Dissimilar phorbol ester binding properties of the individual cysteine-rich motifs of protein kinase D

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Abstract Protein kinase D (PKD) 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, CRD). Here, we examined whether the individual cysteine-rich motifs of the CRD of PKD (referred to as cys1 and cys2) are functionally equivalent in mediating phorbol ester binding both in vivo and in vitro. Our results demonstrate that the cys1 and cys2 motifs of the CRD of PKD are functionally dissimilar, with the cys2 motif responsible for the majority of [³H]phorbol 12,13-dibutyrate (PDB) binding, both in vivo and in vitro.
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Key words: Cysteine-rich domain; Phorbol ester binding; Protein kinase C; Protein kinase D

1. Introduction

Protein kinase C (PKC), a major target for the tumor promoting phorbol esters, plays a critical role in signal transduction through its activation by the second messenger diacylglycerol (DAG) [1,2]. The NH_2-terminal regulatory region of classical (α, β, γ) and novel (δ, ε, η, θ) members of the PKC family contains a tandem repeat of conserved cysteine-rich, zinc finger-like motifs [3–5] that mediates phospholipid-dependent DAG/phorbol ester binding [6–12]. An emerging theme in the elucidation of the role of this domain is that the individual cysteine-rich motifs appear to play non-equivalent functions in mediating phorbol ester binding and translocation [9,13–15].

The newly identified protein kinase D (PKD) is a mouse serine/threonine protein kinase with distinct structural and enzymological properties [16]. The catalytic domain of PKD is distantly related to Ca^{2+}-regulated kinases and shows little similarity to the highly conserved regions of the kinase subdomains of the PKC family [17]. 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 [16,18]. However, the amino-terminal region of PKD contains a tandem repeat of cysteine-rich, zinc finger-like motifs (cys1 and cys2) that binds phorbol esters with high affinity [16]. Immunopurified PKD is markedly stimulated by either biologically active phorbol esters or DAG, in the presence of phosphatidylserine (PS) [18,19]. The human protein kinase PKCμ [20] with 92% homology to PKD is also stimulated by phorbol esters and phospholipids [21]. These in vitro results indicate that PKD/PKCμ is a novel phorbol ester/DAG-stimulated kinase.

Interestingly, there are a number of important differences between the cysteine-rich domain (CRD) of PKCs and PKD. The length of the amino-acid sequence separating the individual cysteine-rich motifs of PKD (95 residues) is substantially longer than that of classical PKCs (28 residues) or novel PKCs (35–36 residues) and the residues leucine-147, alanine-154, alanine-156, methionine-164 and tyrosine-182 in cys1 and residues lysine-298 and cysteine-314 in cys2 differ from the PKC cysteine-rich consensus motif [16]. In contrast to all known PKCs including mammalian, *Drosophila* and yeasts isoforms [22], the NH_2-terminal region of PKD contains a putative transmembrane sequence and a pleckstrin homology (PH) domain interposed between cys2 and the catalytic domain that regulates enzyme activity [23]. Furthermore, PKD does not possess a sequence with homology to a typical PKC autoinhibitory pseudosubstrate motif located upstream of the CRD [16,24]. The striking differences between PKD and PKCs as well as between cys1 and cys2, prompted us to examine whether these cysteine-rich motifs of the PKD CRD play equivalent roles in mediating phorbol ester binding to PKD.

The results presented here demonstrate that the cys1 and cys2 motifs of the CRD of PKD are functionally dissimilar, with the cys2 motif responsible for the majority of [³H]PDB binding, both in vivo and in vitro.

2. Materials and methods

2.1. Deletion mutants and site-directed mutagenesis

Deletion mutants were generated by direct-rapid mutagenesis of large plasmids using PCR with rTth DNA polymerase XL with proof-reading capability (Gene Amp XL PCR Kit, Perkin-Elmer). Deletion of the entire CRD domain, amino acids H145–D353 (ΔCRD), deletion of the cys1 motif, amino acids H145–S223 (Δcys1) and deletion of the cys2 motif, amino acids H277–D353 (Δcys2) were made directly in PKD-cDNA cloned in pBluescript-SK(+) using oligonucleotide primers starting upstream (reverse primer, Rp) and downstream (forward primer, Fp) from the desired deletion and contained the unique restriction site *Asc*I (see Table 1). PCR was performed using a DNA Thermal Cycler (Perkin-Elmer) applying a short number of cycles (9 cycles) starting with a large amount of template DNA (1 μg). The PCR parameters were optimized for the oligonucleotides used. After PCR, template DNA was eliminated by *Dpn*I digestion. Amplified DNA was cut with *Asc*I and religated. The re-

sulting deletion construct was then subcloned into the mammalian expression vector pcDNA3 for transient expression in COS-7 cells.

