Protein kinase D (PKD) is a serine/threonine protein kinase that contains a cysteine-rich repeat sequence homologous to that seen in the regulatory domain of protein kinase C (PKC) and a catalytic domain with only a low degree of sequence similarity to PKCs. PKD also contains a pleckstrin homology (PH) domain inserted between the cysteine-rich motifs and the catalytic domain that is not present in any of the PKCs. To investigate the function of the PH domain in the regulation of PKD activity, we determined the kinase activity of several PKD PH domain mutants immunoprecipitated from lysates of transiently transfected COS-7 cells. Deletion of the entire PH domain (amino acids 429–557) markedly increased the basal activity of the enzyme as assessed by autophosphorylation (−16-fold) and exogenous syn- tide-2 peptide substrate phosphorylation assays (−12- fold). Mutant PKD proteins with partial deletions or single amino acid substitutions within the PH domain (e.g. R447C and W538A) also exhibited increased basal kinase activity. These constitutive active mutants of PKD were only slightly further stimulated by phorbol-12,13-dibutyrate treatment of intact cells. Our results demonstrate, for the first time, that the PKD PH domain plays a negative role in the regulation of enzyme activity.

Protein kinase C (PKC) has been implicated in the signal transduction of a wide range of biological responses including changes in cell morphology, differentiation, and proliferation (1–5). Molecular cloning has demonstrated the presence of multiple related PKC isoforms (3, 6, 7), i.e. classic PKCs (α, β1, β2, and γ), novel PKCs (δ, ε, η, and θ), and atypical PKCs (ζ and λ) all of which possess a highly conserved catalytic domain.

The newly identified PKD is a mouse serine/threonine protein kinase with distinct structural and enzymological properties (8). The catalytic domain of PKD is distantly related to Ca²⁺-regulated kinases and shows little similarity to the highly conserved regions of the kinase subdomains of the PKC family (9). Consistent with this, PKD does not phosphorylate a variety of substrates utilized by PKCs indicating that PKD is a protein kinase with distinct substrate specificity (8, 10). However, the amino-terminal region of PKD contains a tandem repeat of cysteine-rich, zinc finger-like motifs that bind phorbol esters with high affinity (8, 10). Immunopurified PKD is markedly stimulated by PDB or diacylglycerol in the presence of phosphatidylinerine (10). The human protein kinase PKCe (11, 12) with 92% homology to PKD (extending to 98% homology in the catalytic domain) is also stimulated by phorbol esters and phospholipids (12). These in vitro results indicate that PKD/ PKCe is a novel phorbol ester/diacylglycerol-stimulated protein kinase. Recently a new mechanism of PKD activation has been identified (13). Specifically, exposure of intact cells to biologically active phorbol esters and membrane-permeant dia- cylglycerol induces phosphorylation-dependent PKD activation via a PKC-dependent pathway (13). Thus, PKD can function either in parallel to or downstream of PKCs in signal transduction.

The amino-terminal region of PKD also contains a PH domain which is not found in any of the PKCs. PH domains are molecular structures of approximately 120 amino acids with limited identity in sequence but similar three-dimensional structure (14–19). These domains have been identified in a large number of signaling and cytoskeletal proteins (for review see Refs. 18–21). It has been suggested that PH domains mediate intermolecular and/or intramolecular interactions like src homology domain 2 and 3, but their function and binding partners remain unclear. In some cases PH domains have been shown to bind phosphoinositides and their head groups or proteins such as the βγ subunits of heterotrimeric G proteins (19, 22–25). The integrity of the PH domain is critical for the activation and subcellular localization of many PH domain-containing enzymes including Bruton’s tyrosine kinase (14, 26–28), β-adrenergic receptor kinase (25), and the serine/threo- nine kinase encoded by the proto-oncogene c-akt (29). These considerations prompted us to examine the function of the PH domain in the regulation of PKD activity.

