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Identification of calcium-dependent phospholipid-binding proteins in higher plant cells

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Calcium-dependent phospholipid-binding proteins of apparent M_r 33000 and 35000 were isolated from suspension cultures of tomato cells. Two-dimensional gel electrophoresis showed the proteins to have isoelectric points of approx. 5.7 and 5.6, respectively. In the presence of calcium, both proteins bound to liposomes formed from a mixture of phosphatidylserine and phosphatidylcholine, but not to liposomes of phosphatidylcholine alone. Both proteins showed immunological similarities to previously characterized calcium-dependent phospholipid-binding proteins (annexins) from *Torpedo marmorata* and mammalian species. The protein of M_r 33000 cross-reacted with three separate antisera raised to the annexin *Torpedo* calelectrin, whereas that of M_r 35000 cross-reacted with antisera to the bovine annexins p68 and p32/34. We suggest that the two proteins may represent the first identification in higher plants of the annexin family of calcium-dependent phospholipid-binding proteins.

Annexin; Ca^{2+}-dependent phospholipid-binding protein; (Plant cell, *Lycopersicon esculentum*)

1. INTRODUCTION

Annexins constitute a recently identified family of proteins that are characterized by an ability to bind to specific phospholipids in the presence of micromolar calcium concentrations [1–3]. This group of proteins has been isolated from a wide variety of animal cells, including the electric organ of *Torpedo marmorata* [4,5], chicken gizzard [6], various rat [7], porcine [8] and bovine tissues [9,10], and human placenta [11,12]. Annexins include calelectrin from *Torpedo* [4,5], and at least five proteins so far identified in the avian and mammalian tissues including p68 (also termed p70 or 67 kDa calelectrin) [13,14], endonexin [10,15] and the related protein II [16], calpactin I (also termed lipocortin II) [17,18], lipocortin I (also termed calpactin II) [19], and proteins homologous to endonexin II and p32/34 [20–23]. The proteins are highly conserved during evolution from *Torpedo* to human, both immunologically and structurally. Antiserum to *Torpedo* calelectrin cross-reacts with mammalian p68 [10], endonexin [10], calpactin I heavy chain [24] and p32/34 [23], demonstrating the conservation of antigenic determinants. The mammalian proteins all contain four, or eight (p68), repeats of a 70–80 amino acid homologous sequence. Each repeat includes a 17 amino acid consensus sequence which is also present in *Torpedo* calelectrin [15]. Complete amino acid sequence data show 40–60% sequence identity between different annexins [3]. Between species, mouse and human calpactin I heavy chain amino acid sequences show 98% identity, a degree of conservation almost as high as that of actin [17].

In plant cells, calcium has been identified as a second messenger [25] which may be of particular importance since although cAMP is found in higher plants, no role has yet been ascribed to it [26]. Calmodulin is known to be present in plant cells [27], but as in animal cells calmodulin is probably not the sole mediator of the calcium signal. Since annexins are highly conserved in

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animal cells, we have investigated the possibility that plant cells contain similar proteins. We report here the identification of two proteins in cells of tomato suspension cultures which bind to phospholipid in a calcium-dependent manner and are immunologically related to mammalian and *Torpedo* annexins. We suggest that these proteins may represent plant members of the annexin family.

2. MATERIALS AND METHODS

2.1. *Isolation of proteins from tomato suspension cells*

Tomato (*Lycopersicon esculentum*) cells were cultured as a suspension in Murashige and Skoog medium [28] supplemented with 15 mg/l indoleacetic acid, 0.5 mg/l kinetin, 0.2 mg/l thiamine. Cells were harvested by filtration at stationary phase, quick-frozen and ground to a powder in a mortar and pestle using liquid N_2 to keep the cells frozen. The powder (10 g) was homogenized in 50 ml of 0.15 M NaCl/10 mM Hepes/10 mM EGTA (pH 7.4) using a Potter-type homogenizer, and centrifuged for 30 min at 40000 × *g*. The supernatant was removed, and 50 mg bovine brain lipid (Sigma B3635) added, followed by $CaCl_2$ to a final concentration of 15 mM (5 mM excess). After 30 min on ice, the fraction was centrifuged for 30 min at 40000 × *g*. The pellet was washed twice in 0.15 M NaCl/10 mM Hepes/1 mM $CaCl_2$ (pH 7.4). The final pellet was resuspended in 10 ml of 0.15 M NaCl/10 mM Hepes/15 mM EGTA (pH 7.4) and centrifuged for 1.5 h at 100000 × *g*. For use in lipid-binding assays, the final supernatant was concentrated by placing it in dialysis tubing and covering the bag with polyethylene glycol 20000. The concentrated sample was then dialysed exhaustively against 20 mM Hepes (pH 7.4). All steps were performed at 4°C.

