A Novel Conserved Domain Mediates Dimerization of Protein Kinase D (PKD) Isoforms

DIMERIZATION IS ESSENTIAL FOR PKD-DEPENDENT REGULATION OF SECRETION AND INNATE IMMUNITY

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Protein kinase D (PKD) isoforms are protein kinase C effectors in signaling pathways regulated by diacylglycerol. Important physiological processes (including secretion, immune responses, motility, and transcription) are placed under diacylglycerol control by the distinctive substrate specificity and subcellular distribution of PKDs. Potentially, broadly co-expressed PKD polypeptides may interact to generate homo- or hetero-multimeric regulatory complexes. However, the frequency, molecular basis, regulatory significance, and physiological relevance of stable PKD-PKD interactions are largely unknown. Here, we demonstrate that mammalian PKDs 1–3 and the prototypical Caenorhabditis elegans PKD, DKF-2A, are exclusively (homo- or hetero-) dimers in cell extracts and intact cells. We discovered and characterized a novel, highly conserved N-terminal domain, comprising 92 amino acids, which mediates dimerization of PKD1, PKD2, and PKD3 monomers. A similar domain directs DKF-2A homodimerization. Dimerization occurred independently of properties of the regulatory and kinase domains of PKDs. Disruption of PKD dimerization abrogates secretion of PAUF, a protein carried in small trans-Golgi network-derived vesicles. In addition, disruption of DKF-2A homodimerization in C. elegans intestine impaired and degraded the immune defense of the intact animal against an ingested bacterial pathogen. Finally, dimerization was indispensable for the strong, dominant negative effect of catalytically inactive PKDs. Overall, the structural integrity and function of the novel dimerization domain are essential for PKD-mediated regulation of a key aspect of cell physiology, secretion, and innate immunity in vivo.

The membrane-embedded second messenger diacylglycerol (DAG) mediates actions of many hormones and other stimuli by activating two classes of PKC isoforms (1, 2). Conventional PKCs α, βI, βII, and γ are stimulated by DAG and Ca2+ in concert; novel PKCs δ, ε, η, and θ are activated by DAG alone. PKCs regulate many aspects of cell physiology by activating members of another family of Ser/Thr kinases, collectively named PKD (3–6). Like PKCs, PKDs have a C-terminal kinase domain that is preceded by C1a and C1b regulatory domains, which bind DAG and a pharmacological activator, phorbol 12-myristate 13-acetate (PMA). C1 domains mediate translocation of PKDs and PKCs from cytoplasm to DAG/PMA-enriched membranes. DAG/PMA-activated PKC phosphorylates the PKD activation loop (A-loop), thereby switching on catalytic activity of the co-recruited D kinase. Thus, PKDs are PKC effectors in regulatory cascades.

PKDs diversify DAG/PKC signaling by acting on effectors in plasma membrane, the nucleus, cytoplasm, F-actin cytoskeleton, and cytoplasmic surfaces of mitochondria and Golgi membranes. Because PKCs and PKDs phosphorylate different substrates (3, 4, 7), D kinases place distinct effectors and physiological processes under DAG control. For instance, PKDs mediate pro-survival signaling induced by oxidative stress (8); control fission of secretory vesicles from the trans-Golgi network (TGN) (9–13); regulate cofilin-mediated F-actin dynamics underlying cell motility (14, 15); modulate proliferation and intensity of signal transduction in T cells (6, 16, 17); de-repress gene transcription in heart, skeletal muscle, and immune cells by phosphorylating type IIa histone deacetylases (HDACs) (18–22); elicit expression of ~85 genes in Caenorhabditis elegans intestine that coordinately defend animals against bacterial pathogens (23); and enable associative learning in C. elegans by simultaneously transmitting environmental signals to a neuronal circuit and the intestine (24). Dysregulation of PKDs contributes to cancer phenotypes, cardiac hypertrophy, and heart failure (18, 19, 25–27).

Three genes encode mammalian PKD1, PKD2, and PKD3, which contain conserved regulatory and catalytic domains (3, 4). PKDs are broadly expressed, but levels of individual isoforms vary with cell type. In principle, regulatory properties,
substrate/organelle specificity, stability, and functions of PKDs may be markedly affected by formation of complexes containing multiple PKD polypeptides. However, knowledge of the ability of PKDs to oligomerize and functional and regulatory consequences of PKD-PKD interactions is limited and contradictory.

In pioneering studies, Malhotra’s group (28) reported that PKD2-PKD3 dimers control constitutive secretory vesicle biogenesis and fission at the TGN of HeLa cells. The Storz lab (29) subsequently observed that a PKD2-PKD3 complex in HeLa and MDA-MB-468 cells regulates F actin-based, directed motility. These investigations suggest heteromultimeric PKDs may play important roles in coupling DAG signals to regulation of key aspects of cell physiology. Conclusions derived from these narrowly focused studies are consistent with the data, but their general applicability is constrained by the following considerations. Only a fraction of HeLa cell D kinases was detected in PKD2-PKD3 complexes (28, 29). Activities and functions of heteromeric and non-heteromeric PKDs were not segregated and separately analyzed. Thus, it is not definitively known whether critical PKD activity was restricted to heteromeric complexes. Likewise it is not known whether PKD2-PKD3 complexes are dimers or higher order oligomers or whether PKD polypeptides are normally distributed among monomers and multimers or restricted to a single structural species. The possibility that PKD homo-oligomerization is crucial for function has not been studied. Nothing is known about the ability of broadly expressed PKD1 to self-associate or bind with other PKDs. Importantly, intrinsic structural features that mediate assembly of PKD-PKD complexes have not been elucidated.

In BON neuroendocrine cells, vesicle biogenesis at the TGN and chromogranin A secretion were regulated solely by PKD2 (11). PKD1 or PKD3 depletion had no effect. DT40 B lymphocytes express PKDs 1 and 3. Deletion of either PKD gene had no effect on antigen- or PMA-induced phosphorylation and nucleus to cytoplasm translocation of HDAC5 and HDAC7 (22). Disruption of both PKD genes abrogated HDAC phosphorylation/translocation, but expression of either a PKD1 or PKD3 transgene rescued the PKD null phenotype. Thus, in these studies, hetero-oligomeric PKDs were not required to control TGN vesicle fission or gene transcription.

Embryogenesis proceeded normally in mice globally lacking PKD2 activity (6). PKD3-deficient mice exhibited only a mild skeletal deficit. Thus, Golgi vesicle fission, directed cell migration, and other PKD-modulated processes evidently progressed properly in a vast array of cells in the absence of heteromeric PKD2-PKD3. C. elegans PKDs, DKF-2A and DKF-2B, play critical roles in innate immunity and associative learning (23, 24). Because DKF-2A and -2B are expressed in a mutually exclusive manner in intestine and neurons, respectively, hetero-oligomeric kinases are not essential for in vivo regulation in the nematode model.

Overall the frequency, mechanism, regulatory significance, and physiological relevance of PKD-PKD interactions remain largely unknown. To determine whether PKD oligomerization plays a central role in coupling DAG signals to the regulation of cell/tissue physiology, it is necessary to address several pertinent questions: Is widely expressed PKD1 capable of oligomerization? Are native PKDs 1–3 predominantly monomers or are D kinases incorporated into hetero- and homodimers or larger multimers? If PKDs oligomerize, what structural features mediate PKD-PKD interactions? Are scaffold proteins essential to guide oligomerization? What are the consequences of disrupting PKD oligomerization in the context of cell physiology and with respect to PKD-mediated regulation of function in vivo?

We demonstrate that human PKDs 1–3 and the C. elegans PKD, DKF-2A, are homo- or heterodimeric in cell extracts and intact cells. We discovered a novel, conserved domain that mediates dimerization of PKD1, 2, and 3 monomers. A similar domain directs homodimerization of DKF-2A. Disruption of PKD dimerization abrogates secretion of PAUF, a protein carried in small TGN-derived vesicles. Disruption of DKF-2A homodimerization in C. elegans intestine impaired the immune defense of the animals against a bacterial pathogen. Finally, dimerization is indispensable for strong, dominant negative effects of catalytically inactive PKDs.

