It Takes Two to Tango: Activation of Protein Kinase D by Dimerization

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The recent discovery and structure determination of a novel ubiquitin-like dimerization domain in protein kinase D (PKD) has significant implications for its activation. PKD is a serine/threonine kinase activated by the lipid second messenger diacylglycerol (DAG). It is an essential and highly conserved protein that is implicated in plasma membrane directed trafficking processes from the trans-Golgi network. However, many open questions surround its mechanism of activation, its localization, and its role in the biogenesis of cargo transport carriers. In reviewing this field, the focus is primarily on the mechanisms that control the activation of PKD at precise locations in the cell. In light of the new structural findings, the understanding of the mechanisms underlying PKD activation is critically evaluated, with particular emphasis on the role of dimerization in PKD autophosphorylation, and the provenance and recognition of the DAG that activates PKD.

1. Introduction

Protein kinase D (PKD) is a ser/thr kinase of the Ca^{2+}/calmodulin-dependent kinase (CAMK) family, best known for its role in the secretion of cargo from the trans-Golgi network (TGN) to the plasma membrane in mammals.[1–9] The phylogenetic tree of the PKD family (Figure 1) shows that after the urochordates diverged, several rounds of genome duplication led to multiple copies of PKD. The distribution indicates that early chordates had all four PKDs and in many cases, such as birds and some reptiles, the number has been reduced by secondary losses. PKD1 and PKD2 originated from a common ancestor, while PKD3 and PKD4 originated earlier. Teleost fish, which have undergone multiple rounds of genome duplication, have up to 11 PKD genes. Nematode worms encode a second PKD gene, D kinase family-1 (DkF-1), which is divergent from all other PKDs.

PKD binds to and is activated by the lipid second messenger diacylglycerol (DAG),[2,10,11] placing it downstream of phospholipase C (PLC) signaling. Consequently, PKD can be activated by a diverse array of stimuli that trigger PLC activation. Two major pathways drive PLC activation: signaling via G protein-coupled receptors (GPCR) or receptor tyrosine kinases (RTK),[12] both of which activate PKD[13] (Figure 2). Recognition and engagement of DAG by PKD leads to phosphorylation of its activation loop on two serine residues, thereby permitting the phosphorylation of downstream substrates. Both autophosphorylation and trans-phosphorylation by protein kinase C (PKC) have been proposed to drive PKD activation, but a mechanistic understanding of PKD activation at the membrane has been lacking. The recent identification and structure determination of a novel dimerization domain in the N-terminus of PKD[14,15] raises many questions that will guide future efforts to understand the structure, biochemistry, and function of PKD.

In this article, we examine the implications of these findings, primarily focusing on the PKD-intrinsic features that control its activity. We discuss functional redundancy, homo-versus heterodimeric PKD in vertebrates, and dominant negative effects in genetic and cell biological studies. We critically evaluate the use of pharmacological compounds in PKD research, including the use of kinase inhibitors and DAG-mimetics such as phorbol esters. Finally, we discuss the provenance of cellular DAG pools, DAG recognition by PKD, and summarize the open questions that merit further investigation.

2. A Unique Ubiquitin-Like Domain Drives PKD Dimerization

PKD comprises tandem, DAG-binding C1 domains, a pleckstrin homology (PH) domain of unknown function, and a C-terminal kinase domain belonging to the CAMK family. Vertebrate PKD1 and PKD2 additionally encode a C-terminal PDZ motif (Figure 3a). Consistent with a number of studies that have reported a functional requirement for dimerization of PKD,[6,15]
Figure 1. Phylogenetic tree of PKD genes. PKD homologs were obtained using BLAST against the respective genomes at Ensembl/Ensembl-Metazoa and the Hydra 2.0 Genome Portal (https://research.nhgri.nih.gov/hydra/) for *Hydra vulgaris*. The sequences were aligned using MAFFT\textsuperscript{[105]} with the auto-option. Unambiguously aligned regions were discarded from the alignment. The phylogeny was reconstructed using IQ-TREE\textsuperscript{[106]} under the JTT+G model determined using ModelFinder\textsuperscript{[107]} and with 10 000 UFboot samples\textsuperscript{[108]} For readability in the mid-point rooted tree support values inside the PKD-clusters 1–4 are only shown at branches to relevant taxonomic groups. PKD1 (blue), PKD2 (red), PKD3 (green), and PKD4 (purple) clusters are indicated. In addition to Dkf-2, nematodes encode Dkf-1, which is divergent from all other PKDs (gray box). Note that *Xenopus tropicalis* PKD3 and *Pelodiscus sinsensis* PKD3-like have been incorrectly annotated in the genomic databases and in fact cluster with PKD4.
Figure 2. Protein Kinase D signaling. Canonical pathways to PKD activation. GPCRs or RTKs activate PLCβ or PLCγ, respectively, leading to the hydrolysis of PI(4,5)P₂ to DAG and IP₃. PKD is recruited to DAG-containing membranes, leading to its autophosphorylation and activation. DAG-bound, activation loop phosphorylated PKD is competent for downstream substrate phosphorylation.

