Expression and characterization of maize ZBP14, a member of a new family of zinc-binding proteins

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A maize gene (Mz2-12), with a deduced amino acid sequence similar to that of a protein kinase C (PKC) inhibitor from bovine brain, has been expressed in *Escherichia coli* and the protein (ZBP14) purified to homogeneity. The bovine protein was originally identified by Walsh's group and named PKC inhibitor-1 (PKCI-1). The recombinant maize protein (ZBP14) shares characteristics of bovine PKCI-1: it has similar secondary structure, is dimeric, and has a similar affinity for zinc. However, the maize ZBP14 had very little activity as an inhibitor of mammalian brain PKC, thus precluding zinc sequestration as the mechanism of inhibition. The biological role for the maize protein in plant kinase regulation is therefore unclear. In the presence of both maize ZBP14 and 14-3-3 protein (which inhibits PKC in the absence of diacylglycerol), the effects on PKC appeared to be synergistic.

INTRODUCTION

One of the key enzymes in cellular signal transduction is protein kinase C (PKC). PKC has a central role in the control of many cellular processes; its activity is modified by activators such as diacylglycerol [1]. However, putative endogenous protein inhibitors may also be involved in the negative regulation of PKC activity.

Bovine PKCI-1 (PKC inhibitor-1) has been studied extensively by the group of M. P. Walsh [2]. Bovine PKCI-1 was originally purified as a heat-stable, calcium-binding protein [3]. Inhibition is not by phosphatase action [4], is PKC specific, and is independent of calcium concentration [5]. Other calcium-binding proteins have been shown to inhibit PKC, for example calmodulin [6] and members of the annexin family [7]. Bovine PKCI-1 is present in the brain at a sufficient concentration to inhibit all PKC activity, and has a distribution similar to PKC [5]. Structural analysis of bovine PKCI-1 has shown it to be a homodimer of 14 kDa subunits, consisting of 42% β-sheet and 23% α-helix (by circular dichroism) with a pI of 6.0 [2]. The bovine inhibitor has an IC_{50} of 2.2 μM [3]. *In situ* evidence of PKCI-1 inhibition of PKC has been produced by Rane and co-workers [8]. Bovine PKCI-1 is a zinc-binding protein, which exhibits specificity for zinc over other bivalent cations such as calcium or magnesium [2]. Mozier and co-workers [9] have identified the zinc-binding site (His-Val-His-Leu-His). This motif is different from any known zinc-binding protein sequences [9]. Since PKC is also a zinc-binding protein, sequestration of zinc could be a possible mechanism of inhibition. Western blot and ELISA analyses of tissue using polyclonal antibodies raised against bovine PKCI-1 have identified similar proteins in rat liver, chicken gizzard and human platelets [5].

Recently a maize gene (Mz2-12) has been cloned [10], the predicted amino acid sequence of which has high similarity (73% similarity and 53% identity) to bovine PKCI-1. In this study Mz2-12 has been expressed in *Escherichia coli* and the structure and function of the protein product has been analysed. For simplicity this protein product has been named ZBP14 because it is a zinc-binding protein with a molecular mass of 14 kDa.

Database searches have revealed that bovine PKCI-1 and maize ZBP14 belong to a new protein family the members of which have been identified in diverse eukaryotes and prokaryotes. These include the cyanobacterium *Synechococcus* strain AMC084, the nitrogen-fixing bacterium *Azospirillum brasilense*, *Mycoplasma hyorhinis*, *Oryza sativa* (rice), and *Homo sapiens* [11]. The sequences for this family all share regions which encode a conserved, novel zinc-binding site, which may provide a clue to a common functionality.

Since the bovine form has been proposed to be an inhibitor of PKC [3], inhibition by maize ZBP14 was investigated. Although no direct functional equivalent to PKC has been found in higher plants [12], plant homologues of the 14-3-3 family do inhibit mammalian PKC [13], and it is possible that maize ZBP14 may have similar inhibitory activity. The biophysical properties of this maize protein were also analysed.

