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The *Caenorhabditis elegans unc-13* gene product is a phospholipid-dependent high-affinity phorbol ester receptor

Sohail AHMED,*†§ Ichiro N. MARUYAMA,‡‖ Robert KOZMA,*† Joel LEE,*† Sydney BRENNER‡ and Louis LIM*†

*Institute of Molecular and Cell Biology, National University of Singapore, Singapore 0511, †Department of Neurochemistry, Institute of Neurology, 1 Wakefield St., London WC1N 1PJ, U.K., and ‡Medical Research Council Molecular Genetics Unit, Hills Road, Cambridge CB2 2QH, U.K.

The *Caenorhabditis elegans unc-13* mutant is a member of a class of mutants that have *un*-coordinated movement. Mutations of the *unc-13* gene cause diverse defects in *C. elegans*, including abnormal neuronal connections and modified synaptic transmission in the nervous system. *unc-13* cDNA encodes a protein (UNC-13) of 1734 amino acid residues with a predicted molecular mass of 198 kDa and sequence identity to the C1/C2 regions but not to the catalytic domain of the ubiquitously expressed protein kinase C family [Maruyama & Brenner (1991) Proc. Natl. Acad. Sci. U.S.A. **88**, 5729–5733]. To characterize the phorbol ester binding site of the UNC-13 protein, cDNA encoding the C1/C2-like regions (amino acid residues 546–940) was expressed in *Escherichia coli* and the 43 kDa recombinant protein was purified. Phorbol ester binding to the 43 kDa protein was zinc- and phospholipid-dependent, stereospecific and of high affinity (K_d 67 nM). UNC-13 specific antisera detected a protein of approx. 190 kDa in wild-type (N2) but not in mutant (*e1019*) *C. elegans* cell extracts. We conclude that UNC-13 represents a novel class of phorbol ester receptor.

INTRODUCTION

The *Caenorhabditis elegans unc-13* mutations affect the nervous system, leading to slow irregular pharyngeal pumping, abnormal connections between major interneurons, and misplaced or misguided motor and sensory neurons. *unc-13* mutations also lead to the accumulation of high levels of acetylcholine without alterations in the levels of choline and choline acetyltransferase (Chalfie & White, 1988; Hosono *et al.*, 1989; Siddiqui, 1990; I. N. Maruyama, unpublished work). A cDNA that complements the *unc-13* mutant has recently been isolated and sequenced. The *unc-13* cDNA encodes a protein (UNC-13) of 1734 amino acid residues with no overall sequence identity with any protein in the NBRF–PIR database (Maruyama & Brenner, 1991). However, a region comprising amino acid residues 609–846 of UNC-13 has sequence identity to the regulatory domain, regions C1/C2, but not to the catalytic domain, of the multigene protein kinase C (PKC; α–η) family (Nishizuka, 1986, 1988).

The C1 region of PKC contains a cysteine-rich domain (CRD) with the cysteine-rich motif (CRM; $HX_{12}CX_2CX_{10-14}CX_2CX_4HX_2CX_{6/7}C$, where X is any amino acid) which represents a phorbol ester binding site and is duplicated in tandem in all PKC family members apart from PKC-ζ. Similar cysteine-rich sequences are found in n-chimaerin, diacylglycerol kinase (DGK) and the oncogene products RAF and VAV. The CRDs of human proteins, PKC-γ and n-chimaerin have been shown to bind zinc, which is required for phorbol ester binding. DGK also binds zinc but not phorbol ester (Ahmed *et al.*, 1991). The C2 region of PKC has sequence identity to the $p21^{ras}$-GTPase-activating protein (p120), p65, phospholipase C-γ and phospholipase A_2 (see Clark *et al.*, 1991, for sequence alignment). The C2 region, present in two copies in p65 and phospholipase C-γ, is thought to be a Ca^{2+}-dependent lipid-binding domain (Clark *et al.*, 1991) that confers Ca^{2+}-dependence both on phorbol ester binding (C1 region) and on the kinase activity (C3/4 regions) of PKC (Ohno *et al.*, 1988; Ono *et al.*, 1989).