a conserved ubiquitin-like domain (ULD, Figure 3b) that mediates dimerization was recently identified and characterized in human PKD1-3 and Dkf-1, a homolog of PKD in Caenorhabditis elegans. Dkf-1, at just 720 amino acids, has one of the most compact PKD sequences, making it an invaluable template for annotating the core elements required for PKD function (Figure 3a,b). The ULD is unique to PKD, mediating dimerization and trans-autophosphorylation via a hydrophobic surface centered on Phe104 (human PKD1 numbering), the equivalent residue of Ile44 in ubiquitin. While Ile44 mediates the interaction of ubiquitin with the majority of its effector molecules, Phe104 mediates dimerization of PKD. Mutation of Phe104 abrogates the dimerization and trans-autophosphorylation of PKD in cells.

Immediately following the ULD, the C1a domain (Figure 3c) adopts the classical C1 domain fold of two C3H1 zinc fingers with strongest homology to the C1b domain of PKCδ. By homology, the C1b domain adopts the same fold, though no structural information exists for a C1b domain from PKD. The closest structural homolog of the ULD is the ULD-C1a linker, which is very short (≈6 amino acids) and conserved in both length and sequence. This points to a functional significance of the ULD-C1a module, which has yet to be established. The closest structural homolog of the ULD is the Ras-binding domain (RBD) of the Raf kinases. Intriguingly, the Raf RBD is also closely followed by a C1 domain, but there is no obvious functional relationship between the PKD ULD and the Raf RBD. In a recent cryo-electron microscopy structure of autoinhibited Raf, the RBD is not visible, but the C1 domain is buried in intramolecular contacts with the kinase domain and a dimer of 14-3-3 proteins. The authors propose that Ras binding to the exposed RBD is the initial event that recruits Raf to the membrane and extracts the C1 domain to promote membrane binding and formation of the active conformation. Future studies will be required to determine the relationship of the ULD and C1a domains in full-length PKD. In the crystal structure of the ULD-C1a module from C. elegans Dkf-1, the ULD and C1a domains adopt a compact conformation in which the C1a domain packs against a conserved surface of the ULD. In the dimeric arrangement, therefore, the DAG-binding surfaces of the two C1 domains project in almost diametrically opposite directions. Complementary analysis of the structure in solution suggests that the C1 domain can sample an ensemble of restricted conformations. It is an interesting speculation that, in this way, dimeric PKD may recognize a unique membrane conformation.

Solution NMR structures of the PH domains of human PKD2 (Figure 3d) and PKD3 (RIKEN structural genomics/proteomics initiative) reveal strongest homology with the PH domains of dual adaptor of phosphotyrosine and 3-phosphoinositides 1 (DAPP1), tandem PH domain-containing protein 1 (TAPP1), and myosin X (MyoX). However, while each of these domains has been reported to bind to specific phospholipids, the PH domain of PKD exhibits cytosolic localization in J3T fibroblasts and does not translocate to the membrane when the cells are stimulated with a PI3K agonist. It binds nonspecifically to anionic phospholipids in lipid dot blot assays and its deletion does not impair targeting of PKD to the TGN. Comparison of the PH domains of PKD2 and PKD3 with those of DAPP1, TAPP1, and MyoX reveals that, of the four arginine or lysine side chains that coordinate the 3′ or 4′ phosphates of either PI(3,4,5)P₃ or PI(3,4)P₂, two are not conserved in PKD and one is a histidine (Figure 3e). Whether the observed lack of binding to membranes in vitro is due to a weak affinity of the isolated domain, a requirement for the coincident detection of more than one lipid, an avidity effect in the context of dimerization, or the absence of an unknown binding partner remains to be determined. In this respect, early studies identified free Gβγ subunits of the heterotrimeric G-proteins and PKCγ as interaction partners of the PH domain, but these experiments were indirect or based on co-immunoprecipitation, and evidence of a direct interaction with recombinant proteins is still lacking in both cases.

