Tomato Annexins p34 and p35 Bind to F-Actin and Display Nucleotide Phosphodiesterase Activity Inhibited by Phospholipid Binding

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Annexins are a family of proteins found in a range of eukaryotic cell types. They share a characteristic amino acid sequence and a Ca\(^{2+}\)-dependent affinity for specific phospholipids. In plants, proteins with common properties and significant homology with annexins have been identified in a number of species and implicated in diverse cellular functions known to be modulated by Ca\(^{2+}\). This study describes several novel biochemical properties of the tomato annexins p34 and p35 that are relevant to our understanding of their functions in the plant. First, the annexins were found to bind to actin in a calcium- and pH-dependent interaction that was specific for F-actin and not G-actin. Second, an enzyme activity defined as a nucleotide phosphodiesterase activity was found associated with the purified annexin preparation. Selective immunoprecipitation of p34 and p35 strongly suggests that the enzyme activity is a property of the annexins and constitutes 60% of the total soluble activity found in root extracts capable of hydrolyzing free ATP. The substrate specificity of the enzyme within in vitro assays is broad. ATP is the preferred substrate, but nearly identical rates of hydrolysis of GTP and substantial hydrolysis of other nucleotide tri- and diphosphates are observed. The enzyme activity was found to be a property of both p34 and p35, although the specific activity was routinely higher for p34. Third, the enzyme activity of the annexins was not affected by F-actin binding but could be abolished by the specific Ca\(^{2+}\)-dependent interaction of the annexins with phospholipids. Our results showed that p34 and p35 account for substantial enzyme activity in tomato root cells. This activity was exhibited when the proteins were either in soluble form or attached to actin filaments. Enzyme activity was not exhibited when the annexins were bound to phospholipids. These properties suggest a role for the proteins in mediating Ca\(^{2+}\)-dependent events involving interactions of the cytoskeleton and cellular membranes.

INTRODUCTION

Annexins are a family of structurally related proteins characterized by their shared property of Ca\(^{2+}\)-dependent binding to phospholipids. Currently, 13 distinct annexins have been identified in a range of organisms and cell types and have been implicated to function in diverse cellular processes, including channel activity, signal transduction, and membrane vesicle transport and fusion (reviewed in Moss, 1992; Raynal and Pollard, 1994).

The first plant annexins were identified 6 years ago (Boustead et al., 1989) in tomato plants. Subsequently, the proteins were purified from different tissues of the tomato plant, and peptide sequence analysis of p34 and p35 confirmed the presence of an annexin consensus sequence (Smallwood et al., 1990). Further analysis using RNA blots to show gene expression indicated that the most abundant sources were tissues from the roots and stems of the plant (Smallwood et al., 1992). Annexins have now been purified from a range of different plant species, and similar to their counterparts in animal cells, plant annexins have been implicated in a diversity of cellular processes. These include exocytosis (Blackbourn et al., 1992; Clark et al., 1992; Blackbourn and Battey, 1993), regulation of callose synthase (Andrawis et al., 1993), and sensing of cytosolic Ca\(^{2+}\) levels at the vacuolar membrane (Seals et al., 1994). Most recently, an annexin-containing preparation from maize was found to exhibit ATP/GTPase activity (McClung et al., 1994).

Our laboratory has been studying p34 and p35 from tomato plants as potential signal transducers. Tomato is a well-characterized system for analyzing signaling events because a number of defined changes in defense gene activation can be triggered by external stimuli (reviewed in Bowles, 1990). A feature of plant cell signaling that is gaining wider recognition is the association of many key components of transduction pathways with the cytoskeleton (Tan and Boss, 1992; Xu et al., 1992; Drøbak et al., 1994). Annexins I and II have both been shown to bind to F-actin in a Ca\(^{2+}\)-dependent interaction (Gerke and Weber, 1984; Glenney and Glenney, 1985; Schlaepfer and Haigler, 1987; Ikebuchi and Waisman, 1990), and recent

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evidence indicates that the binding of annexin II induces actin bundling (Ma et al., 1994). These data prompted us to analyze whether tomato p34 and p35 could interact with actin and whether the recently described ATP/GTPase activity was involved in the interaction.

Our findings show that p34 and p35 bind to F-actin. Although there is no evidence of a significant role for the enzyme activity in the annexin–actin association, there is evidence of a regulatory relationship between the enzyme activity and the interaction of annexins p34 and p35 with phospholipids.

