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Purification and partial sequence analysis of plant annexins

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A fractionation procedure for annexins involving Ca^{2+}-dependent binding to exogenous phospholipid was applied to tomato suspension culture cells. Two polypeptides (34 kDa and 35.5 kDa) were purified and separated from each other and from contaminant pectic polysaccharide by ion-exchange chromatography. After proteolytic digestion of SDS/PAGE-purified products, *N*-terminal sequencing of the peptide fragments revealed substantial similarity to sequences of known members of the annexin family characterized from a range of animal tissues. In particular, sequence similarity to the 70-amino acid-residue repeat region found in all annexins sequenced to date was present in both of the plant proteins. The data are discussed within the context of annexin involvement in Ca^{2+}-mediated events in higher plants.

INTRODUCTION

Annexins are a family of well-characterized proteins, isolated from animal cells, that share in common a Ca^{2+}-dependent affinity for acidic phospholipid (for reviews see refs. [1–3]). To date at least six distinct members of the family have been identified [4]. All contain a characteristic sequence of about 70 amino acid residues that is repeated four or eight times. It has been suggested that this structure arose from a single ancestral gene by a series of gene duplication events [5].

Despite detailed biochemical studies, the precise role of the annexin family of proteins is unknown, although their properties suggest that they play a fundamental role within the cell. For example, an effector or transducing function in cell signalling is implied by their phosphorylation by tyrosine-specific kinases [6–12] and protein kinase C [13,14]. Phosphorylation, which occurs within the *N*-terminal 30–40 amino acid residues [7], can modulate the affinity of the proteins for phospholipid [15–17] as well as affect their interactions with other cellular components [14]. Since some annexins have been shown to bind cytoskeletal proteins [18–20], a bridging role between the membrane and cytoskeleton has been proposed, and an involvement in exocytosis [21] and Ca^{2+}-dependent vesicle aggregation [22] has been demonstrated.

Ca^{2+} is known to modulate a wide range of metabolic and developmental events in higher plants (for reviews see refs. [23–29]). The central role of Ca^{2+} in signal transduction led us to examine whether annexins also exist in higher plants. In a preliminary analysis we identified two polypeptides in tomato suspension-culture cells that shared some of the features of animal annexins [30]. These included Ca^{2+}-dependent binding to liposomes containing acidic phospholipids and immunological cross-reactivity. In the present paper we describe a refined procedure for isolation of the proteins that allows both removal of contaminant cell-wall polysaccharide and the purification of the two separate polypeptides. Amino acid sequence analysis reveals substantial similarity to sequences of animal annexins. The results are discussed in the light of the Taylor–Geisow model for annexin structure [31] and recent reports in the plant literature where proteins with similar properties to those that we have purified have been identified in studies on cell signalling.

EXPERIMENTAL

Cell culture

Tomato (*Lycopersicon esculentum*, hybrid L2-14, Edinburgh) suspension-culture cells were grown in Murashige and Skoog medium (obtained from Flow Laboratories) supplemented with sucrose (25 g/l), thiamin (0.2 mg/l), indol-3-ylacetic acid (15 mg/l) and kinetin (0.5 mg/l) in 250 ml flasks at 25 °C in a Gallenkamp orbital incubator (100 rev./min). Cells were subcultured weekly.

Purification of annexins

The fractionation procedure described below was applied. Tomato suspension-culture cells were harvested by filtration 7 days after subculture. The cells were resuspended in buffer A (20 mM-Hepes/NaOH buffer, pH 6.8) containing 5 mM-EGTA at 4 °C, shaken for 5 min and collected by filtration. This wash procedure was repeated five times in buffer A containing 5 mM-EGTA, three times in buffer A with no additions and once in buffer A containing 1 mM-$CaCl_2$. The washed cells were quick-frozen in liquid N_2 and stored at −70 °C.

