Human brain *n*-chimaerin cDNA encodes a novel phorbol ester receptor

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A human brain-specific cDNA encoding *n*-chimaerin, a protein of predicted molecular mass 34 kDa, has sequence identity with two different proteins: protein kinase C (PKC) at the *N*-terminus and BCR protein [product of the breakpoint-cluster-region (BCR) gene, involved in Philadelphia chromosome translocation] at the *C*-terminus [Hall, Monfries, Smith, Lim, Kozma, Ahmed, Vannaisungham, Leung & Lim (1990) *J. Mol. Biol.* **211**, 11–16]. The sequence identity of *n*-chimaerin with PKC includes the cysteine-rich motif CX₂CX₁₃CX₂CX₇CX₇C, and amino acids upstream of the first cysteine residue, but not the kinase domain. This region of PKC has been implicated in the binding of diacylglycerol and phorbol esters in a phospholipid-dependent fashion. Part of this cysteine-rich motif (CX₂CX₁₃CX₂C) has the potential of forming a 'Zn-finger' structure. Phorbol esters cause a variety of physiological changes and are among the most potent tumour promoters that have been described. PKC is the only known protein target for these compounds. We now report that *n*-chimaerin cDNA encodes a novel phospholipid-dependent phorbol ester receptor, with the cysteine-rich region being responsible for this activity. This finding has wide implications for previous studies equating phorbol ester binding with the presence of PKC in the brain.

INTRODUCTION

n-Chimaerin cDNA detects a relatively abundant mRNA of 2.2 kb in human brain and 2.3 kb in rat brain. This 2.3 kb mRNA is specifically expressed in rat brain, with highest amounts in the cerebral cortex and hippocampus, and is not detected in a variety of other tissues (Hall *et al.*, 1990). The mRNA has a neuronal distribution. *n*-Chimaerin cDNA encodes a protein of predicted molecular mass 34 kDa. The protein sequence of human *n*-chimaerin cDNA has no direct counterpart in the PIR (Protein Identification Resource) (DNASTAR computer search; Lipman & Pearson, 1985) database. The *N*-terminus contains a stretch of 60 amino acids rich in cysteine residues, with 48% similarity to one half (C1b) of a duplicated sequence found in the C1 region of protein kinase C (PKC) (Parker *et al.*, 1986; Hall *et al.*, 1990). Immediately adjacent to this region there is 42% sequence similarity to the *C*-terminus of BCR protein [product of the breakpoint-cluster-region (BCR) gene involved in Philadelphia (Ph⁺) chromosome translocation (Heisterkamp *et al.*, 1985; Hall *et al.*, 1990)]. The BCR protein has no known function. A homologous rat cDNA has been found to encode a protein with 97.3% sequence similarity to the human *n*-chimaerin (H. H. Lim, unpublished work).

Phorbol esters [analogues of the naturally occurring second messenger diacylglycerol (DAG)] are potent tumour promoters that cause a variety of physiological changes when administered to both cells and tissues (Diamond *et al.*, 1980; Blumberg, 1980; Leach *et al.*, 1983). The only known protein target for phorbol esters is PKC, an enzyme involved in the modulation of ion channels and receptors, gene expression and cell proliferation (Neidel *et al.*, 1983; Nishizuka, 1984, 1988).

The C1 region of PKC binds phorbol esters/DAG in a phospholipid-dependent fashion (Ohno *et al.*, 1988a; Ono *et al.*, 1989). Recently, Nishizuka's group (Ono *et al.*, 1988a) have used

various deletions and point mutations of the rat brain γ -PKC expressed as fusion proteins in *Escherichia coli* to demonstrate the sequence requirements for phospholipid-dependent phorbol ester binding (Ono *et al.*, 1989). From these experiments a consensus sequence for this activity can be obtained. *n*-Chimaerin has 71% (15/21) of the amino acids present in the consensus. In the present study we show that *n*-chimaerin cDNA encodes a protein that binds phorbol esters specifically and in a manner analogous to PKC.

MATERIALS AND METHODS

Subcloning of *n*-chimaerin cDNA into TrpE expression vectors

A 1.7 kb endonuclease-*Pst*I fragment containing full-length *n*-chimaerin coding sequence was purified from pBSCM1 (Hall *et al.*, 1990) and cloned into the *Pst*I site of pATH21 to give pATCM1. pATCM1 was then cut with *Xba*I, which removes 1.2 kb of the 3' end of the cDNA forming pATCM2. pATCM2 encodes the cysteine-rich sequence and lacks most of the domain with sequence identity with BCR protein. A *Pvu*2 fragment obtained from the 1.7 kb *Pst*I fragment was cloned into the *Sma*I site of pATH23 to give pATCM3. pATCM3 encodes the *C*-terminal part of *n*-chimaerin and lacks the cysteine-rich sequence.

