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Minireview

Protein kinase D: a family affair

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Abstract The protein kinase D family of enzymes consists of three isoforms: PKD1/PKCμ, PKD2 and PKD3/PKCν. They all share a similar architecture with regulatory sub-domains that play specific roles in the activation, translocation and function of the enzymes. The PKD enzymes have recently been implicated in very diverse cellular functions, including Golgi organization and plasma membrane directed transport, metastasis, immune responses, apoptosis and cell proliferation.

Key words: Protein kinase D; Protein kinase Cμ; Cell proliferation; Apoptosis; Signal transduction; Golgi apparatus; Stress signaling; Tumor cell invasion

1. Introduction

Second messengers play an important role in the onset of many signaling pathways. Recently, a new family of diacylglycerol (DAG)-stimulated serine/threonine protein kinases has been identified. It comprises three members: PKD1 (protein kinase D1) or its human homolog protein kinase Cμ (PKCμ) [1,2], PKD2 [3] and PKD3 (also named PKCν) [4].

Initially, PKCμ and PKCν were labeled as members of the PKC family [2], creating a new subgroup of the PKCs. However, their only similarity to other PKCs resides in a C1 domain that is homologous to the DAG binding domain of other PKC enzymes, and they lack the C2 domain responsible for Ca^{2+} sensitivity of the conventional PKC subgroup [1,2]. Additionally, in contrast to PKC family members, the catalytic domain of PKD shows a very low homology to the conserved kinase domain of the PKCs and displays a distinct inhibitor and substrate specificity [5–7]. The NH_2-terminal part of PKD contains a pleckstrin homology (PH) domain and lacks the typical autoinhibitory pseudosubstrate motif present in PKCs [1]. These differences made it difficult to classify PKD1, 2 and 3 in the PKC family and recently they were classified as a novel subgroup of the calcium/calmodulin-dependent protein kinase (CAMK) family, based on sequence similarities of the kinase domain [8].

PKD has recently been implicated in the organization of the Golgi apparatus, regulating the fission of vesicles from the trans-Golgi network (TGN) [9]. Several reports identify PKD as a regulator of cell proliferation and apoptosis, proposing a role for the enzyme in carcinogenesis [10–13]. Moreover, PKD appears to be an important regulator of very different intracellular signaling pathways [14–20].

In this minireview we take a closer look at the different members of the PKD family, pointing at their similarities and, when known, at their differences.

2. PKD: structure–function relationship and mechanism of activation

PKD1, 2 and 3 share a similar modular structure (illustrated in Fig. 1), consisting of an N-terminal regulatory domain and a C-terminal kinase domain. The N-terminus of both PKD1 and PKD2 starts with an apolar region, rich in alanine and/or proline residues. In PKD3 this hydrophobic domain is absent. The three isoforms contain two cysteine-rich Zn fingers, separated by an exceptionally long Zn-finger linker region (usually only 14–20 amino acids long in PKCs). Moreover, in PKD2 this linker region contains a serine-rich stretch. In all three isoforms, the Zn fingers precede a region rich in negatively charged amino acids and a PH domain.

The molecular architecture of PKD is without doubt related to the enzyme's regulation. PKD1 kinase activity is regulated intramolecularly, and PKD2 and PKD3, because of their similar structure, may share common interacting factors and regulation characteristics. The regulatory domain of PKD1 exerts an inhibitory effect on the kinase activity, since the deletion of this domain leads to full activation of PKD1 [21]. Moreover, individual regions within the regulatory domain of PKD1 seem to have an inhibitory effect. Mutations in the PH domain lead to full activation of PKD1 [22]. Hence the binding of Gβγ could activate PKD1 [23] by interfering with the interaction of the PH domain with the catalytic subunit, or by displacing the association of an inhibitory ligand. PKD1 is also fully activated when both Zn fingers are deleted [24].

