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Promiscuous and specific phospholipid binding by domains in ZAC, a membrane-associated *Arabidopsis* protein with an ARF GAP zinc finger and a C2 domain

Rikke B. Jensen[1,+], Karin Lykke-Andersen[1,3,+], Gitte I. Frandsen[1], Henrik B. Nielsen[1], Jim Haseloff[2], Hans M. Jespersen[1], John Mundy[1] and Karen Skriver[1,*]

[1]*Institute of Molecular Biology, University of Copenhagen, Øster Farimagsgade 2A, 1353 Copenhagen K, Denmark (*author for correspondence; e-mail: cinacat@Biobase.dk);* [2]*Department of Plant Sciences, University of Cambridge, Downing Street, Cambridge CB2 3EA, UK;* [3]*present address: Department of Molecular, Cellular, and Developmental Biology, Yale University, 165 Prospect Street, New Haven, CT 06520-8104, USA.* [+]*These authors contributed equally to this paper.*

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Abstract

Arabidopsis proteins were predicted which share an 80 residue zinc finger domain known from ADP-ribosylation factor GTPase-activating proteins (ARF GAPs). One of these is a 37 kDa protein, designated ZAC, which has a novel domain structure in which the N-terminal ARF GAP domain and a C-terminal C2 domain are separated by a region without homology to other known proteins. Zac promoter/β-glucuronidase reporter assays revealed highest expression levels in flowering tissue, rosettes and roots. ZAC protein was immuno-detected mainly in association with membranes and fractionated with Golgi and plasma membrane marker proteins. ZAC membrane association was confirmed in assays by a fusion between ZAC and the green fluorescence protein and prompted an analysis of the *in vitro* phospholipid-binding ability of ZAC. Phospholipid dot-blot and liposome-binding assays indicated that fusion proteins containing the ZAC-C2 domain bind anionic phospholipids non-specifically, with some variance in Ca^{2+} and salt dependence. Similar assays demonstrated specific affinity of the ZAC N-terminal region (residues 1–174) for phosphatidylinositol 3-monophosphate (PI-3-P). Binding was dependent in part on an intact zinc finger motif, but proteins containing only the zinc finger domain (residues 1–105) did not bind PI-3-P. Recombinant ZAC possessed GTPase-activating activity on *Arabidopsis* ARF proteins. These data identify a novel PI-3-P-binding protein region and thereby provide evidence that this phosphoinositide is recognized as a signal in plants. A role for ZAC in the regulation of ARF-mediated vesicular transport in plants is discussed.

Abbreviations: ARF, ADP-ribosylation factor; cPLA2, cytosolic phospholipase A2; 1,2-DOG, 1,2-dioleoylglycerol; EST, expressed sequence tag; GAP, GTPase-activating protein; GEF, guanine-nucleotide exchange factor; GSI, 1,4-β-glucan synthase; GSII, 1,3-β-glucan synthase, GST, glutathione *S*-transferase; GUS, β-glucuronidase; MBP, maltose-binding protein; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PH, pleckstrin homology; PI, phosphatidylinositol; PI-3-P, phosphatidylinositol 3-monophosphate; PI-4-P, phosphatidylinositol 4-monophosphate; PI-3,4-P_2, phosphatidylinositol 3,4-bisphosphate; PI-4,5-P_2, phosphatidylinositol 4,5-bisphosphate; PI-3,4,5-P_3, phosphatidylinositol 3,4,5-triphosphate; PKC, protein kinase C; PLC, phosphoinositide-specific phospholipase C; PLD, phospholipase D; PS, phosphatidylserine; smGFP, soluble modified green fluorescent protein; ZFD, zinc finger domain

Introduction

Many of the components of plant signal transduction and cellular trafficking pathways are similar to those of yeast and animal systems. Conserved non-protein molecules include Ca^{2+} and the phosphoinositides phosphatidylinositol 3-monophosphate (PI-3-P), phosphatidylinositol 4-monophosphate (PI-4-P), phosphatidylinositol 3,4-bisphosphate (PI-3,4-P_2), phosphatidylinositol 4,5-bisphosphate (PI-4,5-P_2,) and phosphatidylinositol 3,5-bisphosphate (PI-3,5-P_2,) which are recognized by specific protein domains (reviewed by Munnik *et al.*, 1998; Meijer *et al.*, 1999). One such domain is the Ca^{2+}-binding C2 domain, also identified in plant proteins (Kopka *et al.*, 1998).

C2 domains are typically found in multidomain proteins involved in signal transduction or membrane trafficking (reviewed by Rizo and Südhof, 1998). Although they may bind different ligands, including other proteins, many bind negatively charged phospholipids in a Ca^{2+}-dependent manner. Structural determinations, first performed on a C2 domain from synaptotagmin I (Sutton *et al.*, 1995) and phosphoinositide-specific phospholipase C (PLC)-δ1 (Essen *et al.*, 1996), revealed a common fold comprising an eight-stranded, antiparallel β-sandwich. The Ca^{2+}-binding sites are formed by conserved residues present in interstrand loops at the phospholipid-binding end of the domain (Zhang *et al.*, 1998; Chapman and Davis, 1998). Whereas the interactions of the C2A synaptotagmin I domain with anionic phospholipids are mainly electrostatic and depend on intact Ca^{2+}-binding sites (Zhang *et al.*, 1998; Chapman and Davis, 1998; Davletov *et al.*, 1998), hydrophobic forces seem to dominate the interaction between cytosolic phospholipase A2 (cPLA2) and the zwitterionic target phospholipid phosphatidylcholine (PC; Davletov *et al.*, 1998; Nalefski and Falke, 1998). C2 domains are present in several predicted plant proteins and in plant PLCs and PLDs (Wang, 1997; Kopka *et al.*, 1998), but they have not been characterized in biochemical detail.

The small G proteins, ADP-ribosylation factors (ARFs), represent another group of plant proteins identified mainly by their homology to well-studied proteins from other systems (for references, see Munnik *et al.*, 1998). In other organisms ARFs are involved in the regulation of membrane trafficking and constitute together with coatomer proteins part of the coat of Golgi-derived COPI vesicles. In addition, they can stimulate phospholipase D (PLD; reviewed by Moss and Vaughan, 1998). ARFs are active in their GTP-bound form generated from the inactive GDP-bound form in a reaction catalysed by guanine-nucleotide exchange factors (GEFs), such as *Arabidopsis* GNOM ARF GEF (Steinmann *et al.*, 1999). Inactivation depends on catalysed hydrolysis of GTP to GDP mediated by GTPase-activating proteins (GAPs). GAPs share a ca. 80 residue zinc finger domain (ZFD). Structural integrity of this domain, including conservation of cysteines of the $CX_2CX_{16}CX_2C$ zinc coordination site, is critical for GTPase promoting activity (Cukierman *et al.*, 1995). Several ARF GAPs differing in domain structure, lipid dependence, protein interaction partners and subcellular localization have been described (Randazzo and Kahn, 1994; Antonny *et al.*, 1997; Brown *et al.*, 1998; Premont *et al.*, 1998; Andreev *et al.*, 1998). Recently, the crystal structure of ARF GAP domains from two different proteins, ARF1 GAP (Goldberg, 1999) and PAPβ (Mandiyan *et al.*, 1999), have been determined. The structures are similar for a highly conserved core region, but differ in flanking regions, also of importance for activity. Here we characterize a novel ARF GAP from *Arabidopsis* and show that the linker region between its ARF GAP and C2 domains binds PI-3-P, possibly linking this phosphoinositide to ARF-mediated vesicular transport.

Materials and methods

Database searches and sequence analyses

Zinc-finger-encoding clones were identified in the expressed sequence tag (EST) database by BLAST homology searches (Altschul *et al.*, 1990). Predicted protein sequences were examined for homology, sorting signals and membrane-spanning regions (Nakai and Kanehisa, 1992). DNA sequencing of both strands of all constructs was performed with fluorescent dideoxy dye terminators (Amersham) and analysed on an automated ABI Model 373A DNA sequencer (Perkin Elmer).

Reverse transcription and long-range PCR

Arabidopsis (Col-0) plants were grown and total RNA was isolated as described previously (Jensen *et al.*,

The nucleotide sequence data reported will appear in the GenBank and EBI Nucleotide Sequence Databases under the accession numbers AF177381, AF184144, AF184145 and AF184146.

1998). mRNA expression levels were analysed with an RT-PCR kit (Perkin Elmer Cetus, USA) and Zac specific upstream 5′-ATGAGTTATTCTGGAGCCGGA and downstream 5′-GTTGATTATTTATTGCTCAAG primers. A fragment of 1433 bp upstream of the *Zac* initiation codon (position 38068 on bacterial artificial chromosome F7J7) was amplified from Col-0 genomic DNA by long-range PCR (Mundy *et al.*, 1995) with an upstream *Bam*HI-linker primer, 5′-GACTGGATCCGCAATATCCGCTTTAC, and a downstream *Hin*dIII-linker primer, 5′-CATTAAGCTT CTAAAGAACTAGCAATCATAAACAG.

