

# Protein kinase D activation by deletion of its cysteine-rich motifs

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**Abstract** Protein kinase D is a serine/threonine kinase that binds phorbol esters in a phospholipid-dependent manner via a tandemly repeated cysteine-rich, zinc finger-like motif (the cysteine-rich domain). Here, we examined whether the cysteine-rich domain plays an additional role in the control of the catalytic kinase activity independently of the binding of allosteric effectors. We found that deletion of *cys1*, *cys2* or the entire cysteine-rich domain increases the basal activity of protein kinase D leading to a constitutively active form of this enzyme. Our results demonstrate, for the first time, that the cysteine-rich domain of Protein kinase D plays a negative role in the regulation of protein kinase D kinase activity.

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**Key words:** Cysteine-rich domain; Autoinhibition; Protein kinase C; Protein kinase D regulation

## 1. Introduction

Protein kinase C (PKC), a major target for the tumor promoting phorbol esters, has been implicated in the signal transduction of a wide range of biological responses, including changes in cell morphology, differentiation and proliferation [1,2]. Molecular cloning has demonstrated the presence of multiple related PKC isoforms [2–5], i.e. conventional PKCs ( $\alpha$ ,  $\beta_1$ ,  $\beta_2$  and  $\gamma$ ), novel PKCs ( $\delta$ ,  $\epsilon$ ,  $\eta$  and  $\theta$ ) and atypical PKCs ( $\zeta$  and  $\lambda$ ), all of which possess a highly conserved catalytic domain. The NH<sub>2</sub>-terminal regulatory region of conventional and novel members of the PKC family contains a tandem repeat of conserved cysteine-rich, zinc finger-like motifs [2,6,7] that mediates phospholipid-dependent diacylglycerol (DAG)/phorbol ester binding [8–14]. In contrast, atypical PKCs contain a single cysteine-rich motif, do not bind phorbol esters and are not regulated by DAG. However, other proteins including chimaerin [15] and UNC-13 [16], which possess a single cysteine-rich domain (CRD), bind DAG and phorbol esters. These studies emphasize the complexity of signaling via cysteine-rich motifs and raise the possibility that these motifs may play multiple roles in enzyme regulation.

Protein kinase D (PKD)/PKC $\mu$  is a serine/threonine protein kinase with distinct structural and enzymological properties cloned in two independent laboratories [17,18]. The catalytic domain of PKD is distantly related to Ca<sup>2+</sup>-regulated kinases

and shows little similarity to the highly conserved regions of the kinase subdomains of the PKC family [19]. Consistent with this, PKD does not phosphorylate a variety of substrates utilized by PKCs, indicating that PKD is a protein kinase with a distinct substrate specificity [17,20]. In contrast to all known PKCs, including mammalian, *Drosophila* and yeast isoforms [21], the NH<sub>2</sub>-terminal region of PKD contains a pleckstrin homology (PH) domain that regulates enzyme activity [22] and lacks a sequence with homology to a typical PKC autoinhibitory pseudosubstrate motif [17]. However, the amino-terminal region of PKD contains a tandem repeat of cysteine-rich, zinc finger-like motifs [17,18] that binds phorbol esters with a high affinity [17,23]. Immunopurified PKD is markedly stimulated in vitro by either biologically active phorbol esters or DAG, in the presence of phosphatidylserine [20,24]. PKC $\mu$ , the human homologue of PKD, is also stimulated by phorbol esters and phospholipids in vitro [25], indicating that PKD/PKC $\mu$  is a phorbol ester/DAG-stimulated kinase.

Subsequently, a second mechanism of PKD activation has been identified which involves PKD phosphorylation [26]. Specifically, exposure of intact cells to phorbol esters, cell-permeant DAGs, bryostatin 1, neuropeptide agonists and growth factors induces rapid PKD phosphorylation and activation, which is maintained during cell lysis and immunoprecipitation [22,24,26–29]. PKD activity recovered from cells stimulated with these agents can be measured by kinase assays in the absence of lipid activators. Several lines of evidence, including the use of PKC-specific inhibitors and co-transfection of PKD with constitutively active mutants of PKC, indicate that PKD is activated by phosphorylation through a novel PKC-dependent signal transduction pathway in vivo [24,27,28]. The residues Ser-744 and Ser-748 in the activation loop of PKD have been identified as critical phosphorylation sites in PKD activation [30]. These results indicate that the catalytic activity of PKD can be strikingly activated by multiple mechanisms including allosteric effects and protein phosphorylation.

