The cysteine-rich domain of human proteins, neuronal chimaerin, protein kinase C and diacylglycerol kinase binds zinc

Evidence for the involvement of a zinc-dependent structure in phorbol ester binding

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Diacylglycerol (DG) and its analogue phorbol 12-myristate 13-acetate (PMA) activate the ubiquitous phospholipid/ Ca^{2+} -dependent protein kinase, protein kinase C (PKC), and cause it to become tightly associated with membranes. DG is produced transiently as it is rapidly metabolized by DG kinase (DGK) to phosphatidic acid. Phorbol esters such as PMA are not metabolized and induced a prolonged membrane association of PKC. Until recently, PKC was the only known phorbol ester receptor. We have shown that a novel brain-specific cDNA, neuronal chimaerin (NC), expressed in *Escherichia coli*, binds phorbol ester with high affinity, stereospecificity and a phospholipid requirement [Ahmed, Kozma, Monfries, Hall, Lim, Smith & Lim (1990) *Biochem. J.* 272, 767–773]. The proteins NC, PKC and DGK possess a cysteine-rich domain with the motif $\text{HX}_{11/12}\text{CX}_2\text{CX}_n\text{CX}_2\text{CX}_4\text{HX}_2\text{CX}_{8/7}\text{C}$ (where n varies between 12 and 14). The partial motif, $\text{CX}_2\text{CX}_{13}\text{CX}_2\text{C}$, is present in a number of transcription factors including the steroid hormone receptors and the yeast protein, GAL4, in which zinc plays a structural role of co-ordinating cysteine residues and is essential for DNA binding (protein–nucleic acid interactions). The cysteine-rich domain of NC and PKC is required for phospholipid-dependent phorbol ester binding, suggesting an involvement of this domain in protein–lipid interactions. We have expressed recombinant NC, PKC and DGK glutathione *S*-transferase and TrpE fusion proteins in *E. coli* to investigate the relationship between the cysteine-rich motif, $\text{HX}_{11/12}\text{CX}_2\text{CX}_{10-14}\text{CX}_2\text{CX}_4\text{HX}_2\text{CX}_{8/7}\text{C}$, zinc and phorbol ester binding. The cysteine-rich domain of NC, PKC and DGK bound $^{65}\text{Zn}^{2+}$ but only NC and PKC bound [^3H]phorbol 12,13-dibutyrate. When NC and PKC were subjected to treatments known to remove metal ions from GAL4 and the human glucocorticoid receptor, phorbol ester binding was inhibited. These data provide evidence for the role of a zinc-dependent structure in phorbol ester binding.

INTRODUCTION

Increases in the cellular pools of diacylglycerol (DG) and Ca^{2+} elicited by cell stimulation cause a synergistic activation of the phospholipid-dependent protein kinase C (PKC) (Nishizuka, 1986, 1988). DG is generated transiently as it is metabolized to phosphatidic acid by DG kinases (DGKs) of which there are a number with molecular masses between 80 and 280 kDa (Kanoh *et al.*, 1990). The potent tumour promoters phorbol 12-myristate 13-acetate (PMA) and phorbol dibutyrate (PDBu) also bind to and activate PKC (Nishizuka, 1984). We have isolated and characterized a brain-specific cDNA neuronal chimaerin (NC) which encodes a novel phorbol ester receptor (Hall *et al.*, 1990; Ahmed *et al.*, 1990) and a GTPase-activating protein for $\text{p}21^{\text{rac}}$ (Diekmann *et al.*, 1991). The consequence(s) of phorbol ester binding to NC is unclear.

The human proteins NC (Hall *et al.*, 1990), PKC (Parker *et al.*, 1986; Coussens *et al.*, 1986) and the 80 kDa DGK (Schaap *et al.*, 1990) are related at the primary sequence level as they share a cysteine-rich domain (CRD) with the motif $\text{HX}_{11/12}\text{CX}_2\text{CX}_n\text{CX}_2\text{CX}_4\text{HX}_2\text{CX}_{8/7}\text{C}$ (n varies between 12 and 14). This motif is duplicated in tandem in DGK and PKC α – ϵ and present only once in NC and PKC ζ . The oncogene products RAF (a serine/threonine protein kinase) and VAV (a protein of unknown function) also possess a single copy of this

motif ($n = 9$ and 13 in RAF and VAV respectively; Beck *et al.*, 1987 and Katzav *et al.*, 1989). The cysteine-rich motif (CRM) $\text{HX}_{12}\text{CX}_2\text{CX}_{10-14}\text{CX}_2\text{CX}_4\text{HX}_2\text{CX}_7\text{C}$ is conserved in all PKCs so far sequenced from yeast to man (Ahmed *et al.*, 1990, and references therein).

The presence of clustered cysteines in proteins is indicative of metal-binding domains, where metals, usually zinc, play a structural role (Berg, 1986). The function of these domains include dimer formation (protein–protein interaction) and DNA-binding (protein–nucleic acid interaction; Berg, 1990). The CRM found in the DNA-binding proteins, the *Xenopus laevis* transcription factor IIIA, the human glucocorticoid receptor (HGR) and the fungal protein GAL4 represent examples where the structural role of the metal ions has been elucidated (Klug & Rhodes, 1987; Freedman *et al.*, 1988; Pan & Coleman, 1989, 1990a,b). Treatments that remove Zn^{2+} from these transcription factors lead to loss of DNA-binding activity (Brown *et al.*, 1985; Freedman *et al.*, 1988; Pan & Coleman, 1989, 1990b). NC, PKC and DGK possess the CRM, $\text{CX}_2\text{CX}_{10-14}\text{CX}_2\text{C}$, found in HGR and GAL4 hinting that these proteins may bind DNA. However, in GAL4, HGR and the human oestrogen receptor, the CRM, $\text{CX}_2\text{CX}_{13}\text{CX}_2\text{C}$, represents only a part of the domain involved in DNA binding (Hard *et al.*, 1990; Schwabe *et al.*, 1990; Pan & Coleman, 1990a,b). There is no evidence at present of sequence-specific DNA binding by the CRDs of NC, PKC or DGK.

Abbreviations used: PDBu, phorbol 12,13-dibutyrate; PMA, phorbol 12-myristate 13-acetate ('TPA'); PS, phosphatidylserine; DG, diacylglycerol; AP, alkaline phosphatase; DGK, diacylglycerol kinase; GST, glutathione *S*-transferase; HGR, human glucocorticoid receptor; NC, neuronal chimaerin; PKC, protein kinase C; S.j.26, 26 kDa GST from *Schistosoma japonicum*; IPTG, isopropyl β -thiogalactopyranoside; DTT, dithiothreitol; PBS, phosphate-buffered saline; CRD, cysteine-rich domain; CRM, cysteine-rich motif.