Results

DKF-2A Polypeptides Assemble into Homo-oligomeric Complexes; a Novel Domain Mediates Incorporation of PKD Monomers into Multimers—The possibility that PKD monomers interact to create homo-oligomeric kinases has not been systematically investigated. C. elegans provides a good model for analysis because the animals selectively express two prototypic PKDs, DKF-2A and DKF-2B accumulate in intestinal epithelial cells and a subset of neurons, respectively (23, 24). Thus, DKF-2A and DKF-2B will function either as monomers or homo-oligomers. Transgenes encoding HA- or FLAG-tagged DKF-2A and DKF-2B were expressed in HEK293 cells. PKD-PKD interactions were assessed by co-immunoprecipitation and Western immunoblot analysis (Fig. 1A). Both the 120-kDa WT DKF-2A and constitutively active DKF-2A E (A-loop serines 925 and 929 substituted with phosphomimetic Glu) were incorporated into homomultimers. Dimers are not distinguished from higher order complexes in these assays. In contrast, the 98-kDa DKF-2B polypeptide did not homo-oligomerize or bind with DKF-2A or DKF-2A EE.

DKF-2A and DKF-2B have identical regulatory and kinase domains, but amino acid sequences of their N-terminal regions are markedly divergent. Thus, we examined the ability of the unique N-terminal segment of DKF-2A (amino acids 1–319) to mediate oligomerization. DKF-2A truncation mutants lacking amino acids 1–100, 1–199, 1–215, or 1–227 avidly associated with full-length DKF-2A (Fig. 1, B and C). Elimination of 249 or 404 N-terminal residues ablated complex formation (Fig. 1C). These observations place the N-terminal boundary of a novel PKD oligomerization domain (OD) in a segment of DKF-2A encompassing amino acids 227–249. The OD was more precisely mapped by assaying binding properties of internally deleted DKF-2A mutants (Fig. 1, B and D). Excision of amino acids 228–249 elicited an 80% decrease in complex formation. Binding interactions were suppressed further but still detected in DKF-2A lacking residues 228–271. Elimination of a 73-residue segment of DKF-2A (Δ228–300) generated a D kinase that was virtually incapable of engaging in homomultimeric com-
A novel N-terminal structural module in C. elegans DKF-2A mediates PKD-PKD interactions that generate a homomultimeric kinase. HEK293 cells were co-transfected with transgenes encoding HA or FLAG-tagged DKF-2A (designated 2A), DKF-2B (2B), or constitutively active DKF-2A EE (2A*). After 48 h, the cells were lysed, and PKDs were precipitated from cell extracts by adding anti-HA IgGs and protein G-Sepharose 4B beads. Precipitated and co-precipitated proteins were analyzed by SDS-PAGE and Western blotting, using anti-HA and anti-FLAG IgGs, respectively. The same IgGs detected epitope-tagged PKDs in blots of cell extracts. Tubulin was monitored as a loading control. A schematic diagram depicts mutant DKF-2A proteins and DKF-2A domains that were assayed for oligomerization activity. In C–G, transfection, lysis, immunoprecipitation, and Western blot analysis were performed as indicated in A and “Experimental Procedures.” C, FLAG-DKF-2A or a FLAG-DKF-2A truncation mutant was co-expressed with HA-DKF-2A. D, FLAG-DKF-2A or a FLAG-tagged DKF-2A internal deletion mutant was co-expressed with HA-DKF-2A. E, a DKF-2A 311–545-mCherry fusion protein, which contains the C1a and C1b DAG binding sites, and HA-DKF-2A were co-expressed. The cells were treated with 1 μM PMA or vehicle for 20 min prior to lysis. F, FLAG-DKF-2A or a FLAG-DKF-2A truncation mutant was co-expressed with the DKF-2A 1–319-GFP fusion protein. G, cells were co-transfected with a fixed amount of HA-DKF-2A transgene and increasing amounts of DKF-2A 1–319-mCherry fusion transgene. IB, immunoblot; IP, immunoprecipitation; PH, pleckstrin homology.
plexes. The absence of PKD-PKD interactions in a mutant lacking residues 250–319 directly demonstrated that amino acids located beyond the critical 228–249 segment were also essential for PKD self-association.

To determine whether DKF-2A regulatory and catalytic domains play auxiliary roles in multimerization, we created transgenes in which (a) the N-terminal segment of DKF-2A (residues 1–319) was fused to GFP and (b) C1a and C1b domains (residues 311–545) were coupled to mCherry red fluorescent protein (Fig. 1B). Although C1a and C1b domains fold and function normally in the absence of surrounding N- and C-terminal segments of signaling proteins (30–33), the DKF-2A 311–545-mCherry fusion protein failed to bind with either unstimulated or PMA-PKC-activated DKF-2A (Fig. 1E). In contrast, a DKF-2A 1–319-GFP chimera formed complexes with WT PKD and N terminally truncated D kinases (Δ100, Δ199) that retain the OD (Fig. 1F). Deletion of the critical 228–249 segment of DKF-2A abrogated co-immunoprecipitation with DKF-2A 1–319-GFP.

A modest amount of WT DKF-2A was titrated with increasing concentrations of DKF-2A 1–319-mCherry fusion protein in transfected cells. As the concentration of fusion transgene increased over a 5-fold range, the amount of co-complexed, full-length DKF-2A reached a maximum (Fig. 1G). In parallel, DKF-2A became undetectable in the post immunoprecipitation supernatant solution. Thus, the fusion protein suppressed DKF-2A homo-oligomerization by competitively interacting with the DKF-2A OD. Transfection with higher concentrations of DKF-2A 1–319-mCherry transgene (0.5 µg; Fig. 1G) caused fusion protein expression to exceed the capacity of the anti-mCherry IgGs, thereby artifactually reducing the level of WT DKF-2A precipitated and enabling detection of both binding partners in the supernatant after immunoprecipitation.

Overall, the results indicate that DKF-2A contains a novel domain (OD), encompassing amino acids 228–319, that mediates assembly of PKD homodimers or higher order multimers. The OD apparently folds and functions independently of the conserved DAG binding and kinase domains of DKF-2A. Multimer formation was not affected by conformational changes caused by mimicking A-loop phosphorylation.

A Conserved N-terminal Domain Promotes Homo- and Hetero-oligomerization of Human PKDs—Amino acids comprising the DKF-2A OD aligned with a sequence near the N terminus of PKD2 (Fig. 2A). The 93-residue PKD2 segment is similar in size to the DKF-2A OD and contains 31 identical amino acids (33%) and 24 highly conservative substitutions (Arg/Lys, Asp/Glu, Leu/Ile, etc.), yielding an overall similarity of 59%. The domain identified in PKD2 is highly conserved in PKDs 1 and 3. For example, the corresponding region of PKD1 is 78% identical and 90% similar to the putative PKD2 OD (Fig. 2A). We explored the possibility that the conserved domain mediates formation of homo- and/or hetero-oligomeric mammalian PKDs.

HA- and FLAG-tagged PKDs were co-expressed in HEK293 cells. Robust signals obtained with anti-FLAG IgGs revealed co-precipitated binding partners of HA-tagged PKDs (Fig. 2B). PKD1 and PKD2 efficiently associated into homo-oligomeric complexes (Fig. 2B, upper panel, lanes 2 and 6). HA-PKD3 was poorly expressed, but homomultimers were detected. Each PKD was also incorporated into heteromeric complexes (Fig. 2B, lanes 3–5 and 7–9).

Deletion of amino acids 1–66 in PKD2, which corresponds to the DKF-2A Δ249 mutation, reduced binding with WT PKD2 by 90% (Fig. 2C). The more extensive PKD2 Δ138 deletion, which completely removed the putative OD, abolished the ability of truncated PKD2 to participate in homo-oligomeric complexes. Conversely, expression of a transgene encoding PKD2 amino acids 1–146 fused with mCherry produced a chimeric protein that avidly bound PKD1, PKD2, or PKD3 (Fig. 2D).

Co-immunoprecipitation assays also revealed that the putative PKD3 OD (residues 1–142) strongly associated with intact PKDs 1–3 (Fig. 2E). To verify and further test the generality of our observations, we created PKD1 and PKD1 EE mutants lacking the putative OD by internally deleting amino acids 50–125 (analogous to DKF-2A Δ228–300). PKD1 EE A50–125 did not associate with PKD1, PKD1 EE, or PKD2 (Fig. 2F).

A fusion protein containing the C1a and C1b domains of PKD2 but lacking the predicted OD, did not form complexes with PKDs 1–3 (Fig. 2G). This finding and the results presented above suggest that the DAG/PMA binding modules are not involved in PKD oligomerization.

Our studies show that PKDs 1–3 assemble into stable homo- or heteromultimeric complexes. A conserved but previously uncharacterized OD consisting of ~92 amino acids directs incorporation of PKD monomers into multimers. Multimerization is achieved by interactions between the N termini of PKDs. The recombinant OD, which functions independently and persists in cells, avidly associates with PKDs 1–3. Thus, over-expressed OD could be used to selectively disrupt PKD oligomerization.