In the present study we demonstrate that a PKD mutant lacking the entire PH domain exhibits high basal kinase activity. This active form of PKD binds phorbol esters as well as the wild type protein but is only slightly further activated by treatment with PDB in vivo. PKD mutants lacking part of the PH domain or with single amino acid substitutions within the PH domain also show high basal kinase activity. Our results indicate that the PH domain of PKD plays a negative role in the regulation of PKD kinase activity.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfections**—COS-7 cells were cultured in DMEM supplemented with 10% fetal bovine serum at 37 °C in a humidified atmosphere containing 10% CO₂. Exponentially growing COS-7 cells, 40–60% confluent, were transfected in serum-free medium by using 5 µg of DNA from the various plasmids and 10 µl of Lipofectin reagent (Life Technologies, Inc.) per 60-mm-diameter dish according to the
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TABLE I
Oligonucleotides used for mutagenesis

| Mutant Position | Oligonucleotide sequence |
|-----------------|-------------------------|
| PKDΔPH Fp 1797–1819 | 5′-GTC AGG GCC CCC TCC GGA TCA CAT AAA GA-3′ |
| PKDΔPH Rp 1381–1408 | 5′-GTC AGG GCC CCC TCC TCC CCT GCT TCG TGT GCT TCA-3′ |
| PKDΔPH + PH Fp 1410–1431 | 5′-AGC CTA TAG GGG TAT GGT ACC-3′ |
| PKDΔPH + PH Rp 1778–1797 | 5′-GTC AGG GCC CCC AGC CAT CAG AGG CCT TG-3′ |
| PKDΔα Fp 1410–1431 | 5′-GTC AGG GCC CCC GTG ATG AGA GAA GGG TGG ATG-3′ |
| PKDΔα Rp 1710–1728 | 5′-GTC AGG GCC CCC CCA CAT CGG GAC CAC TGA CTC C-3′ |
| PKDΔ1–4β FP 1548–1573 | 5′-GTC AGG GCC CCC TCA GAA ATA TTA TAT CTA GGA CCA GC-3′ |
| PKDΔ1–4β Rp 1778–1797 | 5′-GTC AGG GCC CCC AGC CAT CAG AGG CCT TG-3′ |

Single amino acid substitutions

| Mutant | Oligonucleotide sequence |
|--------|-------------------------|
| PKD-R447C External Fp, b-SK(+) | 5′-GAC TCA CTA TAG GCC TAA TGG GCT ACC-3′ |
| PKD-R447C Internal Rp, 1457–1478 | 5′-TCT CCA GTA GTG GCA TTT CCC C-3′ |
| PKD-R447C Internal Rp, 1457–1478 | 5′-GGG AAA TGC CAC TAC TGG AGA-3′ |
| PKD-W538A External Fp, b-SK(+) | 5′-GAC TCA CTA TAG GCC TAA TGG GCT ACC-3′ |
| PKD-W538A External Rp, 1727–1767 | 5′-GAT AGG ATG CTC GAT GGC CAC TCT CGC CAT CCT GCG C-3′ |

protocol provided by the manufacturer. Briefly, Lipofectin (10 μl) was diluted to 1 ml with Opti-MEM I medium (Life Technologies, Inc.), left for 30 min, and then mixed with the DNA previously diluted in 1 ml of the same medium. After 15 min, the volume of the DNA-Lipofectin mixture was increased to 2.5 ml with Opti-MEM I and overlaid onto rinsed (once with Opti-MEM I) COS-7 cells. The cultures were incubated at 37 °C for 6 h, and the medium was then replaced with fresh DMEM containing 10% fetal bovine serum. The cells were used for experimental purposes 72 h later.

Deletion Mutants and Site-directed Mutagenesis—A PH domain deletion mutant was generated by direct-rapid mutagenesis of large plasmids using PCR (30) with rTth DNA polymerase XL with proofreading capability (GeneAmp XL PCR Kit, Perkin-Elmer). The PH domain deletion mutant of PKD (PKDΔPH), lacking amino acids Val-429 to Gly-557, was made directly in PKDcDNA cloned in pBluescript-SK (containing the unique restriction site DpnI which is not present in wild-type PKD (Table I, deletion mutant) and PKDΔ1–4β (see below) were reinserted into the PH domain construct where the PH domain was reinserted in the carboxyl terminus. The scheme amplifying the PH domain contains the deduced secondary structure of the PH domain of PKD based on the sequence alignment published by Gibson et al. (18). β1–β4 indicate the seven β-sheets. PKDΔPH is a deletion mutant that lacks amino acids 429–557 encompassing the entire PH domain. PKDΔPH + PH is a construct where the PH domain was reinserted in the deletion mutant PKDΔPH. PKDΔα and PKDΔ1–4β lack the carboxyl-terminal α-helix (amino acids 535–557) or the first amino-terminall four β-sheets (amino acids 429–474), respectively. PKD-R447C carries an arginine to cysteine mutation at position 447 (present in β2), and PKD-W538A carries a tryptophan to alanine mutation at position 538 (present in the α-helix). The mutants were generated by PCR as described in detail under “Experimental Procedures” using the primers listed in Table I.