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Abbreviations: PKD, protein kinase D; PKC, protein kinase C; TGN, trans-Golgi network; PH, pleckstrin homology; DAG, diacylglycerol; PDBu, phorbol dibutyrate; PLC, phospholipase C; PMA, phorbol-12,13-myristate acetate; ERK, extracellular signal regulated kinase; BCR, B-cell receptor; GPCR, G-protein coupled receptor; JNK, Jun N-terminal kinase; PA, phosphatidic acid

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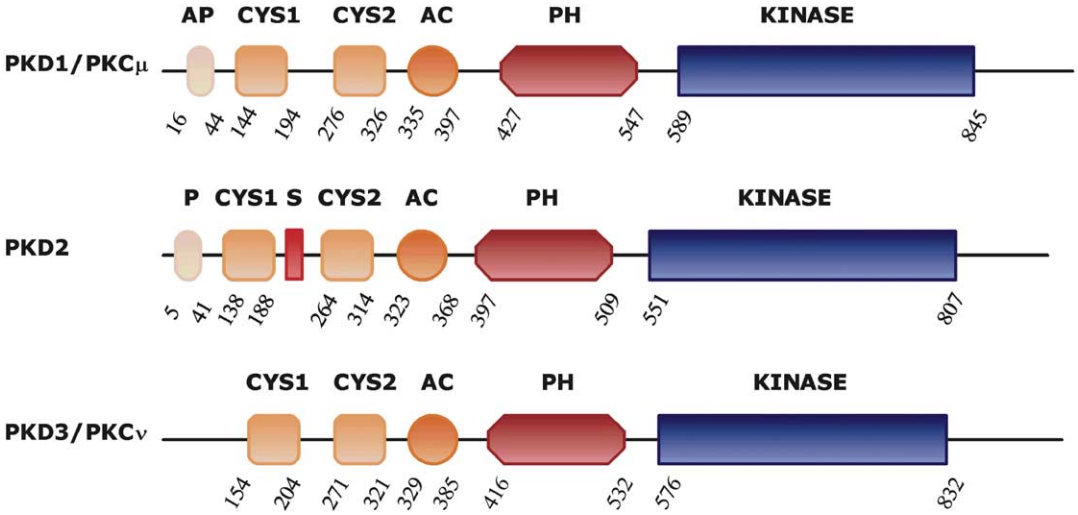

Fig. 1. Modular structure of PKD family members: PKD1/PKCμ, PKD2, PKD3/PKCν. AP, alanine- and proline-rich domain; P, proline-rich domain; S, serine-rich domain; CYS, cysteine-rich Zn finger domain; AC, acidic domain; PH, pleckstrin homology domain; KINASE, kinase catalytic domain.

Recent findings show that only Zn finger 2 binds phorbol dibutyrate (PDBu) with high affinity [25], hence the two Zn fingers are functionally different. Zn finger 1 has a specific inhibitory effect on the catalytic activity of PKD1, but seems to have minor importance in PDBu binding. In contrast, Zn finger 2 is not essential for the PDBu-induced activation of the kinase, but necessary for the PDBu-dependent translocation of PKD1 [24,25]. Consequently, activation and translocation of PKD1 are two separate features. For PKD2 and PKD3, one could predict a similar function for the different sub-domains. However, one should bear in mind that small sequence differences could have profound regulatory or functional consequences.

PKD1 can be activated in vivo by at least three different mechanisms. Several agents are known to activate PKD1 through the activation of phospholipase C (PLC), leading to production of DAG and thereby activating PKCε and/or PKCη, which in turn phosphorylates PKD1 in the activation loop. A recent report has shown that this activation occurs through the release of autoinhibition of the PH domain, and that the activation loop phosphorylation is the mechanistic trigger for this process [26]. Apart from DAG–PLC–PKC-dependent activation of PKD, Gβγ subunits can activate PKD1 through direct interaction with the PH domain [23], thereby regulating the restructuring and function of the Golgi apparatus. A third mechanism for PKD1 activation is through caspase-mediated cleavage during the induction of apoptosis by genotoxic drugs [13].