In the present study we have expressed in *Escherichia coli* and purified (> 95%) a 43 kDa fragment of the UNC-13 protein in order to characterize the phorbol ester binding site. Phorbol ester binding to this 43 kDa protein fragment is phospholipid-dependent, stereospecific and of high affinity. Zinc binds to this UNC-13 protein and is required for phorbol ester binding. In addition, the native *C. elegans* protein has been identified using UNC-13 specific antisera and an *unc-13* mutant. These results show that UNC-13 represents a novel class of phorbol ester receptor.

MATERIALS AND METHODS

Materials

[20(n)-^{3}H]Phorbol 12,13-dibutyrate (PDBu; 1.7 GBq/mg; 46.4 mCi/mg) and $^{65}ZnCl_2$ (37 MBq/mg; 2.5 Ci/mg) were from Amersham International (Amersham, Bucks., U.K.); phorbol esters [phorbol 12-myristate 13-acetate (PMA) and PDBu, α and β analogues] were from LC Services (Woburn, MA, U.S.A.) and Sigma (Poole, Dorset, U.K.). Phospholipids were from Sigma.

Subcloning and expression of *unc-13* cDNA

The T7 system was used for expression of the *unc-13* cDNA in *E. coli* (Studier & Moffat, 1986). To induce expression of the recombinant proteins, *E. coli* BL21 cells containing one of three plasmids (encoding different regions of the UNC-13 protein) or the control plasmid were grown until they reached an A_{500} of 0.5, isopropylthiogalactoside was added to 1 mM final concentration and cells were grown for a further 2–3 h. At this point cells were harvested, washed, concentrated 50–100-fold and frozen at

Abbreviations used: PDBu, phorbol 12,13-dibutyrate; PMA, phorbol 12-myristate 13-acetate; DTT, dithiothreitol; PKC, protein kinase C; DGK, diacylglycerol kinase; Sf9, *Spodoptera fruigiperda* cells; CRD, cysteine-rich domain; CRM, cysteine-rich motif; PMSF, phenylmethanesulphonyl fluoride.

‖ Present address: The Scripps Research Institute, 10666 North Torrey Pines Road, La Jolla, CA 92037, U.S.A.

§ To whom correspondence should be addressed.

−20 °C. The following fragments were subcloned into the pET vector series, directly or with oligonucleotide adaptors: *Bam*HI/*Hin*dIII fragment, residues 389–1287 (899aa; 100 kDa), *Bam*HI/*Eco*RI fragment, residues 389–1090 (702aa; 80 kDa); *Cla*I/*Sph*I fragment, residues 546–940 (395aa; 43 kDa). Proteins were used from both the soluble and insoluble fractions.

Purification and refolding of the UNC-13 43 kDa protein

Pellets were thawed quickly and sonicated using an MSE sonicator for 6 × 5 s at high setting and power on 3. The suspension was then spun in an Eppendorf centrifuge (14000 *g*) for 15 min and the pellet was washed ten times in Tris buffer [10 mM-Tris (pH 8.0)/1 M-NaCl] containing 0.1 % Triton X-100. This washing procedure purifies the inclusion body by removing loosely bound protein. The purified inclusion bodies were then solubilized in 8 M-urea/25 mM-Tris/HCl (pH 7.5)/1 mM-EDTA/1 mM-dithiothreitol (DTT) and then dialysed overnight with two changes against 4 M-urea/1 mM-Tris/acetate (pH 5.0)/1 mM-DTT and 20 mM-Tris/HCl (pH 7.5)/50 mM-NaCl/2 mM-$MgCl_2$/0.2 mM-DTT/0.1 mM-$ZnCl_2$ (Ahmed *et al.*, 1990).