Expression and refolding of TrpE and *n*-chimaerin fusion proteins

The highly inducible TrpE promoter was used to drive expression of the fusion. TrpE fusion proteins partition predominantly into the insoluble fraction, and this characteristic was used to partially purify the proteins (Hoffman *et al.*, 1987). Induction of TrpE and TrpE fusions was carried out essentially as previously described (Hoffman *et al.*, 1987); *E. coli*

Abbreviations used: DAB, 3,3'-diaminobenzidine tetrachloride; PDBu, phorbol 12,13-dibutyrate; PMA, phorbol 12-myristate 13-acetate; PMAAL, 20-oxo-20-deoxyphorbol 12-myristate 13-acetate; PAzBz, phorbol 12-(*p*-azidobenzoate) 13-benzoate; PKC, protein kinase C; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; Ph⁺, Philadelphia; DAG, diacylglycerol; BCR protein, product of the breakpoint-cluster-region gene; DTT, dithiothreitol; PBS, phosphate-buffered saline; PIR, Protein Identification Resource.

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RR1 transformed with pATH23 (TrpE, 37 kDa), pATCM1 (TrpE-*n*-chimaerin fusion, 70 kDa), pATCM2 (TrpE-*N*-terminal *n*-chimaerin fusion, 50 kDa) or pATCM3 (TrpE/*C*-terminal *n*-chimaerin, 55 kDa) were grown overnight in M9 minimal salts with 1 mM-MgSO₄/0.1 mM-CaCl₂/vitamin B-1 (100 µg/ml)/tryptophan (40 µg/ml)/ampicillin (100 µg/ml) or in LB broth with ampicillin. The overnight cultures were diluted 10-fold into 100 ml of the minimal-salts medium, and 3- β -indoleacrylic acid (20 µg/ml) was added when the cultures reached an A_{550} of 0.4. Cells were grown for a further 2–3 h, harvested and then resuspended in 3 ml of 20 mM-Tris/HCl (pH 7.5)/0.25 M-sucrose/2 mM-EDTA/10 mM-EGTA/leupeptin (20 µg/ml)/0.5 % Triton X-100. Pellets were stored at –20 °C before use. Thawed cell pellets were sonicated for 1 min for periods of 5 s with intervals of 15 s on ice and then centrifuged at 20000 *g* for 20 min. The supernatant was used in preliminary binding experiments (this represented the soluble fraction). Cell pellets were solubilized in 3.0 ml of 8 M-urea/25 mM-Tris/HCl (pH 7.5)/1 mM-EDTA/1 mM-dithiothreitol (DTT) and dialysed against 4 M-urea/1 mM-Tris/HCl (pH 5.0)/1 mM-DTT for 1 h and then against 20 mM-Tris/HCl (pH 7.5)/50 mM-NaCl/2 mM-MgCl₂/200 µM-DTT/100 µM-ZnCl₂ overnight with two changes of buffer (the insoluble fraction was used to produce all the results presented in this study). This procedure should allow the refolding of the potential Zn-dependent structure. The cell extracts were stored as a 30 % (v/v) glycerol solution at 4 °C and used within 24 h or stored for longer times at –20 °C.

Immunological analysis

Antibodies were raised against TrpE-*n*-chimaerin *C*-terminal fusion protein using the following protocol: the insoluble fraction from *E. coli* cells transformed with pATCM3 was prepared as described above and electrophoresed on SDS/polyacrylamide gels (Laemmli, 1970). The gel was then incubated with 0.1 M-KCl for 1 h at 4 °C, and the visible TrpE-fusion protein band excised. The gel slice containing TrpE-fusion protein was placed in a dialysis sac and electroeluted for 2 h at 180 V in a horizontal electrophoresis tank. The TrpE fusion protein was then acetone-precipitated and resuspended in PBS, yielding a protein with > 95 % purity and at a concentration of ~1 mg/ml. The immunization procedure consisted of injection of fusion protein (100 µg) in complete Freund's adjuvant, followed by boosts (every 4 weeks) of fusion protein (100 µg) in incomplete Freund's adjuvant. Rabbits were bled from an ear vein 1 week after the third boost and subsequent injections. Proteins were revealed either by running cell extracts (5 µl) on SDS/polyacrylamide gels in Laemmli buffer and staining with Coomassie Blue (0.1 %) or by blotting on to nitrocellulose and incubating with primary antibody (*n*-chimaerin antiserum obtained as described above). Gels were blotted on to nitrocellulose by using the Sartoblot 11-S (Sartorius) for 1 h in 25 mM-Tris (pH 8.3)/192 mM-glycine/20 % methanol at 100 mA. Immunoreactive bands were revealed by using the biotin-streptavidin system (Amersham International), with horseradish peroxidase pre-formed complex and staining with DAB (3,3'-diaminobenzidine tetrachloride). Non-specific binding sites were blocked with 5 % (w/v) Marvel non-fat milk, followed by washing with phosphate-buffered saline (PBS; 0.01 M-sodium phosphate/0.15 M-NaCl, pH 7.2)/0.1 % Tween-20. *n*-Chimaerin antiserum was purified by absorbing out TrpE-reacting material on a CNBr-activated Sepharose 4B column of the insoluble fraction from induced *E. coli* pATH23. The flow-through was then passed over a second Sepharose 4B column of partially purified full and *C*-terminal fusion proteins. Elution was carried out with 0.5 M-acetic acid and/or 4 M-guanidinium chloride; resulting fractions were dialysed against water for 24 h and then freeze-dried. The dried material was

