Protein kinase D (PKD) isoforms are protein kinase C (PKC) effectors in diacylglycerol (DAG)-regulated signaling pathways. Key physiological processes are placed under DAG control by the distinctive substrate specificity and intracellular distribution of PKDs. Comprehension of the roles of PKDs in homeostasis and signal transduction requires further knowledge of regulatory interplay among PKD and PKC isoforms, analysis of PKC-independent PKD activation, and characterization of functions controlled by PKDs.

Cellular, molecular, and physiological complications of type II diabetes (6–11). Upon PLC activation, diacylglycerol (DAG) binding modules (C1 domains) ligate membrane-intercalated DAG. Consequently, PKCs translocate from cytoplasm to a membrane surface enriched in phosphatidylserine (PS), a PKC activator. Association of PKCs with DAG and PS elicits expulsion of the pseudosubstrate domain (12) from the catalytic cleft and enables expression of intrinsic kinase activity (3, 13, 14). Ligand binding with >100 types of cell surface receptors triggers production of DAG in numerous mammalian cells. Knowledge of structures, activation mechanisms, and intracellular trafficking of PKCs is extensive. In contrast, information about in situ regulation, downstream effectors, interacting modulatory proteins, and the precise physiological roles of individual PKC isoforms is more limited.

The discovery of protein kinase D (PKD) isoforms (PKD, PKD2, and PKD3) revealed a new domain in DAG-mediated signal transduction (15–18). PKDs are composed of ~900 amino acids and contain tandem C1 domains and a Ser/Thr protein kinase segment, which are homologous with domains in classical PKDs. DKF-1 and PKDs have similar substrate specificities. Phorbol 12-myristate 13-acetate (PMA) switches on DKF-1 catalytic activity in situ by promoting phosphorylation of a single amino acid Thr588 in the activation loop. DKF-1 phosphorylation and activation are unaffected when PKC activity is eliminated by inhibitors. Both phosphorylation and kinase activity of DKF-1 are extinguished by substituting Ala for Thr588 or Gln for Lys455 (“kinase dead”) or incubating with protein phosphatase 2C. Thus, DKF-1 is a PKA-activated, PKC-independent D kinase. In vivo, dkf-1 gene promoter activity is evident in neurons. Both dkf-1 gene disruption (null phenotype) and RNA interference-mediated depletion of DKF-1 protein cause lower body paralysis. Targeted DKF-1 expression corrected this locomotory defect in dkf-1 null animals. Supraphysiological expression of DKF-1 limited C. elegans growth to ~60% of normal length.

A group of eight protein kinase C (PKC) isoforms mediates actions of hormones and growth factors (GFs) that stimulate phospholipases (PLCs) β, γ, and ε (1–5). PKCs phosphorylate specific Ser/Thr hydroxyl groups in substrate/effectors proteins, thereby modulating ion transport, secretion, cell growth, differentiation, gene transcription, and other physiological processes. Aberrant activation of PKCs is linked to tumor promotion, myocardial hypertrophy and infarction, and pathophysiological complications of type II diabetes (6–11). Upon PLC activation, diacylglycerol (DAG) binding modules (C1 domains) ligate membrane-intercalated DAG. Consequently, PKCs translocate from cytoplasm to a membrane surface enriched in phosphatidylserine (PS), a PKC activator. Association of PKCs with DAG and PS elicits expulsion of the pseudosubstrate domain (12) from the catalytic cleft and enables expression of intrinsic kinase activity (3, 13, 14). Ligand binding with >100 types of cell surface receptors triggers production of DAG in numerous mammalian cells. Knowledge of structures, activation mechanisms, and intracellular trafficking of PKCs is extensive. In contrast, information about in situ regulation, downstream effectors, interacting modulatory proteins, and the precise physiological roles of individual PKC isoforms is more limited.

The discovery of protein kinase D (PKD) isoforms (PKD, PKD2, and PKD3) revealed a new domain in DAG-mediated signal transduction (15–18). PKDs are composed of ~900 amino acids and contain tandem C1 domains and a Ser/Thr protein kinase module near their N- and C termini, respectively. PMA or DAG promotes redistribution of PKDs from cytoplasm to membranes and concomitant activation of PKD catalytic activity (19–21). PKDs are distinguished from PKCs by (a) the presence of a pseudostein homology (PH) domain, (b) the absence of a pseudosubstrate site, and (c) divergent substrate specificity (18–23). Application of PKC-selective inhibitors and expression of constitutively active PKCs in transfected cells has disclosed that PKCs govern PKD activation (24–26). Thus, PKDs are candidate physiological effectors for DAG-stimulated PKCs.

In mammals, PKD activation is associated with cell replication, tumorigenesis, Golgi vesicle fission and trafficking, adhesion, responses to stress, NF-κB activation, modulation of JNK activity, and other processes (5, 19–21). Current concepts of regulation and physiological consequences of PKD activation are based predominantly on expression of wild type (WT) and mutant PKDs expressed in transiently transfected cells. Important insights into the properties of PKDs were acquired via transient transfection analysis, but inherent limitations of the methodology merit consideration. Typically, supraphysiological levels of WT/mutant PKDs accumulate in transfected cells. Thus, the potential for (a) mislocalization of activated or inhibitory (“dominant negative,” “kinase dead”) PKDs, (b) promiscuous phosphorylation of minor or nonrelevant substrates, and/or (c) association of PKD with atypical binding partners is increased. High level PKD expression may ablate counter-regulatory processes such as effector dephosphorylation or hinder operation of negative feedback loops. If these factors apply, data interpretation could be compromised and predictions of PKD physiological roles might be qualitatively or quantitatively imprecise.

Individual domains may specify subcellular location, level of catalytic activity, nuclear entry and exit, membrane association, or other attributes of PKDs (27–33). However, reports of complex or contradic-
PMA, Translocation, and Phosphorylation Regulate DKF-1

Isozyme structure/function relationships have also confounded straightforward assignment of particular roles to specific domains in several instances. For example, both PKD that is diphosphorylated at Ser residues in the activation loop and PKD lacking phospho-Ser in the same loop are proposed as candidate mediators of NFkB activation (25, 34–36). Thus, a lack of information derived from (a) direct assessment or depletion of endogenous PKDs or (b) expression of near physiological levels of WT and mutant D kinases precludes definitive answers to the question of whether observed DAG/PKC/PKD signaling pathways are major or auxiliary (cell-specific or ubiquitous, etc.) contributors to overall cell/organism physiology. Full comprehension of effects of PKDs in homeostasis and hormone/GF actions will require more advanced knowledge of the complex regulatory interplay among PKD and PKC isoforms, rigorous analysis of modes of PKC-independent PKD regulation/activation, and discovery of critical physiological changes in vivo that are specifically linked to alterations in PKD concentration and/or activity. At present, many central questions about PKDs remain unresolved including the following. 1) Are all PKD isoforms PKC effectors? 2) Can PKDs independently receive, amplify, and disseminate signals carried by DAG? 3) Are individual PKDs indispensable for specific physiological processes in intact animals? If so, which functions? 4) Will targeted overexpression reveal novel physiological roles for PKDs?