Plant transformation, histochemical analysis, and β-glucuronidase (GUS) activity assay

The 5′-upstream *Bam*HI/*Hin*dIII fragment containing the *Zac* gene promoter was ligated in those sites of pGEM-T GUS NosT which contain the *Escherichia coli* UidA reporter followed by the nopaline synthase terminator (Jefferson *et al.*, 1987). The resultant Zac-Gus-NosT was isolated by digestion with *Eco*RI/*Bam*HI, introduced into these sites in the binary pCambia 2300 vector (Cambia, Australia), and used to stably transform *Arabidopsis* Col-0 plants by vacuum infiltration (Bechtold *et al.*, 1993). GUS activity was assayed in plants by histochemical staining with 1 mM potassium ferri/ferrocyanide, and GUS activity in selected tissues was quantified with 4-methylumbelliferyl β-D-glucuronide substrate in a Wallac Victor II fluorometer at 0, 30, 60, 90 and 120 min (Jefferson *et al.*, 1987).

Construction of expression vectors and production of recombinant proteins

A *Zac* fragment corresponding to residues 1–174, containing the ZFD and linker region, was synthesized by PCR with an upstream *Bam*HI linker (5′-GAACGCGGATCCATGAGTTATTCTGGAGCC) and a downstream *Sal*I linker primer (5′-GAACGGTCGAC TTATTCAAGTTGCGGCT) and cloned into the pMal-c2 expression vector (New England Biolabs). Site-directed mutagenesis was performed with primers containing the desired mutation together with the *Bam*HI and *Sal*I linker primers. Recombinant maltose-binding protein (MBP) and MBP fusion proteins were purified by amylose-agarose affinity followed by anion exchange chromatography on a Q Sepharose column (Amersham Pharmacia Biotech). DNA fragments encoding full-length ZAC (residues 1–337), the ZFD (residues 1–105), the ZFD and linker region (residues 1–174) and the C2 domain (residues 148–337 and 167–337) were PCR-amplified by using upstream *Bam*HI-linker primers 5′-GAACGCGGATCCATGAGTTATTCTGGAGCC (GST-ZAC(1–337), GST-ZAC(1–174), GST-ZAC(1–105)), 5′-TAGAGGATCCGCATTTCTTTCATCGAGT CTA (GST-ZAC(148–337)) or 5′-ATGAGGATCCTCA TCGCAACAGCCGCAACTT (GST-ZAC(167–337)) and downstream *Sal*I-linker primer 5′-GGACGGTCG ACTTAAAGAGCTACCTTCAGGAATA (GST-ZAC (1–105)), *Eco*RI-linker primer 5′-GAACGGAATTCTT ATTCAAGTTGCGGCT (GST-ZAC(1–174)) or *Xho*I-linker primer 5′-CCGCTCGAGTTATTGCTCAAGAG GTAGCCA (GST-ZAC(1–337), (148–337) and (167–337)) and cloned in frame with the glutathione *S*-transferase (GST) gene in pGEX-4T-1 (Amersham Pharmacia Biotech). Recombinant GST fusion proteins were purified by glutathione affinity chromatography and cleaved with thrombin or purified further by anion exchange chromatography.

Immunodetection of ZAC in tissue extracts by western blotting

For generation of polyclonal rabbit antibodies, 1.5 mg freeze-dried GST-ZAC was resuspended in 0.3 ml Freund's complete adjuvant before injection. The antiserum was purified on GST-ZAC(1–337) bound to glutathione Sepharose 4B. *Arabidopsis* cellular fractions were prepared by homogenizing freshly harvested tissue in 0.25 M sucrose, 0.1 M tricine pH 7.9, 50 mM NaCl, 2 mM dithiothreitol, 5 mg/ml polyvinylpolypyrrolidone, 5 μg/ml antipain, 5 μg/ml leupeptin and 1 mM phenylmethylsulfonyl fluoride with or without EGTA and Ca^{2+}. The homogenate was stirred for 1 h and filtered through a 100 μm nylon membrane, and insoluble debris removed by centrifugation at 8000 $\times$ *g*. The supernatant was collected and microsomes pelleted by centrifugation at 264 000 $\times$ *g* for 75 min at 4 °C. Aliquots of 10 μg protein, determined in a bicinchoninic acid assay (Pierce), were separated by SDS-PAGE, transferred onto polyvinylidene difluoride membranes (Immobilon P; Millipore) and blocked with 1% w/v non-fat dry milk before incubation with anti-GST-ZAC. Swine anti-IgG horseradish peroxidase conjugate (DAKO) was used as the secondary antibody and reactions detected by chemiluminescence (Amersham Pharmacia Biotech).

Sucrose gradient fractionation

Fractionation of cellular membranes was performed on microsomes from 40 g *Arabidopsis* Col-0 green siliques. Microsomes were pelleted at 185 000 × *g* and resuspended in 1 ml 0.3 M sucrose ingradient buffer (25 mM MOPS-Bis-Tris-propane pH 7.2, 50 mM KCl). A 5 mg portion of protein was placed on top of a continuous (1.7–0.9 M) sucrose gradient, and the sucrose concentration of fractions collected from the bottom of the gradient measured by birefraction (Askerlund, 1997). The following enzymatic markers were assayed: cytochrome-*c* oxidase (mitochondrial inner membrane; Wigge and Gardestrom, 1987); 1,3-β-glucan synthase (plasma membrane) in a modified solution containing 50 mM HEPES-KOH pH 7.3, 0.2 mM $CaCl_2$, 2 mM cellobiose, 0.3 M sucrose, 0.015% w/v digitonin, 10 mM unlabelled UDP-glucose and 5 μCi ^{3}H-UDP-glucose (29 Ci/mmol, Amersham Pharmacia Biotech), and 1,4-β-glucan synthase (Golgi apparatus) in a modified solution containing 50 mM HEPES-KOH pH 7.3, 0.33 M sucrose, 10 mM $MgCl_2$, 0.5 μM unlabelled UDP-glucose and 5 μCi ^{3}H-UDP-glucose under conditions that may also detect 1,3-β-glucan synthase (Widell and Larsson, 1990). Fractions were also analysed by western blotting with antisera against tobacco BiP (endoplasmatic reticulum; Denecke *et al.*, 1991), and α-TIP (protein storage vacuole; Johnson *et al.*, 1989).

Confocal microscopy

The ZAC ORF was fused to the soluble modified green fluorescent protein (smGFP; accession number U70495) by strand overlap extension PCR with the overlap primers 5′-GCATGGATGAACTATACAAAATGAGTTATTCTGGAGCCG and 5′-CGGCTCCAGAATAACTCATTTTGTATAGTTCATCCATGC, and the smGFP upstream *Bam*HI-linker primer 5′-GGAAGAGGATCCAAGGAGATATAACAATGAG and the ZAC downstream *Sac*I-linker primer 5′-GGAAGAGAGCTCTTATTGCTCAAGAGGTAGCCAC and the fragment inserted into the *Bam*HI-*Sac*I sites of a pBI121-derived vector (Jefferson *et al.*, 1987). The plasmids were transformed into *Agrobacterium tumefaciens* strain LBA4044 by electroporation, and roots of *Arabidopsis* (ecotype C24) transformed as described by Valvekens *et al.* (1988). Transient expression of the smGFPs in roots was detected from day 3 to day 6, and transgenic material mounted in water under glass coverslips for microscopy. Images were acquired with a Leica TCS SP confocal scanning microscope equipped with laser and filter sets suitable for the detection of smGFP fluorescence.

Zinc blot assay

Protein blot zinc-binding assays were performed as described recently (Raventos *et al.*, 1998) with a change of the refolding buffer (to 150 mM NaCl, 1 mM $CaCl_2$, 2 mM dithiothreitol, 100 mM Tris-HCl pH 6.8) to reduce background binding.

Phospholipid dot-blot assay

Initial screening for phospholipid binding was performed by using a dot-blot assay. The indicated amounts in 5 μl of the lipids (from Avanti Polar Lipids, Sigma or Matreya) phosphatidic acid (PA), PC, phosphatidylethanolamine (PE), phosphatidylserine (PS), 1,2-dioleoylglycerol (1,2-DOG), phosphatidylinositol (PI), PI-3-P, PI-3,4-P_2, PI-4-P, PI-4,5-P_2, and phosphatidylinositol 3,4,5-trisphosphate (PI-3,4,5-P_3) were spotted onto nitrocellulose (NitroBind 0.45 μm, MSI). The membrane was dried, blocked, incubated with protein and washed as described by Stevenson *et al.* (1998) before incubation for 2 h at room temperature with polyclonal goat antibodies against GST (Amersham Pharmacia Biotech). Incubation with protein and the following steps were performed in TBST (10 mM Tris pH 8.0, 150 mM NaCl, 0.1% v/v Tween 20) with or without 200 μM $CaCl_2$ or 2 mM EGTA. Immunodetection was performed with goat anti-IgG horseradish peroxidase conjugate (Amersham Pharmacia Biotech) as secondary antibody.