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The CRD of PKC and NC appears to be responsible for the high-affinity phospholipid-dependent binding of phorbol esters (Ono *et al.*, 1989a; Ahmed *et al.*, 1990). Since DG is the natural analogue of phorbol ester and competes for binding, it follows that these CRDs may also bind DG. Its presence in DGK would support this. Thus the CRD of NC, PKC and DGK is implicated in protein-lipid interactions. Evidence for the functional involvement of Zn^{2+} with this CRD includes the following: (i) phorbol ester binding and membrane localization of PKC in cell cultures and homogenates are affected by treatments that modulate the levels of Zn^{2+} (Csermely *et al.*, 1988; Forbes *et al.*, 1989, 1990); (ii) site-directed mutagenesis of bacterially expressed rat PKC γ has shown that cysteine residues 3 and 4 of the CRM (potential Zn^{2+} -binding residues) are essential for phorbol ester binding (Ono *et al.*, 1989a); (iii) urea-denatured TrpE/NC fusion protein binds phorbol ester only if refolded in the presence of $ZnCl_2$ (Ahmed *et al.*, 1990).

In the present study, we have used *Escherichia coli* expression systems [TrpE (pATH21-23) and glutathione *S*-transferase (pGEX1-3X); Dieckmann & Tzagoloff (1985); Smith & Johnson (1988)] and the $^{65}Zn^{2+}$ -blotting technique (Mazen *et al.*, 1988; Schiff *et al.*, 1988a) to investigate the amino acid sequence requirements and the role of zinc in phospholipid-dependent phorbol ester binding. The CRD of NC, PKC and DGK can bind $^{65}Zn^{2+}$ but only NC and PKC bind phorbol ester. Phorbol ester binding to NC and PKC is inhibited by treatments known to remove metal ions from proteins.

MATERIALS AND METHODS

Materials

Proteins (molecular mass in kDa) [myosin (205), β -galactosidase (116), phosphorylase *b* (94), BSA (67), alkaline phosphatase (58), enolase (48), ovalbumin (43), carbonic anhydrase (30), soyabean trypsin inhibitor (20) and lysozyme (14)], phosphatidylserine and chexel 100 resin were obtained from Sigma Chemical Co. $^{65}ZnCl_2$ (37 MBq/mg; 2.5 Ci/mg) and $[20(n)^3H]$ phorbol 12,13-dibutyrate (1.72 GBq/mg; 46.4 mCi/mg), antibody detection kits and nitrocellulose filters were obtained from Amersham International (Amersham, Bucks., U.K.). Purified rat brain PKC was from Lipidex (Westfield, NJ, U.S.A.). Glass-fibre filters (GF/C) were from BDH, and pGEX1, 2T and 3X expression vectors and Sepharose 4B columns were from Pharmacia. DNA-modification enzymes and pUC sequencing kit were obtained from Boehringer-Mannheim. Human PKC γ clone (phPKC γ 5) in pUC13 was from the American Tissue Culture Collection (Rockville, MD, U.S.A.). *E. coli* XL-1 Blue was from Stratagene (Cambridge, U.K.), and X-OMAT XAR film and hyperfilm were from Kodak.

Subcloning of cDNAs into pGEX1, 2T and 3X expression vectors

The construction of vectors (pATCM1-3) encoding TrpE/NC fusion proteins was as previously described (Ahmed *et al.*, 1990). Vectors encoding GST fusion proteins were constructed using human PKC γ , NC and DGK cDNAs (Coussens *et al.*, 1986; Hall *et al.*, 1990; Schaap *et al.*, 1990 respectively) and the pGEX (1, 2T and 3X) plasmid series. These vectors allow the expression of cDNAs downstream of *S.j.26* glutathione *S*-transferase (GST) DNA. Three sites are available for subcloning (*Bam*HI, *Sma*I and *Eco*RI) and these are followed by stop codons in all three reading frames (Smith & Johnson, 1988).

Subcloning of NC cDNA. A 1.7 kb *Pst*I fragment from pBSCM1 was purified and the 3'-protruding ends digested with T4 DNA polymerase to produce blunt ends. This fragment was

then subcloned into the *Sma*I site of pGEX-3X to give pGECM1 (coding nucleotides 501-1456). pGECM1 was then restricted with *Xba*I and *Eco*RI (both unique sites) to delete the C-terminal coding nucleotides. After blunt-ending by filling in with DNA polymerase Klenow fragment the plasmid was religated, generating pGEAM1 (coding nucleotides 501-958). The GST/NC and GST/NC N-terminal fusion proteins contain 19 amino acid residues upstream of the first methionine. To construct the NC C-terminal fusion protein the 1.7 kb purified *Pst*I fragment was cut with *Pvu*II and the *Pvu*II fragment subcloned into the *Sma*I site of pGEX-2T giving pGECM3 (coding nucleotides 878-1456).

Subcloning PKC cDNA. The PKC γ clone is a truncated cDNA lacking DNA encoding the C3/C4 regions (Coussens *et al.*, 1986). A 1.2 kb *Bam*HI fragment was purified from pUC13 clone (ATCC) and subcloned into the *Bam*HI site of pGEX-1 to give pGESA3 (correct orientation, coding nucleotides 165-1146) and pGESA4 (opposite orientation, nucleotides 1365-165). pGESA3 encodes the C1 and C2 regions while pGESA4 encodes a small fusion protein from the non-coding strand 3'-end of PKC γ . The cDNA encoding the C2 region in pGESA3 was deleted by cutting with *Sst*I and *Eco*RI (unique sites), blunt-ended with T4 DNA polymerase and religated to give pGESA5 which encodes the C1 region only (coding nucleotides 165-669). The GST/PKC fusion proteins contain seven amino acid residues upstream of the initiating methionine.

Subcloning DGK cDNA. A *Bam*HI/*Eco*RI 2.1 kb fragment was purified and subcloned into the *Bam*HI/*Eco*RI sites of pGEX-3X to give pGESA1 (coding nucleotides 486-2564) encoding a protein that lacks the first 113 amino acids. pGESA1 was then cleaved with *Nco*I and *Eco*RI (unique sites), treated with T4 DNA polymerase to blunt-end and re-ligated giving pGESA2 (coding nucleotides 486-975). The plasmid pGESA2 encodes two CRMs (C1a and C1b) and an EF-hand motif.

Expression of proteins

E. coli XL-1 Blue was transformed with the above plasmids (pGESA1-5, pGECM1/3, pGEAM1 and pGEX-3X). Overnight cultures of *E. coli* XL-1 Blue were diluted 100-fold into 400 ml of Luria broth/ampicillin (50 μ g/ml) and grown for 2-3 h to an A_{600} of 0.4, when 1 mM-isopropyl- β -thiogalactopyranoside (IPTG) was added and growth allowed to continue for another 3-4 h. Occasionally, cells were grown for 4-5 h after dilution and then induced with IPTG for 1 h. Cells were then harvested, concentrated approximately 100-fold in 20 mM-Tris/HCl (pH 7.5)/0.25 M-sucrose/2 mM-EDTA/10 mM-EGTA/leupeptin (20 μ g/ml)/0.5% Triton X-100 and stored in 1 ml portions at -20 °C. Immunological analysis confirmed expression of the correct reading frame for the different fusion proteins. NC fusion proteins were analysed with antisera raised against TrpE/NC fusion protein (pATCM1) as described previously (Ahmed *et al.*, 1990); PKC proteins with the monoclonal antibody 5A2 which detects the epitope GVDHTERRGRIYL (Cazaubon *et al.*, 1990); DGK proteins with antisera raised against a C-terminal peptide MGPPRSTNFFGFLS (D. Schapp, unpublished work). Proteins were also detected in the soluble fraction by the relevant antibodies.

