Protein kinase D (PKD) isoforms are effectors in signaling pathways controlled by diacylglycerol. PKDs contain conserved diacylglycerol binding (C1a, C1b), pleckstrin homology (PH), and Ser/Thr kinase domains. However, the properties of conserved domains may vary within the context of distinct PKD polypeptides. Such functional/structural malleability (plasticity) was explored by studying *Caenorhabditis elegans* D kinase family-1 (DKF-1), a PKD that governs locomotion in vivo. Phorbol ester binding with C1b alone activates classical PKDs by relieving C1-mediated inhibition. In contrast, C1a avidly ligated phorbol 12-myristate 13-acetate (PMA) and anchored DKF-1 at the plasma membrane. C1b bound PMA (moderate affinity) and cooperated with C1a in targeting DKF-1 to membranes. Mutations at a “Pro” position in C1 domains were inactivating; kinase activity was minimal at PMA concentrations that stimulated wild type DKF-1 ~10-fold. DKF-1 mutants exhibited unchanged, maximum kinase activity after cells were incubated with high PMA concentrations. Titration *in situ* revealed that translocation and activation of wild type and mutant DKF-1 were tightly and quantitatively linked at all PMA concentrations. Thus, C1 domains positively regulated phosphotransferase activity by docking DKF-1 with pools of activating lipid. A PH domain inhibits kinase activity in classical PKDs. The DKF-1 PH module neither inhibited catalytic activity nor bound phosphoinositides. Consequently, the PH module is an obligatory, positive regulator of DKF-1 activity that is compromised by mutation of Lys or Trp. Phosphorylation of Thr switched on DKF-1 kinase activity. Persistent phosphorylation of Thr (activation loop) promoted ubiquitination and proteasome-mediated degradation of DKF-1. Each DKF-1 domain displayed novel properties indicative of functional malleability (plasticity).

The protein kinase D (PKD) family of Ser/Thr protein kinases (PKD, PKD2, and PKD3) mediates transmission and targeting of signals carried by the second messenger, diacylglycerol (DAG) (1–3). PKD activation is correlated with enhanced NFκB-mediated gene transcription, oxidative stress responses, changes in cell adhesion, Golgi vesicle generation and trafficking, apoptosis, and T-cell activation and secretion. The functions of PKDs are governed by four conserved structural modules (4–7). Tandem C1a and C1b domains, located near the PKD N terminus, bind DAG or phorbol esters (e.g. phorbol 12-myristate 13-acetate (PMA)) that mimic DAG. A central PH domain inhibits kinase activity (8) but also ligates PKD-activating proteins (9–11). Catalytic activity of the C-terminal kinase domain is expressed when two Ser hydroxyl groups in the activation loop undergo trans phosphorylation (12, 13). DAG/PMA-activated protein kinase C (PKC) isoforms phosphorylate the PKD activation loop (1–3). Thus, PKDs are PKC effectors in signaling pathways governed by hormones or growth factors that activate phospholipases C or C, PKDs disseminate and diversify DAG/PKC signals by phosphorylating a distinct constellation of substrates that reside in plasma membrane, Golgi membranes, nucleus, mitochondria, and cytoplasm (1–3, 14–19).

PKDs are activated at the cell periphery (1, 20, 21). Subsequent release from plasma membrane, intracellular transit to organelles, phosphorylation of substrates, and quenching of kinase activity must be precisely orchestrated in time and intracellular space to produce integrated physiological responses to signals carried by DAG. Dissemination and integration of DAG signals are accomplished by the division of tasks among PKD domains. According to one model, C1b alone binds DAG/PMA at the cell surface (21), whereas C1a routes PKD to Golgi membranes (22). Nuclear import of PKD is governed by C1b, but the PH motif mediates nuclear egress (23). Activation loop phosphorylation (catalyzed by PKC) switches on PKD activity (12, 13). Physiological consequences of coupled PKC-PKD pathways are determined, in part, by the substrate specificity of PKDs.

A growing body of discordant evidence challenges current ideas about specific functions of individual PKD domains and, hence, the overall model. For example, recent studies show that: (a) both C1a and C1b (or C1a and C1b preceded by a complete N terminus) are essential for targeting PKD to Golgi membranes (24, 25); (b) C1a, not C1b, binds DAG (26); (c) the PH domain is a critical scaffold for a group of signaling proteins (PKCs, Gβγ subunits, tyrosine protein kinases, etc.) that activate PKDs (because these signaling proteins lack common, conserved domains/sequence motifs, it appears that the PH region is polyvalent and multifunctional (9–11, 27)); (d) PKDs can be activated in cytoplasm without translocation to plasma membrane (28, 29); and (e) PKDs regulate physiological functions in the absence of activation loop phosphorylation (30–33), a mode of regulation that depends upon tyrosine phosphorylation in the PH domain or Ser/Thr phosphorylation at sites distant from the kinase domain.

A possible explanation for observations that diverge from a unifying model is that domains comprising PKDs can accommodate more than one ligand and/or perform multiple tasks, thereby enabling functional flexibility (plasticity). Properties of multitasking domains could be further modulated by (a) cell-specific expression of interacting regulatory
molecules, (b) intramolecular binding with isoform-specific segments of PKDs, and/or (c) “induced” plasticity generated by physical interaction with regulatory proteins or higher order molecular complexes in organelles or cytoskeleton. Ultimately, exhaustive characterization of domains in mammalian PKD isoforms will provide stringent testing of these ideas.

An approach that synergizes with studies on classical PKDs, involves analysis of conserved and divergent properties of domains in a new member of the PKD family, DKF-1 (66). DKF-1 mediates DAG signaling in Caenorhabditis elegans, a model organism that shares conserved signaling molecules, mechanisms, and pathways with mammals. We discovered that DKF-1 has the domain composition and organization as well as the substrate specificity and amino acid sequence conservation expected for an authentic PKD isoform (66). DKF-1 is indispensable in vivo for neuromuscular coordination underlying locomotion. In addition, persistent elevation of DKF-1 content markedly reduces post-embryonic cell growth and overall organism size (66). Thorough studies on domain functions of DKF-1 may yield novel insights into conserved and divergent features of the C1α, C1β, PH, and kinase modules. DKF-1 can be studied facilely in vitro, in intact cultured cells, and in vivo. In principle, this strategy should promote progress in defining strictly conserved domain features, while simultaneously illuminating novel but physiologically crucial tasks governed and enabled by domain plasticity. Here we report that functional plasticity is superimposed on core conserved domain features. This enables the C1α, C1β, and PH domains of DKF-1 to support novel functions and mechanisms. In addition, we demonstrate that phosphorylation of Thr588 in the kinase domain provides a “switch” and “timer” mechanism that controls DKF-1 activity and concentration when cells receive transient or persistent DAG input signals.