Recently, we demonstrated that the *cys1* and *cys2* motifs of the PKD CRD differ in their ability to mediate specific phorbol ester binding to this kinase [23]. Mutational analysis of the CRD of PKD followed by in vivo and in vitro binding studies indicated that the major site for phorbol ester binding to PKD lies within the *cys2* motif. Here, we examined whether the CRD plays a role in the control of the catalytic kinase activity independently of the binding of allosteric effectors.

## 2. Materials and methods

### 2.1. cDNA constructs

Deletion of the entire CRD domain, amino acids H-145–D-353, (PKD $\Delta$ CRD), deletion of the *cys1* motif, amino acids H-145–S-223,

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**Abbreviations:** CRD, cysteine-rich domain; DAG, diacylglycerol; PAGE, polyacrylamide gel electrophoresis; PDB, phorbol 12,13-dibutyrate; PH, pleckstrin homology; PKC, protein kinase C; PKD, protein kinase D

(PKD $\Delta$ cys1) and deletion of the cys2 motif, amino acids H-277–D-353, (PKD $\Delta$ cys2) were generated by PCR with rTth DNA polymerase XL with proof-reading capability (GeneAmp XL PCR kit, Perkin-Elmer). Site-specific mutations within the CRD domain of PKD resulting in single amino acid substitutions (P-155-G, P-287-G and P-155/287-G) were made by overlap PCR. The details of the generation of all these mutants has been previously described [23] and Fig. 1 shows a scheme of the different mutants used in this study.

## 2.2. Cell culture and transient transfection

COS-7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37°C in an humidified atmosphere containing 10% CO<sub>2</sub>. Exponentially growing COS-7 cells, 40–60% confluent, were transfected with PKD mammalian expression plasmids in serum-free medium by using Lipofectin (Life Technologies) as previously described [22].

## 2.3. Immunoprecipitation

Transfected COS-7 cells were lysed in buffer A (50 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)-benzenesulfonyl fluoride hydrochloride and 1% Triton X-100). PKD was immunoprecipitated at 4°C for 3 h with the PA-1 antiserum (1:50 dilution) raised against the synthetic peptide EEREMKALSERSVIL that corresponds to the C-terminal region of PKD, as previously described [20]. The immune complexes were recovered using protein A coupled to agarose.

## 2.4. Autophosphorylation assay

PKD autophosphorylation was determined in an *in vitro* kinase assay as previously described [24]. 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, 10 mM MgCl<sub>2</sub>) and 20 µl of PKD immune complexes was mixed with 20 µl kinase buffer containing 100 µM final concentration of [ $\gamma$ -<sup>32</sup>P]ATP (specific activity 400–600 cpm/pmol) for 10 min at 30°C. The reaction was then stopped by adding an equal volume of 2×SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer (1 M Tris-HCl, pH 6.8, 0.1 mM Na<sub>3</sub>VO<sub>4</sub>, 6% SDS, 0.5 M EDTA, 4% 2-mercaptoethanol, 10% glycerol) and analyzed by SDS-PAGE. The gels were dried and exposed to get the autoradiographs.

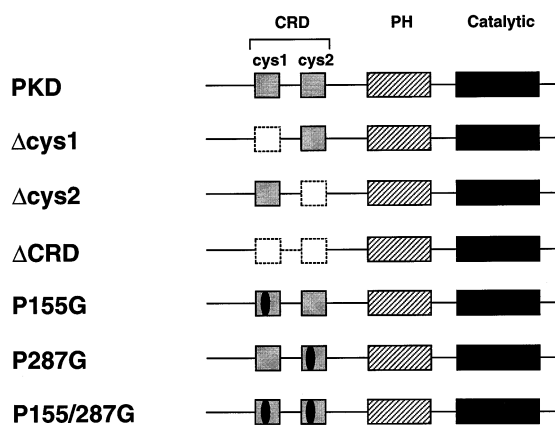


Fig. 1. Schematic representation of wild-type PKD and the different PKD CRD mutants. The CRD, comprising cys1 and cys2, is localized in the amino-terminal region of wild-type PKD (amino acids H-145–D-353). The PH domain is interposed between cys1 and the catalytic domain present in the carboxy-terminal end. PKD $\Delta$ cys1 and PKD $\Delta$ cys2 lack the first (amino acids H-145–S-233) or the second (amino acids H-277–D-353) cysteine-rich region. PKD $\Delta$ CRD is a construct where the whole CRD was deleted (amino acids H-145–D-353). PKD P-155-G and PKD P-287-G carry a proline to glycine mutation at positions 155 and 287, within cys1 or cys2, respectively. PKD P-155/287-G is a double mutant in which both prolines have been substituted by glycine.