Fractionation and refolding of cell pellets containing fusion proteins

Frozen cell pellets (1 ml) were thawed quickly followed by sonication (for 5 s with intervals of 15 s) until pellet suspensions were translucent. The samples were then centrifuged at 12000 g for 1 min and separated into insoluble (pellet) and soluble (supernatant) fractions. The soluble fraction was then used directly in binding assays. The insoluble fraction was either used immediately for SDS/PAGE (Laemmli, 1970) and immuno-

	Molecular mass (kDa)	(Amino acids)	GST	<i>n</i> -Chimaerin	[³ H]PDBu binding (c.p.m./10 μ l of extract)	
					Refolded	Supernatant
pGECM1	59 (52)	(-19-299)			16635 $\pm$ 712 (4)	8846 $\pm$ 350 (5)
pGEAM1	39 (37)	(-19-133)			19472 $\pm$ 660 (5)	5775 $\pm$ 1235 (5)
pGECM3	44 (40)	(106-299)			926 $\pm$ 301 (5)	1285 $\pm$ 744 (4)
pGEX1-3	26 (20)	—			961 $\pm$ 161 (5)	854 $\pm$ 309 (6)
pGESA3	59 (46)	(-7-323)			21519 $\pm$ 3310 (5)	5836 $\pm$ 1277 (5)
pGESA5	43 (38)	(-7-163)			19080 $\pm$ 4366 (5)	7670 $\pm$ 722 (5)
pGESA4	— (22)*	—			798 $\pm$ 271 (5)	1046 $\pm$ 256 (5)
pGESA1	87 (82)	(113-727)			1220 $\pm$ 380 (5)	706 $\pm$ 244 (3)
pGESA2	46 (40)	(113-276)			1076 $\pm$ 280 (5)	572 $\pm$ 43 (3)

Fig. 1. Expression and phorbol-ester-binding ability of NC, PKC and DGK GST fusion proteins

NC, PKC and DGK cDNA were cloned into pGEX1-3X vectors as described in the Materials and Methods section. The encoded proteins were pGECM1-NC, pGEAM1-NC *N*-terminal, pGECM3-NC *C*-terminal, pGEX1-3X-GST, pGESA1-DGK, pGESA2-DGK *N*-terminal, pGESA3-PKC C1-C2, pGESA4-PKC* (cDNA is cloned in the opposite orientation with the non-coding 3' strand of cDNA expressed), pGESA5-PKC C1. Cell extracts from *E. coli* XL-1 Blue transformed with these plasmids were analysed on SDS/PAGE. The predicted sizes of the NC, PKC and DGK fusion proteins are shown with the estimated sizes in parentheses. The larger fusion proteins (encoded by plasmids pGECM1, pGESA3 and pGESA1) were susceptible to breakdown, possibly due to the host strain (XL-1 Blue) used for transformation (GST is 26 kDa but runs 6 kDa less than predicted). Schematic structure of proteins derived from the various pGEX/cDNA expression vectors is shown with GST hatched and each vertical line represents a cysteine residue in the motif CX₂CX₁₂₋₁₄CX₂CX_{6,7}C. The motif is not present in proteins encoded by pGECM3, pGEX1-3 and pGESA4. For pGESA4 the size cannot be predicted as the sequence is not known. However, the fusion protein generated is slightly bigger than GST and is found predominantly in the insoluble fraction. Amino acid numbers for NC, PKC and DGK are from Hall *et al.* (1990), Coussens *et al.* (1986) and Schaap *et al.* (1990) respectively. Negative amino acid numbers indicate that the fusion protein contains amino acids present upstream of the proposed start codon. Phorbol ester binding was determined for both soluble (supernatant, refolded *in vivo*, in *E. coli*) and insoluble fractions (refolded *in vitro*) by incubating cell extracts with 33 nM-[³H]PDBu for 30 min at room temperature and then 30 min at 4 °C, followed by rapid filtration on GF/C filters (for details see the Materials and Methods section). Background/non-specific binding was not subtracted from the data presented (means $\pm$ S.D.; *n* values in parentheses).

blotting or washed ($\times 4-5$) with 2 mM-EDTA/0.5% Triton X-100 (Nagai & Thøgersen, 1987) or 1 M-NaCl/10 mM-Tris/HCl (pH 8.0) to increase the purity of fusion proteins. In all cases the purity of the fusion proteins was above 50%. The partially pure fusion proteins were solubilized in 5 ml of 8 M-urea/25 mM-Tris/HCl (pH 7.5)/1 mM-EDTA/1 mM-dithiothreitol (DTT) and dialysed against 400 ml of 4 M-urea/1 mM-Tris/HCl (pH 5.0)/1 mM-DTT for 1-2 h and then against 2 litres of 20 mM-Tris/HCl (pH 7.5)/50 mM-NaCl/2 mM-MgCl₂/200 μ M-DTT/100 μ M-ZnCl₂ overnight with two changes of buffer. These cell extracts were then concentrated using Centricon filtration to a volume equivalent to that of the supernatant and either used immediately or stored with 15% glycerol at -20 °C and used within 2 weeks.

Ligand binding

Cell extracts from either soluble or insoluble (refolded as described above) fractions were incubated in 25 mM-Tris/HCl (pH 7.5)/BSA (4 mg/ml) with phosphatidylserine (100 μ g/ml) and 33 nM-[³H]PDBu (Amersham) for 30 min at room temperature. Samples were then placed at 4 °C for a further 30 min before rapid filtration on glass-fibre G/FC filters. The filters were washed with 3 $\times$ 3 ml of ice-cold 25 mM-Tris/HCl (pH 7.5), air-dried and radioassayed in 3 ml of scintillation fluid (Ready Safe, Beckman). For experiments on the effect of zinc, all glassware

was washed with acid and buffers treated with chelex 100 resin. Background binding was estimated by carrying out parallel incubations in the presence of unlabelled 10 μ M-PDBu and varied in the range 800-1200 c.p.m./sample. The error ($\pm$) in binding experiments is expressed as S.D.