### 3. PKD Is Regulated by Dimerization-Mediated Trans-Autophosphorylation

The C-terminal kinase domain of PKD can dimerize and trans-autophosphorylate in its activation loop. With a sequence identity of 38% over the kinase domain, PKD is most closely related to checkpoint kinase 2 (Chk2). While the isolated kinase domain of PKD exhibits no activity against its own activation loop sequence supplied in trans, autophosphorylation of Ser742 occurs robustly and specifically, and is dramatically enhanced in the context of a dimeric GST-fusion protein. Like PKD, the kinase domain of Chk2 dimerizes (Figure 3f) and trans-autophosphorylates on a non-consensus sequence in its activation loop, though the mechanism by which full-length Chk2 dimerizes is entirely different. In the case of Chk2, an N-terminal forhead-associated ( FHA) domain mediates symmetric dimerization by binding to the N-lobe of the kinase domain in a domain-swapped dimer. A mutation in the hydrophobic interface between the FHA and kinase domains gives rise to the cancer predisposition Li-Fraumeni syndrome. Similar activity against non-consensus activation loop sequences has been reported for STE20-like kinase, lymphocyte-originated kinase (LOK), and death-associated protein kinase 3 (DAPK3), leading to the proposal of a mechanism...
Figure 3. Structure and regulation of PKD. a) Cartoon schematic of PKD domain organization and reported post-translational modifications (PTMs) and their corresponding upstream kinases. ULD = green, C1a = cyan, C1b = blue, PH = orange, kinase domain = light pink (N-lobe)/magenta (C-lobe). Invariant PTM residues are indicated with a solid red line, those conserved in metazoan PKDs with solid black lines, and those that are not conserved are indicated with dashed black lines. The C-terminal PDZ motif, conserved invertebrate PKD1 and PKD2, is colored in light blue. b) Crystal structure of the ULD of $C.$ elegans Dkf-1 (PDB 6RAO). The ULD is a homodimer, for which the dimer axis is indicated with a dashed line. F59 (F104 in HsPKD1), shown in red, sits at the center of the dimer interface. c) Crystal structure of the C1a domain of $C.$ elegans Dkf-1 (PDB 6RAO). The C1a domain adopts the classical C1 domain structure held together by two C3H1 zinc fingers, which create a cleft into which the headgroup of DAG binds. d) NMR structure of the PH domain of human PKD2 (PDB 2COA). The domain is color coded by surface conservation and the putative phospholipid binding pocket is indicated. One surface of the domain exhibits considerably greater surface conservation than the other. e) Alignment of the PKD2 PH domain with known phospholipid binding PH domains with strongest structural homology. The position of residues involved in phosphoinositide binding are highlighted in red and the position of the phosphate on the inositol ring that is coordinated is indicated below the alignment. The degree of conservation of each coordinating residue in PKD is indicated above the alignment. f) Crystal structure of the kinase domain of the evolutionarily related kinase Chk2 (PDB 3I6U). Chk2 forms a homodimer in which the kinase domains are arranged in a head-to-head fashion such that the activation loops of each kinase domain project toward the active site of the other protomer (disordered in the structure). ATP is bound between the N- (light pink) and C-lobes (magenta) of the kinase domain. g) Close-up of the active site of CaMKII illustrating the network of hydrogen bonds stabilizing the phospho-transfer competent enzyme:substrate complex. Thr177 in the activation loop, which is the equivalent of T387 in Chk2, stabilizes the orientation of the catalytic aspartate (D136).
for effective activation loop trans-autophosphorylation shared by a wide array of diverse kinases.\cite{29} In each case, autophosphorylation occurs on the primary phospho-acceptor serine or threonine in the activation loop (Ser742 in PKD1) that is common to all eukaryotic protein kinases that are regulated by activation loop phosphorylation.\cite{30} In both Chk2 and PKD, activation loop phosphorylation blocks dimerization of the kinase domain,\cite{31,32} which is necessary in order to promote the binding to and phosphorylation of downstream substrates.

ULD-mediated dimerization of PKD explains the strong, dominant negative effect of overexpression of catalytically inactive PKD or truncated PKD comprising only its regulatory domain.\cite{33,34} These dominant negative effects may also extend to cross-isoform inhibition under conditions of ectopic overexpression, since the ULD dimerization interface is invariant across PKD1, 2, and 3.\cite{35} While multiple studies have confirmed the ability of over-expressed PKD1, 2, and 3 to heterodimerize in cells,\cite{36,37,38} whether isoform specificity in PKD exists at endogenous expression levels is still open to debate. Since PKD expression levels are in the low nanomolar range in mammalian cells,\cite{39,40} other mechanisms that control PKD localization, such as engagement of its C-terminal PDZ motif with scaffolding proteins, may play an as yet under-appreciated role in targeting specific PKD isoforms to discrete subcellular locations.