Site-specific mutations within the CRD domain of PKD resulting in single amino acid substitutions (P155G and P287G) were generated by overlap PCR using pBluescript SK(+)-PKD cDNA (pBS-PKD). A sequence upstream of PKD cDNA corresponding to pBSK and close to the polylinker and a sequence near to the *Sph*I site (at nucleotide 1758) within PKD cDNA were used as external forward and reverse primers together with internal reverse and forward primers, respectively, containing the residue substitutions P155(CCA) to G155(GGA) and/or P287(CCC) to G287(GGC) (see Table 1). After the second PCR reaction the amplified fragment was digested with *Xho*I and *Sph*I. pBSK-PKD was also digested with these two restriction enzymes and the wild-type PKD-*Xho*I/*Sph*I fragment was then replaced by the PCR product containing the desired mutation(s). Constructs were subcloned into the mammalian expression vector pcDNA3 for transient expression in COS-7 cells. All constructs were analyzed by DNA restriction enzyme analysis and sequencing and were shown to contain the desired deletion or mutations.

2.2. Glutathione S-transferase (GST)-fusion protein expression and purification

The sequences spanning the entire CRD domain of PKD (residues 105–353) were amplified by PCR from either wild-type (GST-CRD WT) or mutant (GST-CRD P155G; GST-CRD P287G; GST-CRD P155/287G) cDNA sequences using the specific oligonucleotide primers 5′-GCCGGATCCGATCCTGCCTCCGACAAC and 3′-GCGGAATTCCATCACTCCCTTCTTCCAT. PCR products were then subcloned into the pGEX4T3 vector (Pharmacia) using the *Bam*-HI and *Eco*RI restriction sites underlined. All constructs were checked by sequencing. Expression of GST-fusion proteins in *E. coli* was induced by the addition of isopropyl-β-D-thiogalactopyranoside at a final concentration of 0.4 mM for 3 h at 37°C. Bacteria were harvested by centrifugation and lysed in 10 ml lysis buffer containing 1% Triton X-100, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin in phosphate buffered saline (PBS). Lysates were sonicated on ice, clarified by centrifugation and GST-fusion proteins were purified by incubation with glutathione agarose beads for 2 h at 4°C. The beads were then washed and GST-fusion proteins were eluted with 25 mM reduced glutathione. The eluted fractions were dialyzed against PBS before they were stored at −20°C.

2.3. Cell culture and transient transfection

COS-7 cells were plated in 60-mm dishes at 3×10^5 cells/dish in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and used for transfection 1 day later. Exponentially growing COS-7 cells, 40–60% confluent, were transfected with PKD mammalian expression plasmids using Lipofectin (Life Technologies) as previously described [23,25].

2.4. [³H]Phorbol 12,13-dibutyrate (PDB) binding assays

[³H]PDB binding to intact COS-7 cells was performed as described previously [26]. Briefly, cultures were washed twice with DMEM and incubated with binding medium (DMEM containing 1 mg/ml bovine serum albumin) and 10 nM [³H]PDB at 37°C for 30 min. The cultures were then rapidly washed at 4°C with PBS containing 1 mg/ml bovine serum albumin and lysed with NaOH-SDS. Bound radioactivity was determined by liquid scintillation counting. Non-specific binding was determined in the presence of 10 μM unlabeled PDB.

In vitro binding of [³H]PDB to GST-fusion proteins was analyzed by incubating 100 ng of GST-fusion proteins with various concentrations of [³H]PDB and 125 μg/ml PS in the presence of 20 mM Tris-HCl (pH 7.5) in a final volume of 200 μl for 60 min at 4°C. Fusion proteins were recovered by the addition of 300 μl of DE52 cellulose equilibrated with 20 mM Tris-HCl, pH 7.5 (50:50 suspension) for 20 min at 4°C. Incubations were terminated by rapid filtration on a Whatman GF/F glass filter disc followed by rapid washing with 20 mM Tris-HCl, pH 7.5, at 4°C. Bound radioactivity was determined by liquid scintillation counting. Non-specific binding was determined by the addition of 10 μM unlabeled PDB to the incubation mixture. PS was sonicated for 1 min at 4°C to form phospholipid micelles.