Fig. 1. Schematic representation of wild type PKD and the different PKD-PH domain mutants described in these studies. Wild type PKD contains a PH domain (PH, 429–557 amino acids) inserted between the cysteine-rich domain and the catalytic domain present in the carboxyl terminus. The scheme amplifying the PH domain shows the deduced secondary structure of the PH domain of PKD based on the sequence alignment published by Gibson et al. (18). β1–β4 indicate the seven β-sheets. PKDΔPH is a deletion mutant that lacks amino acids 429–557 encompassing the entire PH domain. PKDΔPH + PH is a construct where the PH domain was reinserted in the deletion mutant PKDΔPH. PKDΔα and PKDΔ1–4β lack the carboxyl-terminal α-helix (amino acids 535–557) or the first amino-terminal four β-sheets (amino acids 429–474), respectively. PKD-R447C carries an arginine to cysteine mutation at position 447 (present in β2), and PKD-W538A carries a tryptophan to alanine mutation at position 538 (present in the α-helix). The mutants were generated by PCR as described in detail under “Experimental Procedures” using the primers listed in Table I.

Fig. 1 shows a scheme of the different mutants. After the second PCR reaction the amplified fragment was cut with XhoI and SphI. pBSK-PKD was also digested with these two restriction enzymes, and the wild type PKD-XhoI/SphI fragment was then replaced by the PCR product containing the desired mutation. For PKD-W538A, which alters the only invariant amino acid in the carboxyl-terminal α-helix of the PH domain, the same external forward primer and a reverse primer containing both the W538(TGG) to A538(GCG) mutation and the
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PKD mutants expressed in transfected COS-7 cells, 50 μM unlabeled PDB and PKD-PH in COS-7 Cells—Western blot analysis and [3H]PDB binding. Right panel, binding of [3H]PDB to COS-7 cells expressing PKD and PKD-PH. COS-7 cells were transiently transfected with pcDNA3 vector, pcDNA3-PKD, or pcDNA3-PKD-PH. After 72 h, the cultures were washed twice with PBS and incubated for 4 h at 30 °C. Then cultures were rapidly washed at 4 °C with PBS containing 1 mg/ml bovine serum albumin and lysed with NaOH/SDS, and bound radioactivity was determined by scintillation counting. Non-specific binding was determined in the presence of 10 μM unlabeled PDB.

Materials—[γ-32P]ATP (370 MBq/ml), 125I-labeled protein A (15 mCi/ml), and enhanced chemiluminescence reagents were from Amersham Int. (United Kingdom). PDB was obtained from Sigma. The inhibitor GF I was from LC Laboratories. Protein A-agarose was from Boehringer Mannheim. Other items were from standard suppliers or as indicated in the text.