MATERIALS AND METHODS

Materials

All reagents were of analytical grade, obtained from BDH, Sigma or Boehringer. Protein assay reagent was obtained from Bio-Rad. Oligonucleotides were synthesized by the Sequencing and Synthesis Service, N.I.M.R., using a Beckman Oligo 1000 DNA Synthesizer.

[α]^{35}S-labelled dATP (9.25 MBq/25 μl) was obtained from Amersham. X-ograph Blue X-ray film was obtained from X-graph Ltd. Western blot antigens were visualized with the Enhanced Chemiluminescence (ECL) detection system from Amersham.

pMz2-12 (pUC13 plasmid containing the maize gene Mz2-12) was a kind gift from G. G. Simpson (Scottish Crop Research Institute, Dundee, U.K.), who cloned the gene in the course of

Abbreviations used: ECL, enhanced chemiluminescence; ES-MS, electrospray MS; GST, glutathione S-transferase; NP-40, Nonidet P-40; PKC, protein kinase C; PKCI, protein kinase C inhibitor; ZBP14, zinc-binding protein with molecular mass of approx. 14 kDa; MARCKS, myristoylated alanine-rich C-kinase substrate; MARCKS peptide, 21-residue peptide consisting of the phosphorylatable domain of MARCKS.

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screening a lambda gt11 cDNA expression library of *Zea mays* (maize) A619 endosperm with a cDNA probe for the human 70 kDa protein of the U1 ribonucleoprotein particle [14]. Polyclonal antiserum raised against bovine PKCI-1 was kindly provided by M. P. Walsh, Calgary, Canada.

Nucleotide and amino acid sequences were analysed by the Genetics Computer Group package [15].

DNA manipulation

DNA manipulation and protein expression were carried out in *E. coli* TB1 (New England Biolabs). The expression vector was pGEX-2T plasmid (Pharmacia) which produces a glutathione S-transferase (GST) fusion protein expressed from a *tac* promoter. Cells were grown at 37 °C in L-broth or L-agar, in the presence of penicillin (100 μg/ml in L-broth and 300 μg/ml in L-agar), when necessary.

The Mz2-12 gene was amplified from pUC13 for insertion into pGEX-2T, using the PCR. Primers, 5′-GATC GGA TCC ATG TCG TCG GAG AAG GAG GCG (containing the start codon and *Bam*HI site) and 5′-CG GAA TTC TTA GCC TGG GGG CCA GTT (containing the stop codon and *Eco*RI site), were used. The restriction sites facilitated the subsequent insertion into pGEX-2T. The PCR reaction was as follows: 10 mM Tris/HCl, pH 8.5, 50 mM KCl, 2 mM $MgCl_2$, 0.1 mg/ml gelatin, 0.2 mM each dNTP, 100 pmol of each primer, 100 ng of pMz2-12, 1.25 units of *Taq* polymerase, which was added when the reaction was at 94 °C. Each cycle included denaturation for 1 min, annealing of DNA primers at 52 °C for 2 min and DNA extension at 72 °C for 1 min 45 s. The product was electrophoresed on a 1% TAE agarose gel [16], and DNA fragments were purified using the Gene-clean kit from Bio 101. After ligation into pGEX-2T, the PCR product was sequenced by the dideoxy chain termination method [17] using a Sequenase 2.0 kit supplied by USB.

Protein purification

E. coli containing the overexpressing plasmid were grown overnight in L-broth. Cultures were diluted 2-fold with fresh medium containing 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG). After a further 4 h of growth the bacteria were harvested, resuspended in 60 ml of PBS (magnesium- and calcium-free), and sonicated on ice at 18 μm amplitude for 6 × 30 s with 30 s rest intervals, using a MSE Soniprep 150 (9.5 mm probe).