PKDs Oligomerize in Intact Cells—It was possible that multi-PKD complexes assembled when previously segregated PKDs intermingled after detergent mediated cell lysis. Thus, we used cell-permeable, irreversible cross-linkers to assay PKD-PKD association within cells. 1,5-Difluoro-2,4-dinitrobenzene (DFDNB) and disuccinimidyl suberate (DSS) are homo-bifunctional compounds that react with Lys e-amino groups. DFDNB has a short spacer arm that enables selective cross-linking of protein subunits, whereas a longer spacer in DSS facilitates cross-linking of both subunits and binding partners.

Transfected cells expressing HA- and FLAG-tagged PKDs were incubated with cross-linker before lysis. Treatment with DFDNB or DSS sharply diminished the content of monomeric DKF-2A (Fig. 3A). Simultaneously, covalently linked, high molecular weight DKF-2A oligomers accumulated. Endogenous PKD2 was also incorporated into high molecular weight complexes (Fig. 3B). Like PKDs, PKCa and PKCδ contain tandem C1a and C1b regulatory domains and a C-terminal kinase module. However, PKCs are monomeric proteins. Levels of PKCa and PKCδ monomers were not affected by incubating cells with DFDNB or DSS (Fig. 3B); neither PKC appeared in a high molecular complex. The results confirm the validity and specificity of the approach.

PKD multimerization was also probed with the reversible, Lys-directed cross-linker dithiobis(succinimidyl propio-
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A

PKD2

| 45 VSFHIQGLTFRLPA-ASELAHVKQLACISVFQRFPEGFGYLYKDLIFFKHDPTSA 103 |

| DKF-2A |

| 227 LTFRLGQSIHKSIAVEGTEALDRDLNEALQFKEIEYPEKGCSSLEDHLYKLHDLRST 286 |

| PKD2 |

| NLLQVRGGDQLEDVLEVLSASATFEDQIRP 138 |

| DKF-2A |

| NLLQITSSDVTGLVEVIGSCQNERIVVHP 321 |

| PKD1 |

| 49 ISFHLGSLQREPVLQDSSGSLAHVREMCMIVSQVQFPEGFGYLYKDLIFFKHDPT 100 |

| PKD2 |

| 45 VSFHIQRGLTFRLPA---SEMAVQKLACISVFQRFPEGFGYLYKDLIFFKHDPT 100 |

| PKD1 |

| TSNLQVRGGDQLEDVLEVLSASATFEDQIRP 146 |

| PKD2 |

| TSNLQVRGGDQLEDVLEVLSASATFEDQIRP 138 |

nate) (DSP). DSP permeates cells and has a disulfide bond located between the two Lys-reactive moieties. Thus, reduction with β-mercaptoethanol will regenerate monomers from cross-linked proteins. Treatment of cells with DSP eliminated DKF-2A monomers and generated multimeric complexes (Fig. 3C, lower panel). Incubation of cell extracts with β-mercaptoethanol, under denaturing conditions, regenerated DKF-2A monomers (Fig. 3C, upper panel). A similar result was obtained for endogenous human PKDs, as illustrated for PKD2 (Fig. 3D). Internal deletion of the OD in the PKD1 Δ50–125 mutant dramatically suppressed cross-linker-mediated loss of monomers and simultaneously blocked production of higher order multi-

FIGURE 2. Human PKDs assemble into homo- and hetero-oligomers; a conserved domain mediates PKD multimerization. A, human PKD2 and PKD1 have a conserved domain that shares substantial amino acid sequence identity (**) and similarity (:) with the DKF-2A OD. B–G, transfection, lysis, immunoprecipitation and Western blot analysis were performed as indicated in Fig. 1A. B, pairs of HA and FLAG-tagged PKD isoforms or empty vector (–) were co-expressed in HEK293 cells. C, FLAG-PKD2 or a FLAG-PKD2 N-terminal truncation mutant was co-expressed with HA-PKD2. D, HA-PKD1, HA-PKD2, or HA-PKD3 was co-expressed with a chimeric protein containing amino acids 1–146 of PKD2 fused with mCherry. E, HA-PKD1, HA-PKD2, or HA-PKD3 was co-expressed with a FLAG-tagged protein containing amino acids 1–142 of PKD3. F, FLAG-PKD1, FLAG-PKD2, or FLAG-PKD1 EE was co-expressed with an internal deletion mutant of HA-PKD1 EE (PKD1 EE Δ50–125). G, a protein that contains the C1α and C1β binding domains of PKD2 (amino acids 128–328) fused to mCherry was co-expressed with HA-tagged PKD1, PKD2, or PKD3. IB, immunoblot; IP, immunoprecipitation.
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FIGURE 3. PKDs oligomerize in intact cells. A, cells co-expressing HA-DKF-2A and FLAG-DKF-2A were incubated with the indicated concentrations of DFDNB or DSS cross-linker or vehicle. Subsequently cross-linking was quenched, the cells were lysed, and PKD oligomerization was monitored by SDS-PAGE and Western blot analysis as described under “Experimental Procedures.” Positions of monomeric DKF-2A (M) and high molecular weight DKF-2A oligomers (O) are marked. B, the Western blot in A was stripped and reprobed with IgGs directed against endogenous PKD2, PKCδ, and PKCε. C, cells expressing HA-DKF-2A and FLAG-DKF-2A were treated with reversible cross-linker DSP as described in A. Replicate samples of Triton X-100-soluble proteins were denatured in SDS-PAGE loading buffer containing or lacking 5% 2-mercaptoethanol. Oligomerization was monitored as described in A. An asterisk marks the position of a nonspecific band. D, the Western blot in C was stripped and reprobed with IgGs directed against endogenous PKD2. PKD2 monomers (M) and oligomers (O) are indicated. E, cells expressing HA-PKD1 Δ50–125 were incubated in the absence or presence of 0.3 mM DFDNB. Extracted proteins were analyzed as described for A. The position of monomeric PKD1 Δ50–125 (M) is marked; no oligomers were detected. IB, immunoblot.

mers (Fig. 3E). Thus, the conserved OD mediates and is essential for PKD oligomerization in intact cells.

Overall, the data suggest that most intracellular PKD molecules are in very close proximity (i.e. in complexes) with partner PKDs in the physiological environment of intact cells. Thus, PKDs are multimeric signaling proteins.

**DKF-2A and Human PKDs Are Dimers—** Gel filtration chromatography and sucrose density gradient sedimentation can provide accurate measurements of the Stokes radius (R_s) and sedimentation coefficient (S_{20,w}) of PKDs in minimally manipulated cell extracts (34, 35). R_s and S_{20,w} values are then used to calculate the native PKD molecular weight (M_r) via the Svedberg equation (see “Experimental Procedures”). Comparison of native M_r with monomer M_1 (determined from genomic DNA or cDNA databases) reveals the number of monomers in the complex.

Lysates of cells expressing HA- and FLAG-tagged DKF-2A were analyzed by FPLC gel filtration chromatography on a column of Superose 6 and sucrose density gradient centrifugation. The column and gradients were calibrated with purified proteins having previously established R_s and S_{20,w} values (Fig. 4, A and B). Upon gel filtration, DKF-2A appeared in fractions that correspond to elution peaks of 600-kDa globular proteins (Fig. 4C, bottom panel). However, large globular proteins and nonglobular proteins with elongated shapes but much smaller molecular mass can exhibit similar R_s values. Introduction of the S_{20,w} value in the M_r calculation corrects for the contribution of protein shape. The measured R_s (7.3 nm) and S_{20,w} (8.8) parameters (Fig. 4, C and D) yielded an estimated M_r for DKF-2A that best accommodates two monomers (Table 1).

Thus, the C. elegans PKD is a dimer. Using the same approach, R_s, S_{20,w}, and M_r values were determined for endogenous PKD1 and PKD2 (Fig. 4, C and D, and Table 1). The hydrodynamic behavior of human PKDs 1 and 2 indicates that these kinases are also dimers. The M_r and subunit composition of two internal control proteins, endogenous PKCδ (monomer) and GAPDH (tetramer), were correctly determined (Fig. 4, A–D, and Table 1), thereby validating the methodology. Based on the preceding results and S_{max}/S_{20,w} ratios that exceed 1.5 (see “Experimental Procedures” and Table 1), we conclude that DKF-2A and human PKDs 1 and 2 are elongated dimers.