Phospholipid liposome-binding measurements

Quantitative measurements of phospholipid binding were based on a frequently used strategy (Davletov and Südhof, 1993) with minor modifications. PC alone or mixed with the indicated molar fraction of another phospholipid and a trace amount of ^{3}H-labelled PC (1.0 mCi/ml, Amersham Pharmacia Biotech) was dried under nitrogen, resuspended in 50 mM HEPES-NaOH pH 7.4, 100 mM NaCl (buffer A) by vortexing and sonicated for 2 min. For each assay, 0.6 nmol protein (determined by amino acid analysis) was preincubated with 20 μl glutathione-Sepharose 4B or 30 μl amylose-agarose resin on ice before a 20 min incubation with shaking at room temperature with 20 μg phospholipid mixture in buffer A with 200 μM Ca^{2+} or 2 mM EGTA as indicated. After incubation beads were washed three times in 0.5 ml of the same buffer

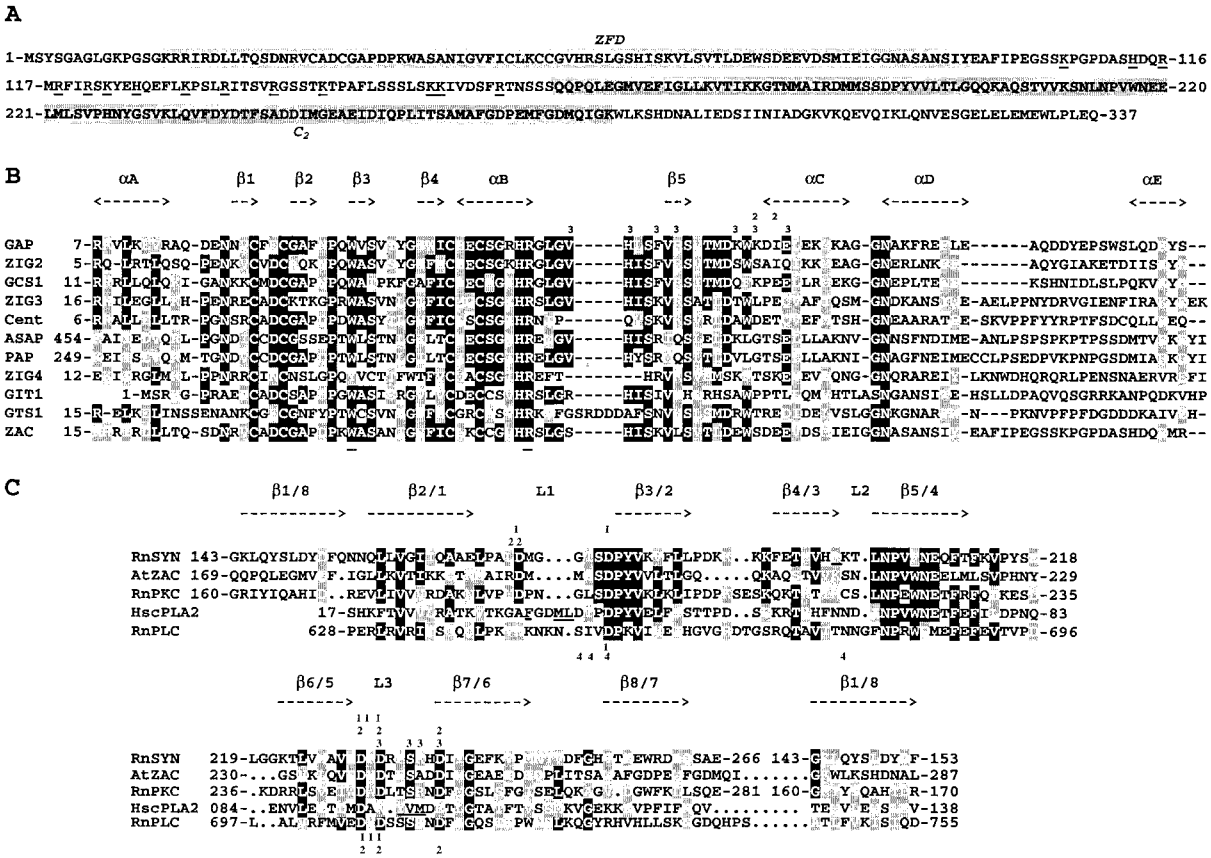

Figure 1. Sequence and domain structure of ZAC. A. Deduced amino acid sequence of ZAC. The approximate position of the zinc finger (ZFD) and C2 domains are in grey, and basic residues in the linker region are underlined. B. Alignment of the ZFD and C-terminal extension from eleven proteins. The positions of α-helices, β-strands and residues interacting with GDP-bound ARF1 are as shown for ARF1 GAP (Goldberg, 1999). The positions of residues in PAPβ suggested to be important for catalytic activity (Arg-292) or interaction with ARF (Trp-274) (Mandiyan *et al.*, 1999) are underlined. ZAC and ZIG2–4, predicted *Arabidopsis* proteins; GAP, rat ARF1 GAP (accession number U35776); GCS1 and GTS1, yeast regulatory Glo proteins (P35197 and P40956); Cent, rat α-centaurin (AAC52683); ASAP, mouse Src-associated ARF GAP protein (AAC98349); PAP, mouse Pyk2 target protein (PAPβ; 6730310); GIT1, rat G protein-coupled receptor kinase-associated ARF GAP (AAD28046). C. Alignment of the sequence of the ZAC-C2 domain with the sequence of four Ca^{2+}-binding C2 domains of known structure: RnSYN, first C2 domain in rat synaptotagmin I (Sutton *et al.*, 1995); RnPKC, rat protein kinase C-β (PDB ID code 1A25); HscPLA2, human cytosolic phospholipase A2 (PDB ID code 1BCI or 1RLW); RnPLC, rat phospholipase C-δ1 (Essen *et al.*, 1996). Approximate positions of β-strands and residues forming specific Ca^{2+}-binding sites (1–4) in PLC-δ1 and synaptotagmin I-C_2A are indicated above and below the alignment (Essen *et al.*, 1997; Rizo and Südhof, 1998). Residues suggested to be involved in the interactions of synaptotagmin I-C2A (Zhang *et al.*, 1998) and cPLA2 (Davletov *et al.*, 1998; Nalefski and Falke, 1998) with phospholipids are underlined. To facilitate sequence comparison, the sequences of β-strand 1 from synaptotagmin I and PKC-β have been repeated below β-strand 8 from PLC-δ1 and cPLA2. Residues conserved or chemically similar in 50% or more of the sequences are surrounded by black and grey, respectively.

and lipid binding quantified by liquid scintillation counting.

GTPase assay

Truncated soluble versions of *Arabidopsis* ARF1 (18–181; accession number P36397) and ARF3 (18–182; X77385) were used to test for GAP activity (Paris *et al.*, 1997; Goldberg, 1999). EST clones, obtained from the Arabidopsis Biological Resource Center, Ohio State University, were PCR-amplified with the *Nco*I-linker primer 5′-CGAGCCCCATGGGTATTCTGATGGTTGG and the *Bam*HI-linker primer 5′-TATTATGGATCCTTATGCC TTGCTTGCGATGTT (ARF1) or the *Nco*I-linker primer 5′-CGAGCCCCATGGGTATTCTGATGGTTG G and the *Bam*HI-linker primer 5′-CGAGCCGGATCC TTATTAGCCACTTCCCGACTTC (ARF3), and cloned into pET-30a (Novagen) to generate poly-His ARF recombinant proteins (His_6-ARF1Δ17 and His_6-ARF3Δ17). Purified His_6-ARFΔ17 (5 μg) was

immobilized per μl of HiTrap chelating beads (Amersham Pharmacia Biotech) and each 1 μl bead was equilibrated with 10 μl buffer consisting of 20 mM Tris-Cl pH 8.0 (Morinaga *et al.*, 1997) at 4 °C, 50 μM GTP containing [α-^{32}P]- or [γ-^{32}P]-labelled GTP as tracer (NEN Life Science Products), 1 mM $MgCl_2$, 150 mM KCl and 2.5 mM 2-mercaptoethanol. Nucleotide exchange was catalysed for 12 h at 4 °C by 1.2 μM Sec7 GEF domain produced in *E. coli* (Goldberg, 1998). The mixture was washed to remove unbound nucleotides before His_6-ARFΔ17 was eluted in 20 mM Tris-Cl, 300 mM NaCl, 5 mM $MgCl_2$, 2.5 mM 2-mercaptoethanol, 250 mM imidazole. The protein was diluted into 25 mM HEPES pH 7.4, 100 mM KCl, 1 mM dithiothreitol, 5 mM $MgCl_2$ to reduce the imidazole concentration. His_6-ARFΔ17 (20 μM) was incubated with 9 μM recombinant ZAC at room temperature for the indicated times in 30 μl of the dilution buffer with or without 0.2 mM $CaCl_2$. Reactions were stopped by dilution into ice-cold buffer and protein-bound nucleotides collected by filtering on nitrocellulose and counted (Goldberg, 1999).