Ligand binding

Phorbol ester binding and zinc blotting were carried out essentially as described previously (Ahmed *et al.*, 1991). [^{3}H]-PDBu was incubated with protein samples under appropriate conditions for 30 min at room temperature, followed by 30 min at 4 °C, and the protein was separated from the surrounding buffer by rapid filtration on Whatman GF/C filters. Phorbol ester binding in cell extracts was measured as follows. A 10 ml induced culture was centrifuged at 12000 *g* for 10 min and the cell pellet was resuspended in 100 μl of lysis buffer [20 mM-Tris/HCl (pH 7.4)/0.25 M-sucrose/2 mM-DTT/2 mM-phenylmethanesulphonyl fluoride (PMSF)/lysozyme (0.6 mg/ml)] and sonicated for 1 min. A 10 μl sample of lysed cell suspension was then diluted 20-fold into 50 mM-Tris/HCl, pH 7.4, and used in binding assays as described above. Phorbol ester binding to refolded protein was carried out in 25 mM-Tris/HCl (pH 7.5)/BSA (4 mg/ml). For zinc blotting, protein was run on SDS/PAGE (Laemmli, 1970), washed in 5 % β-mercaptoethanol and then in Western transfer buffer, and transferred to nitrocellulose on a Sartoblot. Filters were incubated in binding buffer [100 mM-Tris/HCl (pH 6.8)/50 mM-NaCl/0.5 mM-$MgCl_2$/0.5 mM-$CaCl_2$/5 mM-DTT] for 2 h at room temperature before probing with $^{65}ZnCl_2$ in binding buffer without DTT. After autoradiographs of the zinc blots had been made, filters were stained with Amido Black to quantify the amount of protein transferred. Quantification was by image analysis.

Immunological analysis

Antibodies were raised against the 43 kDa UNC-13 recombinant protein fragment (*Cla*I-*Sph*I restriction fragment; amino acid residues 546–940) essentially as described in Harlow & Lane (1988). The 43 kDa protein was purified by repeated washing of inclusion bodies as described above. The protein was then run on SDS/PAGE, visualized by staining with 0.3 M-$CuCl_2$ and excised. The gel slice was dried by lyophilization for 24 h, ground into a fine powder and mixed with a volume of water equal to the original volume. A 100 μg portion of antigen (> 95 % purity) in 150 μl was injected subcutaneously into rabbits after making an emulsion with 150 μl of complete Freund's adjuvant. Booster injections were given every 4 weeks with the same antigen in incomplete Freund's adjuvant. Blood was collected from the ear vein 10 days after the third booster injection.

C. elegans cell extracts were probed with anti-UNC-13 antiserum as follows. *C. elegans* from a 6 cm diameter culture plate were washed off with M9 buffer (20 mM-KH_2PO_4/40 mM-Na_2HPO_4/90 mM-NaCl/1 mM-$MgCl_2$) and suspended in 50 μl of 50 mM-Tris/HCl (pH 7.4)/2 mM-PMSF/0.1 % SDS/0.14 M-β-mercaptoethanol/1 mM-EDTA. The *C. elegans* suspension was sonicated for 1 min and then centrifuged at 14000 *g* for 10 min. Supernatant (10 μl) was mixed with an equal volume of sample buffer [10 mM-Tris/HCl (pH 6.8)/20 % glycerol/2 % β-mercaptoethanol/2 % SDS/Bromophenol Blue (1 mg/ml)], boiled for 3 min and separated on SDS/PAGE (10 % gels). The gel was either stained with Coomassie Blue or blotted on to nitrocellulose membrane (Towbin *et al.*, 1979) for immunostaining. Nitrocellulose membranes were incubated in non-fat milk followed by anti-UNC-13 antiserum and then by anti-rabbit IgG conjugated with alkaline phosphatase. For colour development the membrane was incubated with 0.3 mg of Nitro Blue Tetrazolium/ml and 0.17 mg of bromochloroindoyl phosphate/ml in alkaline phosphatase buffer [100 mM-Tris/HCl (pH 9.5)/100 mM-NaCl/5 mM-$MgCl_2$].