The possibility that repeated transient interactions with scaffolds or modulatory proteins are required to assemble and sustain dimeric PKDs was rigorously evaluated. We constructed recombinant baculovirus that contains cDNA encoding GST-His_6–DKF-2A under control of the powerful polyhedrin promoter. The fusion protein was expressed in infected Sf9 insect cells and initially purified by metal ion affinity chromatography on Ni^{2+}-NTA-agarose beads (Fig. 5A). Additional purification of doubly tagged DKF-2A was achieved by performing affinity chromatography on GSH-Sepharose 4B. Next, GST and His_6 tags were removed by digestion with thrombin, which cleaves at a unique site that precedes the N terminus of DKF-2A. Finally, the D kinase was further purified and characterized by Superose 6 gel filtration chromatography. Both silver staining (Fig. 5B) and Western immunoblot analysis (Fig. 5C) showed that the
FIGURE 4. DKF-2A and PKDs 1 and 2 are dimeric proteins. A, a 1 × 30-cm Superose 6 gel filtration column was calibrated with purified proteins with established Stokes radii: thyroglobulin (8.5 nm), apoferritin (6.2 nm), ferritin (6.2 nm), catalase (5.2 nm), aldolase (4.8 nm), ADH (4.5 nm), albumin (3.6 nm), and myoglobin (2.0 nm). $K_{av} = V_e - V_o/V_t - V_o$, where $V_e$ is the elution volume, $V_o$ is the void volume, and $V_t$ is the total column volume. The log-linear relationship of the plot enables accurate interpolation of experimental $R_s$ values by using Graph Pad Prism 6 linear regression software. The details are provided under “Experimental Procedures.” B, a 5–30% sucrose density gradient was calibrated for determination of $S_{20,W}$ values of PKDs. Purified proteins with established $S_{20,W}$ values, catalase (11.3), aldolase (7.4), ADH (7.3), albumin (4.6), and myoglobin (1.9), were centrifuged at 36,000 rpm for 26 h at 5 °C in an SW41 Ti rotor. Proteins in fractions collected from the gradient were characterized by SDS-PAGE and Coomassie Blue staining. Graph Pad Prism 6 linear regression software was used to determine experimental $S_{20,W}$ values (see “Experimental Procedures: and Refs. 34 and 35). C, detergent-soluble proteins were extracted from cells expressing FLAG-DKF-2A and HA-DKF-2A. Proteins were fractionated according to $R_s$ by gel filtration on Superose 6. DKF-2A and endogenous PKD1, PKD2, and GAPDH were detected by assaying aliquots of column fractions via Western immunoblotting. Yellow dots show positions of protein peaks. Experimentally determined $S_{20,W}$ values are listed in Table 1. Native molecular weights of calibrating proteins (red arrows) are shown to illustrate that PKDs appear to be large oligomers in the absence of information about $S_{20,W}$ values. Only relevant fractions are shown; $V_o$ was collected in fractions 1–8. $T$ indicates the signal obtained from a sample of total protein extract. D, a sample of lysate described in C was fractionated in a sucrose gradient. Fractions were collected and analyzed as described under “Experimental Procedures.” Protein peaks are marked with yellow dots. Peaks of calibrating proteins are indicated with red arrows. Experimentally determined $S_{20,W}$ values are given in Table 1.
Stokes radius of highly purified DKF-2A was similar to that determined for DKF-2A in cell extracts (Fig. 4 and Table 1). Furthermore, examination of the complete silver-stained SDS-PAGE gel (Fig. 5) revealed that purified DKF-2A eluted from the FPLC gel filtration column appears to be essentially a single entity. No associated proteins are evident. His6-PKD2 was also expressed in Sf9 cells, affinity-purified, and characterized on the Superose 6 column. The $R_s$ value determined from the elution peak (Fig. 5D) was similar to that obtained for PKD2 in cell extracts (Table 1). Peak fractions from the Superose 6 column contained only PKD2 polypeptides or minor amounts of large, proteolytically nicked PKD2 fragments. No interacting proteins co-purified with the kinase. Neither DKF-2A nor PKD2 monomers were detected during the analysis. Overall, the results indicate that dimerization is an independent, intrinsic property of nematode and mammalian PKD proteins.

**Dimerization Is Required for PKD-mediated Protein Secretion**—PKDs regulate the biogenesis and fission of TGN-derived vesicles known as CARTS (36). CARTS selectively incorporate and transport relatively small, secreted proteins to plasma membrane, thereby facilitating constitutive and/or regulated exocytosis. PAUF (pancreatic adenocarcinoma up-regulated factor) is a representative granin family glycoprotein that is incorporated into CARTS at the TGN and then directed to the cell surface (36, 37). Secreted PAUF is a multifunctional regulatory protein that stimulates cell growth and motility, increases pancreatic tumor cell invasiveness, triggers cytokine production in immune cells, and

**TABLE 1**

| Complex | $M_r$ calculated/actual | $M_r$ (calculated) | $S_{max}$ | $S_{max}/S_{20,w}$ | $K_{av}$ | $R_s$ | $S_{20,w}$ | $M_r$ (actual) | $K_{av}$ | $R_s$ | $S_{20,w}$ |
|---------|-------------------------|--------------------|-----------|-------------------|--------|-------|-----------|----------------|--------|-------|-----------|
| DKF-2A  | 1.13                    | 241,100            | 1.59      | 271,700           | 0.338  | 7.3   | 8.8       | 87,200         | 0.406  | 6.1   | 7.9       |
| PKD1    | 1.15                    | 203,400            | 1.53      | 233,600           | 0.368  | 6.8   | 8.2       | 87,200         | 0.513  | 4.3   | 4.8       |
| PKD2    | 1.05                    | 193,400            | 1.53      | 203,700           | 0.406  | 6.1   | 7.9       | 87,200         | 0.513  | 4.3   | 4.8       |
| PKCδ    | 1.12                    | 77,500             | 1.37      | 87,200            | 0.513  | 4.3   | 4.8       | 87,200         | 0.513  | 4.3   | 4.8       |
| GAPDH   | 0.94                    | 144,200            | 1.28      | 135,000           | 0.513  | 4.3   | 7.5       | 135,000        | 0.513  | 4.3   | 7.5       |

FIGURE 5. **Dimerization is an intrinsic property of PKDs.** (A) GST-His$_6$-DKF-2A was expressed in Sf9 cells infected with recombinant baculovirus and then purified by affinity chromatography on a column of Ni$^{2+}$-NTA-agarose (see “Experimental Procedures”). Samples of total detergent-soluble protein (ST), flow through (FT) proteins, and proteins collected during washing of the resin and elution of GST-His$_6$-DKF-2A with 0.25 M imidazole were analyzed by SDS-PAGE and staining with Coomassie Blue. B, after subsequent affinity chromatography on GSH-Sepharose 4B and cleavage of the GST-His$_6$ tag by thrombin, DKF-2A was further purified and characterized by FPLC gel filtration on Superose 6. Aliquots of fractions were analyzed by SDS-PAGE, silver staining (C), and Western blotting with anti-DKF-2A IgGs (C, D). His-PKD2 was expressed, purified on Ni$^{2+}$-NTA-agarose, and characterized as described above. Western immunoblot analyses of aliquots of fractions collected from the Superose 6 column are shown.
potently elicits angiogenesis by activating signal transduction in endothelial cells (37–39).

The principal intracellular pool of PAUF consists of two partially processed polypeptides with apparent $M_r$ values of 22 and 24 kDa. Mature, secreted PAUF has an apparent $M_r$ of ~28 kDa, but it is visualized as a diffuse band (on Western blots) because of variations in length and composition of its $N$-linked oligosaccharide and other modifications. Because CARTS biogenesis and PAUF secretion are PKD-dependent (36), this secretion system provides a good model for analysis of the physiological relevance of D kinase dimerization.

Partially processed PAUF precursors accumulated, and mature PAUF was secreted in HEK293 cells that overexpressed PKD1 (Fig. 6A, lane 2). More robust (5–10-fold) PAUF secretion was detected in cells expressing constitutively active PKD1 EE (Fig. 6A, lane 4). Both dominant negative PKD1 KD and a selective PKD inhibitor NX-6 (40) eliminated PKD-mediated PAUF secretion (Fig. 6A, lanes 3, 5, and 6). Thus, PAUF secretion machinery is present, functioning and regulated by PKDs in HEK293 cells. Further, cells overexpressing WT or constitutively activated PKDs can be used to explore the role of D kinase dimerization in basal and regulated PAUF secretion, respectively.
Disruption of PKD1 EE homodimerization, by abundantly co-expressing the OD of PKD2 (PKD2 1–146-mCherry) or PKD3 (FLAG-PKD3 1–142) strongly suppressed regulated secretion of PAUF (Fig. 6B). Basal secretion, promoted by elevated PKD1, was also inhibited by expressing OD domains (Fig. 6C). Similarly, secretion of PAUF was sharply reduced when excess OD domain interfered with PKD2 EE dimerization (Fig. 6D).