Results

Identification and sequence characteristics of ZAC

Our interest in structure-function aspects of zinc-finger proteins encouraged a search in the *Arabidopsis* EST database for clones encoding specific zinc-finger domains (Jensen *et al.*, 1998). This revealed several ESTs encoding sequences with similarity to the ZFD of ARF GAP proteins. Corresponding cDNAs were obtained from the Arabidopsis Biological Resource Center and sequenced. One of the clones (B83TP) encodes a 37 kDa predicted protein we term ZAC (zinc- and calcium-binding; Figure 1A). Comparison of the protein encoded by the cDNA and that annotated for the gene (position 38068 on bacterial artificial chromosome F7J7) showed that the latter is incorrect due to faulty intron prediction. The N-terminal ZFD is followed by a region of ca. 70 amino acids with no clear sequence homology to known proteins, which is in turn followed by a C-terminal C2 domain.

The ZAC-ZFD shows significant similarity to ARF GAP proteins from animals and fungi. The sequences of *Arabidopsis* ESTs encoding proteins with similar domains were determined, and the three most divergent ones (ZIG2–4) included in a sequence comparison (Figure 1B). The sequences are similar in the ca. 80 residue ZFD corresponding to a structurally conserved core (Goldberg, 1999; Mandiyan *et al.*, 1999). Evidence is accumulating that proteins containing this core function as GAPs for ARFs (Randazzo and Kahn, 1994; Brown *et al.*, 1998; Premont *et al.*, 1998). Residues in the ARF GAP, PAPβ (Andreev *et al.*, 1999), suggested to be important for catalytic activity (Arg-292) or to interact with ARF (Trp-274; Mandiyan *et al.*, 1999), are conserved in ZAC, further supporting a GTPase-activating function. The pronounced sequence similarity between ZAC and ARF1 GAP does not completely span the 120 residue region needed for the GAP activity of ARF1 GAP (Cukierman *et al.*, 1995). In contrast, *Arabidopsis* ZIG2 shows distant global homology to ARF1 GAP and may be an orthologue. The proteins represented in Figure 1B are only related by their ZFD and have different domain patterns. Only ZAC contains a C2 domain and thus has a novel domain combination.

The ZAC-C2 domain was aligned with well-characterized C2 domains (Figure 1C; Rizo and Südhof, 1998). Two different topologies have been found for the strands in the C2-domain β-sandwich (Essen *et al.*, 1996) with the first β-strand of the synaptotagmin I C2A-like topology I occupying the same structural position as the eighth β-strand of the PLC-δ1-like topology II. However, since ZAC shows no obvious sequence similarity in the β7/β8 and the β8/β1 regions, it is not possible to assign a topology to ZAC-C2 based on sequence comparison alone. The position of the residues forming the three Ca^{2+}-binding sites (sites 1–3) in synaptotagmin I-C2A (Zhang *et al.*, 1998; Ubach *et al.*, 1998) are marked in Figure 1C, as are the residues likely to form three Ca^{2+}-binding sites (sites 1, 2 and 4) in PLC-δ (Essen *et al.*, 1997; Rizo and Südhof, 1998). The Ca^{2+}-chelating side-chains of sites 1–3, all important for binding anionic phospholipids by synaptotagmin I, but not of site 4, are conserved in ZAC. Positively charged residues, especially Arg-199, also contribute to the electrostatic interaction between synaptotagmin I-C2A and target phospholipids (Zhang *et al.*, 1998), and ZAC contains a lysine in the corresponding position. In contrast, the hydrophobicity of loops 1 and 3, mediating membrane association in cPLA2 (Davletov *et al.*, 1998; Nalefski and Falke, 1998), is not pronounced in ZAC. This suggests that ZAC-C2 will bind negatively charged phospholipids in a Ca^{2+}-concentration-dependent manner.

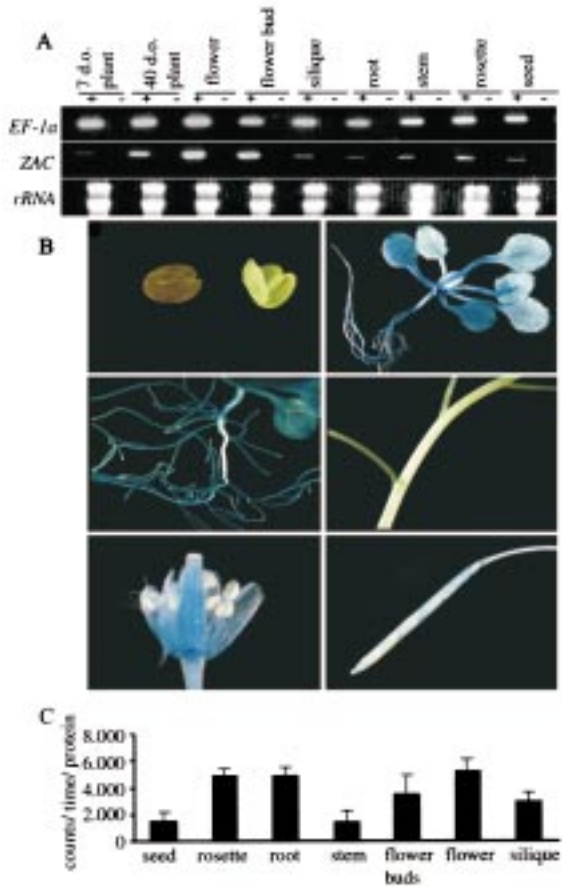

Figure 2. Accumulation of *Zac* mRNA and expression of the *Zac* gene. A. Reverse-transcription PCR analysis of the accumulation of *EF-1α* (top) and *Zac* (middle) mRNA performed in the presence (+) and absence (−) of reverse transcriptase. Total *Arabidopsis* RNAs used as template were isolated from the organs indicated. d.o., days old. 'rRNA' shows 10 μg of the RNA template sample stained with ethidium bromide. PCR products separated in a 2% agarose gel are shown. B. β-glucuronidase activity from the *Zac* promoter assayed histochemically in different tissues. Panels, from top left to bottom right, are: seed coat and embryo; whole plantlet; root; flowering stem; flower; silique. C. β-glucuronidase activity from the *Zac* promoter assayed *in vitro* with the fluorometric substrate 4-methylumbelliferyl β-D-glucuronidate.

Expression of the ZAC *gene*

The distribution of *Zac* mRNA was analysed by reverse-transcription PCR from total RNA representing the material used for generating the library containing the *Zac* EST (Figure 2A). A single 1.0 kb band, the size expected from the *Zac* cDNA, was obtained for all samples, with the strongest bands appearing in flower tissue. Amplification of contaminating genomic DNA can be excluded since the *Zac*-specific primers span the intron in the corresponding gene. Amplification of constitutively expressed EF-1α mRNA (accession number AAB07882) was used as control and resulted in stronger bands than for *Zac*. The results suggest that *Zac* is expressed at a low level in several different organs.

These patterns of *Zac* mRNA accumulation were confirmed by analysis of transgenic plants expressing the GUS reporter gene under control of the *Zac* promoter. Histochemical localization revealed very low levels in seeds and stem, higher levels in siliques and highest levels in rosette leaves, secondary roots and flowering tissues (Figure 2B). This was also reflected in the levels of GUS activity quantified in extracts from various tissues (Figure 2C).

Accumulation and membrane association of ZAC

Antibodies against recombinant GST-ZAC(1–337) fusion protein were used to examine ZAC accumulation in cell extracts and fractions. Single bands of the expected 37 kDa protein were immunodetected in a homogenate of *Arabidopsis* flowers and for recombinant ZAC (Figure 3A). A band of roughly the same intensity was also detected in a microsomal membrane fraction from flowers, whereas only a weak band was detected in the soluble fraction. A similar pattern was seen when anti-GST-ZAC(1–174) and anti-GST-ZAC(148–337) (Figure 4A) were used for detection. This confirms that the protein detected by the antibody is ZAC. Examination of ZAC tissue distribution detected the strongest bands in extracts and microsomal fractions of flower tissue, and weak bands in stem and seed samples. These results indicate that ZAC protein and mRNAs levels and tissue distribution are similar.