reconstituted in 1–2 ml of PBS/1 % BSA and stored in aliquots at –20 °C. Molecular masses were determined by SDS/PAGE with biotinylated standard proteins (Sigma).

Ligand binding

Phorbol ester binding was measured using 20 nM-[³H]phorbol 12,13-dibutyrate (PDBu) [19 Ci/mmol (Amersham) and 20 Ci/mmol (NEN)]. Cell extracts (5 µl) were incubated in 50 mM-Tris (pH 7.5)/phosphatidylserine (PS; 100 µg/ml)/2 mM-CaCl₂/BSA (4 mg/ml) for 1 h at room temperature and then placed on ice for 10–30 min before filtration. Samples were filtered on pre-wetted GF/C (Whatman) discs followed by washing with 5 × 2 ml of ice-cold 20 mM-Tris, pH 7.5. The filters were dried and then counted for radioactivity in emulsifier-safe scintillant (Packard). Non-specific binding was estimated by the addition of 10 µM unlabelled PDBu in parallel with the other experiments and was found to be 521 ± 86 c.p.m. (*n* = 14). This meant that there was negligible PDBu binding to the extracts containing TrpE or the *C*-terminal *n*-chimaerin fusion proteins. Binding was determined from between nine and 12 experiments. For the fusions containing the consensus sequence, binding was proportional to protein concentration over the range 0.3–5 µl of cell extract. For the extracts containing TrpE or *C*-terminal fusion proteins, binding was independent of protein concentration over the same range and essentially the same as that in the absence of protein. All errors in ligand-binding studies are expressed as standard deviations (S.D.).

RESULTS

Comparison of PKC structure with *n*-chimaerin

There are seven known members of the PKC family (Coussens *et al.*, 1986; Parker *et al.*, 1986; Ono *et al.*, 1987, 1988*a,b*), and these fall into two groups I (α , β I, β II and γ) and II (δ , ϵ and ζ) the main difference being the absence of the C2 region in the latter. More recently, it has been shown that ϵ' and ζ PKC have only one cysteine-rich motif (Ono *et al.*, 1988*b*; Huang, 1989). The schematic presentation of PKC structure highlights the four conserved regions of the protein family, C1–C4 (Fig. 1). The regulatory domain is composed of C1 and C2. The C1 region is cysteine-rich and contains two adjacent motifs CX₂CX₁₃CX₂CX₇CX₇C, (a and b). Part of this motif, CX₂CX₁₃CX₂C, has the potential of forming a 'Zn-finger' (Klug & Rhodes, 1987; Freedman *et al.*, 1988). This partial motif has a role in DNA binding of steroid-hormone receptors (Green & Chambon, 1988) and certain fungal transcription factors (Johnstone, 1987). The C2 region, when present, is responsible for the Ca²⁺-dependence of both kinase activity and phorbol ester binding (Ohno *et al.*, 1988*a*; Ono *et al.*, 1989). C3 contains the ATP-binding consensus sequence (GXGX₂G-K) and together with the C4 forms the kinase domain of PKC (Parker *et al.*, 1986). *n*-Chimaerin consists of a single cysteine-rich motif and no apparent kinase domain (Fig. 1). Instead the *C*-terminus of the protein has similarity to the *C*-terminus of BCR protein (Hall *et al.*, 1990). The cysteine-rich sequence of *n*-chimaerin has 38 and 48 % sequence identity with the C1a and C1b regions of human brain β PKC respectively (Hall *et al.*, 1990).

