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Domains, Amino Acid Residues, and New Isoforms of *Caenorhabditis elegans* Diacylglycerol Kinase 1 (DGK-1) Important for Terminating Diacylglycerol Signaling *in Vivo**[S]

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Diacylglycerol kinases (DGKs) inhibit diacylglycerol (DAG) signaling by phosphorylating DAG. DGK-1, the *Caenorhabditis elegans* ortholog of human neuronal DGK*θ*, inhibits neurotransmission to control behavior. DGK-1, like DGK*θ*, has three cysteine-rich domains (CRDs), a pleckstrin homology domain, and a kinase domain. To identify DGK domains and amino acid residues critical for terminating DAG signaling *in vivo*, we analyzed 20 *dgk-1* mutants defective in DGK-1-controlled behaviors. We found by sequencing that the mutations included nine amino acid substitutions and seven premature stop codons that impair the physiological functions of DGK-1. All nine amino acid substitutions are in the second CRD, the third CRD, or the kinase domain. Thus, these domains are important for the termination of DAG signaling by DGK-1 *in vivo*. Seven of the substituted amino acid residues are present in all human DGKs and likely define key residues required for the function of all DGKs. An ATP-binding site mutation expected to inactivate the kinase domain retained very little physiological function, but we found two stop codon mutants predicted to truncate DGK-1 before its kinase domain that retained significantly more function. We detected novel splice forms of *dgk-1* that can reconcile this apparent conflict, as they skip exons containing the stop codons to produce DGK-1 isoforms that contain the kinase domain. Two of these isoforms lack an intact pleckstrin homology domain and yet appear to have significant function. Additional novel isoform(s) account for all of the DGK-1 function necessary for one behavior, dopamine response.

Diacylglycerol kinases (DGKs)[1] phosphorylate diacylglycerol (DAG), thus attenuating signaling by this important second messenger (1, 2). DAG activates protein kinase C to control numerous cellular processes mediated by neurotransmitters, growth factors, and hormones (3). DAG also activates the synaptic vesicle priming protein UNC-13 (4, 5) to control neurotransmission and certain transient receptor potential cation channels (6). In humans, nine DGK isozymes have been identified (DGKα, β, γ, δ, η, ϵ, ζ, ι, and θ), but their physiological functions remain largely unknown (1, 2).

C. elegans DGK-1 provides a genetically tractable model for elucidating the physiological functions of diacylglycerol kinases. DGK-1 is expressed in neurons and is 38% identical to the human brain enzyme DGKθ (7). Genetic analysis of DGK-1 has shown that it depletes DAG generated by $G\alpha_q$ signaling in response to the neurotransmitters dopamine and serotonin (7, 8). Because the synaptic vesicle priming protein UNC-13 requires DAG to promote neurotransmission, this depletion of DAG by DGK-1 results in reduced neurotransmission (7). Loss of DGK-1 leads to increased neurotransmission and, thus, strong behavioral defects, including defects in dopamine-controlled locomotion behavior and defects in serotonin-controlled egg-laying behavior (7, 8). Similar behavioral defects are also caused by loss of *C. elegans* $G\alpha_o$, a neural G protein, due to an increase of UNC-13 at synaptic release sites (7). Furthermore, the loss of DGK-1 suppresses the behavioral defects caused by the overexpression of constitutively active $G\alpha_o$ (9). These genetic data have led to the hypothesis that $G\alpha_o$ signaling may activate DGK-1 to deplete DAG levels.

All human DGKs have a kinase domain and two or three cysteine-rich domains (CRDs) (1, 2). Most DGK isozymes contain additional distinct conserved domains. The diversity of DGKs is further increased by the fact that in six DGK isozymes alternative splicing has been shown to produce isoforms with altered domain structures (10–15). DGK-1, like its human ortholog DGKθ, has three CRDs, a pleckstrin homology (PH) domain, and a kinase domain (7, 16).

The functions of the various conserved domains in DGKs remain poorly understood. Their roles have been examined by transgenically expressing DGKs in yeast, COS7, or other cells and assessing the effects of mutations on enzymatic activity and subcellular localization (17–20). The CRDs are similar to the DAG-binding domains of protein kinase C, suggesting that the CRDs might bind DAG and present it for phosphorylation (21). Consistent with this hypothesis, mutating CRDs results in the dramatic loss of *in vitro* kinase activity (17–20). However, it appears that not all CRDs are able to bind DAG (22), and the CRDs of some DGK isozymes are not absolutely required for *in vitro* kinase activity (19, 23). Another proposed function of the CRDs is based on the observation that certain DGK isozymes translocate to plasma membranes to act on DAG generated in response to extracellular signals, and in DGKζ the CRDs are required for such translocation (18). PH domains are,

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[S] The on-line version of this article (available at http://www.jbc.org) contains further information on this subject in the form of supplemental experimental procedures, references, two figures, and a table.

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[1] The abbreviations used are: DGK, diacylglycerol kinase; CRD, cysteine-rich domain; DAG, diacylglycerol; MBP, maltose-binding protein; PA, phosphatidic acid; PH, pleckstrin homology; RT, reverse transcriptase.

in general, involved in protein-protein or lipid-protein interactions (24). The roles of PH domains in DGKs remain unclear, though the DGKδ PH domain has been shown to bind phospholipids (25) and to be required for translocation to the plasma membrane (13). The kinase ("catalytic") domain has a weak similarity to phosphofructokinases (26), and for two isozymes the expression of this domain alone was sufficient to produce diacylglycerol kinase activity (19, 23). However, the levels of activity produced were lower than those of the full-length enzymes, suggesting that other domains also contribute to catalysis.

We carried out a structure/function analysis of *C. elegans* DGK-1 in its physiological setting. By analyzing a panel of *dgk-1* mutant strains generated in genetic screens for animals defective in DGK-1 controlled behaviors, we found that the second CRD, the third CRD, and the kinase domain of DGK-1 are important for attenuating DAG signaling *in vivo*. Seven conserved amino acid residues in these domains are critical for DGK-1 function and likely define key residues required for the function of all DGKs *in vivo*. Our analysis also led us to detect novel DGK-1 isoforms that arise from alternative splicing and to show that these isoforms have significant physiological functions.

EXPERIMENTAL PROCEDURES

Construction of Plasmids and Recombinant Protein Purification—The *dgk-1a* cDNA from pKP137 (7) was subcloned into pFastBacHTb (Invitrogen) to generate a baculovirus expressing N-terminally His-tagged DGK-1 (His-DGK-1), which was purified from baculovirus-infected High Five (Invitrogen) insect cell extracts using nickel-nitrilotriacetic acid agarose affinity chromatography (Qiagen). DNA encoding amino acids 633–950 of DGK-1a was cloned into pET19b (Novagen) to express an N-terminally His-tagged DGK-1 kinase domain (KD) fragment, His-KD, in BL21(DE3) cells (Novagen). His-KD was purified in the presence of 8 M urea.

C. elegans Alleles, Transgenes, and Culture—*C. elegans* strains used for behavioral assays were cultured at 20 °C under standard conditions (27), except where otherwise noted. All 20 *dgk-1* alleles were outcrossed four times to the wild-type strain (Bristol N2) before behavioral analysis. *vs8*, *vs9*, *vs24*, and *vs55* are *dgk-1* alleles obtained from a screen for mutants that lay eggs at an early developmental stage (28). *vs67* and *vs71* are *dgk-1* alleles obtained from a screen for mutants resistant to paralysis by dopamine (8). *sy424*, *sy425*, *sy426*, *sy428*, *sy429*, *sy435*, *sy436*, *sy442*, *sy453*, *sy454*, *sy455, sy456*, and *sy512* are *dgk-1* alleles obtained from a screen for suppressors of the locomotion defect in animals expressing constitutively active GOA-1(Q205L) from a transgene (9, 29). These suppressor alleles were separated from the transgene during the outcrosses. The gonad of *dgk-1(nu62)* null mutant animals was injected with a mix of 10 ng/μl *myo-2-gfp* coinjection marker (gift from A. Fire, Stanford University) and 10 ng/μl test construct pAJ4, pAJ6, or pAJ8 to generate transgenic lines.