Since ZAC levels were low in supernatant fractions, most ZAC protein is either associated with membranes, or cytosolic ZAC is more exposed to degradation than membrane-associated ZAC. ZAC does not contain any hydrophobic, putative membrane-spanning regions, typical of intrinsic membrane proteins, or sequence signals for lipid membrane-anchor attachment. However, C2 domains can mediate association with membranes. We therefore tested whether ZAC association with membrane fractions was dependent on Ca^{2+} (Figure 3B) by ZAC immunodetection in soluble and membrane flower fractions prepared in the absence or presence of Ca^{2+} (kept by buffering; Davletov and Südhof, 1993). This indicated that ZAC fractionates with particulate material – both in the presence and absence of available Ca^{2+}. To examine the nature of the association of ZAC

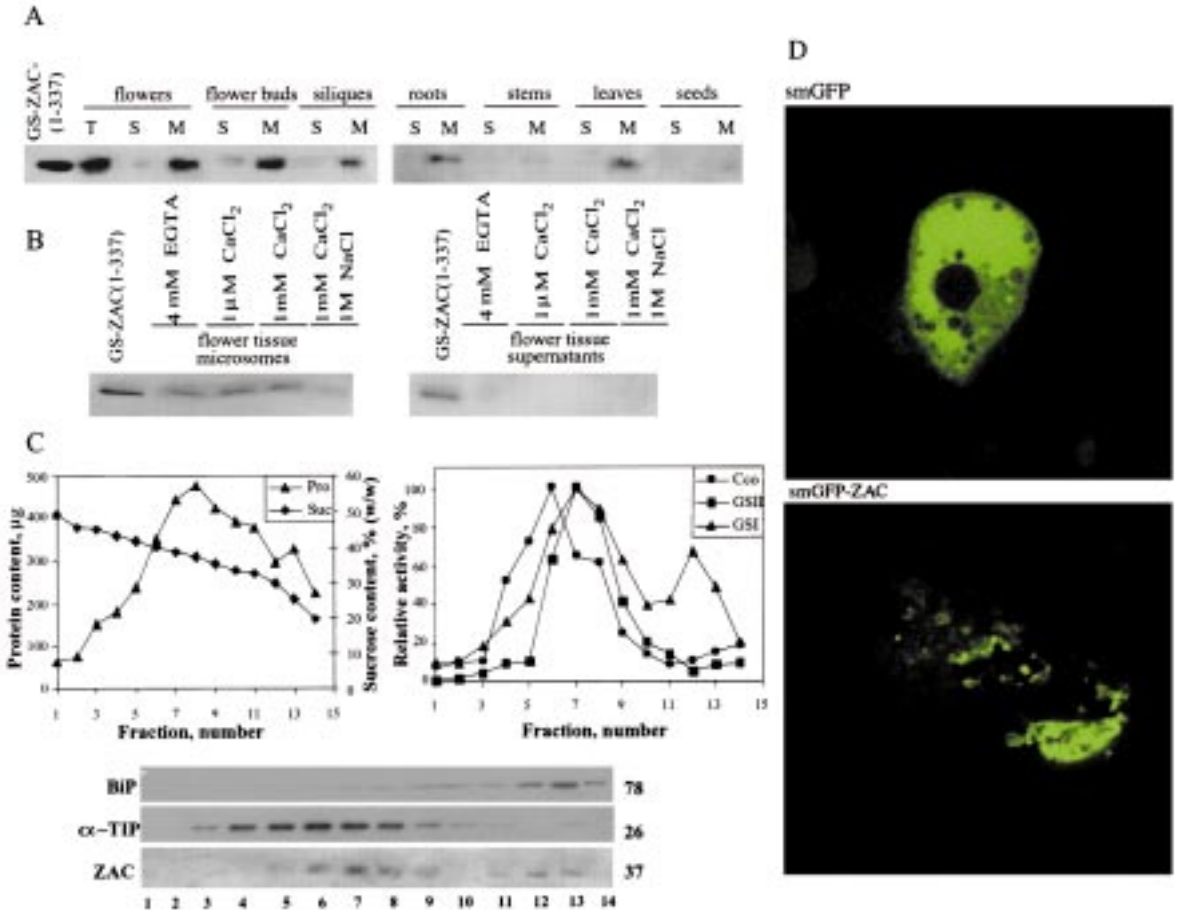

Figure 3. Subcellular localization of ZAC. A. Western blot analysis of 10 μg total (T), soluble (S), and microsomal (M) protein derived from *Arabidopsis* tissues probed with anti-ZAC antibodies. The electrophoretic mobility was compared to that of recombinant GS-ZAC(1–337) derived from GST-ZAC(1–337) by proteolytic cleavage. B. Western blot analysis of flower tissues probed with anti-ZAC antibodies. Protein of flower homogenates (10 μg) containing 4 mM EGTA, 1 μM Ca^{2+} (kept by buffering), 1 mM Ca^{2+}, or 1 mM Ca^{2+} and 1 M NaCl were separated into microsomal and cytosolic (supernatant) fractions by centrifugation. C. Continuous sucrose gradient separation of microsomal proteins extracted from green siliques. Upper left panel, percentage (w/w) of sucrose and protein levels; upper right panel, relative activity of the markers cytochrome *c* oxidase (Cco; mitochondrial inner membrane), 1,3-β-glucan synthase (GSII; plasma membrane), and 1,4-β-glucan synthase (GSI; Golgi apparatus); lower panel, western blot analysis with antisera against BiP (endoplasmatic reticulum), α-TIP (storage vacuole), and ZAC. A volume corresponding to 30 μl of each fraction was loaded on the gel. D. Confocal microscopy of callus tissue expressing smGFP or smGFP-ZAC(1–337). *Arabidopsis* roots transformed with the corresponding constructs using *Agrobacterium* were mounted on glass slides and images recorded by a confocal microscope. A representative section of a cell expressing smGFP or smGFP-ZAC(1–337) is shown.

with membranes, a flower homogenate was treated with 1 M NaCl which should abolish electrostatic interactions. In this case the intensity of the band corresponding to the microsomal sample was decreased in response to the treatment. Again, ZAC could not be immunodetected in the supernatant. Since this may be explained by degradation the results suggest that the interaction is sensitive to ionic strength.

The association of ZAC with membranes was further analysed in sucrose density gradient fractions of microsomes and compared to various marker proteins (Figure 3C). A single ZAC band, corresponding in size to full-length ZAC, was most strongly immunodetected in fractions 7 and 12. These fractions exhibited maximum activity for the plasma membrane marker GSII (fraction 7) and the Golgi marker GSI (fraction 12). Since these fractions do not represent those with the highest protein content, this indicates that ZAC is associated both with the plasma membrane and the Golgi apparatus.

The membrane association was further confirmed by *Agrobacterium*-mediated transformation of a fu-

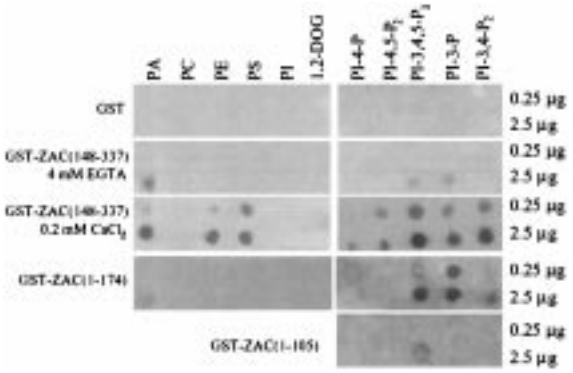

Figure 4. Phospholipid dot-blot assay with recombinant ZAC-proteins. The indicated amounts of the lipids PA, PC, PE, PS and 1,2-DOG and the phosphoinositides PI, PI-4-P, PI-4,5-P_2, PI-3,4,5-P_3, PI-3-P and PI-3,4-P_2 were spotted onto nitrocellulose. Blots were incubated with 2.0 μg/ml of the proteins in a buffer containing 2 mM EGTA or 0.2 mM $CaCl_2$ when indicated. Binding was monitored immunologically with a polyclonal antibody against GST. The blots are representative of two independent experiments showing the same binding characteristics.