The sonicate was centrifuged at 140500 *g*, 4 °C for 2 h; the supernatant was loaded at 2 ml/min on to a PBS-equilibrated glutathione–Sepharose matrix (10 ml, Pharmacia). The glutathione–Sepharose affinity support bound the GST–maize ZBP14 fusion protein. The matrix was removed from the column, was treated with 17.2 μl (80 units) of human thrombin (Sigma, cat. no. T3010), and was incubated overnight with continuous agitation. The thrombin cleaved the maize ZBP14 from the GST bound to the matrix. Released maize ZBP14 was separated from the matrix by centrifugation at 470 *g* at 4 °C for 5 min. The supernatant containing maize ZBP14 was treated with 5 mg of amidinophenylmethanesulphonyl fluoride (APMSF) (a soluble form of PMSF) to inhibit further thrombin activity. Sample was buffer-exchanged into 20 mM Tris/HCl, pH 7.5, using PD10 columns (Pharmacia).

Anion-exchange chromatography was used to concentrate the sample and as a final purification step. Sample was loaded on to a Mono Q 5/5 column (Pharmacia), pre-equilibrated in 20 mM Tris/HCl, pH 7.5, at a flow rate of 1 ml/min by FPLC. Protein was eluted with a 60 min linear gradient (0 to 0.6 M NaCl), and 1 ml fractions were collected. Position of elution and purity of maize ZBP14 was determined by analysis on SDS/15%-PAGE, electrophoresed under reducing conditions [18]. Protein concentration was estimated by using the Bio-Rad protein assay. Fractions containing maize ZBP14 were stored at −70 °C.

14-3-3 was isolated from sheep brain by a combination of anion-exchange and hydrophobic-interaction chromatography by the method described [19].

Western blotting

Supernatant from induced, sonicated *E. coli* expressing GST–maize ZBP14 fusion protein was run on SDS/15%-PAGE. This material was electro-eluted on to nitrocellulose using a semi-dry blotting apparatus (LKB 2117-250 Novablot). The SDS/PAGE gel was soaked for 20–30 min in 3-cyclohexylaminopropanesulphonic acid (CAPS, pH 11, 10 mM)/10% methanol transfer buffer. Transfer was carried out at 2 W per 100 cm^2 of gel for 1 h. The nitrocellulose was blocked using 1% (w/v) non-fat milk protein in Tris-buffered saline [20 mM Tris/HCl, pH 7.6, 150 mM NaCl, 0.1% Nonidet P-40 (NP-40)] overnight at 4 °C. The anti-(bovine PKCI-1) antibody was used at 0.7 mg/ml, the secondary antibody [peroxidase-coupled goat anti-(rabbit IgG), Bio-Rad] was used at a 1:5000 dilution. Non-specifically bound antibody was washed off with five 100 ml changes of Tris-buffered saline containing 0.1% NP-40 (v/v). Antigens were visualized with the ECL detection system (Amersham, U.K.) and briefly exposed to X-ograph Blue X-ray film.

Isoelectric focusing

Isoelectric focusing was carried out using a Multiphor II Isoelectric Focusing Unit (Pharmacia). Isogels (Flowgen Instruments Ltd.) are a kit form of prepoured isoelectric focusing gels. Between 2 and 5 μl of 2–10 mg/ml sample was loaded per track. Gels were prefocused at 1 W for 10 min and focused at 1000 V, 25 W for 40 min. Coomassie Blue staining was as detailed in the manufacturer's instructions.

Circular dichroism (CD)

Far-UV CD spectra were recorded from 260 to 195 nm using a Jasco J-600 spectropolarimeter operated with an instrument time constant of 0.5 s. The spectra were recorded at 22 °C for protein solutions (20 mM Tris/HCl, pH 7.5, 100 mM NaCl) at concentrations in the range 0.1 to 1.0 mg/ml in 1 or 2 mm fused silica cuvettes. The spectra shown represent the averages of at least four scans. All data are reported in terms of the molar CD absorption coefficient, ΔE, based upon an assumed mean residue weight of 111. The residue molar ellipticity, $[\theta]_{mrw}$, may be obtained from the relationship $[\theta]_{mrw} = \Delta E \times 3300$.

The dissociation constant for maize ZBP14 was calculated using a non-linear least-squares analysis which assumed formation of a 1:1 complex.