⁶⁵Zn²⁺ binding to proteins transferred to nitrocellulose

The ⁶⁵ZnCl₂-binding assay was carried out as essentially described in Groissman & Laimins (1989). Briefly, proteins were electrophoresed on 10% SDS/PAGE gels for approx. 1 h at 160 V and then incubated for 10 min with 5% 2-mercaptoethanol, washed for 5 min in Western transfer buffer, and then placed on a Sartoblot for transfer to nitrocellulose as described previously (Ahmed *et al.*, 1990). After transfer, filters were incubated in binding buffer [100 mM-Tris/HCl (pH 6.8)/50 mM-NaCl/0.5 mM-MgCl₂/0.5 mM-CaCl₂/5 mM-DTT] for 2 h at room temperature. Variations in pH and DTT were used and are discussed in the Results section. Filters were next washed ($\times 2$) in binding buffer without DTT and then probed with ⁶⁵ZnCl₂ (6 μ M; 1.32 μ Ci/ml; 1 mCi/mg) for 30 min. Filters were then washed ($\times 2$) in binding buffer pH 6.8 and then (4 $\times$ 5 min) at pH 5.8. In competition experiments the appropriate metal chlorides, freshly made, were included at the relevant concentration in the buffer containing ⁶⁵Zn²⁺ and all wash buffers.

Table 1. Effect of zinc removal and reconstitution and site-directed mutagenesis on phorbol ester binding to NC and PKC fusion proteins

Phorbol ester binding was determined as described in Fig. 1 for fusion proteins (encoded by plasmids), TrpE/NC (pATCM1), GST/NC *N*-terminal (pGEAM1) and GST/PKC C1 (pGESA5). (A) The chelators, EDTA, EGTA and 1,10-phenanthroline were incubated with proteins for 30 min before addition of phosphatidylserine (100 µg/ml) and [³H]PDBu (33 nM). The incubation was continued for 30 min and then tubes were placed at 4 °C for 30 min before filtration. Incubation with 1,10-phenanthroline was at pH 6.0 (Zahradka & Ebisuzaki, 1984). (B) The insoluble fraction was solubilized and refolded as described in the Materials and Methods section. Equal volumes of refolded proteins were dialysed for 2 h either in 2 litres of 20 mM-Tris/HCl (pH 7.5)/50 mM-NaCl/200 µM-DTT/100 µM-ZnCl₂ or 2 litres of 20 mM-Tris/HCl (pH 5.0)/20 mM-EDTA/50 mM-NaCl/200 µM-DTT followed by dialysis in buffer at pH 7.5 without ZnCl₂, the latter two buffers being chelex-treated. Ligand binding was carried out either with or without ZnCl₂ (100 µM). (C) Refolding of the 8 M-urea-solubilized inclusion bodies involves two steps, dialysis in 4 M-urea (pH 5.0) followed by dialysis in 20 mM-Tris/HCl (pH 7.5) with 100 µM-ZnCl₂. Equal volumes of urea-solubilized proteins were, (i) refolded normally, (ii) refolded by omitting the 4 M-urea step or (iii) refolded in the absence of ZnCl₂ in chelex-treated buffer. Again ligand binding was carried out either in the presence or absence of ZnCl₂. (D) The mutant cDNA pATSA5 encodes a TrpE/NC fusion protein with cysteines 3 and 4 of the CRM altered to glycine residues. Refolded protein was used in the ligand-binding assay. For (A–D), the data presented are from a single experiment carried out in triplicate (two to three other experiments gave similar results). 100 % binding was in the range presented in Fig. 1. Data are presented as the percentile activity remaining after the different manipulations. Background/non-specific binding was subtracted from the data presented (means ± S.D., *n* = 3). n.d., not determined.

Experimental variation	[³ H]PDBu binding remaining (%)		
	TrpE/NC (pATCM1)	GST/NC <i>N</i> -terminal (pGEAM1)	GST/PKC C1 (pGESA5)
(A) Metal chelators			
EDTA (2 mM)	108 ± 10	97 ± 5	88 ± 11
EGTA (2 mM)	111 ± 11	114 ± 7	74 ± 6
1,10-phenanthroline (0.1 mM)	n.d.	59 ± 7	46 ± 8
1,10-phenanthroline (1.0 mM)	n.d.	27 ± 3	15 ± 6
(B) Dialysis			
Against Tris/HCl, pH 7.5	91 ± 6	85 ± 13	102 ± 3
Against EDTA (20 mM), pH 5.0	10 ± 4	11 ± 4	10 ± 4
Against EDTA (20 mM), pH 5.0 and then incubation with zinc	36 ± 5	26 ± 5	25 ± 2
(C) Refolding			
Without 4 M-urea step	n.d.	59 ± 15	67 ± 3
In the absence of zinc	4 ± 3	4 ± 1	2 ± 1
In the absence of zinc and then incubation with zinc	n.d.	18 ± 4	19 ± 2
(D) Mutagenesis			
Cys-(3 and 4) to Gly/Gly	3 ± 2	n.d.	*n.d.

* Ono *et al.* (1989a) have mutated cysteine residues 3 and 4 of the CRM in rat PKCγ to valine residues. When expressed in *E. coli* this mutant does not bind phorbol ester.

Filters were then dried and exposed¹ to X-Omat X-ray film for 24–48 h at –80 °C with intensifying screens.

Some ⁶⁵Zn²⁺-binding was carried out using dot-blotting instead of SDS/PAGE. Nitrocellulose sheets were presoaked in phosphate-buffered saline (PBS) and placed on a 96-well Bethesda Research Laboratory apparatus under vacuum section. Protein samples (0.2–5 µg) all in 100 µl were then filtered. The sheets were left to dry before being probed with ⁶⁵Zn²⁺ as described above.

Densitometry using image analysis

After autoradiography the protein blots were stained with Amido Black [0.1 % Amido Black dye in methanol/acetic acid/water (9:2:9, by vol.)]. The autoradiographs and Amido Black-stained filters were then analysed on a Quantimet 570 image analyser (Cambridge Instruments) using the black-and-white mode of the camera and under constant-detection threshold. The areas of grey images corresponding to protein bands on the Amido Black-stained blots and exposed areas on the autoradiograms were measured in arbitrary units. Protein concentration was expressed as a function of molecular mass in arbitrary units.

Site-directed mutagenesis

Site-directed mutagenesis of NC was carried out on the clone pBSCM1 (Hall *et al.*, 1990) by the method of Kunkel *et al.*

(1987). The plasmid pBSCM1 contains a 2.1 kb cDNA clone which encodes NC with both 5' and 3' non-coding sequences. *E. coli* CJ236 (*dut*hngl*relA*-1) was transformed with this plasmid and single-strand UTP-incorporated DNA isolated using the helper phage M13 K07. The mutagenic primer 5'-GTG AAA GGT GCA GAT GGT GGT TTG-3' (24-mer) changes cysteine residues 3 and 4 in the CRM to two glycine residues. The 24-mer was used to prime second strand synthesis with the enzymes T4 DNA polymerase and gene 32 protein, followed by ligation with T4 DNA ligase. The DNA generated was then transformed into *E. coli* XL-1 Blue and 4/5 clones were isolated and sequenced to confirm the mutagenesis. A 1.7 kb *Pst*I fragment was purified from one such mutant and subcloned into the *Pst*I site of pATH23 (TrpE expression vector, as previously described; Ahmed *et al.*, 1990) to generate pATSA5. The sequence of this mutant was confirmed by plasmid (ds) sequencing using the pUC kit.