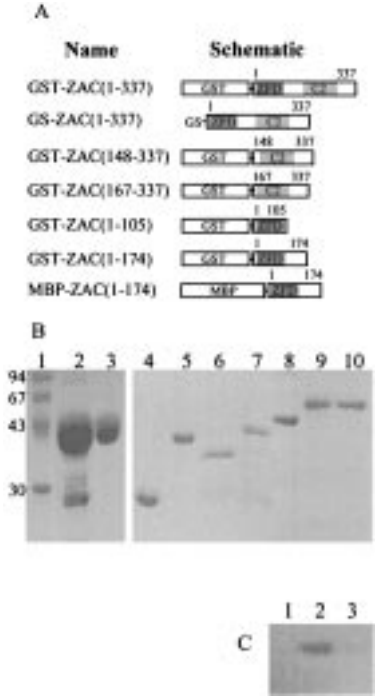

Figure 5. Recombinant ZAC proteins. A. Names and schematic of ZAC recombinant proteins. The zinc-finger (ZFD) and C2 (C2) domains are shown in grey. GS represents additional residues present in recombinant ZAC derived from GST-ZAC(1–337) by proteolytic cleavage. B. SDS-PAGE and Coomassie blue staining of recombinant proteins used for phospholipid-binding assays. Molecular mass standard (lane 1); GST-ZAC(148–337) eluted from glutathione Sepharose beads (lane 2); GST-ZAC(148–337) purified further by anion exchange chromatography (lane 3); GST (lane 4); GST-ZAC(167–337) (lane 5); GST-ZAC(1–105) (lane 6); GST-ZAC(1–174) (lane 7); MBP (lane 8); MBP-ZAC(1–174) (lane 9); MBP-ZAC(1–174)C33 → S (lane 10). C. $^{65}Zn^{2+}$ binding blot assay of MBP (lane 1); MBP-ZAC(1–174) (lane 2); and MBP-ZAC(1-174)C33 → S (lane 3), shown by Coomassie blue staining under B in lanes 8–10.

sion between *ZAC* and *smGFP* into *Arabidopsis* roots followed by visualization of the transient expression in callus tissue by confocal microscopy. Whereas smGFP always exibited a cytoplasmic localization with dark spots indicating exclusion from subcellular organelles (Figure 3D), optical sections showed that smGFP-ZAC had a punctuate localization pattern consistent with compartmentalization and/or membrane association of the protein (Figure 3D).

Recombinant ZAC fusion proteins

ZAC domain function was initially examined for two recombinant fusion proteins. The first, GST-ZAC(1–174), contains the ZFD and a C-terminal extension to the beginning of the C2 domain, since regions close to the ZFD may be essential for both interactions with lipids and for GAP activity (Antonny *et al.*, 1997; Brown *et al.*, 1998; Mandiyan *et al.*, 1999). The second, GST-ZAC(148–337), contains the C2 domain and an N-terminal extension to provide flexibility between GST and the C2 domain (Figure 4A). Shorter proteins containing just the ZFD (GST-ZAC(1–105)) or the C2 domain (GST-ZAC(167–337)), fused to GST, were also synthesized. The GST fusion proteins purified by glutathione affinity chromatography were contaminated with GST (Figure 4B) and were purified further by anion exchange chromatography. To use Zn^{2+} binding as a probe for folding of the ZFD, the ability of ZAC(1–174) and ZAC(1–174)C33 → S, containing a substitution of a putative Zn^{2+}-chelating residue, to bind Zn^{2+} was compared in a blot assay. Since GST bound $^{65}Zn^{2+}$ in the assay, MBP was used as a fusion partner for these experiments. In the assays, $^{65}Zn^{2+}$ bound to MBP-ZAC(1–174), but not to MBP nor to MBP-ZAC(1–174)C33 → S (Figure 4C). This shows that ZAC(1–174) is able to bind Zn^{2+} and therefore likely to fold correctly despite an N-terminal fusion partner.

Phospholipid binding by ZAC

Since both the C2 domain and the ZFD are putative lipid-binding domains, the ability of ZAC regions to bind specific lipids *in vitro* was examined. Initial screening was performed in a dot-blot assay by binding phospholipids to nitrocellulose, incubation

with specific proteins, and immunological detection of the interaction (Stevenson *et al.*, 1998). The assay included phospholipids known to be constituents of plant membranes (PC, PE, PA, and PS; Larsson *et al.*, 1990), known or expected to be involved in plant signal transduction or membrane trafficking (PA, PI-3-P, PI-4-P, PI-3,4-P_2, and PI-4,5-P_2; Munnik *et al.*, 1998) or differing in the number and position of negative charges on the phospholipid head group (PI, PI-3-P, PI-4-P, PI-4,5-P_2, PI-3,4-P_2, and PI-3,4,5-P_3), as well as the diacylglycerol lipid 1,2-DOG, previously shown to bind to and activate ARF1 GAP (Antonny *et al.*, 1997). No binding of GST, used as a control, to immobilized lipids was observed (Figure 5). In contrast, weak binding to some of the phospholipids, including PA, was observed when GST-ZAC(148–337) was used for the incubation performed in the presence of EDTA. The assay revealed a decrease in the amount of PA needed for detectable binding in the presence of 0.2 mM Ca^{2+}. Binding of PE, PS, PI-3-P, PI-4-P, PI-3,4-P, PI-4,5-P_2 and PI-3,4,5-P_3 was strong at the same Ca^{2+} concentration. These results show that the C2 domain in GST-ZAC(148–337) binds promiscuously to regions with immobilized anionic phospholipids in the presence of Ca^{2+}.

Weak binding of GST-ZAC(1–174), containing the ZFD, to PA was also observed, but for this fusion protein no binding to the membrane building blocks PE and PS was observed. However, the protein bound to 3-phosphoinositides, exhibiting strongest affinity for PI-3-P. GST-ZAC(1–105), which is C-terminally truncated compared to GST-ZAC(1–174) and contains just the ZFD, lacked this affinity (Figure 4A). Since this recombinant protein was apparently stable during synthesis and purification (Figure 4B), the changed binding affinity was more likely due to removal of binding determinants present in GST-ZAC(1–174) than to structural obstruction due to the truncation.

The ability of purified GST-ZAC(148–337) to bind phospholipids *in vitro* was further analysed in a standard assay for C2 domain interactions with membrane-building phospholipids (Davletov and Südhof, 1993). Liposomes consisting either entirely of PC or PC and 25% mol/mol of a different phospholipid were tested for their ability to bind to ZAC regions (Figure 6A). In the absence of free Ca^{2+}, GST-ZAC(148–337) showed significant binding of only PA- and PI-4,5-P_2-containing vesicles. In contrast, 0.2 mM Ca^{2+} significantly induced binding of all of the phospholipids tested, except PC and PI. GST-ZAC(167–337) showed the same pattern of binding of PC/PA liposomes, suggesting that regions within the C2 domain determine the characteristic binding of PA. Increasing the NaCl concentration in the incubation and washing buffers from 100 mM to 1.0 M reduced, by roughly 75%, binding of PC/PS liposomes to GST-ZAC(148–337). In contrast, binding of PA-containing vesicles was reduced much less by increased NaCl (Figure 6A). When the PA concentration in the PC-based liposomes was decreased to 7% mol/mol, binding in the presence of Ca^{2+}, but not EGTA, was still significant for both GST-ZAC(148–337) and GST-ZAC(167–337) (Figure 6B). Binding of liposomes consisting of the same molar percentage of the phosphoinositides PI, PI-3-P, PI-4-P, PI-4,5-P_2, PI-3,4-P_2 and PI-3,4,5-P_3, the lower limit for comparable, significant binding (Figure 6C), also indicated that ZAC-C2 binds anionic phospholipids promiscuously.

The ability of GST-ZAC(1–174) to bind specific phospholipids present at 4% mol/mol in PC-based liposomes was tested for phospholipids selected in the screening assay (Figure 7A). A low molar percentage of target phospholipid was used to decrease signals from unspecific binding, and because a specific phosphoinositide signal may be present at a low level in a natural membrane system. Again, binding of PI-3-P by GST-ZAC(1–174) was most significant, and PI-3-P was not bound by GST-ZAC(1–105). To examine if an intact zinc-chelating motif is necessary for the PI-3-P affinity, similar assays were performed with the MBP fusions MBP-ZAC(1–174) and MBP(1–174)C33 → S (Figure 7B). A pronounced decrease in binding was observed for the substituted recombinant protein, although binding was more efficient than when MBP alone was used as bait. This indicates that structural integrity of the ZFD and the C-terminal extension is important for the interaction.