C. elegans Protein Extracts and Fractionation—The *C. elegans* strains used for Western blotting and biochemical experiments were grown in liquid culture at 20 °C as mixed stage populations. Worms were purified by floatation on 30% sucrose and transferred to lysis buffer (50 mM HEPES pH 7.4, 1 mM EDTA, 150 mM NaCl, 10% glycerol, 1 μM dithiothreitol, 1 μM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, and 1 μg/ml pepstatin) and lysed by passing three times through a French press. Debris was removed by centrifugation at 2,000 rpm in an IEC clinical centrifuge. The resulting total (T) lysates were flash-frozen in liquid nitrogen and stored at −80 °C. Protein concentrations were determined by Bradford analysis (Bio-Rad). When required, total lysates were fractionated before flash-freezing into soluble and pellet fractions by centrifugation at 100,000 × *g* for 45 min.

Antibodies and Western Blotting—Anti-DGK-1 antibodies were affinity purified using purified His-KD as described (30). Proteins were separated by SDS-PAGE and then blotted onto nitrocellulose filters. The anti-β-tubulin antibody was E7 (developed by Michael Klymkowsky and obtained from the Developmental Studies Hybridoma Bank, University of Iowa). Western blots were developed using chemiluminescence detection reagents (Pierce) and BioMax MR film. The Western blots in Fig. 3 are representative of four blots with at least two independently prepared extracts per genotype. The mobility on SDS-PAGE of the proteins studied was 110 kDa for His-DGK-1 and DGK-1 and 55 kDa for β-tubulin. In DGK-1 Western blots, the presence of nonspecific bands prevented detection of any possible DGK-1 products of lower molecular mass than the full-length 110-kDa protein.

Behavioral Assays—Proportion of eggs laid at a late developmental stage was measured as in Bany *et al.* (31) using animals that were aged 36 h past their fourth larval stage. We measured susceptibility to paralysis by dopamine using a modification of the method in Chase *et al.* (8) by placing animals on agar plates containing 30 mM dopamine and determining the fraction of animals paralyzed (unable to move in a 1-min interval). The error bars on all measures of behavior denote 95% confidence intervals for a single proportion calculated using Wilson's estimates, and *p* values for comparison of two proportions were calculated using the proportion of pooled values (32). We analyzed five independent transgenic lines for each test construct to test susceptibility to paralysis by dopamine (number of animals assayed per line, > 40). *dgk-1*(*sy512*) was strongly defective in both behaviors tested but had no mutations in the *dgk-1* coding regions and no detectable DGK-1 protein on a Western blot (data not shown).

In Vitro DGK-1 Activity Assay—The DGK-1 activity assay was adapted from that developed for its human ortholog, DGKθ (16). 125 ng of purified His-DGK-1 in 20 μl (or an equivalent volume of buffer) was added to 100 mM HEPES, pH 8, 10 mM $MgCl_2$, 0.7 mM dithiothreitol, 2 mM *n*-octyl β-D-glucopyranoside (Sigma), 40 μM L-phosphatidylserine (Avanti Polar Lipids), and either 40 μM 1,2-dioleoyl-*sn*-glycerol (Avanti Polar Lipids) or the substrate indicated (40 μM) in Table I to a final volume of 246 μl. 2 mM [γ-^{32}P]ATP at 55 × 10^4 dpm/pmol in 4 μl was added to start the reaction. The reaction was stopped after 20 min at 25 °C by adding 500 μl of 0.5 M EDTA. The lipids were extracted using 2 ml of a 2:1 mixture of $CHCl_3$/CH_3OH, and either the radioactive product in 100 μl of the extracted liquid was quantitated using scintillation counting or the extracted liquid was dried down to ~10 μl and separated by thin layer chromatography. The reaction product, phosphatidic acid (PA), was visualized as a radioactive spot after thin layer chromatography using BioMax MR film. For reference, 5 μg of PA (Avanti Polar Lipids) was also separated on the same thin layer chromatography plate and detected using phosphomolybdic acid (Sigma). For an assay of native DGK activity in soluble lysates of *C. elegans*, two independent lysates were prepared from each strain analyzed, and 100 μg of protein was assayed as described above, except that the specific activity of radiolabeled ATP was increased 4-fold, and the concentration of the diacylglycerol substrate was increased 2-fold.

Sequence Similarity—Genomic sequences spanning exons 1–14 of *dgk-1a* from *C. elegans* and *Caenorhabditis briggsae* were compared by NCBI-BLAST using the default settings, and regions of extended similarity (>7 bp) outside of and contiguous to known exons were noted. Amino acid sequences were compared using ClustalW and Megalign (Lasergene). The GenBank™ accession numbers of the sequences used were NM_075790 (DGK-1, REFSEQ), NM_001347 (DGKθ, REFSEQ), NM_001345 (DGKα, REFSEQ), NM_003648 (DGKδ, REFSEQ), NM_003647 (DGKε, REFSEQ), and U51477 (DGKζ).

Splice Form Analysis—Reverse transcriptase (RT) reactions were performed using a primer complementary to exon 10 of DGK-1. Single-stranded cDNA products were then used as templates in a PCR with various 5′-primers and a 3′-primer that overlapped the RT primer. No products were detected when RT-PCR reactions were attempted with 5′-primers in the region between splice site *ii* and putative splice site *iii*.

RESULTS

DGK-1 Phosphorylates DAG in Vitro and Is Predominantly a Soluble Enzyme—Because there has been little molecular characterization of DGK-1, we first examined some of the properties of this enzyme. We expressed His_6 affinity-tagged recombinant DGK-1 in baculovirus-infected insect cells and used nickel affinity chromatography to obtain a substantially purified enzyme (Fig. 1*A*). Purified His-DGK-1 phosphorylated the diacylglycerol 1,2-dioleoyl-*sn*-glycerol *in vitro* to make PA (Fig. 1*B*). The specific activity of this protein was 80 nmol/mg/min (data not shown), which is ~135-fold less than that of the purified rat DGK-1 ortholog in a comparable *in vitro* assay (33). We also purified a maltose binding protein (MBP) affinity-tagged DGK-1 fusion protein that had a low specific activity similar to that of the His-DGK-1 protein. To test whether this relatively low specific activity may be due to *C. elegans* DGK-1 having a different substrate specificity than the mammalian enzyme, we examined the ability of crude cell lysates expressing MBP-DGK-1 to phosphorylate different species of diacylglycerols and