2.2. *Isolation of bovine annexins p68 and p32/34*

Proteins were isolated from bovine lung as described [23]. The identity of p68 was confirmed by immunoblotting using antiserum to bovine liver p68 [10].

2.3. *Protein assay*

Protein concentrations were measured using BCA reagent (Pierce, Rockford, IL) according to the manufacturer's instructions.

2.4. *Electrophoresis and immunoblotting*

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli [29], except that the electrode buffer used was 188 mM Tris/188 mM glycine/0.1% SDS. M_r standards used were bovine albumin (M_r 66000), egg albumin (M_r 45000), carbonic anhydrase (M_r 29000) and trypsin inhibitor (M_r 20100) (Sigma, Poole, England). Two-dimensional gel electrophoresis was according to O'Farrell [30] as modified by Anderson and Anderson [31]. Ampholytes (2D Pharmalytes, Pharmacia, Uppsala) were pH 3–10. Conditions for SDS-PAGE separation were as above. Gels were stained with Coomassie brilliant blue. Protein separated by SDS-PAGE was electrophoretically transferred to nitrocellulose as described [32]. Nitrocellulose blots were stained for protein with 0.5% Ponceau Red in 1.0% acetic acid and the position of the protein bands marked. The nitrocellulose was then further processed for immunoblotting essentially as in [33]. Rabbit antisera to *Torpedo* calelectrin [4] and bovine p68 [10] have been characterized previously. Sheep antiserum to *Torpedo* calelectrin was raised using the same immunization schedule as used with rabbits. The antiserum obtained was identical in specificity to previously described rabbit antiserum [4]. Rabbit antiserum to bovine p32/34 was prepared following the protocol for antiserum to p68 in [10] using protein purified as in [23]. The antisera were specific when tested by immunoblotting of total tissues. All antisera were used at a dilution of 1:50 for 2 h at 20°C and visualised with peroxidase-conjugated second antibody (ICN Biomedicals, High Wycombe, England) and 3,3′-diaminobenzidine. Pre-immune serum controls were blank.

2.5. *Phospholipid binding*

Phospholipid vesicles were made in the presence of 240 mM sucrose from phosphatidylcholine (PC) alone, or a 1:1 mixture of PC and phosphatidylserine (PS) (Sigma) [34]. The liposomes were harvested by adding 2 vols EGTA buffer (100 mM KCl/2 mM $MgCl_2$/1 mM EGTA/20 mM Hepes, pH 7.4) or calcium buffer (EGTA buffer plus 2 mM $CaCl_2$ to give 1 mM free Ca^{2+}) and centrifuging for 10 min at 12000 × *g*. Following two further washes in EGTA or calcium buffer, binding of the proteins to liposomes was measured. 10 μg protein was incubated for 15 min at 20°C with liposomes (150 μg phospholipid) in a total volume of 500 μl EGTA or calcium buffer. After centrifugation for 10 min at 12000 × *g*, the supernatants were removed and protein precipitated with an equal volume of 20% trichloroacetic acid. The pellets were washed once in 500 μl EGTA or calcium buffer, extracted with 300 μl acetone at −20°C for 30 min, and centrifuged for 10 min at 12000 × *g*. Equal proportions of the supernatants and pellets were analysed by 10% SDS-PAGE.