Answers to the posed questions may be acquired by coupling investigations on mammalian systems with complementary studies on PKDs in a model organism. Caenorhabditis elegans is an attractive choice because its cell biology, development, and physiology are characterized in exceptional detail (37–43). Moreover, C. elegans employs signaling molecules, mechanisms, and pathways that are conserved among eukaryotes (44–49). Techniques for gene disruption, RNA interference (RNAi), and targeted mRNA/protein expression in specific cells are optimized for in vivo analysis (50–54). Typically, information and insights derived from experimentation on C. elegans signal transduction networks complement and synergize with data and concepts obtained from investigations on parallel signaling pathways in mammals (39, 41, 44–49). We now report the discovery, detailed characterization, and consequences of in vivo depletion of a novel D kinase family isoform (DKF-1). DKF-1 is a new prototype within the PKD family. In an accompanying article (89), we report on a series of unique regulatory properties in the C1a, C1b, PH, and kinase domains of DKF-1.

EXPERIMENTAL PROCEDURES

Isolation of cDNAs Encoding DKF-1—A BLAST search of the GenBank™ identified C. elegans expressed sequence tags (ESTs) that are homologous with cDNAs encoding human PKDs. Alignments of ESTs and cDNAs enabled design of a cDNA probe optimized for library screening. A 45-mer oligonucleotide (5'-CTGGATATGTGGTCTGT-TGGTGTCTATT-TATGTCAGTTATCA-3') was synthesized, end-labeled with [32P]P, and used to screen a C. elegans cDNA library in Agt10 bacteriophage as described by Hu and Rubin (55). Positive cDNA inserts were excised from recombinant phages and cloned in the plasmid pGEM7Z (+) (Promega). One partial cDNA insert was repeatedly retrieved. Consequently, this (1.6 kbp) cDNA was excised by digestion with EagI and EcoRV, purified, and radiolabeled by random priming. A C. elegans cDNA library in bacteriophage AZAPII (Stratagene) was hybridized with the [32P]-labeled cDNA fragment. pBluescript SK phagemids that contained hybridizing cDNA inserts were excised from phage in Escherichia coli (in situ), amplified, and purified. Cloned cDNAs (2.3 kbp), which contain the complete coding sequence and 3'-untranslated region of DKF-1 mRNA, were isolated.

DNA Sequencing and Analysis—Genomic and PCR-amplified DNA and DKF-1 cDNAs were sequenced as described previously (56). Sequence comparisons and data base searches were performed with programs provided by NCBI servers at National Institutes of Health, Bethesda, MD. Protein domains were identified and characterized by using the SMART Web site (European Molecular Biology Laboratories, Heidelberg, Germany) (57). ClustalW programs were accessed at European Bioinformatics Institute, Hinxton, UK.

Purification of DKF-1 Fusion Protein Expressed in Escherichia coli—A DKF-1 cDNA fragment encoding amino acids 2–154 (Fig. 1A) was synthesized via PCR as reported previously (58, 59) using the high fidelity DNA polymerase PfuTurbo (Stratagene). Unique EcoRI (5') and Xhol (3') restriction enzyme sites were incorporated into the PCR primers. Product DNA was digested with EcoRI and Xhol and cloned into the bacterial expression plasmid pGEX-3X (GE Healthcare). Vector DNA encoding Schistosoma japonicum glutathione S-transferase (GST) precedes the cDNA insert. Fusion gene transcription/translation is controlled by an iso- propyl-1-thio-β-D-galactopyranoside (IPTG)-inducible tac promoter. Subsequent steps in fusion protein production are described in detail by Land et al. (60). Approximately 1.5 mg of highly purified DKF-1 fusion protein was isolated from a 1-liter culture of E. coli.

Production of Antiserum Directed against DKF-1—DKF-1 fusion protein was injected into rabbits (an 0.4-mg initial injection; 0.2 mg for each of four booster injections) at Covance Laboratories (Vienna, VA) at 3-week intervals. Antiserum was collected at 3-week intervals.

Affinity Purification of Anti-DKF-Immunoglobulins (IgGs)—Three milligrams of GST-DKF-1 (2–154) was coupled to 2 ml of Sepharose 4B (GE Healthcare) as described previously (61). Anti-DKF-1 IgGs were then purified from 2 ml of immune serum by affinity chromatography as published previously (60, 62). Purified IgGs were dialyzed against phosphate-buffered saline containing 50% glycerol and stored at −20 °C.

Growth and Synchronization of C. elegans—Bristol N2 WT C. elegans was grown, synchronized, harvested, and pulverized into powder in a mortar cooled with liquid N2. L1 larvae were harvested 6 h after hatching, L2 larvae at 20 h, L3 larvae at 29 h, L4 larvae at 40 h, and young adult C. elegans at 52 h; egg-laying adult animals were collected at 78 h. A complete description of the procedures is given in Hu and Rubin (55).

Preparation of Particulate and Cytosolic Fractions from C. elegans—Frozen, powdered nematodes were suspended in buffer and disrupted as reported previously (60). Homogenates were centrifuged at 100,000 × g to separate and isolate supernatant solution (cytosol) and an insoluble pellet fraction. Cytosol and resuspended pellet were stored in liquid nitrogen. Details are provided in Ref. 60.

In Vitro Synthesis of DKF-1—A coupled transcription-translation lysate translation system (TNT™, Promega Corp.) was programmed with 1 µg of recombinant pBluescript that contains full-length DKF-1 cDNA. Incubation, denaturing electrophoresis, and autoradiography were performed as published previously (55, 62).

Cell Culture—A cell line derived from a hamster subcutaneous tumor (AV-12) and human HEK293 cells were obtained from the American Type Culture Collection. Cells were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum in an atmosphere of 7% CO2 and 93% air.

Electrophoresis of Proteins and Western Immunoblot Assays—Cytosol and particulate proteins were isolated from mammalian cells as stated previously (63). Triton X-100 was added or omitted from cell lysis buffer as indicated in the legend for Fig. 4 and under “Results.” Proteins
were size-fractionated by electrophoresis in a denaturing polyacrylamide (8% or otherwise indicated) gel as reported previously (64). BenchMarker™ prestained proteins (9–182 kDa, Invitrogen) or Precision Plus Protein™ (10–250 kDa, Bio-Rad) polypeptides were used as molecular size standards. Western blots of size-fractionated proteins were prepared, blocked, incubated with anti-DKF-1 IgGs (1:1000), and washed as indicated in previous studies (63, 64). Unless noted otherwise, each lane in Western blots received 30 μg of protein. Antibody-antibody complexes were visualized and quantified by using an enhanced chemiluminescence procedure and image analysis software (ImageQuant, GE Healthcare).

**Purification of DKF-1 Fusion Protein Expressed in Sf9 Cells**—Full-length DKF-1 cDNA was cloned downstream from a GST gene in a baculovirus transfer vector (pAcGHLT, Pharmingen). Recombinant baculovirus encoding GST-DKF-1 was generated and amplified as described in Islas-Trejo et al. (65). Procedures for infection, growth, and lysis of Sf9 cells are elaborated in detail elsewhere (65, 66). Fusion protein was purified by affinity chromatography on GSH-Sepharose 4B (60). Fractions containing high levels of DKF-1 were pooled and stored at −80 °C.