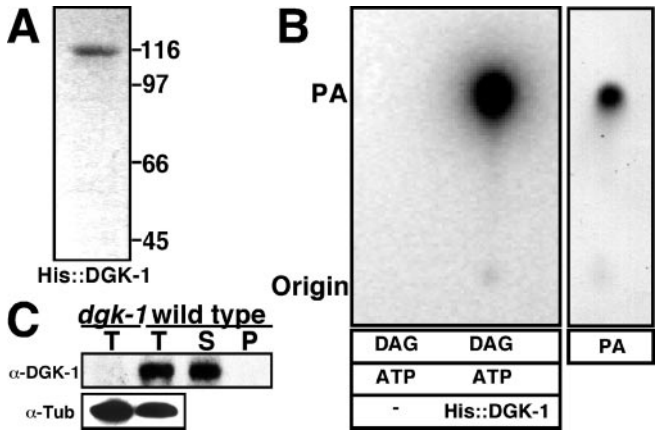


FIG. 1. ***In vitro* activity of recombinant DGK-1 and the fractionation of native DGK-1.** *A*, Coomassie-stained gel of recombinant His-tagged DGK-1 purified from insect cells. A single band is detected at ~110 kDa. *B*, *in vitro* DGK activity assay. *Left*, autoradiograph of a thin layer chromatogram showing PA generated upon phosphorylation of a DAG (1,2-dioleoyl-*sn*-glycerol) by purified recombinant His-DGK-1 with [γ-^{32}P]ATP (*ATP*). His-DGK-1 (*His::DGK-1*) (125 ng) or no enzyme was added to the reaction as indicated. *Right*, chemically stained reference PA separated on the same thin layer chromatography plate. The positions of the origin and the PA spot are indicated. *C*, DGK-1 Western blot of fractionated *C. elegans* extracts. Total (*T*), soluble (*S*), and pellet (*P*) fractions of *C. elegans* extracts are shown. *α-DGK-1*, anti-DGK-1 Western blot. *α-Tub*, anti-tubulin Western used to verify similar loading of total extracts. *dgk-1* denotes protein extract from *dgk-1*(*nu62*) null mutant worms. DGK-1 is a predominantly soluble protein.

monoacylglycerols (Table I). We found that DGK-1 prefers 1,2-diacyl-*sn*-glycerols to 1,3-diacyl-*sn*-glycerols and 1-monoacylglycerols, similarly to its mammalian ortholog (16). We found that the low activity of purified DGK-1 can be explained by ~95% of the protein being present as inactive aggregates (supplemental Fig. 1). When monomeric MBP-DGK-1 was separated from the aggregates by gel filtration, its specific activity was ~7,500 nmol/min/mg, comparable with that of the rat DGK-1 ortholog in a similar *in vitro* assay (33). Purified monomeric MBP-DGK-1 or His-DGK-1 protein isolated by gel filtration, however, aggregated over time (data not shown), precluding a detailed biochemical analysis of the recombinant protein. This instability of recombinant purified DGK-1 fusion proteins may be due to a lack of either post-translational modifications or cofactors that are present in the native DGK-1 protein in *C. elegans*.

To analyze native DGK-1 in *C. elegans,* we developed a DGK-1 antibody directed against part of the kinase domain. On Western blots, this antibody detected a single specific band (~110 kDa) in wild-type worm extracts that was absent in *dgk-1* null mutant extracts (Fig. 1*C*). This band had a similar mobility in SDS-PAGE as purified His-DGK-1 (Fig. 1*A*). We pelleted membranes from *C. elegans* extracts by centrifugation at 100,000 × *g* for 45 min and found that the native DGK-1, like its mammalian ortholog DGKθ (33), remained in the soluble fraction (Fig. 1*C*) although one of its substrates, diacylglycerol, is expected to be present in the pellet fraction with membranes. Wild-type soluble protein extracts had a measurable albeit low level of DGK activity (0.003 ± 0.0008 nmol/mg/min). The DGK activity present in soluble protein extracts from *dgk-1* null mutants (0.002 ± 0.0014 nmol/mg/min) was not significantly lower than the low DGK activity present in wild-type protein extracts. Thus DGK-1 is likely not the major soluble DGK activity in *C. elegans*, which encodes four other DGKs in its genome (1). In conclusion, DGK-1 is a predominantly soluble enzyme that, like DGKθ (34), likely translocates to membranes to inactivate the second messenger DAG by phosphorylation.

TABLE I
Activity of DGK-1 fusion protein expressed in insect cells

30 h after insect cell infection with a baculovirus encoding MBP or MBP-DGK-1, soluble fractions of cell lysates were assayed for DAG kinase activity with the substrates indicated. Assays were done in triplicate.

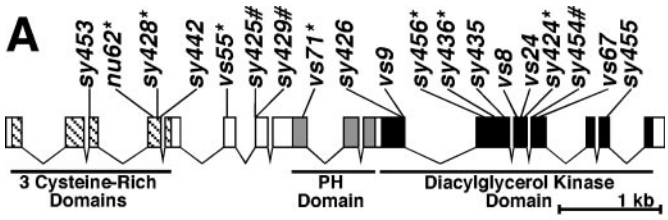
Substrate	Substrate phosphorylation	
	MBP	MBP-DGK-1
	nmol/mg/min	
1,2-Dioleoyl-*sn*-glycerol	0.06 ± 0.003	1.9 ± 0.03
1-Stearoyl-2-arachidonoyl-*sn*-glycerol	0.02 ± 0.002	2.1 ± 0.04
1,3-Dioleoyl-*sn*-glycerol	0.006 ± 0.0004	0.08 ± 0.002
1-Oleoyl-*rac*-glycerol	0.005 ± 0.0006	0.01 ± 0.0003

DGK-1 May Terminate DAG Signaling Independent of Gα_o Signaling—Genetic analysis in *C. elegans* has led to the hypothesis that DGK-1 is activated by the Gα_o G protein GOA-1 (9). We evaluated this hypothesis. We failed to detect translocation of DGK-1 to membranes in response to either expression of activated GOA-1 from a transgene (supplemental Fig. 2*A*, available in the on-line version of this article) or genetic mutations that alter GOA-1 signaling (supplemental Fig. 2*B*). We also failed to pull down DGK-1 from *C. elegans* extracts using purified, affinity-tagged GOA-1 whether the G protein was in its active state or not (supplemental Fig. 1*C*). Thus, we found no evidence that GOA-1 signaling affects DGK-1, suggesting that DGK-1 may act independently of GOA-1 to attenuate neural DAG signaling.

dgk-1 Mutants Define 19 Molecular Lesions That Compromise DGK-1 Function in Vivo—To identify DGK domains and amino acid residues critical for attenuating neural DAG signaling *in vivo*, we analyzed 20 *dgk-1* mutants that were previously isolated based on defects in DGK-1-controlled behaviors (7–9, 28). We sequenced the *dgk-1* coding regions from genomic DNA for each mutant and identified molecular lesions in 19 of the 20 mutants (Fig. 2*A*, and supplemental Table I). These molecular lesions included nine missense mutations, seven stop codon mutations, and three mutations that affect *dgk-1* splice sites (Fig. 2*B*). No mutations were detected in the *dgk-1* coding region of one mutant, *dgk-1*(*sy512*), which probably carries a mutation in the promoter or the regulatory regions of *dgk-1* that were not sequenced. The *nu62* (7) and *sy428* mutations both result in stop codons very early in the DGK-1 open reading frame and therefore likely represent null alleles.

In *C. elegans*, Gα_q signals to generate DAG and thereby promotes egg laying and opposes the paralysis induced by exogenously applied dopamine (8, 35). DGK-1 functions to attenuate DAG signaling, and mutants lacking DGK-1 show enhanced Gα_q signaling, *i.e.* hyperactive egg laying and resistance to dopamine-induced paralysis (7, 8). We measured the impact of molecular lesions on DGK-1 function *in vivo* by quantifying these behaviors in our panel of *dgk-1* mutants (Fig. 3). We first measured defects in egg-laying behavior. Whereas wild-type animals retain fertilized eggs for >2 h and lay 95% of their eggs at late developmental stages (>8 cells), *dgk-1(nu62)* null mutants retain fertilized eggs for a shorter time and lay all of their eggs prematurely (before the 8-cell stage) (28). The percentage of late-stage eggs laid can be used as a measure of the residual DGK-1 function present in mutants. All *dgk-1* mutants tested, except *dgk-1*(*vs55*), were almost completely defective for egg laying and laid <5% of their eggs at late developmental stages (Fig. 3). *dgk-1*(*vs55*) was the weakest *dgk-1* mutant and retained significant DGK-1 function by this measure ($p < 0.0001$).