## 2.5. Exogenous substrate phosphorylation

The phosphorylation of syntide-2 peptide by immunoprecipitated wild-type PKD or mutants was carried out under the same conditions as in the *in vitro* kinase assay adding a final concentration of 2.5 mg/ml syntide-2. After 10 min at 30°C, the reaction was terminated by adding 75 mM H<sub>3</sub>PO<sub>4</sub> and spotting the supernatant on P-81 phosphocellulose paper and free [ $\gamma$ -<sup>32</sup>P]ATP was separated from the labelled substrate by washing in 75 mM H<sub>3</sub>PO<sub>4</sub> as described elsewhere [20]. The P-81 papers were dried and incorporation was determined by Cerenkov counting.

## 2.6. Western blot analysis

For Western blotting analysis of PKD or PKD CRD mutants expression levels, 50 µg of protein from lysates of COS-7-transfected cells was boiled for 10 min in 2×SDS-PAGE sample buffer and analyzed by SDS-PAGE followed by transfer to Immobilon membranes at 100 V, 0.4 A at 4°C for 4 h, as previously described (XX). Membranes were blocked, incubated with the PA-1 antibody and immunoreactive bands were visualized using [<sup>125</sup>I]protein A and autoradiography.

## 2.7. Materials

Snot [<sup>125</sup>I]-labelled protein A (15 mCi/ml) and ECL reagents were from Amersham International (UK). Phorbol 12,13-dibutyrate (PDB) was obtained from Sigma. Protein A agarose was from Boehringer Mannheim. All other items were from standard suppliers or as indicated in the text.

## 3. Results

### 3.1. Deletion of the CRD causes PKD activation

To determine the contribution of the individual cysteine-rich motifs to the regulation of PKD activity *in vivo*, we used PKD deletion mutants lacking the first ( $\Delta$ cys1) or the second ( $\Delta$ cys2) cysteine-rich motif of the PKD CRD as well as the entire CRD ( $\Delta$ CRD) of PKD (described in Fig. 1). COS-7 cells, transiently transfected with wild-type or CRD deletion mutants of PKD, 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 [ $\gamma$ -<sup>32</sup>P]ATP and analyzed by SDS-PAGE and autoradiography to determine the level of PKD autophosphorylation (Fig. 2B, lower panel).

In agreement with previous results, wild-type PKD isolated from unstimulated cells had a low catalytic activity that was markedly activated by PDB stimulation of intact cells. In striking contrast, PKD lacking either the entire CRD or the individual cysteine-rich motifs (cys1 and cys2) exhibited a high level of basal catalytic activity (15–20-fold increase compared with unstimulated PKD) which was not further enhanced by treatment with PDB (Fig. 2B, lower panel). All PKD constructs (PKD,  $\Delta$ cys1,  $\Delta$ cys2 and  $\Delta$ CRD) exhibited similar levels of expression, as shown by Western blotting with a specific antibody directed against the C-terminal region of PKD (Fig. 2A).

Subsequently, we determined whether a high level of basal activity of the PKD forms carrying deletions of its CRD could also be demonstrated using an exogenous substrate. The synthetic peptide syntide-2 has been identified as an efficient substrate for the catalytic domain of PKD and for the full-length PKD. As shown in Fig. 2B (upper panel), the PKD deletion mutants lacking cys1, cys2 or the CRD displayed a high basal syntide-2 kinase activity (8–10-fold increase) that was not further increased by PDB stimulation of intact cells. The results corroborated our conclusion that deletion of the CRDs of PKD leads to a constitutively active state of this enzyme.