GTPase activating activity of ZAC

Initial attempts to demonstrate a GTPase-activating activity for ZAC in assays using bovine ARF1 were not successful (data not shown). However, although the ARF sequences are highly conserved, the *Arabidopsis* sequences diverge in a regions proposed to interact with GAPs (data not shown; Goldberg, 1999) making the use of *Arabidopsis* ARFs appropriate. Two *Arabidopsis* proteins, previously named ARF1 (Regad *et al.*, 1993) and ARF3 (Lebas and Axelos, 1994), were selected among several predicted ARFs and ARF-like proteins (ARL) since they represent different branches of the protein family and are, based

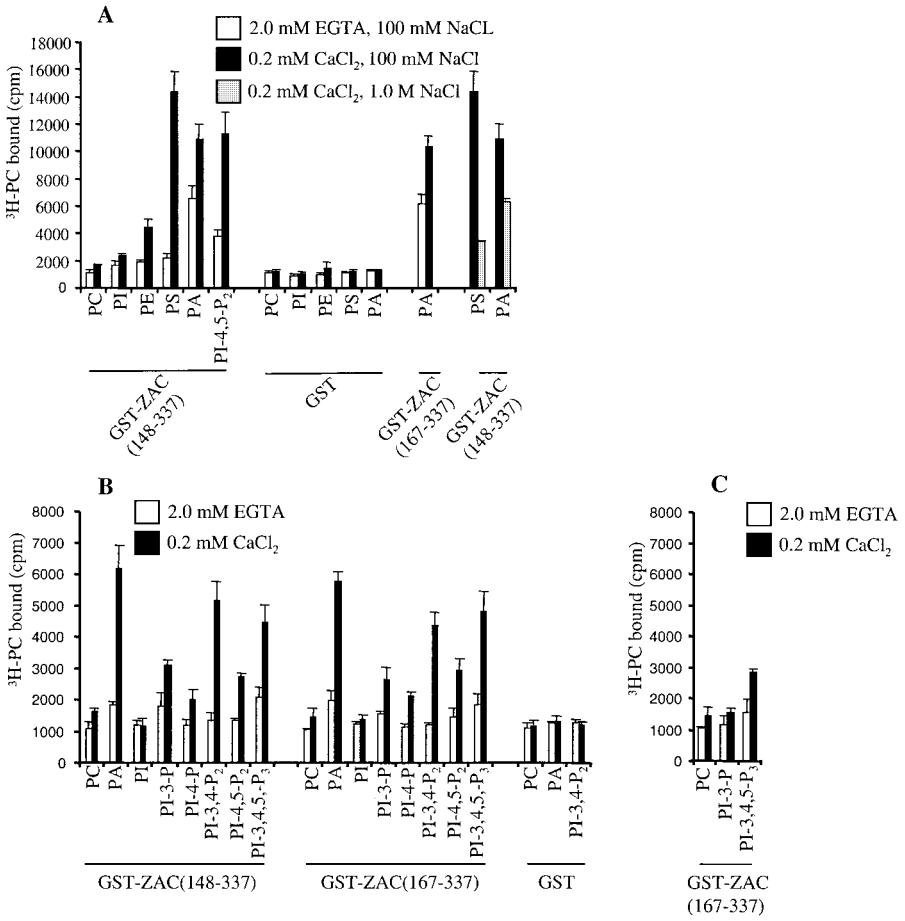

Figure 6. Binding of phospholipid vesicles by recombinant ZAC-C2 proteins. A. Binding of ^{3}H-PC-labelled vesicles consisting of PC or 75% mol/mol PC and 25% mol/mol PI, PE, PS, PA or PI-4,5-P_2 to GST-ZAC(148–337), GST-ZAC(167–337) or GST. Protein samples were incubated with ^{3}H-PC-labelled liposomes in the presence of either 2.0 mM EGTA, 0.2 mM $CaCl_2$ or 0.2 mM $CaCl_2$ and 1.0 M NaCl. B. Binding of GST-ZAC(148–337) and GST-ZAC(167–337) to ^{3}H-PC-labelled vesicles consisting of PC or 93% mol/mol PC and 7% mol/mol PA or one of the phosphoinositides PI, PI-3-P, PI-4-P, PI-3,4-P_2, PI-4,5-P_2 or PI-3,4,5-P_3. C. Binding of GST-ZAC(167–337) to ^{3}H-PC-labelled vesicles consisting of PC or 96% mol/mol PC and 4% mol/mol PI-3-P or PI-3,4,5-P_3. Bars indicate the standard errors of the mean from triplicate determinations.

on the number of representative ESTs, likely to be abundant.

No or low levels of recombinant full-length ARF1 and ARF3 were obtained from heterologous expression in *E. coli* (data not shown). Therefore, N-terminally truncated (residues 1–17 removed) versions fused to a poly histidine tag were produced (named His_6-ARF1Δ17 and His_6-ARF3Δ17; Figure 8A). A similarily truncated form of human ARF1 was soluble and active in GEF assays (Paris *et al.*, 1997) and used to measure GTPase-promoting activity for ARF1GAP in an assay exploiting the ability to immobilize the His_6-ARF fusion protein to a resin, thereby avoiding the membrane requirement (Goldberg, 1999).

Whereas the His_6-ARF1Δ17 and His_6-ARF3Δ17 fusion proteins have no detectable GTPase activity, incubation of His_6-ARF1Δ17 with both GST-ZAC(1–174) and GST-ZAC(1–337) induced the activity (Figure 8B). When 200 μM $CaCl_2$ was included in the buffer to ensure folding of the loop regions in the C2 domain, the effect of GST-ZAC(1–337) was increased. However, this assay does not allow the use of EGTA buffering to control the Ca^{2+} concentration. Therefore, further experiments are needed to determine if the increased catalytic activity of GST-ZAC(1–337) compared to GST-ZAC(1–174), and the increased effect of GST-ZAC(1–337) in the presence of $CaCl_2$, reflect an effect of the C2 domain. In addition, weak

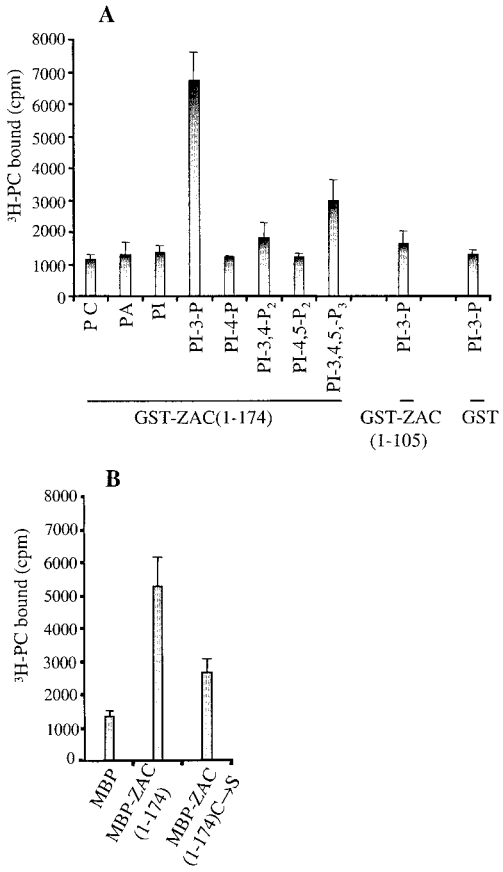

Figure 7. Binding of phospholipid vesicles by recombinant ZAC-ZFD. A. Binding of ^{3}H-PC-labelled vesicles to GST-ZAC(1–174), GST-ZAC(1–105) or GST. The liposomes consisted of PC or 96% mol/mol PC and 4% mol/mol of PA, PI, PI-3-P, PI-4-P, PI-3,4-P_2, PI-4,5-P_2 or PI-3,4,5-P_3. B. Binding of ^{3}H-PC-labelled vesicles consisting of 96% mol/mol PC and 4% mol/mol PI-3-P to MBP-ZAC(1–174), MBP-ZAC(1–174)C33 → S or MBP. Bars indicate the standard errors of the mean from triplicate determinations.

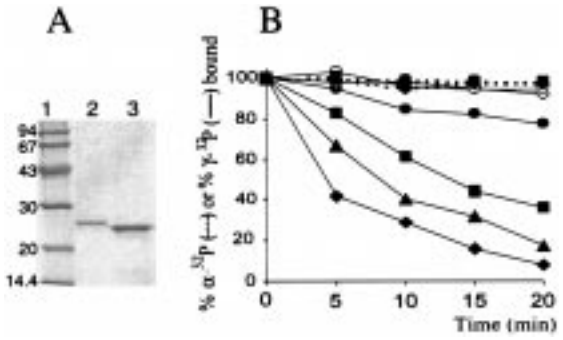

Figure 8. A. SDS-PAGE and Coomassie blue staining of recombinant *Arabidopsis*-derived His_6-ARF1Δ17 and His_6-ARF3Δ17 proteins used for ARF GAP assays. B. Stimulation of GTPase activity of His_6-ARF1Δ17 and His_6-ARF3Δ17 by GST-ZAC(1–174) or GST-ZAC(1–337) measured as the decrease in filter-bound [γ-^{32}P]GTP. 20 μM of [α-^{32}P]GTP- (as control of dissociation, broken line) or [γ-^{32}P]GTP (solid line)-bound His_6-ARF1Δ17 was incubated alone (white diamonds), with 9 μM GST-ZAC(1–174) (black squares) or with 9 μM GST-ZAC(1–337) without added $CaCl_2$ (black triangles) or with 200 μM $CaCl_2$ (black diamonds). 20 μM His_6-ARF3Δ17 was incubated alone (white circles) or with 9 μM GST-ZAC(1–174) (black circles).

GTPase activity was detected for His_6-ARF3Δ17 in the presence of GST-ZAC(1–337).

