kinase 1 (SK1), *Homo sapiens*, 11464967; sphingosine kinase (SK), *D. melanogaster*, 17862170; ceramide kinase (CERK), *Homo sapiens*, 21624340.

(Figure 2C). Lysates of yeast transformed with the pYCDE2 vector did not express detectable background DAGK activity (Figure 2B). These findings suggest that a direct interaction with the catalytic domain may contribute to PS stimulation of the DAGK α activity. Because of the low activity of the Δ 332 mutant, these results do not exclude an additional interaction of PS with the C1 domains during activation of the full-length enzyme.

Site-directed mutagenesis of the DAGK α catalytic domain

Results with the DAGK $\alpha \Delta 332$ mutant demonstrate that the C1 domains are not absolutely required for DAG phosphorylation. The first half of DAGK α C-terminal region, corresponding to residues 375–501, has been designated as the DAGK catalytic domain (SMART ID: DAGKc), whereas the second half has been designated as the DAGK accessory domain (SMART ID: DAGKa). These DAGK α sequences can be aligned, not only with other

DAGKs, but also with sphingosine kinases, ceramide kinases and a prokaryotic protein family of unknown function (Figure 3). Moreover, as described in the Discussion section, some conserved sequence blocks in this alignment are distantly related to sequences in the active sites of PFKs and NAD kinases. Six aspartate residues are conserved among all DAGK sequences (Figure 3). Although not all of these aspartate residues are conserved in the sphingosine/ceramide kinases and the bacterial homologues, all are located in conserved stretches in the aligned sequences (Figure 3). Aspartate residues play critical roles in many kinases, including serving as nucleophilic catalysts during phospho transfer, co-ordinating bivalent metal ions and participating in substrate binding [33–35]. Some of these aspartate residues may thus play key roles in the catalytic mechanism of DAGKs.

The six aspartate residues were individually converted into alanine or asparagine in the full-length DAGK α . All the mutant

Table 1 Activities of DGK α aspartate mutants

Assays were performed as described in the Experimental section using the deoxycholate assay and 20 μ I (100–200 μ g) of DEAE-purified extract of yeast expressing wild-type DGK α or the indicated point mutants. Reaction products were extracted into CHCl₃/ethanol and the solvent-evaporated under a stream of nitrogen. The residue was dissolved in CHCl₃/methanol (9:1, ν /ν) and spotted on to silica gel 60 plates together with authentic PA standard. Plates were developed with chloroform/methanol/acetic acid (13:3:1, by vol.). The PA standard was detected with iodine vapour and the ³²P by scintillation counting. Product co-migrating with the PA standard was readily detected with all the DGKs, but not with the pYCDE2 vector. All activities are expressed as a percentage of the activity measured in parallel assays with wild-type DGK α .

	Percentage of wild-	-type DGK activity
	$D \rightarrow A$	$D\toN$
Wild-type	100	100
D434	n.d.*	n.d.*
D465	n.d.*	0.1
D497	n.d.*	0.9
D529	1.1	5.5
D650	n.d.*	n.d.*
D607	14	4.0

*n.d., not detectable. Activity with the D434A, D465A, D497A, D650A, D434N and D650N mutants was indistinguishable from the background. Moreover, radioactivity co-migrating on TLC with the PA standard could not be detected with these six mutants, even after prolonged autoradiography.

Table 2 Stimulation of DGK α mutants by calcium and phosphatidylserine

Assays were performed as described in the Experimental section using the OG/PS method and 20 μ l (100–200 μ g) of DEAE-purified extract of yeast expressing wild-type DGK α and the indicated D \rightarrow N mutants. Reaction products were quantified by TLC as described in the legend to Table 1. Calcium stimulation is the ratio of activity in OG, 20 mol % PS and 20 μ M Ca²⁺ to the activity in the same OG and PS with no Ca²⁺. PS stimulation is the ratio of activity in 20 mol % PS when compared with 10 mol % PS. The D465N mutant activity, which was extremely low in the deoxycholate-based assay, could not be detected in the OG/PS assay used for these stimulation studies.

	Stimulation (fo	Stimulation (fold)										
	Ca ²⁺	PS										
Wild-type DGK α	36.1	9.5										
DGKa D497N	6.2	11.2										
DGKa D529N	36.2	11.6										
DGK α D697N	4.7	12.4										