Washed cells were ground to a fine powder while frozen under liquid N_2 and allowed to thaw in buffer B (20 mM-Hepes/NaOH buffer, pH 7.4, containing 0.15 M-NaCl) (fresh weight/buffer ratio 1:3, w/v), and filtered through one layer of Miracloth. EGTA (0.2 M) was added to the filtrate to give a final concentration of 10 mM before centrifugation in a Sorvall SS34 rotor at 15000 rev./min (27000 $g_{av.}$) for 30 min at 4 °C. $CaCl_2$ (2 M) was added to the supernatant (S1) to give a final concentration of 15 mM. A fresh suspension of phospholipid was prepared by homogenization of bovine brain extract (type VII; Sigma Chemical Co.) in 1.8 ml of distilled water in a small Potter homogenizer. Then 0.2 ml of 10-fold-strength buffer B containing 50 mM-$CaCl_2$ was added. The suspension was mixed with the supernatant (S1) to give 2.5 mg of lipid/g wet wt. of starting material. After incubation on ice for 30 min and centrifugation in a Sorvall SS34 rotor at 18000 rev./min (39000 $g_{av.}$) for 30 min at 4 °C, the pellet was retained for further purification of the annexins. This involved two wash steps, firstly in buffer B containing 3 mM-$CaCl_2$ and secondly in buffer C (20 mM-Hepes/NaOH buffer, pH 6.95) also containing 3 mM-$CaCl_2$. The washed pellet was

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then resuspended in buffer C containing 15 mM-EGTA and incubated on ice for 15 min before centrifugation in a Beckman SW50 rotor at 35000 rev./min (100000 g_{av}) for 1.5 h at 4 °C.

The supernatant (S5) was applied directly on to an ion-exchange matrix (DEAE-Sephacel, 10 ml bed volume; flow rate 11 ml/h at 4 °C) pre-equilibrated in buffer C containing 1 mM-EGTA. Then 200 ml of a linear salt gradient (0–0.3 M-NaCl in buffer C containing 1 mM-EGTA) was applied, and the flow-though eluate was collected in 2.7 ml fractions. The fractions were assayed for protein by the method of Sedmark & Grossberg [32], with a 0–20 μg BSA standard curve, and for pectin [33]. A 0.5 ml portion of every alternate fraction was precipitated in ice-cold 10% (w/v) trichloroacetic acid together with 25 μg of DNA as 'carrier' for 30 min, and then centrifuged at 13000 rev./min (10000 g) for 10 min in a Microfuge. Pellets were washed three times in ice-cold acetone and resuspended in 25 μl of SDS/PAGE sample buffer [34], and 4 μl portions were applied to 0.5 mm 10% gels.

Pectin assay

A modified version of the Blumenkrantz & Absoe-Hanson [33] assay for uronic acids was used to detect pectin. Briefly, 200 μl of the solution (ice-cold) under test was added slowly to 1 ml of ice-cold 25 mM-$Na_2B_4O_7$ in conc. H_2SO_4 in Eppendorf tubes. The solutions were heated for 10 min in a boiling-water bath, then cooled, and 20 μl of fresh 0.15% (w/v) 3-phenyl-4,4′-biphenol was added to each. The assay mixture was allowed to develop at room temperature for 30 min and the absorbance was then read at 520 nm. All assays were performed in duplicate. A standard curve with galacturonic acid (5–80 μg/ml) was constructed on each occasion that pectin was quantified. The assay detects down to approx. 5 μg of galacturonic acid/ml (25 μM).

Peptide mapping

Annexins purified to the S5 stage of the isolation procedure were subjected to SDS/PAGE on 0.75 mm 7.5–10%-gradient gels. Bands were excised and subjected to enzymic digestion by *Staphylococcus aureus* V8 proteinase (300 ng/track) by the 'in-gel' method of Cleveland [35]. LKB Midget system gels (1.5 mm thick, 4.5% stacking gel, 15% separation gel) were used for peptide mapping.