PAUF secretion was low or undetectable in unstimulated HEK293 cells (Fig. 6, A and G) because basal activities and amounts of endogenous PKDs are modest. However, incubation with PMA, which promotes PKC-mediated activation of PKDs, elicited substantial PAUF secretion (Fig. 6, E–G). PMA-induced secretion was strongly inhibited by dominant negative PKD1 KD or PKD2 KD proteins (Fig. 6E). In contrast, PKD1 KD protein lacking a dimerization domain (HA-PKD1 KD Δ50–125) only minimally reduced PAUF exocytosis (Fig. 6F). Expression of a dimerization domain-mCherry fusion protein (PKD2 1–146-mCherry), which competitively inhibits normal PKD dimerization, blocked PMA-induced PAUF secretion as effectively as dominant negative PKDs (Fig. 6F).

PAUF precursors are absent or low in various lanes of the Fig. 6 (A, D, and E), where PKD activity is impaired. Observation of limited PAUF precursor accumulation in these instances is consistent with reports from the laboratories of Seufferlein (11), Ricci (12), and Evers (41). They demonstrated that PKDs control not only signal-dependent secretion of PAUF, chromogranin A (a PAUF-related glycoprotein), insulin, and neurotensin but also biogenesis of specialized secretory vesicles that transport these secreted proteins from the TGN to the cell surface. When PKD-mediated vesicle biogenesis was blocked, both secretion and intracellular levels of secretory proteins (and their precursors) declined coordinately and precipitously. Secretory protein synthesis was not inhibited. Instead, secretory protein precursors that were not properly packaged and processed in the absence of vesicles were rapidly degraded. This current model suggests the following scenario. When a strong dimerization disruptor or PKD KD mutant is expressed in cells (Fig. 6, A–E), PAUF precursors are synthesized, but few CARTS vesicles are properly generated. Consequently, PAUF precursors that are not packaged in CARTS are degraded, whereas secretion is simultaneously inhibited.

To directly determine whether the results obtained from transient transfection experiments (Fig. 6, A–F) were affected by variations in PAUF precursor levels or stability, we generated and studied cloned HEK293 cells that contain a stably integrated, abundantly expressed PAUF transgene. Fig. 6G shows PAUF precursor levels were elevated and relatively constant under all conditions tested. PAUF was not released into the medium under basal conditions. However, stimulation of endogenous PKDs with PMA elicited robust PAUF secretion. Again, expression of the PKD2 OD or dominant negative PKD1 KD sharply suppressed PMA-stimulated secretion (Fig. 6G). Thus, dimerization of endogenous PKDs is required for signal-dependent, D kinase-mediated regulation of PAUF exocytosis. Observations on PAUF secretion presented in Fig. 6G are in excellent agreement with results obtained from transient transfections depicted in Fig. 6 (A–F).

Overall, the data indicate that native PKD dimers are essential for regulation of CARTS-mediated secretion. Thus, the newly described PKD dimerization domain is indispensable for proper control of a key physiological process. Dimerization is also essential for potent inhibitory effects exerted by catalytically inactive PKDs.

**Impaired Dimerization Impedes PKD-catalyzed Phosphorylation of a Substrate Effector**—Because PAUF secretion is an indirect assay, we also analyzed the role of dimerization in phosphorylation of a *bona fide* PKD substrate effector, phosphatidylinositol 4-kinase IIIβ (PI4KIIIβ) (13). The cells were co-transfected with plasmids encoding GFP-PI4KIIIβ and either FLAG-PKD2 or FLAG-PKD2 mutants containing defective dimerization domains. GFP-PI4KIIIβ was isolated from cell extracts by immunoprecipitation with IgGs directed against GFP. Phosphorylation of PI4KIIIβ was monitored by using anti-phospho-(Ser/Thr) PKD substrate antibody as described under “Experimental Procedures” and Ref. 13. PMA promoted phosphorylation of GFP-PI4KIIIβ in cells expressing wild type PKD2 (Fig. 6H). In contrast, little or no phosphorylation of GFP-PI4KIIIβ was observed in cells expressing dimerization defective PKD2 mutants (PKD2 Δ66 or Δ138). These data demonstrate that dimerization plays a central role in coupling upstream signals to PKD-mediated phosphorylation of a representative target effector protein.

**FIGURE 6. Disruption of dimerization blocks PKD-mediated secretion.** A, cells co-expressing PAUF-Myc and either HA-PKD1, HA-PKD1 KD, or HA-PKD ED were assayed for accumulation of immature PAUF in cell extracts and secreted PAUF in serum-free OptiMEM medium as described under “Experimental Procedures.” A PKD-selective inhibitor NX-6 was added as indicated. Immune and secreted PAUF were detected by Western blot analysis using anti-Myc IgGs. B, triply transfected HEK293 cells co-expressing PAUF-Myc and HA-PKD1 EE, along with either the PKD2 OD (PKD2 1–146-mCherry), the PKD3 OD (FLAG-PKD3 1–142), or empty vector were assayed for PAUF production and secretion. C, cells co-expressing PAUF-Myc and HA-PKD1, along with either the PKD2 OD, the PKD3 OD, or empty vector were assayed for PAUF secretion. D, cells co-expressing PAUF-Myc and HA-PKD2 EE, along with either the PKD2 OD or empty vector were assayed for PAUF secretion. E, cells co-expressing PAUF-Myc and either HA-PKD1 KD, HA-PKD2 KD, or empty vector were assayed for PAUF secretion. PMA was added to OptiMEM secretion medium. F, cells co-expressing PAUF-Myc and either HA-PKD1 KD, HA-PKD1 KD Δ50–125, PKD2 1–146-mCherry, or empty vector were assayed for PAUF secretion. G, cloned HEK293 cells stably expressing a PAUF-Myc transgene were transiently transfected with empty vector or transgenes encoding HA-PKD1 KD or PKD2 1–146-mCherry. The cells were assayed for PAUF secretion as above. H, extracts of cells co-expressing GFP-PI4KIIIβ and either FLAG-PKD2 or a dimerization-defective FLAG-PKD2 mutant (Δ66 or Δ138) were assayed for PI4KIIIβ phosphorylation. GFP-PI4KIIIβ was isolated from cell extracts by immunoprecipitation with anti-GFP IgG; Western immunoblot analysis was performed using anti-phospho-PKD substrate IgGs as a primary antibody. The cells were treated with 1 μM PMA or vehicle for 20 min prior to lysis. l, cells expressing both GFP-PI4KIIIβ and HA-DKF-2A were co-transfected with a transgene encoding DFK-2A 1–319-mCherry (dimerization domain) or empty vector and were assayed for PI4KIIIβ phosphorylation as described in H above. I, cells expressing GFP-PI4KIIIβ were co-transfected with transgenes encoding HA-DKF-2A EE and DFK-2A 1–319-mCherry (dimerization domain) or empty vector, as indicated. Phosphorylation of PI4KIIIβ was assayed via Western immunoblotting as described in H above. All experiments were repeated three times; each replication yielded similar results. IB, immunoblot; IP, immunoprecipitation.
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To assess the relevance of C. elegans DKF-2A dimerization to substrate phosphorylation, we exploited our observation that the nematode D kinase robustly phosphorylates PI4KIIIβ in transfected HEK293 cells. Consequently, the cells were cotransfected with transgenes encoding GFP-PI4KIIIβ and either wild type HA-DKF-2A or constitutively active HA-DKF-2A EE. PMA elicited strong phosphorylation of GFP-PI4KIIIβ by WT DKF-2A (Fig. 6I). Disruption of DKF-2A dimerization via overexpression of DKF-2A 1–319-mCherry sharply reduced PI4KIIIβ phosphorylation. As expected, HA-DKF-2A EE efficiently phosphorylated PI4KIIIβ in the absence of stimulus (Fig. 6J). However, constitutive phosphorylation was also strongly inhibited when dimerization of DKF-2A EE was impaired. These observations show that dimerization of the nematode PKD, like mammalian PKDs, is essential for optimal phosphorylation of a target effector protein in the context of intact cells.