2.5. Western blot analysis

COS-7 cells were washed twice in ice-cold PBS and lysed in 50 mM Tris-HCl, pH 7.5, 2 mM EGTA, 2 mM EDTA, 1 mM dithiothreitol, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM 4-(2′-aminoethyl)-benzenesulfonyl fluoride hydrochloride and 1% Triton X-100 (buffer A). For Western blotting of wild-type and mutant PKD expression levels, 50 μg of protein from lysates were extracted for 10 min at 95°C in 2× SDS-PAGE sample buffer and analyzed by polyacrylamide gel electrophoresis (SDS-PAGE) followed by transfer to Immobilon membranes at 100 V, 0.4 A at 4°C for 4 h using a Bio-Rad transfer apparatus. The composition of the transfer buffer was 200 mM glycine, 25 mM Tris, 0.01% SDS and 20% CH₃OH. Membranes were blocked using 5% non-fat dried milk in PBS, pH 7.2 and incubated with PA-1 antiserum (1:500) at room temperature for 3 h in PBS containing 3% non-fat dried milk. Immunoreactive bands were visualized using ¹²⁵I-protein A and autoradiography.

2.6. Materials

[³H]PDB (0.25 mCi/ml) and ¹²⁵I-protein A (15 mCi/ml) were from Amersham International (UK). PDB was obtained from Sigma. Protein A agarose was from Boehringer Mannheim. Oligonucleotide primers were synthesized at the Imperial Cancer Research Fund. All other items were from standard suppliers or as indicated in the text.

Table 1
Oligonucleotides used for mutagenesis

Mutant		Position	Oligonucleotide Sequence
Deletion mutant	ΔCys1	Fp: 795–820	5′-GTC AGG CGC GCC GTC CCT GAT GAG CCT TTA CTG TCT C-3′
		Rp: 540–557	5′-GTC AGG CGC GCC AGG GCC GGA TCT GGA AGT C-3′
	ΔCys2	Fp: 1185–1207	5′-GTC AGG CGC GCC AAT GAC AGT GAG CGG AAC AGT GG-3′
		Rp: 931–952	5′-GTC AGG CGC GCC AAG GCA CCT TCA CCT TAG ACA TCA-3′
	ΔCRD	Fp: 1185–1207	5′-GTC AGG CGC GCC AAT GAC AGT GAG CGG AAC AGT GG-3′
		Rp: 540–557	5′-GTC AGG CGC GCC AGG GCC GGA TCT GGA AGT C-3′
Single amino acid substitutions	P155G	External Fp: pB-SK(+)	5′-GAC TCA CTA TAG GGC GAA TTG GGT ACC G-3′
		Internal Rp: 577–598	5′-CA GAA AGC T**CC** GGC TC TGT ATG-3′
		Internal Fp: 577–598	5′-CA TAC AGA GCC **GG**A GCT TTC TG-3′
		External Rp: 1758–1781	5′-CTT GGG GAT GAC GGG CAT AAG AGC-3′
	P287G	External Fp: pB-SK(+)	5′-GAC TCA CTA TAG GGC GAA TTG GGT ACC G-3′
		Internal Rp: 974–995	5′-GCA GAC CGT G**CC** CCG TGT GTA G-3′
		Internal Fp: 974–995	5′-C TAC ACA CGG **GG**C ACG TCT GC-3′
		External Rp: 1758–1781	5′-CTT GGG GAT GAC GGG CAT AAG AGC-3′

Position and sequence of the oligonucleotides annealing to PKD cDNA for the construction of different mutants: Fp (forward primer) and Rp (reverse primer). Nucleotides of *Asc*I restriction site are underlined. Specific nucleotides changed for single amino acid substitutions are in bold.

3. Results and discussion

3.1. Specific [³H]PDB binding to COS-7 cells transfected with wild-type PKD or PKD with deletions of cys1, cys2 or the entire CRD

To determine the contribution of the individual cysteine-rich motifs to high affinity phorbol ester binding by PKD in vivo, we deleted the first (Δcys1) or the second (Δcys2) cysteine-rich motif as well as the entire CRD (ΔCRD) of PKD (Fig. 1A). The cDNAs encoding wild-type and the mutant PKDs were subcloned into the mammalian expression vector pcDNA3 and transiently transfected into COS-7 cells. As illustrated in Fig. 1B, specific binding of 10 nM [³H]PDB to COS-7 cells expressing wild-type PKD showed a 12-fold increase over vector control levels. COS-7 cells expressing a PKD mutant lacking the cys1 motif (Δcys1) retained a significant degree of specific [³H]PDB binding ($\sim$50% as compared to wild-type PKD). In striking contrast, COS-7 cells expressing a PKD mutant lacking cys2 (Δcys2) did not exhibit any significant increase in specific [³H]PDB over control (pcDNA3) levels. In fact, a deletion of the cys2 motif was as effective as a deletion of the entire CRD (ΔCRD) in eliminating specific [³H]PDB binding to PKD in vivo (Fig. 1B).