RESULTS

Phorbol-ester-binding ability of NC, PKC and DGK GST fusion proteins

To estimate the phorbol-ester-binding capacity of NC, PKC and DGK, proteins in the insoluble fraction were refolded and used in parallel with the soluble fraction in binding assays (Fig.

Fig. 2. $^{65}\text{Zn}^{2+}$ binding to known metalloproteins and non-metalloproteins

Proteins (approx. 10 $\mu\text{g}/\text{sample}$) were electrophoresed on SDS/PAGE and then transferred to nitrocellulose. Lanes: 1,1', enolase; 2,2', alkaline phosphatase; 3,3', BSA. (a) The blots were probed with 6 μM - $^{65}\text{Zn}^{2+}$ (lanes 1'–3') followed by Amido Black staining to estimate the amount of protein (lanes 1–3) as described in the Materials and Methods section. (b) Experiments were carried out as in (a) except that a range of protein concentrations (2–20 $\mu\text{g}/\text{sample}$) for enolase (■), alkaline phosphatase (●) and BSA (○) was used and the data analysed by image analysis. Arbitrary units are used for both $^{65}\text{Zn}^{2+}$ binding and protein. Results presented are from a typical experiment; similar results were obtained from two to three other experiments.

1). GST/NC and NC *N*-terminal fusion proteins containing the CRD bind phorbol ester. GST/PKC C1–C2 and C1 fusion proteins possessing two copies of the CRD were also able to bind phorbol ester. This binding was not significantly affected by deletion of the C2 region. The level of phorbol ester binding to PKC and NC was in the same range for proteins from both the insoluble (folded *in vitro*) and the soluble phases (Fig. 1). The plasmid pGES4 has the PKC γ cDNA cloned in the opposite orientation to that of pGES3 and encodes a small fusion protein found predominantly in the insoluble fraction which does not bind phorbol ester. In addition, GST protein (present only in the soluble phase) affinity-purified on glutathione-agarose did not bind phorbol ester (Fig. 1).

The alignment of amino acid sequence of the CRD from rat PKC γ and NC allows a consensus sequence for phorbol ester binding to be derived. DGK possesses two CRDs (C1a and b) which have 87% (13/15) and 67% (10/15) sequence identity with this consensus sequence respectively (see the Discussion section). However, unlike PKC and NC, DGK fusion proteins did not bind phorbol ester specifically (Fig. 1). The amount of phorbol ester associated with DGK was independent of protein concentration and was accounted for by background binding. Phosphatidylcholine and/or deoxycholate did not increase this binding (results not shown).

Effect of metal ion chelation and site-directed mutagenesis on phorbol ester binding to NC and PKC fusion proteins

The phorbol ester binding consensus sequence stretches over 50 amino acid residues. Within this sequence there are six cysteine residues and two histidine residues forming the motif $\text{HX}_{12}\text{CX}_2\text{CX}_{13}\text{CX}_2\text{CX}_4\text{HX}_2\text{CX}_7\text{C}$ (Ahmed *et al.*, 1990). GST/NC *N*-terminal, TrpE/NC and GST/PKC C1 fusion proteins were used to investigate the relationship between phorbol ester binding and metal binding. The C2 domain which confers Ca^{2+} -dependence on [^3H]PDBu binding and enzyme activity is not present in this PKC fusion protein. Incubation of refolded proteins with either EDTA or EGTA (both at 2 mM) for 30 min

before assay did not significantly affect the level of phorbol ester binding to NC. However, with PKC, binding was somewhat inhibited (12–26%; Table 1). This result suggests that the metal ion, if present, is tightly bound to the fusion proteins. In the case of GAL4 and HGR proteins, which have similar CRMs to that present in the phorbol ester binding consensus, harsher treatments are necessary to remove bound metal (Freedman *et al.*, 1988; Pan & Coleman, 1989, 1990b). Interestingly, at pH 6.0, 1 mM of the high-affinity Zn^{2+} -specific chelator, 1,10-phenanthroline, was sufficient to inhibit binding to NC and PKC by 73 and 85% respectively. Dialysis of fusion proteins against 20 mM-EDTA, pH 5.0, was also sufficient to inhibit binding to NC and PKC, this time by 89 and 90% respectively. These effects could be partially reversed by adding ZnCl_2 to the assay buffer (25–36% binding; Table 1). Phorbol ester binding to native rat brain PKC was also inhibited by dialysis against 20 mM-EDTA, pH 5.0 (results not shown).

Fusion proteins partially purified from the insoluble fraction require denaturing and refolding to bind phorbol ester. The refolding procedure consists of two steps (dialysis against 4 M-urea and then against a Tris/ ZnCl_2 buffer; see the Materials and Methods section for details) after denaturation in 8 M-urea. The refolding procedure was varied to investigate the possible role of zinc in phorbol ester binding. The omission of the 4 M-urea step during refolding decreased phorbol ester binding to 59 and 67% with NC *N*-terminal and PKC C1 fusion proteins respectively (Table 1). We have previously shown that refolding of TrpE/NC in the absence of ZnCl_2 led to loss of phorbol-ester-binding activity (Ahmed *et al.*, 1990). Treatment of the dialysis buffer (without added ZnCl_2) with chelex resin before refolding of TrpE/NC, GST/NC *N*-terminal and GST/PKC C1 fusion proteins decreased phorbol ester binding to 4, 4 and 2% respectively. When ZnCl_2 was added to protein extracts containing GST/NC *N*-terminal and GST/PKC C1, 18 and 19% of the binding activity was recovered respectively (Table 1). The reconstitution of phorbol-ester-binding activity to NC and PKC 'apoproteins' by ZnCl_2 addition probably reflects the ease with which this domain can refold.

Lastly, we tested the ability of a mutant of the TrpE/NC fusion protein (pATSA5) in which cysteines 3 and 4 of the CRM (potential metal-binding sites) were replaced by two glycine residues. As previously observed for rat PKC γ , this mutation results in loss of phorbol ester binding to NC (Table 1; Ono *et al.*, 1989a).

$^{65}\text{Zn}^{2+}$ binding to control proteins and rat brain PKC using the $^{65}\text{Zn}^{2+}$ -blot assay

The most direct method to examine whether a protein binds metal ions is to use atomic absorption to estimate the amount of metal associating with purified proteins. Relatively high protein concentrations are required to overcome contaminating Zn^{2+} in assay buffers. Another method involves transferring denatured proteins on to nitrocellulose and allowing them to refold in the presence of radioactive ZnCl_2 ($^{65}\text{Zn}^{2+}$; Mazen *et al.*, 1988; Schiff *et al.*, 1988b). This method has the advantage that lower protein concentrations are required and that partially pure proteins can be used. However, a disadvantage of this method is that proteins require to refold after transferring to nitrocellulose. Certain proteins are able to refold when immobilized on nitrocellulose filters, including those with potential 'zinc-finger-like' structures, poly(ADP-ribose) polymerase, papillomavirus E6 and E7 and the reovirus outer capsid protein sigma 3 (Schiff *et al.*, 1988b; Barbosa *et al.*, 1989; Groissman & Laimins, 1989; Gradwohl *et al.*, 1990).