RESULTS

F-Actin Binding Properties of Annexins p34 and p35

Initial experiments involved the use of a dot blot assay to determine whether the annexins could associate with immobilized actin. Figure 1A shows that the presence of annexins was detected using polyclonal antisera that reacted with both p34 and p35. The annexins bound to the actin in a Ca$^{2+}$-dependent interaction, and no binding could be detected in the presence of EGTA. Mg$^{2+}$ could not be used as a substitute for Ca$^{2+}$ (Figure 1A). The annexins did not interact with two other Ca$^{2+}$-containing proteins, calmodulin and filamin, suggesting that their interaction with actin was more specific than merely through recognition of bound calcium (data not shown). Interestingly, the binding of the annexins to actin appeared to be reduced in the presence of ATP (Figure 1A). A limitation of the dot blot assay is the inability to select the polymerization state of the actin population. Thus, actin immobilized to Sepharose was used to determine whether the binding of the annexins was specific for F-actin or G-actin. This method has been used previously to analyze the interactions of actin peptides (Lebart et al., 1993). The specificity and the stability of the F-actin matrices were confirmed through the use of α-actinin, a protein known to bind to F-actin and not to G-actin. As shown in Figure 1B, α-actinin interacted specifically with the immobilized F-actin. Also shown in Figure 1B is the selective binding of p34/p35 to the F-actin matrix. Further analyses shown in Figures 1C and 1D confirm the specificity of binding and show that the interaction was dependent on Ca$^{2+}$, with both proteins eluted from the F-actin matrix by the addition of EGTA. Mg$^{2+}$ could not substitute for Ca$^{2+}$ in annexin binding to F-actin (data not shown).

The amount of free Ca$^{2+}$ required for annexin binding of F-actin was estimated using a low-speed cosedimentation assay. Differential Ca$^{2+}$ requirements were observed, with p34 requiring 100 nM Ca$^{2+}$, whereas p35 only bound at higher (300 nM) Ca$^{2+}$ concentrations (Figure 2). When the pH dependence of annexin binding to F-actin was investigated, both annexins bound at the physiological pH range of 6 to 8 (data not shown).

Nucleotide Phosphodiesterase Activity of Annexins

Given that the presence of ATP in the dot blot assay reduced binding of the annexins to actin, and in light of recent data presented by McClung et al. (1994), the proteins prepared from tomato roots were assayed for ATPase activity. Figure 3 shows...
the pH curve, with optimal activity found at pH 6 to 7. Routinely, the enzyme activity of the tomato annexins was found to be in the range of 26 to 30 μmol of Pi mg⁻¹ hr⁻¹, at pH 7, when assayed within 24 hr of extraction.

Although p34 and p35 are the only two polypeptides visualized by protein staining in annexin preparations, it remained a possibility that the enzyme activity reflected a contaminant protein in amounts below the level of detection. Two approaches were selected to address this possibility. Polyclonal antisera raised to the tomato annexins did not cross-react with any other polypeptides in a total extract from tomato roots (Figure 4). After incubating the antisera with the purified annexin preparation, the annexin-depleted supernatant was assayed for ATPase activity. As shown in Table 1, a reduction of 94% in soluble enzyme activity was observed. To confirm that the antibodies precipitating the ATPase activity corresponded to those specific for p34 and p35, the two polypeptides were SDS-PAGE purified and blotted onto nitrocellulose, and that region of the blot was used to remove cross-reacting antibodies from the antisera. After this treatment, immunoprecipitation had no effect on the solubility of the activity associated with the annexin preparation. As a further control, a monoclonal antibody recognizing an epitope common to p34 and p35 was used for immunoprecipitation. Soluble ATPase activity remaining after this treatment was substantially reduced to 10%. These immunological approaches strongly suggest that the enzyme activities are properties of the p34/p35.

In a second approach, detergent and high salt were added to the annexins to disrupt any potential attachment of any contaminating enzymes to p34 or p35, and the preparation was applied to an F-actin matrix. After washing, the annexins were eluted by EGTA and analyzed for enzyme activity. Results in Figure 5 confirm that the eluted annexins exhibited ATPase activity. These data provide additional evidence that the enzyme activity is a property of the annexin polypeptides p34/p35.