**Expression of DKF-1 in Mammalian Cells**—Full-length DKF-1 cDNA, excised from recombinant pBluescript plasmids (see above) by digestion with XbaI and Asp718, was cloned in the mammalian expression vector pCDNA3.1 (+) (Invitrogen, Carlsbad CA). Hamster AV-12 or human HEK293 cells were transfected with the recombinant DKF-1 transgene using Lipofectamine® (Invitrogen). Stable cell lines were expanded and verified by independently determining DKF-1 protein levels and measuring PKC-stimulated kinase activity in cell extracts (see below).

**Protein Determination**—Protein concentrations were determined with the Bio-Rad DC protein assay reagents. Bovine albumin was employed as a standard.

**Immunoprecipitation and in Vitro DKF-1 Kinase Assays**—AV-12 cells were transfected by calcium phosphate precipitation (58, 63) or uptake of Lipofectamine™-recombinant DNA complexes. After 24 h, cells were harvested and homogenized in lysis buffer as reported previously (63). All operations (except kinase assays) were performed at 4 °C. Cell lysates were centrifuged at 40,000 × g for 30 min, and samples of supernatant solution (0.1–0.3 mg of protein) received 2 μl (~1 μg) of affinity-purified anti-DKF-1 IgG. Incubation for 3 h yielded an optimal level of antigen-IgG complex formation. Subsequently, 25 μl of protein A (or G)-Sepharose 4B beads (Zymed Laboratories Inc.) was added, and the incubation was continued for 60 min. Next, bead-bound immune complexes were washed three times with lysis buffer and then twice with kinase buffer containing 25 mM Tris-HCl, pH 7.4, 5 mM MgCl₂, and 1 mM dithiothreitol. Catalytic activity of DKF-1 was quantified by measuring incorporation of [32P]radioactivity from [γ-32P]ATP into Syntide-2 peptide substrate (Calbiochem). Reaction buffer (30 μl, containing 25 mM Tris-HCl, pH 7.4, 30 μM peptide substrate, 100 μM [γ-32P]ATP (~150 cpm/pmol), 5 mM MgCl₂, 0.5 mM EGTA, and 1 mM dithiothreitol) was added to immune complexes, and samples were incubated at 30 °C for 10 min. Assays were terminated by the addition of 5 μl of 0.2 M EDTA, pH 8.0, containing 60 mM NaF. Reaction mixtures were applied to P81 filter papers (Whatman) and subjected to extensive washing in 75 mM H₃PO₄ (32P-Syntide-2 binds P81 filters under acidic conditions, whereas 32P, and [γ-32P]ATP are washed away). After filters were washed and air-dried, 32P radioactivity incorporated into Syntide-2 was measured in a scintillation counter. Phosphotransferase activity of purified GST-DKF-1 fusion protein from Sf9 cells was assayed as described above, except that 3 μl of enzyme (~50 ng protein) was used instead of an immunoprecipitate. Various combinations of PS, DAG, and Ca²⁺ were added to kinase buffer to test their effects on catalytic activity.

PKCε peptide RFAKRGSGLRQKNV, PKCγ peptide YRGRSSRWWK-KIY, Syntide-2 peptide PLARTLSVAGLPKK, an optimum PKD peptide designed by Cantley et al. (23), AALVRQMSVAFFF, and myelin basic protein were tested as substrates for DKF-1. Potential phosphorylation sites are shown (above) in bold underlined italics.

To test the effects of PKC inhibitors on DKF-1 catalytic activity in situ, AV-12 cells were incubated with various concentrations of bisindolylmaleimide I (GF109203X) or bisindolylmaleimide IX (RO31-8220) (Axxora, LLC) for 1 h. Cells were then treated with specified concentrations of PMA for 10 min. In vitro kinase assays were performed on immunoprecipitated DKF-1 as described above. PKC inhibitors were tested in vitro by adding various amounts of GF109203X or RO31-8220 directly to the kinase reaction mixture.

**Single Worm PCR Amplification of a Disrupted dkf-1 Gene**—Deletions of 1–3 kbp in target genes were generated by mutagenesis with Psoralen and ultraviolet light by Liao Teng Wang in the laboratory of Dr. Judith Kimble (Department of Genetics, University of Wisconsin). Individual adult worms from potential dkf-1 knock-out lines were extracted with incubation with 5 μl of lysozyme buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.2, 2.5 mM MgCl₂, 0.45% Nonidet P-40, 0.45% Tween 20, 0.01% gelatin, and 5% proteinase K) at 65 °C for 1 h. Large fragments of the dkf-1 gene were amplified by nested PCR using genomic DNA in diluted C elegans extracts as template. PCR was first executed with an "external left" primer (5’-TCCGAAGAAGATGATCCAGG-3’) and an "external right" primer (5’-CAATTGTGGAACACTGTC-3’) pair. A second round of PCR used internal left (5’-AACACAGTGGAGGCCAG-3’) and internal right (5’-AATCGGCACCAATTCAGG-3’) primers. Sizes of amplified DNA segments were estimated by electrophoresis in a 1% agarose gel. Exact deletion boundaries were determined by sequencing the amplified genomic DNA.

**Preparation of Transgenic C. elegans**—A DNA fragment (2.5 kbp) that precedes the 5′ end of the dkf-1 structural gene was ligated to cDNA encoding the complete DKF-1 open reading frame in pBluescript SK by standard procedures. Subsequently, the fusion DNA (promoter + open reading frame) was cloned into pH95.77, a C elegans expression vector (52). A DKF-1 cDNA translation stop codon was eliminated by mutagenesis to permit in-frame ligation with DNA encoding green fluorescent protein (GFP). The GFP coding region is followed by translation termination and poly(A) addition signals. DKF-1 promoter/enhancer DNA ensures normal temporal developmental and cell-specific regulation and expression of DKF-1-GFP protein. Fluorescence signals emanating from individual cells of living or fixed worms are recorded via microscopy systems equipped for epifluorescence.

**C. elegans** was transformed by microinjecting the DKF-1-GFP kinase-reporter plasmid (dkf-1::DKF-1-GFP) as described previously (66). Transgenic C. elegans were identified by fluorescence microscopy and transferred to new plates to establish cloned populations. The dkf-1::DKF-1-GFP chimeric gene was chromosomally integrated as described in a protocol provided by Dr. Michael Koelle (Department of Molecular Biophysics and Biochemistry, Yale University). Expression of DKF-1-GFP in C. elegans allows visualization of authentic promoter activation in individual cells in vivo. Fluorescence signals were captured as reported previously (60, 65).

**Ablation of DKF-1 Protein and Function by RNAi**—Full-length DKF-1 cDNA was cloned into PPD129.36 (generously provided by Dr. A. Fire, Departments of Pathology and Genetics, Stanford University
School of Medicine), a vector that has two opposing promoters for T7 RNA polymerase on sense and antisense DNA strands (67–69). *E. coli* HT115 (DE3) was transformed with recombinant plasmid, grown to 
\[ A_{595}/H11005 = 0.4 \] and seeded on plates containing 0.8 mM IPTG. L4-stage worms were transferred to plates containing transformed bacteria.

**Double-stranded RNAi ingested by *C. elegans* is disseminated to cells throughout the nematode via double-stranded RNA transporters (67–69).** After 30–36 h at 20 °C, individual adult worms were transferred to fresh plates, and phenotypes of adults and offspring were observed by light microscopy.