RESULTS AND DISCUSSION

Expression of UNC-13 protein fragments

E. coli extracts containing a T7/UNC-13 80 kDa protein fragment (amino acid residues 389–1090 containing the C1/C2 PKC-like regions) have been shown to bind phorbol ester in a Ca^{2+}-dependent manner (Maruyama & Brenner, 1991). However, the level of phorbol ester binding to extracts containing the 80 kDa protein was approx. 50-fold lower than that found with extracts containing T7/PKC fusion protein (Ono *et al.*, 1989), hampering the characterization of its phorbol ester binding site. A phospholipid-dependence of phorbol ester binding to UNC-13 was also not demonstrated in this study (Maruyama & Brenner, 1991). A possible reason for this is that, because of low expression in *E. coli*, there is limited availability of the 80 kDa protein and sufficient contaminating phospholipid is present in the cell extract to support phorbol ester binding. To increase the level of expression of the recombinant UNC-13 protein, two further constructs were made encoding proteins of 43 kDa and 100 kDa (residues 546–940 and 389–1287 respectively). The 43 kDa protein was greatly overexpressed and could be easily visualized by Coomassie Blue staining of SDS/polyacrylamide gels (Fig. 1*a*). Both 43 and 100 kDa recombinant proteins from cell extracts bound phorbol ester to approximately the same level as did the 80 kDa protein and in a Ca^{2+}-dependent manner (Fig. 1*b*), but still without a phospholipid requirement. The addition of phosphatidylserine to these extracts did not increase the level of phorbol ester binding (results not shown). Fractionation of the cell extracts into soluble and insoluble phases revealed that the majority of the 43 kDa protein was insoluble (as were the 80 and 100 kDa proteins). When inclusion bodies from the 43 kDa protein expressing *E. coli* were isolated and washed, essentially pure protein could be obtained in high yield (Fig. 2*a*). The purified 43 kDa protein was solubilized in urea and then refolded to investigate phorbol ester binding characteristics of UNC-13.

Characteristics of phorbol ester binding to the UNC-13 43 kDa protein fragment

Phosphatidylserine was found to stimulate phorbol ester binding to the refolded 43 kDa protein (Fig. 2*b*, row 1). The presence of phosphatidylcholine and phosphatidylethanolamine did not significantly affect binding. However, phosphatidylinositol did stimulate phorbol ester binding, but to a level lower than with PS (Fig. 2*b*, rows 2–5). The phorbol ester binding in the presence of phosphatidylserine was stereospecific, as β-analogues of PMA and PDBu were inhibitory while the α-

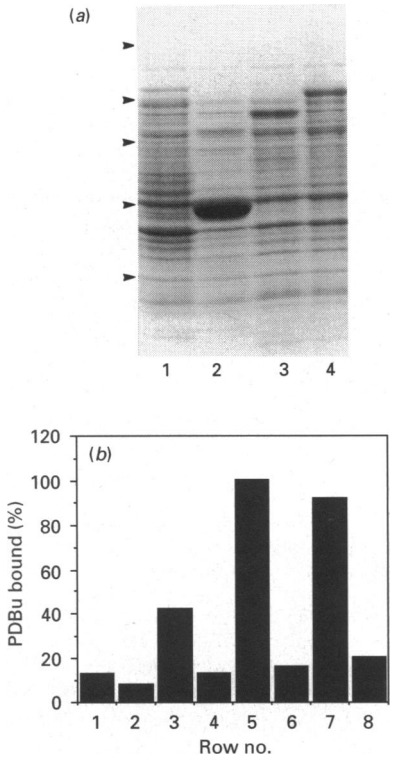

Fig. 1. Expression of and phorbol ester binding to UNC-13 proteins

(*a*) SDS/PAGE of total cell extracts from *E. coli* cells transformed with T7 expression vectors. Lane 1, empty vector; 2, 43 kDa protein; 3, 80 kDa protein; 4, 100 kDa protein. Molecular weight markers from the top (all in kDa): 200, 97.4, 68, 43 and 20. (*b*) Phorbol ester binding to cell extracts expressing empty vector peptide (1, 2) and UNC-13 proteins [43 (3, 4), 80 (5, 6) and 100 kDa (7, 8)] in the absence of (2, 4, 6 and 8) or presence of Ca^{2+} (1, 3, 5 and 7). 100% phorbol ester binding is equal to 3700 c.p.m./ml of total cell extract and the data represent means ± S.E.M. of 3–6 independent experiments using separately prepared samples. Data in rows 5 and 6 have been previously published (Maruyama & Brenner, 1991) and are shown here only for completeness.