To gain insight into the size of the active enzyme, the purified annexins were subjected to gel electrophoresis under native conditions. The results are shown in Figure 6A and indicate that only one diffuse band of 70 kD can be visualized in the native gel by protein staining. Unstained regions of the gel were sliced, and ATPase activity was measured in each slice. Figure 6B shows that the enzyme activity coelutes with the stained protein. Very low levels were also detected at the interface of the stacking and running gel, most probably indicating that some aggregation had occurred. When the region of the gel corresponding to the protein band and enzyme activity was analyzed by SDS-PAGE, the annexin polypeptides p34 and p35 were present, as shown in Figure 6C. The resolution of the native gel was insufficient to distinguish between two proteins at 68 and 70 kD, that is, homodimers, or one at 69 kD, that is, the heterodimer.

The ATPase activity reported for the maize annexin preparation is not affected by the presence of EDTA (McClung et al., 1994). When the effect of EDTA on tomato annexins was examined, the results in Table 2 show that no reduction in enzyme activity was observed. Using the presence of EDTA to distinguish between Mg²⁺-dependent and Mg²⁺-independent activity, the relative contribution of annexin ATPase to the total cytosolic ATPase was determined. The results in Table 2 indicate that selective removal of the annexin by immunoprecipitation led to a substantial reduction of 60% in Mg²⁺-independent ATPase activity in the soluble fraction, whereas Mg²⁺-dependent activity was only slightly affected (6%). This result provides evidence that the annexins contribute to a significant proportion of the cytosolic enzyme activity able to hydrolyze free ATP.

**Figure 3.** pH Optima of Annexin ATPase Activity.

Purified annexins were preincubated in reaction medium (30 mM MgCl₂, 50 mM KCl, 5 mM bis-tris propane-2-(N-morpholino)ethanesulfonic acid buffer) for 5 min, and the reaction was initiated by adding 30 mM ATP. Each point is the mean of three duplicate experiments ± SEM.

**Figure 4.** Specificity of Anti-Annexin Antibody.

A whole-cell extract of tomato suspension cells was subjected to SDS-PAGE and transferred to nitrocellulose. The polypeptides were probed with annexin antisera, and the annexins p34 and p35 are labeled.
Table 1. Residual ATPase Activity after Immunoprecipitationa

<table>
<thead>
<tr>
<th>Assay</th>
<th>Specific Activity (μmol Pi mg⁻¹ hr⁻¹)</th>
<th>Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Annexin controlb</td>
<td>265 ± 0.6</td>
<td>100</td>
</tr>
<tr>
<td>Antisera controlb</td>
<td>270 ± 0.5</td>
<td>102</td>
</tr>
<tr>
<td>Annexin-depleted supernatant</td>
<td>1.6 ± 0.3</td>
<td>6</td>
</tr>
</tbody>
</table>

a Immunoprecipitation by anti-annexin polyclonal antiserum was performed as detailed in Methods.
b An aliquot of annexin was taken through the same experimental procedure, with buffer substituted for the antiserum. The immunoprecipitated supernatant was assayed for ATPase activity at pH 7.0.
c Antisera were affinity depleted of anti-annexin antibodies and then used in the immunoprecipitation assay.

The results in Table 3 illustrate the ability of the annexins to hydrolyze a range of related substrates. Although ATP was the preferred substrate, as indicated by the higher rate of hydrolysis at high and low substrate concentrations, the rate of hydrolysis of GTP was found to be nearly identical to that of ATP. Lower hydrolytic rates of the other nucleotide tri- and diphosphates were also observed, but in contrast, the annexins displayed only very limited hydrolysis of hexose-phosphates, inorganic pyrophosphate, and AMP. A dose–response curve for the ATPase activity of the annexin preparation is depicted in Figure 7. It shows that the concentration of ATP required for half-maximal activity was 0.19 ± 0.06 mM ATP.

The results in Table 4 show that the enzyme activity is a property of both p34 and p35 in the annexin preparation, although the specific activity of p34 was always found to be higher than that of p35. The substrate specificity was found to be identical for the two proteins (Table 4), together with the pH optimum and effect of EDTA.

**Effect of F-Actin and Phospholipids on Nucleotide Phosphodiesterase Activity**

Given that the enzyme activity was not affected by the addition of EDTA, which implies that Mg2⁺ is not required for activity, and that this property is also shared by myosin ATPases, we decided to investigate the effect of F-actin on ATP/GTP hydrolysis. Results indicated that the ATPase activity was not affected significantly by preincubation of the annexins with 2 to 10 μg of F-actin in the presence of Ca2⁺ over the pH range of 6 to 8 (Table 5). Similarly, no effect on GTPase activity was found (data not shown).