Several conditions critical for specific $^{65}\text{Zn}^{2+}$ binding in this assay were established. Firstly treatment of the proteins on gels

Fig. 3. $^{65}\text{Zn}^{2+}$ binding by dot-blotting

Proteins were dot-blotted on to nitrocellulose and then probed with $6\ \mu\text{M}$ - $^{65}\text{Zn}^{2+}$ as described for gels in Fig. 2. (a) Lanes: 1,1', alkaline phosphatase; 2,2', TrpE; 3,3', enolase; 4,4', rat brain PKC. Lanes 1–4, Amido Black stained and 1'–4', $^{65}\text{Zn}^{2+}$ binding. (b) Proteins (0.2–2 μg /sample) were dot-blotted and analysed by image analysis. Rat brain PKC (●), alkaline phosphatase (○), enolase (■) and TrpE (□) are shown. Protein and $^{65}\text{Zn}^{2+}$ binding are expressed in arbitrary units. Results presented are from a typical experiment; similar results were obtained from two to three other experiments.

with 2-mercaptoethanol and on blots with DTT was essential to detect significant amounts of $^{65}\text{Zn}^{2+}$ binding by autoradiography. Secondly, amounts of protein lower than 20 μg /sample were used to avoid non-specific binding. Thirdly, $^{65}\text{Zn}^{2+}$ blots were washed at low pH (pH 5.8–6.0, high-stringency) to eliminate non-specific interactions (results not shown).

Using the ^{65}Zn -blot assay, metalloproteins (BSA, alkaline phosphatase, carbonic anhydrase, metallothionein) can be distinguished from non-metalloproteins (enolase, lysozyme,

ovalbumin, β -galactosidase, myosin, phosphorylase *b* and soya-bean trypsin inhibitor) (Fig. 2a and results not shown). The effect of protein concentration on the amount of $^{65}\text{Zn}^{2+}$ bound is shown in Fig. 2(b). The stoichiometry of $^{65}\text{Zn}^{2+}$ binding to BSA was greater than that to alkaline phosphatase and carbonic anhydrase. In previous studies with the $^{65}\text{Zn}^{2+}$ -blot assay, alkaline phosphatase and carbonic anhydrase were found to bind more $^{65}\text{Zn}^{2+}$ than BSA (Schiff *et al.*, 1988a,b). In the present study, treatment of gels with 2-mercaptoethanol and blots with DTT probably optimize $^{65}\text{Zn}^{2+}$ binding to proteins containing cysteine residues in their metal-binding sites. $^{65}\text{Zn}^{2+}$ binding to BSA and alkaline phosphatase was not affected by the presence of 10 mM- CaCl_2 or $-\text{MgCl}_2$ in the binding buffer while 100 μM - ZnCl_2 was sufficient to eliminate binding (results not shown).

With dot-blotting, the quantity of protein required in this more sensitive assay was between 0.2 and 5.0 μg /sample. Control proteins (TrpE, enolase and alkaline phosphatase) behaved as expected. Purified rat brain PKC was found to bind $^{65}\text{Zn}^{2+}$ and the amount of $^{65}\text{Zn}^{2+}$ binding was proportional to protein concentration, as with alkaline phosphatase (Fig. 3).

$^{65}\text{Zn}^{2+}$ binding to NC, PKC and DGK fusion proteins

Only proteins containing the CRD (TrpE/NC, TrpE/NC *N*-terminal, GST/NC, GST/NC *N*-terminal, GST/PKC C1–C2, GST/PKC C1, GST/DGK and GST/DGK *N*-terminal) bound $^{65}\text{Zn}^{2+}$. Deletion of the *C*-terminals of these fusion proteins did not significantly affect $^{65}\text{Zn}^{2+}$ binding (Fig. 4). The other proteins, GST and TrpE, NC *C*-terminal constructs and the product of the opposite orientation clone of PKC, did not bind $^{65}\text{Zn}^{2+}$ (Fig. 4). $^{65}\text{Zn}^{2+}$ binding to fusion proteins lacking the CRD was independent of protein concentration. With the CRD-containing fusion proteins there was a near-linear relationship between protein concentration and amount of $^{65}\text{Zn}^{2+}$ bound (Fig. 5). The ratio $^{65}\text{Zn}^{2+}$ bound/protein was derived from the slopes of the curves presented in Fig. 5. Slope values obtained for NC (0.64 ± 0.35 , $n = 4$), PKC (1.22 ± 1.1 , $n = 2$) and DGK (0.85 ± 0.2 ,

Fig. 4. $^{65}\text{Zn}^{2+}$ binding to NC, PKC and DGK TrpE/GST fusion proteins

TrpE vectors and encoded proteins were as previously described (Ahmed *et al.*, 1990). TrpE/GST fusion proteins (approx. 10 μg /sample) were electrophoresed on SDS/PAGE and probed with $6\ \mu\text{M}$ - $^{65}\text{Zn}^{2+}$ (lanes 1'–13'). After autoradiography, proteins on nitrocellulose were stained with Amido Black (lanes 1–13) as described in the Materials and Methods section. TrpE/NC fusion proteins: 1,1', NC *N*-terminal (pATCM2); 2,2', TrpE (pATH23); 3,3' NC (pATCM1); 4,4', NC *C*-terminal (pATCM3). GST fusion proteins: 5,5', NC *N*-terminal (pGEAM1); 6,6', GST (pGEX3X); 7,7', NC (pGECM1); 8,8', NC *C*-terminal (pGECM2); 9,9', PKC C1–C2 (pGESA3); 10,10', PKC C1 (pGESA3); 11,11', PKC opposite orientation (pGESA4) and 12/12, DGK (pGESA1); 13/13', DGK *N*-terminal (pGESA2).

Fig. 5. Protein titration of $^{65}\text{Zn}^{2+}$ binding to TrpE/NC and GST/NC, GST/PKC and GST/DGK fusion proteins

Details are as in Fig. 4 except that a range (2–10 μg) of protein concentrations was used. Bands were quantified using an image analyser. (a) TrpE/NC (pATCM1) (○), TrpE/NC N-terminal (pATCM2) (●), TrpE/NC C-terminal (pATCM3) (■), TrpE (pATH21) (□). (b) GST/PKC C1–C2 (pGESA3) (△), GST/PKC C1 (pGESA5) (▲), GST/PKC opposite orientation (pGESA4) (■). (c) GST/DGK (pGESA1) (△), GST/DGK N-terminal (pGESA2) (▲), GST (pGEX2X) (□). (d) GST/NC (pGECM1) (○), GST/NC N-terminal (pGEAM1) (●), GST/NC C-terminal (pGECM3) (■). Protein and $^{65}\text{Zn}^{2+}$ bound are given in arbitrary units.