### 4. Is PKD Also Regulated by PKC-Dependent Phosphorylation?

It has previously been reported that PKD can also be activated by phosphorylation of a second residue in the activation loop, Ser738, by the novel PKCs.\cite{41–44} Phosphorylation of additional residues both upstream and downstream of the primary phosphorylation site has been reported for a number of eukaryotic protein kinases\cite{45} including Chk2.\cite{46} In the MAPK ERK2, for example, phosphorylation of both primary and secondary sites is required for full catalytic activity\cite{47,48} and individual mutation of the primary and secondary sites in Chk2 to alanine results in loss of kinase activity against Cdc25 in vitro.\cite{49} However, while phosphorylation of Chk2 at T383 (primary site) has been directly observed and clear biochemical data support its activating function, the phosphorylation of T387 (secondary site) has not been directly observed. From mutagenesis experiments in which T387 was substituted with alanine it was erroneously concluded that the phosphorylation blocks dimerization of the kinase domain\cite{50} and mechanistic insight into their function is still lacking.\cite{51,52} Since the scope of this article is confined to the core mechanisms controlling PKD activity, we will not discuss most of these regulatory sites further. A recent study has implicated the phosphorylation of an invariant tyrosine in the P+1 loop in PKD2 (Tyr717, Tyr749 in PKD1; Figure 3a) by Abl tyrosine kinase under oxidative stress conditions.\cite{53} Despite high sequence homology between PKD1, 2, and 3, the phosphorylation (as determined by Western blotting with a phospho-specific antibody) is variable between isoforms and the absolute levels with respect to unphosphorylated or Ser742-phosphorylated PKD are not shown.

**Regulation of PKD activity by phosphorylation of residues outside of the catalytic domain has been reported in multiple studies, though the majority of these residues are not conserved (Figure 3a) and mechanistic insight into their function is still lacking.** Since the scope of this article is confined to the core mechanisms controlling PKD activity, we will not discuss most of these regulatory sites further. A recent study has implicated the phosphorylation of an invariant tyrosine in the P+1 loop in PKD2 (Tyr717, Tyr749 in PKD1; Figure 3a) by Abl tyrosine kinase under oxidative stress conditions. Despite high sequence homology between PKD1, 2, and 3, the phosphorylation (as determined by Western blotting with a phospho-specific antibody) is variable between isoforms and the absolute levels with respect to unphosphorylated or Ser742-phosphorylated PKD are not shown.
A second study from the same group suggests that PKD is itself capable of autophosphorylation at the same site,[56] though rather counter-intuitively this is reduced upon PKD activation with phorbol esters (a tumor-promoting compound derived from plants and potent cellular activator of PKC and PKD) and the only precedent for its phosphorylation is its qualitative observation in proteomic studies. Given also that there is no structural or biochemical rationale behind regulation by tyrosine phosphorylation in the P+1 loop for any protein kinase, whether tyrosine phosphorylation is physiologically relevant is not clear. Our own in vitro autophosphorylation assays with highly purified, recombinant PKD1 kinase domain showed almost negligible phosphorylation of Tyr749.[14]

Precisely what autophosphorylation of the PDZ ligand mimetic glutamates, indicating that the event is autocatalytic in the activation loop Ser738 and Ser742 with phospho-kinase-inactivating mutation and basally enhanced by substitution of cellswithphorbolesters; phosphorylation is blocked by Ile-Leu of human PKD1 (Ser876 in PKD2) accompanies stimulation of both DAG-rich membranes.[32] Regulation of binding by phosphorylation of the PDZ motif has also been widely reported.[57] In studies requiring affinity tags or fluorescent fusion proteins, it is therefore advisable to place the tag at the N-terminus of PKD1/2 so as to avoid interfering with the interaction of the C-terminus with PDZ domain-containing proteins and its regulation by autophosphorylation.

The isolated C-terminal peptides of PKD1 and PKD2 have been shown to interact with the PDZ domain-containing protein NHERF1 (Na+/H+ exchanger regulatory factor 1) in vitro, while treatment of cells with phorbol ester decreased the interaction between PKD and NHERF1 in FRET-based experiments.[60] Whilst the authors inferred from the kinetics that the loss of interaction might reflect autophosphorylation of the C-terminus, there remain considerable questions about the physiological role of NHERF1 in PKD signaling and cellular FRET studies in which both partners are over-expressed should be treated cautiously.

