FEBS Letters 400 (1997) 226–232 FEBS 18001

Cysteine-rich regions of protein kinase Cδ are functionally non-equivalent

Differences between cysteine-rich regions of non-calcium-dependent protein kinase Cδ and calcium-dependent protein kinase Cγ

Martin Hunn, Andrew F.G. Quest*

Institute of Biochemistry, University of Lausanne, Ch. des Boveresses 155, CH-1066 Epalinges, Switzerland

Received 25 September 1996; revised version received 17 November 1996

Abstract Regulatory domain elements of the non-calcium-dependent protein kinase Cδ (nPKCδ), including either or both of the cysteine-rich regions Cys1(δ) and Cys2(δ), were expressed as fusion proteins with glutathione-S-transferase and characterized using liposomal or mixed micellar phorbol ester binding assays. Fusion proteins containing Cys2(δ) bound phorbol-12,13-dibutyrate (PDBu) efficiently in the assay employing phosphatidylserine (PS) vesicles, while no significant binding was seen for proteins containing only Cys1(δ). Likewise, in mixed micellar assays, fusion proteins with Cys2(δ) bound PDBu with high affinity (K_d: 14–37 nM) and to significant stoichiometric levels (0.23–0.66 mol/mol), but no binding could be detected for proteins with Cys1(δ) only. The PS dependence of PDBu binding to Cys2(δ) was highly cooperative with Hill numbers lying in the range of 2.5–5.2. These results demonstrate the presence of striking functional differences between the cysteine-rich regions of nPKCδ and the calcium-dependent isoform, cPKCγ, where both cysteine-rich regions represent functional PDBu binding elements.

Key words: Protein kinase C isoform; Protein kinase C delta; Protein kinase C gamma; Cysteine-rich region; Phosphatidylserine; Phorbol ester

1. Introduction

Protein kinase C (PKC) is a family of at least 11 serine/threonine kinases critically implicated in the control of many cellular processes including important growth-related events like proliferation, differentiation or cell transformation. PKCs are divided into three groups: the calcium-dependent cPKCs (α, β_I, β_{II}, γ), non-calcium-dependent nPKCs (δ, ε, η(L), Θ), atypical aPKCs (ζ, λ/ι), and the novel atypical PKCμ. All in common is the presence of an NH_2-terminal regulatory domain that maintains the kinase in an inactive state in the absence of stimuli [1–5].

A physiologically relevant mechanism of cPKC and nPKC activation involves multiple simultaneous interactions of the regulatory domain with the essential acidic phospholipid co-factor phosphatidylserine (PS) and the neutral lipid activator diacylglycerol (DAG). These interactions induce conformational changes required to release autoinhibition of catalytic domain activity [3–6]. cPKCs and nPKCs are also activated by phorbol esters which, in the presence of PS, bind to a site similar if not identical to that occupied by DAG [5,7–10]. aPKCs, by contrast, neither bind to nor are activated by either phorbol esters or DAGs [5,11–13].

A hallmark of all PKC isoforms characterized so far is the presence of 50–51 amino acid cysteine-rich regions with the general formula H–X_{12}–C–X_2–C–X_n–C–X_2–C–X_4–H–X_2–C–X_7–C, where C = cysteine, H = histidine, X = variable amino acids and n = 13–14 for PKCs [11,14]. The six conserved cysteines and two conserved histidines (C_6H_2) found in all cysteine–rich regions are required for coordination of two atoms of zinc and, with the exception of the most COOH-terminal conserved cysteine, are crucial for these sequence elements to function as phorbol ester binding sites [15,16]. In addition to the C_6H_2 consensus, a conserved proline residue at position 11 has been identified in cysteine-rich regions of cPKCs and nPKCs, which is altered to glycine in aPKCζ. Upon mutation of this conserved proline to glycine in the second cysteine-rich region of PKCδ, phorbol ester binding is abolished [15]. This result provides a rationale to understand the highly distinct regulatory properties of aPKCs, but most of all underscores the importance of cysteine-rich regions as key distinguishing elements between PKC isoforms.

Previous studies characterizing lipid interactions with the regulatory domain elements of PKCγ indicated that Cys1(γ) and Cys2(γ) are functionally indistinguishable when expressed individually as COOH-terminal fusion proteins with glutathione-S-transferase (GST). However, when together as a GST–Cys1Cys2(γ) fusion protein, two binding sites with distinct PDBu binding affinities were detected in the presence of calcium, one of which bound PDBu with an affinity comparable to that of intact PKCγ [17]. The inference that only one of the two cysteine-rich regions present in cPKCs is ultimately responsible for phorbol ester- or DAG-dependent PKC acti-

*Corresponding author. Fax: (41) 21-692-57-05.
E-mail: aquest@eliot.unil.ch

Abbreviations: aPKC, atypical PKC; AT, antisense; C1, first conserved region of PKC; C2, second conserved region of cPKC; C_6H_2, six conserved cysteines and two conserved histidines of cysteine-rich regions; cPKC, calcium-dependent PKC; Cys1, first cysteine-rich region of PKC; Cys2, second cysteine-rich region of PKC; DAG, diacylglycerol; EGTA, ethyleneglycol-bis-(2-aminoethyl)-tetraacetic acid; GST, glutathione-S-transferase; GST-PKCδ, COOH-terminal fusion proteins of GST with regulatory domain elements of PKCδ (see Fig. 1); HEPES, *N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid]; IPTG, isopropyl-1-thio-β-D-galactopyranoside; nPKC, non-calcium-dependent PKC; PKC, protein kinase C; PDBu, 4β-phorbol-12,13-dibutyrate; PMA, phorbol 12-myristate 13-acetate; PS, 1,2-dioleoyl-L-3-phosphatidyl-L-serine; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SN, sense; V1, first variable region of PKC; V2, second variable region of PKC; ψ, pseudosubstrate region of PKC