RESULTS

Expression of PKDΔPH in COS-7 Cells—The region comprising nucleotides 1408–1796 in the cDNA encoding PKD (corresponding to amino acids 429–557 coding for the PH domain) was deleted and the mutated PKD, lacking the entire PH domain, was subcloned into the mammalian expression vector pcDNA3. The resulting construct (PKDΔPH) was transiently transfected into COS-7 cells. To examine the expression of PKDΔPH, lysates from these cells were analyzed by Western blotting with the PA-1 antibody directed against the carboxyl-terminal region of PKD, as described previously (10). The immunoreactive bands corresponding to autophosphorylated PKD or the different mutants was determined in an in vitro kinase assay as described previously (13). Briefly, the immunoprecipitates were washed once with buffer A, twice with buffer B (buffer A minus Triton X-100), twice with kinase buffer (30 mM Tris-HCl, pH 7.6, 2 mM EGTA, 2 mM EDTA, 1 mM dithiothreitol, 10 μg/ml aprotinin, 100 μg/ml leupeptin, 1 mM 4-(2-aminoethyl)-benzenesulfon fluoride hydrochloride, and 1% Triton X-100). PKD was immunoprecipitated at 4 °C for 4 h using a Bio-Rad transfer apparatus. The transfer buffer composition was 200 mM glycine, 25 mM Tris, 0.01% SDS, and 20% CH3OH. Membranes were blocked for 1 h at room temperature in 5% non-fat dried milk in PBS, pH 7.2, and incubated for 3 h with PA-1 antiserum (1:50 dilution) raised against the synthetic peptide EEREMKALSERVSIL that corresponds to the carboxyl-terminal region of PKD, as described previously (10). The immunoreactive bands were visualized using either 125I-labeled protein A (0.1 μCi/ml) and autoradiography or horseradish peroxidase-conjugated anti-rabbit IgG and subsequent enhanced chemiluminescence detection.

RESULTS

Expression of PKDΔPH in COS-7 Cells—The region comprising nucleotides 1408–1796 in the cDNA encoding PKD (corresponding to amino acids 429–557 coding for the PH domain) was deleted and the mutated PKD, lacking the entire PH domain, was subcloned into the mammalian expression vector pcDNA3. The resulting construct (PKDΔPH) was transiently transfected into COS-7 cells. To examine the expression of PKDΔPH, lysates from these cells were analyzed by Western blotting with the PA-1 antibody directed against the carboxyl-terminal region of PKD. As can be seen in Fig. 2 (left), PKDΔPH migrates in SDS-PAGE gels with an apparent molecular mass of ~96 kDa instead of 110 kDa as expected after deletion of the 12 amino acids corresponding to the PH domain. The level of immunoreactive PKDΔPH was comparable to that of PKD (Fig. 2, left). In addition, cells transfected with either pcDNA3-PKD or pcDNA3-PKD-PH showed a similar increase (5–6-fold) in specific [3H]PDB binding as compared with that obtained in COS-7 cells transfected with the vector alone (Fig. 2, right).

Thus, the level of expression of PKDΔPH, judged either by immunoblotting or [3H]PDB binding, is similar to that of PKD in transiently transfected COS-7 cells.

Deletion of the PH Domain Causes PKD Activation—To ex-
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The function of the PH domain in the regulation of PKD activity, COS-7 cells transiently transfected with pcDNA3-PKD or pcDNA3-PKDPH were treated with or without 200 nM PDB for 10 min and then lysed and immunoprecipitated with the PA-1 antibody. The immune complexes were incubated with [γ-32P]ATP and analyzed by SDS-PAGE, autoradiography, and scanning densitometry to determine the level of PKD autophosphorylation. In agreement with previous results (10, 13), PKD isolated from unstimulated cells had low catalytic activity that was markedly activated by PDB stimulation of intact cells. In striking contrast, PKDΔPH exhibited a high level of basal catalytic activity (16-fold increase compared with unstimulated PKD) which was not affected by treatment with PDB (Fig. 3A).

Subsequently, we determined whether a high level of basal PKDΔPH activity could also be demonstrated using an exogenous substrate. The synthetic peptide syntide-2 (13, 32, 33) has been identified as an efficient substrate for the catalytic domain of PKD and for the full-length PKD (8, 10, 13). As shown in the Fig. 3B, PKDΔPH, unlike PKD, displayed high basal syntide-2 kinase activity (8.5-fold increase) that was only slightly further enhanced by PDB stimulation of intact cells. These results corroborated that deletion of the PH domain of PKD leads to a constitutively active state of this enzyme.

The catalytic domain of PKD or the full-length enzyme efficiently phosphorylate syntide-2, but they phosphorylate myelin basic protein inefficiently and they do not phosphorylate histones or a peptide based on the pseudosubstrate motif of PKCs which is a substrate for all members of the PKC family (34, 35). As shown in Table II, this characteristic substrate specificity was not altered by deletion of the PH domain from PKD.