Recent findings from our [21] and other [27] laboratories have shown that PKD1 is phosphorylated on multiple sites during in vivo activation. Five phosphorylation sites have been identified in PKD1: two sites in the regulatory domain, two in the catalytic domain, and one at the C-terminus. Ser744 and Ser748 (both in the activation loop) play a crucial role in the activation of PKD1. Substitution of these amino acids with alanine completely blocks PKD activation, while substitution with glutamic acid (mimicking phosphorylation) causes a constitutive activation [28]. Ser916 (C-terminus) is an autophosphorylation site, not required for activation but rather regulating the conformation of PKD1. Ser203 (regulatory domain) is an autophosphorylation site and is located in the region that interacts with 14-3-3 proteins. Ser255 (in the regulatory domain) is a transphosphorylation site, targeted by PKC or a PKC-activated kinase. Remarkably, in vivo stimulation with PDBu of a PKD S255E mutant no longer requires PKC phosphorylation.

The exact role of each phosphorylation event in the activation process of PKD1 is still speculative. Ser744/748 are thought to be phosphorylated by PKCε, PKCη or PKCθ, but in a recent report [26] it is shown that Ser748 can be slowly autophosphorylated, and that PKCε can serve as a second site kinase for Ser744. Perhaps autophosphorylation at Ser748 can positively influence Ser744 for transphosphorylation by PKCε. In addition, the PKC isoform(s) responsible for the phosphorylation of PKD will likely be cell type-dependent, simply because not all PKC isozymes are expressed in each cell type.

As for PKD1, it has recently been shown that PKD2 also becomes activated downstream of G-protein coupled receptors (GPCRs), through the activation of PLCγ, which in turn activates PKCα, PKCε, or PKCη [29]. For PKD2, three phosphorylation sites have been identified so far: Ser876 corresponding to Ser916 in PKD1 [3], and Ser706 and Ser710 (activation loop) corresponding to Ser744 and Ser748 in PKD1 [29]. Very recently it has been found that PKD3 is also activated through a DAG–PLC–PKC-dependent pathway in B-cells [30]. PKCε, PKCθ, and PKCη are candidates for the transphosphorylation of the activation loop Ser731 and Ser735 of PKD3 in this activation event, since cotransfection studies with constitutively active and kinase-dead PKC isoforms revealed PKD3 activation loop phosphorylation by these three novel PKCs. In contrast to PKD1 and PKD2, PKD3 does not seem to have an autophosphorylation site at its C-terminus.

3. Intracellular localization of PKD and translocation to different organelles

The association of PKD with other proteins or lipids through its regulatory subdomains is important for its subcel-

lular localization. Through these domains PKD can be recruited to multiple cellular compartments (Fig. 2), such as the plasma membrane, the Golgi apparatus or the nucleus, in response to specific stimuli. In resting cells a large fraction of PKD1 resides in the cytosol, while a smaller fraction can be found in the Golgi. In some specialized cells, PKD1 has also been found in mitochondria [31] and in secretory granules [32]. The two Zn fingers of PKD1 have different lipid binding specificities [25], which has consequences for targeting PKD1 to different cellular locations. Upon stimulation of cells with mitogenic GPCR agonists, PKD1 is translocated initially from the cytosol to the plasma membrane (through its second Zn finger), followed by a rapid dissociation from the plasma membrane, which requires PKC activation and phosphorylation of the PKD1 activation loop. Finally, PKD1 transiently accumulates in the nucleus (mediated by the second Zn finger). The export from the nucleus requires the PH domain [33]. PKD localization studies should be interpreted with some caution because they were performed by overexpressing green fluorescent protein–PKD constructs. PKD1 has also been reported to bind to the TGN. This association is mediated by the first cysteine-rich domain of PKD1 and is dependent on local DAG production [34,35]. Unlike other PH domain-containing kinases, such as PKB/Akt, PKD1 does not need its PH domain for membrane translocation [32,36], nor for Golgi localization [34]. This is in agreement with the lack of evidence for interaction of the PKD1 PH domain with those phosphorylated inositol lipids that are important for the recruitment of PKB to the plasma membrane. To discover the exact relationship between the spatio-temporal regulation of PKD1 translocations and its biological functions, more detailed investigations are needed.