Disruption of PKD Dimerization Compromises C. elegans Innate Immunity—C. elegans feeds on microbes and normally lives ~3 weeks on a diet of Escherichia coli OP50, a rich source of nutrients. When C. elegans persistently ingests Pseudomonas aeruginosa 14 (PA14), this pathogenic bacterium colonizes the intestine and causes toxicity, which kills the nematodes in 4–5 days. If PA14 and OP50 are simultaneously available, C. elegans will produce a complex array of immune effectors that cooperate with pathogen avoidance and foraging behaviors to protect animals against transient infection, restore health, and reinitiate reproduction. The C. elegans innate immune system, which is located principally in intestinal epithelial cells, defends against PA14 by synthesizing and secreting antimicrobial proteins and lectins and increasing the concentration of detoxifying enzymes and proteins that sustain the intestinal lining.

PA14 infection triggers DKF-2A activation in intestine (23). DKF-2A (in concert with p38 MAP kinase) suppresses PA14 toxicity by promoting induction of ~85 mRNAs encoding protective, anti-microbial proteins. C. elegans lacking DKF-2A (dkf-2 null) is hypersensitive to killing by PA14. Hypersensitivity is rescued by targeting expression of a DKF-2A-GFP transgene to intestinal cells in dkf-2 null animals (23). Moreover, resistance to PA14 increased markedly (super-resistance) in both WT and dkf-2 null animals when a DKF-2A transgene was modestly overexpressed in intestine. Thus, we investigated the physiological significance of PKD dimerization in vivo by assaying PA14 sensitivity of transgenic animals in which (a) dimerization of endogenous DKF-2A is disrupted or (b) a DKF-2A mutant lacking a dimerization domain is expressed in intestine of dkf-2 null animals.

Synchronized animals are grown on OP50 and then transferred to plates seeded with PA14. Because PA14 is the sole food source, all animals eventually succumb to infection. However, the rate of PA14-mediated killing will be fast in populations of hypersensitive animals with a compromised immune system and relatively slow in animals with WT or enhanced immune systems. Thus, consequences of impaired PKD dimerization on a critical function, immune defense against a life-threatening pathogen, can be elucidated by determining PA14 killing kinetics (survival curves).

A transgene, dkh-2A::DKF-2A 1–319-GFP (using the notation promoter:: cDNA), encoding the GFP-tagged dimerization region of DKF-2A was expressed in intestinal cells of WT animals. Authentic dkh-2A promoter/enhancer DNA ensures that the kinetics and cellular specificity of transgene expression parallel the timing and intestine-specific expression of endogenous Dkh-2A throughout development (23). A few WT and transgenic animals were killed by PA14 during the initial 32 h of pathogen ingestion. Subsequently, survival curves diverged markedly (Fig. 7A). Intestinal accumulation of DKF-2A 1–319-GFP, which disrupts DKF-2A dimerization, generated animals that were hypersensitive to PA14 pathogen. After feeding on PA14 for 66 h, only 20% of transgenic animals were alive; 9% of animals expressing DKF-2A 1–319-GFP survived for 72 h, and the entire population was killed within 80 h. In contrast, 52, 39, and 28% of WT animals were viable at 66, 72, and 80 h, respectively. PA14 hypersensitivity caused by inhibiting DKF-2A homodimerization resembles the phenotype observed in DKF-2A depleted (dkf-2 null) animals (23) (Fig. 7B). In marked contrast, expression of a 3.5-fold excess of WT DKF-2A dimers in intestinal cells (23) conferred super-resistance to PA14 (Fig. 7A), as illustrated by survival of 75 and 55% of the transgenic...
animals after ingesting pathogen for 66 and 80 h, respectively (Fig. 7A). Thus, DKF-2A homodimerization is essential for pathogen-induced, D kinase-mediated immune responses in vivo.

Super-resistance to PA14 was also conferred on DKF-2A depleted (dkf-2 null) animals when intestinal DKF-2A content was raised 3.5-fold (relative to WT DKF-2A content) via targeted expression of a dkf-2A::DKF-2A-GFP transgene (23) (Fig. 7B). Expression of DKF-2A Δ228–300-GFP, a dimerization-domain-deficient PKD in intestinal cells of dkf-2 null animals, failed to ameliorate hypersensitivity to PA14 (Fig. 7B). Thus, a DKF-2A variant, which contains normal DAG binding and kinase domains, is unable to up-regulate innate immunity when dimerization is impaired. This confirms that PKD dimerization in vivo is indispensable for a central physiological function.

Discussion

Current knowledge of the occurrence, molecular basis, and biological significance of PKD multimers is extremely limited and somewhat contradictory. We addressed these issues by determining the oligomerization status of PKDs and elucidating relationships between PKD dimerization and physiological functions. We discovered and characterized a novel domain that mediates dimerization of PKDs. Dimerization is essential for PKD-mediated secretion of PAUF, PKD-dependent activation of the C. elegans innate immune system and potent, dominant negative effects of catalytically inactive PKD mutants. Virtually nothing was previously known about assembly and functions of homo-oligomeric PKDs. Consequently, initial studies focused on DKF-2A, which regulates innate immunity in the absence of other PKD isoforms. Co-immunoprecipitation of DKF-2A polypeptides labeled with different epitope tags revealed robust homo-oligomerization. Chemical cross-linking showed that DKF-2A monomers were closely apposed within intact cells. The highly efficient conversion of DKF-2A monomers to oligomers by cross-linkers contrasted starkly with results obtained for PKCs α and δ. Like PKDs, PKCs have N-terminal DAG binding sites and a C-terminal kinase domain, but they remained monomeric at all cross-linker concentrations. The results also indicate that PKD oligomers detected in cell extracts were not generated artificially after detergent-based lysis, e.g. by liberating segregated PKD monomers from binding sites on different organelles.

Characterization of DKF-2A in cell extracts by gel filtration and sucrose density gradient sedimentation yielded an estimated M, value corresponding to a homodimer. Stokes radii of highly purified DKF-2A and DKF-2A in extracts are similar, indicating that stable, high affinity binding between two DKF-2A monomers is an intrinsic property of the PKD. Overall, DKF-2A is preponderantly (if not exclusively) dimeric.

Deletion mutagenesis disclosed that a domain composed of 92 amino acids (residues 228–319) that precedes the C1a DAG binding site mediates DKF-2A dimerization. A DKF-2A 1–319-GFP fusion protein accumulated in transfected cells and avidly bound DKF-2A and DKF-2A mutants that retain amino acids 228–319. Robust formation of these complexes shows that the newly discovered domain folds and functions independently of conserved, regulatory, and catalytic regions of PKDs.

Successful analysis of dimerization of the prototypical C. elegans PKD provided guidance and an experimental framework for investigations on oligomerization of mammalian PKDs. Co-immunoprecipitation analysis, chemical cross-linking, mutagenesis, and hydrodynamic studies revealed that PKDs 1–3 form stable heterodimers and homodimers. This may diversify PKD functions. For instance, cells expressing all PKD isoforms can generate three homodimers and three heterodimers. The six distinct PKDs could potentially interact differentially with upstream PKCs or downstream substrates, exhibit qualitatively or quantitatively different patterns of intracellular localization, have different susceptibilities to degradation, etc. A highly conserved but previously unappreciated domain (59% similar to amino acids 228–319 in DKF-2A) directs incorporation of monomeric PKD1, PKD2, and PKD3 polypeptides into stable homo- or heterodimeric complexes. The 92-residue domain precedes the C1a module in PKD polypeptides, indicating that dimerization depends on protein-protein interactions occurring near the N termini of D kinases. FLAG or mCherry fusion proteins that contain the PKD dimerization domain alone avidly ligated PKDs 1–3 or self-associated. The data suggest that the dimerization domain is both necessary and sufficient to establish and sustain the quaternary structure of PKDs.

Homodimeric and heterodimeric complexes were generated by various combinations of WT, PMA-activated WT (data not shown), and constitutively active PKD EE isoforms. The results suggest that the dimerization domain acts independently and is not influenced by configuration of the A-loop, DAG/PMA binding, or catalytic activity. In contrast, constitutively active PKD1 EE Δ50–125, which lacks a dimerization region, was neither susceptible to chemical cross-linking nor co-immunoprecipitation with WT PKDs or full-length PKD1 EE. Dimerization was also essential for physiological effects of PKDs (see below).