PII S0014-5793(96)01395-6

vation is entirely consistent with data from the literature showing that binding of either one molecule of phorbol ester or DAG is sufficient for complete PKC activation [17–19].

cPKCs contain a C2 region COOH-terminal to the second cysteine-rich region (Cys2), which is implicated in mediating calcium-dependent activation of these isoforms [20–22] and directly coordinates two calcium atoms between conserved aspartic acid residues [23]. In nPKCs an element homologous to the C2 region, but lacking the aspartic acid residues required for calcium binding, is found NH_2-terminal to the first cysteine-rich region (Cys1) [24,25]. To investigate the regulation of nPKCs and identify differences between cPKCs and nPKCs not necessarily linked to the presence of the C2 region, regulatory domain elements of PKCδ expressed as GST fusion proteins were purified and subsequently characterized. Here we show that, unlike the cysteine-rich regions of cPKCγ [17], Cys1(δ) and Cys2(δ) differ very dramatically in their ability to bind PDBu in the presence of PS. While Cys2(δ) was indistinguishable from the two cysteine-rich regions of PKCγ, Cys1(δ) lacked the capacity to bind PDBu, suggesting that Cys2(δ) is the regulatory domain element responsible for PDBu/DAG-dependent activation of PKCδ.

2. Materials and methods

2.1. Materials

Heat-stable Taq polymerase was from Perkin Elmer, the Geneclean kit from Bio101, 4β-phorbol-12,13-dibutyrate and glutathione agarose from Sigma, 20-[^{3}H]-4β-phorbol-12,13-dibutyrate from DuPont NEN, the 1,2-dioleoyl-L-3-phosphatidyl-L-serine from Avanti Polar Lipids Inc., Triton X-100 from Pierce Chemical Co., the gel filtration matrix Sephacryl S-200-HR from Pharmacia Biotech, and prestained molecular size standards were from BioRad. All other reagents were of the highest quality available.

2.2. Expression of GST-PKCδ fusion proteins

To obtain large quantities of protein for characterization, PKCδ regulatory domain elements were expressed in *Escherichia coli* as COOH-terminal fusion proteins with GST as described [26]. Briefly, elements of the regulatory domain of rat PKCδ [21] were amplified by the polymerase chain reaction (PCR) with 5′ end primers (SN) containing *Bam*HI and *Bgl*II restriction sites and 3′ end primers (AT) containing *Kpn*I and *Eco*RI restriction sites. The following list shows all primers used in PCR experiments with their respective abbreviations indicating either the first (SN primers at 5′ end) or last amino acid residue (AT primers at 3′ end) of PKCδ present with the restriction sites underlined:

delta/A2 (SN): aa gga tcc aga tct gca ccg ttc ctg cg
delta/Q150 (SN): cgc cgt gga tcc aga tct cag gcc aag att
delta/Q221 (SN): agc cgg gga tcc aga tct cag aaa gaa cgc
delta/K157 (AT): t ggc gat gaa ttc ggt acc ctt gat gta gtg
delta/R216 (AT): t ctg gaa ttc ggt acc ccg gct att gg
delta/T295 (AT): tgg ctt ccg gaa ttc ggt acc ggt cac ttg gtt c
delta/T331 (AT): at ctt gaa ttc ggt acc gtt gtt gtc tgg gat

DNA amplification was carried out for 35 cycles, each at 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min. Amplification products were digested with *Bam*HI/*Eco*RI, analyzed on 2% agarose gels, isolated using Geneclean® (from Bio101) following standard protocols provided by the manufacturer, and ligated into *Bam*HI/*Eco*RI double-digested pGEX2T plasmid (Pharmacia Biotech). Following ligation, *E. coli* XL1B (Stratagene) were transformed with the plasmids by electroporation (2.5 kV for 4–5 s). Positive clones were initially selected by restriction analysis and subsequently confirmed by sequencing. For all plasmids encoding Cys2(δ), a single base change in comparison to the originally published sequence [21] was detected leading to a replacement of threonine by serine at position 249 of PKCδ. Fusion proteins were expressed in *E. coli* at 20°C for 16 h after addition of IPTG to a final concentration of 0.2 mM as described [26]. Cells were harvested by centrifugation and cell pellets were stored frozen at −20°C.

2.3. Purification of GST fusion proteins

GST fusion proteins were purified from *E. coli* cell lysates in the presence of protease inhibitors by affinity chromatography using glutathione-agarose (Sigma) and subsequently stored at −20°C in the elution buffer [26]. Proteins were quantitated by the Amido Black dye binding assay [27] using fatty acid-free bovine serum albumin as a standard.

2.4. [^{3}H]PDBu-binding assay

PDBu binding was measured either in the vesicle assay in the presence of 100 nM [^{3}H]PDBu and 1 mM $CaCl_2$ [10,28] or in the mixed micellar assay as described [17,19]. To determine PDBu binding in mixed micelle experiments, the [^{3}H]PDBu concentration in the reaction mixture was varied from 10 to 200 nM in the presence of either 1 mM $CaCl_2$ or 1 mM EGTA and 30 mol% PS. For measurements of PS concentration-dependent PDBu binding, the PDBu concentration was held constant at 50 nM and the PS concentration in mixed micelles was varied from 4 to 30 mol% in the presence of either 1 mM $CaCl_2$, 1 mM $MgCl_2$ or 1 mM EGTA. Protein-bound and free PDBu was separated by gel filtration in Sephacryl S-200-HR columns (bed volume: 2 ml).