**EXPERIMENTAL PROCEDURES**

DKF-1 *Down-regulation*—AV-12 (or HEK293) cells that stably express DKF-1 were incubated in the presence or absence of 1 μM PMA for 18 h (or for otherwise indicated time intervals). Cytosol and particulate proteins were isolated from mammalian cells as stated previously (34). Triton X-100 was added or omitted from cell lysis buffer as indicated in the legends for Figs. 2, 4, 7, and 8 and under “Results.” Proteins were size-fractionated by electrophoresis in a denaturing polyacrylamide (8% or otherwise indicated) gel as reported previously (35). 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 (1:1,000), and washed as indicated in previous studies (34, 36). Unless noted otherwise, each lane in Western blots received 30 μg of protein. Antigen-antibody complexes were visualized and quantified by recording enhanced chemiluminescence signals and utilizing image analysis software (ImageQuant, GE Healthcare) as reported previously (34, 36).

To study the effects of proteasome inhibitors on DKF-1 down-regulation, stably transfected AV-12 cells were preincubated with 30 μM MG132 or 10 μM lactacystin (a highly specific inhibitor of proteasome-mediated degradation) for 30 min. Subsequently, cells were maintained in medium containing one of the indicated inhibitors plus 1 μM PMA for time intervals provided in the legends for Figs. 7 and 8. Levels of DKF-1 and certain PKCs were estimated as described above.

**Deletion and Site-directed Mutagenesis**—Mutagenesis was performed with methods and strategies described in detail in several publications (37–39). All mutants were verified by DNA sequencing as reported in earlier studies (37).

Confocal Immunofluorescence Microscopy—AV-12 cells stably transfected with a DKF-1 transgene were grown on glass coverslips. Cells were fixed, permeabilized with saponin, and blocked as described previously (40). Next, samples were incubated sequentially with anti-DKF-1 IgGs (1:100 dilution) and 10 μg/ml fluorescein isothiocyanate (FITC)-conjugated goat IgGs directed against rabbit immunoglobulins. Cells were washed with phosphate-buffered saline, 0.2% Tween 20 between and after incubations. After air-drying, 15 μl of 50% glycerol in phosphate-buffered saline containing 1 mg/ml p-phenylene diamine (anti-bleaching agent) was placed on the specimens, and coverslips were mounted on slides. Fluorescence signals corresponding to antigen-antibody complexes were collected with a Bio-Rad MRC 600 laser-scanning confocal microscope (Image Analysis Facility, Albert Einstein College of Medicine) as described by Li and Rubin (40).

**Assay for DKF-1 Ubiquitinylation in Vivo**—A line of transgenic (TG) C. elegans that expresses DKF-1-GFP was created. Wild type (WT) or TG animals were suspended in 3 volumes of disruption buffer, 50 mM Hepes-NAOH buffer, pH 7.7, containing 50 mM potassium acetate, 1 mM EDTA, 0.25 M sucrose, 10 mM N-ethylmaleimide, and a mixture of protease inhibitors (10 μg/ml leupeptin, 5 μg/ml chymostatin, 3 μg/ml elastatin, and 1 μg/ml pepstatin A). Nematodes were disrupted in a French press using air at 5,000 p.s.i. All operations were performed at 4 °C. Cytosol was separated from total particulate proteins by centrifugation at 100,000 × g for 30 min. Pellets were extracted with 3 volumes of disruption buffer supplemented with 1% Triton X-100 to solubilize membrane proteins. Samples were centrifuged at 100,000 × g for 30 min and detergent-soluble membrane proteins were recovered in the supernatant solution. Rabbit polyclonal anti-GFP IgGs (Clontech) (1.5 μg) were added to samples containing 500 μg of total cytosolic or membrane proteins. Samples were incubated for 4 h before DKF-1-GFP-IgG complexes were precipitated with protein G-Sepharose 4B beads (GE Healthcare). After extensive washing with disruption buffer containing 0.5% Triton X-100, precipitated proteins were size-fractionated by electrophoresis in a denaturing 8% polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. To ensure that covalently bound ubiquitin was fully denatured, the membrane was incubated in 20 mM Tris-HCl buffer, pH 7.5, containing 6 M guanidine-HCl and 5 mM β-mercaptoethanol for 1 h. After washing the membrane six times with phosphate-buffered saline (10 min/wash), the blot was incubated sequentially with monoclonal anti-ubiquitin IgG (Zymed Laboratories Inc.) (1 μg/ml) and secondary antibody coupled to peroxidase. Ubiquitin-IgG complexes were visualized by using enhanced chemiluminescence methodology, and signals were recorded on x-ray film as described previously (36, 38). Samples (30 μg) of total cytosolic and Triton X-100-solubilized membrane proteins, derived from WT and TG C. elegans, were assayed for DKF-1-GFP content by Western immunoblot analysis (as described in Refs. 34 and 38) using monoclonal anti-GFP IgG (Roche Applied Science) as primary antibody.

**Immunoprecipitation and in Vitro Kinase Assays for Total DKF-1 or PKD**—Transfected cells were lysed in buffer containing 1% Triton X-100 as reported (34). Operations were performed at 4 °C. DKF-1 (or endogenous PKD) was isolated by immunoprecipitation with monospecific affinity-purified antibodies. (Tests for antibody cross-reactivity were negative.) After centrifugation (40,000 × g, 30 min) the supernatant solution (0.3 mg of protein) received 1 μg of anti-DKF-1 IgG (66) (or anti-PKD IgG (Santa Cruz Biotechnology)), and samples were incubated for 3 h. Next, 25 μl of protein G-Sepharose 4B beads was added, and incubations were extended 60 min. Subsequently, bead-bound immune complexes were washed three times with lysis buffer and twice with kinase buffer containing 25 mM Tris-HCl, pH 7.4, 5 mM MgCl₂ and...
Mechanistic and Functional Plasticity in PKD Domains

Detergent-soluble DKF-1 was purified from extracts of control and PMA-treated cells via immunoprecipitation. Subsequent kinase assays revealed that PMA (or DAG) elicits a substantial, stable increase (typically 8–20-fold) in DKF-1 catalytic activity in situ (Fig. 1C). Moreover, in the context of a shared intracellular milieu, both transgene-encoded, modestly expressed DKF-1 and endogenous PKD are activated to a similar degree (fold) by PMA (Fig. 1C). After activation in intact cells, DKF-1 expresses high level phosphotransferase activity (in vitro) that is not altered by inclusion or omission of phosphatidylerine, PMA, or detergent in the assay buffer. Parallel studies on HEK293 cells confirmed observations made using AV-12 cells (data not shown).