Discussion

Genomic and cDNA sequence data is a rich reservoir for gene discovery and functional characterization. We exploited this resource to predict a novel *Arabidopsis* protein, ZAC. Although the combination of ARF GAP and C2 domains in ZAC has not been reported previously, ZFD and C2 domains are found separately in proteins involved in trafficking and/or signal transduction. With the aim of determining putative ZAC function(s), we examined its patterns of expression and functional aspects of its separate domains. This showed that the *Zac* gene was widely expressed to the highest levels in flowering tissue, rosettes and roots.

ZAC protein displayed sedimentation behaviour consistent with an association with membranes. The complete and partial persistence of this association after treatment with EGTA or high salt suggested that ZAC is not only associated with membranes by a Ca^{2+}-dependent electrostatic interaction of the C2 domain. A dot-blot assay was initially used to screen for ZAC domain binding to different phospholipids, and binding was confirmed and quantified in a phopholipid liposome-binding assay. Although the results obtained in the two assays were generally in agreement, minor discrepancies may reflect the limitation of the qualitative blotting screen. Furthermore, since exposure times differed in the individual experiments of the dot-blot assay, comparison of absolute spot intensity is only valid within a single binding series. It was therefore relevant to use both assays.

The ZAC-C2 domain bound anionic phospholipids non-specifically in the presence of Ca^{2+}, as expected from sequence comparison (Figure 1C). Several plant proteins with a C2 domain have been predicted (Kopka *et al.*, 1998), making a biochemical characterization of an isolated C2 domain of plant origin relevant. Binding of PS containing PC vesicles showed half-saturation at a concentration of ca. 6 μM free Ca^{2+} (data not shown), a value similar to that obtained for

the C2A domain in synaptotagmin (4–6 μM; Davletov and Südhof, 1993).

Comparison of the binding preferences for PI, PI-3-P, PI-4-P, PI-3,4-P_2, PI-4,5-P_2 and PI-3,4,5-P_3 indicated dependence on charge density, as suggested previously for synaptotagmin I C2A (Zhang *et al.*, 1998). However, additional factors may be involved in binding. For example, a marginal preference of 3- over 4-phosphoinositides was observed in both assays. This may represent stereospecific binding, although it is unlikely to be of functional significance unless more pronounced *in vivo*. ZAC-C2 also shows significant interactions with PE and no binding of PC. PE and PC both lack net charge, but PC contains three methyl groups not present in PE, suggesting steric exclusion from binding. Furthermore, the interaction with PS-containing vesicles is more sensitive to ionic strength than the interaction with PA-containing vesicles (Figure 6A). The relative contribution of electrostatic and hydrophobic interactions may be different for the interactions with these two phospholipids. One possible explanation is that the C2 domain penetrates more deeply into membranes when interacting with PA than with PS due to less steric hindrance. Although ZAC loop region 1 (Figure 1C), which corresponds to a region important for hydrophobic interactions between cPLA2 and membranes (Davletov *et al.*, 1998; Nalefski and Falke, 1998), is short, it contains two methionines which could participate in interactions with membranes – possibly also in a Ca^{2+}-independent manner.

Recombinant proteins containing the ZAC-C2 domain showed Ca^{2+}-independent binding to PA, and GST fusions to the ZFD also showed some binding to PA (Figures 5 and 6A). Other plant lipid-binding domains, such as the plekstrin homology (PH) domain from *Arabidopsis* PI 4-kinase (Stevenson *et al.*, 1998), also binds PA. While this may be fortuitous, it may also reflect the importance of PA in plants. Our results suggest that ZAC is associated with the plasma membrane and the Golgi apparatus. While PA is a minor constituent of mammalian membranes, it is often third to PC and PE in abundance in plant plasma membranes and may be a true constituent of these membranes (Larsson *et al.*, 1990). However, PA can also originate from intensive PLD activity (Wang, 1997) and the coupled action of PLC and diacylglycerol kinase and may therefore function as a signal in a number of different plant processes (Munnik *et al.*, 1998). Since ARF can stimulate PLD activity a regulatory link between ARF/ZAC and PA is also possible.

The PI-3-P-specific binding to the ZFD-containing protein GST-ZAC(1–174) was confirmed in a liposome-binding assay containing a low molar percentage (4%) of phosphoinositide which was not sufficient to sustain PI-3-P binding by GST-ZAC(167–337) (Figure 6C). This suggests that the interaction is real and not just due to non-specific electrostatic interactions with negatively charged surfaces. GST-ZAC(1–105), representing a C-terminal truncation of GST-ZAC(1–174), did not interact with PI-3-P. This truncation removes a region with a high content of potentially positively charged residues (Figure 1A). Based on analogy to other phosphoinositide-binding domains, such as the PH and the FYVE zinc-finger domains, such residues would be essential for interactions with phospholipids (Fruman *et al.*, 1999). That structural integrity of the ZFD is also important for binding was shown by the decreased binding capacity of the mutated protein lacking one of the conserved cysteines likely to chelate Zn^{2+}. This may be explained by a direct contact between the ZFD and PI-3-P or by a role for Zn^{2+} in structural stabilization extending beyond the ZFD.

PI-3-P is known to be present in plants, but its roles remain elusive. In yeast and mammals, PI-3-P is produced through phosphorylation of PI by phosphoinositide 3-kinases. In plants, *in vitro* phosphoinositide 3-kinase activity has been demonstrated and corresponding enzymes cloned (Munnik *et al.*, 1998). The FYVE domain is known for its ability to bind PI-3-P specifically (Fruman *et al.*, 1999) and is also found in plant proteins (Gaullier *et al.*, 1998). Although the FYVE finger may be the predominant PI-3-P-binding protein domain, other less widely distributed PI-3-P targets may exist. In this study we have identified a novel PI-3-P-binding domain or region in a plant protein further supporting that PI-3-P functions as a signal which is recognized by specific protein domains in plants. Although the region needed for PI-3-P binding by ZAC does not show pronounced sequence similarity to known proteins it may still represent a structurally conserved phosphoinositide-binding motif.

ZAC functions as a GAP for two different *Arabidopsis* ARFs, representing evolutionarily distant ARFA/ARL proteins. Since the catalytic ability was determined using truncated ARFs as substrates, we have not as yet measured the effects of specific phospholipids, such as PI-3-P or PA, on ZAC activity in detail. It has recently been shown that enhancement of ARF GAP activity by a specific phosphoinositide can depend on an intact N-terminus of the target ARF

(Kam *et al.*, 2000). It is also possible that phospholipid binding enhances the catalytic activity of ARF GAPs by co-localizing them with myristoylated membrane-associated ARF at specific membranes (Antonny *et al.*, 1997).

Several potential *Arabidopsis* ARF GAPs and a range of ARFs/ARLs were identified by examination of the databases (Figure 1B, data not shown). That ARFs and their regulators play important roles in plants was recently demonstrated by studies of *Arabidopsis gnom* embryos, characterized by a defective ARF GEF. It was suggested that GNOM-dependent vesicular trafficking establishes cell polarity and, thereby, polar auxin transport (Steinmann *et al.*, 1999). Mammalian ARFs are mostly known from their involvement in the regulation of PLD and COP vesicular transport (Moss and Vaughan, 1998). Although the existence of COP vesicles in plants has not been conclusively demonstrated, homologues of COP-I components have been identified (Robinson *et al.*, 1998). Mammalian ARFs function at the Golgi apparatus or the plasma membrane, and the Golgi/endosome is the most likely localization of PI-3-P (Wurmser *et al.*, 1999), suggesting a possible physical/functional link between ZAC and ARFs. Based on structural criteria (Figure 1B), several *Arabidopsis* ARF regulators exist. However, the *in vivo* role and subcellular localization of these regulators remain to be established.

ZAC contains two putative membrane-targeting domains, and it is possible that its function requires a bimodal interaction with membranes, and hence an interplay between PI-3-P and another phospholipid. It is tempting to speculate that the ZAC-C2 domain provides interaction with membranes in general, whereas high-affinity binding to PI-3-P may direct binding to specific locations or mediate further tethering to membranes. Alternatively, the C2 could be an effector domain, which interacts with a different type of macromolecule (Rizo and Südhof, 1998) or with other domains in ZAC, which would explain the Ca^{2+}dependence of its activity. PAPβ, which contains an evolutionarily conserved PH-ARF GAP-ankyrin repeat core (Brown *et al.*, 1998; Andreev *et al.*, 1999), has an extensive interface between the ARF GAP domain and the first two ankyrin repeats (Mandiyan *et al.*, 1999). ARF1 GAP interacts through its non-catalytic C-terminal domain with the KDEL receptor at the target membrane (Aoe *et al.*, 1999). Therefore, based on analogy, a function for the C2 domain in ZAC different from or in addition to membrane binding, is likely. In any event, we have functionally characterized the domains of ZAC and shown that a domain or region binds PI-3-P with high specificity. Further study of ZAC and other plant proteins may reveal how PI-3-P functions in (ARF) vesicle trafficking in plants.

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