We also measured the susceptibility of the various mutants to the paralytic effect of dopamine. Whereas wild-type worms were 100% paralyzed when placed on agar medium containing



B

Allele	Change in DGK-1
sy453	Cys 115 to Tyr
*nu62**	Arg 167 to Stop
*sy428**	Arg 180 to Stop
sy442	Cys 184 to Tyr
*vs55**	Gln 246 to Stop
sy425#	Splice acceptor
sy429#	Splice acceptor
*vs71**	Gln 422 to Stop
sy426	Gly 606 to Glu
vs9	Gly 609 to Glu
*sy456**	Trp 646 to Stop
*sy436**	Trp 674 to Stop
sy435	Ala 722 to Val
vs8	Pro 736 to Ser
vs24	Asn 745 to Ile
*sy424**	Trp 767 to Stop
sy454#	Splice acceptor
vs67	Gly 796 to Arg
sy455	Ser 880 to Leu
sy512	No mutations in the coding sequence

FIG. 2. **Molecular lesions that compromise DGK-1 function *in vivo*.** *A*, locations of *dgk-1* mutations that compromise DGK-1 function *in vivo*. Exons of *dgk-1a* are indicated with *boxes*. *Hatches*, *gray boxes*, and *black boxes* represent sequences encoding cysteine-rich domains, the PH domain, and the kinase domain, respectively. Mutations are indicated with their allele names. Stop codons are denoted with an *asterisk* (*), and splicing mutations are indicated with a *pound sign* (#). *B*, the molecular lesions in the DGK-1 protein corresponding to the mutations shown in *panel A*. The amino acid numbers are with respect to DGK-1a.

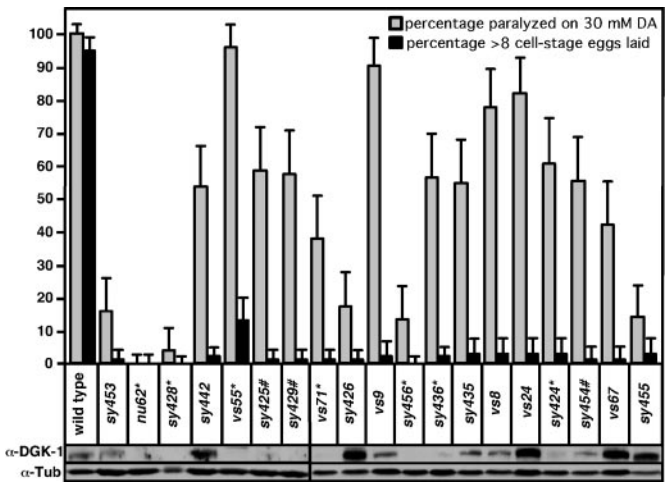


FIG. 3. **Effects of *dgk-1* mutations on *C. elegans* behavior and the DGK-1 protein.** Two behaviors were measured, namely paralysis induced by the neurotransmitter dopamine (*DA*) and the laying of late stage (>8 cell) eggs. For dopamine-induced paralysis $n = 50$, and for the laying of late stage eggs $n = 100$. *Error bars* indicate 95% confidence interval. Western blots detecting full-length DGK-1 and the loading control tubulin (*α-Tub*) in total protein extracts of the wild-type and mutants are also shown. In the egg-laying assay, *vs55* was the only mutant with significantly greater function than the null mutant *nu62* ($p < 0.0001$). In the dopamine response assay, every mutant except *sy428* showed significantly greater function than *nu62* ($p < 0.002$).

30 mM dopamine, *dgk-1*(*nu62*) null mutants were 0% paralyzed (Fig. 3). The remaining mutants showed different susceptibilities to paralysis, ranging from 96% paralyzed in *dgk-1*(*vs55*) animals to 4% paralyzed in *dgk-1*(*sy428*). Thus, dopamine-induced paralysis allows a range of levels of DGK-1 function to be detected in the mutants, possibly because lower levels of DGK-1 function are required for dopamine response than for egg laying. In principle, DGK-1 might function differently in egg laying than in dopamine-induced paralysis so that certain molecular lesions could result in defects in one behavior but not in the other. However, the mutant least defective in dopamine-induced paralysis, *dgk-1*(*vs55*), was also the least defective in egg laying. Furthermore, all missense mutants that were strongly defective in dopamine-induced paralysis (<30% paralyzed, *e.g. dgk-1*(*sy453*) and *dgk-1*(*sy426*)) were also strongly defective in egg laying (<4% of the eggs laid at late developmental stages). Hence, there was no evidence for molecular lesions that differentially affected the two behaviors.

Seven Missense Mutations Identify Conserved Amino Acid Residues Crucial for DGK-1 Function—Missense mutants can be used to identify amino acid residues important for the *in vivo* function of DGK-1. However, defects in some missense mutants may not reflect a role for the affected amino acid residue in enzymatic activity but may be due to misfolding of the mutant protein. Because misfolding can lead to protein degradation, we tested if there was detectable DGK-1 protein in extracts of *dgk-1* mutants (Fig. 3, *bottom*). Whereas all stop codon mutants had little if any detectable full-length DGK-1 protein (Fig. 3, *bottom*), we found that all missense mutants had detectable full-length DGK-1 protein. In Fig. 3, some missense mutant extracts appear to have more DGK-1 than wild-type extracts, but this variation in the Western blot signal was not reproducible (data not shown). Thus, the missense mutations likely do not destabilize the DGK-1 protein, and they potentially define the amino acid residues of DGK-1 required for function of the folded protein.

All nine missense mutations are in the second CRD, the third CRD, or the kinase domain. To examine the conservation of the mutated amino acid residues, we performed a multiple sequence alignment of the affected domains (Fig. 4). Whereas most DGKs have only two CRDs, DGK-1 and its mammalian ortholog DGKθ have three CRDs, among which the second and third are most similar to the CRDs of other DGKs (21) and are therefore shown in our alignment.

Two missense mutations affected the CRDs. In *dgk-1*(*sy453*) and *dgk-1*(*sy442*), the mutant DGK-1 protein made has conserved Cys residues replaced by Tyr residues in the second and third CRDs, respectively (Fig. 4*A*). These mutants have no detectable function in the egg-laying assay but do show reduced albeit significant function in the dopamine response assay (Fig. 3). Thus, the second and third CRDs are important for the physiological functions of DGK-1.

Seven missense mutations affected the kinase domain. We identified two mutations, *sy426* and *vs9*, in the conserved Gly-Glu-Gly-Xaa-Xaa-Gly ATP binding motif of the DGK kinase domain that change the second and third conserved Gly of this motif to Glu, respectively (1) (Fig. 4*B*). In other DGKs, changes in the amino acid residue corresponding to that changed by *sy426* (Fig. 4) abolished *in vitro* kinase activity (1, 36). We found three additional mutations (*sy435*, *vs8*, and *vs24*) in the kinase domain that change amino acid residues that are absolutely conserved among all DGKs (Fig. 4*B*). Finally, we found two mutations, *vs67* and *sy455*, in the kinase domain that alter amino acid residues that were not conserved among all DGKs and may represent residues that are important for the function of only a subset of known DGKs. The mutation *sy455* alters a Ser residue that might be phosphorylated because it is located in a possible protein kinase C phosphorylation site (37), and the human DGK-1 ortholog DGKθ can be phosphorylated *in vitro* by protein kinase C (1).