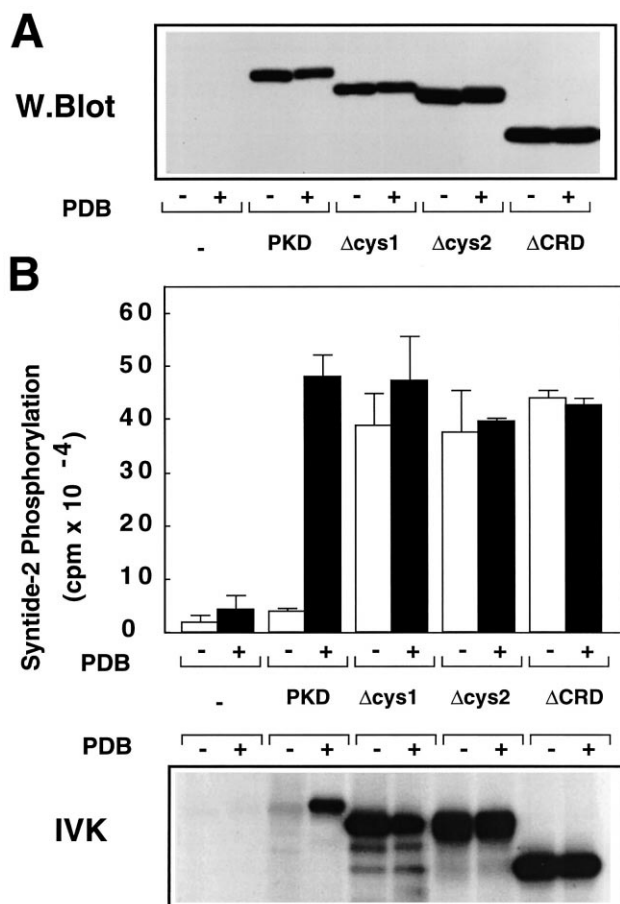


Fig. 2. Deletion mutants are constitutively active. COS-7 cells were transfected with either pcDNA3 (–), pcDNA3-PKD wild-type (PKD), pcDNA3-PKD $\Delta$ cys1 ( $\Delta$ cys1), pcDNA3-PKD $\Delta$ cys2 ( $\Delta$ cys2) or pcDNA3-PKD $\Delta$ CRD ( $\Delta$ CRD) deletion mutants. After 72 h, the cultures were incubated for 10 min in the absence (–, open bars) or presence (+, closed bars) of 200 nM PDB (as indicated) and lysed. PKD was immunoprecipitated from the lysates using the PA-1 antiserum. (A) Western blot (W. Blot) showing the expression of the different PKD mutants in transfected cell lysates. 50  $\mu$ g of total protein from lysates was separated by SDS-PAGE and immunoblotted with the PA-1 antiserum as specified in Section 2. (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 in Section 2. Upper panel: syntide-2 phosphorylation in immune complexes. Results represent the means  $\pm$  S.E.M. from three experiments, each performed in duplicate. Lower panel (IVK): the autoradiogram shown is representative of three independent experiments with similar results.

### 3.2. Kinase activity of PKD with single or double amino acid substitutions within cys1 and cys2

The increase in the basal activity of PKD induced by deletion of cys1, cys2 or the entire CRD prompted us to examine whether single amino acid substitutions in the individual cysteine-rich motifs (cys1 and cys2, Fig. 1) or in both motifs were sufficient to increase the basal activity of PKD. Since a highly conserved proline residue present in the cysteine-rich motifs of all DAG/phorbol ester sensitive proteins has previously been shown to be critical for high affinity phorbol ester binding [23], we used PKD mutants which contained single amino acid substitutions at this site in the first (P-155-G), second

(P-287-G) or in both (P-155/287-G) cysteine-rich motifs of PKD (illustrated in Fig. 1).

As shown in Fig. 3, the level of activity of a PKD mutant with a proline to glycine mutation in cys2 (P-287-G) was comparable to that seen in wild-type PKD, as shown by autophosphorylation or syntide-2 kinase assays (Fig. 3B). In contrast, the basal level of activity of a PKD mutant with a proline to glycine mutation in cys1 (P-155-G) was significantly increased over wild-type PKD (3-fold increase), despite similar levels of expression of all these constructs (Fig. 3A). The results presented in Fig. 3 suggested that the cys1 and cys2 motifs of PKD are non-equivalent in their ability to repress the activity of PKD.

We also examined the catalytic activity of PKD carrying proline to glycine mutations in both cys1 and cys2. As shown