C1 Domains Mediate Accumulation and Activation of DKF-1 at the Cell Surface—Typical C1 domains are composed of ~50 amino acids and include a conserved Pro residue at position 11 (41–44). Pro11 stabilizes a molecular configuration of the Cys-rich, zinc-finger module that avidly binds DAG and/or PMA. The DKF-1 C1a finger contains the conserved Pro (Pro109). However, an atypical amino acid, Phe197, occupies a corresponding position in DKF-1 C1b. This lack of amino acid conservation and an absence of knowledge concerning functional consequences of incorporation of aromatic amino acids at the “Pro11” position were addressed experimentally to illuminate roles for C1 domains in DKF-1.

Cells expressing WT or mutant DKF-1 polypeptides were incubated for 10 min in the presence or absence of PMA. Subsequently, disrupted cells were separated into cytosol and total membrane fractions by centrifugation at 120,000 × g. All preparations of cytosol contained basal kinase activity that was not altered by phorbol ester. In contrast, accumulation of PMA-activated DKF-1 in membranes enabled independent determinations of "in vivo dose-response curves" for (a) phorbol ester activation of DKF-1 kinase activity (Fig. 2A) and (b) PMA-promoted transfer of DKF-1 protein from cytoplasm to cell surface (Fig. 2, B and C). The regulated pool of DKF-1 was isolated by first extracting membranes with buffer supplemented with 1% Triton X-100 and then by immunoprecipitation with affinity-purified IgGs. Subsequent kinase assays revealed that incubation of cells with 2 μM PMA elicited a 21-fold increase in catalytic activity of WT membrane-associated DKF-1 (Fig. 2A). The level of kinase activity evidently reflects saturation of a regulatory C1 binding site(s) by stimulatory ligand (PMA/DAG), because no further increases in DKF-1 catalytic activity were detected when higher concentrations of phorbol ester were tested.
Mechanistic and Functional Plasticity in PKD Domains

FIGURE 2. Effects of defective C1 domains on activation and translocation of DKF-1. AV-12 cells were stably transfected with WT or mutant DKF-1 transgenes as labeled on the graphs and plots. In A, cells were exposed to the indicated range of PMA concentrations for 10 min. Disrupted cells were fractionated into soluble cytosol (S) and particulate membrane (P) proteins as described under "Experimental Procedures." WT and mutant DKF-1 polypeptides were solubilized from membrane fractions by extraction with buffer containing 1% Triton X-100. After purification and precipitation with affinity-purified anti-DKF-1 IgGs, variant and WT DKFs were assayed for protein kinase activity. Phosphate incorporation into Synctide-2 peptide was calculated and plotted with Prism software (GraphPad). B and C show typical Western immunoblots that document recruitment (translocation) of WT or mutant DKF-1 proteins to membranes as a function of various concentrations of PMA (see "Experimental Procedures"). The time of exposure to phorbol ester was 10 min. These experiments were repeated twice, and similar results were obtained in each instance. Representative data are shown.

(data not shown). Approximately 130 nM external PMA is sufficient for half-maximal activation ($K_a$) of WT DKF-1 in intact cells (Fig. 2A). PMA-induced kinase activation (Fig. 2A) is directly proportional to the amounts of DKF-1 polypeptide recruited to membranes (Fig. 2B) over a range of phorbol ester concentrations that spans two orders of magnitude (0.01–2 μM PMA). Thus, the translocation of DKF-1 from cytoplasm to membranes and kinase activation are closely and quantitatively linked processes.

Substitution of Gly for Pro$^{109}$ generated a DKF-1 mutant (DKF-1[Gly$^{109}$]) that was inactive at 0.1 μM PMA and only minimally stimulated in cells incubated with 0.3 μM drug (Fig. 2A and C). Nevertheless, DKF-1[Gly$^{109}$] activity increased ~7-fold when cells were treated with 0.5 μM PMA. A maximal value of ~20-fold stimulation was achieved when the phorbol ester concentration was raised to 10 μM. The $K_a$ (apparent half-maximal activation) for DKF-1[Gly$^{109}$] increased 6-fold (relative to WT kinase) to ~800 nM. In contrast, replacement of Phe$^{197}$ with Gly in the C1b domain did not compromise DKF-1 activation or translocation to membranes (Fig. 2A, A and B). Instead, the mutation slightly increased (~2-fold) sensitivity of DKF-1[Gly$^{109}$] to PMA-mediated activation in situ ($K_a$ ~70 nM). The gain in sensitivity did not reflect a change in relationship between DKF-1 translocation and the degree of PMA-induced kinase activation. The fraction of total PMA-dependent kinase activity and total DKF-1 protein transferred to the cell periphery varied in parallel with PMA concentration for DKF-1[Gly$^{197}$], DKF-1[Gly$^{109}$], WT DKF-1 (see above), and a third DKF-1 variant (described below) that contains mutations in both C1 domains (Fig. 2, compare A with B and C).

The Gly$^{197}$ mutation in C1b was combined with the corresponding substitution in C1a (Gly$^{109}$) to generate a DKF-1[Gly$^{109}$,Gly$^{197}$] variant that undergoes limited activation/translocation (~10% of maximum) when stably transfected AV-12 cells are incubated with 0.5 μM PMA (Fig. 2, A and C). However, supplementation of culture medium with atypically high amounts of phorbol ester enabled recruitment and activation of doubly mutated DKF-1 at the cell surface. For example, exposure of cells to 10 μM PMA caused activation/translocation of a high proportion of DKF-1[Gly$^{109}$,Gly$^{197}$] (Fig. 2, A and C). Complete translocation to membranes and maximal 24-fold activation of DKF-1[Gly$^{109}$,Gly$^{197}$]-mediated catalysis were achieved at 35 μM PMA (data not shown). The apparent $K_a$ value for DKF-1[Gly$^{109}$,Gly$^{197}$] activation by PMA (~2500 nM) is 19-fold higher than the level of phorbol ester required for half-maximal stimulation of WT kinase.