2.5. Data analysis

PDBu concentration-dependent PDBu binding was evaluated by Scatchard analysis as described [10,17]. PS concentration-dependent PDBu binding was analyzed by a non-linear least squares to fit to a modified Hill equation as described [10,17]:

$$y = a\left(\frac{x^{n_H}}{k^{n_H} + x^{n_H}}\right)$$

where y is the amount of PDBu binding measured, a the maximum PDBu binding value, x the concentration of PS, k the concentration of PS resulting in half-maximal binding, and n_H the Hill coefficient.

2.6. Analysis by SDS-PAGE/immunoblots

Fusion proteins were characterized by SDS-PAGE on 10% minigels (BioRad) and identified with an anti-GST antibody [17] after transfer to nitrocellulose [29].

3. Results

3.1. Purification and characterization of GST-PKCδ fusion proteins

In order to gain deeper insight into lipid interactions in the regulatory domain of nPKCδ relevant to activation, different elements (see Fig. 1) were expressed in *E. coli* as GST fusion proteins and purified following protocols established for PKCγ [10,17,26]. In contrast to PKCγ [17,26] all GST-PKCδ fusion proteins listed (Fig. 1) were readily expressed as soluble proteins without severe degradation in the strain XL1B. Purification by affinity chromatography on glutathione agarose yielded in several cases homogeneous protein preparations as judged by Coomassie Blue staining (Fig. 2A; lanes 1, 2, 5 and 8). For other fusion proteins, multiple bands were visible (Fig. 2A; lanes 3, 4, 6, 7 and 9), but also here a major band of the anticipated molecular mass was clearly identifiable (see arrows). The less abundant protein species in these preparations generally migrated with somewhat lower apparent molecular weight compared to the major species, suggesting that the former might represent degradation products of the latter.

To further confirm the identity of the expressed and purified fusion proteins, these were transferred to nitrocellulose and probed with an anti-GST antibody (Fig. 2B). The major bands visible after Coomassie Blue staining, which migrated with apparent molecular masses anticipated for the respective

GST fusion protein (Code Nr./name)	PKCδ amino acids present	Primers used for PCR (sense/antisense)	Predicted M_r (x 10^{-3}) of fusion protein
1 / GST-V1	2 - 157	A2(SN) / K157(AT)	45.3
2 /GST-V1Cys1	2 - 216	A2(SN) / R216(AT)	52.0
3 / GST-V1Cys1Cys2	2 - 295	A2(SN) / T295(AT)	61.1
4 / GST-V1Cys1Cys2hinge	2 - 331	A2(SN) / T331(AT)	65.0
5 / GST-Cys1	150 - 216	Q150(SN) / R216(AT)	35.2
6 / GST-Cys1Cys2	150 - 295	Q150(SN) / T295(AT)	44.3
7 / GST-Cy1Cys2hinge	150 - 331	Q150(SN) / T331(AT)	48.1
8 / GST-Cys2	221 - 295	Q221(SN) / T295(AT)	36.1
9 / GST-Cys2hinge	221- 331	Q221(SN) / T331(AT)	40.0

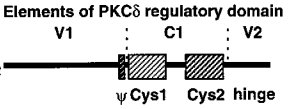

Fig. 1. Expression of PKCδ regulatory domain elements as GST fusion proteins. GST fusion proteins with PKCδ regulatory domain elements were generated by PCR using the indicated combinations of sense (SN) and antisense (AT) primers, followed by ligation into pGEX2T, and subsequent expression in the *E. coli* strain XL1B (see Section 2). Amino acids of PKCδ present and predicted molecular weights (M_r) of the respective GST-PKCδ fusion proteins are shown. Regulatory domain elements of PKCδ present in the respective fusion proteins are illustrated schematically: V1, first variable region containing the pseudosubstrate region (ψ); C1, first conserved region with the two cysteine-rich regions (Cys1 and Cys2); V2, second variable region or hinge region.

fusion proteins (Fig. 1), were also detected by this antibody (Fig. 2A,B; see arrows). In those preparations where multiple bands were visible after Coomassie Blue staining (Fig. 2A; lanes 3, 4, 6, 7 and 9), corresponding additional bands migrating ahead of the major band, were also detected by the antibody, supporting the notion that apparent impurity of the preparations was predominantly due to degradation of the respective fusion protein. However, it is important to note that degradation affected mainly those proteins containing both cysteine-rich regions (Fig. 2; lanes 3, 4, 6 and 7) or Cys2(δ) together with the hinge region (Fig. 2; lane 9) but not those with V1Cys1(δ), Cys1(δ) or Cys2(δ) (Fig. 2; lanes 2, 5 and 8). Protease cleavage sites are present within the hinge regions of PKCs [30,31] and thus fusion proteins containing this element (Fig. 2; lanes 4, 7 and 9) are expected to be more susceptible to degradation.