Size-exclusion chromatography

Pure maize ZBP14 samples were applied on to a size-exclusion column (Superdex 75 HR 10/30, Pharmacia), under the following conditions: (a) 6 nmol (75 μl, moles calculated for monomer) of pure maize ZBP14 was loaded on to the column equilibrated with 20 mM Tris/HCl, pH 7.5, 0.1 M NaCl; and (b) 4 nmol (50 μl, moles calculated for monomer) of pure maize ZBP14 pretreated with 1 mM zinc sulphate was loaded on to the column equilibrated with 20 mM Tris/HCl, pH 7.5, 0.1 M NaCl, 1 mM zinc sulphate.

The column was calibrated with the following protein standards: BSA, carbonic anhydrase and myoglobin.

Protein sequencing

Sequencing of the N-terminal 34 residues of purified maize ZBP14 was carried out by the Sequencing and Synthesis Service, N.I.M.R., on a 477A pulsed liquid-phase peptide/protein sequencer with an Applied Biosystems 120A on-line phenylthiohydantoin analyser. Data collection and analysis were performed with an Applied Biosystems 900A module calibrated with 25 pmol phenylthiohydantoin standards.

Electrospray MS

Electrospray MS (ES-MS) was carried out on a Fisons VG Platform instrument, at a needle voltage of 2.9 kV, extraction and focus lenses were set at −30 V and −37 V respectively. Nitrogen was used as the nebulizing gas at a flow rate of 15 l/h and as drying gas at 300 l/h. Purified maize ZBP14 (100 pmol) was introduced to the ES-MS source. On-line trapping [20,21], comprising a 0.75 mm PEEK column slurry packed with 8 μm × 300 Å PLRP-S packing material (Polymer Laboratories), was used to desalt the sample before introduction to the ES-MS source.

PKC assay

PKC purification and assay was as detailed previously [22]. PKC activity is defined as 1 nmol of phosphate incorporated into myristoylated alanine-rich C-kinase substrate (MARCKS) peptide in 1 min per ml of PKC at 30 °C, in the presence of saturating concentrations of phosphatidylserine. The assay was carried out in the presence and absence of phosphatidylserine, and expressed as phosphatidylserine-dependent kinase activity, in order to correct for PKM (the proteolysed, cofactor-independent form of PKC). Inhibition of PKC by 14-3-3 is carried out in the absence of diacylglycerol and phorbol ester.

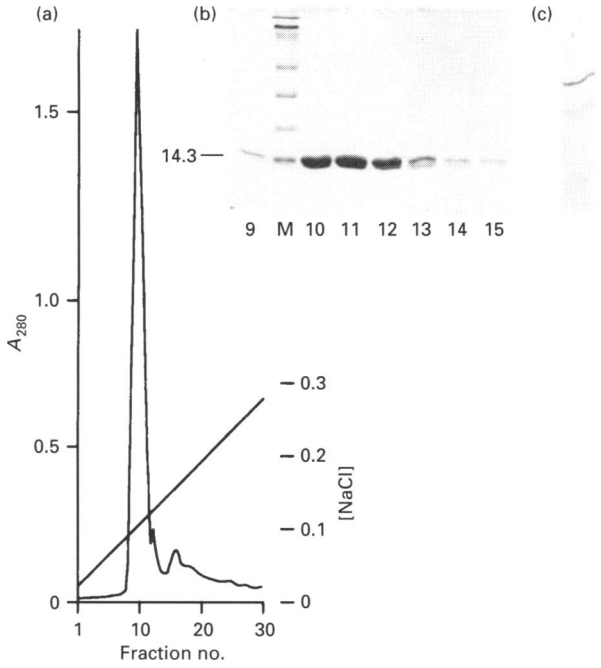

Figure 1 Purification of maize ZBP14

(**a**) Mono Q (Pharmacia) anion-exchange chromatogram of maize ZBP14. The diagonal line shows the NaCl concentration across the gradient. Due to the delay between the flow cell in the detector and the fraction collector, the peak of protein was collected in fractions 10 and 11. (**b**) Coomassie Brilliant Blue-stained SDS/15%-PAGE (under reducing conditions) of Mono Q fractions (numbered 9–15) and molecular-mass markers (M) ranging from 14.3 kDa to 200 kDa. (**c**) Western blot of supernatant from *E. coli* TB1 expressing the 43 kDa maize ZBP14–GST fusion protein, probed with bovine PKCI-1 antiserum.