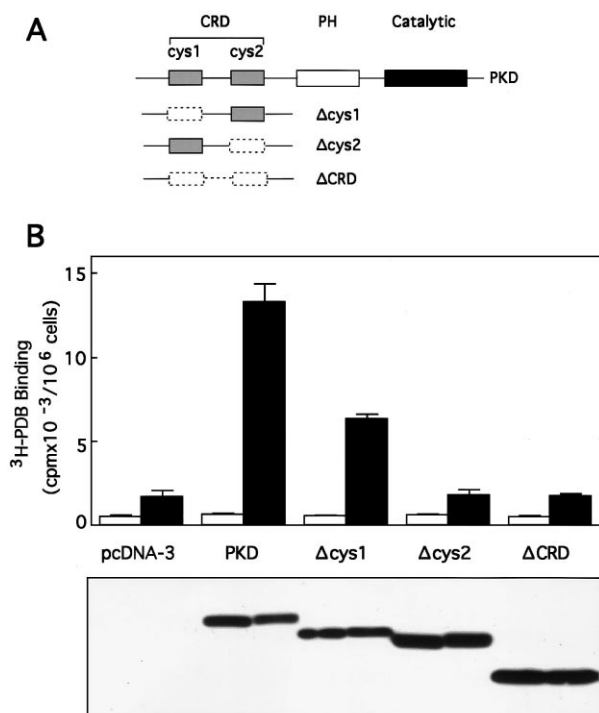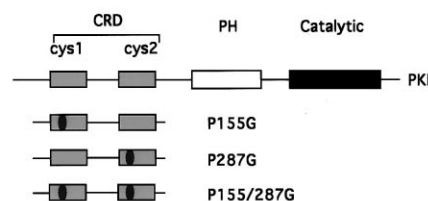Fig. 1. Binding of [³H]PDB to COS-7 cells expressing wild-type and deletion mutants of PKD. A: Schematic representation of the PKD expression constructs containing deletions of either the cys1 (Δcys1) or cys2 (Δcys2) motifs or of the entire CRD (ΔCRD). B: Upper panel: Binding of [³H]PDB to PKD CRD domain deletion mutants expressed in COS-7 cells. Total (filled columns) and non-specific binding (open columns) of 10 nM [³H]PDB to COS-7 cells expressing vector control (pcDNA3), wild-type (PKD) or deletion mutants (Δcys1, Δcys2 and ΔCRD) of PKD are shown, expressed as cpm $\times 10^{-3}/10^6$ cells. Non-specific binding was measured in the presence of 10 μM unlabeled PDB. Values shown are the mean±S.E. from three independent experiments. Lower panel: Western blot showing the expression of the different PKD CRD deletion mutants in transfected cells. Fifty μg of total protein from the cell lysates were separated by SDS-PAGE and immunoblotted with the PA-1 antiserum as specified in Section 2.
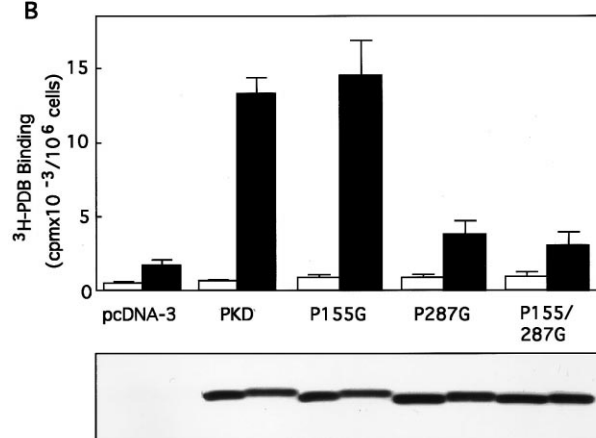