Consensus sequence for phorbol ester binding

A consensus sequence for phospholipid-dependent phorbol ester binding can be derived from recently published experiments (Ono *et al.*, 1989). Either one of the two cysteine-rich sequences (C1a or C1b) is able to bind phorbol esters in a phospholipid-dependent fashion (Ono *et al.*, 1989). This consensus sequence represents a maximal requirement, since there may be residues which are not essential for the activity. Alignment of the cysteine-

Fig. 1. Schematic comparison of PKC structure with *n*-chimaerin

The PKC family members are divided into structural groups (I and II) based on the absence of the C2 region in group II. *n*-Chimaerin shares sequence similarity to the C1a/b region, but not to regions C2, C3 or C4. Each vertical line represents one cysteine residue.

Fig. 2. Alignment of the N-terminus of *n*-chimaerin with the phorbol-ester-binding consensus sequence

Amino acid residues 40–100 of *n*-chimaerin (Hall *et al.*, 1990) are aligned to the phorbol-ester-binding consensus derived from rat γ PKC (Ono *et al.*, 1989). This consensus sequence is sufficient for lipid-dependent phorbol ester binding. Broken lines indicate no amino acid preference. Solid boxes indicate identity and empty boxes non-identity.

rich sequence of *n*-chimaerin with the consensus sequence for phospholipid-dependent phorbol ester binding is shown in Fig. 2. *n*-Chimaerin contains 15 of the 21 amino acids present in the consensus, giving 71 % sequence identity.

Expression of *n*-chimaerin fusion proteins and their ability to bind phorbol esters

To test whether *n*-chimaerin may embody a novel phorbol ester receptor, we made use of TrpE expression vectors (Dieckmann & Tzagoloff, 1985; Fig. 3). *E. coli* cells containing plasmids pATH23 (TrpE, control) and pATCM1, pATCM2 and pATCM3 (TrpE-*n*-chimaerin constructs) were induced to express fusion proteins containing (pATCM1 and pATCM2) or lacking (pATCM3) the consensus sequence. These three constructs comprised the entire coding region, the N-terminal region and the C-terminal region respectively. Most of the fusion protein was in the insoluble form, permitting one-step partial purification (Fig. 4). The insoluble fractions were solubilized in 8 M-urea and then renatured in the presence of ZnCl₂ to allow the refolding of the potential 'Zn-finger' or Zn-dependent structure. The purity of the fusion proteins was approx. 80 % on the basis of Coomassie Blue-stained gels (Fig. 4, lanes 1–4). The Western analysis demonstrates that the presence of vectors pATCM1, pATCM2 and pATCM3 in *E. coli* allowed the expression of fusion proteins consisting of both TrpE and *n*-chimaerin moieties of the expected size (Fig. 4). The cell extracts containing the partially pure proteins were then used to investigate [³H]PDBu binding activity of the three *n*-chimaerin fusion proteins and TrpE. After subtracting background radioactivity (approx. 500 c.p.m.), PDBu binding was seen only in the cell extracts expressing fusion proteins containing the consensus sequence (Fig. 5). *E. coli* proteins/lipids alone did not bind any phorbol ester. Fusion protein (pATCM1) refolded in the absence of ZnCl₂ did not possess binding activity (results not shown). PDBu binding was also seen with β -galactosidase-*n*-chimaerin fusion

proteins containing the consensus sequence, but not in those lacking this domain [using the pUR vector series (Koenen *et al.*, 1985) to generate fusions; S. Ahmed, R. Kozma & C. Monfries, unpublished work]. These fusions were more unstable than the TrpE fusions.

Characteristics of phorbol ester binding

The binding of phorbol ester to TrpE-*n*-chimaerin fusion protein (pATCM1) was dependent on the presence of the phospholipid PS. The presence of other phospholipids, namely phosphatidylinositol (PI), phosphatidylcholine (PC) and phosphatidylethanolamine (PE) did not significantly affect basal binding activity (Fig. 6, lanes 1–5). Inhibitors of PDBu binding to PKC, dioleoin (dioleoylglycerol) and PMA, inhibited the binding of [³H]PDBu to TrpE-*n*-chimaerin protein. PMA was significantly more potent than dioleoin, with 100 nM giving 92 % inhibition of binding (Fig. 6, lanes 6 and 8; Table 1). Interestingly, the PMA analogue PMAAL, which lacks the important C-20 hydroxy group (Weinstein *et al.*, 1984), does not affect binding of [³H]PDBu at between 1 and 100 nM (Fig. 6, lane 7; Table 1). Table 1 also shows the effect of a range of other phorbol ester analogues on [³H]PDBu binding. 4 α -PDBu/PMA do not cause inhibition of binding, whereas the azido analogue, PAzBz, is nearly as potent in inhibiting binding as PMA. Since *n*-chimaerin does not have sequence identity with the calcium-binding C2 region of PKC, PDBu-binding would be predicted to be Ca²⁺-independent (Ohno *et al.*, 1988a; Ono *et al.*, 1989). This was confirmed when the binding assay was carried out in the absence of CaCl₂ or in the presence of 2 mM-EGTA (Fig. 6, lanes 9 and 10). The soluble fraction exhibited binding characteristics similar to those of the insoluble fraction (i.e. binding only occurred when the consensus sequence was present, and was phospholipid-dependent but Ca²⁺-independent). The level of specific binding (c.p.m./5 μ l of cell extract) in the soluble fraction was 1516 $\pm$ 850 (n = 5) compared with 15703 $\pm$ 2106 (n = 11) in the insoluble