**RESULTS**

**Characterization of cDNA Encoding *C. elegans* DKF-1**—A 2,317-bp cDNA that encodes a novel *C. elegans* PKD (DKF-1) was obtained by a strategy described under “Experimental Procedures.” An open reading frame of 2,166 bp begins at ATG in a *C. elegans* consensus motif for translation initiation, (A/G)NN\_ATG (boldface indicates the initiator Met codon), and ends with a translation termination codon at nucleotides 2167–2169 (see GenBank™ accession number MN_060989). The coding sequence precedes a 3\_untranslated region comprising 132 nucleotides. An atypical but functional poly(A) addition signal (GATAAA, nucleotides 2280–2285) (70) is located 13 nucleotides upstream from the poly(A) tail. Translation of the long open reading frame predicts that DKF-1 is composed of 722 amino acids (Fig. 1 A) and has a \[ M_{r} \] of \[ 81,000 \].

*In vitro* transcription/translation programmed by the 2.3-kbp cDNA yielded an 81-kDa polypeptide (Fig. 2 D). This polypeptide has the same apparent \[ M_{r} \] and shares epitopes with DKF-1 protein that (a) accumulates in cultured cells expressing a DKF-1 transgene (Fig. 2 C) and (b) is abundant (endogenously) in vivo in a subset of cells in *C. elegans* (Figs. 2, B and E, 7, and 8, A and D).

**Structure/Function Relationships in DKF-1**—A C-terminal region (amino acids 426–685, Fig. 1 A) of DKF-1 contains sequence motifs that are conserved in catalytic domains of Ser/Thr protein kinases. Functions for these conserved amino acids were established by biochemical
analysis, mutagenesis, comparisons with sequences of several hundred phosphotransferases, and insights derived from crystal structures of prototypic Ser/Thr protein kinases (71–74). Sequences in DKF-1 are tentatively linked to specific functions by analogy. A GXXGXXG\(_{16-}^{\perp}K\) motif (residues 433–455, Fig. 1A) provides sites that bind with phosphates of the substrate MgATP. Lys\(^{455}\) is essential for catalysis and binding of ATP. A DFG tripeptide (residues 573–575, Fig. 1A) contributes a carboxyl side chain (Asp\(^{573}\)) that can mediate binding of divalent metal and \(\gamma\) phosphate of MgATP and stabilize pentavalent phosphorus in the transition state for the kinase reaction. Glu\(^{399}\), which is part of a conserved PPE motif (APE in other protein kinases), as well as Asp\(^{511}\) and Arg\(^{573}\), stabilizes the catalytic core region. An XDDLLKKX(N/D) motif is a Ser/Thr protein kinase “signature” sequence that guides protein substrate into an orientation favorable for catalysis (71–74). The DKF-1 catalytic loop sequence, HCDLKPEN (residues 549–556), diverges from the corresponding loop (YRDLLKDN) present in all members of the related PKC family. However, the HCDLKPEN motif is conserved among PKD isoforms (15–18).

The N-terminal portion of DKF-1 contains two Cys (and His)-rich sequences (HX\(_{12}\)CX\(_{3-}\)CX\(_{1-}\)CX\(_{3-}\)HX\(_{12}\)CX\(_{3-}\)C, residues 99–148 and residues 187–236, Fig. 1A) that conserve conserved regulatory regions (C1 domains) in DAG-activated PKCs (Fig. 1B). C1 domains fold into “finger-like” structures. Conserved spacing of six Cys and two His residues enables binding of two atoms of zinc in each finger. Zinc is essential for proper folding and function of PKCs.

In PKC isoforms, a pseudosubstrate motif (Ala flanked by basic amino acids) is located \(\sim 15\) amino acids upstream from the first C1 domain (C1a). DKF-1 lacks a classical pseudosubstrate sequence. Unlike PKCs, DKF-1 contains a PH domain (residues 279–407, Fig. 1A), which is \(\sim 33\%\) identical (50% similarity) with an analogous domain in mammalian PKD. DKF-1 also lacks a calcium-binding domain (C2) that is conserved in cPKC isoforms (Fig. 1B).

Sequence Conservation and Divergence between DKF-1 and Human PKD Isoforms—The amino acid sequences of DKF-1 and protein kinases in standard data bases were aligned. DKF-1 is most closely related to mammalian PKD, PKD2, and PKD3 isoforms (50% similarity; \(\geq 38\%)\) identical amino acids with each isoform) (Table 1). No other protein kinases share \(\geq 26\%\) overall identity with DKF-1. Sequence homology varies markedly among domains (Table 1). The catalytic domains of human PKD isoforms and nematode DKF-1 are maximally conserved (60% identity). DKF-1 and PKDs also share high levels of homology in the C1a and C1b regulatory regions (44–58% identity, Table 1). Four His and 12 Cys residues, which are crucial for organizing/stabilizing zinc finger structures and binding physiological activators (DAG, PS) to C1 domains of PKCs and PKDs, are retained in perfect register in DKF-1. The PH domain and extreme C-terminal region of DKF-1 have lower but significant levels of sequence identity with corresponding segments in human PKDs (\(\sim 30–33\)% identity plus \(\sim 20\%)\) conserved amino acid substitutions, Table 1). Conservation of amino acid sequences and the relative positions of domains along the polypeptide chain (Fig. 1B, Table 1) indicate that DKF-1 is a member of the PKD family. However, major differences in structure/function relationships and mode of activation (elaborated in detail below and in an accompanying article (89)) indicate that DKF-1 is a novel, previously uncharacterized PKD isotype.

A C. elegans gene named dkl-2 encodes another PKD isoform.\(^5\) Conservation between amino acid sequences of DKF-1 and DKL-2 proteins parallels the relationship between DKF-1 and mammalian PKDs. The C1a, C1b, PH, and catalytic domains of DKF-1 and DKL-2 are 56, 50, 34, and 57% identical, respectively.

Organization of the C. elegans dkl-1 Gene—During the course of our investigations on DKF-1, the C. elegans Genome Project deposited genomic DNA sequence data for cosmid W09C5 in GenBank\(^{274}\) and Wormbase. We established the organization of exons and introns for the dkl-1 structural gene by identifying genomic DNA sequences in recombinant cosmids that overlap with segments of DKF-1 cDNA. Our results and subsequent analysis by the C. elegans Genome Consortium are in agreement (Table 2). The dkl-1 structural gene contains 11 exons and spans \(\sim 8\) kbp of DNA (Table 2).