Fig. 2. Binding of [³H]PDB to COS-7 cells expressing wild-type and point mutants of PKD. A: Schematic representation of the PKD expression constructs containing single (P155G, P287G) or double (P155/287G) amino-acid substitutions in the cys1 and cys2 motifs of the PKD CRD. B, upper panel: Binding of [³H]PDB to PKD wild-type or mutant PKDs with single amino acid substitutions expressed in COS-7 cells. Total (filled columns) and non-specific binding (open columns) of 10 nM [³H]PDB to COS-7 cells expressing vector control (pcDNA3), wild-type PKD (PKD) or PKD CRD mutants (P155G, P287G and P155/287G) are shown, expressed as cpm $\times 10^{-3}/10^6$ cells. Non-specific binding was measured in the presence of 10 μM unlabeled PDB. Values shown are the mean±S.E. from three individual experiments. Lower panel: Western blot showing the expression of the different PKD CRD mutants in transfected cells. Fifty μg of total protein from lysates were separated by SDS-PAGE and immunoblotted with the PA-1 antiserum as specified in Section 2.

All PKD constructs (PKD, Δcys1, Δcys2 and ΔCRD) exhibited similar levels of expression, as shown by Western blotting with a specific antibody directed against the C-terminal region of PKD (Fig. 1B, lower panel).

3.2. Specific ³[H]PDB binding to COS-7 cells transfected with wild-type PKD or PKD with single amino-acid substitutions within cys1, cys2 or both

The results presented in Fig. 1 suggested that the cys1 and cys2 motifs of PKD have dissimilar [³H]PDB binding properties. Alternatively, these results could arise from conformational changes introduced into the structure of PKD by these deletions. Consequently, we examined the in vivo binding of [³H]PDB to COS-7 cells expressing wild-type and mutant forms of PKD containing single amino acid substitutions in cys1, cys2 or in both cysteine-rich motifs. 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 [11], we generated PKD mutants which contained single ami-
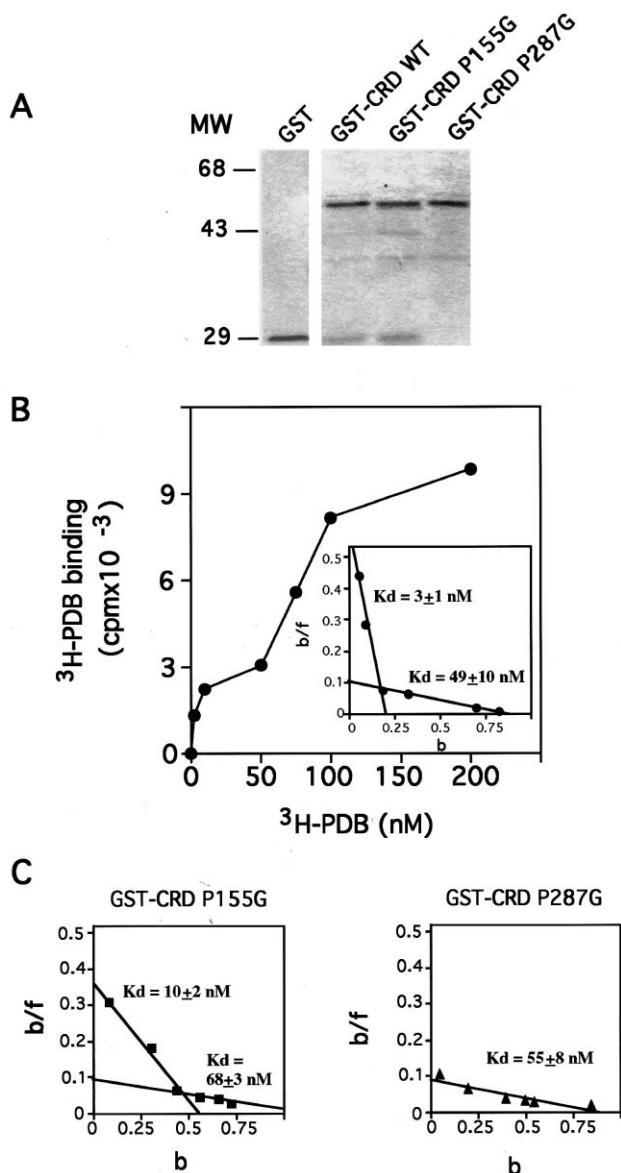
Fig. 3. In vitro binding of [³H]PDB to GST-fusion proteins of the PKD CRD. A: GST-fusion proteins of wild-type or mutant CRDs of PKD were generated as described in Section 2. Fusion protein purification was analyzed by SDS-PAGE and Coomassie blue staining. B: In vitro binding of [³H]PDB (2–200 nM) to 100 ng of GST-CRD WT was carried out as described in Section 2. The results shown are expressed as specific cpm bound. Non-specific binding was <10% of the total binding. All binding measurements were carried out in duplicate. Inset: Scatchard analysis (bound/free vs. bound) of the binding curve for GST-CRD WT. The K_d values for [³H]PDB were determined by linear regression analysis. Results shown are representative of four independent experiments and the K_d values are the mean±S.E. of the four experiments. C: Scatchard analysis of the in vitro [³H]PDB binding curves for GST-CRD P155G and GST-CRD P287G. Results shown (bound/free vs. bound) were determined as for panel B and are representative of three independent experiments. The K_d values of [³H]PDB binding to these mutant CRD fusion proteins were calculated by linear regression analysis and the results shown are the mean±S.E. of three experiments.