Fig. 3. Construction of TrpE-*n*-chimaerin expression vectors

pATH vectors (Dieckmann & Tzagoloff, 1985) (pATH21 and pATH23) allow the expression of TrpE fusion proteins of DNA cloned 3' to TrpE coding sequence in the multiple cloning site (black box). *n*-Chimaerin cDNA (2.2 kb) was subcloned as an *Eco*RI fragment from a human retinal library (Nathans *et al.*, 1986) λ gt 10 clone into the Bluescript vector SK M13, generating pBSCM1. The *n*-chimaerin cDNA contains an open reading frame from nucleotides 454 to 1449 (just preceding the *Pst*I site to the *Dra*I site). The first methionine residue of the open reading frame is at nucleotide 553. The cysteine-rich sequence spans nucleotides 727–838 (Hall *et al.*, 1990). *n*-Chimaerin cDNA fragments (*Pst*I–*Pst*I, *Pst*I–*Xba*I and *Pvu*II–*Pvu*II) were cloned into the appropriate reading frame in either pATH21 or pATH23 vector to generate three different TrpE fusions.

Fig. 4. Expression and immunoblots of TrpE-*n*-chimaerin fusion proteins

Cell extracts were made from *E. coli* cultures (as described in the Materials and methods section), induced to express TrpE (control) or TrpE-*n*-chimaerin fusion proteins and analysed on SDS/polyacrylamide gels (lanes 1–4). Molecular-mass markers are shown in lane 5. The gels were then immunoblotted using either antisera (1:10000 dilution) produced from rabbits injected with the purified C-terminal fusion protein (lanes 6–9) or the same antisera (1:200 dilution) with nearly all TrpE-reacting material removed (lanes 10–13). TrpE protein (pATH23, lanes 1, 6 and 10), TrpE-*n*-chimaerin (pATCM1, lanes 2, 7 and 11), TrpE-C-terminal *n*-chimaerin (pATCM3, lanes 3, 8 and 12) and TrpE-N-terminal chimaerin (pATCM2, lanes 4, 9 and 13). This purified antisera also reacted with β -galactosidase-*n*-chimaerin (generated using pUR vectors; Koenen *et al.*, 1985) fusion protein, but not against the β -galactosidase protein itself (C. Monfries, unpublished work). The purified *n*-chimaerin antisera reacted weakly with the N-terminal fusion, as this protein contains only a small portion (28 amino acids) of the C-terminal fusion used for immunization. Revelation of immunoreactive bands was carried out as described in the Materials and methods section.

fraction. This reflected the partitioning of the TrpE fusion proteins between the soluble and insoluble fractions.

Specificity of phorbol ester binding

To show the specificity of interaction of the phorbol ester with the TrpE-*n*-chimaerin fusion protein (pATCM1) the effects of increasing PDBu concentration on binding were determined

(Fig. 7). The binding showed saturation characteristics typical of protein receptors. Scatchard analysis of the data suggests the presence of a single receptor binding site of high affinity with a K_d of 29 ± 7 nM ($n = 6$; β -galactosidase-*n*-chimaerin fusions gave a K_d of 50 nM). The stoichiometry of phorbol ester binding to the TrpE-*n*-chimaerin fusion protein was 0.24 ± 0.04 ($n = 6$) using maximum binding (B_{max}) values of 3–4.5 pmol (derived from the

Fig. 5. Phorbol ester binding by refolded TrpE-*n*-chimaerin fusion proteins

A schematic diagram of the fusion proteins generated using pATH vectors is shown. pATCM1 and pATCM2 contain the consensus sequence, whereas pATCM3 and pATH23 do not. Cysteine residues are shown as vertical lines forming the motif CX₂CX₁₃CX₂CX₇C (as in Fig. 1). Background/non-specific binding was not subtracted from the data presented (means ± s.d.; *n* values in parenthesis).