A common property of all annexins is the ability of the proteins to bind to phospholipids. The specificity of the interaction of p34 and p35 has already been shown (Smallwood, 1992) and indicates that the annexins bound to phosphatidylserine but did not interact with phosphatidylcholine. The effects of phospholipids on the enzyme activity of p34 and p35 are shown in Table 6. The results indicate that ATP/GTP hydrolysis was affected by the presence of phospholipids. The inhibition was dependent on the type of phospholipid and the presence of Ca2⁺. Whereas phosphatidylserine reduced the rate of hydrolysis by >90%, the same amount of phosphatidylycholine had negligible effect. The addition of brain extract lipid, containing a mixture of phospholipids, was found to have an intermediate effect. Identical effects on GTPase activity were also observed (data not shown). The Ca2⁺ dependence of the inhibition induced by the phospholipids, together with the selectivity of the effect, strongly implies that the enzyme activity and phospholipid binding activity of the tomato annexins cannot be exhibited simultaneously.

**DISCUSSION**

Annexins represent a highly defined family of proteins that all share a characteristic peptide sequence of 70 amino acids, which is repeated four or eight times in the various proteins that have been characterized. The repeats are known to be involved in the property that is common to all annexins, namely, the Ca2⁺-dependent interaction of the proteins with acidic phospholipids. The N-terminal region of the different annexins is more diverse, and this region has been implicated in conferring the specific properties associated with the individual members of the family.

In plants, a number of proteins have been identified that contain a partial peptide sequence homologous to regions within the 70–amino acid repeat region of the well-characterized animal annexins. This homology, together with their properties...
of calcium-dependent phospholipid binding, provides the basis for defining the proteins as annexins. Thus, proteins with these features have been identified in tomato (Smallwood et al., 1990), maize (Blackbourn et al., 1992), and pea (Clark et al., 1992). Cross-reactive species sharing common epitopes with the maize protein have been identified in *Tradescantia* and tobacco, and use of the tomato annexin DNA probe shows homologs in potato, soybean, and barley (Smallwood et al., 1992). In addition, a protein binding to vacuolar membranes in celery (Seals et al., 1994) and three proteins in cotton that affect callose synthase activity have also been shown to contain partial peptide sequences indicative of the 70–amino acid annexin repeat (Andrawis et al., 1993).

To further our understanding of the potential role of annexins in calcium-mediated events within plant cells, we have been defining the range of biochemical properties exhibited by the tomato annexins p34 and p35. This study shows that the two proteins bind to F-actin filaments in a calcium-dependent interaction. Our data with the tomato annexins are in contrast with the negative results obtained with maize p33 and p35, which indicated no interaction with purified actin under a range of calcium concentrations (Blackbourn et al., 1992). In that study, however, the method for analyzing actin–annexin interactions involved centrifugation to pellet the F-actin. Under those circumstances, substantial amounts of actin were shown to remain in the supernatant, and one explanation for their data might be the interaction of the maize annexins with the unpelleted actin. Another problem with the methodology employed in the study of Blackbourn et al. (1992) was that the concentration of Ca\(^{2+}\) was shown to promote annexin precipitation. The use of actin matrices as described in this study overcomes both of these problems.

In studies with animal annexins, actin binding has been described for only two of the 13 members of the annexin family: annexin 1 and annexin 2 (Gerke and Weber, 1984, 1985; Glenney and Glenney, 1985; Glenney, 1986; Glenney et al., 1987; Schlaepfer and Haigler, 1987; Ikebuchi and Waisman, 1990; Jones et al., 1992). The binding of these two annexins to actin has been shown to be Ca\(^{2+}\) dependent and selective.

### Table 2. ATPase Activity of the Soluble Fraction of Tomato Roots\(^a\)

| ATPase Assay \(^b\) | Specific Activity (umol Pi mg\(^{-1}\) hr\(^{-1}\)) | Reduction (%)
|---------------------|-----------------------------------------------|------------------
| + Mg\(^{2+}\) soluble fraction | 90 ± 9 | NA\(^c\) |
| + Mg\(^{2+}\) after immunoprecipitation\(^d\) | 85 ± 6 | 6 |
| + EDTA soluble fraction | 37 ± 2 | NA |
| + EDTA after immunoprecipitation | 15 ± 2 | 61 |

\(^a\) Soluble fraction was the supernatant from a 100,000g centrifugation.