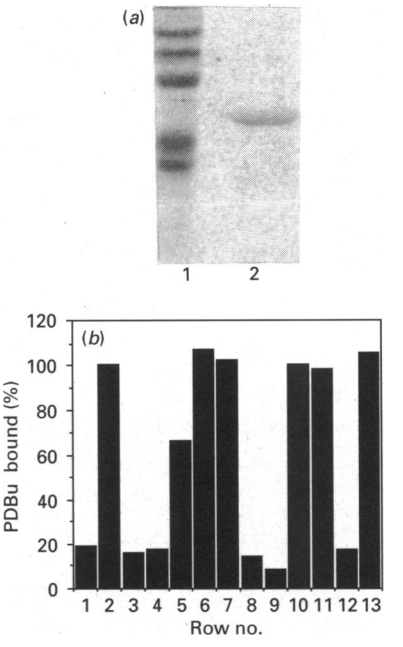

Fig. 2. Purification and characteristics of phorbol ester binding to refolded 43 kDa UNC-13 protein

(*a*) SDS/PAGE of molecular mass markers from the top (all in kDa): 84, 58, 48.5, 36.5 and 26.6 (lane 1) and purified 43 kDa UNC-13 protein (lane 2). Inclusion bodies were isolated and washed (× 10) in Tris/0.1% Triton X-100 buffer and the protein was refolded as described in the Materials and methods section. (*b*) Phorbol ester binding was measured as described in the Materials and methods section. Rows: 1, no lipid; 2, +phosphatidylserine (PS; 100 μg/ml); 3, +phosphatidylcholine (100 μg/ml); 4, +phosphatidylethanolamine (100 μg/ml); 5, +phosphatidylinositol (100 μg/ml); 6, PS+α-PDBu (1 μM); 7, PS+α-PMA (1 μM); 8, PS+β-PDBu (1 μM); 9, PS+β-PMA (1 μM); 10, PS+Ca^{2+} (2 mM); 11, PS+EGTA (2 mM); 12, +PS and refolded in Zn^{2+}-free buffer; 13, +PS and refolded in buffers containing Zn^{2+}. Experiments 12 and 13 were carried out in parallel. Data were obtained in triplicate and represent averages (S.D.s varied between 1 and 8% for the data presented). Two or three other experiments gave similar results. 100% binding was 10640 c.p.m./20 μl of protein. Background binding in the presence of 30 μM-PDBu has been subtracted from the data obtained.

analogues were not (Fig. 2*b*, rows 6–9). Interestingly, the Ca^{2+}-dependence of binding was not seen with refolded protein. Phorbol ester binding was not significantly affected by the presence of 2 mM-Ca^{2+} or EGTA (Fig. 2*b*, rows 10 and 11). Phorbol ester binding to refolded PKC-γ C1/C2–glutathione *S*-transferase fusion protein is also independent of Ca^{2+} (results not shown). These results may explain why Perin *et al.* (1990) did not detect $^{45}Ca^{2+}$ binding to the p65 protein on nitrocellulose blots, as under these conditions the protein would be refolded. The loss of the Ca^{2+}-dependence of the refolded proteins suggests that normally the C2 domain masks the phorbol ester binding site and that Ca^{2+} binding causes a conformational change allowing phorbol esters access to the C1 domain. When refolding of the protein fragment was carried out without addition of $ZnCl_2$ and in Chelex-treated buffers, phorbol ester binding was significantly reduced (Fig. 2*b*, compare rows 12 and 13).