DAGK α polypeptides were expressed at levels comparable with the wild-type enzyme, as judged from immunoblots. All had significantly decreased DAGK activities (Table 1). No activity was detected with mutants at D434 (Asp⁴³⁴) or D650. Enzyme activity was also abolished in the D465A and D497A mutants, whereas D465N and D497N expressed very low activities. Activity was readily detectable with the DAGK α D529 and D697 mutants, albeit still much decreased as compared with wild-type DAGK α . None of the mutant activities were altered by increasing the Mg-ATP, DAG or free Mg²⁺ concentrations in the assay (results not shown). Calcium activation of the D529N mutant, expressed as the ratio of activity in $20 \,\mu M \, \text{Ca}^{2+}$ to activity in the absence of Ca²⁺, was identical with wild-type DAGK α (Table 2 and Figure 4). However, Ca^{2+} activation of the DAGK α D497N and D697N mutants was markedly impaired (Table 2 and Figure 4). PS activated the D497N, D529N and D697N mutants similarly to wild-type DAGK α . Activity of the D465N mutant, which was extremely low in the deoxycholate assay (Table 1), could not be detected in the OG-based assays used for Ca2+ and PS stimulation studies. Normal PS stimulation of the D497N and D697N mutants,



Figure 4 Impaired calcium activation of DAGKα D697N

Wild-type DAGK α , DAGK α D497N, DAGK α D529N and DAGK α D697N were prepared in *S. cerevisiae* as described in the Experimental section. Calcium-dependent activation of these preparations was examined in OG with 20 mol % PS. Activities are given relative to the activity of the same preparation in the absence of calcium. These results are representative from two separate experiments with independent DAGK preparations. \bullet , Wild-type DAGK α ; \checkmark , DAGK α D497N; \bigtriangledown , DAGK α D529N; \bigcirc , DAGK α D697N.

both of which exhibited impaired Ca²⁺-dependent activation, provides further evidence that Ca²⁺ and acidic phospholipids activate DAGK α via distinct mechanisms.

DISCUSSION

DAGKs, sphingosine, kinases, ceramide kinases and the related prokaryotic protein family are distantly related to PFKs and NAD kinases [12]. Conserved blocks from DAGKs and related protein families are shown in Figure 5. Structures are available for three homo-oligomeric PFKs from bacteria [36-38]. The subunits consist of two lobes, each with a central β -sheet sandwiched between two layers of α -helices. The PFK active site resides in a cleft between these two lobes, with most of the catalytic residues located on turns between β -strands and α -helices. The conserved segments in Figure 5 all correspond to sequences in the PFK active site. This suggests that folding around the active site is conserved between DAGKs and PFKs. Based on these alignments, D434 in DAGK α corresponds to D104 of PFK1, which interacts with the ATP-bound Mg^{2+} [36]. The other essential aspartate residue, D650, corresponds to E223 in PFK1. This glutamate residue, which is conserved in all PFKs, forms a hydrogen bond with O(4) of fructose 6-phosphate [36]. The D465N mutant had extremely low activity. The corresponding residue in PFK1, D130, interacts with the ATP-bound Mg²⁺ via a water molecule [36]. Mutations of D104 or D130 in PFK1 have markedly decreased kinase activities [39]. DAGK residues D529 and D697 align near R172 and R253 of Escherichia coli PFK1. These two arginine residues interact with the 1-phosphate, 6-phosphate and O(3) of the fructose 1,6-bisphosphate product. The final conserved aspartate residue, D497, resides halfway between D465 and D529. In PFKs, a long α -helix extends from the loop corresponding to D465 away from the active site and a β -strand returns, ending in the loop corresponding to D529. It is possible that the fold of DAGKs differs from PFKs in this region, permitting D497 to reside near the active site. However, as