7. Is PKD Constitutively Dimeric or Is Dimerization DAG-Dependent?

It is now clear that PKD activation and downstream signaling necessitates its dimerization and autophosphorylation, but an important question mark still surrounds the nature of the PKD dimer—namely, whether it is constitutive or driven by local concentration on DAG-rich membranes.

While the ULD is necessary and sufficient for dimerization of both Dkf-1 and human PKD1, it has a relatively weak affinity for self-association (≈0.5–2 µm) and PKD is expressed at low nanomolar levels in mammalian cells.[32,33] This suggests that PKD dimerization is likely to be regulated by its local concentration on membranes by DAG. Consistent with this model, a point mutant in the ULD, which prohibits dimerization, translocates with identical kinetics to wild-type PKD (Figure 4b). A dimer of PKD would be expected to translocate faster than a monomer by virtue of the avidity effect of having four C1 domains compared with two. However, size-exclusion chromatography and sucrose gradient centrifugation of detergent-solubilized cell lysates containing C. elegans Dkf-2 or human PKD1/PKD2 yielded elongated dimers in each case, leading the authors to conclude that PKD is constitutively dimeric.[33] It should be noted, though, that over-expression may have driven dimer formation in these cells and that detergent-solubilization could recover dimeric PKD in an active (and hence more extended) conformation.

6. DAG Activates PKD by Relieving Autoinhibition of Its Kinase Domain

Like many protein kinases, PKD exists in unstimulated cells in an inactive, unphosphorylated conformation in which its regulatory domains inhibit the activity of its kinase domain (Figure 4a). The inactive state of PKD is characterized by sequestration of its DAG binding domains such that the DAG-binding surfaces are less accessible,[14] a feature also shared by PKC.[61,62] Agonist-elicited increases in cellular DAG concentration or treatment of cells with phorbol esters results in membrane translocation of PKD, concomitant activation loop phosphorylation, and downstream substrate phosphorylation.[2,10,11,14,33,37] Deletion of its regulatory C1 domains results in constitutive kinase activity that cannot be further stimulated with exogenous phorbol ester,[63] though contradictory reports surround the effect on kinase activity of mutation or deletion of the PH domain.[64,65] The isolated C1 domains translocate rapidly to the plasma membrane in phorbol ester-treated cells, in contrast to full-length PKD, which requires conformational changes to expose the DAG-binding surfaces (Figure 4b). These conformational changes may arise from an equilibrium between conformational states and subsequent sampling of the membrane as has previously been proposed for Akt,[66] or as a consequence of the allosteric sensing of additional membrane-based signals. The precise conformational activation trajectory, however, is still being worked out in the much more extensively studied PKCs.

Deletion of the ULD modestly accelerates membrane translocation, suggesting that it also contributes to the stability of the autoinhibited state[14] (Figure 4b). Similar to Akt and PKC, small molecule inhibitors that bind to the active site paradoxically lead to activation loop phosphorylation as well as re-localization of PKD to basal DAG pools,[67] indicating that inhibitors can dramatically alter the conformational landscape of protein kinases and that phosphorylation should be treated cautiously as a proxy for kinase activity. In summary, the regulatory domains maintain the kinase domain in an inactive conformation in the absence of DAG, analogous to what has been observed for PKC.[68]
DAG-regulated dimerization necessitates that the resulting 2D search problem in the plane of the membrane can be solved sufficiently rapidly so as to mediate robust signal transduction. While lateral diffusion in the plane of the membrane has been suggested as the mechanism by which the conventional PKCs encounter and bind DAG following Ca\(^{2+}\)-mediated translocation to the plasma membrane, there are considerable barriers to the diffusion of both lipids and peripheral membrane proteins in the membrane.\(^{[69,70]}\) Such a model is perhaps only conceivable if PKDs are targeted to precisely defined membrane environments, such that the probability of self-association is high. Characterizing the composition and properties of those target membranes will therefore be of great importance in understanding PKD regulation in the cell.

In conclusion, we propose the following model for PKD activation. Upon DAG production in the membrane, binding of PKD elicits conformational changes that permit ULD-mediated dimerization, followed by trans-autophosphorylation of its kinase domains. Ser742 phosphorylation results in kinase domain dissociation and primes PKD for activity against downstream substrates (Figure 4c).