RESULTS AND DISCUSSION

Expression and purification

The maize Mz2-12 gene was expressed in the plasmid pGEX-2T (Pharmacia) in *E. coli* as a fusion protein of GST covalently bound to maize ZBP14. The GST was cleaved by thrombin, leaving two additional amino acids at the N-terminus: glycine and serine.

Maize ZBP14 eluted as a single peak from Mono Q (Figure 1a), and apparent purity was confirmed by SDS/PAGE (Figure 1b). Peak fraction(s) were typically 2–3 mg/ml. An antiserum against mammalian protein specifically recognized the plant protein homologue, even in the form of the fusion protein, in the crude mixture of unfractionated *E. coli* cytosolic proteins (Figure 1c). This antiserum could therefore prove useful in identifying homologues, including those that have recently been proposed in other diverse species [10,11].

The theoretical pI of maize ZBP14 was 6.01; however, isoelectric focusing analysis (results not shown) indicated a pI of approx. 5.2. The difference between experimental and theoretical pI values is probably due to the influence of residues on the surface of the native dimeric molecule.

Zinc binding

Secondary structure was analysed by CD. The spectrum confirmed a secondary structure similar to that of bovine PKCI-1 (approx. 23 % α-helix; [2]), thereby suggesting correct folding of the recombinant maize protein. Interestingly, when zinc was added there was a large shift in the secondary structure towards formation of β-sheet at the expense of α-helix, which returned to the original conformation when EDTA was added to chelate the zinc (Figure 2a). This shift in conformation was also emulated by cadmium (data not shown). In order to estimate the affinity of maize ZBP14 for zinc, $ZnSO_4$ was added stepwise to the protein until no further shift in CD was detectable (Figure 2b). The results indicate that one Zn^{2+} is bound per monomer of maize ZBP14. A non-linear least-squares analysis, which assumed formation of a 1:1 complex, gave a value of K_d equal to 0.65 ± 0.25 μM. Although CD is not the ideal method for determining K_d values, this figure gives a good approximation (within 1–2 μM). These results compare with equilibrium gel penetration studies, which suggest that bovine PKCI-1 binds one zinc ion per monomer, with a K_d value of 4.3 μM [9].

The quaternary structure of maize ZBP14 was analysed by size-exclusion chromatography. The results indicated that the maize ZBP14, like the bovine form, is dimeric; this provides additional evidence that the recombinant maize protein is folded correctly. A shift to a later elution position was observed when the column was equilibrated with zinc (Figure 3), indicating that maize ZBP14 dimer exists in a more extended structure when zinc is absent.

Sequence analysis

The precise nucleotide sequence of the original maize Mz2-12 gene was unclear due to a severe compression approx. 90 bp from the ATG start codon. Direct automated sequencing of the purified maize recombinant ZBP14 protein showed the sequence in this region was $L^{30}AAVL^{34}$ and this was confirmed by ES-MS