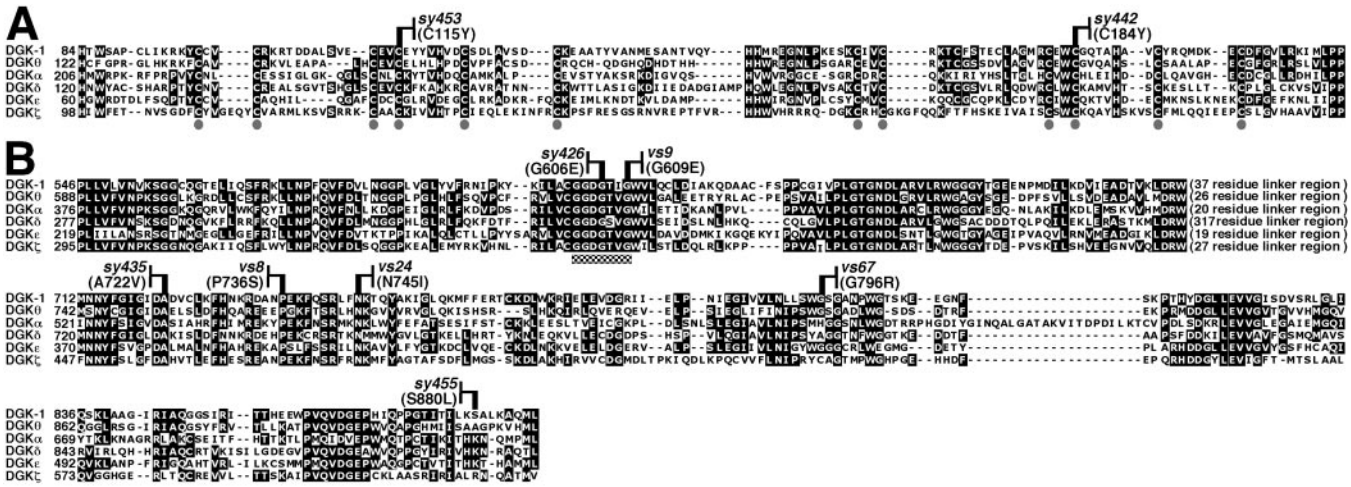


FIG. 4. **DGK-1 missense mutations and alignment with human DGKs.** *A*, multiple sequence alignment of the second and third DGK-1 CRDs, with CRDs from one member of each human DGK subfamily (1). *Gray circles* identify the six conserved cysteines in each CRD (21). *B*, alignment of kinase domains. *Checkered box* denotes the ATP binding motif. A variable length non-conserved linker region is indicated. Allele names and molecular lesions of *dgk-1* missense mutants are indicated above the alignments. Amino acids identical in three or more sequences are in *black boxes*. Seven of the nine DGK-1 missense mutations are in residues conserved in all human DGKs.

All of the kinase domain missense mutations severely reduced DGK-1 function in the egg-laying assay and also reduced DGK-1 function to varying extents in the dopamine response assay, indicating that the kinase domain is important for the physiological functions of DGK-1. We note that none of these missense mutations produced defects as severe as those in the *dgk-1* null mutants *nu62* and *sy428*. In addition, three stop codon mutations in the kinase domain (*sy456*, *sy436*, and *sy424*) also produced defects less severe than those in the null mutants (Fig. 3). The residual function present in all kinase domain mutants was only evident in the dopamine response assay, which is apparently more sensitive than the egg-laying assay. These results suggest that the kinase domain of DGK-1 may not be absolutely required for all of its physiological functions.

Two Stop Codon Mutants Predicted to Lack a Kinase Domain Retain Significant Function in Vivo—Two early stop codon mutants, *vs55* and *vs71*, are predicted to produce truncated DGK-1 containing the CRDs but lacking the kinase domain. They retain significantly more function than the ATP-binding site mutant *sy426* (Fig. 3). Indeed, *vs55* mutants have greater function in the dopamine response assay than any kinase domain mutant and are actually indistinguishable from the wild-type in this assay ($p > 0.999$). These results raise the following question. How can a mutation that completely eliminates the kinase domain perturb function less than any mutation within the kinase domain? One possibility is that *vs55* and *vs71* actually do not completely eliminate the kinase domain. This could occur if there are alternative splice forms of *dgk-1* that skip the exons containing the *vs55* and *vs71* stop codons to produce novel DGK-1 isoforms that include the kinase domain

New dgk-1 Splice Forms May Explain Residual Function in dgk-1(vs55) and dgk-1(vs71) Stop Codon Mutants—To detect the proposed novel splice forms, we used RT-PCR to amplify cDNAs from the region surrounding the two mutations. We found four different splice forms named *dgk-1a*, *dgk-1b*, *dgk-1c*, and *dgk-1d* (Fig. 5*A*). *dgk-1a* and *dgk-1b* have been reported earlier (7) and differ in using alternative splice donors (*i* and *ii*) from exon 5. *dgk-1c* and *dgk-1d* are novel splice forms identified in this study that use splice donors *i* or *ii* from exon 5, include exon 6, and then skip exons 7 and 8. Because *vs71* lies in exon 8, these two novel splice forms are undisrupted in the *dgk-1*(*vs71*) mutant. The DGK-1c and DGK-1d isoforms do contain the kinase domain and thus may account for the significant DGK-1 function in the *dgk-1*(*vs71*) mutant (Fig. 3).