3.2. PDBu binding in the vesicle assay

In the presence of PS liposomes, all fusion proteins containing either Cys2(δ) alone (Fig. 3; fusion proteins 8 and 9) or Cys1(δ) and Cys2(δ) together (Fig. 3; fusion proteins 3, 4, 6 and 7) readily bound PDBu, while those with only Cys1(δ) (Fig. 3; fusion proteins 2 and 5) were essentially non-functional, as was also a fusion protein lacking any of the cysteine-rich regions (Fig. 3; fusion protein 1). Highest levels of binding, comparable to values previously reported for GST–PKCγ fusion proteins [17,26] were measured for GST–PKCδ fusion proteins containing both cysteine-rich regions, despite more apparent proteolytic degradation in these preparations (Fig. 2; lanes 3, 4, 6 and 7).

3.3. PDBu binding in the mixed micellar assay: PDBu concentration dependence

To investigate these differences in greater detail, PDBu binding was also measured using the mixed micellar assay, which permits the determination of binding affinities and stoichiometries (Fig. 4 and Table 1). As anticipated based upon the above results, the fusion proteins GST–Cys2(δ) (Fig. 4A,B) or GST–Cys1Cys2(δ) (Fig. 4E,F) bound PDBu with high affinity (19.1 ± 2.6 nM and 18.2 ± 3.3 nM, respectively) and comparable stoichiometries (0.37 ± 0.05 and 0.49 ± 0.09), while no significant binding could be detected with Cys1(δ) alone (Fig. 4C,D).

All purified fusion proteins (Figs. 1 and 2) were characterized in the manner shown in Fig. 4, either in the presence of

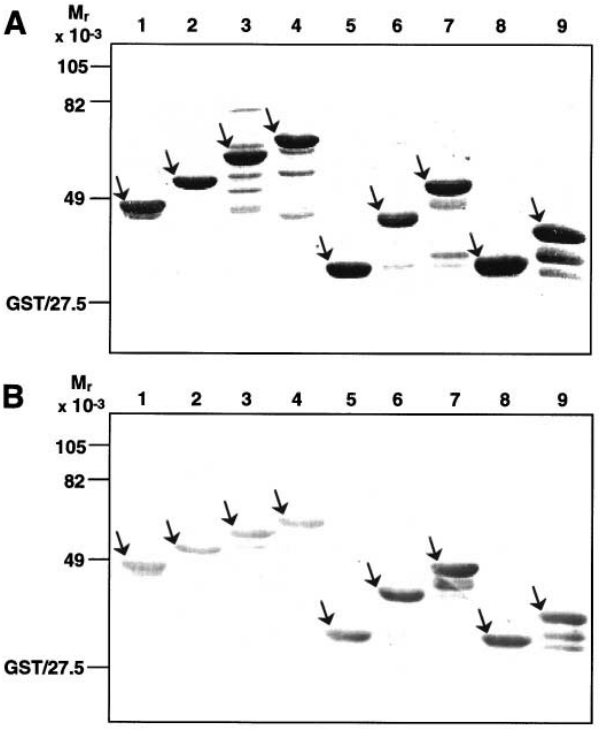

Fig. 2. Characterization of GST-PKCδ fusion proteins. Affinity-purified GST-PKCδ fusion proteins (3 μg/lane) resolved by SDS-PAGE on 10% gels were stained with Coomassie Blue (A) or transferred to nitrocellulose, where they were probed with a polyclonal rabbit anti-GST antibody (B): lane 1, GST–V1; lane 2, GST–V1Cys1; lane 3, GST–V1Cys1Cys2; lane 4, GST–V1Cys1Cys2hinge; lane 5, GST–Cys1; lane 6, GST–Cys1Cys2; lane 7, GST–Cys1Cys2hinge; lane 8, GST–Cys2; lane 9, GST–Cys2hinge. Bands corresponding in size to the anticipated M_r values of the GST–fusion proteins are marked with an arrow. Values of prestained molecular weight markers are indicated on the left. Additionally the migration position of GST alone (27.5 kDa) is indicated.

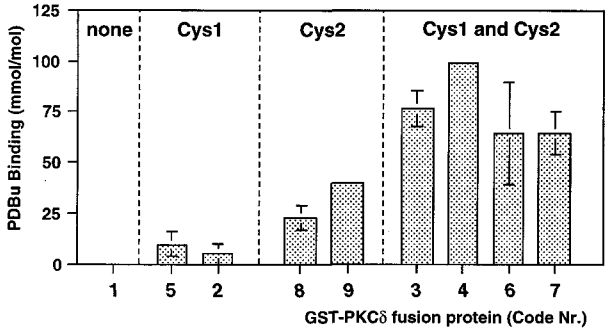

Fig. 3. Comparison of PDBu binding of GST–PKCδ fusion proteins in the vesicle assay. [^{3}H]PDBu binding of PKCδ fusion proteins (mmol PDBu/mol protein) is depicted. Errors indicated are standard deviations of at least three independent experiments. Standard deviations below the threshold values of 5 are not visible on the graph. Fusion proteins are abbreviated by code numbers shown in Fig. 1. Results are grouped according to the presence of cysteine-rich regions: none, fusion proteins lacking cysteine-rich regions; Cys1, fusion proteins with only Cys1; Cys2, fusion proteins with only Cys2; Cys1 and Cys2, fusion proteins with both cysteine-rich regions. PDBu binding values (mmol PDBu/mol protein) for fusion proteins, listed with the respective code numbers in brackets, are as follows: V1 (1) 0; V1Cys1 (2) 5±5; Cys1 (5) 9±6; Cys2 (8) 23±6; Cys2hinge (9) 40±2; V1Cys1Cys2 (3) 77±5; V1Cys1Cys2hinge (4) 99±1; Cys1Cys2 (6) 65±21; Cys1Cys2hinge (7) 65±11.