3. RESULTS

Proteins were isolated from the tomato suspension cells using a slightly modified version of a procedure developed for the purification of calcium-dependent membrane-binding proteins from animal cells [23]. Lipids were added to the initial supernatant together with the calcium to ensure that sufficient was available for the precipitation of calcium-dependent phospholipid-binding proteins. Typically, from 10 g fresh wt starting material, 0.35 mg protein was recovered in the final supernatant. When this fraction was separated by SDS-PAGE and stained with Coomassie blue, two major polypeptides, of apparent M_r 33000 and 35000, were observed. A third minor Coomassie-staining band, which was less sharply resolved, was detectable at an apparent M_r of approx. 36000 (fig.1a). Fig.1b shows the

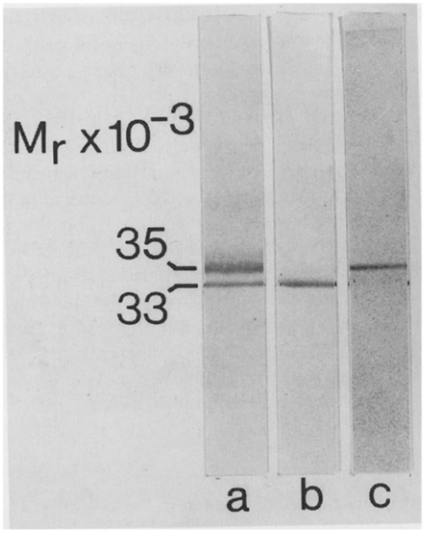

Fig.1. (a) Coomassie blue-stained 10% SDS-PAGE of the final supernatant of the tomato cell preparation. (b,c) Immunoblots of fractions similar to (a) using antiserum to *Torpedo* calelectrin (b) or bovine p68 (c).

result of an immunoblot of a similar fraction with a rabbit antiserum raised to *Torpedo* calelectrin. The antiserum cross-reacts with the polypeptide of M_r 33000, but not that of M_r 35000. Identical results were obtained with a sheep antiserum and a further rabbit antiserum to *Torpedo* calelectrin (not shown). The polypeptide of M_r 35000 cross-reacted on immunoblots with an antiserum to the bovine annexin p68 (fig.1c) and with an antiserum to bovine p32/34 (not shown). No response to the polypeptide of M_r 33000 was detectable using these antibodies. None of the antisera used showed any cross-reactivity with the minor component at M_r 36000.

As a further characterization of the two major polypeptides, they were analysed by two-dimensional gel electrophoresis. Fig.2 shows the separation of the two plant polypeptides alone, or with the addition of bovine p68 and p32/34 to provide internal p*I* standards (5.85 and 5.1/5.0, respectively [10,23]). The apparent isoelectric points of the polypeptides of M_r 33000 and 35000 were approx. 5.7 and 5.6, respectively.

To investigate their lipid-binding properties, the proteins were incubated in the presence or absence of calcium with sucrose-loaded liposomes prepared from either PC alone or a 1:1 mixture of PC and

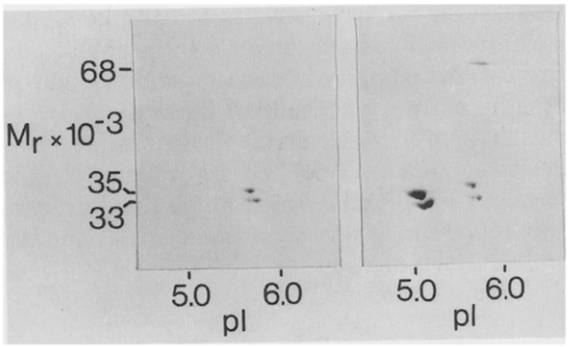

Fig.2. Coomassie blue-stained two-dimensional gel electrophoresis of the isolated plant polypeptides, either alone (left) or in combination with bovine p68 and p32/34 (right). Only the relevant sections of the gels are shown.