Recently, PKD3 was also shown to translocate rapidly to the plasma membrane in response to phorbol-12,13-myristate acetate (PMA), where it becomes active. In response to PMA, PKD3 stays at the plasma membrane even at late time points, >10 min. B-cell receptor (BCR) cross-linking also induced translocation and activation of PKD3, but very shortly after translocation (<30 s) PKD3 is found both at the plasma membrane and in the cytosol [30].

4. Substrate and inhibitor specificity of PKD

Little is known about direct targets downstream of the PKD family members, and the lack of specific inhibitors makes the research in this field even more difficult.

Only a few physiological substrates of PKD1 are identified. Kidins220 (kinase D interacting substrate of 220 kDa), an integral membrane protein selectively expressed in brain and neuroendocrine cells, is phosphorylated by PKD1 at serine 919 [16]. PKD1 was also suggested to phosphorylate c-Jun at sites different from those phosphorylated by Jun N-terminal kinase (JNK), downregulating in this manner the JNK pathway [37]. Further investigations, however, are required to prove that this is the in vivo mechanism. Recently, another in vivo substrate for PKD1 was identified. The RAS effector

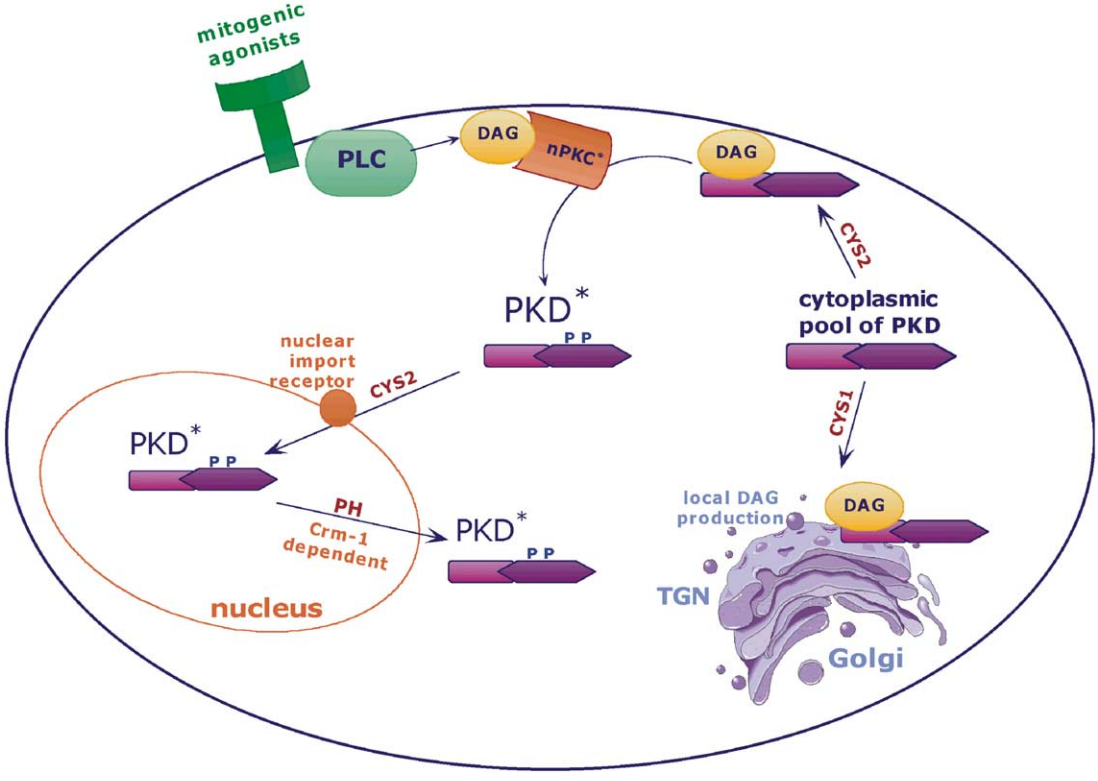

Fig. 2. Nucleoplasmatic shuttling and Golgi translocation. Upon stimulation of cells with mitogenic GPCR agonists PKD is initially translocated from the cytosol to the plasma membrane through its second cysteine-rich Zn finger. PKD dissociates rapidly from the plasma membrane when it is activated and phosphorylated at the activation loop through PKC. Relocation in the cytosol is followed by accumulation of PKD in the nucleus, mediated by its second cysteine-rich Zn finger. Export from the nucleus requires an intact PH domain and a Crm-1-dependent nuclear export mechanism. Recruitment of PKD to the TGN is mediated by its first cysteine-rich Zn finger domain and depends on locally produced DAG. PKD*, activated PKD; nPKC, novel PKCs; PLC, phospholipase C; Cys, cysteine-rich domain; PH, pleckstrin homology domain; P, phosphate.

RIN1 is phosphorylated by PKD1 at serine 351, thereby causing dissociation from RAS and stimulating binding of RIN to 14-3-3 proteins. This in turn allows Raf to interact with RAS [15].

Since no other endogenous substrates have been identified so far, little is known about the substrate specificity of the PKD family members. Using an oriented peptide library approach, substrate specificities were determined for the different PKC isoforms including PKCμ/PKD1 [6]. PKD1 prefers a basic residue at position −3 and a hydrophobic residue (valine, in contrast to the phenylalanine for PKCs) at position +1 with respect to the phosphorylatable serine. PKD1 was unique in that it selected for substrates with hydrophobic residues at −4, +2, +3, +4, and +5, where PKCs prefer more basic residues, and the most critical residue for selectivity of PKD1 is a leucine at −5.