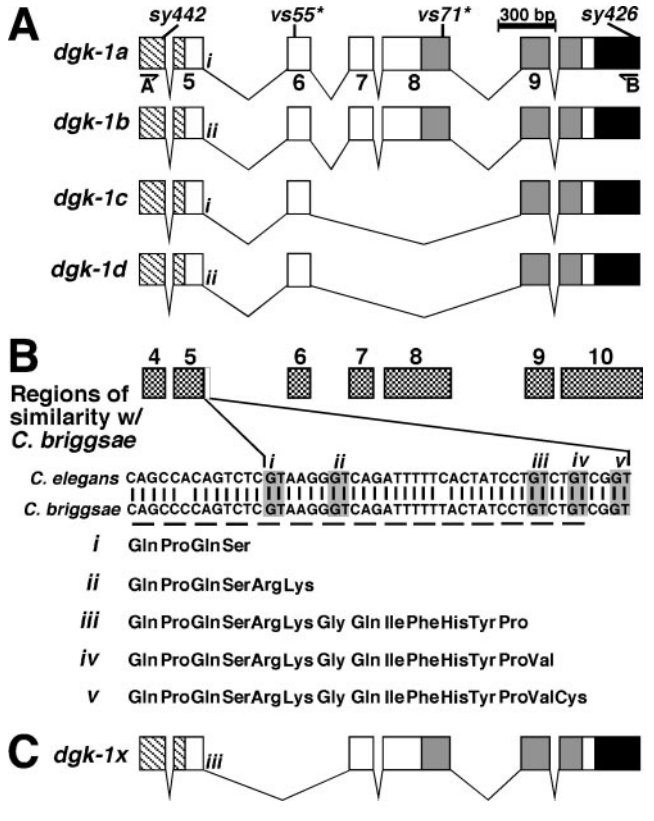


FIG. 5. **Detection of alternative splice forms of *dgk-1*.** *A*, schematics showing splice forms of *dgk-1* identified experimentally. Exons 4 through 10 of *dgk-1a* are schematized, with conserved domain coding sequences *shaded* and the mutations indicated as in Fig. 2*A*. Positions of the outer primers used for RT-PCR are indicated (*A* and *B underneath harpoons*). Different splice donor sites after exon 5 are indicated with either *i* or *ii*. *B*, comparison of *C. elegans* and *C. briggsae* sequences to detect possible novel exons of *dgk-1*. The regions of sequence similarity between *C. elegans* and *C. briggsae* are schematized by *boxes* that are aligned with the exon schematics in *panel A*. *Checkered boxes* correspond to the exons of *dgk-1a* as *numbered*. A region of similarity beyond exon 5 of *dgk-1a* is indicated with a *white box* and an expanded view showing the *C. elegans* and *C. briggsae* sequences. Conserved GT dinucleotides of potential splice donors are shown in *gray boxes* and are numbered *i–v*. The amino acids encoded by the potential exon 5 extensions are indicated below the DNA sequence. *C*, schematic showing a potential *dgk-1* splice form that skips exon 6 (which contains *vs55)* and would make a DGK-1 isoform with a kinase domain.

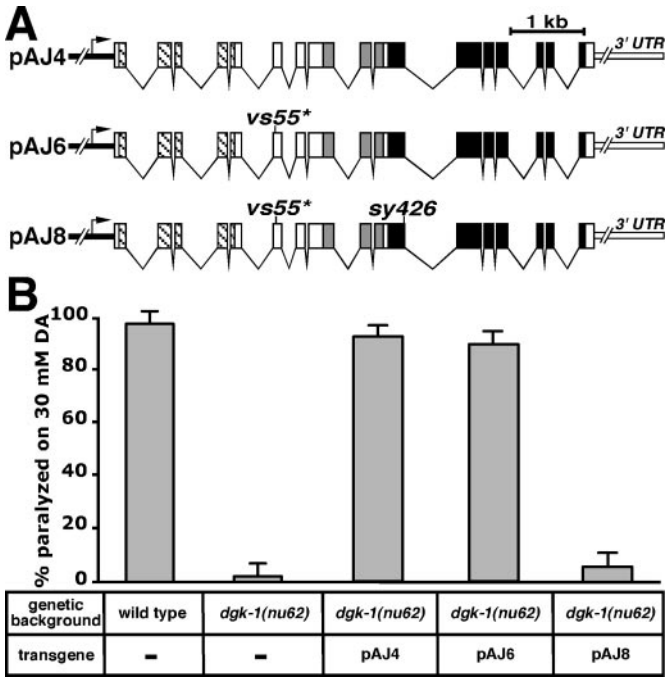


FIG. 6. **Use of transgenes to test if a kinase domain is required for *dgk-1*(*vs55*) function.** *A*, schematics showing the transgenes used. *Thick black line* indicates the *dgk-1* promoter region (~4.1 kb). *Arrowhead* represents the start and direction of transcription. The *dgk-1* gene, the sequences encoding conserved domains of DGK-1, and the *vs55* and *sy426* mutations are schematized as in Fig. 2*A*. *Thick white line* indicates the 3′-untranslated region (*UTR)* of *dgk-1*. *B*, effect of transgene expression on susceptibility to paralysis by dopamine (*DA*). The genetic backgrounds and transgenes used are indicated. *Error bars* indicate 95% confidence interval.

We did not detect any DGK-1 isoforms that could similarly account for the significant function in *dgk-1(vs55)* (Fig. 3 and data not shown). To determine whether such isoforms might exist, we compared the genomic sequence of *C. elegans dgk-1* with that of the related species, *C. briggsae* (38), to identify possible alternative splice forms (Fig. 5*B*). Coding sequences, and not introns, are highly conserved between these two species. Outside of the known *dgk-1* coding sequences, we found only a single region of similarity >7 bp. This 30-bp region extended 3′ of previously known splice donors from exon 5 (Fig. 5*B*) and can extend the coding potential of exon 5 through the use of additional splice donors. A similar analysis was used to successfully identify an analogous exon extension allowing the subsequent detection of a novel splice form of the programmed cell death gene *ced-4* (39). Three conserved splice donor sites within the potential extension of *dgk-1* exon 5 (Fig. 5*B*, *iii–v*) could allow splicing to downstream exons, skipping the *vs55* mutation to make functional DGK-1 proteins with kinase domains. For example, use of splice donor *iii* would require skipping exon 6 (which contains *vs55*) to maintain the *dgk-1* reading frame. By splicing instead to exon 7, a protein with the kinase domain could be made (Fig. 5*C*).

It is possible that such a splice form is made at low levels and, hence, eluded detection. Alternatively, the *dgk-1*(*vs55*) mutant may produce a highly functional truncated protein lacking the kinase domain and containing only the three CRDs. To distinguish between these two possibilities, we evaluated the requirement for the kinase domain in the *dgk-1(vs55*) mutant (Fig. 6). We constructed a transgene, pAJ4, containing the wild-type *dgk-1* gene and its regulatory regions (Fig. 6*A*). This transgene was able to restore DGK-1 function in the *dgk-1* null mutant *dgk-1(nu62)* to wild-type levels (Fig. 6*B*). An analogous transgene, pAJ6, with the *vs55* stop codon mutation (Fig. 6*A*) was also able to restore significant DGK-1 function to the *dgk-1* null mutant (Fig. 6*B*). This was as expected, because the *dgk-1*(*vs55*) mutant animals showed significant DGK-1 function (Fig. 3). We reasoned that if pAJ6 confers function by producing DGK-1 isoforms containing the kinase domain but lacking the *vs55* stop codon because of alternative splicing, we should be able to inactivate the kinase domain of these isoforms by adding the ATP-binding site mutation *sy426* to pAJ6. Alternatively, if pAJ6 confers function by producing a truncated DGK-1 protein lacking the kinase domain, then adding the *sy426* mutation to pAJ6 should have no effect. We constructed the transgene pAJ8, containing both the *vs55* and *sy426* mutations (Fig. 6*A*). pAJ8 had no detectable function (Fig. 6*B*). Thus the *dgk-1*(*vs55*) mutant likely makes a functional DGK-1 isoform with a kinase domain due to alternative splicing.

DISCUSSION

In Vivo Structure/Function Analysis—The physiological functions of human DGKs are largely unknown. The readout of function that has been used in earlier structure/function studies of DGKs has involved transgenically expressing altered enzymes in cultured cells and measuring localization and/or *in vitro* kinase activity (17–20). In contrast, we carried out a structure/function study of the *C. elegans* DGK enzyme, DGK-1, by measuring defects in its physiological functions using animals in which the endogenous enzyme was altered by mutations.

In a structure/function study of human DGKθ (ortholog of DGK-1), virtually all alterations of the enzyme led to complete abolishment of *in vitro* activity (20). It is possible that the various altered DGKθ enzymes tested had differing levels of activity, all of which were below the detection limit of the *in vitro* assay used. We measured the behavioral defects in *dgk-1* mutants using two DGK-1-controlled behaviors, egg laying and dopamine-induced paralysis. Most of the mutants were indistinguishable by the egg-laying assay, as they showed no DGK-1 function by this measure. However, by measuring susceptibility to the paralytic effects of dopamine, we were able to finely discriminate between the defects due to the various molecular lesions of the DGK-1 protein, possibly because this assay, unlike the egg-laying assay, allowed detection of even very low levels of DGK-1 function.