1 mM $CaCl_2$ or 1 mM EGTA. As suspected for regulatory domain elements of an nPKC, where calcium is not required for activation, no significant differences in either stoichiometry (averaged values: 0.51±0.10 and 0.42±0.25, respectively) or affinity (averaged values: 24.3±4.5 nM and 20.6±8.6 nM, respectively) of binding were noticeable. Binding was seen with fusion proteins containing the Cys2(δ) region (Table 1, fusion proteins 3, 4, 6, 7, 8, 9) but not with those in which only Cys1(δ) was present (Table 1, fusion proteins 2 and 5). Amongst Cys2(δ)-containing proteins no distinction was possible between those containing only Cys2(δ) (fusion proteins 8, 9: averaged affinity, 19.5±4.4; stoichiometry, 0.42±0.10) and those with both Cys1(δ) and Cys2(δ) (fusion proteins 3, 4, 6, 7: averaged affinity, 23.8±7.7; stoichiometry, 0.49±0.23), suggesting that Cys2(δ) represents the element in the regulatory domain of PKCδ exclusively responsible for PDBu binding and, by inference, DAG-dependent PKCδ activation.

3.4. PDBu binding in the mixed micellar assay: PS concentration dependence

Experiments assessing the dependence of PDBu binding on the PS concentration in the presence of 1 mM $CaCl_2$, 1 mM $MgCl_2$ or 1 mM EGTA are shown for GST–Cys2(δ)hinge (Fig. 5). Values for the PS concentration required for half maximal binding varied depending on the conditions, being similar in the presence of 1 mM $CaCl_2$ or 1 mM EGTA (13.5±1.7 and 11.3±0.6 mol%, respectively), but slightly higher in the presence of magnesium (17.0±6.1 mol%), where also total PDBu binding doubled. An increase in PDBu binding in the presence of magnesium, but without alteration in the PS concentration required for half maximal binding was reported for GST–PKCγ fusion proteins [17]. Alternatively, the Hill numbers (n_H) calculated for PS concentration-dependent PDBu binding are similar in the presence of 1 mM $CaCl_2$ or 1 mM $MgCl_2$ (n_H = 2.5±0.5 and 2.6±1.3, respectively), but somewhat higher in the presence of 1 mM EGTA (n_H = 5.2±1.4), following the trend previously seen for cysteine-rich regions of cPKCγ [17], although values obtained for Cys2hinge of PKCδ under the different conditions are slightly lower.

4. Discussion

A common feature of cPKCs and nPKCs is their activation by DAGs and phorbol esters such as phorbol 12-myristate 13-acetate (PMA) or PDBu in the presence of PS, as well as the presence of two cysteine-rich regions in tandem within the regulatory domain. While cysteine-rich regions are essential for lipid-dependent PKC activation, recent experiments in particular with cPKCs indicate that they are not functionally equivalent in the context of the intact regulatory domain. For instance, two distinct phorbol ester binding sites have been detected in PKCα that differ in their PDBu binding affinity [11] and their ability to bind the fluorescent phorbol ester analog sapintoxin-D [32]. Also, cysteine-rich regions of

Table 1
Summary of PDBu binding values determined in the mixed micellar assay for GST–PKCδ fusion proteins

GST–PKCδ fusion protein		+Ca		+EGTA	
Code no.	Name	K_d (nM±SD)	Stoichiometry (mol/mol±SD)	K_d (nM±SD)	Stoichiometry (mol/mol±SD)
1	V1	nb		nb	
5	Cys1	nb		nb	
2	V1Cys1	nb		nb	
3	V1Cys1Cys2	28.6±4.7	0.41±0.16	14.3±2.4	0.23±0.04
4	V1Cys1Cys2hinge	28.3±0.7	0.57±0.01	16.3±4.5	0.38±0.10
6	Cys1Cys2	18.2±3.3	0.49±0.09	21.3±4.8	0.25±0.06
7	Cys1Cys2hinge	26.7±5.9	0.66±0.15	37.0±12.0	0.92±0.29
8	Cys2	19.1±2.6	0.37±0.05	14.3±1.8	0.33±0.04
9	Cys2hinge	25.0±1.5	0.56±0.03	nd	nd
Average		24.3±4.5	0.51±0.10	20.6±8.6	0.42±0.25

PDBu binding was measured in the presence of either 1 mM $CaCl_2$ (Ca) or 1 mM EGTA (EGTA) and Triton X-100 mixed micelles containing 30 mol% PS. PDBu binding affinities (K_d, nM) and stoichiometries (n, mol/mol protein) were calculated by Scatchard analysis. Errors indicated are equivalent to the standard deviations (SD) obtained by regression analysis. Fusion proteins lacking Cys2 did not bind PDBu (nb). Average values for PDBu binding affinity and stoichiometry of all fusion proteins containing Cys2 were 24.3±4.5 nM, n = 0.51±0.10 mol/mol (Ca), and 20.6±9.6 nM, n = 0.42±0.28 mol/mol (EGTA). For GST fusion proteins having both cysteine-rich regions (V1Cys1Cys2, V1Cys1Cys2hinge, Cys1Cys2, Cys1Cys2hinge), the average values for PDBu binding affinity and stoichiometry were 23.8±7.7 nM and n = 0.49±0.23 mol/mol. For Cys2 and Cys2hinge, averaged PDBu affinity and stoichiometry values were 19.5±4.4 nM and n = 0.42±0.10 mol/mol.