Immunoblotting

The products of *S. aureus*-V8-proteinase from peptide-mapping gels were electrophoretically transferred on to nitrocellulose as described by Towbin *et al.* [36]. A sheep antiserum raised against *Torpedo* calelectrin has previously been shown to cross-react with the 35.5 kDa polypeptide found in tomato cells [30]. An IgG fraction from this antiserum (kindly given by Dr. J. H. Walker, University of Leeds) was used to detect immunoreactive peptides.

SDS/PAGE

SDS/PAGE was carried out according to the procedure of Laemmli [34] on LKB Midget system gels (column-fraction analysis, peptide mapping) or Bio-Rad Protean 2 system gels (separation of polypeptides for peptide mapping). Densitometric analysis of gels stained with 0.1% Coomassie Brilliant Blue in acetic acid/methanol/water (1:4:5, by vol.) and destained in propan-2-ol/acetic acid/water (5:4:31, by vol.) was performed on an LKB Ultroscan XL Laser densitometer.

Amino acid sequencing

Coomassie Brilliant Blue-stained peptides derived from *S. aureus*-V8-proteinase proteolysis were excised from the gel and electro-eluted, dialysed and freeze-dried as described by Bowles *et al.* [37]. Peptides were subjected to amino acid sequence analysis according to the procedures of Findlay *et al.* [38] in the micro-sequence facility built by the Protein Sequence Unit, Department of Biochemistry, University of Leeds.

Computer analysis of sequence

The partial protein sequences determined were used as probes to search the OWL database for proteins with similar sequences with the SWEEP program, part of the Leeds/Birkbeck ISIS (integrated sequences/integrated structures) package.

RESULTS

Purification

Two polypeptides of apparent molecular masses 35.5 kDa (P35.5) and 34 kDa (P34) on SDS/PAGE were purified from tomato suspension-culture cells by using a fractionation procedure adapted from one used for purification of annexins from animal tissues (see the Experimental section for details). Isolation of the polypeptides was based on extraction from a cell homogenate in EGTA followed by Ca^{2+}-dependent precipitation with exogenous phospholipid. Fig. 1 illustrates SDS/PAGE analysis of stages in the purification.

A typical purification yielded 0.5 mg of protein in the final supernatant (S5) from 50 g wet wt. of starting material. This represents approx. 0.6% of the total protein detected in the filtered homogenate. Densitometric scanning of Coomassie Brilliant Blue-stained gels demonstrated that 68% of the protein present in supernatant S5 was located in the P34 and P35.5 bands (44% and 24% respectively).

A problem in the isolation procedure was the co-purification of pectin, which was also extracted by EGTA and precipitated in the presence of Ca^{2+}. Tomato suspension-culture cells secrete large quantities of pectin most of which appears to be loosely attached to cells in a Ca^{2+}-dependent manner. Washing of freshly harvested cells in buffer containing EGTA displaced much of the polysaccharide before entry into the purification schedule. Filtration of the homogenate before addition of EGTA removed most of the remainder. The residue that co-purified during the fractionation was separated from P35.5 and P34 by using ion-exchange chromatography, as illustrated in Fig. 2. This step also partially separated the two polypeptides.

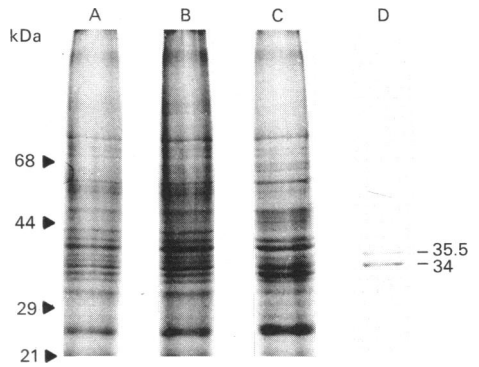

Fig. 1. SDS/PAGE analysis of stages in the fractionation procedure

Lane A, homogenate (H); lane B, supernatant from first centrifugation in the presence of EGTA (S1); lane C, supernatant after selective precipitation of P35.5 and P34 from supernatant S1 with Ca^{2+} and exogenous phospholipid (S2); lane D, final supernatant, EGTA extract of washed phospholipid pellet containing P35.5 and P34 (S5).