Fig. 6. Characteristics of phorbol ester binding to TrpE-*n*-chimaerin protein (pATCM1)

Effects of lipid (1–5), inhibitors (6–8) and Ca²⁺ (9, 10) are shown. Cell extracts (1.5–2.0 μl) were incubated in buffers of various compositions for 1 h with 20 nM-[³H]PDBu, and binding was measured as described in the Materials and methods section; 100% binding was taken as the value obtained in the presence of PS and Ca²⁺ (1). Binding was determined in the absence of phospholipids (2) or in the presence of PC (3), PE (4), PI (5), all at 100 μg/ml. Diolein (6), PMAAL (7) and PMA (8) were present at 10 μg/ml, 100 nM and 100 nM respectively. Binding was determined in the presence of PS without Ca²⁺ (9) and PS with 2 mM-EGTA (10). Similar experiments as in 9 and 10 with the other lipids (PI, PC and PE) also showed no dependence on Ca²⁺ (results not shown). Determinations were carried out in triplicate and represent the mean results obtained from two or three experiments. (± s.d., *n* = 6–9). Non-specific binding (~ 500 c.p.m.) was subtracted from the data.

Table 1. Inhibition of [³H]PDBu binding to *n*-chimaerin fusion protein (pATCM1) by phorbol ester analogues and diolein

Experimental details for phorbol ester binding were as in Fig. 3 and the Materials and methods section.

Phorbol ester analogue	Concn. (nM)	Inhibition of binding (%)
PMAAL	1	0
	10	7
	100	0
4α-PMA	1	3
	10	1
	100	0
4α-PDBu	1	0
	10	0
	100	5
PAzBz	1	8
	10	57
	100	72
PMA	1	15
	10	75
	100	92
Diolein	1 μg/ml	0
	10 μg/ml	10
	100 μg/ml	31

this motif, with six of the 21 amino acids being *N*-terminal to the first cysteine residue. In addition to these cysteine residues there are two histidine residues (conserved in all PKC family members) that may be involved in metal binding, forming the larger motif HX₁₂CX₂CX₁₃CX₂CX₄HX₂CX₇C (Table 2). Site-directed mutagenesis has shown that cysteine-3 and -4 of the cysteine-rich motif of rat brain γ-PKC are essential for phorbol ester binding (Ono *et al.*, 1989). In the present study, the urea-denatured *n*-chimaerin fusion protein was refolded in a manner which should allow a potential Zn-dependent structure to form (Nagai *et al.*, 1988). When refolding was attempted under conditions in which Zn was not available, the fusion protein (pATCM1) was unable to bind phorbol esters. These results suggest that the formation of a Zn-dependent structure was required to allow *n*-chimaerin/PKC to bind to phorbol esters and/or lipids.

Cysteine-rich motifs similar to those found in the phorbol-ester-binding consensus are present in a variety of transcription factors, including GAL4, E1A and the steroid-hormone receptors (Berg, 1986; Green & Chambon, 1988; Pan & Coleman, 1989; Table 2). Alignment of the amino acid sequence of these transcription factors with the phorbol-ester-binding consensus

Scatchard plot). Stoichiometries of between 0.46 and 0.94 have been obtained for native PKC (Bazzi & Nelsestuen, 1988). The higher values obtained for PKC could be explained by it having two cysteine-rich motifs, which may allow it to bind twice as much phorbol ester, and/or the bacterial *n*-chimaerin being possibly not as active as native PKC.

DISCUSSION

The consensus sequence for phorbol ester binding derived from rat brain γ-PKC (Ono *et al.*, 1988a, 1989) is cysteine-rich and composed of 21 amino acids interspersed within a stretch of 50 amino acids. The cysteine residues form the motif CX₂CX₁₃CX₂CX₇C. The consensus sequence extends beyond

Fig. 7. Analysis of $[^3\text{H}]\text{PDBu}$ binding to TrpE-*n*-chimaerin protein (pATCM1)

The effect of increasing concentrations of $[^3\text{H}]\text{PDBu}$ on binding is shown. $[^3\text{H}]\text{PDBu}$ was increased over the concentration range 12.5–400 nM using NEN $[^3\text{H}]\text{PDBu}$ (20 Ci/mmol) at constant specific radioactivity. Cell extracts (1.5 μl) were incubated with different concentrations of phorbol ester for 1 h and then binding was determined by filtration on GF/C filters as described in the Materials and methods section. Results presented are of a typical experiment, with each point determined in triplicate (mean $\pm$ S.D., $n = 3$). Six other experiments were carried out, yielding similar results. Non-specific binding was determined using cell extracts expressing the C-terminal fusion protein and was subtracted from the data. The non-specific binding varied from approx. 400 to 4800 c.p.m. over the concentration range used, giving a background of 8–25% of total radioactivity. This represented binding to glass-fibre filters and not to bacterial protein, as the same level of non-specific binding was obtained in the absence of *E. coli* cell extracts or in the presence of unlabelled PDBu (10 μM).