\(^5\) H. Feng and C. S. Rubin, unpublished observations.
Production and Specificity of Anti-DKF-1 Immunoglobulins—A fusion protein that consists of 153 amino acids from DKF-1 (residues 2–154, Fig. 1A) appended to the C terminus of GST was produced in *E. coli* and purified as described under Experimental Procedures.” Anti-sera directed against the 43-kDa DKF-1 fusion protein (Fig. 2A) were produced in rabbits. IgGs that bind DKF-1 were purified from serum by affinity chromatography using Sepharose 4B beads derivatized with partial DKF-1 protein. Affinity-purified anti-DKF-1 IgGs bound (**Fig. 2B, lane 1**) to native or recombinant DKF-1 yielded essentially the same results under all conditions.

| Domain in DKF-1 | PKD | PKD2 | PKD3 | PKCa | PKCe |
|----------------|-----|------|------|------|------|
| N-terminal (residues 1–98) | 12 | 21 | 15 | 8 | 7 |
| C1a (residues 99–148) | 56 | 56 | 58 | 30 (44)* | 28 (48)* |
| Linker between C1a and C1b (residues 149–186) | 10 | 10 | 10 | 26 | 13 |
| C1b (residues 187–236) | 50 | 48 | 44 | 42 (32)* | 46 (32)* |
| Linker between C1b and PH (residues 237–278) | 19 | 19 | 16 | 5 | 7 |
| PH domain (residues 279–407) | 34 | 30 | 33 | 4 | 6 |
| Linker between PH and catalytic (residues 408–425) | 11 | 11 | 11 | 16 | 11 |
| Catalytic (residues 426–685) | 60 | 61 | 61 | 26 | 25 |
| C-terminal (residues 686–722) | 29 | 29 | 29 | 5 | 8 |
| Overall | 39 | 42 | 38 | 18 | 17 |

*DKF1 C1a alignment with PKC C1a (DKF1 C1b alignment with PKC C1b).*

* Fractions indicate that exons begin or end within a codon. If 1 nucleotide is present, it is 1/3 of a complete codon.

**Fractions indicate that exons begin or end within a codon and 132 bp of 5′-untranslated sequence.**
Steady-state kinetic analysis of DFK-1-catalyzed phosphorylation yielded apparent $K_m$ values of 11.7 ± 1.2 μM and 45.3 ± 3.5 μM for Syntide-2 and MgATP, respectively (Fig. 3B).

**FIGURE 3.**

**A**

MBP

ε peptide

ζ peptide

Syn tide-2

AALVRQMSVAFFF

Phosphate incorporated (pmol)

0 10 25 50 75 100

**B**

velocity (pmol phosphate incorporated/min)

substrate (μM)

0 50 100 150 200 250 300

**C**

Basal

PMA

GF109203X + PMA

Staurosporine + PMA

**D**

phosphate incorporated (pmol)

0 10 20 30 40 50 60

**TABLE 3**

**In vitro activation of C. elegans DFK-1**

Phosphotransferase activity of GST-DKF-1 was measured as described under “Experimental Procedures.” The specified concentrations of PS, DAG, and/or Ca$^{2+}$ were added as indicated. For immunodepletion, a sample of DFK-1 was immunoprecipitated with 0.5 μg of affinity purified anti-DKF-1 IgG that was bound to protein A-Sepharose 4B. Kinase activity remaining in the supernatant solution was then quantified. All assays were repeated three times. Typical results are shown.

| Activators tested       | P-Syntide-2 pmol |
|-------------------------|-----------------|
| PS (20 μg/ml)           | 1.2             |
| DAG (8 μg/ml)           | 0.6             |
| PS (20 μg/ml) + DAG (8 μg/ml) | 14.6         |
| PS (20 μg/ml) + DAG (8 μg/ml) + Ca$^{2+}$ (0.2 mM) | 13.7         |
| PS (20 μg/ml) + DAG (8 μg/ml) after immunodepletion | 0.7           |

(b) an ability of members of the PKD family to remain active for an extended time after DAG levels decline. Bombesin-induced translocation of DFK-1 to membranes is documented in a companion article (89).

Treatment of cells with a high concentration of PKC inhibitor (GF109203X) did not affect bombesin-stimulated DFK-1 activation (Fig. 4B). In contrast, a PLC inhibitor (U73122) diminished bombesin-controlled DFK-1 activation by ~70% (Fig. 4B). Thus, bombesin and bombesin receptors trigger PLC/DAG-mediated, PKC-independent activation of DFK-1.

**Tnr**<sup>1108</sup> Regulates DFK-1 Catalytic Activity—Activation loops in mammalian PKD isoforms contain the sequence SFRRSV (amino acids...
FIGURE 4. DKF-1 is activated via bombesin receptors and PLC. A, transfected AV-12 cells expressing bombesin BB2 receptors and DKF-1 were incubated with 100 nM bombesin for the indicated time intervals. Subsequently, cells were lysed in buffer containing 1% Triton X-100, DKF-1 was isolated and purified by precipitation with anti-DKF-1 IgGs bound to protein G-Sepharose 4B beads, and kinase assays were performed (see “Experimental Procedures”). The inset is a Western immunoblot showing that similar amounts of DKF-1 protein were immunoprecipitated and assayed at each time point during the course of bombesin treatment. The blot was probed sequentially with anti-DKF-1 IgGs and secondary antibodies coupled to peroxidase. Chemiluminescence signals were detected using the ECL reagent. The data presented represent the average values obtained from three independent experiments. B, transfected AV12 cells described in A were incubated with 5 μM GF109203X, 5 μM U73122, or vehicle for 1 h. Subsequently, cells were treated with 100 nM bombesin or buffer (control) for 2 min. DKF-1 was isolated from cell extracts by immunoprecipitation with affinity-purified anti-DKF-1 IgGs and secondary antibodies coupled to peroxidase. Chemiluminescence signals were detected using the ECL reagent. The data presented represent the average values obtained from three independent experiments.

PMA, Translocation, and Phosphorylation Regulate DKF-1

Like DKF-1(Asp588), DKF-1(Asp588) exhibits high basal activity that is comparable to WT DKF-1. In contrast, phorbol ester stimulation of AV-12 cells transfected with DKF-1(Asp588) produced a larger increase in specific kinase activity than WT DKF-1. These results support the idea that phosphorylation of Thr588 is required for maximal catalytic activity of DKF-1.

Additional mutations (e.g., Glu588) were introduced into the DKF-1 transgenes to mimic negative charge (P-Thr588) that might be associated with PMA-activated WT DKF-1. These mutant proteins, WT DKF-1, and the Ser588 and Ala588 DKF-1 variants were expressed at similar levels in AV-12 cells (Fig. 5A, upper panel). However, in non-stimulated cells, the specific activity of DKF-1(Glu588) was elevated ~3-fold relative to WT DKF-1 (Fig. 5B, lanes 2 and 9). Incubation of transfected cells with PMA caused a large increase in specific kinase activity of WT DKF-1 (Fig. 5B, lanes 2 and 3). In contrast, phorbol ester treatment did not alter DKF-1(Glu588) activity (Fig. 5B, lanes 9 and 10). Like DKF-1(Glu588), DKF-1(Asp588) exhibits high basal activity that is not further enhanced by PMA. Replacement of Thr588 with nonacidic amino acids (e.g. Ala) yielded DKF-1 mutants that exhibit minimal basal activity and are unresponsive to PMA (Fig. 5B, lanes 7 and 8).

Possible linkage between phosphorylation of DKF-1 and expression of maximal DKF-1 catalytic activity with an optimal exogenous substrate was explored via a dephosphorylation strategy. PMA-activated WT DKF-1 was immunoprecipitated from cell extracts and incubated with highly purified protein phosphatase 2C (PP2C). Treatment with the phosphatase diminished DKF-1 phosphotransferase activity in a time- and temperature-dependent manner (Fig. 6). Under optimal conditions ~90% of the kinase activity was abolished by PP2C.