Cross-linking experiments and determinations of Stokes radius, S_{20,w}, and native molecular weight values revealed that endogenous PKD1 and PKD2, like recombinant D kinases 1–3 and C. elegans DKF-2A, are exclusively dimers. Previous studies by the Malhotra group (28) and Storz group (29) documented the formation of endogenous PKD2-PKD3 complexes. In addition, disruption of endogenous PKD dimerization, caused by expressing a PKD2 1–146-mCherry transgene in HEK 293 cells, strongly suppressed PMA-induced PAUF secretion. Collectively, these observations show that the newly characterized, conserved dimerization domain mediates stable formation of endogenous PKD homodimers and heterodimers.

Dimerization of endogenous PKDs is essential for D kinase-mediated regulation of a key cell function, secretion from the TGN.

Our studies provide a molecular basis for the generation of PKD2-PKD3 complexes that affect secretion and motility in HeLa cells (28, 29): high affinity binding between conserved dimerization domains that are embedded in each isoform. PKD-PKD association appears to be limited to dimer formation; no monomers were detected, and small amounts of high molecular weight PKD observed by gel filtration appeared to be nonspecific aggregates. No evidence was obtained for trimers, tetramers, or higher order PKD complexes. A report indicating
that nearly all tissues in PKD2-deficient or PKD3-defective mice develop and function normally (6) argues against an essential requirement for PKD2-PKD3 heterodimers in broadly regulating TGN fission or F-actin-based motility in vivo. Only PKD2 was required for constitutive and regulated production of TGN-derived vesicles that mediate chromogranin A secretion from BON endocrine cells (11). PKD1 or PKD3, presumably acting as monomers or homo-oligomers, can independently regulate phosphorylation and nucleus to cytoplasm translocation of HDAC7 in B cells (22). The preceding observations raise a central question: is dimerization critical for PKD-mediated regulation of key physiological processes?

Disruption of endogenous DKF-2A dimerization in C. elegans intestinal cells markedly impaired immune responses that defend against an ingested pathogenic bacterium PA14. Further, targeted intestinal expression of a dimerization-deficient DKF-2A variant, which has normal DAG binding and kinase domains, failed to rescue PA14 hypersensitivity of dkf-2 null C. elegans. In contrast, modest overexpression of WT DKF-2A dimers in intestine conferred super-resistance to PA14 in dkf-2 null or WT animals (23) (Fig. 7). Thus, homodimerization is essential for a critical PKD-regulated function, activation of the immune system in intact animals.

In transfected cells, PKD1 EE promoted PAUF secretion, which is mediated by TGN-derived vesicles designated CARTS (36). PAUF secretion was abrogated when the PKD1 EE dimerization domain was deleted. PMA/PKC-induced activation of endogenous PKDs in HEK293 cells triggered robust PAUF secretion. However, abundant expression of a PKD dimerization domain fusion protein (PKD 1–146-mCherry), which forms stable complexes with full-length PKDs 1–3, potently inhibited PMA-stimulated PAUF secretion. The phenotype caused by competitively interfering with dimerization of endogenous PKDs resembled suppression of PAUF secretion elicited by a dominant negative PKD mutant. Classically, a single dominant negative PKD isoform, which contains a mutation that sharply diminishes ATP binding at the catalytic site, is sufficient to suppress actions of all intracellular PKDs. The ability of dominant negative PKD1 to suppress PAUF exocytosis was neutralized when dimerization was impaired by a deletion mutation (PKD1 KD ∆50–125). Loss of dimerization capacity may also explain why some N-terminal mutations compromise intracellular targeting of PKDs (33). Overall, dimerization is critical for both dominant negative PKD-mediated ablation and WT PKD-mediated up-regulation of an important physiological process, secretion.

The discovery of the dimerization domain and characterization of the dimeric structure of PKDs provide platforms for further analysis. It is of interest to determine whether homo- and heterodimers are assembled according to intrinsic binding affinities and PKD isoform concentrations or through regulated processes controlled by signal transduction. Signal-induced activation of PKDs could potentially elicit isoform-specific changes in proportions of homodimers versus various heterodimers, whereas total PKD dimer concentration remains constant. Future investigations on this topic may yield new insights into mechanisms of D kinase regulation. Site-directed mutagenesis and structural elucidation of the dimerization domain should reveal individual amino acids and features of secondary and tertiary structure that govern formation of complexes between monomeric PKD polypeptides. The possibility that the dimerization domain affects intracellular localization of PKDs or binding with regulatory proteins merits exploration. Biochemical, molecular, and genetic approaches could be used to separate and characterize various PKD dimers, thereby determining their redundant and unique properties and providing clues regarding which isoforms regulate specific physiological processes. The dimerization domain appears to occur only in PKDs. Thus, development of peptido-mimetic compounds that selectively disrupt dimerization or co-associate activating or inhibitory modulators with PKD monomers might be useful for pharmacological intervention in certain diseases, such as tumors in which the epithelial to mesenchymal transition, proliferation, and/or metastasis are driven by altered activities of PKDs (42–46).

**Experimental Procedures**

**Cell Culture**—Human HEK293 cells were grown as previously described (47). Cells (6-cm plates) were transfected with recombinant plasmid DNA (3 μg) complexed with polyethyleneimine (12 μg) (48). Cloned cell lines stably expressing transgenes were selected with 1 mg/ml G418 (49). Effects of WT and mutant PKDs on secretory vesicle biogenesis and export from the TGN were assayed by monitoring both intracellular accumulation and secretion of PAUF, a granin family glycoprotein (36, 37). Growth medium was removed 40–48 h post-transfection, and cells were incubated in Opti-MEM I (serum-free) medium (Life Technologies). After 5 h, the medium was collected, and cells were lysed. Aliquots of medium and detergent-soluble cell proteins were assayed for PAUF content by Western immunoblot analysis.

**Transgene Construction**—cDNAs encoding WT or mutant PKDs 1–3, DKF-2A, DKF-2B, and PAUF were amplified and tagged with restriction sites by the polymerase chain reaction. Product DNA was cloned into a modified pCDNA3.1 expression plasmid (Invitrogen), which appends an N-terminal HA or FLAG tag to the protein. C-terminal Myc was added to PAUF. To express fusion proteins tagged with C-terminal GFP, cDNAs were cloned into the pEGFP-N1 vector (Clontech). In some constructs, GFP was replaced by mCherry. Site-directed and internal deletion mutagenesis were performed with the Q5® mutagenesis kit (New England BioLabs) according to the manufacturer’s protocols. Full-length PI4KIIIβ cDNA was obtained from the DNASU plasmid repository at Arizona State University. The cDNA insert was provided in the pLP-EGFP-C1 vector, which appends a GFP tag at the N terminus of PI4KIIIβ.

**Cell Lysis and Western Immunoblot Analysis**—Cells (6-cm plate) were lysed on ice in 0.3 ml of 25 mM Tris-HCl, pH 7.4, containing 0.15 mM NaCl, 0.2 mM EGTA, 1 mM dithiothreitol, 0.8% Triton X-100, 2% glycerol, and cocktails of protease (Roche) and phosphatase (Sigma) inhibitors. Lysates were sonicated for 5 s and clarified by centrifugation at 44,000 × g for 15 min at 4 °C. Proteins were size-fractionated by denaturing electrophoresis and transferred to a polyvinylidene difluoride membrane (50, 51). The blots were probed with primary IgGs.
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specified in the Figs. 1–6. Antigen-antibody complexes were visualized by using peroxidase-coupled secondary antibodies and an enhanced chemiluminescence procedure (49–51). Chemiluminescence signals were captured on x-ray film. Assays were repeated three times, and similar results were obtained.

PKD Inhibitor—A selective, potent pan-PKD inhibitor, 2′-cyclohexylamino-6-piperazine-1-yl[2,4′]bibpyridinyl-4-carboxylic acid amide (40), was provided by Novartis Institutes for BioMedical Research (Cambridge, MA). The inhibitor, also known as NVP-LDM321-NX-6, is compound 12A in Ref. 39. In text and figures, the inhibitor is designated NX-6.

Antibodies—IgGs directed against GFP, C-terminal, and dihydrophosphorylated A-loop peptides of DKF-2A, and mCherry were produced in rabbits and affinity-purified as previously reported (24, 52–54). IgGs that recognize the following antigens were acquired as follows: PKD1 (D-20, sc-935), PKCδ (C-20, sc-937), and c-Myc (9E10, sc-40) were purchased from Santa Cruz; PKD2 (ST1042) and FLAG (F3165) were obtained from Millipore and Sigma, respectively; HA (HA.C5, T002) and GAPDH (GA1R, L001) were purchased from Epitope Biotech. PKD2 monomers and oligomers were assessed by Western blot analysis. When a reversible cross-linker, DSP, 1.2-nm spacer, was incubated with 3 ml of quench buffer (20 mM Tris-HCl, pH 7.4, 0.13 M NaCl) for 30 min at 20 °C. Two irreversible cross-linkers were employed: DFDNB, 0.3-nm spacer; and DSS, 1.14-nm spacer. DMSO was the vehicle.