Immunofluorescence microscopy enabled visualization of the location of WT and mutant DKF-1 polypeptides in situ (Figs. 1B and 3B). DKF-1 variants and WT DKF-1 were dispersed in the cytoplasm of unstimulated cells. A large proportion of WT DKF-1 and DKF-1[Gly$^{197}$] accumulated at the cell periphery after incubation with 0.2 μM PMA for 10 min (Figs. 1B and 3E). Replacement of conserved Pro$^{109}$ with Gly produced a kinase that was affected minimally by 0.2 μM phorbol ester (Fig. 3A, A and D). Some enrichment of DKF-1[Gly$^{109}$] was evident at sites of cell-cell contact, but the bulk of the signaling protein remained dispersed in cytoplasm. Doubly mutated DKF-1[Gly$^{109}$,Gly$^{197}$] was uniformly distributed in cytoplasm in the absence or presence of PMA (Fig. 3, C and F). However, both immunofluorescence microscopy (data not shown) and biochemical fractionation (Fig. 2, B and C) show that incubation of cells with a high concentration of PMA (≥10 μM) causes rapid and efficient accumulation of DKF-1[Gly$^{109}$] and DKF-1[Gly$^{109}$,Gly$^{197}$] mutants at the plasma membrane. Evidently, substitution of Pro$^{109}$ and/or Phe$^{197}$ with Gly does not grossly alter folding of the C1 domains or adjoining segments of the DKF-1 protein. Rather, these amino acids appear to be involved in mediating high (Pro$^{109}$) and low (Phe$^{197}$) affinity binding of plasma membrane DAG/PMA or other ligands by C1a and C1b, respectively.

FIGURE 3. C1 domains govern PMA-induced recruitment of DKF-1 to the cell periphery. AV-12 cells that stably express DKF-1[Pro$^{109}$ to Gly] (A and D), DKF-1[Pro$^{109}$ to Gly] (B and E), DKF-1[F197 to Gly] (C, F) or WT DKF-1 (see Fig. 2B) transgenes were incubated in the presence (D–F) or absence (A–C) of 1 μM PMA for 10 min. Cells were then washed, fixed, permeabilized, and incubated sequentially with anti-DKF-1 IgGs (1:100), and secondary antibodies that were tagged with FITC, as described previously. Results of immunofluorescence analyses are shown. Signals were obtained by confocal microscopy. Experiments were performed three times, and each replication yielded similar results.
C1 Domains Mediate Bombesin-induced Activation and Translocation of DKF-1—Physiological activators of PLC promote rapid but transient accumulation of DAG in the plasma membrane. Thus, pertinent questions are the following. 1) Is DKF-1 activated and routed to the cell periphery by a hormone that stimulates PLC? 2) If so, is association of DKF-1 with the membrane stable or transient? 3) Do C1a and C1b domains govern hormone-induced activation/translocation in accord with their roles in binding PMA (Figs. 2 and 3)? Transfected cells expressing the bombesin BB2 receptor (45, 46) were used to address these questions. Upon ligation of bombesin peptide, the BB2 receptor mediates loading of GTP onto the α subunit of Gq, a heterotrimeric G protein. Gqα3-GTP binds and activates PLCβ, thereby stimulating a transient increase in plasma membrane DAG content. Bombesin-occupied BB2 receptors efficiently and selectively stimulate DAG synthesis in a broad spectrum of cells and tissues (45, 46). Furthermore, bombesin-mediated activation of mammalian PKCs and PKDs is a well-documented phenomenon (21, 47).

Cells expressing DKF-1 and bombesin BB2 receptors were treated with various concentrations of bombesin peptide. After incubation for 2 min, DKF-1 was isolated from cell extracts via immunoprecipitation, and kinase activity was measured by following phosphorylation of Synthide-2 (Fig. 4A). Phosphotransferase activity of DKF-1 was elevated ~3-fold in cells exposed to 1 nM bombesin; maximal DKF-1 activity (typically 7–12-fold above the basal level) was obtained after incubation
Mechanistic and Functional Plasticity in PKD Domains

of cells with 100 nM hormone. The dose-response curve (Fig. 4A), high level induction, and $K_v$ value of 2.5 nM (for in situ activation) indicate that PLCβ-coupled BB2 receptors are potent activators of DKF-1 in the physiological range of bombesin concentration.

Experiments reported in a companion article (66) demonstrate that DKF-1 activation is PKC-independent but is suppressed by inhibition of PLC. DKF-1 kinase activity peaks 2 min after treatment with 100 nM bombesin (66). Subsequently, kinase activity declines by ~50% over 20 min. Restoration of basal DKF-1 activity is achieved by extending the incubation time to 150 min.

Bombesin treatment caused transient migration of DKF-1 from cytosol to cell membranes (Fig. 4B). Maximal association of DKF-1 with membranes was detected 2 min after addition of bombesin to cells. The amount of membrane-associated DKF-1 declined ~50% over the succeeding 3 min and reached a near basal level 15 min after initiation of hormone treatment (Fig. 4B). Immunofluorescence microscopy extended the analysis to intact cells (Fig. 4C). Bombesin promoted a striking optimal accumulation of DKF-1 at the cell periphery within 2 min. Partial redistribution of DKF-1 to cytoplasm was evident at 5 min and essentially completed 15 min after bombesin addition.

Results presented in Fig. 4, B and C, and the kinetics of DKF-1 activation (66) demonstrate that membrane translocation and activation of the kinase are tightly coupled during an early phase of cell responses to bombesin, a physiological activator. In contrast to the stable accumulation of PMA in membranes, DAG levels vary continuously during the course of bombesin treatment. Moreover, PKDs that contain a phosphorylated activation loop remain active in the absence of DAG. Thus, transient accumulation/activation of DKF-1 in membranes is followed by an “uncoupled” late phase in which a pool of activated DKF-partitions into cytoplasm and, perhaps, other intracellular compartments.