Table 2. Competition of $^{65}\text{Zn}^{2+}$ binding to NC, PKC and DGK GST fusion proteins by zinc, cadmium and cobalt

The $^{65}\text{Zn}^{2+}$ -blot assay was carried out essentially as described in the Materials and Methods section with fusion proteins (encoded by plasmids), PKC C1 (pGESA5), DGK N-terminal (pGESA2) and NC N-terminal (pGEAM1). Freshly prepared metal chlorides were diluted to the appropriate concentration (10–100 μM) in the $^{65}\text{Zn}^{2+}$ -probing buffer and all wash buffers. Data presented are of a typical experiment. Two or three other experiments yielded qualitatively similar results. >95% indicates the limit of detection by image analysis. 100% indicates the complete absence of labelling on autoradiographs.

Metal	Concn. (μM)	Inhibition of $^{65}\text{Zn}^{2+}$ -binding to GST fusion proteins (%)		
		GST/PKC C1 (pGESA5)	GST/DGK N-terminal (pGESA2)	GST/NC N-terminal (pGEAM1)
Zinc	10	89	75	95
	50	> 95	> 95	> 95
	100	100	100	100
Cadmium	10	31	25	19
	50	100	100	100
	100	100	100	100
Cobalt	10	24	44	76
	50	43	73	83
	100	> 95	> 95	> 95

$n = 2$) suggest that more $^{65}\text{Zn}^{2+}$ binds to the latter two proteins. The specificity of $^{65}\text{Zn}^{2+}$ binding was investigated by carrying out competition assays with the ions Ca^{2+} , Mg^{2+} , Zn^{2+} , Cd^{2+} and Co^{2+} and with the GST/NC N-terminal, GST/PKC C1 and GST/DGK N-terminal fusion proteins. As with the control proteins (BSA and alkaline phosphatase), Ca^{2+} and Mg^{2+} up to 10 mM did not affect the $^{65}\text{Zn}^{2+}$ binding to the three fusion

proteins (results not shown). Zn^{2+} was the most effective competitor, followed closely by cadmium. Cobalt was least effective and 100 μM was necessary to decrease binding to > 95% (Table 2).

DISCUSSION

Phorbol ester binding to recombinant NC and PKC proteins expressed in *E. coli* has now been demonstrated with four different fusion protein constructs [TrpE, β -galactosidase, T7 leader and GST (Ono *et al.*, 1989a; Ahmed *et al.*, 1990; present study)]. These results support the view that the CRD is required to allow the PKC and NC fusion proteins to bind phorbol ester and that this domain folds independently of adjacent amino acid sequences. Phorbol ester binding to the CRDs of human proteins, NC and PKC, was phosphatidylserine-dependent with both proteins folded *in vitro* and *in vivo* (in *E. coli*) indicating that post-translational modifications are not necessary for this activity. *E. coli* extracts expressing GST protein alone did not bind phorbol esters, even at high protein concentrations (500 μl of cell extract). This suggests that *E. coli* either lacks or contains very low concentrations of an endogenous phorbol-ester-binding protein. This finding opens up the possibility of using expression libraries to search for novel phorbol-ester-binding proteins by ligand screening or dilution cloning (Harada *et al.*, 1990).

Three CRDs, rat brain PKC γ C1a and b regions and NC, when expressed in *E. coli* bind phorbol esters with high affinity. Alignment of a stretch of 50 amino acid residues of these CRDs starting with histidine and ending with cysteine with the derived consensus is shown in Fig. 6. There are no conserved amino acid

Fig. 6. Sequence alignment of the CRDs of NC, PKC γ , DGK, PKC ζ , VAV and RAF; consensus sequence for phorbol ester binding

The amino acid sequences of rat PKC γ (C1a/b, 37–87/97–147), human DGK (C1a/b, 205–253/269–319), NC (40–100), PKC ζ (131–180), RAF (93–146) and VAV (465–515) cysteine-rich regions are aligned (Beck *et al.*, 1987; Ono *et al.*, 1988, 1989b; Katzav *et al.*, 1989; Hall *et al.*, 1990; Schaap *et al.*, 1990). The human PKC γ sequence used in this study is identical with the rat sequence presented. The CRD of NC, the C1a or b region of PKC γ have been shown to bind phorbol esters (Ono *et al.*, 1989a; Ahmed *et al.*, 1990). When the sequences of these three regions are aligned there are no conserved residues either upstream of the first histidine (position 1) or downstream of the last cysteine (position 51). Therefore this stretch of 50 amino acid residues has been designated as the domain responsible for phospholipid-dependent phorbol ester binding. Invariant residues of the alignment of these three sequences are shown forming a consensus sequence. Boxed residues above the consensus show overall similarity between NC and the C1a/b regions of PKC γ . Boxed residues below the consensus show invariant amino acid residues present in DGK C1a/b regions, PKC ζ , VAV and RAF. Overall identity of the C1a/b regions of DGK, PKC ζ , VAV and RAF with the consensus sequence is 13/15 (87%) and 10/15 (67%), 14/15 (93%), 13/15 (87%) and 11/15 (73%) respectively. '.' represents non-conserved residues.

residues *N*-terminal or *C*-terminal of this 50-mer peptide. The consensus sequence includes the six cysteine and two histidine residues potentially involved in metal binding (see below) and seven other residues (Fig. 6). DGK contains two copies of the CRD (C1a and b) as for PKC with sequence identities to the phorbol-ester-binding consensus being 87% (13/15) and 67% (10/15) respectively. Recombinant DGK expressed in *E. coli* does not bind phorbol esters under a variety of conditions. However, the possibility that DGK fusion protein did not fold correctly cannot be ruled out. To address this possibility we have expressed full-length DGK cDNA in COS7 cells and found that, although DGK activity is increased 6–7-fold (Sharp *et al.*, 1980), there is no increase in phorbol ester binding (S. Ahmed, R. Kozma & D. Schaap, unpublished work). The lack of effect of 1 μ M-PMA on purified native porcine DGK enzyme activity supports the view that DGK is not a specific phorbol ester receptor (Sakane *et al.*, 1990).

Several PKC isoenzymes encoded by cDNAs for human (α , β , γ , ϵ , η), mouse (η), rabbit (α , β I, β II, γ , ϵ) and rat (α , β I, β II, γ , δ) bind phorbol esters when expressed transiently in COS cells (Knopf *et al.*, 1986; Ono *et al.*, 1987; Kikkawa *et al.*, 1987; Ohno *et al.*, 1988; Akita *et al.*, 1990; Osada *et al.*, 1990; Bacher *et al.*, 1991). However, rat PKC ζ cDNA uniquely encodes only one CRM and when expressed in COS cells does not bind phorbol ester (Ono *et al.*, 1989b). Sequence alignment of rat PKC ζ with the binding consensus (Fig. 6) reveals that 14/15 (93%) of the consensus amino acid residues are present. The differences between DGK and PKC ζ and the phorbol ester binding consensus indicate the importance of certain amino acids for this activity. Nevertheless, it will be necessary to use site-directed mutagenesis to elucidate the precise role of individual amino acids in phospholipid-dependent phorbol ester binding.