Immunoprecipitation—Proteins were isolated by immunoprecipitation with monospecific, affinity-purified antibodies and protein G-Sepharose 4B beads (53). Precipitated proteins were characterized by denaturing electrophoresis and Western immunoblotting conditions suggested by the manufacturer.

Cross-linking Assays—Plates (6 cm) of transfected HEK293 cells were incubated with cross-linker in 3 ml of PBS-8 (20 mM sodium phosphate buffer, pH 8.0, 0.13 M NaCl) for 30 min at 20 °C. Two irreversible cross-linkers were employed: DFDNB, 0.3-nm spacer; and DSS, 1.14-nm spacer. DMSO was the vehicle control. After removing PBS-8 and cross-linker, the cells were incubated with 3 ml of quench buffer (20 mM Tris-HCl, pH 7.4, 0.13 M NaCl) for 15 min at 20 °C. Subsequently, the cells were lysed, and samples of detergent-soluble proteins were resolved by denaturing electrophoresis (6% gel). Amounts of PKD monomers and oligomers were assessed by Western blot analysis. When a reversible cross-linker, DSP, 1.2-nm spacer, was used, dithiothreitol was omitted from cell lysis buffer. Duplicate samples of extracted proteins were denatured in the presence or absence of 5% β-mercaptoethanol prior to denaturing electrophoresis and Western blot analysis.

Determination of Native Mₙ of PKDs—Experimental measurements of Stokes radius and sedimentation coefficient (S₂₀,w) enable calculation of native Mₙ of proteins via the Svedberg equation (34, 35): M = Sₙₐₓₓ(6πηRₛ)/c(1 – vₛ/Rₛ). M is protein mass in Da, S is the sedimentation coefficient, Nₐₓₓ is Avogadro’s number, η is the solvent viscosity, Rₛ is the Stokes radius, vₛ is the partial specific volume, and ρ is the solvent density. Insertion of standard values for constants (Nₐₓₓ, η, vₛ, and ρ) yields a simplified equation: M = 4205(S₂₀,w) (34). Comparison of native Mₙ with the monomeric Mᵣ, derived from genomic DNA or cDNA sequences, reveals whether a protein is a monomer, dimer, or higher order oligomer.

Rₛ values were determined by FPLC gel filtration on a Superose 6 10/300 GL column (GE-Pharmacia Biotech) equilibrated with GF buffer (20 mM Hepes-NaOH, pH 7.5, 0.15 M NaCl, 0.5 mM EDTA, 0.1% CHAPS, 0.01% Brij-35, and 2% glycerol). Samples (0.4 ml) of lysates or purified PKD were applied and eluted with GF buffer (0.5-ml fractions) using a flow rate of 0.25 ml/min. Elution volumes (Vₑ) corresponding to maximal concentrations of DKF-2A, PKD1, PKD2, or other proteins were determined by analyzing samples of each fraction by denaturing electrophoresis and Western immunoblotting. The legend for Fig. 4A describes column calibration and explains how Rₛ is obtained from Vₑ.

S₂₀,w values were measured by velocity centrifugation through a 5–30% sucrose density gradient prepared in 11 ml of GF buffer. 320 μl of experimental sample or calibration proteins were applied to each gradient. Ultracentrifugation conditions are given in Fig. 4B. Fractions (285 μl) were collected from the bottom of centrifuge tubes. Volumes corresponding to maximal concentrations of PKDs or other proteins were determined by analyzing samples from each fraction by denaturing electrophoresis and Western immunoblotting. Calibrating proteins were detected and quantified by denaturing electrophoresis and Coomassie Blue staining. Experimental S₂₀,w values were obtained as indicated in Fig. 4B.

Extended, elongated proteins are distinguished from classical globular proteins by the Sₘₐₓ/S₂₀,w ratio (34). Sₘₐₓ is readily calculated and corresponds to S₂₀,w for a sphere with a specified Mₙ. The spherical shape maximizes S₂₀,w. Globular proteins have Sₘₐₓ/S₂₀,w ratios of 1.2–1.3; ratios of >1.5 are characteristic of elongated non-globular proteins.

Purification of PKDs from Sf9 Cells—Sf9 insect cells were grown statically at 27 °C in TC-100 insect medium (Sigma) supplemented with 10% fetal calf serum. Full-length DFK-2A and PKD2 cDNAs were cloned into baculovirus transfer vectors pAcGHLT and pAcSG-His-NT (BD Biosciences), respectively. Recombinant baculovirus encoding GST-His₆-DKF-2A or His₆-PKD2 was collected from the medium 65 h after the cells were co-transfected with recombinant transfer plasmid and linearized ProGreen Baculoviral DNA (AB Vector, San Diego, CA) using the Protectin transfection reagent. The virus stock was amplified according to protocols provided by AB Vector.

His₆-tagged PKDs were expressed by infecting 12 × 10⁶ Sf9 cells (per 10-cm dish) with 360 μl of high titer, recombinant virus. After 65 h, GST-His₆-DKF-2A or His₆-PKD2 was purified by affinity chromatography on Ni²⁺-NTA-agarose (Novagen), using procedures given by the Novagen user protocol TB273 (EMD-Millipore). Detergents were added to buffers as follows: lysis/binding buffer (1% Triton X-100); initial wash buffer (0.5% Triton X-100); and final wash buffer (0.1% CHAPS, 0.01% Brij-35). Affinity elution was performed with 50 mM sodium phosphate buffer, pH 7.8, containing 0.3 M NaCl, 0.25 M imidazole, 2 mM 2-mercaptoethanol, 0.1% CHAPS, and 0.01% Brij-35. GST-His₆-DKF-2A was further purified by affinity chromatography on GSH-Sepharose 4B as previously described (52). Column fractions were analyzed by denaturing electrophoresis, Coomassie Blue (or silver) staining, and Western
immunoblotting. Subsequent purification and characterization of PKDs were achieved by gel filtration on Superose 6 (as described above) after elution buffer was exchanged for GF buffer on a spin desalting column (Thermo-Zeba). GST and His$_2$ tags were removed from DKF-2A by incubating the fusion protein with thrombin (Millipore).

*C. elegans Culture and Strains—C. elegans* strains were cultivated at 20 °C on agar plates seeded with *E. coli* OP50. dkf-2(${pr}$3) null animals were described previously (23, 53).

**Construction of Transgenes and Transgenic Animals**—A dkf-2A::DKF-2A-GFP transgene (DKF-2A-GFP mRNA transcription driven by the authentic dkf-2A promoter-enhancer) was previously cloned into the pPD95.79 *C. elegans* expression plasmid (23). Deletion mutants were generated from this template. One transgene, dkf-2A::DKF-2A 1–319-GFP, was expressed in the WT background, dkf-2A::DKF-2A Δ228–300-GFP, was expressed in DKF-2A-deficient animals (dkf-2(${pr}$3) strain). Standard protocols were used to generate transgenic animals (47, 55). The dkf-2A promoter targets protein expression to intestinal cells.

**Pathogen-mediated Killing—P. aeruginosa** (PA14) was seeded on agar plates containing *C. elegans* growth medium. A group of 120 synchronized L4 animals was transferred from normal food (*E. coli* OP50) to PA14 pathogen plates. The plates were incubated at 25 °C, and the number of living worms was determined at 8-h intervals. Immobile worms unresponsive to touch were scored as dead.

**Author Contributions**—C. S. R. and C. A. R. conceived and designed the studies, analyzed and interpreted results, and wrote the paper. C. A. R. performed the experiments presented in Figs. 1–6. C. S. R. contributed by collaboratively participating in the performance of experiments reported in Figs. 4 and 5. S. D. Q. H. provided technical assistance for many experiments, performed and analyzed a subset of experiments shown in Fig. 6, and created WT and mutant versions of transgenes encoding epitope-tagged and chimeric PKDs and DKF-2A, which were used in experiments presented in Figs. 1–7. M. L. designed, performed, and analyzed experiments shown in Fig. 7 (A and B). All authors reviewed the results and approved the final version of the manuscript.

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