744–749 in PKD). Hormones or GFs that increase DAG content in membranes promote phosphorylation of both Ser544 and Ser549 (29, 30). Moreover, incorporation of phosphate at both target sites is essential for generating maximal protein phosphotransferase activity in the adjacent catalytic loop (amino acids 710–720 in PKD). In contrast, only Thr588 can be phosphorylated in the corresponding region of the predicted DKF-1 activation loop (QFRKTV, residues 584–589). This raised the possibility that DKF-1 and PKDs are activated by different mechanisms. Mutations were designed to test the importance of Thr588 in kinase activation; replacement of Thr588 with Ala will eliminate interactions mediated by the polar hydroxyl group and extinguish phosphorylation at the putative target site, whereas substitution of Thr588 with Ser might sustain normal function and regulation. Mutation of Thr588 to Ala sharply suppressed (>85%) basal and phorbol ester-stimulated DKF-1 phosphotransferase activity in situ (Fig. 5B, compare lanes 2 and 3 with lanes 7 and 8), whereas interchange of Ser for Thr588 produced a PMA-activated kinase that was indistinguishable from WT DKF-1 (Fig. 5B, compare lanes 2 and 3 with lanes 11 and 12). The results demonstrate the central importance of Thr588 in elevating and controlling DKF-1 kinase activity. Both WT DKF-1 and the Ala588 mutant were targeted to membranes upon incubation of cells with PMA for 10 min (Fig. 5C, and immunolocalization data shown in an accompanying article (89)).
WT and DKF-1(Ser<sup>588</sup>) activities were not inhibited by GF109203X or other PKC inhibitors (Fig. 5B, lanes 4–6). Thus, DKF-1 and PKDs are regulated by distinct upstream activators and different mechanisms.

Substitution of Thr<sup>588</sup> with Ser did not affect properties of DKF-1. Both wild type and variant (DKF-1(Ser<sup>588</sup>)) kinases were stimulated ~8-fold in cells incubated with PMA (Fig. 5B, lanes 2, 3, 11, and 12). Preincubation of cells with 3.5 μM GF109203X did not alter the magnitude of activation of DKF-1 or DKF-1(Ser<sup>588</sup>) by subsequent addition of PMA. Thus, the presence of Thr or Ser at amino acid 588 in the activation loop (QFRK(T/S)V) enables PKC-independent activation of DKF-1.

dkf-1 Gene Promoter Activity and Cognate DKF-1 Protein Kinase Are Selectively Expressed in a Subset of Cells in Vivo—Promoter/enhancer DNA that precedes the authentic dkf-1 structural gene was coupled to the 5’ end of full-length DKF-1 cDNA. The resulting dkf-1p::DKF-1 minigene was inserted upstream from GFP reporter cDNA in a C. elegans expression plasmid. Subsequently, the dkf-1p::DKF-1-GFP fusion gene was integrated into C. elegans genomic DNA, and stable transgenic lines of C. elegans were propagated. Levels of DKF-1-GFP were monitored by Western immunoblotting (Fig. 7A), and expression of DKF-1 fusion protein in individual cells in vivo was assessed by fluorescence microscopy (Fig. 7, B–D). A highly reproducible pattern of DKF-1 expression was observed as animals matured from embryo to adult. Intense fluorescence signals corresponding to DKF-1-GFP revealed robust kinase accumulation in both (a) a region bounded by the anterior and posterior bulbs of the pharynx (Fig. 7, B, B2, and C) and (b) a tail area that contains lumbar, dorsoventral and pre-anal ganglia (Fig. 7D).

Specifically, DKF-1 is differentially enriched in a cluster of cells that are immediately adjacent to the posterior pharyngeal bulb (Fig. 7B). Strong signals also emanate from cells positioned along the lateral surface of this bulb in animals carrying the dkf-1p::DKF-1-GFP transgene (Fig. 7B2, arrows). At the anterior pharyngeal bulb, DKF-1 accumulates selectively in bodies and in very thin processes (dendrites and axons) of two neurons (Fig. 7C). High cell density and complex organization of neuronal ganglia in the head (and tail) region of C. elegans preclude determination of the exact identities of individual cells that exhibit elevated dkf-1 promoter activity and, therefore, enrichment in DKF-1-GFP polypeptide.

Despite the limitations listed above, a comparison of our current results with the C. elegans anatomy data base is quite informative. This approach focuses attention on a small group of “candidate DKF-1 positive cells” that will guide further studies. Nearly all cells expressing DKF-1 in Fig. 7, B, B2, C, and D, appear to be neurons. Two fluorescent cells with similar sizes and locations (at the anterior edge of the isthmus-posterior bulb, Fig. 7, BN and B, arrows) may be M2 motor neurons. M2 neurons innervate muscles in the anterior bulb of the pharynx. The location of the more posterior fluorescent neuron in Fig. 7C (upper arrow) approximates the position of the cell body of an NSM neuron. NSM is a multifunctional neuron involved in sensing food and physical stretch, locomotory behavior, and secretion of growth factors and hormones. DKF-1 also accumulates in a cell resembling II (Fig. 7C, lower arrow), an interneuron that links sensory and effector neurons. Other candidate DKF-1-enriched cells in the pharyngeal region include: the AWB, ADL, and ADF chemosensory neurons; and AVB and AIA interneurons, which process sensory inputs and determine (directly or indirectly) the course of motor neuron actions at neuromuscular junctions, thereby governing body movement.

In C. elegans tail, DKF-1-GFP expression is differentially elevated in neurons located within the dense neuropile of several tail ganglia (Fig. 7D). This arrangement suggests that activation of the DKF-1 might...
PMA, Translocation, and Phosphorylation Regulate DKF-1

FIGURE 7. Expression and localization of DKF-1 in C. elegans. Lines of transgenic C. elegans that express full-length DKF-1-GFP under control of the d kf-1 gene promoter/enhancer were created as indicated under “Experimental Procedures.” A, mixed populations of WT and transgenic animals were extracted with SDS-PAGE loading buffer. Proteins were separated by denaturing electrophoresis. Expression of DKF-1 fusion protein was assessed by Western immunoblot analysis as described under “Experimental Procedures.” Endogenous DKF-1 (81 kDa) as well as DKF-1-GFP fusion protein (107 kDa) are detected by anti-DKF-1 IgGs. DKF-1-GFP is not present in WT animals. B-D, cells enriched in DKF-1 protein were identified by fluorescence microscopy (>600 magnification). B, DKF-1 is expressed prominently in cells associated with the posterior bulb of the pharynx. Nomarski interference images of worms in cells associated with the posterior bulb of the pharynx illustrate the isolation of animals with a disrupted d kf-1 gene (see Fig. 8 and under “Results” below).

Depletion or Elimination of DKF-1 Protein in Vivo Reveals a Novel Physiological Role for a Member of the PKD Family—DKF-1 mRNA and protein levels were diminished in vivo using RNAi methodology. Full-length DKF-1 cDNA was cloned into an RNAi vector (pPD139.06). The cDNA insert is located between opposing, IPTG-inducible, T7 promoters on sense and antisense DNA strands. E. coli was transformed with recombimant plasmid and double-stranded RNA (dsRNA) production was induced by adding IPTG. C. elegans cells take up dsRNA from ingested, transformed E. coli. Ubiquitous dsRNA transporters disseminate “silencing” reagent (RNAi) throughout the tissues of C. elegans (79, 80). Cells in the nematodes contain enzymes that cleave high molecular weight dsRNAs into small fragments (~25 bp) that efficiently and selectively mediate degradation of a target transcript (DKF-1 mRNA in this instance) (81, 82).