DGKx 375 PLLVFVNPKSGG (39) DYRILVCGCDCTCGCTVGWILE (12) VAVLPLGTCKVLLAG VAVLPLGTCK DA DGKz 219 PLIILANSRSGT (40) SARVLVCGCGCTVGWVLD (16) VAVLPLGTCK DA DS DGK1 361 PLLVFINKKSGA (39) HFRVLVCGCCGCTVGWVLD (12) VAVLPLGTCKDLS DS DgK1 334 VLVFVSKGG (37) HFRVLVCGCGCGTVGWVLD (12) VAVLPLGTCKDLS DS bmvU 5 KALLIHNGNAAN (42) VDTVFLLGCGCGTVGWLP (10) LGVTPVGTTKOGSGCA DFA yar0 3 R RTIVNPTSCR (43) FDLITCAGCGCGTNHEVN (10) LGVTPVGTTKOGSGCA DFA yar0 3 R RTIVNPTSCR (44) VDTVFLLGCGCGTNHEVN (10) LGVTPVGTKAAN DFA SK1 15 RVLUNPRGCK (44) VDTLVVSGCGCGTNLEVN (10) LGVTPAGAYNDFS DFA SK1 15 RVLUNPRGCK (44) VDTLVVSGCGCGTNLLYASS (3) GSVPPWAALAAN DFA NADK, E.coli 7 CGVVSGCGASN GGTNLGAAN (3)	DGK α sequence			38	30									430						44)							460	I					
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yiri 3 H W F F I I N P T A G H (46) L K R L I V I G Q D G T M H E V V N (9) L S E V P A G A Y N D F S R LCBK1 228 S I L V I I N P H G G K (44) Y D T I A C A S G D G I P Y E V I N (16) V T Q L P C G S G N A M S I A S G D G L M H E V V N (15) L C S L P A G S G N A M S I A S A S C D G L M H E V N (15) L C S L P A G S G N A M S I A S A S C D G L M H E V N (15) L C S L P A G S G N A M S I A S A S A S A A L A A N A D K, E C M T I A G G P G T L L Y A S S G D G L M H E V N (15) L C S L P A G S G N A M S I A S A S A S A A L A A N A D K, E C M T I A G S V L V I K K M R D A S (57) I D F I I C L G G D G T L L Y A S S (3) G S V P P V M A F H L G S L P K S C A D A V V I G G D G S L T G A N I G A N I G C P K T I D G D L K C Y G G D G S L T G A N I G A N I G C P K T I D G D L K C Y G G D G S L T G A N I G A N I G C P K T I D N D F C G O F K I I D N D F C G O F K I S G G D A P (81) I T N L C V I G G D G S L T G A N I G A N I G C P K T I D N D F C G O F K I S G G D A P (79) I D A L V V I G G D G S L T G A N I G A N I G A N I G A N I G A N I G A N I G A N I G A N I Y G S G G A A C A A S A N I D F C G G C S G A G A G N I N Y G G C C G A V A G A R (8) C I G L P G T I D N D F C G C A C A A G A N I G A N I G A N I G A G C N G A N I A S A G A N I Y N I G Y G C D A X A A G A S N I A N Y N I G Y G C D A X A A G A S N I M N N Y F S Y G P D A L M A (87) R H D D G L L E V G G (34) L P M Q I D C P C A Y D G C P W A S 2 G A Y A G A S N A Y A I Y I N Y N I G Y G C D A X Y A (90) S N I D K I V V Y O G C D A Y	verQ	3	RA	R I	Ν,	(14	P	т 5 с	S R	(4	3)	F	DI		A 4	I G	c D	e	τì	N	EM	V N	à	0)		c	v	I P	N	G 1	·т	NC	D E .	R
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Figure 5 Motifs conserved in the catalytic domains of DAGKs and other proteins

Catalytic domain sequences of DAGKs, sphingosine/ceramide kinases, prokaryotic lipid kinase homologues, PFKs and NAD kinases (NADKs) were examined for conserved motifs using Blockmaker [29] and MEME [28]. Alignments of conserved blocks from these families are shown. The alignment is numbered at the top according to the DAGKα sequence and at the bottom according to *E. coli* PFK1. Similar amino acids are shaded light grey and aspartate residues conserved in DAGKs are shown by white letters on black background. GenBank[®] accession numbers for the sequences shown are as follows: DAGKα, *Sus scrufa*, 125323; DAGK*ε*, *Homo sapiens*, 4503313; DAGK1, *A. thaliana*, 2494034; Type V DAGK, *Dictyostelium discoideum*, 24061802; bmrU, *B. subtilis*, 728972; yerQ, *B. subtilis*, 7474554; ytlR, *B. subtilis*, 16080046; LCBK1, *S. cerevisiae*, 6324745; SK1, *Homo sapiens*, 11464967; NADK, *E. coli*, 26248979; NADK, *Homo sapiens*, 8480400; PFK *β* chain, *A. thaliana*, 15221156; PFK liver, *Homo sapiens*, 14286326; PFK1, *E. coli*, 15804505.

discussed below, this aspartate residue may play a non-catalytic role in enzyme regulation. An N-terminal loop in PFKs is also conserved in DAGKs (Figure 5). G12 of this loop hydrogen bonds the ATP β -phosphate in PFKs, suggesting that this motif also forms part of the DAGK active site. Overall, our findings indicate that the entire C-terminal region of DAGK α comprises its catalytic domain and that the structure around the active site is related to that of PFKs. Conserved blocks in the more highly conserved N-terminal half of the catalytic domain (SMART ID: DAGKc) correspond to PFK sequences involved in Mg-ATP binding. Conserved segments in the C-terminal half of DAGK catalytic domains (SMART ID: DAGKa) do not correspond to PFK sequences involved in Mg-ATP binding, and quite probably include residues required for DAG binding.