Given that PKD is activated by PDB in intact cells through a PKC-dependent pathway (13), we considered the possibility that the active state of PKDΔPH is also mediated through PKC. To examine this possibility, we assessed the effect of the PKC inhibitor GF I (also known as bisindolylmaleimide I or GF 109203X; Ref. 36) on the activity of PKDΔPH immunoprecipitated from lysates of COS-7 cells pretreated with or without PDB. Exposure to 3.5 μM GF I for 90 min did not affect the high activity of PKDΔPH recovered from either control or PDB-treated cells as shown by autophosphorylation and syntide-2 phosphorylation assays (Fig. 4). In contrast, an identical treatment (3.5 μM GF I for 90 min) markedly inhibited the activation of PKD induced by PDB. These results indicate that the active state of PKDΔPH is not mediated by PKC.

**Partial Deletions and Single Amino Acid Substitutions within the PH Domain Also Lead to PKD Activation**—The preceding results suggest that deletion of the PKD PH domain leads to an active form of PKD. To substantiate this conclusion, we analyzed the activity of a set of PKD mutants containing partial deletions or single amino acid substitutions within the PH domain. The partially deleted PKD mutants were generated by reinserting the PH domain sequence of PKD containing partial deletions into PKDΔPH to produce PKDΔm and PKDΔ1–4β. As a control, the entire sequence of the PH domain was also reinserted into PKDΔPH (PKDΔPH+PH). Fig. 1 shows a schematic representation of the different mutants.

COS-7 cells transiently transfected with these mutants were incubated in the absence or presence of PDB for 10 min, and kinase activity was determined after immunoprecipitation by measuring either syntide-2 phosphorylation or autophosphorylation. The expression of these mutants was verified by Western blotting (Fig. 5).

When the complete PH sequence was reinserted into PKDΔPH, the basal kinase activity of the new construct PKDΔPH+PH was markedly reduced as compared with the unstimulated activity of PKDΔPH. Treatment with PDB of intact cells transiently transfected with PKDΔPH+PH induced marked kinase activation. The level of syntide-2 phosphorylation by PKDΔPH+PH activated by PDB was comparable to that obtained in the wild type PKD (Fig. 5B). In contrast, the partial deletion mutants (i.e. PKDΔm, PKDΔ1–4β) showed high basal activity as judged either by syntide-2 phosphorylation or autophosphorylation assays (Fig. 5B). Treatment with PDB of the cells transfected with these mutants caused only a small further increase in kinase activity as compared with PKD or


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COS-7 cells were transiently transfected with pcDNA3-PKD or pcDNA3-PKDΔPH. After 72 h, the cultures were incubated for 10 min in the absence or presence of 200 nM PDB and lysed. PKD was immunoprecipitated from the lysates using the PA-1 antiserum. The immune complexes were incubated for 10 min with different protein kinase substrates under the conditions described under “Experimental Procedures.” The results are expressed as the percentage of the incorporation of 32P in syntide-2.

| Substrate          | PKD - | PDB | PKDΔPH - | PDB |
|--------------------|-------|-----|----------|-----|
| Syntide-2          | 9.50  | 100.00 | 86.00 | 100.00 |
| Myelin basic protein | 3.90  | 24.46 | 14.43 | 19.98 |
| PKC pseudosubstrate ε | 1.86  | 4.65  | 2.26  | 2.95  |
| Histone II S       | 1.81  | 3.45  | 1.68  | 1.90  |
| Histone II S       | 1.96  | 5.04  | 3.48  | 3.51  |
| Histone VII        | 0.99  | 3.23  | 1.77  | 2.13  |

**DISCUSSION**

Although the tertiary structure of several PH domains, some bound to ligands, has been determined, the function of this domain remains incompletely understood (18–21). The PH domains of a variety of enzymes have been mutated or deleted in an effort to elucidate their function. Recent studies with Akt (23, 29, 37), G protein-coupled receptor kinase (38), Bruton’s tyrosine kinase (14, 26–28), β-adrenergic receptor kinase (25), the Ras exchange factor Sos (39–42), and phospholipase C-δ (43) have demonstrated that the PH domain plays an important role in the regulation of the enzyme activity. Most studies have shown that the integrity of the PH domain is necessary for PKDΔPH + PH.