Table 2. Sequence identity of some cysteine-rich proteins with the phorbol-ester-binding consensus

The proteins shown are rat PKC family α - ζ (Ono *et al.*, 1987, 1988a,b), human *n*-chimaerin (Hall *et al.*, 1990), human A-Raf-1 (Beck *et al.*, 1987), porcine DAG kinase (Sakane *et al.*, 1990), *Saccharomyces cerevisiae* GAL4 (Keegan *et al.*, 1986), adenoviral E1A (Berg, 1986), human glucocorticoid receptor (HGR) (Green *et al.*, 1988). The motif $\text{HX}_{12}\text{CX}_2\text{CX}_{13/14}\text{CX}_2\text{CX}_4\text{HX}_2\text{CX}_7\text{C}$ present in the rat PKC family above is also found in all PKC isoenzymes so far sequenced, including: human and bovine α - τ , rabbit α - ϵ , *Drosophila melanogaster* and *Caenorhabditis elegans tpa-1* (Coussens *et al.*, 1986; Parker *et al.*, 1986; Ohno *et al.*, 1987, 1988a,b; Rosenthal *et al.*, 1987; Schaeffer *et al.*, 1989; Tabuse *et al.*, 1989). The sequence-identity values for PKC and DAG kinase are taken from both C1a and 1b regions of these proteins

Protein (species)	Sequence identity (%)	Motif
PKC (rat)	71–100	$\text{HX}_{12}\text{CX}_2\text{CX}_{13/14}\text{CX}_2\text{CX}_4\text{HX}_2\text{CX}_7\text{C}$
<i>n</i> -Chimaerin (human)	71	$\text{HX}_{12}\text{CX}_2\text{CX}_{13}\text{CX}_2\text{CX}_4\text{HX}_2\text{CX}_7\text{C}$
A-Raf-1 (human)	52	$\text{HX}_{12}\text{CX}_2\text{CX}_9\text{CX}_2\text{CX}_4\text{HX}_2\text{CX}_7\text{C}$
DAG kinase (porcine)	42–62	$\text{HX}_{11/12}\text{CX}_2\text{CX}_{12/14}\text{CX}_2\text{CX}_4\text{HX}_2\text{CX}_{6/7}\text{C}$
HGR	30	$\text{CX}_2\text{CX}_{15}\text{CX}_2\text{CX}_{14}\text{C}$
GAL4 (<i>S. cerevisiae</i>)	19	$\text{CX}_2\text{CX}_6\text{CX}_6\text{CX}_2\text{CX}_6\text{C}$
E1A (adenovirus)	19	$\text{CX}_2\text{CX}_{15}\text{CX}_2\text{C}$

reveals the similarity (19–30%) between the sequences to be restricted almost exclusively to the cysteine residues. In the case of the glucocorticoid receptor, tetrahedral Zn co-ordination has been demonstrated using e.x.a.f.s. spectroscopy, supporting the presence of a Zn-finger structure in this protein (Freedman *et al.*, 1988). However, with GAL4, ^{113}Cd - and two-dimensional ^1H -n.m.r. data suggest the formation of a binuclear cluster structure with the six cysteine residues being co-ordinated by two Zn atoms (Pan & Coleman, 1990). The cysteine-rich motif found in *n*-chimaerin, PKC, DAG kinase and A-Raf-1 is different from both that found in GAL4 and that in the glucocorticoid receptor, suggesting that a structure distinctive from a Zn-finger or binuclear cluster is formed in these proteins (Table 2). Thus, although Zn-dependent structures can potentially form in all proteins containing cysteine-rich motifs, the exact structure formed seems to be determined by the nature of the motif. Moreover, the activity conferred by these Zn-dependent structures is probably determined by those amino acids designated 'X' and those flanking the motif (Johnstone & Dover, 1988; Mader *et al.*, 1989).