L4 stage worms were fed with bacteria producing RNAi directed against DKF-1, and phenotypes of these worms and their progeny were observed with a dissecting microscope. DKF-1 protein content decreased dramatically (~90%) in vivo when cognate RNAi was ingested (Fig. 8A, upper panel). In contrast, the concentration of a control protein (PKC-1) was not altered (Fig. 8A, lower panel). A distinctive phenotype was discovered in DKF-1-deficient C. elegans. Normal C. elegans traverse the agar medium on culture plates with a sinusoidal motion. This pattern is compromised by DKF-1 RNAi; worms exhibited a "zigzag" motility pattern with markedly altered amplitude (Compare Fig. 8B, panel 1 with panels 2 and 3). Two days after RNAi feeding, the mobility of adult worms was impaired. Severe or complete loss of muscle contraction near the anus caused the partially paralyzed tail region of C. elegans to "zigzag" and lose coordination with the rest of the body. The concentration of a control protein (PKC-1) was not altered (Fig. 8A, lower panel). A distinctive phenotype was discovered in DKF-1-deficient C. elegans. Normal C. elegans traverse the agar medium on culture plates with a sinusoidal motion. This pattern is compromised by DKF-1 RNAi; worms exhibited a "zigzag" motility pattern with markedly altered amplitude (Compare Fig. 8B, panel 1 with panels 2 and 3). Two days after RNAi feeding, the mobility of adult worms was impaired. Severe or complete loss of muscle contraction near the anus caused the partially paralyzed tail region of the animals to be dragged along by the upper body. Thus, DKF-1 deficiency apparently causes a neuromuscular defect (uncordinated (unc) phenotype) in the tail region.

A C. elegans gene disruption project in the laboratory of Judith Kimble (Department of Genetics, University of Wisconsin) facilitated the isolation of animals with a disrupted d kf-1 gene (generously provided by Liaoeteng Wang, University of Wisconsin). Back-crossing with wild type C. elegans eliminated extraneous mutations and yielded viable worms with a d kf-1(−/−) genotype. Fragments of the disrupted d kf-1 gene were generated by PCR amplification using genomic DNA tem-
plates from individual worms in concert with a set of nested primers (Fig. 8C). Sequencing of PCR-amplified DNA revealed that a 1366-bp segment was deleted from the dkf-1 gene. This deletion corresponds to nucleotides 12736–14281 in the sequence of cosmid W09C5. Comparison of cosmid (genomic) and cDNA sequences indicates that exon 3 and portions of two introns are deleted from the dkf-1 gene. The expected splicing of exon 2 to exon 4 in the disrupted gene will cause a frameshift in DKF-1 mRNA that places a translation stop codon after amino acid 83. The resulting transcript will encode a chimeric protein composed of amino acids 1–78 of DKF-1 plus five C-terminal amino acids from an incorrect reading frame. The calculated Mr for the chimera is 9,600. Because the truncated DKF-1 polypeptide terminates before the first Cys-rich domain (C1a) and also lacks a kinase domain, the animals have a null phenotype for all established functional domains in DKF-1. dkf-1(–/–) C. elegans and WT worms that contain DKF-1 RNAi have the same “uncoordinated” phenotype (Fig. 8B, panels 3 and 2, respectively). Null mutants move normally from the time of hatching to the egg-laying stage and have no obvious defects in embryogenesis. Adult dkf-1(–/–) C. elegans display uncoordinated tail movement at day 5 (2 days after the final larval stage, L4) of the life cycle. The phenotype persists until death. A link between DKF-1 protein and null phenotype (unc) was rigorously confirmed by demonstrating that introduction of a dkf-1::DKF-1-GFP fusion gene into dkf-1(–/–) animals corrected the movement disorder. Cell-specific expression of the gene restored normal sinusoidal motion (data not shown). Western immunoblots demonstrated that the predicted 107-kDa DKF-1-GFP fusion protein accumulates in rescued transgenic nematodes. Endogenous DKF-1 protein expression was examined by Western immunoblotting. Samples of total protein (30 μg) from dkf-1(–/–) null animals (lane 1), WT worms (lane 2), dkf-1(–/–) null animals rescued with DKF-1-GFP (lane 3), and transgenic worms that overexpress DKF-1-GFP in a WT background (lane 4) were analyzed. The protein blot was probed with anti-DKF-1 IgGs, and polypeptides were visualized as described under “Experimental Procedures.” Note that an occasionally observed nonspecific band in lane 1 migrates slightly faster than 81-kDa DKF-1.

Although uncoordinated movement was compensated by synthesis of DKF-1-GFP, rescued C. elegans adults were distinguished from WT animals by size. The rescued nematodes have small cells and are substantially reduced in overall body size (Fig. 9A, b). In particular, abundant DKF-1 attenuates growth that occurs upon the L4 to adult transition. This idea was validated by creating stable transgenic C. elegans that overexpress DKF-1-GFP in appropriate cells in the context of a WT background (Fig. 9A, d). C. elegans body length was measured at L4 and adult stages. WT animals, dkf-1 null nematodes rescued with a dkf-1-gfp transgene, and WT nematodes overexpressing DKF-1 fusion protein were studied in parallel (Fig. 9B). At the last larval stage (L4), animals containing excess DKF-1 are ~20% shorter than WT worms. During the next 2 days, wild type C. elegans increased >50% in length. In contrast, worms with elevated DKF-1 content elongated only ~10% (Fig. 9B). Consequently, mature animals with constitutively elevated DKF-1 content are only ~60% as long as their wild type counterparts. Because the small worms are also thin relative to wild type C. elegans, their total internal volumes are likely to be diminished by >50%.

**DISCUSSION**

The C. elegans dkf-1 gene encodes a novel PMA/DAG-regulated Ser/Thr protein kinase. Like cPKCs and nPKCs, the 81-kDa DKF-1 polypeptide possesses tandem N-terminal C1 domains that control phosphotransferase activity (see accompanying article (89)) in a C-terminal kinase module. Several divergent features distinguish DKF-1 from PKCs. DKF-1 lacks (a) a Ca2+-binding C2 domain inherent in cPKCs, (b) an N-terminal (C2-like) extension region that supports Ca2+-independent lipid binding in nPKCs, and (c) a pseudosubstrate inhibitory segment that is present in all PKC isoforms. DKF-1 contains a PH domain (a structural module not found in PKCs) and phosphorylates different effector proteins, because its optimal target amino acid sequence (LVRQMSVAF, critical amino acids Leu(−5), Arg(−3), and Met(−1)) precede the C-terminal target Ser(0)) is not related to established PKC phosphorylation sites. Peptides and proteins that are effi-