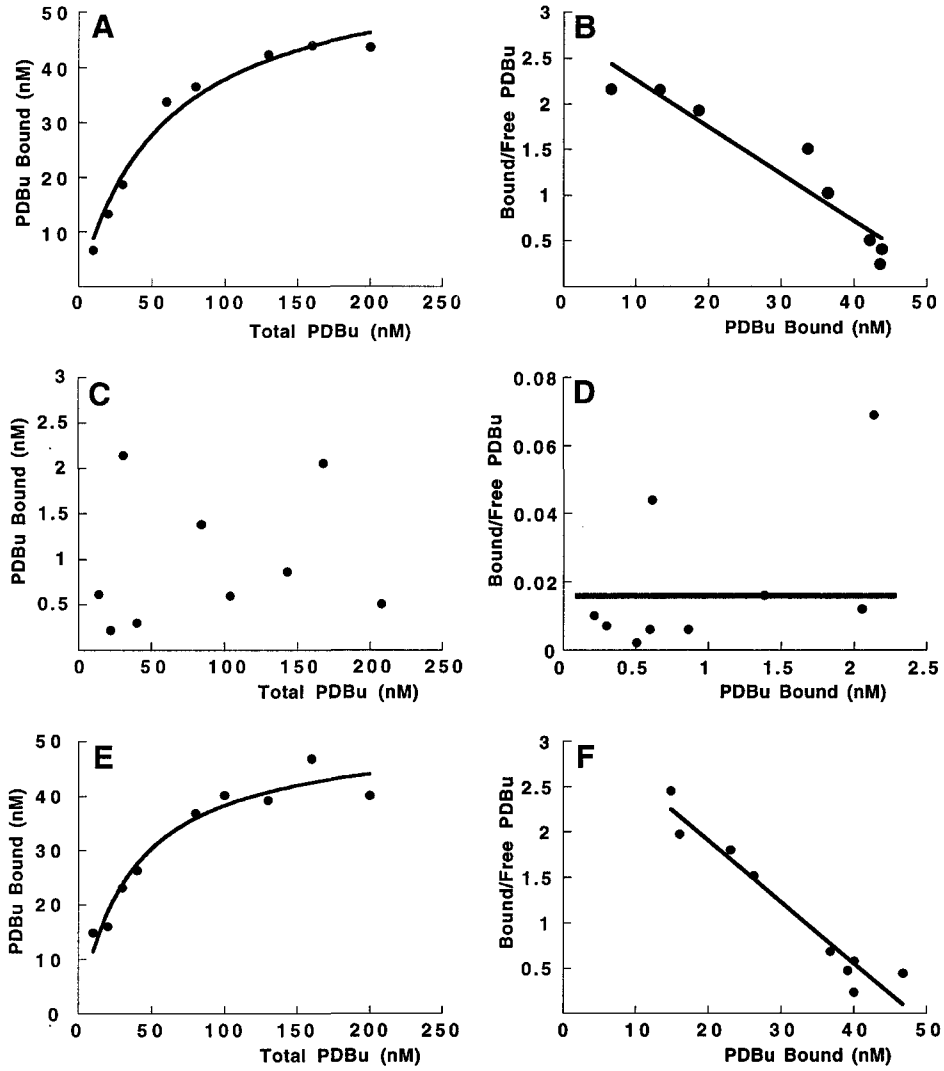

Fig. 4. Comparison of PDBu binding of GST–PKCδ fusion proteins GST–Cys1, GST–Cys2, and GST–Cys1Cys2 in the mixed micellar assay. PDBu binding of GST–Cys2 (A,B), GST–Cys1 (C,D), or GST–Cys1Cys2 (E, F) were measured in the presence of 1 mM $CaCl_2$ and Triton X-100 mixed micelles containing 30 mol% PS. [^{3}H]PDBu binding is depicted as a function of the PDBu concentration present (A,C and E) or the corresponding Scatchard plots are shown (B,D and F). Binding affinity (K_d) as well as the stoichiometry (n) for GST–Cys2 ($K_d = 19.1 \pm 2.6$ nM, $n = 0.37 \pm 0.05$ mol/mol) and GST–Cys1Cys2 ($K_d = 18.2 \pm 3.3$ nM, $n = 0.49 \pm 0.09$ mol/mol) were determined by regression analysis.

PKCγ which are functionally equivalent when expressed individually, behaved very differently in a GST–Cys1Cys2γ fusion protein [17]. Finally, one molecule of either phorbol ester or DAG is sufficient for maximum PKC activation and binding stoichiometries for either PDBu or DAG are around one [7,18,19]. Taken together, these results indicate that the context in which a cysteine-rich region is present within the regulatory domain of cPKCs dictates whether it directly mediates binding of either DAGs or phorbol esters critical for activation.

In the work presented here, we have characterized GST–PKCδ fusion proteins, as previously described for PKCγ, with particular emphasis on identifying differences between cysteine-rich regions. In the vesicle assay, significant PDBu binding was seen with any fusion protein containing Cys2(δ), but not with those in which only Cys1(δ) was present (Fig. 3), although the quality of non-functional Cys1(δ) containing fusion proteins was comparable to those containing Cys2(δ) (see SDS-PAGE analysis in Fig. 2). Elevated PDBu binding in fusion proteins with both cysteine-rich regions in the vesicle assay (Fig. 3) indicated that Cys1(δ) might promote PDBu binding in fusion proteins containing both cysteine-rich regions. Measurements in the mixed micellar assay clearly confirmed that the presence of Cys2(δ) was essential for high-affinity PDBu binding (Fig. 4). Furthermore, no significant differences in either PDBu binding affinity or stoichiometry were apparent between any of the Cys2(δ)-containing fusion proteins (Table 1). Thus, the presence of Cys1(δ) did not facilitate PDBu binding to Cys2(δ)-containing fusion proteins in the mixed micellar assay by any of these criteria.