The dose-response curves shown in Fig. 4A reveal that amounts of DAG generated via BB2 receptors-Gαs-PLCβ are insufficient to activate a DKF-1 variant (DKF-1(Gly109,Gly197)) that contains substitutions at the Pro11 position in both C1 domains. Replacement of Phe197 with Gly in C1b generates a DKF-1 mutant that exhibits only a modest reduction in sensitivity to bombesin (Fig. 4B). PKD treatment (Fig. 4B). Immunofluorescence microscopy extended the analysis to intact cells (Fig. 4C). Bombesin stimulated a striking optimal accumulation of DKF-1 at the cell periphery within 2 min. Partial redistribution of DKF-1 to cytoplasm was evident at 5 min and essentially completed 15 min after bombesin addition.

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Properties of C1 domain evident in Figs. 2 and 3 reflect binding interactions with an invariant concentration of phosphor ester in membranes under near equilibrium conditions. The roles of C1a and C1b domains in mediating bombesin-induced DKF-1 activation and transient translocation mirror a much more dynamic situation in which on-off kinetics may dominate. Quantitative comparisons between C1 domain properties in PMA and bombesin-treated cells are also limited by: (a) continuously variable (principally declining after 2 min) cellular responses to physiological ligand with increasing time; (b) regulatory complexity introduced by the possibility that levels of $G_\alpha$, PLC, BB2 receptors or diacylglycerol kinase (and not bombesin concentration) may be rate-limiting for accumulation of DAG in plasma membrane; and (c) continuously variable partial loss and partial persistence of active DKF-1 after the kinase departs from plasma membrane. Despite these caveats and complexities, the results shown in Fig. 4, A-D, demonstrate that key observations derived from studies on PMA-activated WT and mutant DKF-1 proteins apply to mechanisms underlying DKF-1 activation by a physiological regulator (bombesin). Thus, C1a is essential for high sensitivity to bombesin; C1b appears to cooperate with C1a in promoting delivery of DKF-1 to a membrane surface that contains DAG and the constitutively available enzyme activator phosphatidylyserine. Introduction of mutations at sites in C1a and C1b that mediate PMA/DAG ligation generates a variant kinase that is not activated in the physiological range of bombesin concentrations.

A PH Domain Is Crucial for DKF-1 Activation; Neither Mutation nor Deletion of the PH Domain Impairs DKF-1 Translocation—PH domains direct a subset of signaling proteins to membranes by binding with phosphatidylinositol 3,4,5-trisphosphate ($PIP_3$) and/or $PIP_2$ metabolites (42, 48). A segment of DKF-1 (residues 279–407) has properties conserved in classical PH domains; the predicted secondary structure includes seven β-strands that generate an electrostatically polarized β-sandwich, which terminates in an α-helix. The predicted β-strands and α-helix in DKF-1 are aligned with corresponding regions of authentic PH domains in Fig. 5A. Mapping amino acids 279–407 from DKF-1 onto structures determined for PH domains of Bruton’s tyrosine kinase (Btk), PLCβ, and Akt-1 (49–51) identified Lys298 as an analog of a critical, positively charged residue (Lys/Arg) that engages phosphoinositides, other phospholipids, or membrane proteins via electrostatic interactions. Trp396 in DKF-1 corresponds to the only amino acid conserved in all PH domains (48), suggesting that this aromatic residue plays an indispensable role in PH domain integrity, folding, and/or function.

Mutation of either Lys298 or Trp396 (to Ala) or PH domain excision (∆PH) diminished in situ PMA-induced stimulation of DKF-1 catalytic activity by ~85–90% (Fig. 6A, lower panel, and B, lower panel). Likewise, treatment of cells with PMA conferred apparent “autophosphorylation” activity, an alternative indicator of kinase activation, on WT but not PH domain-deficient DKF-1 (Fig. 6B, upper panel). (At present, it is not known whether apparent DKF-1 autophosphorylation is controlled by intramolecular catalysis or an upstream protein kinase.) In contrast, PMA-induced translocation of cytoplasmic DKF-1 to membranes was not altered by PH domain mutation or deletion (Fig. 5B, lanes 1–12; accumulation of DKF-1 and DKF-1 mutant proteins in membranes is evident in lanes 4, 8, 12, and 16). Translocation and activation of WT DKF-1 are shown as positive controls (Figs. 5B, lanes 13–16, and 6A, lower panel). Confocal immunofluorescence microscopy confirmed that DKF-1 PH domain mutants were efficiently recruited to the cell periphery (data not shown). Thus, a fully functional PH domain is essential for expression of maximal PMA/DAG-stimulated DKF-1 kinase activity. However, the PH module is not involved in recruitment of DKF-1 from cytoplasm to plasma membrane.

PMA Elicits Down-regulation of DKF-1—Prolonged exposure of cells to PMA causes “down-regulation” of activated PKC isoforms via proteolytic degradation (52–54). This mode of regulation persists persistent, deleterious activation of DAG-controlled signaling pathways. Like PKCs, DKF-1 is sharply down-regulated (70–90% depletion) when stably transfected cells are treated with 1 μM PMA for 18 h (Fig. 7A, lanes 17 and 18, and B, lanes 1–3). Determination of a detailed time course for association of DKF-1 with cell membrane disclosed three temporal phases in the translocation-down-regulation process (Fig. 7A). An initial recruitment phase is both rapid and efficient (Fig. 7A, lanes 1–10). Substantial accumulation of DKF-1 at plasma membrane is readily detected after incubation of cells with PMA for only 30 s. Almost all of the DKF-1 polypeptide is located at the cell periphery when drug treatment is extended to 5 or 10 min (Fig. 7A, lanes 9–12, and Fig. 1B, panel 2). A second phase of DKF-1-membrane association is evident between 5 and 30 min. During this period, a high concentration of (presumably) activated DKF-1 remains tightly bound to membranes, and net degradation of the kinase is limited (Fig. 7A, lanes 9–14). A slow decline in DKF-1 content begins during the 30–60 min interval, and the amount...
Mechanistic and Functional Plasticity in PKD Domains

FIGURE 5. Effects of PH domain mutations on expression and intracellular targeting of DKF-1. A, the amino acid sequence of a predicted DKF-1 PH domain is aligned with corresponding regions in Btk, PLCδ, and Akt. The positions of documented or predicted β-strands and α-helices are shown above the amino acid sequences (bold arrows). Arg/Lys (bold and boxed) in the second β-sheet (β2) promotes phosphoinositide binding with Btk, PLCδ, and Akt-1. Trp (bold italic and boxed) in the C-terminal α-helix is conserved in all PH domains. B, a representative Western immunoblot containing cytosol (S) and membrane (P) proteins derived from AV-12 cells stably transfected with WT or mutated (K298A, W396A, and ΔPH) DKF-1 transgenes. Cells were incubated with 1 μM PMA (+) or vehicle (−) for 10 min as indicated. WT and mutant kinases were detected via Western immunoblotting using anti-DKF-1 IgGs and chemiluminescence as described previously (54, 38, 66). Note that the apparent M, values for WT DKF-1 and DKF-1 ΔPH are 81,000 and 66,000, respectively. Experiments were replicated as indicated in the legend for Fig. 3.