\(^b\) ATPase activity was measured in the presence or absence of Mg\(^{2+}\).

\(^c\) NA, not applicable.

\(^d\) Immunoprecipitation by annexin antisera was performed as described in Methods.
The activity with each substrate is compared with ATPase activity, which is assigned activity of 100%.

Table 3. Comparative Rates of Substrate Hydrolysis

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Specific Activity (μmol Pi/mg protein/hr)</th>
<th>Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>28.7 ± 0.6 [3.0 mM], 16.1 ± 0.7 [0.3 mM]</td>
<td>100 [3.0 mM], 100 [0.3 mM]</td>
</tr>
<tr>
<td>ADP</td>
<td>18.3 ± 0.9 ND [3.0 mM], ND [0.3 mM]</td>
<td>64 NA [3.0 mM], NA [0.3 mM]</td>
</tr>
<tr>
<td>AMP</td>
<td>7.4 ± 2.0 ND [3.0 mM], ND [0.3 mM]</td>
<td>26 NA [3.0 mM], NA [0.3 mM]</td>
</tr>
<tr>
<td>GTP</td>
<td>26.0 ± 1.5 ND [3.0 mM], 12.7 ± 0.6 [0.3 mM]</td>
<td>91 79</td>
</tr>
<tr>
<td>CTP</td>
<td>23.0 ± 1.8 10.0 ± 1.4 [0.3 mM]</td>
<td>80 63</td>
</tr>
<tr>
<td>TTP</td>
<td>17.0 ± 1.7 8.0 ± 1.2 [0.3 mM]</td>
<td>59 50</td>
</tr>
<tr>
<td>PPI</td>
<td>6.3 ± 0.45 ND [3.0 mM], 22 NA [0.3 mM]</td>
<td>15 NA [3.0 mM], NA [0.3 mM]</td>
</tr>
<tr>
<td>Fructose 1-P</td>
<td>4.2 ± 0.16 ND [3.0 mM], 15 NA [0.3 mM]</td>
<td>5 NA [3.0 mM], NA [0.3 mM]</td>
</tr>
<tr>
<td>Glucose 1,6-P</td>
<td>1.5 ± 0.14 ND [3.0 mM], 5 NA [0.3 mM]</td>
<td>9 NA [3.0 mM], NA [0.3 mM]</td>
</tr>
<tr>
<td>Inositol 1,4,5-P</td>
<td>2.5 ± 0.3 ND [3.0 mM], 9 NA [0.3 mM]</td>
<td>9 NA [3.0 mM], NA [0.3 mM]</td>
</tr>
</tbody>
</table>

(a) Substrates were added at the given concentration, and liberation of Pi was determined as detailed in Methods.

(b) The activity with each substrate is compared with ATPase activity, which is assigned activity of 100%.

(c) ND, not determined.

(d) NA, not applicable.

McClung et al. (1994) recently demonstrated that a maize extract ~70% pure in annexin proteins p33 and p35 expressed an ability to hydrolyze a range of nucleotides. The native enzyme in tomato of ~70 kD is clearly a dimer that can be separated on SDS-PAGE into p34 and p45. Due to the resolution of native gels, we cannot ascertain whether there are two molecular species of 68 and 70 kD corresponding to homodimers of each annexin or whether there is a heterodimer consisting of p34 and p35. However, our analysis contrasts with that of the study of maize annexins in which McClung et al. (1994) interpreted their data as indicating that the enzyme exhibited a denatured size of 68 kD. Their gel filtration profile showed the presence of three polypeptides (68, 35, and 33 kD) in the exclusion volume. If this result represented an aggregate that was not fully denatured before SDS-PAGE, then the difference in our two sets of data on the molecular size of the enzyme would be resolved. Although described by the authors as an ATPase, the data showed a higher rate of hydrolysis of GTP, with substantial release of Pi also from other nucleotide tri- and diphosphates. Because we had observed a slight reduction in annexin binding to actin on immunoblots in the presence of ATP, the data of McClung et al. (1994) led us to question whether the tomato annexins would be capable of hydrolyzing nucleotides and whether that activity was related in some way to their interaction with F-actin.