PS concentration dependence of PKC activation and PDBu binding is highly cooperative for cPKCs with Hill numbers ranging from 4 to 11, indicative of multiple PS interactions being critical for activation [18,33,34]. Experiments analyzing cysteine-rich regions indicate that for PKCγ the PS binding sites critical for activation are present within an individual cysteine-rich region. PS concentration-dependent PDBu binding experiments with GST–Cys2(δ)hinge (Fig. 5) showed that

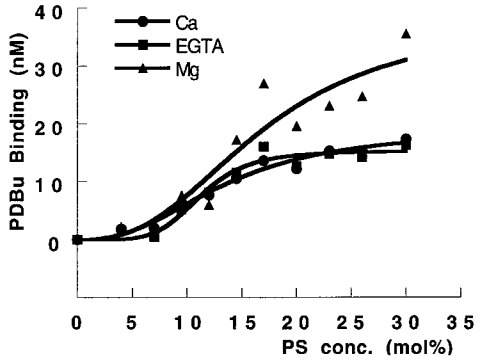

Fig. 5. PS concentration dependent PDBu binding of GST–Cys2-hinge in the mixed micellar assay. [^{3}H]PDBu binding is shown as a function of the PS concentration (4–30 mol%) in the presence of 1 mM $CaCl_2$ (●), 1 mM $MgCl_2$ (▲), or 1 mM EGTA (■). Binding data were analyzed by non-linear regression analysis and curves representing best fits are shown. PS concentrations for half-maximal binding as well as Hill numbers (n_H) calculated for these curves are as follows: 13.5±1.7 mol% and n_H = 2.5±0.5 (Ca); 11.3±0.6 mol% and n_H = 5.2±1.4 (EGTA); 17.0±6.1 mol% and n_H = 2.6±1.3 (Mg).

PDBu binding was also highly cooperative, yielding Hill coefficients (n_H=2.5–5.2) and PS concentrations for half maximal binding (11.3–17 mol%) comparable to those reported for both Cys1(γ) and Cys2(γ) [17]. Analysis of GST–Cys1Cys2(δ) in the same manner yielded similar results (data not shown) providing additional evidence for the absence in Cys1(δ) of lipid interactions sites critical for PDBu binding.

A possible concern regarding these experiments is that PDBu binding stoichiometries for Cys2(δ) containing fusion proteins were significantly lower than 1, suggesting that, although soluble, fusion proteins might not necessarily be functional. In this case, the inability of Cys1(δ) to bind phorbol esters with detectable affinity could reflect a problem of Cys1(δ) folding related to the expression system rather than a property inherent to this particular cysteine-rich region, and thus also to PKC(δ). Here, however, two points are worth noting. First, binding stoichiometries for Cys2(δ) are higher, on an average, than those values reported for both cysteine-rich regions of PKCγ [17,26]. Second, PDBu binding stoichiometries are generally lower than one. Even with purified PKC (rat brain or recombinant), where PDBu binding affinities are an order of magnitude higher, stoichiometries detected may vary considerably depending on the preparation and the assay conditions employed, generally lying anywhere between 0.5 and 1.0 for recombinant PKC [17] and sometimes even lower for PKC purified from rat brain (A. Quest, unpublished data). Such variations may be attributed to differences in enzyme source and preparation, as well as the fact that phorbol ester binding is not entirely irreversible [17]. PDBu binding stoichiometries can vary somewhat in the mixed micelle assay depending upon the affinity, as is illustrated by results obtained with recombinant PKCγ in the presence or absence of calcium, where PDBu binding affinities vary between 3 and 30 nM and binding stoichiometries roughly between 1.0 and 0.6, respectively, for comparable PKC preparations [17]. Hence, PDBu binding stoichiometries determined for fusion proteins containing Cys2(δ) correspond to values that might be expected based on the PDBu binding affinities determined for these proteins, indicating that the majority of soluble, purified Cys2(δ)-containing fusion proteins do represent functional PDBu binding sites. Taken together, therefore, our results strongly suggest that the inability of Cys1(δ) to bind PDBu represents a property inherent to this particular cysteine-rich motif.

In summary, the results presented here highlight a further striking difference in the regulatory domain between cPKCs and nPKCs, in addition to the presence or absence of a C2 region. While Cys2(δ) is a functional PDBu binding site, with properties very similar to those reported for both cysteine-rich regions of PKCγ, Cys1(δ) is essentially non-functional in this respect. Thus, Cys2(δ) most likely represents the region in PKCδ responsible for phorbol ester- and DAG-dependent activation. Studies are currently on the way in our laboratory to identify alternative roles for Cys1(δ) in PKCδ regulation.

The above conclusion is corroborated in a recent report by Blumberg and coworkers using a different approach. Conserved prolines [15] in cysteine-rich regions of PKCδ were converted to glycine and the ability of resulting PKCδ mutants to bind PMA was assessed after expression in NIH3T3 cells. Mutations in either Cys2(δ) alone or both Cys1(δ) and Cys2(δ), but not Cys1(δ) alone, reduced PMA-induced membrane translocation of PKCδ [35].