An important feature in the search for downstream pathways and more specific substrates of the PKD family members is the need for specific inhibitors. So far, no specific inhibitor is known for any of the isozymes. In several reports the combination of Gö6976 and Gö6983 (staurosporine-related compounds) was used; the former is an inhibitor of cPKCs and of PKD1 (IC_{50} = 20 nM), the latter is an inhibitor of cPKCs and nPKCs but a very weak inhibitor for PKD1 (IC_{50} = 20 μM) [7]. Remarkably, the staurosporine-derived compound, bisindolylmaleimide I (GF-109203X), which is only a weak inhibitor for PKD1 [7], inhibits the autokinase activity of PKD2 by about 75% [29]. Thus, despite the high percentage of homology in the catalytic subunit of PKD1 and PKD2, there seems to be a functional difference with respect to their inhibitor sensitivity.

5. Cellular signaling and importance in biological functions

5.1. Signaling pathways activating PKD

Many agents activate PKD in vivo through a PKC-dependent pathway [38–41]: tumor-promoting phorbol esters, DAG and analogs, bryostatin, platelet-derived growth factor (PDGF), neuropeptides, oxidative stress, convulxin and thrombin, ATP and gastrin or cholecystokinin. Recently, it has been shown that PKD2 is also activated via PKCα, PKCε or PKCη through the CCK_B/gastrin receptor in human gastric cancer cells [29]. In addition, it was shown that PKD1 is activated through B- and T-cell cross-linking [17] and through an IgE plus Ag stimulus in mast cells [42]. A recent report also shows that PKD3 is activated through BCR cross-linking [30]. Tumor necrosis factor α (TNF-α) [12], doxorubicin [43] and other genotoxic chemotherapeutic agents also activate PKD1 [13], but the role of PKC in these signaling pathways is unknown.

Clearly, the PLC–DAG–PKC pathway plays a major role in the activation of PKD by several agents. Although the second cysteine-rich domain of PKD binds directly to DAG, this is not the main activation mechanism following stimulation of PLC. In case of classical and novel PKCs, a pseudosubstrate sequence is replaced from the substrate binding site during DAG binding, creating an active enzyme. However, this PKC activation is not retained during immunoprecipitation, because DAG is released. Since PKD preserves its activity after being immunoprecipitated, and because of the absence of such a pseudosubstrate domain, it seems that DAG binding in PKD mainly serves to localize the enzyme to specific cellular targets. The activation itself is predominantly mediated by phosphorylation events. This is reminiscent of the activation mechanism of PKB/Akt. After PI3-kinase stimulation, PKB is recruited to the membrane through interaction of its PH domain with PtdIns(3,4,5)P_3 or PtdIns(3,4)P_2. The interaction alters its conformation and PKB becomes accessible for phosphorylation and activation by PDK1 and PDK2 [44]. Although PKD possesses a PH domain, this domain is not responsible for its recruitment to the plasma membrane, but the parallel with the mechanism of PKB activation can be drawn: DAG, produced after PLC activation, binds to Zn finger 2 of PKD and ensures the recruitment of the kinase at the plasma membrane, where it can be phosphorylated by PKCs.