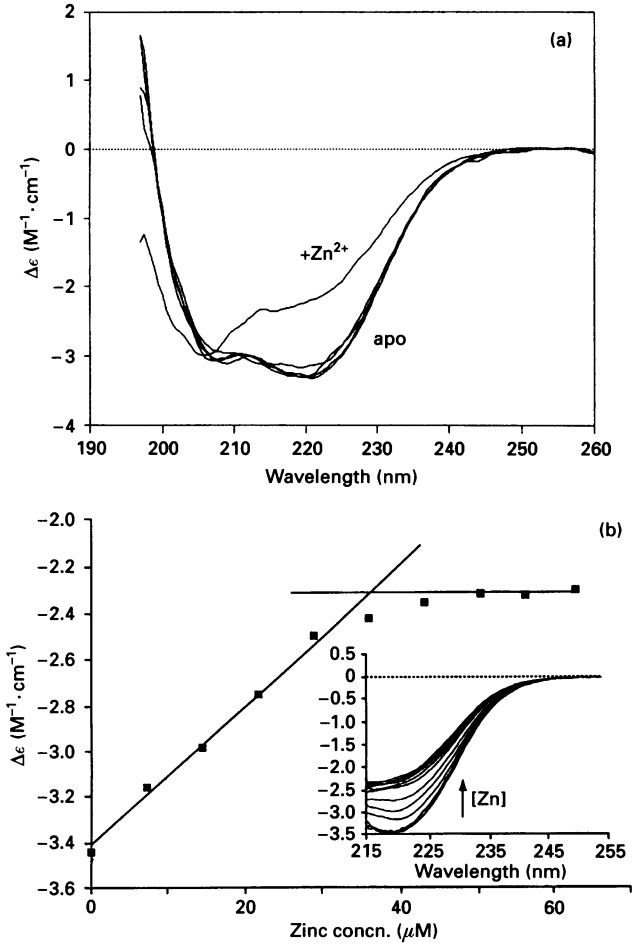

Figure 2 Circular dichroism of maize ZBP14

(**a**) CD spectra of two samples of recombinant maize ZBP14 (8.1 μM) in a 1 mm cuvette. Addition of 2 mM EDTA produced no change in the spectrum (labelled apo). To a fresh sample of 8.1 μM maize ZBP14, 1 mM zinc sulphate was added. This caused a shift in the spectrum (labelled $+Zn^{2+}$) which was consistent with the formation of β-sheet at the expense of α-helix. This was reversed by the chelation of the zinc on addition of 2 mM EDTA. The first three and the last analyses therefore gave the four superimposable spectra shown (apo). (**b**) Spectra of recombinant maize ZBP14 in the presence of a range of zinc concentrations were recorded over a limited range of 260–215 nm, in a 2 mm cuvette. Zinc sulphate was added stepwise, in 7.5 μM increments to 44.7 μM maize ZBP14 (monomer concentration), which was re-scanned at each step (insert). A saturation point was reached where increasing the zinc concentration further no longer caused a shift in the spectrum. Zinc concentration was plotted against average change in CD at 219, 219.5, 220 and 220.5 nm. Saturation point was at 1:1 in terms of the monomer protein concentration. A non-linear least-squares analysis, which assumed formation of a 1:1 complex, gave a value of K_d equal to 0.65 ± 0.25 μM.

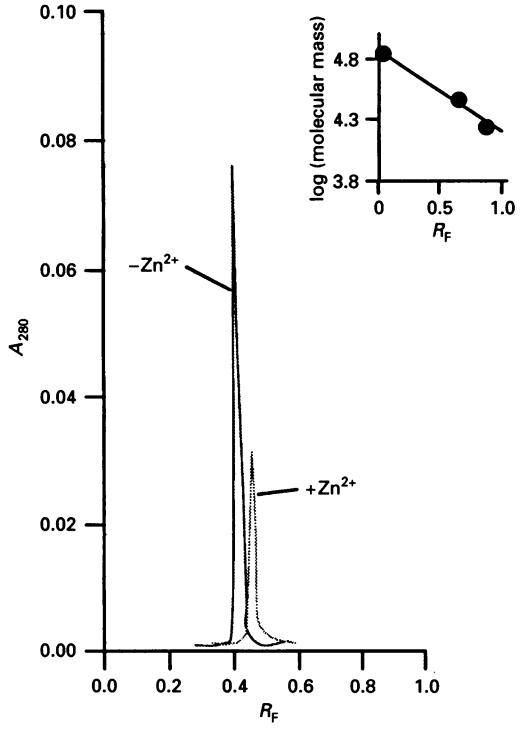

Figure 3 Size-exclusion chromatography of maize ZBP14

In separate analyses, maize ZBP14 protein was applied to a Superdex 75 HR column (Pharmacia) equilibrated in the presence ($+Zn^{2+}$) and absence ($-Zn^{2+}$) of 1 mM zinc sulphate. The presence of zinc sulphate caused maize ZBP14 to be eluted slightly later, suggesting a change in the dimer to a more globular shape. The insert shows the elution position of molecular-mass markers (BSA, 66 kDa; carbonic anhydrase, 29 kDa; myoglobin, 17.2 kDa).