of phosphotransferase is markedly diminished (> 90% in Fig. 7A, lanes 17 and 18) after cells are incubated for 18 h with phosphor bet. (The complex intracellular itinerary of activated DKF-1 may include several membrane compartments. Detailed studies on this subject will be reported in another article). Chronic, long term exposure to PMA provoked similar decrements in levels of endogenous PKCe, PLCδ (Fig. 7B, lanes 2 and 3) and other DAG-controlled PKC isoforms (data not shown). Immunofluorescence microscopy demonstrated that the PKA-dependent decline in DKF-1 content occurs uniformly at the level of individual cells (Fig. 7C). Long term incubation of cells with ligands for endogenous, PLC-coupled hormone and growth factor receptors caused similar declines in DFK-1 content. A similar series of results was obtained when studies described above were performed with HEK293 cells that constitutively express a DKF-1 transgene (data not shown).

Down-regulation of DKF-1 Is Controlled by Ubiquitinylation and Phosphorylation—The decline in DKF-1 is partially blocked by a potent proteasome inhibitor, MG132 (Fig. 7B, lane 7). Lactacystin, a more specific inhibitor of proteasome-mediated degradation, is also highly effective in reducing the rate of DKF-1 proteolysis. For example, substantial protection of DKF-1 from proteasome-catalyzed digestion is clearly evident after cells are exposed to PMA and lactacystin for 6 h (Fig. 7D, lanes 3 and 4). These results suggested that DKF-1 and endogenous PKC isoforms (Fig. 7B) are probably degraded through a ubiquitin-triggered proteasome pathway. Robust ubiquitinylation of DFK-1 (mediated by endogenous ubiquitin ligases and ubiquitin) was visualized by Western immunoblotting when PMA-treated cells were co-incubated with MG132 (Fig. 8A, lanes 4–6) or lactacystin (data not shown).

The preceding results suggest that DKF-1 may be a target for ubiquitinylation in vivo. Like mammals, C. elegans expresses ubiquitin, ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzymes (E2), and ubiquitin ligases (E3) (55). E2-E3 complexes mediate the addition of ubiquitin (and polyubiquitin) to proteins by catalyzing synthesis of isopeptide bonds between the C terminus of ubiquitin and ε-amino groups of Lys residues in specific substrates. Moreover, ubiquitinated C. elegans proteins are recognized and degraded by a conserved 26 S proteasome complex. 75 of 76 amino acids are identical in human and C. elegans ubiquitins (55, 56). Thus, antibodies directed against human ubiquitin can be used to assess incorporation of C. elegans ubiquitin into DKF-1 protein in vivo.

Because it is difficult to determine and calibrate the amounts of pharmacological agents (e.g. PMA, MG132, lactacystin) that penetrate the nonporous collagen coat of C. elegans, we adopted a strategy developed by Burba et al. (57). A GFP-tagged full-length C.elegans protein is expressed in intact animals. This generates a pool of labile, ubiquitinylated fusion protein (in the absence of proteasome inhibitors) sufficient for detection by a combination of immunoprecipitation (anti-GFP) and Western blot analysis (anti-ubiquitin) with highly specific and sensitive IgGs. Burba and colleagues (57) also demonstrate that sites in the C. elegans protein are ubiquitinated, whereas the GFP tag is not modified.

We generated transgenic C. elegans that expresses DKF-1-GFP protein under the control of the authentic dkf-1 promoter. The DKF-1 fusion is fully functional because it rescues the DKF-1 null phenotype (66); it also enables efficient detection and selection of TG animals via immunofluorescence microscopy. WT and TG animals were disrupted in a French press, and extracts were separated into cytosol and membrane fractions by centrifugation. Membrane proteins were then solubilized with buffer containing 1% Triton X-100. Similar levels of DKF-1 fusion protein were detected in cytosol and membranes derived from TG animals (Fig. 8B). As anticipated, no signals were observed when corresponding fractions from WT C. elegans (no transgene) were assayed by probing a Western blot with IgGs directed against GFP (Fig. 8B). This provides a negative control and also documents antibody specificity. Only a small fraction of DKF-1 protein was expected to be in the labile, ubiquitinylated form at steady-state. Thus, DKF-1-GFP was enriched by immunoprecipitation prior to determination of ubiquitin content. Western immunoblot analysis revealed that DKF-1 is ubiquitinated in vivo (Fig. 8C). The modified kinase accumulated differentially in the membrane fraction. Anti-DKF-1 IgGs also bound (Fig. 8B) and precipitated (Fig. 8C) the same proteins as anti-GFP IgGs (data not shown).
Mechanistic and Functional Plasticity in PKD Domains

**DISCUSSION**

*Caenorhabditis elegans* DKF-1 is a novel, PMA/DAG-regulated Ser/Thr protein kinase that lies within the CAMK branch of the kinome (58). Evidence presented under “Results” and in an accompanying article (66) supports the inclusion of DKF-1 as an authentic, new member of the PKD family of protein phosphotransferases. DKF-1 shares several structural properties with PKCs. For instance, N-terminal C1 domains regulate phosphotransferase activity in a C-terminal kinase module. C1 domains mediate translocation of cytoplasmic PKD-1 by binding DAG or PMA at the cell periphery. Lipid-C1 interaction relaxes inhibitory constraints and enables activation loop phosphorylation and expression of DKF-1 catalytic activity. Recruitment to the cell periphery also juxtaposes the kinase with phosphatidylinositol, a DKF-1 co-activator that is enriched in the inner leaflet of plasma membrane. Thus, DKF-1 C1 domains link localized generation of lipid second messenger to triggering of effector protein phosphorylation. Binding and targeting properties embedded in C1 modules ensure that cells produce precisely quantified and integrated physiological responses to dynamic fluctuations in concentrations of hormones and growth factors that activate PLCs. Normally PLC...
Mechanistic and Functional Plasticity in PKD Domains