The specificity of phorbol ester binding was investigated by measuring the amount bound as a function of its concentration (Fig. 3). Scatchard plot analysis revealed that the binding was consistent with the presence of a single class of receptor of high affinity (K_d 67 nM).

The characteristics of phorbol ester binding, including K_d values, of *E. coli*-expressed proteins shown above for UNC-13 resemble those seen for PKC and n-chimaerin (the K_d values of 67, 5 and 29 nM respectively; present study; Cazaubon *et al.*, 1990; Ahmed *et al.*, 1990). The PKC-γCRD expressed (in Sf9 cells) singly or in tandem has been found to have K_d values in the range 21–41 nM for phorbol ester binding (Burns & Bell, 1991). Phorbol ester binding to PKC and n-chimaerin has been found with a variety of fusion protein constructs, including T7, β-galactosidase, TrpE (anthranilate synthase), glutathione *S*-transferase and maltose-binding protein (Ono *et al.*, 1989; Ahmed *et al.*, 1990, 1991; S. Ahmed, unpublished work). These results suggest that the phorbol ester binding CRD folds autonomously and makes it likely that the full-length/native UNC-13 will also bind phorbol ester.

Zinc binding

We have previously shown that the CRD of n-chimaerin, PKC and DGK binds zinc using a $^{65}Zn^{2+}$-blotting technique (Ahmed *et al.*, 1991). Subsequently, Hendrickson's group (Hubbard *et al.*, 1991) showed by atomic absorption spectroscopy that recombinant PKC-β partially purified from Sf9 cells contains 4 mol of Zn^{2+}/mol of protein. To analyse further the zinc interaction with UNC-13 we probed the 43 kDa protein on nitrocellulose blots with $^{65}Zn^{2+}$ under conditions shown previously to detect specific binding (Ahmed *et al.*, 1991) (Fig. 4).

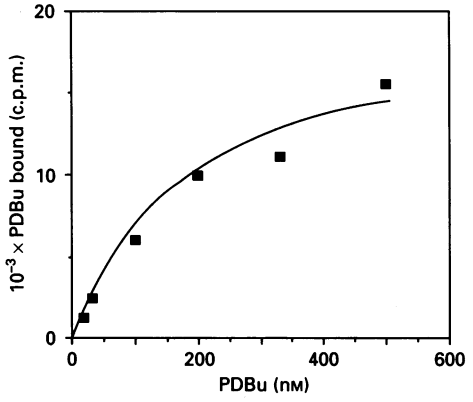

Fig. 3. Specificity of phorbol ester binding to the 43 kDa UNC-13 protein

Phorbol ester binding was measured as described in the Materials and methods section with increasing amounts of [^{3}H]PDBu at constant specific radioactivity. Each assay contained 5 μl of protein. Three other experiments gave similar results.

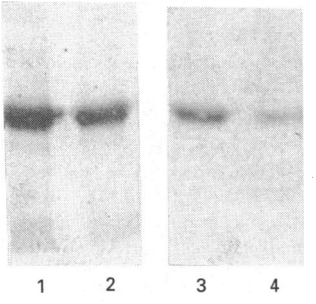

Fig. 4. $^{65}Zn^{2+}$ binding to the 43 kDa UNC-13 protein

Lanes 1 and 2, Amido Black-stained protein; 3 and 4, $^{65}Zn^{2+}$-probed proteins, both on nitrocellulose at two different protein concentrations (10 and 5 μg/lane respectively). Refolded 43 kDa protein was run on SDS/PAGE and transferred to nitrocellulose as described in Ahmed *et al.* (1991). The filters were probed with $^{65}Zn^{2+}$, autoradiographs made and then filters stained with Amido Black. Control proteins (enolase and glutathione *S*-transferase) did not bind zinc under similar conditions.