8. Not All DAG Signals

Signaling lipids are typically a minor component in a sea of lipids that define the target membrane of signal transducers such as PKD. Eukaryotic cells contain more than 1000 lipid species, which, in combination with transmembrane and membrane binding proteins, confer unique physico-chemical properties like curvature, surface charge, fluidity, and permeability that are essential for controlling the transport of molecules, fission and fusion of cellular compartments, and signal transduction.\(^{[71]}\) In the following section, we will review the physico-chemical properties of DAGs, and how these impact their recognition by signaling proteins, including PKD, that contain specific DAG-binding C1 domains. Finally, we will examine the subcellular localization of PKD and the signaling pathways that lead to its activation.
DAG is a neutral lipid with a glycerol backbone esterified with two acyl chains. In addition to its role as a signaling lipid, DAG is a biosynthetic intermediate in triglyceride (TG) synthesis and fat storage, as well as a bulk component of endomembranes. In the cell, multiple pathways control its production and consumption in response to the metabolic and signaling needs of the cell.[72] Consequently, not all DAG is equal: TG hydrolysis yields primarily sn-1,3- or sn-2,3 diacylglycerol, in contrast to PLC, which generates exclusively sn-1,2-diacylglycerol from PI(4,5)P2.[73] (Figure 5a). While sn-1,2-diacylglycerol specifically activates PKC, sn-1,3- and sn-2,3-diacylglycerols do not.[74]

Mammalian cells contain between 25 and 50 chemically distinct species of sn-1,2-diacylglycerol in which the fatty acid substituents vary in length and may be saturated or unsaturated to varying degrees.[75,76] For example, the majority of DAG generated by the action of phosphoinositide (PI)-PLCs in the cell will be esterified with stearic acid (18:0) and arachidonic acid (20:4) in the sn1 and sn2 positions, respectively, since PIs exhibit a relatively homogeneous 18:0–20:4 composition[77] (Figure 5a). In contrast, DAG derived from the combined actions of phospholipase D (PLD) (whose substrate is the more abundant phosphatidylcholine (PC)) and phosphatidic acid phosphatase (PAP) is more likely to have saturated or mono-unsaturated acyl chains in the sn2 position.[77] (Figure 5a). Similarly, sphingomyelin synthase (SMS), which generates DAG as a by-product of sphingomyelin synthesis from ceramides, utilizes PC as the choline donor.[73] PLD-derived diacylglycerol, in contrast to that derived from PLC, reportedly does not activate PKC.[78,79] though equivalent studies do not exist for PKD. Photo-uncaging of synthetic DAGs also highlights the ability of 18:0-20:4 DAG, but not other DAGs, to signal through the PKCa pathway.[80]

Termination of DAG signaling at the plasma membrane is driven by diacylglycerol kinase (DGK)-mediated phosphorylation of DAG to phosphatidic acid.[79] Of the ten eukaryotic DGK isoforms, DGKε is specific for arachidonic acid (20:4) chains at the sn2 position and generates 18:0-20:4 phosphatidic acid, the preferred substrate of CDP-DAG synthase 2 (CDS2), which produces CDP-DAG at the endoplasmic reticulum,[81] where it is converted into phosphatidylinositol (PI). Transport of PI back to the plasma membrane (reviewed in)[82] permits its re-phosphorylation by PI4K and PI5K, whereupon PI(4,5)P2 is recycled. This cycle of 18:0–20:4 specific processing of phosphatidylinositol precursors maintains their characteristic acyl chain signature (Figure 5a).

The chemical differences between DAG species of different origins have a large impact on their physico-chemical properties. DAG is a so-called conical lipid with the highest propensity to induce negative membrane curvature on account of its small headgroup compared to other glycerophospholipids. Consequently, both DAG and PKD are implicated in membrane trafficking processes such as vesicle fission and fusion that require significant membrane bending at the site where two bilayers are brought into close apposition.[83]

In the context of signal transduction, however, this poses a significant problem to DAG recognition. In contrast to the phosphoinositides, with their bulky headgroup that favors positive membrane curvature, the headgroup of DAG is small and easily obscured by the headgroups of neighboring lipids (umbrella effect). Consequently, recognition of DAG by cellular effectors
depends largely on the introduction of packing defects in the membrane that promote exposure of both DAG and the apolar regions of neighboring lipids.[71]

9. C1 Domains Are Context-Dependent Co-Incident Detectors of DAG

All known effectors of DAG signaling possess at least one, and often two, C1 domains. First identified in PKC, these 50 amino acid domains consist of two zinc fingers that organize two small beta sheets and an alpha helix. The DAG-binding cleft is formed by two beta strands that are maintained in an unzipped arrangement by a conserved proline, mutation of which leads to a loss of DAG/phorbol ester binding[86] and, in the case of PKD, loss of recruitment to the TGN.[23]