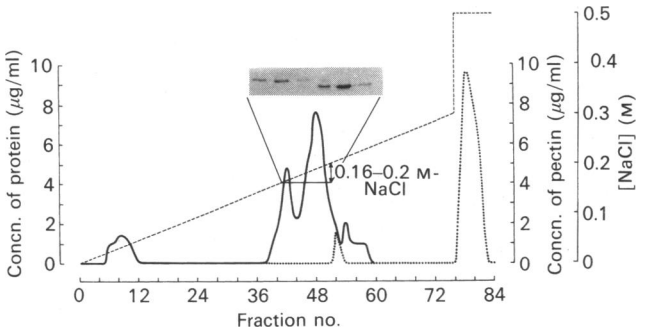

Fig. 2. Elution profile of proteins present in supernatant S5 from a DEAE-Sephacel column

A 7 ml portion of supernatant S5 derived from 35 g wet wt. of starting material was applied. See the Experimental section for details. The inset shows Coomassie Brilliant Blue-stained SDS/PAGE gels of the P35.5/P34 peak fractions.

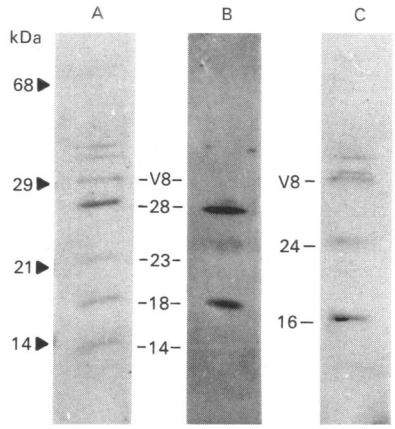

Fig. 3. Cleveland maps of P35.5 and P34 digested with *S. aureus* V8 proteinase

Lane A, digest of P35.5 stained with Coomassie Brilliant Blue; lane B, Western blot of the P35.5 map stained with antiserum raised against calelectrin derived from *Torpedo marmorata*; lane C, digest of P34 stained with Coomassie Brilliant Blue.

Peptide maps

From preliminary analyses P34 and P35.5 were shown to be *N*-terminally blocked (results not shown). In order to obtain sequence data from the two polypeptides, partial digestion by the 'in-gel' method of Cleveland [35] were performed. Cleveland maps are shown in Fig. 3. Proteolysis with *S. aureus* V8 proteinase produced four major peptides from P35.5 (track A) and two from P34 (track C). The differences between the two maps confirm P34 as a distinct polypeptide rather than a proteolytic product of P35.5. Two of the P35.5 fragments, with apparent molecular masses 28 kDa and 18 kDa, cross-reacted with antiserum raised against calelectrin isolated from *Torpedo marmorata*. The immunoblot is illustrated in track B of Fig. 3. Neither whole P34 nor fragments derived from it cross-reacted with this antiserum. P34 has previously been shown to cross-react with antisera raised against other animal annexins [30] that do not cross-react with P35.5.

All the major peptides shown in Fig. 3 were subjected to amino acid sequence analysis. The 28 kDa and 18 kDa cross-reactive fragments obtained from P35.5 and the 16 kDa fragment from P34 gave the sequence data shown in Fig. 4. The remaining peptides were found to be *N*-terminally blocked.