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**FIGURE B. Depletion of endogenous DKF-1 by RNAi or gene disruption causes an impaired movement defect.** A, L4 worms were fed with bacteria that produce DKF-1 RNAi as described under “Experimental Procedures.” DKF-1 protein expression was examined by Western immunoblotting. Left lane, worms fed with E. coli expressing control dsRNA. Right lane, worms fed with bacteria producing dsDKF-1 RNAi. Endogenous DKF-1 protein content was markedly reduced by feeding dsRNA; the amount of a nontargeted protein, PKC1 (a DAG-activated PKC (60)), was not altered. Note that the phosphorylation state of PKC1 (indicated by bands >90 kDa) varies, but total PKC1 protein is unchanged. B, worm phenotypes were revealed by light microscopy. Panel 1 shows tracks of WT C. elegans on an agar plate. Panels 2 and 3 depict highly atypical tracks produced by (homozygous null) dkf-1(–/–) animals and worms fed with DKF-1 RNAi, respectively. C, PCR analysis was performed on genomic DNA templates from individual WT and putative DKF-1 null C. elegans (see “Experimental Procedures”). Nested primers that hybridize only with the dkf-1 gene were used. Amplified DNA products were analyzed in a 1% agarose gel. Ethidium bromide-stained DNA is shown. A deletion of ~1.4 kbp is evident in the lane labeled dkf-1(–/–). Lane M received DNA size markers; lane N2 contains amplified WT DNA. D, DKF-1-GFP fusion proteins and endogenous DKF-1 were detected by immunoblotting. Samples of total protein (30 μg) from dkf-1(–/–) null animals (lane 1), WT worms (lane 2), dkf-1(–/–) null animals rescued with DKF-1-GFP (lane 3), and transgenic worms that overexpress DKF-1-GFP in a WT background (lane 4) were analyzed. The protein blot was probed with anti-DKF-1 IgGs, and polypeptides were visualized as described under “Experimental Procedures.” Note that an occasionally observed nonspecific band in lane 1 migrates slightly faster than 81-kDa DKF-1.
PMA, Translocation, and Phosphorylation Regulate DKF-1

FIGURE 9. DKF-1 overexpression causes a dramatic reduction in C. elegans body size. Synchronized L4 worms from the indicated lines of C. elegans were grown for 2 days, thereby becoming mature adults of maximum size. Worm images were taken by phase contrast microscopy at ×40 magnification. A, images of a WT nematode (a); dkh-1(-/-) C. elegans that expresses a DKF-1-GFP transgene at a supraphysiological level (b); dkh-1(-/-) worm that expresses a high level of a distinct DKF isoform (DKF-2) (H. Feng and C. S. Rubin, unpublished observations) by transcribing a DKF-2-GFP transgene (c); WT C. elegans that expresses a high level of DKF-1-GFP (d); and a WT worm overexpressing a DKF-2-GFP transgene (e). Relative body length was measured at L4 and adult stages by ImageJ software; results are presented in B.

A

B

DKF-2 Rescue

DKF-2 Overexpression

DKF-1 Rescue

DKF-1 Overexpression

WT

L4

Adult

2 Days after L4

0

100

200

300

400

Worm length (arbitrary units)

DKF-1 is not activated by PKCs. Thr588 in the DKF-1 activation loop is critical for an 8–20-fold enhancement in kinase activity. However, treatment of cells with PKC inhibitors (at concentrations that fully suppress PKD activation by hormones and DAG and/or PMA) has no effect on PMA-stimulated activation of DKF-1. We conclude that DKF-1 is an example (perhaps the first) of a PKC-independent, DAG-activated protein kinase. Thus, DKF-1 may exert control over a subset of target effectors that receive no input from PKC-mediated signaling pathways. DKF-1-regulated signal transduction could also antagonize or synergize with signaling pathways governed by activated PKCs when cells containing both kinases initiate DAG production. Further investigation is needed to determine whether Thr588 is a target for auto- and/or transphosphorylation. In either case, the DKF-1 enzyme and viable DKF-1 null animals are key components of a potentially important model system that may simplify future analysis of PKC-independent, PKD-mediated cell regulation. This minimally explored topic is especially relevant in view of reports that document bone morphogenetic protein-induced, PKD-mediated activation of stress kinases (84), H2O2-promoted, PKD-regulated stimulation of the ASK1/JNK pathway (36), PKD2-controlled activation of NFκB activity downstream from Bcr-Abl (34) and PKD3-mediated basal glucose transport in myotubes (85). These processes proceed in the absence of PKC activation and without phosphorylation of Ser residues in the PKD activation loop.

Mammalian PKDs influence a diverse group of cell functions and pathologies, including extracellular signal-regulated kinase (ERK) and JNK signaling, induction of NFκB by stress, transport vesicle budding from the trans-Golgi network, vesicular trafficking, integrin recruitment at focal adhesions, chromatin organization, apoptosis, cardiac myocyte hypertrophy, and cancer cell invasiveness (19–21, 86, 87). Caveats and cautions apply when PKD physiological relevance is predicted principally from analysis of transfected, cultured cells. Inputs from PKD modulators (e.g. protein phosphatases, binding partners, DAG kinase, etc.), opposing or synergistic signaling pathways, redundancy introduced by other PKD isoforms, etc. can be compromised when supraphysiological levels of WT or mutant PKDs accumulate in transfected cells. Consequently, physiological effects elicited by PKD transgenes may reflect major or minor, unique or redundant, properly targeted or mislocalized signal transduction pathways. Moreover, the...
candidate PKD-controlled physiological processes listed above are also regulated by established (apparently) PKD-independent mechanisms as well. Thus, the observations summarized above provide invaluable guidance for further analysis of PKD physiological relevance, but definitive conclusions must be deferred until D kinases are linked to specific physiological functions in intact tissues and organisms. At present there are no published reports of experimental manipulation of PKD gene structure/expression in vertebrates in vivo.

We discovered that DKF-1 null C. elegans has a movement disorder (uncoordinated or unc) characterized by paralysis in the tail region. The phenotype is consistent with temporal and cellular patterns of DKF-1 expression and is reversed by restoration of normal DKF-1 content in animals carrying an integrated dki-1 transgene. Thus, an indispensable connection between a PKD family member and a tissue function is established in vivo. The precise molecular basis for the unc phenotype is not yet known. A speculative possibility is that DKF-1-mediated phosphorylation/regulation controls contractile protein or ion channel functions in posterior body muscle (or transcription factors that regulate expression of these proteins), thereby accounting for paralysis. Other scenarios consistent with paralysis include (a) pre synaptic dysfunction (e.g. lack of neurotransmitter release) in neurons or (b) post-synaptic aberrations in neurotransmitter receptors or downstream signaling proteins in muscle as consequences of the lack of DKF-1-catalyzed phosphorylation at neuromuscular junctions.

By exploiting authentic enhancer/promoter DNA and transgenesis, WT DKF-1 protein was modestly overexpressed in the appropriate cells and with normal kinetics during the nematode life cycle. A second stable phenotype was created; constitutively elevated expression and is reversed by restoration of normal DKF-1 content in animals carrying an integrated dki-1 transgene. Thus, an indispensable connection between a PKD family member and a tissue function is established in vivo. The precise molecular basis for the unc phenotype is not yet known. A speculative possibility is that DKF-1-mediated phosphorylation/regulation controls contractile protein or ion channel functions in posterior body muscle (or transcription factors that regulate expression of these proteins), thereby accounting for paralysis. Other scenarios consistent with paralysis include (a) pre synaptic dysfunction (e.g. lack of neurotransmitter release) in neurons or (b) post-synaptic aberrations in neurotransmitter receptors or downstream signaling proteins in muscle as consequences of the lack of DKF-1-catalyzed phosphorylation at neuromuscular junctions.

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