Conserved Domains and Amino Acid Residues Critical for the in Vivo Function of DGK-1 and Possibly All DGKs—The CRDs of DGKs are characterized by a series of six conserved cysteine residues (Fig. 4*A*, *gray circles*) (21). *sy453* and *sy442* change the fourth conserved Cys in the second and the third CRDs of DGK-1, respectively, indicating that these CRDs are important for DGK-1 function *in vivo*. Because we did not find any missense mutations in the first CRD of DGK-1 within our panel of mutants that had been selected for defects in DGK-1 controlled behaviors, it is possible that this domain is not essential for the behaviors measured. Consistent with this suggestion, in a structure/function study of DGKθ the only altered enzyme that retained significant *in vitro* activity was an N-terminal truncation that removed the first CRD (20).

The kinase domain of DGKs is distantly related to that of phosphofructokinases (26). Based on the structure of bacterial phosphofructokinase (40) and on sequence alignments, it has been suggested that DGK kinase domains consist of two conserved subdomains, a DGKc (catalytic) subdomain that binds ATP and a DGKa (accessory) subdomain that binds DAG (19).

The DGKc subdomain contains a conserved Gly-Glu-Gly-Xaa-Xaa-Gly ATP binding motif. Mutation of the second Gly in this motif has been shown to result in defective DGKs that cannot phosphorylate DAG *in vitro* (1, 36). Consistent with this finding, we found that a mutation that alters the corresponding

residue of DGK-1 caused severe defects in both behaviors assayed (*sy426* in Figs. 3 and 4). We note that the *sy426* mutant retained a low but measurable amount of function in the dopamine response assay, suggesting that kinase activity may not be absolutely required for all of the DGK-1 function *in vivo*. Mutating the third Gly residue in the ATP binding motif has a less dramatic effect on DGK-1 function *in vivo* (*vs9* in Figs. 3 and 4). This may be explained by the fact that the third Gly is not conserved in phosphofructokinases (19) and, thus, may not be essential for binding ATP.

Within a 34-amino acid stretch of the DGKa subdomain of DGK-1 we found three mutations that alter residues conserved among all DGKs (*sy435*, *vs8*, and *vs24* in Figs. 3 and 4). Thus, these mutations identify amino acid residues in a novel motif that may be required for DAG binding by all DGKs.

Physiological Role of DGK Isoforms Generated through Alternative Splicing—We found two early stop codon mutants (*vs55* and *vs71*) that were expected to produce a DGK-1 protein truncated before the kinase domain retained greater function than the ATP-binding site mutant *sy426* (Fig. 3). The genes encoding six human DGK isozymes (DGKβ, γ, δ, ι, η, and ζ) are alternatively spliced to generate novel isoforms (10–15), and we hypothesized that the significant function retained in *vs55* and *vs71* was possibly due to unidentified *dgk-1* splice forms that skip these stop codon mutations through alternative splicing. We identified two such splice forms that skip the exon containing the *vs71* mutation to make two novel DGK-1 isoforms lacking part of the PH domain but containing the kinase domain. These DGK-1 isoforms likely account for the function seen in the *dgk-1*(*vs71*) mutant. Because *dgk-1*(*vs71*) had no detectable function in the egg-laying assay and only affects DGK-1 isoforms containing an intact PH domain, these isoforms must play the major role in regulating egg laying. In contrast, the significant function of *dgk-1*(*vs71*) in the dopamine response assay suggests that the DGK-1 isoforms lacking an intact PH domain play a significant role in dopamine response, because these isoforms are unaffected by *vs71*.

The *vs55* stop codon mutant was indistinguishable from the wild-type in the dopamine response assay and also retained significant function in the egg-laying assay. Using transgenes containing both the *vs55* stop codon mutation and an inactivating mutation in the downstream kinase domain, we showed that the exon containing the *vs55* mutation is likely skipped to generate functional DGK-1 isoform(s) containing a kinase domain. We were unable to detect such splice form(s) by RT-PCR, suggesting these forms are of low abundance. It is possible that these splice form(s) are expressed only in a small subset of cells in the animal, such as the motor neurons that respond to dopamine (8). Thus, our genetic approach has allowed us to deduce the presence of physiologically important DGK-1 isoform(s) whose very low abundance might have otherwise prevented their discovery by molecular approaches.

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SUPPLEMENTAL MATERIAL

SUPPLEMENTAL FIGURE LEGENDS

SUPPLEMENTAL FIG. 1. **Examination of aggregate formation by recombinant DGK-1**. MBP::DGK-1 (~ 160 kDa) was purified to a similar extent as His::DGK-1 (Fig 1*A*) using amylose-affinity chromatography. This was centrifuged at 100,000xg for 30 min and the supernatant was immediately run on a gel filtration column. The resulting chromatogram indicating protein concentration (as measured by A_{280}) along with DGK activity present in fractions 9 through 29 is shown. The fractionation of molecular weight standards run on the same column is indicated above the graph as reference. A large peak of activity in fraction 23 likely corresponds to the monomeric DGK-1 fusion protein. We estimate the specific activity in this fraction to be 7,500 nmol/mg/min. A second smaller activity peak (fraction 18) likely corresponds to dimeric DGK-1 fusion protein. The majority of the protein fractionated as much larger species that showed little activity, presumably representing inactive aggregates.

SUPPLEMENTAL FIG. 2. **Biochemical evaluation of possible interaction between G$_o$ (GOA-1) and DGK-1.** *A*, Effect of overexpression of constitutively active GOA-1(Q205L) on the fractionation of DGK-1. Anti-DGK-1, anti-GOA-1, and anti-tubulin Western blots are indicated by □-DGK-1, □-GOA-1, and □-Tub, respectively. Supernatant (S) and pellet (P) fractions of a 100,000xg 45 min centrifugation are indicated. *hs* denotes extracts of worms that express GOA-1(Q205L) under the control of a heat shock promoter. *wt* denotes control extracts of wild-type worms that were also heat shocked. Anti-tubulin Western blot is a loading control. DGK-1 remained predominantly soluble after expression of activated GOA-1. *B*, Effects of GOA-1 signaling mutations on the fractionation of DGK-1. Anti-DGK-1 and anti-tubulin Western blots

of supernatant (S) and pellet (P) fractions are shown. *wt*, extracts of control wild-type worms; *goa-1*, extracts of *goa-1* null mutant worms; *egl-10*, extracts of null mutants of *egl-10*, the negative regulator of GOA-1 (1). DGK-1 remained predominantly soluble in the mutant extracts. *C*, Affinity pull-down of DGK-1 from worm extracts using recombinant GST::GOA-1 fusion protein in different G protein activation states. *Top panel*, Anti-DGK-1 Western blot. *Bottom panel*, Ponceau S stain showing GST::GOA-1 that was pulled down. The GOA-1 fusion protein was used in either its inactive state (GDP-bound, GDP) or its active state (AlF_4^-·GDP- or GTPγS-bound, AlF_4^- or GTPγS). DGK-1 was not pulled down under any condition tested.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

GST::GOA-1 pull downs - pGEX-2T (Amersham Biosciences) was used to express Glutathione-S-Transferase (GST). The GOA-1 cDNA was cloned into pGEX-2T to express recombinant affinity-tagged GST::GOA-1 in *E. coli*. Cells were lysed in lysis buffer (50 mM HEPES pH 7.4, 1 mM EDTA, 150 mM NaCl, 10% glycerol, 1 μM DTT, 1 μM PMSF and 1 μg/ml leupeptin, and 1 μg/ml pepstatin) and purified using Glutathione Sepharose 4B (Amersham Biosciences) affinity chromatography followed by gel filtration. GST::GOA-1 and GST were used to attempt to pull down DGK-1 from *C. elegans* extracts. 120 nmoles of GST::GOA-1 or GST were pre-bound to Glutathione Sepharose 4B and pre-incubated for 2 hr at room temperature (RT) in lysis buffer containing 20 mM $MgCl_2$ and one of the following: GDP, 5 μM GDP; GTPγS, 5 μM GTPγS; or AlF_4^-, 5 μM GDP, 10 μM $AlCl_3$, and 5 mM NaF. Then 167 μl of ~1.5 mg/ml soluble protein extracts in the corresponding buffer was added to the pre-incubated GST::GOA-1 bound Glutathione Sepharose 4B and was incubated for 1 hr at RT. The Glutathione Sepharose 4B resins were then washed 3 times under the same conditions as above, centrifuged, separated by SDS-PAGE, and transferred to membranes. The blots were probed with anti-DGK-1 to detect DGK-1 and stained with Ponceau S to visualize GST::GOA-1.