Amino acid sequence

Alignment of sequences derived from the plant polypeptides with repeat regions from six annexin family members isolated from animals revealed substantial sequence similarity between P35.5 and P34 and the annexin family of proteins. The alignments are shown in Fig. 4, in which the three plant peptides (one from P34, two from P35.5) are each compared with animal annexin repeat sequences (Figs. 4*a*–4*c*) and with each other together with a proposed annexin consensus sequence (Fig. 4*d*). Optimized scores for the best matching repeats obtained by using a 250 PAM mutation data matrix are: P34 (16 kDa fragment) and pig endonexin (first repeat), 29%; P35.5 (28 kDa fragment) and human calpactin 1 (second repeat), 38%; P35.5 (18 kDa fragment) and chicken anchorin (third repeat), 24%. These values compare with 30% sequence identity between individual repeated regions of animal annexin family members [39]. The plant sequence similarity to any single annexin repeat is of a similar value, though that of the P35.5 18 kDa fragment is relatively low. When they are compared with a number of repeats, as in Figs. 4(*a*)–4(*c*), the similarity to the annexin family becomes clearer. For example, 26 of the 56 known residues in P34 are identical with the corresponding residues in at least one of the six annexins aligned in Fig. 4(*a*); of the remaining 30 residues, 13 are represented by conservative substitutions in at least two of the aligned repeats.

Fig. 4(*d*) shows the plant sequences aligned with each other and a consensus sequence for the repeated region of the annexin family. Of the 32 residues in the consensus sequence 28 are identical or conserved in at least one of the plant sequences aligned above. This alignment also provides evidence for repeated structure within P35.5. The overlapping sequences of the P35.5 18 kDa and 28 kDa fragments are not identical but do resemble each other. The presence of more than one copy of the annexin repeat region within P35.5 gives further backing to the hypothesis that the polypeptides discussed here belong to the annexin family. Indeed, the only family of proteins consistently found on sweeping the OWL database (details in the Experimental section) with all of the three plant peptides was the annexins. Individually, the P34 fragment also showed sequence similarity to intermediate-filament proteins, as did the P35.5 18 kDa fragment. Some similarity was identified between the P35.5 28 kDa peptide and the conserved subdomain 10 of protein kinase C as defined by Hanks *et al.* [40].

DISCUSSION

The annexin family of proteins exhibits a number of interesting biochemical properties that have led to speculation concerning an essential role in Ca^{2+}-mediated events within animal cells. Sequence analysis of the two polypeptides that we have purified from tomato cells in the present investigation provides good evidence that annexins also exist in higher plants. Of particular interest in assigning these polypeptides to the annexin family are the locations of an arginine residue at position 26 and a tyrosine residue at position 38 (Fig. 4*d*). It is clear from the alignments shown in Figs. 4(*a*)–4(*c*) that the arginine residue is totally conserved in annexin repeat regions from diverse members of the annexin family. It has been speculated that it is involved in phospholipid binding [5] or could form a counterion for acidic groups involved in Ca^{2+} binding [31]. Similarly a residue with a phenolic side group is invariably found at position 38 (marked *c* Fig. 4*d*). It has been suggested that the tyrosine residue, or equivalent, could be the site of interaction with other cellular components or have a structural role in packaging [31].

Within the Taylor–Geisow model for annexin structure [31] two acidic side-chain Ca^{2+} contacts are postulated at positions 15