There is a growing number of reports which suggest that phorbol esters can exert their cellular effects independently of PKC (Hockberger *et al.*, 1989; Sha'afi, 1989). Our results, showing that *n*-chimaerin cDNA encodes a phorbol ester receptor, would support this. Addition of DAG analogues to sensory neurons has been shown to block Ca^{2+} currents (Rane & Dunlap, 1986). However, specific inhibitors of PKC were only 80% effective in reversing this effect (Rane *et al.*, 1989). Two possible explanations of these results are (i) that the inhibition of PKC is not complete or (ii) that other phorbol ester receptors, such as *n*-chimaerin, are involved in the modulation of Ca^{2+} channels. *n*-Chimaerin, PKC, A-Raf-1 and DAG kinase have in common the motif $\text{HX}_{12}\text{CX}_2\text{CX}_n\text{CX}_2\text{CX}_4\text{HX}_2\text{CX}_7\text{C}$ (where n varies between 9 and 14). The presence of this motif and the sequence identity with the phorbol-ester-binding consensus in A-Raf-1 and DAG kinase (52 and 42–62% respectively; spacings in DAG kinase cysteine-rich motifs are slightly different; see Table 2) suggests that these proteins have a Zn-dependent structure and are phorbol ester receptors (Table 2). Taking these results together, the mechanism determining the effect of phorbol esters on cellular activities and phenotypes must be more complicated than initially imagined.

The binding of phorbol esters to PKC and *n*-chimaerin is dependent on the presence of the phospholipids with specificity for PS. The amino acid sequence requirements for the binding of both substrates is found in the consensus sequence presented in Fig. 2. The K_d for PDBu binding to PKC is in the nanomolar range (e.g., 7 nM found by Niedel *et al.*, 1983). In the present study, *n*-chimaerin expressed in *E. coli* has a K_d of 29 nM, falling in the range found for PKC. We further investigated this binding by allowing $[^3\text{H}]\text{PDBu}$ to compete in binding to *n*-chimaerin with five phorbol ester analogues and diolein (Table 1). PMA (Neidel *et al.*, 1983) and PAzBz (Delcos *et al.*, 1983) have been shown to be potent inhibitors of PDBu binding to PKC, with K_i values lower than the K_d for binding. Diolein, on the other hand, is a weak inhibitor of PDBu binding to PKC. PMAAL and 4α -PMA/ 4α -PDBu are biologically inactive, owing to changes in the phorbol ester molecule thought to be essential for high-affinity binding to PKC (Weinstein *et al.*, 1984; C-20 hydroxy group to an aldehyde group in PMAAL and C-4 hydroxy group β -isomer to α -isomer in 4α -PMA/ 4α -PDBu). The inhibition characteristics of the five phorbol ester analogues for PDBu binding to *n*-chimaerin followed the pattern (Table 1) previously described for PKC (Niedel *et al.*, 1983; Delcos *et al.*, 1983; Weinstein *et al.*, 1984). However, diolein was less effective as an inhibitor of PDBu binding to *n*-chimaerin. This may reflect

differences in substrate-specificity between *n*-chimaerin and PKC. The characteristics of phorbol ester binding to *n*-chimaerin, namely its stereospecificity and its phospholipid-dependence and Ca²⁺-independence, mirror those of the PKC II group (Ohno *et al.*, 1988a; Ono *et al.*, 1989; Fig. 1). The physiological consequence of phorbol ester/DAG binding to PKC is to stimulate kinase activity and possibly to cause translocation of PKC from the cytoplasm to the plasma membrane (Nishizuka, 1984, 1988). *n*-Chimaerin may be an additional protein whose activity and location is affected by the second messenger DAG.

The purified *n*-chimaerin antiserum used in the present study has been found to stain specifically neurons in the hippocampus and other brain regions (C. Monfries, unpublished work) that have been shown previously to express *n*-chimaerin mRNA (Hall *et al.*, 1990). This antiserum also detects four proteins of molecular masses 20–45 kDa in human and rat brain cytosol. These proteins may represent additional members of the *n*-chimaerin family or be post-translation modifications of the same protein (C. Hall, R. Kozma and C. Monfries, unpublished work). The elucidation of the activity encoded by the BCR-related region of *n*-chimaerin will be critical in understanding the exact role this molecule plays in the signal transduction.

Note added in proof (received 6 November 1990)

A yeast (*Saccharomyces cerevisiae*) PKC gene (PKC1) has been isolated and sequenced (Levin *et al.*, 1990). The yeast PKC1 protein has the duplicated cysteine-rich sequence (C1a and 1b) with the motifs HX₁₂CX₂CX₁₀CX₂CX₄HX₂CX₇C and HX₁₂CX₂CX₁₃CX₂CX₄HX₂CX₇C. The sequence identity of this region of PKC1 with the phorbol-ester-binding consensus sequence is 52–62%.

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