FIGURE 8. Phosphorylation of Thr18528 promotes ubiquitylation and subsequent degradation of DKF-1. AV-12 cells that express C-terminal myc-tagged DKF-1 were incubated with vehicle or 1 μM PMA for the indicated time period. To assess possible ubiquitylation of DKF-1, replicate batches of cells were simultaneously treated with 30 μM MG132 (see labeling in A). After incubation, cells were lysed in buffer containing 1% Triton X-100. Affinity-purified IgGs (1.5 μg) directed against myc peptide were added to samples containing 300 μg of detergent-soluble protein and IgG/DKF-1 complexes were isolated as described previously (34, 38). Immunoprecipitated DKF-1 was purified by denaturing electrophoresis and transferred to a polyvinylidene difluoride membrane (35). Western blots were probed with affinity-purified primary antibodies directed against DKF-1 (lanes 1–3) or ubiquitin (Zymed Laboratories Inc.) (lanes 4–6). Blots were developed as described previously (34, 38), and chemiluminescence signals, recorded on x-ray film, are shown. The positions of molecular mass standards are marked on the left. Note that anti-DKF-1 IgGs react weakly with ubiquitylated antigen (lanes 1–3). The smeared signal, corresponding to multiple forms of polyubiquitylated DKF-1 (lanes 5 and 6), is a typical result for proteins modified by ligation of ubiquitin to subsets of a large population of target lys ε-amino groups (52, 54). Immunoblotting (IB) and immunoprecipitation (IP) controls are described under “Experimental Procedures.”

A. IB: Anti-DKF-1
- + + - + + MG132
- - - - - - PMA 18 h

B. DKB-1-GFP

C. IP: Anti-myc

D. DKB-1 S858E
- - + - - - MG132
PMA 18 h

E. DKB-1 A858
- - + - - - Control
PMA

F. T58A
- - - - - + S P PM

G. T58A
- - - - - - S P PM

activation is transient. When association of activated kinases with plasma membrane persists, DKF-1 protein concentration is sharply reduced by enhanced proteolytic degradation (Figs. 7 and 8). Expansion of the PKD family by addition of DKF-1 prompted examination of functions of apparently equivalent structural domains. Does ordered incorporation of C1, PH, and kinase domains into a polypeptide chain configure a signaling enzyme with invariant biochemical properties? Alternatively, are homologous PKD structural domains inherently malleable (“plastic”) and able to subserve different functions and mechanisms within the context of distinct polypeptides encoded by various PKD genes? Igleisas et al. (59) reported that C1b accounts for all phorbol ester binding activity. Thus, DKF-1 C1a links ligand binding to enzymatic catalytic activity. However, substitution of Pro109 to Ala (C1a) and Pro287 with Gly (the critical Pro11 sites in C1 domains (41, 43)) yielded PKD variants that express maximal kinase activity in the absence of ligands (60). Phorbol ester binding activity was extinguished in the mutant Pro287 with Gly (C1a), or substitution of both Pro155 (C1a) and Pro287 with Gly (the critical Pro11 sites in C1 domains (41, 43)) yielded PKD variants that express maximal kinase activity in the absence of stimuli (60). Phorbol ester binding activity was extinguished in the mutant PKD lacking both Pro155 and Pro287 (8). A key conclusion is that the C1a-C1b region directly suppresses catalytic activity of PKD in manner analogous to the pseudosubstrate site in PFKs (59, 60).

We discovered substantial variations in functions and mechanisms associated with “hallmark” domains of the PKD family. For instance, both C1 modules in DKF-1 bind PMA (Fig. 2). C1a ligates phorbol ester with high affinity (apparent K_a ~ 70 nM) and is the dominant mediator of DKF-1 accumulation at the cell periphery. Translocation to plasma membrane is essential for (and synchronous with) expression of DKF-1 catalytic activity. Thus, DKF-1 C1a links ligand binding to enzymatic catalytic activity and enables phosphorylation of effector proteins co-localized with the kinase at membranes or adjacent cytoskeleton. When C1b alone is compromised by mutation (Phe197 to Gly), the apparent affinity of DKF-1 for PMA doubled. However, substitution of Phe197 with Gly synergistically decreased affinity for PMA (K_a increases from ~800 nM to ~2500 nM) when coupled with a partial loss-of-function mutation (Pro109 to Gly) in the C1a module. Consequently, DKF-1 C1b sequesters PMA with low affinity. Nevertheless, the doubly mutated C1a-C1b binding region directs catalytically competent DKF-1 to plasma membrane when PMA levels are elevated to the 5–35 μM range. Because C1b is covalently tethered to C1a, its accessibility to PMA is greatly enhanced when C1a docks at the cell surface. DKF-1 C1b may cooperate with the partner C1a domain by providing additional binding energy when excess PMA/DAG is
Mechanistic and Functional Plasticity in PKD Domains

available at the membrane. It is also possible that C1b mediates initial contact of DKF-1 with PMA/DAG in membranes, whereupon a conformational change permits previously occluded C1a to anchor the kinase with high binding affinity (see Oancea and Meyer (61) and Oancea et al. (62) for data and discussions supporting this scenario). WT C1b may limit net avidity of DKF-1 for DAG by acting as a modest C1a competitor when PMA/DAG concentrations are low. This would enhance the release of activated DKF-1 from plasma membrane when cell surface DAG levels decline. DKF-1 may remain active for minutes to hours in the absence of C1 ligand (1, 28). Consequently, hormonal signals can be disseminated to additional effectors when dissociated, active DKF-1 binds with DAG or docking proteins at various intracellular membranes.

Amino acids corresponding to Pro155 and Pro287 in mammalian PKD were mutated to Gly in DKF-1. DKF-1(Gly109) and DKF-1(Gly109,Gly197) are not constitutively active. On the contrary, their low basal catalytic activities were not altered by incubating cells with 100 nM PMA, which induces half-maximal activation of the WT kinase. Broad range dose-response curves revealed that the DKF-1 variants had sharply diminished sensitivity to PMA. Single amino acid substitution in one (C1a) or both C1 domains caused 6- and 19-fold increases, respectively, in $K_{\text{m}}$ values for PMA-induced kinase activation. However, DKF-1 mutants exhibited near WT levels of kinase activity when cells were exposed to high levels of PMA (Fig. 2). Recruitment of WT and mutant DKF-1 proteins to the membrane and kinase activation were in synchrony at all PMA concentrations. Thus, core structural features required for the accommodation of PMA molecules within C1 domains are retained in DKF-1 mutants. C1 domains (principally C1a) are highly positive regulators of DKF-1 catalytic activity. In addition, Pro109 and Phe197 have been identified as crucial amino acids that together govern high affinity binding of PMA by DKF-1.