Fractionation of mutant and transgenic C. elegans *extracts - egl-10(md176)* and *goa-1(sa734)* are null mutants that fail to produce EGL-10 and GOA-1 proteins, respectively (1, 2). For Supplemental Fig. 1*A* wild-type worms and worms expressing constitutively active GOA-1(Q205L) from a heat shock promoter (3) were heat shocked for 30 min at 33°C and then grown for an additional 2.5 hr at 20°C before harvesting. The GOA-1(Q205L) worms exhibited lethargy and bloating with eggs after heat shock (data not shown), demonstrating the effects of activated

GOA-1 (4). Rabbit anti-GOA-1 (5) was used to detect GOA-1. The mobility on SDS-PAGE of the proteins studied were as follows: GOA-1, 40 kDa; and GST::GOA-1, 66 kDa.

Construction of plasmids, recombinant protein purification, and gel filtration analysis- The cDNA of the MalE maltose binding protein alone or the *dgk-1a* cDNA from pKP137 (7) as well as the cDNA of MalE, and were subcloned into pFastBacHTb (GibcoBRL) to generate a baculovirus encoding Maltose Binding Protein (MBP) or MBP-tagged DGK-1 (MBP::DGK-1) respectively. MBP::DGK-1 was purified from baculovirus-infected High Five (Invitrogen) insect cell soluble extracts (100,000xg 45 min supernatant) using amylose-affinity chromatography (New England Biolabs). Monomeric MBP::DGK-1 was separated form higher order complexes using a Superdex 200 HR 10/30 (Amersham Pharmacia Biotech) column. Large aggregates of the recombinant protein were removed by centrifugation at 100,000xg for 30 min prior to loading the column. 300 □l fractions were collected and DGK-1 activity assays were performed on 150 □l of each fraction.

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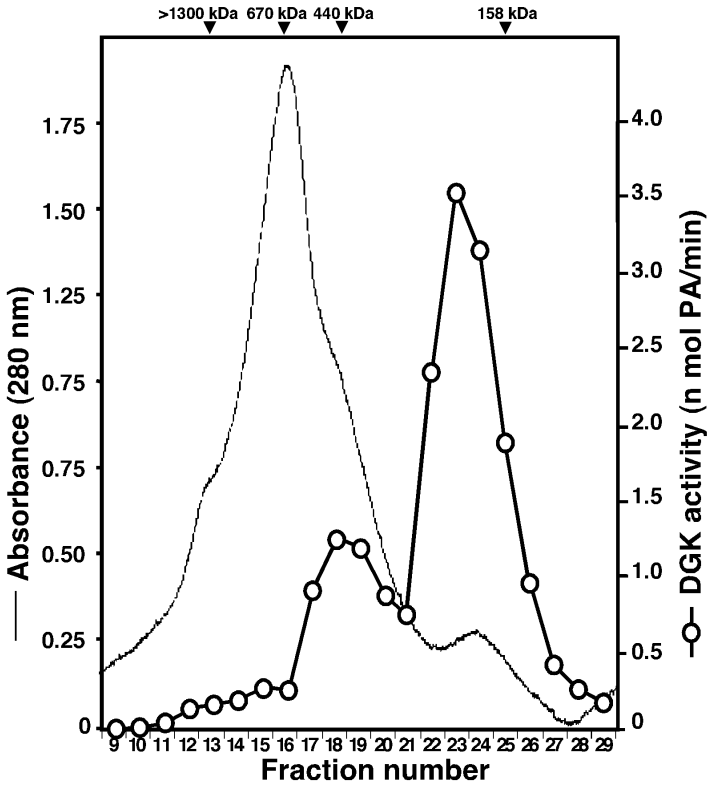
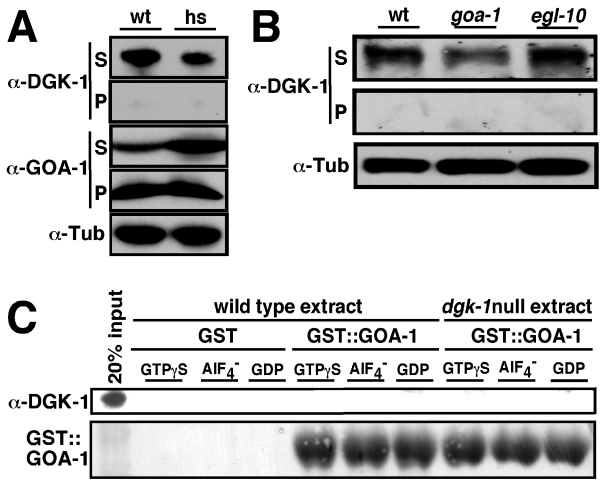


Supplemental Figure 1



Supplemental Figure 2

Supplemental Table 1. Change in the DNA sequence of the different *dgk-1* mutant alleles

Allele	wild type sequence[1]	mutant sequence[1]
sy453	tttcagtttGcgagtacta	tttcagtttAcgagtacta
*nu62**	attgtttgcCgaaaaacgt	attgtttgcTgaaaaacgt
*sy428**	gccggaatgCgatgtgaat	gccggaatgTgatgtgaat
sy442	gtgaatggtGtggtcagac	gtgaatggtAtggtcagac
*vs55**	tcaaaaattCaagcggatg	tcaaaaattTaagcggatg
sy425#	ccatttctaGatttcgcgc	ccatttctaAatttcgcgc
sy429#	ccatttctaGatttcgcgc	ccatttctaAatttcgcgc
*vs71**	tgcaatccaCaagagaatg	tgcaatccaTaagagaatg
sy426	gtggagatgGaacaattgg	gtggagatgAaacaattgg
vs9	gaacaattgGatgggtact	gaacaattgAatgggtact
*sy456**	tctccggtgGggtggtggt	tctccggtgAggtggtggt
*sy436**	tagacagatGggctgtagt	tagacagatAggctgtagt
sy435	gaattgatgCagatgtttg	gaattgatgTagatgtttg
vs8	gacgcgaatCcggaaaaat	gacgcgaatTcggaaaaat
vs24[2]	aAcaaaacacaatatgcAa	aTcaaaacacaatatgcGa
*sy424**	aagatttatGgaaacgtat	aagatttatAgaaacgtat
sy454#[3]	tataaAactttcaGgttga	tataaCactttcGaAgttga
vs67	ctcagttggGgaagtggtg	ctcagttggAgaagtggtg
sy455	tattgaaatCagctttgaa	tattgaaatTagctttgaa
sy512	No mutations in the coding sequence	

[1] Changed bases are indicated in upper case

[2] *vs24* has two changes

[3] *sy454#* has two changes and a one base pair insertion