DAGKs also differ from PFKs in important respects. PFKs are oligomeric. However, size-exclusion profiles of DAGK α , DAGK ε and sphingosine kinase-1 indicate that these enzymes are monomeric [32,40,41]. Also, substrate kinetics of these enzymes reveal no evidence of the co-operativity seen in PFKs [23,32,41]. Lack of oligomeric structure in DAGKs is of particular interest because two arginine residues from an adjacent subunit, R163

Figure 3), suggesting that this feature of PFKs is not conserved in DAGKs. Two DAGKa mutants, D497N and D697N, exhibited impaired activation by Ca2+, suggesting some interaction with the regulatory region of the enzyme. Within PFK subunits, the transition from the active R-state to the inactive T-state is accompanied by a 1.2 Å (1 Å = 0.1 nm) translation of two α -helices across the adjacent β -sheet and rearrangement of two loops [37]. D497 is located halfway between D465 and D529, which places it in or near the first of these two loops. Several mutations spanning this region alter the allosteric regulation of PFKs [43–45]. Impaired activation of DAGK α D697N is also of much interest. This aspartate residue aligns near R253 of PFK1 (Figure 5). In the R-state, R253 binds the 6-phosphate moiety, whereas in the T-state it interacts with a glutamate residue from an adjacent subunit. [37]. Mutation of this arginine residue profoundly alters not only fructose 6-phosphate binding, but

and R244 in E. coli PFK1, extend into the active site to bind

the 6-phosphate moiety of fructose 6-phosphate [42]. These two

arginine residues are 8-10 residues N-terminal of the loops that

correspond to D529 and D697 in DAGK α . DAGKs do not show

strong similarity in these regions, even among themselves (see

also the allosteric activation of the enzyme [46]. The other four conserved DAGK aspartate residues do not correspond to PFK sequences involved in its allosteric transition. Impaired Ca^{2+} activation of the D497N and D697N mutants thus suggests that activation of DAGK α via its Ca^{2+} -binding regulatory region in some way reflects the allosteric activation of PFKs.

DAGK α is activated by PS and other acidic phospholipids. This activation can be demonstrated both in the presence and absence of added Ca²⁺. Observations that PS binding to C1 domains facilitates membrane targeting of some proteins suggested that the low DAGK α Δ 332 activity may reflect loss of binding to the micelle surface [3]. The $\triangle 332$ activity exhibited PS activation which, expressed as fold increase, was similar to wild-type DAGK α . This observation suggests a direct interaction of PS with the catalytic domain. Such an interaction might serve to orient the catalytic site on the membrane/micelle surface for DAG phosphorylation. Owing to the low activity of the $\Delta 332$ mutant, these results cannot exclude an additional interaction of PS with the C1 domains in the full-length enzyme. The D497N and D697N mutants, in which Ca2+-dependent activation was impaired, exhibited normal PS activation. These findings clearly demonstrate that the mechanism of PS activation is distinct from that of Ca²⁺, and suggest that it involves, at least in part, a direct interaction of PS with the catalytic domain.

The relatively low activity of the DAGK $\alpha \Delta 332$ mutant, nearly 100-fold less compared with the constitutive Δ 196 mutant, suggests a critical role for the C1 domains in physiological DAGK α activation. DAG phosphorylation by the $\Delta 332$ mutant excludes any essential role of C1 domain in catalytic DAG binding, as does the existence of well-characterized plant DAGKs, which lack C1 domains [19]. Our results do not preclude the possibility that the C1 domains target DAGKs to membranes or micelles in a PSdependent or -independent manner. Indeed, other results indicate that the first C1 domain of DAGK γ is required to target it to membranes in stimulated cells [18]. There is also evidence that the first C1 domains of DAGK β and DAGK γ bind phorbol esters [14,15]. This suggests that DAGK C1 domains may also bind DAG. Such DAG binding might contribute to targeting of DAGKs to DAG-rich regions of membranes where phosphoinositide turnover is occurring. Low activity of the DAGK α Δ 332 mutant also raises the possibility that one or both of its C1 domains directly stimulates the catalytic domain. If DAGKs undergo an activating conformational change, as occurs in PFKs, then the C1 domains could be favouring the active conformation.

It is of interest that bacteria express homologues of eukaryotic DAGKs. Prokaryotic DAGKs are unrelated to those of eukaryotes [47], and null mutants of bacterial DAGKs are devoid of DAGK activity [48]. Thus these proteins are unlikely to phosphorylate DAG. Up to three of these proteins can be expressed by a single species, suggesting that they may include multiple families with distinct metabolic functions. For example, Bacillus subtilis expresses bmrU, yerQ and ytlS (see Figure 5). Expression of bmrU is induced by energy deprivation and other stresses [49]. AcoX, a homologue in Alcaligenes eutrophus, is part of an operon induced during growth on acetoin [50]. Some actinobacterial homologues (GenBank[®] accession nos. 1666180, 1666188 and 21224814) have an N-terminal domain similar to acid phosphatases, and a homologue in blue-green algae (GenBank® accession no. 16331996) has a domain similar to methylglyoxal synthetases. These observations suggest that, whereas their specific substrates remain unknown, prokaryotic DAGK homologues may be kinases involved in utilization of alternative carbon sources.

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