Acknowledgements: Parts of this work have previously been presented in abstract form (*Experientia* 52, A36 (1996) and PW11.19, 24th FEBS Meeting, Barcelona, 1996). This work was supported by the Swiss National Science Foundation (SNF 3100-040477 to A.F.G. Quest). The authors are grateful to Dr. Y. Nishizuka for providing the PKCδ template cDNA. Furthermore, Drs. Lisette Leyton, Claude Bron and Stefan Demotz are acknowledged for careful reading of the manuscript.

References

[1] Nishizuka, Y. (1984) Nature 308, 693–698.
[2] Nishizuka, Y. (1988) Nature 334, 661–665.
[3] Nishizuka, Y. (1992) Science 258, 607–614.
[4] Newton, A.C. (1995) J. Biol. Chem. 270, 28495–28498.
[5] Quest, A.F.G. (1996) Enzyme Prot. 49, in press.
[6] Takai, Y., Kishimoto, A., Inoue, M. and Nishizuka, Y. (1977) J. Biol. Chem. 252, 7603–7609.
[7] Koenig, B., Di Nitto, P.A. and Blumberg, P.M. (1985) J. Cell. Biochem. 29, 37–44.
[8] Sharkey, N.A., Leach, K.L. and Blumberg, P.A. (1984) Proc. Natl. Acad. Sci. USA 81, 607–610.
[9] Sharkey, N.A. and Blumberg, P.M. (1985) Biochem. Biophys. Res. Commun. 133, 1051–1056.
[10] Quest, A.F.G., Bardes, E.S.G. and Bell, R.M. (1994) J. Biol. Chem. 269, 2953–2960.
[11] Kazanietz, M.G., Krausz, K.W. and Blumberg, P.M. (1992) J. Biol. Chem. 267, 20878–20886.
[12] Ono, Y., Fujii, T., Ogita, K., Kikkawa, U., Igarashi, K. and Nishizuka, Y. (1989) Proc. Natl. Acad. Sci. USA 86, 3099–3103.
[13] Ways, D.K., Cook, P.P., Webster, C. and Parker, P.J. (1992) J. Biol. Chem. 267, 4799–4805.
[14] Ahmed, S., Kozma, R., Lee, J., Monfries, C., Harden, N. and Lim, L. (1991) Biochem. J. 280, 233–241.
[15] Kazanietz, M.G., Wang, S., Milne, G.W.A., Lewin, N.E., Liu, H.L. and Blumberg, P.M. (1995) J. Biol. Chem. 270, 21852–21859.
[16] Quest, A.F.G., Bardes, E.S.G. and Bell, R.M. (1994) J. Biol. Chem. 269, 2961–2970.
[17] Quest, A.F.G. and Bell, R.M. (1994) J. Biol. Chem. 269, 20000–20012.
[18] Hannun, Y.A., Loomis, C.R. and Bell, R.M. (1985) J. Biol. Chem. 260, 10039–10043.
[19] Hannun, Y.A. and Bell, R.M. (1986) J. Biol. Chem. 261, 9341–9347.
[20] Ohno, S., Akita, Y., Konno, Y., Imajoh, S. and Suzuki, K. (1988) Cell 53, 731–741.

[21] Ono, Y., Fujii, T., Ogita, K., Kikkawa, U., Igarashi, K. and Nishizuka, Y. (1988) J. Biol. Chem. 263, 6927–6932.
[22] Clark, J.D., Lin, L.L., Kriz, R.W., Ramesha, C.S., Sultzman, L.A., Lin, A.Y., Milona, N. and Knopf, J.L. (1991) Cell 65, 1043–1051.
[23] Shao, X., Davletov, B.A., Sutton, R.B., Suedhof, T.C. and Rizo, J. (1996) Science 273, 248–251.
[24] Sossin, W.S. and Schwartz, J.H. (1993) Trends Biochem. Sci. 18, 207–208.
[25] Newton, A.C. (1995) Curr. Biol. 5, 973–976.
[26] Quest, A.F.G., Bardes, E.S.G., Xie, W.Q., Willot, E., Borchardt, R.A. and Bell, R.M. (1995) Methods Enzymol. 252, 153–167.
[27] Schaffner, W. and Weissmann, C. (1973) Anal. Biochem. 56, 502–514.
[28] Ebeling, J.G., Vandenbark, G.R., Kuhn, L.J., Ganong, B.R., Bell, R.M. and Niedel, J.E. (1985) Proc. Natl. Acad. Sci. USA 82, 815–819.
[29] Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
[30] Hug, H. and Sarre, T.F. (1993) Biochem. J. 291, 329–343.
[31] Lee, M.H. and Bell, R.M. (1986) J. Biol. Chem. 261, 14867–14870.
[32] Slater, S.J., Ho, C., Kelly, M.B., Larkin, J.D., Taddeo, F.J., Yeager, M.D. and Stubbs, C.D. (1996) J. Biol. Chem. 271, 4627–4631.
[33] Burns, D.J., Bloomenthal, J., Lee, M.-H. and Bell, R.M. (1990) J. Biol. Chem. 265, 12044–12051.
[34] Newton, A.C. and Koshland, D.E. Jr. (1989) J. Biol. Chem. 264, 14909–14915.
[35] Szallasi, Z., Bogi, K., Gohari, S., Biro, T., Acs, P. and Blumberg, P.M. (1996) J. Biol. Chem. 271, 18299–18301.