no-acid substitutions at this site in the first (P155G), second (P287G) or in both (P155/287G) cysteine-rich motifs of PKD (Fig. 2A).

The level of specific binding of 10 nM [³H]PDB to COS-7 cells expressing a PKD mutant with a proline to glycine mutation in cys1 (P155G) was comparable to that seen in COS-7 cells expressing wild-type PKD (Fig. 2B). In striking contrast, specific [³H]PDB binding to COS-7 cells expressing the P287G or the P155/287G mutants of PKD was not significantly increased over COS-7 cells expressing vector alone, despite similar levels of expression of all these constructs (Fig. 2B). Together, these results indicate that the majority of specific [³H]PDB binding to PKD in vivo is mediated through the cys2 motif.

3.3. Specific [³H]PDB binding to wild-type and mutant GST-fusion proteins comprising the entire CRD of PKD

We subsequently used a different approach to corroborate the non-equivalent roles of cys1 and cys2 of PKD in phorbol ester binding. We produced GST-fusion proteins of the CRD domain of PKD, either wild-type or containing single or double mutations at the conserved proline residues within the cys1 and cys2 motifs described above. The GST-fusion protein preparations used in this study migrated on SDS-PAGE gels at the predicted MW (Fig. 3A). The specific binding of increasing concentrations of [³H]PDB (2–200 nM) to a GST-fusion protein of the wild-type PKD CRD (GST-CRD WT) is shown in Fig. 3B. Scatchard analysis of this binding curve showed two classes of binding sites (Fig. 3B, inset), a pattern observed in four independent experiments (K_d values of 3 and 49 nM, respectively). In view of the results presented in Figs. 1 and 2, the biphasic Scatchard plot could be the consequence of dissimilar affinities of cys1 and cys2 for [³H]PDB in the context of the PKD CRD, e.g. cys2 mediates high affinity binding whereas cys1 provides the low affinity site. In agreement with this hypothesis, a fusion protein of the CRD containing a proline to glycine mutation within the cys2 motif (GST-CRD P287G) had a greatly reduced [³H]PDB binding affinity ($K_d = 55 \pm 8$ nM; Fig. 3C). In contrast, a GST-fusion protein of the PKD CRD containing the comparable proline to glycine mutation within the cys1 motif (GST-CRD P155G) retained the high affinity component for [³H]PDB binding ($K_d = 10 \pm 2$ nM; Fig. 3C). No significant [³H]PDB binding to the double mutant (GST-CRD P155G/287G) could be detected using a range of fusion protein amounts (0.2–2 µg) or [³H]PDB concentrations (2–200 nM; data not shown). GST alone did not bind [³H]PDB.

3.4. Conclusions

Our results demonstrate that the cys1 and cys2 motifs of the PKD CRD differ in their ability to mediate specific phorbol ester binding to this kinase. Mutational analysis of the CRD of PKD followed by in vivo and in vitro binding studies indicate that the major site for phorbol ester binding to PKD lies within the cys2 motif. We conclude that the cys1 and cys2 motifs play distinct roles in the regulation of PKD.

It is increasingly recognized that the individual cysteine-rich motifs of classical and novel PKCs play non-equivalent functions in mediating phorbol ester binding to these enzymes [9,13–15]. Our findings with PKD, which contains a CRD that differs from those of classical and novel PKCs (see Section 1 for details), suggests that this functional non-equivalence of cysteine-rich motifs may be a conserved feature of DAG/phorbol ester sensitive proteins that contain a tandem repeat of these motifs.

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