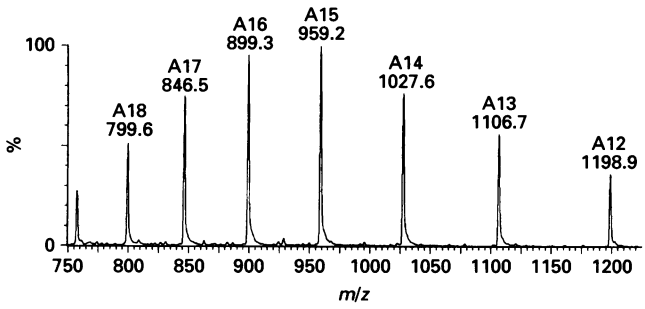

Figure 4 Electrospray mass spectrometry of maize ZBP14

ES-MS was carried out on a Fisons VG Platform instrument [21]. Purified maize ZBP14 (100 pmol) was introduced to the ES-MS source. Analysis of the data gave a molecular mass of 14373.34 ± 0.48 Da. This mass confirmed the deduced amino acid sequence (calculated to be 14373.44 Da) and that maize ZBP14 was a single molecular species. The recombinant protein (unlike that from bovine brain [2]) is not N-acetylated but is expressed with an additional Gly-Ser- sequence at the N-terminus. A12–A18 refers to the number of charges on the particular species. Each corresponding *m/z* value is also indicated.

(Figure 4). This also helped to assign the correct nucleotide sequence to the original Mz2-12 cDNA [10].

Maize ZBP14 inhibition of PKC

Maize ZBP14 inhibited PKC to a maximum of 20% (Figure 5). This analysis was carried out under a number of assay conditions and effects were similar with both a dispersion assay and a mixed micelle assay as described previously [22]. The conditions of the mixed micelle assay (data not shown) were the same as now used by the group of Walsh and co-workers for bovine PKCI-1 (M. P. Walsh, personal communication). The analysis was also carried out with various PKC substrates (equimolar MARCKS peptide, histone IIIS, histone H1, histone H2B and histone H3) and against both recombinant ϵ PKC and mixed brain PKC. However, maximum inhibition of 20% was obtained in each case (results not shown).

The bovine PKCI-1 is heat-treated during purification [3], and it may be that this prior heat treatment is required to potentiate