The discoveries that (a) translocation and activation of DKF-1 are directly proportional at various PMA concentrations and (b) C1 domain mutations do not change basal DKF-1 catalytic activity indicate that DKF-1 and PKD are controlled in fundamentally different ways. This may reflect plasticity in C1 domains. In DKF-1, C1a is dominant; C1a and C1b cooperatively promote kinase activation, the C1 region does not suppress catalytic activity, and the C1 domains enable kinase activation by routing DKF-1 to a pool of lipid activator in membranes. In PKD, C1b is dominant; C1b alone binds phorbol esters, the C1 region directly and potently inhibits PKD phosphotransferase activity, and C1-mediated delivery of PKD to the cell periphery relieves pseudosubstrate-like inhibition of the kinase. Thus, the positive mode of DKF-1 regulation by PMA-occupied C1 domains diverges markedly from the negative suppression model developed for PKD.

Accumulation of DKF-1 at plasma membrane is obligatory for kinase activation. Membrane-bound DKF-1 is evidently the physiologically relevant target for concurrent or subsequent trans and/or cis phosphorylation that generates a conformation which supports maximal phosphotransferase activity. Because kinetics and levels of cell surface accumulation of WT and “kinase dead” DKF-1 proteins are indistinguishable, it is also apparent that targeting of DKF-1 to plasma membrane can precede enzyme activation. Moreover, routing of DKF-1 to the cell periphery is not dependent on its intrinsic kinase activity.

Experimental conditions may account for some differences between the functions of C1 modules in DKF-1 and PKD. PKD C1 domains have been studied in transiently transfected cells (59, 60). This procedure can generate supraphysiological levels of recombinant protein in the subpopulation of cells that internalize exogenous expression vector. Studies on phorbol dibutyrate binding by PKD have been performed with individual, recombinant C1 domains that were removed from the context of the intact PKD polypeptide. In contrast, cloned, stably transfected cells that uniformly produce modest, near physiological amounts of enzyme were used for studies on DKF-1. Other investigators have documented functional complexity and versatility of C1 domains. Baron and Malhotra (26) report the binding of DAG with the PKD C1a module. This domain guides PKD to the cytoplasmic surface of Golgi membranes (22). Stable association of PKD with plasma membrane is facilitated by direct binding of $G_{\alpha_1}$ with C1b (62). In addition, Hauser et al. (24) found that an N-terminal segment cooperates with both C1 domains to achieve proper intracellular targeting of PKD.

PH domains are 15-kDa structural modules (7 β-strands preceding an α-helix) that mediate binding of PIP$_3$ and some PIP$_3$ metabolites with certain signaling proteins (48). For example, Akt is recruited to plasma membrane by its PH domain when PIP$_3$ is generated by activated phosphatidylinositol 3-kinase (64). However, ~90% of proteins that contain PH domains, including PKDs, do not bind PIP$_3$ or other phosphoinositides (65). PH domain deletion or mutation of a single critical amino acid (Arg447 to Cys or Trp538 to Ala in PKD) within the domain generates PKD variants with high level, constitutive kinase activity (8). Thus, the PH domain evidently inhibits PKD catalytic activity in the absence of DAG in situ. Inhibition may be due to direct steric occlusion or distortion of the PKD catalytic cleft by a segment of the PH module. PKC isoforms (e.g. PKCs ε and η) that phosphorylate and activate PKD associate with the PH domain (11). By subunits of heterotrimeric G proteins and H$_2$O$_2$-activated tyrosine protein kinases (Src/Abl pathway) promote PKD activation by binding or phosphorylating (Tyr463) the PH module (10, 67). Thus, the PKD PH module may be a malleable multitasking scaffold that organizes signaling proteins.

The DKF-1 PH domain does not ligate phosphoinositides. However, its role in regulating DKF-1 kinase activity is inverted relative to PH module function in PKDs. PH domain deletion or mutation of amino acids (Lys398 or Trp596) corresponding to Arg447 or Trp538 in PKD generates defective DKF-1 proteins that have low basal activity and are poorly activated by PMA. Thus, the DKF-1 PH domain provides a striking example of functional flexibility/plasticity in a conserved structural module that is embedded in all PKD polypeptides. In DKF-1 the PH module is an essential, positive modulator of kinase activity (Fig. 6). A reasonable speculation is that the PH domain (with critical contributions from Lys398 and Trp596) has an indispensable structural role in supporting ligand (PMA/DAG, phosphatidylserine)-induced remodeling of DKF-1 structure to generate an active conformation in the catalytic cleft. Recent reports demonstrate that PH domains in guanine nucleotide exchange factors for Rac, Cdc42, and RhoA are essential for GTP loading onto p21 G proteins (63, 68). GTP loading activity is enhanced by conformation-specific binding between portions of the PH domain and flexible regions of the exchanger active site. Phosphoinositide ligation is not required for these interactions (63, 68). The DKF-1 PH domain may function in an analogous manner. Determination of the molecular basis (e.g. interaction with various partner proteins) for divergent properties of PH domains among PKD isoforms is a high priority topic for further analysis.

The preceding observations and a companion report (66) yield insights that enable formulation of a model for DKF-1 activation-inactivation. 1) DKF-1 translocates to plasma membrane when DAG
is elevated at the inner surface of the bilayer, and 2) A DAG-induced conformational change enables phosphorylation of Thr588 in the activation loop. 3) In concert, DAG binding with C1 domains (predominantly C1a), Thr588 phosphorylation, and the protein configuration within the PH domain create a conformation that allows expression of maximal kinase activity. 4) Activated DKF-1 migrates from the cell periphery to various intracellular locations to engage additional substrate/effector proteins. 5) Negative regulation intervenes to prevent prolonged, potentially toxic, hyperphosphorylation of DKF-1 substrates. Persistence of phosphate on Thr588 targets activated DKF-1 for ubiquitinylation and degradation by the 26 S proteasome.

The final point in the model was explored further by substituting Thr588 with Glu to mimic negative charge associated with phosphorylation of the DKF-1 activation loop. Stably transfected, unstimulated cells contain a low concentration of DKF-1(Glu588) compared with lev-

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