The 43 kDa UNC-13 protein clearly binds zinc. The zinc binding was competed effectively by micromolar Zn^{2+} but not by millimolar Ca^{2+} or Mg^{2+}, and was linearly related to protein concentration. In preliminary experiments, zinc measured by atomic absorption spectroscopy has been detected in refolded n-chimaerin, PKC, DGK and the 43 kDa UNC-13 protein (S. Ahmed & J. Lee, unpublished work). Extended X-ray fine structure analysis of PKC has not been able to establish the co-ordination ligands for the two zinc atoms present per motif. However, the results support a structure different from and possibly more complex than a 'zinc-finger' or 'zinc-cluster' in which the two histidine residues could play a role (Hubbard *et al.*, 1991). It should be possible to use ^{113}Cd-n.m.r. of refolded proteins to distinguish between the various models put forward for zinc co-ordination (Hubbard *et al.*, 1991).

Identification of the *unc-13* gene product

The *unc-13* cDNA was isolated by complementation of a *C. elegans* mutant. To further substantiate the existence of the *unc-13* gene product, antibodies to the purified 43 kDa protein fragment were raised (see the Materials and methods section for details). This anti-UNC-13 antiserum was specific, as it detected the 80 and 100 kDa recombinant proteins in *E. coli* cell extracts.

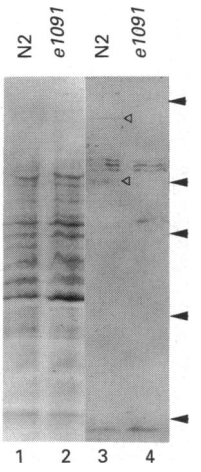

Fig. 5. Identification of the *unc-13* gene product by Western analysis

Protein extracts from wild-type (N2, lanes 1 and 3) and mutant (*e1091*, lanes 2 and 4) mixed population cultures were separated by SDS/PAGE on 10% gels as described in the Materials and methods section. Gels were processed either by staining with Coomassie Blue (lanes 1 and 2) or by transferring proteins to nitrocellulose membrane and then reacting with anti-UNC-13 serum (lanes 3 and 4). Specific immunoreactive UNC-13 bands at approx. 190 and 97 kDa are shown with open arrowheads. Molecular mass markers from the top (200, 97.4, 68, 43 and 29 kDa) are shown with solid arrowheads.

Fig. 5 shows SDS/PAGE and Western analysis (using the anti-UNC-13 serum) of *C. elegans* cell extracts from a mixed population of wild-type (N2) and a mutant (*e1091*). *unc-13 e1091* is a point mutation (G-to-A transition) in the Trp (TGG) codon that generates a stop (TAG) codon at amino acid 431 of the protein (Maruyama & Brenner, 1991) and prevents the synthesis of an UNC-13 protein containing the C1/C2-like regions (residues 609–846). The anti-UNC-13 serum detected at least seven protein bands ranging in size between 70 and 190 kDa from *C. elegans* cell extracts of N2. The protein bands at 97.4 kDa and 190 kDa are not detected by the preimmune serum and, most importantly, are absent from the *e1091* mutant (Fig. 5). These results strongly suggest that the 190 kDa protein represents the full-length *unc-13* gene product. The protein at 97.4 kDa could either be a proteolytic fragment of the 190 kDa protein or be an alternatively spliced *unc-13* gene product. Interestingly, the UNC-13-specific antiserum used in this study has detected a protein of approx. 90 kDa in a variety of mammalian cell lines including the neuroblastoma NE-108 (I. Maruyama, R. Kozma & S. Ahmed, unpublished work). This protein was not detected by the pre-immune serum. Further work with purified antisera and monoclonal antibodies will be necessary to establish the existence of mammalian homologues of the UNC-13 protein.