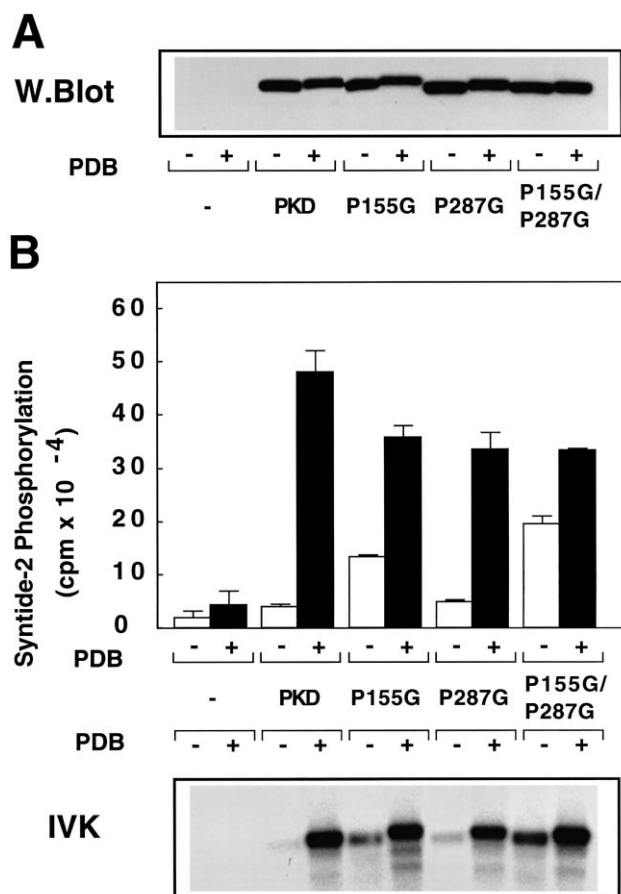


Fig. 3. Single amino acid substitutions in the PKD CRD (P-155-G and P-155/287-G) also induce high basal PKD kinase activity. pcDNA3 (–), pcDNA3-PKD (PKD), pcDNA3-PKD-P-155-G (P155G), pcDNA3-PKD-P-287-G (P287G) or pcDNA3-PKD-P-155/287-G (P155/287G) were transiently transfected in COS-7 cells. After 72 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 point mutants in transfected cell lysates. 50  $\mu$ g of total protein from lysates was separated by SDS-PAGE and immunoblotted with the PA-1 antiserum as specified in Section 2. (B) PKD was immunoprecipitated from cell lysates and assayed for kinase activity with the synthetic peptide syntide-2 (upper panel) or by autophosphorylation (lower panel) as described in Section 2. Upper panel: syntide-2 phosphorylation in immune complexes. Results are the means  $\pm$  S.E.M. from three experiments, each done in duplicate. Lower panel (IVK): the autoradiogram shown is representative of three independent experiments with similar results.

in Fig. 3, the double mutant (P-155/287-G) of PKD displayed a marked increase in kinase basal activity, as shown by autophosphorylation and syntide-2 phosphorylation assays. The activity of PKD P-155/287-G was modestly further increased by treatment of the intact cells with PDB (Fig. 3B).

#### 4. Discussion

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 [31–34]. For example, all members of the PKC family from yeast to human PKCs possess an autoinhibitory pseudosubstrate motif that is located upstream of the first CRD [2]. In contrast, PKD does not contain a typical pseudosubstrate region in a comparable position [17]. Recently, we demonstrated that the PH domain of PKD, which is interposed between cys2 and the catalytic domain, could play a role in maintaining unstimulated PKD in a state of low catalytic kinase activity, acting like an autoinhibitory domain [22]. These results, however, did not exclude the possibility that other regions of the regulatory domain of PKD could also contribute to stabilize an inactive state of this enzyme.

Here, we found that deletion of cys1, cys2 or the entire CRD increases the basal activity of PKD leading to a constitutively active form of this enzyme. Our results demonstrate, for the first time, that the CRD of PKD plays a negative role in the regulation of PKD activity. Previously, we demonstrated that treatment of intact cells with PDB induces rapid PKD activation that was maintained during cell disruption and immunoprecipitation through a PKC-dependent signal transduction pathway [24,27,28]. The results presented here demonstrate that PKDs carrying deletions of the CRD domain were not stimulated further by treatment of the cells with PDB, implying that PKD rendered active by CRD domain mutation is already fully activated. In addition, amino acid substitutions within cys1 and cys2 in the double mutant P-155/287-G also lead to a constitutive active form of PKD. Our results suggest that the CRD domain, like the PH domain, contributes to maintain PKD in a state of low catalytic kinase activity.

Recent results in other systems also indicate that the CRD can play a role in negative regulation of enzyme activity. For example, mutations located in the CRD of Raf-1 increase its biological and enzymatic activity, as shown with *Drosophila* [35] as well as mammalian Raf-1 [34]. In fact, a current model of Raf-1 autoregulation envisages that the CRD directly interacts with the catalytic domain to maintain a repressed state of Raf-1 [34]. At present, it is not clear whether the PKD CRD acts as an autoinhibitory domain, like in Raf-1, or alternatively, binds an inhibitory ligand(s). Future studies should attempt to distinguish between these alternative models as well as to define whether the CRD and the PH domains act in a cooperative fashion to keep unstimulated PKD in a state of low catalytic activity.

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