5.2. Signaling pathways affected by PKD, and their biological importance

5.2.1. PKD: role in control of cell growth and survival versus apoptosis. PKD1 expression is correlated with the proliferative state of mouse keratinocytes. Moreover, mouse skin carcinomas display an increased PKD1 expression level and for NIH3T3 fibroblasts overexpressing PKD1, the correlation with proliferation could also be observed [10]. In Swiss3T3 cells, overexpression of PKD1 potentiates DNA synthesis and cell growth induced by bombesin, vasopressin, or phorbol esters [11].

PKD1 seems to have a prominent role in the mitogen-activated ERK (extracellular signal regulated kinase) and JNK pathways. A model for the activation of ERK through PKD1 has recently been proposed [38]: PKC-dependent activation of PKD1 induces translocation of PKD1 to the plasma membrane, where it can phosphorylate RIN1, a protein that associates with Ras and 14-3-3 proteins (also a binding partner for PKD1). Through phosphorylation of RIN1, the association with 14-3-3 could become more intense; therefore Ras can dissociate and is free to be activated such that it can stimulate the Raf–MEK–ERK pathway. The inhibitory effect of PKD1 on JNK signaling has been reported before and has been implicated in the PDGF-mediated suppression of the epidermal growth factor (EGF) receptor signaling to JNK. PKD1 overexpression in HEK293 cells causes the phosphorylation of the EGF receptor, which prevents the further signaling to JNK [45]. Another study reported a negative regulation of JNK through its physical interaction with activated PKD1, leading to the inhibition of JNK and c-*jun* phosphorylation at sites distinct from those phosphorylated by JNK, thereby inhibiting the ability of c-Jun to further regulate proliferation and differentiation [37]. It is possible that both mechanisms of inhibition work in parallel. One should bear in mind that these observations rely on overexpression studies, and hence the physiological importance is unknown.

Besides its function in proliferation, PKD1 seems to have an important role in cell survival: PKD1 is activated by oxidative stress and induces survival pathways. It has been suggested that oxidative stress leads to activation of Src and PLC, which stimulates several PKCs, leading to PKD1 activation [46]. Recently, a slightly different model has been proposed: reactive oxygen species activate the Src–Abl signaling pathway, leading to tyrosine phosphorylation of PKD1 in the PH domain and subsequent activation; at the same time oxidative stress could also lead to activation of PKCs, which activate PKD1 through activation-loop phosphorylation.

These two events would result in a fully activated PKD1, mediating activation of IKKβ and degradation of IκBα, resulting in NF-κB activation and cell survival [20]. Besides controlling the activation of PKD1, the tyrosine phosphorylation of the PH domain in PKD1 could also serve as a recognition site for interacting proteins (through SH_2 or PTB domains), thereby bringing these protein(s) in the proximity of their upstream and/or downstream effectors. Surely, further work is required to examine these possibilities. This pro-survival effect of PKD, however, disappears when PKD undergoes proteolysis upon treatment of cells with β-D-arabinofuranosyl cytosine (ara-C) or other genotoxic drugs [13]. When human U-937 myeloid leukemia cells are treated with these agents, PKD1 is cleaved by caspases and this further sensitizes the cells to apoptosis.