The structure of the C1b domain from PKCδ in complex with a phorbol ester revealed that phorbol binds between two beta strands and its C20 hydroxyl is coordinated at the base of the cleft by hydrogen bonds to main chain amide and carbonyl groups, thereby bridging the strands.[17] A recent molecular dynamics study indicates that a conserved glutamine in the cleft (Figure 5b) plays a critical role in mediating DAG binding (but not phorbol ester),[83] consistent with experimental results reported (but not actually published) in a previous study.[86] Although the structure of a C1 domain in complex with DAG has not been determined to date, the C20 hydroxyl of phorbol is considered to mimic the 3-hydroxyl of DAG. Phorbol esters are undoubtedly a powerful tool for probing the structure and conformation of PKCs and PKDs in cells,[14,61] but their different membrane partitioning properties (compared to DAG) and their ability to induce chronic accumulation and constitutive activation of both PKC and PKD at the plasma membrane due to their higher binding affinity and slower turnover (reviewed in),[87] compromise their utility in studying physiological processes.

The structure of the C1a domain of C. elegans Dkf-1 is highly homologous to that of the PKCδ C1b domain, with a r.m.s.d. of 1.05 Å over all Cα atoms.[14] C1 domains are characterized by an almost exclusively hydrophobic surface of ~800 Å² that caps the DAG binding cleft, and a surface below the cap composed primarily of basic residues that are poised to interact with the headgroups of acidic phospholipids (Figure 5b). In the C1a domain of PKD, this highly conserved basic surface forms a cavity at the level of the headgroup layer, likely indicating a functional role in the context-specific recognition of DAG. A homologous surface on the C1b domain is not obvious from surface conservation and electrostatics analysis. Early in vitro experiments indicated a synergistic stimulation of PKD activity by phorbol ester and phosphatidylserine (PS).[10] though whether PS or another anionic phospholipid is physiologically relevant in PKD activation is unknown. However, the role of PS in promoting PKC binding to DAG- or phorbol ester-containing membranes has been well documented.[88–90]

While very little is known about the membrane binding properties of PKD C1 domains, molecular dynamics (MD) studies, in combination with experimental methods, have provided insight into the recognition of DAG by the C1 domains of PKCs. MD simulations of PKD C1 domains have not been possible until very recently[14] given the absence of a high-resolution structure. Substitution of a conserved tyrosine with tryptophan in the hydrophobic surface of the C1b domain of conventional PKCs promotes a 100-fold increase in the affinity for DAG micelles,[86] mirroring its increased translocation efficiency to the plasma membrane in vivo.[91] In addition, MD simulations of the tandem C1a and C1b domains of PKCα illustrate the non-equivalence of the two domains with respect to DAG binding,[85] a phenomenon that has also been observed in PKD.[23] These findings, however, must be tempered with the knowledge that computational resources and MD force fields do not currently permit an exhaustive sampling of all lipid combinations and stoichiometries. The context-specific recognition of two DAG molecules (in the case of tandem C1 domains such as those found in PKD) is infinitely more complex than simply recognizing one.

10. Where in the Cell Is PKD Activated?

The subcellular localization of PKD has been addressed in numerous studies, with varying results. Immunostaining of endogenous PKD showed it to be homogeneously cytosolic, associated with punctate structures, or restricted to the TGN.[92–95] Ectopic overexpression of wild-type PKD showed it to be predominantly cytosolic, with varying degrees of enrichment on the TGN and endosomal structures.[1–4,51,59,92–98] while kinase inactivating mutations of PKD consistently localize to the TGN and result in its tubulation.[1,3,4,96] Tubulation of the TGN has been linked to the inhibition of cargo transport.[1,3] In response to receptor stimulation on the cell surface, PKD translocates from the cytoplasm to the plasma membrane in a PLC-dependent manner.[51,92,95,97,98] In summary, the identity of the tag, placement of the tag (N- or C-terminal), cell line, PKD expression level, PKD mutational status, and cell state all appear to influence the subcellular localization of PKD. Future studies must consider these factors carefully in determining precisely where in the cell individual PKD isoforms are active.

The C1a domain has been proposed to be the primary DAG-binding C1 domain[2] responsible for recruitment of cytosolic GST-PKD1 to the TGN.[23] However, it has also been reported that the C1a domain drives PKD translocation to the plasma membrane in response to carbachol,[98] it should be noted that the C1a domain-containing construct employed in this study also contained a flanking sequence encoding the recently identified ULD.[14] Neither the C1a nor the C1b domain alone translocated to the membrane in response to carbachol. As such, the authors concluded that the C1a and C1b domains respond differentially to DAG production when in fact they were likely observing (at least in part) an avidity effect stemming from the ULD-mediated dimerization of the C1a domain.