The oncogenes RAF and VAV and the *Saccharomyces cerevisiae* PKC1 gene products possess the CRM found in NC, PKC and DGK. Alignment of the CRDs of RAF, VAV and PKC1 with the binding consensus gives 73% (11/15), 87% (13/15) and 73% (11/15, for both C1a and b regions) sequence identity respectively. Apart from rat PKC ζ all other mammalian PKC isoenzymes so far sequenced possess at least one CRD with 100% identity with the phorbol ester binding consensus. It is unlikely that RAF, VAV or PKC1 proteins will bind phorbol ester with high affinity.

The presence of the CRM $\text{HX}_{11/12}\text{CX}_2\text{CX}_{13/14}\text{CX}_2\text{CX}_4\text{HX}_2\text{CX}_{6/7}\text{C}$ is indicative of a metal-binding site (Berg, 1986, 1990). Since phorbol ester binding is also associated with this motif, treatments that remove metals may inhibit this activity. With the transcription factors HGR and GAL4, Zn^{2+} has been found to play a structural role and conditions for removal of this Zn^{2+} have been described (Freedman *et al.*, 1988; Pan & Coleman, 1989, 1990b). To explore the effect of treatments that interfere with metal binding on phorbol ester binding, fusion proteins with *C*-terminal deletions (pGEAM1 and pGESA5) were used. This was particularly important in the case of PKC because of the possible interaction that might occur between the C1 and C2 regions. Harsh treatments (1,10-phenanthroline/dialysis against EDTA at low pH) that chelate metal did indeed inhibit phorbol ester binding to NC and PKC while mild chelation (EDTA/EGTA incubation) did not (although PKC was somewhat affected by mild chelation). When the yeast transcription factor SWI5 is refolded in the absence of ZnCl_2 , sequence-specific DNA binding is not recovered (Nagai *et al.*, 1988). When NC and PKC were refolded in the absence of ZnCl_2 , phorbol-ester-binding activity was lost or decreased when the 4 M-urea step was omitted. Optimal phorbol ester binding was recovered when both the 4 M-urea step and dialysis against ZnCl_2 were used. Interestingly, with 'apoproteins' of NC and PKC (where

metal has been removed either by chelation or by refolding in the absence of ZnCl_2) binding activity was partially restored by addition of ZnCl_2 before the ligand-binding assay. Addition of ZnCl_2 to the 'apoprotein' of the transcription factor Sp1 partially restored sequence-specific DNA binding (Kadonaga *et al.*, 1987). Mutation of two of the cysteine residues (potential metal-binding residues) in the consensus sequence to glycine showed them to be required for phorbol ester binding. Site-directed mutagenesis of the equivalent cysteine residues to valine in rat brain PKC γ also caused loss of phorbol-ester-binding activity (Ono *et al.*, 1989a). Taken together, and by analogy with similar experiments carried out on transcription factors, Sp1, HGR, SWI5 and GAL4 (Kadonaga *et al.*, 1987; Freedman *et al.*, 1988; Nagai *et al.*, 1988; Pan & Coleman, 1989, 1990b), these results suggest that there is a Zn^{2+} requirement for phorbol ester binding and that this requirement is probably a structural one.

The CRD of NC, PKC and DGK as well as purified rat brain PKC binds $^{65}\text{Zn}^{2+}$ after transfer to nitrocellulose. The stoichiometry of $^{65}\text{Zn}^{2+}$ binding was measured by varying the protein concentration and estimating the change in the amount of $^{65}\text{Zn}^{2+}$ bound. When slopes of the curves for PKC and DGK proteins were compared with those obtained with NC, the former two proteins bound significantly more $^{65}\text{Zn}^{2+}$ (1.5-fold) than NC, possibly reflecting the presence of two CRMs in these proteins. The $^{65}\text{Zn}^{2+}$ binding to PKC, NC and DGK in this assay was specific, of high affinity and competitive with cadmium and cobalt. This may allow the Zn^{2+} -binding site of these proteins to be investigated with ^{113}Cd n.m.r. and visible Co^{2+} spectroscopy (Pan & Coleman, 1990a; Parraga *et al.*, 1990) and is consistent with a tetrahedral type of co-ordination of the metal.

It can be envisaged that Zn^{2+} co-ordinates the cysteine and/or histidine residues of the CRM present in NC, PKC and DGK, forming a primitive structure (since the motif is present in yeast PKC; Levin *et al.*, 1990) that can interact with phorbol ester/phosphatidylserine or with the lipids DG/phosphatidylserine. The presence of this CRM in RAF and VAV makes it highly probable that a similar zinc co-ordination occurs in these proteins leading to the formation of a similar structure. The CRM present in these proteins is distinct from that found in transcription factor IIIA, HGR and GAL4, and therefore probably forms a distinct structure. The fact that NC and PKC bind phorbol ester with high affinity while DGK does not can be interpreted in at least two ways. Firstly, it may reflect the manner in which substrate-binding sites and activating sites differ, allowing on the one hand a chemical modification and on the other a change in protein conformation. Secondly, it could reflect the different substrate specificities of the two sites. In other words, each CRD may have unique interactions with different DGs, this being seen by some binding phorbol ester while others do not. The finding that PKC ζ expressed in COS cells does not bind phorbol ester (Ono *et al.*, 1989b) would support the latter interpretation. The role of the CRD in RAF and VAV is less clear. RAF has been found complexed to the platelet-derived growth factor receptor (Kaplan *et al.*, 1990), and on cell stimulation translocates to the nucleus (Rapp *et al.*, 1988). One may then speculate that the RAF CRD may be involved in membrane localization, and after cell stimulation this membrane interaction is in some way altered (possibly by phosphorylation) causing it to translocate to the nucleus.

In conclusion, we have demonstrated that the CRD with the motif $\text{HX}_{11/12}\text{CX}_2\text{CX}_{13-14}\text{CX}_2\text{CX}_4\text{HX}_2\text{CX}_{6/7}\text{C}$ in NC, PKC and DGK binds Zn^{2+} and that this binding is required for phorbol ester binding by NC and PKC. DGK fusion proteins did not bind phorbol ester. The results presented here provide the first evidence for the involvement of a zinc-dependent structure in protein-lipid interactions.

Note added in proof (received 29 August 1991)

The *Caenorhabditis elegans unc-13* gene product has recently been shown to encode a phorbol-ester receptor (Maruyama & Brenner, 1991). The UNC-13 protein possesses a CRD with the motif $HX_{12}CX_2CX_{12}CX_2CX_4HX_2CX_2C$ that has 93% (14/15) sequence identity with the phorbol-ester-binding consensus. The single change being a conservative leucine-for-valine substitution at position 47 of the CRD.

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