5.2.2. PKD function in the immune response. PKD's role in immune regulation is supported by several studies: BCR or T-cell receptor (TCR) cross-linking causes activation of PKD1 [17]; PKD1 rapidly relocalizes to the plasma membrane in B-cells and mast cells after antigen receptor triggering [32], and endogenous PKD1 exhibits a constitutive association with Btk [47]. The activation of PKD1 in BCR cross-linking depends on Syk and PLCγ activity and is also regulated by Btk. It was suggested that PKD1 could function in a feedback loop, negatively regulating the BCR by reducing the ability of Syk to phosphorylate PLCγ. Recently, it was found that PKD3 is abundantly expressed in B-cells, and as for PKD1, BCR engagement results in strong activation of PKD3. PLCγ and novel PKC isoforms are required in this activation process [30].

5.2.3. PKD importance in Golgi organization and function. PKD1 has been implicated in the regulation of Golgi organization [35]. The marine sponge metabolite ilimaquinone and Gβγ both cause complete fragmentation of the pericentriolar Golgi membranes. This process is effectively inhibited by PKD1 peptide substrates. Moreover, immunoprecipitated PKD1 shows stimulated kinase activity due to direct interaction of the PH domain with Gβγ. Altogether, these data suggest that PKD1 is a component of the Golgi fragmentation pathway. In the same study it was found that PKD1 activity is required for protein transport to the plasma membrane. Vesicular stomatitis virus G-protein transport was inhibited by PKD1 peptide substrate or by the PH domain of PKD1. The PKD1 involvement in fission of transport carriers from the TGN to the plasma membrane has gained extra support by the finding that a kinase-inactive form of PKD1 causes extensive tubulation of the TGN and inhibits the detachment of cargo-containing vesicles from the TGN to the cell surface [9]. It remains unclear how PKD1 regulates this fission process, but recently a model has been proposed [48]: lipid metabolism, PKD1 activity and its recruitment of effectors are all thought to play a pivotal role in a vesicle budding machine. In this respect, the interaction of PI-4-kinase and PI-4,5-kinase with the Zn finger region of active PKD1 could be of importance [49]. PKD1 might act as a scaffold to recruit these enzymes to the Golgi, where they produce phosphorylated inositol lipids. Phosphatidic acid (PA) is also implicated in fission promotion [50]. DAG, which recruits PKD1 to the TGN through its binding to Zn finger 1, could, in a second step, become the substrate for DAG kinases that convert DAG to PA. The production of these phosphorylated inositol lipids and/or PA would lead to the formation of a vesicle, budding off from the TGN membrane.

5.2.4. Possible role for PKD in cell shape regulation and invasion. In invasive breast cancer cells PKD1 forms a complex with cortactin and paxillin, associated with invadopodial membranes that extend into the extracellular matrix [51]. Invadopodia are actin-containing protrusions extending into the matrix and participating in active proteolytic matrix degradation. Cortactin has been suggested to play a role in cell motility and cellular invasion, while the exact role of paxillin remains mainly unidentified. The function of cortactin might be modulated by paxillin and PKD1 in this invasion-related complex. Further studies should clarify questions such as: What is the exact function of PKD1 in this complex? Is PKD1 a modulator of complex formation or does it regulate the protease activity? Is PKD1 phosphorylating substrates in these invadopodia?

On top of the findings that PKD1 could play a role in cell shape modulation of invasive cells, PKD1 was also shown to phosphorylate Kidins220, a protein abundant in neurite tips and growth cones of PC12 pheochromocytoma cells, which could possibly point to a role for PKD1 in cytoskeletal reorganization [H].

6. Perspectives

While we currently have a reasonable understanding of the regulation of PKD1 enzyme activity by PKC-dependent pathways, much remains to be discovered regarding the PKD enzyme family. First, it can be expected that the different family members will not all have the same tissue distribution. The functional roles for PKD1, 2 and 3 in different tissues still need a lot of exploration. Second, intracellular localization studies have so far focused only on PKD1. It is very well possible that localization of PKD2 and 3 could be regulated in subtly different fashions. Third, sufficient differences exist in the regulatory domain of the PKDs to already predict the existence of common, but also subtype-specific interactors for these enzymes. These still largely remain to be identified. And finally, a much better understanding of the role of this kinase family in cellular signaling will be gained from a systematic exploration of their in vivo substrates.

Nevertheless, the discovery of the PKD family of enzymes has put a very important novel set of players on the map of signal transduction.

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