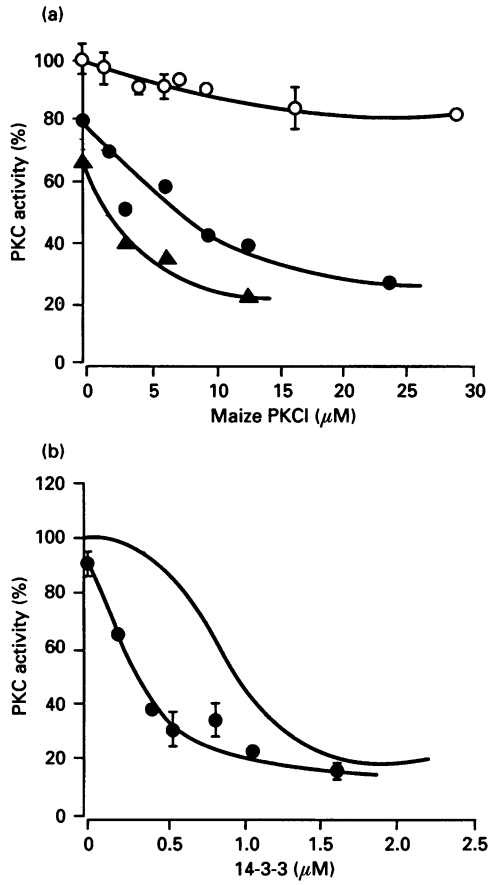

Figure 5 Maize ZBP14 inhibition of PKC

Mixed brain PKC was incubated in the dispersion assay, in the absence of diacylglycerol, with MARCKS peptide as substrate with: (**a**) a range of recombinant maize ZBP14 concentrations in the absence of 14-3-3 (○) and in the presence of sufficient 14-3-3 (see Figure 5b) to give 20% inhibition (●) or sufficient 14-3-3 to give 34% inhibition (▲). Half maximal inhibition by ZBP14 was obtained at a concentration of 6.6±3.4 μM; or (**b**) a range of 14-3-3 concentrations in the presence (●) and absence (smooth curve; from ref. [19]) of 6.6 μM maize ZBP14. Height of bars represents double the standard error at each concentration point.

inhibitory activity. However, after heating for 2 min at 80 °C, maize ZBP14 still failed to significantly inhibit PKC (results not shown). Maize ZBP14 may lack a second interaction site, present on the bovine form, which is required to totally block PKC activity.

PKC is also a zinc-binding protein [23], therefore PKC may be inhibited by sequestration of zinc ions. Like bovine PKCI-1, maize ZBP14 has an affinity for zinc in the micromolar range, although it is a poor inhibitor of PKC. However, the presence of 25-fold excess zinc to maize ZBP14 in the assay had no effect on inhibition (results not shown). This confirms the results of Mozier and co-workers [9] who found that a peptide of bovine PKCI-1 that contained the zinc-binding region failed to inhibit PKC.

When 14-3-3 protein, which inhibits PKC in the absence of diacylglycerol [19,22], and maize ZBP14 were assayed together for inhibition of PKC, their combined effect was greater than the sum of their individual inhibitory activities (Figure 5). The 14-3-3 inhibition curve shifted to the left in the presence of 6.6 μM maize ZBP14 (Figure 5b). One possible explanation for this phenomenon is that ZBP14 does interact with PKC (but at a site distinct to that of 14-3-3), causing a conformational change which allows 14-3-3 easier access to block the active site. The 14-3-3 protein family has been shown to interact with PKC at the diacylglycerol-binding zinc-finger domain [22]. Mammalian and yeast 14-3-3 isoforms have recently been shown to activate Raf protein kinase [24,25]. The principal region of Raf with which 14-3-3 isoforms interact contains the zinc-finger domain [24]. There is, however, no evidence that the zinc-binding properties of ZBP14 are somehow involved in enhancing the association of 14-3-3 with PKC. Maize ZBP14 protein has now been crystallized (B. Xiao, unpublished work) and tertiary structure analysis of these two proteins may be able to provide evidence for their contrasting and complementary mechanisms of PKC inhibition. Comparative modelling of the mammalian and plant homologues may indicate differences in potential PKC interaction site(s).

In summary, maize ZBP14 has been expressed and purified to homogeneity by affinity and anion-exchange chromatography. Biochemical analysis has shown that the recombinant protein is a zinc-binding protein with a native pI of 5.2, and a monomeric mass of 14373 Da by ES-MS, although the protein was a dimer on size-exclusion chromatography. These features, as well as the CD spectra, are similar to the bovine form; therefore the recombinant molecule appeared to be folded correctly. Maize ZBP14 has very little activity as a direct inhibitor of mammalian brain PKC. In the presence of both 14-3-3 and maize ZBP14, the effects on PKC appeared to be synergistic. The results from this study suggest that the physiological role of this new protein family may be connected to its zinc-binding function rather than PKC inhibition.

Note added in proof (received 10 February 1995)

A *Saccharomyces cerevisiae* open reading frame called HIT1 has been identified that is also a member of the ZBP14 family (called HIT family by Seraphin [26]). This sequence had shown up during a search of one of our databases but not in the others. It was inadvertently omitted in the line-up of sequences in [11] because of confusion with an unrelated yeast gene also called HIT1. Subsequent to the submission of our paper, a 17 residue fragment of an *E. coli* sequence with a high level of similarity to the N-terminus of this family(ies) has appeared in Swissprot database (ycff_ecoli). Our thanks to Professor B. Baum who drew our attention to these sequences.

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