PS. Following centrifugation, equal proportions of the supernatant and liposome-containing pellet were analysed by SDS-PAGE. Fig.3 shows that in the presence of EGTA, no association of the proteins with the PS-containing liposomes was detectable. However, in the presence of 1 mM Ca^{2+}, both major polypeptides were found in the

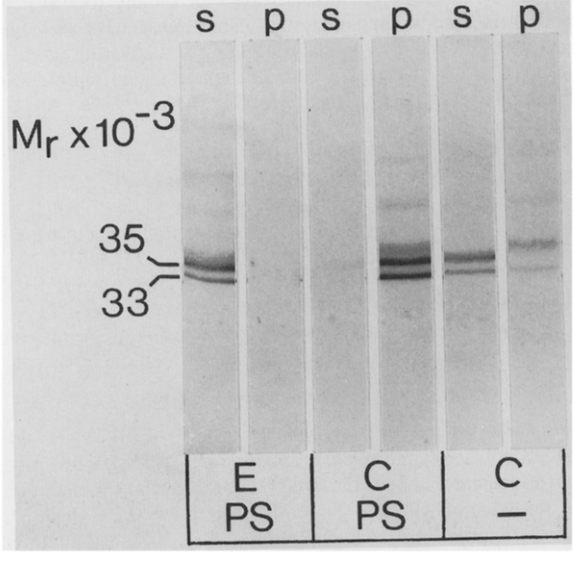

Fig.3. Phospholipid-binding properties of the plant proteins. Proteins were incubated either with liposomes (1:1 phosphatidylserine/phosphatidylcholine) (PS) or in the absence of lipid (−) in buffer containing 1 mM EGTA (E) or 1 mM Ca^{2+} (C). Following centrifugation equal proportions of the supernatants (s) and liposome-containing pellets (p) were analysed by 10% SDS-PAGE and stained with Coomassie blue.

liposome pellet. As a control for calcium-induced aggregation of the proteins, the experiment was also performed in the absence of liposomes. In this case, the majority of the two major polypeptides remained in the supernatant. However, the minor component at M_r 36000, and a number of faintly detectable polypeptides of higher M_r, were precipitated by the addition of calcium alone. The same result was obtained when liposomes of pure PC and calcium buffer were used (not shown). The presence of the polypeptides of M_r 33000 and 35000 in the supernatant in this case demonstrates that they do not bind to PC in the presence of calcium.

4. DISCUSSION

We describe here two proteins from tomato suspension cells which can be isolated by utilising their calcium-dependent affinity for lipids. The properties of these proteins show several similarities to the annexin family of calcium-dependent phospholipid-binding proteins from animal cells. The M_r values of the plant proteins are similar to those of animal annexins which, with the exception of p68 (M_r ~68000) and the small subunit (of M_r 10000) of calpactin I [35] lie between M_r 32000 (endonexin) [10] and M_r 37000 (lipocortin I) [19]. The isoelectric points are also typical of those reported for animal annexins which range from p*I* 5.0 (p32/34) [23] to p*I* 7.4 (calpactin I heavy chain) [35]. Both proteins show immunological cross-reaction with antisera raised to annexins from *Torpedo* and bovine tissues. The antisera to *Torpedo* calelectrin are known to cross-react with mammalian endonexin [10], p70 [10], calpactin I [24] and p32/34 [23] and have not been observed to cross-react with any proteins other than these members of the annexin family. The antisera to p68 and p32/34 are each specific when tested on immunoblots of bovine tissues. This suggests that the plant proteins share common antigenic determinants with animal annexins. Further similarities are seen in the lipid-binding properties. In common with the mammalian annexins so far described, the two major plant polypeptides bind to PS but not to PC in the presence of calcium [9,23,36–39].

The third minor component appears not to belong to the annexin family since no cross-reactivity was detectable with the antisera used. Also, it was precipitated by calcium in the absence of liposomes under conditions where mammalian annexins and the plant polypeptides of M_r 33000 and 35000 remain in the supernatant [23,36–39].

The work reported here therefore identifies two novel calcium-dependent phospholipid-binding proteins in higher plants, and suggests the existence of annexins in the plant, as well as the animal kingdom. Although the physiological role of the annexins is not yet clear, this further evidence for their evolutionary conservation points to an essential function in calcium-regulated processes within the cell.

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