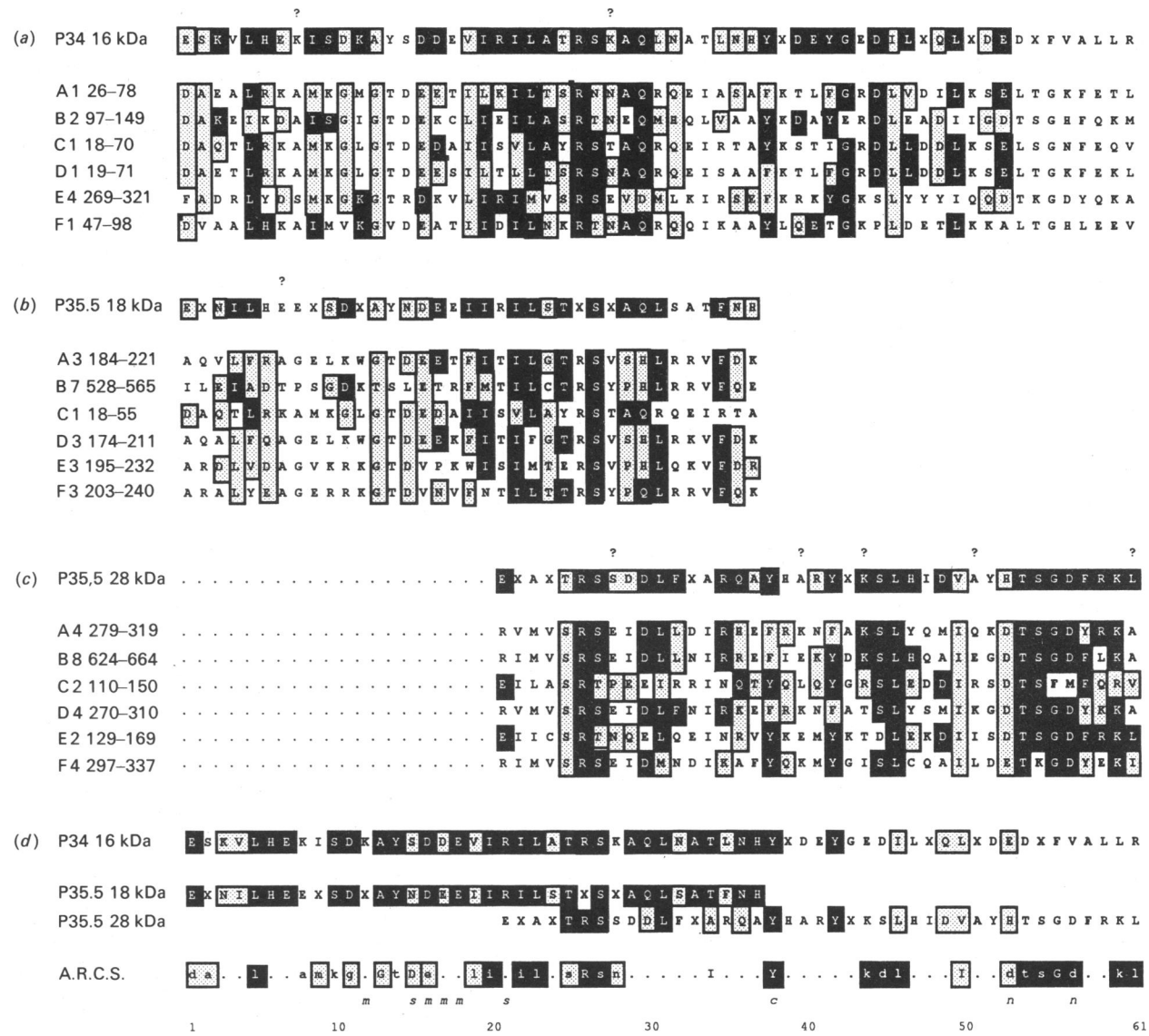

Fig. 4. Alignments of plant derived sequences with each other and annexin repeat sequences