Next, we examined whether single amino acid substitutions of the PH domain were sufficient to increase the basal activity of PKD. We mutated arginine 447 to cysteine (PKD-R447C) localized in the second β-sheet because comparable mutations in the PH domains of Bruton’s tyrosine kinase (14, 26, 27) and Akt (29, 37) interfered with their function. Tryptophan 538 was mutated to alanine (PKD-W538A) because this residue is highly conserved in the α-helix of most known PH domains. These two mutants exhibited higher basal activity when compared with wild type PKD either in syntide-2 phosphorylation or autophosphorylation assays (Fig. 6B). The increases in the basal syntide-2 kinase activity of PKD-R447C and PKD-W538A were 3.3- and 8-fold, respectively. The high constitutive activity of PKD-W538A was comparable to that of PKD mutants carrying partial or complete deletions of the PH domain.

![Fig. 5. PKD carrying partially deleted PH domains show high basal activity.](image)

**Fig. 5.** PKD carrying partially deleted PH domains show high basal activity. pcDNA3 (−) or pcDNA3-containing wild type PKD (PKD) or the different deletion mutants PKDΔPH (ΔPH), PKDΔPH + PH (ΔPH + PH), PKDΔα (Δα) and PKDΔ1–4β (Δ1–4β) were transiently transfected in COS-7 cells. After 72 h, cells were unstimulated (−, open bars) or stimulated (+, closed bars) with 200 nM PDB for 10 min. A, Western blot (W. Blot) showing the expression of the different PKD mutants in transfected cell lysates. 50 μg of total protein from lysates were separated by SDS-PAGE and immunoblotted with the PA-1 antiserum as specified under “Experimental Procedures.” B, PKD wild type and mutants were immunoprecipitated from cell lysates and assayed by phosphorylation of the synthetic peptide syntide-2 (upper panel) or by autophosphorylation (lower panel) as described under “Experimental Procedures.” Upper panel, syntide-2 phosphorylation in immune complexes. Results represent the means ± S.E. from three experiments, each performed in duplicate. Lower panel (IVK), the autoradiogram shown is representative of five independent experiments with similar results.
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Recent results demonstrated that treatment of intact cells with PDB, cell-permeant diacylglycerols, or bryostatin induces rapid PKD activation that was maintained during cell disruption and immunoprecipitation (13, 44). Several lines of evidence including the use of PKC inhibitors and cotransfection of PKD with constitutively activated mutants of PKCe and -g (13) indicate that PKD can be activated by phosphorylation in intact cells through a PKC-dependent signal transduction pathway. Our results demonstrate that PKD carrying deletions of the PH domain or the single amino acid substitution W538A within the a-helix of the PH domain were only slightly stimulated further by treatment of the cells with PDB, implying that PKD rendered active by PH domain mutation is already fully activated. We conclude that the integrity of the PH domain is critical for maintaining unstimulated PKD in a state of low catalytic kinase activity.

The low basal activity of many protein kinases is maintained by the interaction between an autoinhibitory domain located within the enzyme with its catalytic site, thereby preventing the binding of substrates (45). For example, all members of the PKC family from yeast to human PKCs possess an autoinhibitory motif that is located upstream of the first cysteine-rich domain (46). In contrast, PKD does not contain a pseudosubstrate region in a comparable position (9). Although it is conceivable that PKD is also regulated by autoinhibition, as many other regulated protein kinases, the putative autoinhibitory region has not been identified yet. In view of our results, it is tempting to speculate that the PH domain of PKD has an inactivating function by functioning as an autoinhibitory domain. This suggests a novel role of PH domains in enzyme regulation. In the context of this model, partial or complete deletions of the PH domain of PKD or single amino acid substitutions within this domain should stabilize an active conformation of PKD as, in fact, it is shown by the mutational analysis presented here. Alternatively, our results cannot exclude the possibility that the PH domain of PKD could bind an inhibitory ligand(s) that is released by allosteric stimulation or by phosphorylation-dependent activation induced by treatment of intact cells with PDB. However, PH domain ligands such as phosphoinositides and bγ subunits of G proteins promote correct subcellular localization and enzyme activation rather than inhibition of enzyme activity (18–21). Future studies should attempt to distinguish between these alternative models. Regardless of the precise mechanism(s), our results demonstrate, for the first time, that the PH domain of PKD plays a negative role in the regulation of PKD kinase activity.

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