Consensus sequence for high-affinity phorbol ester binding

The sequence alignment of a stretch of 50 amino acid residues from PKC, n-chimaerin and UNC-13 shown in Fig. 6 reveals a consensus sequence for phorbol ester binding, with six cysteines, two histidines and six additional amino acids. All residues *N*-terminal and *C*-terminal to this 50-mer peptide are variant. The only change from the previously proposed consensus (Ahmed *et al.*, 1991), imposed by UNC-13, is the conservative valine-to-leucine substitution at position 47. The motif found in these proteins, $HX_{12}CX_2CX_{9-14}CX_2CX_4HX_2CX_{6/7}C$, and in DGK, the yeast PKC and the oncogene products RAF and VAV, is unique to these cysteine-rich proteins (Ahmed *et al.*, 1990, 1991). The physiological significance of the finding that the CRDs of PKC,

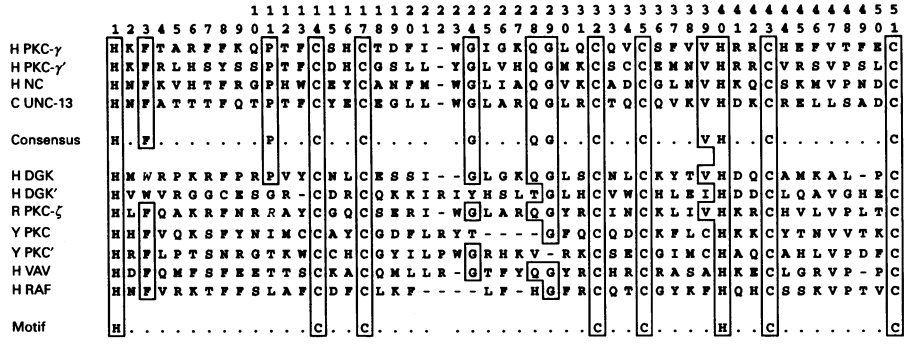

Fig. 6. Consensus sequence for phospholipid-dependent high-affinity phorbol ester binding

When the cysteine-rich regions of PKC, n-chimaerin (NC) and UNC-13 are aligned there is no sequence identity *N*-terminal of H (position 1) or *C*-terminal of C (position 51). This 50-mer polypeptide is referred to as the CRD. The CRDs of PKC (rat-γ), n-chimaerin and UNC-13 when expressed in *E. coli* bind phorbol ester with high affinity and in a phospholipid-dependent manner. These CRDs have been shown to bind zinc which is required for phorbol ester binding. The CRD of DGK (first CRD) and PKC-ζ have 92% (13/14 amino acid residues) sequence identity with the consensus but do not bind phorbol ester. The CRD of DGK binds zinc (PKC-ζ has not been tested for zinc binding). In proteins with two CRDs the second sequence is designated with a prime. The W residue at position 3 in the DGK CRD and the R residue at position 11 in PKC-ζ are shown in italic to emphasize the single difference between these CRDs and the phorbol ester binding consensus. Since the sequence identity of the CRD of vav, raf and yeast PKC with the consensus is 86% (12/14), 64% (9/14) and 71% (10/14) respectively, these proteins would be predicted not to bind phorbol ester with high affinity. H, human; Y, yeast; R, rat; C, *C. elegans*. The alignment shows invariant residues forming a consensus sequence. The motif highlights the potential zinc co-ordinating residues. Variant residues are indicated with –.

n-chimaerin and UNC-13 bind phorbol esters is that this domain then represents the target for the second messenger diacylglycerol. Thus the diacylglycerol signalling pathway may involve not only the PKC (α–η) family but also n-chimaerin, UNC-13, DGK, RAF and VAV. The presence of the CRD of the PKC family in the oncogene products RAF and VAV, and in DGK, albeit without 100% identity with the consensus sequence (Fig. 6) may indicate the presence of low-affinity phorbol ester binding sites in these proteins. It is therefore possible that phorbol esters influence the activity of these proteins. For example, phorbol esters do affect DGK activity when present in the micromolar range (Sakane *et al.*, 1990).

In conclusion, the data presented in this study show that UNC-13 is a specific phospholipid-dependent phorbol ester receptor. UNC-13 is distinct from the PKC family and n-chimaerin. A protein from HL-60 (human promyelocytic leukaemia) cells has been found to bind phorbol ester specifically and upon binding translocates to the nucleus. This protein did not have kinase activity (Hashimoto & Shudo, 1990). Thus the use of phorbol esters as agents to affect specifically (activate and down-regulate) only the PKC family and the mode of action of these agents must be re-evaluated.

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