The one-letter convention is used. The sources of the plant sequences are identified by the molecular mass of the polypeptide from which they are derived and the molecular mass of the *S. aureus* V8 proteinase fragment (see Fig. 3). An 'X' in the plant sequence indicates an unidentified residue; ones with a '?' above have not been unequivocally identified. *Italicized* letters below aligned sequences identify residues to which functions have been attributed by the Taylor & Geisow [31] model: *m*, main-chain carbonyl ligand to bound Ca^{2+}; *s*, side-chain ligand (carboxylic acid oxygen atom); *c*, possible site for interaction with cellular component; *n*, possible ligand to charged nitrogen atom of phospholipid. (*a*)–(*c*) The individual plant sequences are aligned with sequences from the repeated region of six members of the annexin family derived from animals. Residues shown as white letters in black boxes are identical in the plant sequence and at least one of the annexin sequences aligned below. Boxed and shaded letters are residues with a conservation of > 1 according to a mutation data matrix (250 PAM). Letters and numbers to the left of aligned sequences indicate sequence source, repeat number and residue numbers, e.g. 'A 1 26–78' indicates anchorin, repeat 1, residues 26–78. Key: A, chicken anchorin [41]; B, human P68 [42]; C, pig endonexin [43]; D, human placental anti-coagulant protein [44]; E, human lipocortin II [45]; F, human lipocortin 1 [45]. (*d*) The plant sequences are aligned against each other and a consensus sequence for the annexin repeated region (A.R.C.S.). Residues shown as white letters in black boxes are identical in at least two of the aligned plant sequences. Boxed and shaded letters are residues that are conserved in at least two of the plant sequences. Dots (.) in the consensus sequence are residues that are relatively unconserved, letters in lower case are conserved, letters in upper case are totally conserved. In the consensus sequence white letters in black boxes indicate that an identical residue is found in at least one of the plant sequences aligned above; boxed and shaded residues show a conservative substitution in a plant sequence.

and 21 (Fig. 4*d*). In the plant proteins one of the acidic residues lying at positions 16–18 could substitute for the first contact, and a glutamic acid residue is found at position 21 in the P35.5 28 kDa fragment. The same model suggests that residues 54–61 (Fig. 4*d*) may be important in lipid binding. Unlike the P35.5 fragment, the P34 peptide shows no sequence similarity to annexins over this region. This may reflect a structure and function of the plant proteins unrelated to those of animal annexins.

The sequence data show clearly that P35.5 and P34 are very closely related structurally. The degree of sequence identity between the 18 kDa peptide from P35.5 and the 16 kDa peptide from P34 is in excess of 80%. Since this degree of identity is much greater than that found between other fragments (for example, the two peptides derived from P35.5), it is possible that the 18 kDa and 16 kDa peptides represent the same domain within the two proteins, P35.5 and P34. The structural relationship between these two plant annexins should become clearer when their corresponding genes have been sequenced.

A property of animal annexins that has attracted considerable attention over recent years is their ability to inhibit phospholipase A_2, although the mechanism of inhibition is as yet unclear [46–49]. In this context it may be noted that the action of phospholipase A_2 is increasingly recognized in plant cells [26], where there is some evidence to indicate it forms a link in the signal-transduction pathway involving auxin [50]. For example Scherer & André have recently demonstrated an increased phospholipase A_2 activity in response to auxin stimulation of soya-bean suspension-culture cells and zucchini hypocotyls [50]. Similarly there is growing evidence that lysophospholipid acts as a signalling molecule in higher plants [51–53]. This product of phospholipase A_2 activity has been demonstrated to activate a proton-translocating ATPase via a soluble protein kinase [52,53]. It is therefore possible that the very rapid changes in proton extrusion in response to auxin may occur via this mechanism.

Within this context, and in view of the putative modulation of annexin inhibition of phospholipase A_2 activity by phosphorylation, it is of interest that a protein of similar molecular mass and isoelectric point to the tomato annexins was found to be phosphorylated in response to lysophospholipid or Ca^{2+} stimulation of a microsomal fraction prepared from zucchini hypocotyls [51]. Similarly Tognoli & Basso identified a 33 kDa protein in sycamore suspension-culture cells that was phosphorylated in response to fusicoccin treatment of the cells [54,55]. The sycamore phosphoprotein partitioned mainly in the soluble fraction in the presence of EGTA/EDTA, but was recovered in the microsomal fraction when chelators were omitted from the homogenization buffers. An identical pattern of partitioning is found for the tomato annexins discussed in the present paper. These features raise the possibility that the proteins identified in the other contexts may well be annexins.

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