Cell biological evidence implicates PKD in the fission of secretory granules at the TGN, though multiple reports also indicate PKD activity at the plasma membrane. The principle question therefore pertains to the sources of DAG in these membranes and how PKD recognizes context-specific pools of DAG.

Mammals encode six families of PLC enzymes that hydrolyze PI(4,5)P2 to DAG and inositol-1,4,5-trisphosphate (IP3). The structure, biochemistry, and regulation of mammalian PLCs has been comprehensively reviewed elsewhere,[12] so our focus is restricted to what is known about PLC signaling upstream of PKD.
PDGF-mediated activation of PLCγ activates PKD1 in Swiss 3T3 fibroblasts expressing endogenous PDGFR and A341 cells reconstituted with PDGFR.[13,37] Neuropeptide agonists of GPCRs elicit PKD activation via Gaq-mediated PLCβ activity.[13,99] while carbachol drives PLCβ activation (and downstream PKD) via the muscarinic M1 receptor in HEK293 cells.[98] PLCβ3 has been implicated in vesicle trafficking from the TGN to the plasma membrane.[100] Knockdown of PLCβ3, but not other PLCβ isoforms, PLCδ, γ or ε, impaired the trafficking of VSVG to the plasma membrane in HeLa cells. Interestingly, knockdown of PLCβ3 impaired the autophosphorylation of ectopically expressed GST-PKD1, indicating that constitutive dimerization does not override the requirement for DAG. Nevertheless, given the recent characterization of a dimerization domain in the N-terminal of PKD required for its activation, we would advise against the use of constitutive dimeric affinity tags such as GST.

In C. elegans, Dkf-1, but not Dkf-2, has been implicated in the immune response to pathogen infection. Like mammalian PLCs, the PLCβ homolog EGL-8 is activated by EGL-30, a homolog of the Gaq subunit of the heterotrimeric G-proteins. Perturbation of EGL-30 or Dkf-1, but not EGL-8 impairs the translocation of the transcription factor EB (TFEB) to the nucleus, where it controls the expression of essential host defense genes.[101] In place of PLCβ (EGL-8), the authors identified a PLCε homolog (PLC-1) as the PLC upstream of Dkf-1 in this pathway. Activation of mammalian PKD by thrombin, lysophosphatidic acid, and sphingosine 1-phosphate, but not carbachol, has also been reported to be dependent on PLCε.[102] However, in contrast to the well-characterized mechanism of Gaq signaling to PLCβ,[12] the mechanism linking Gaq to PLCε signaling remains to be determined.

In summary, while both GPCR and RTK pathways are coupled to PKD activation at the plasma membrane, the provenance of the DAG that activates PKD at the TGN is less well established. While calcium drives PKD activation at the plasma membrane via PLC,[103] the link between calcium signaling and PKD activation at the Golgi is not clear; neither PI-PLC nor PAP inhibition affects DAG accumulation in response to thapsigargin-induced increased intracellular calcium concentration.[104] Further studies will undoubtedly be required to dissect the mechanistic links between receptor stimulation at the plasma membrane and DAG signaling through PKD at the TGN.

11. Conclusions and Future Directions

Where do we now stand in our efforts to understand PKD signaling? Before answering that question, we must first separate the biological processes that PKD controls from the signal transduction mediated by PKD itself. In this review we have not tried to deal with the first question, but rather concerned ourselves with the mechanisms by which PKD transduces signals (in the form of DAG) into activity against downstream effectors.

First of all, the discovery of the ULD in PKD provides mechanistic insights into its activation by dimerization and trans-autophosphorylation. But where does that leave PKCs in the activation of PKD? Careful biochemical characterization of purified kinase domain is now required to establish the consequences of Ser738 phosphorylation. Only then, and assuming that it has an activating effect, will it make sense to revisit the question of which kinase is responsible for its phosphorylation. The conservation of the ULD dimerization interface explains heterodimerization of ectopically expressed PKDs in cells, but is it physiological? Future studies will be required to examine the precise localization of PKD isoforms at endogenous expression levels and without tags that have the potential to interfere with localization or activity. Is dimerization constitutive or regulated? In this respect, characterization of full-length, recombinant PKD would be helpful, though this has so far proved challenging. Finally, how and where does PKD recognize DAG in the cell? Determining the role of the PH domain in targeting PKD as well as the contextual recognition of DAG by the C1 domains will undoubtedly provide clues as to which subcellular pools of DAG are accessible.

In summary, much work remains to be done to answer the questions of where, when, and how PKD is activated in the cell. The discovery of a new, ubiquitin-like domain in PKD and its mechanistic consequences will hopefully help frame the next chapter in PKD research.

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Conflict of Interest

The authors declare no conflict of interest.

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