The results described in this study show that an enzyme activity is associated with the tomato annexins. All of the control experiments suggest that the activity is a property of the annexin proteins rather than a contaminant in the preparation. Importantly, immuno-depletion from a total soluble fraction indicates that the annexin enzyme activity accounts for 60% of a total soluble activity capable of hydrolyzing free ATP. By using...
Table 4. Comparison of Enzyme Activity of p34 and p35

<table>
<thead>
<tr>
<th>Assaya</th>
<th>Substrate</th>
<th>p34 (μmol Pi mg⁻¹ hr⁻¹)</th>
<th>p35 (μmol Pi mg⁻¹ hr⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ATP</td>
<td>25.1 ± 0.7</td>
<td>15.7 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>ADP</td>
<td>20.8 ± 0.8</td>
<td>11.1 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>GTP</td>
<td>233 ± 1.6</td>
<td>15.7 ± 0.3</td>
</tr>
<tr>
<td>Inhibitors of ATP hydrolysis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>N-ethylmaleimide</td>
<td>11.9 ± 0.8</td>
<td>73 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>EDTA</td>
<td>24.2 ± 0.4</td>
<td>16.2 ± 2.0</td>
</tr>
<tr>
<td></td>
<td>Molybdate</td>
<td>19.2 ± 1.4</td>
<td>13.2 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>Vanadate</td>
<td>20.8 ± 0.8</td>
<td>10.0 ± 1.2</td>
</tr>
</tbody>
</table>

a Annexins p34 and p35 were separated by DEAE–Sephacel ion-exchange chromatography. Their individual activities were then assayed at pH 7.0.

the in vitro assay, we showed that the tomato annexin enzyme activity indicated a broad specificity toward nucleotide triphosphates and diphosphates, leading us to define the enzyme activity as a nucleotide phosphodiesterase. The nature of the substrate(s) in vivo must remain an open question. If the enzyme activity in the cell also uses free ATP as the preferred substrate, the data indicate that 0.2 mM ATP would be required for half-maximal activity, which is within the range of free ATP concentrations calculated for plant cells (Davies et al., 1993).

Given that the tomato annexins purified in soluble form from the cells exhibited an enzyme activity, it became important for us to determine whether the activity would be affected when the proteins were interacting with actin filaments or phospholipids. The data indicate that annexins attached to actin filaments still hydrolyze substrate, because no effect on the rate of hydrolysis of ATP or GTP was observed. It is possible that the energy provided by ATP hydrolysis is important to annexin function, for example, if annexins' role in the cell were to act as motor proteins. Comparisons between annexin ATPase and myosin ATPase have been highlighted due to their shared ability to hydrolyze free ATP (McClung et al., 1994); however, F-actin is known to stimulate myosin ATPase (Kabsch and Vandekerckhove, 1992), and yet we have demonstrated that F-actin has no effect on annexin ATPase.

One of the most significant features described in this study is the inhibition of annexin enzyme activity by phospholipids. The effect is dependent on the presence of calcium and on the type of phospholipid. Tomato p34 and p35 have previously been shown to exhibit characteristic properties of phospholipid binding, with a calcium dependence of <100 μM and a specificity that distinguished phosphatidylserine from phosphatidylcholine (Smallwood, 1992). Thus, our data indicate that when tomato annexins are attached to phospholipids, they are incapable of hydrolyzing their substrates. From studies on animal annexins, the proteins are known to be highly flexible molecules with large conformational changes induced under different conditions, such as in the presence or absence of Ca²⁺ (Huber et al., 1990; Concha et al., 1993) or during binding to phosphatidylserine (Pigault et al., 1990). It is possible, therefore, that the effect is allosteric, but additional work is required to investigate fully the nature of the inhibition. Given that phospholipid binding is a universal property of all annexins, it will be interesting to discover whether an enzyme activity modulated by phospholipids is also a common feature of other plant and animal members of the family.

Together, our results suggest that when p34 and p35 are in soluble form in plant cells or are attached to actin filaments, they are also enzymes. In contrast, when the annexins are bound to phospholipids, such as the components of cellular membranes, their enzyme activity is inhibited. Future studies are required to understand better these biochemical properties within the context of their cellular function.

METHODS

Preparation of Plant Tissue and Purification of Annexin Proteins

Tomato (Lycopersicon esculentum cv Money Maker) plants were grown from seed on compost at a density of 35 seeds per tray, with a 16-hr photoperiod of light intensity of 580 μE m⁻² sec⁻¹. The temperature regime was 22°C (light) and 18°C (dark), with relative humidity at 60%. Root material was collected after 21 days.

Annexins were prepared from root tissue on the basis of their Ca²⁺-dependent phospholipid binding, as described by Smallwood et al. (1990, 1992). Purified annexins were exchanged into buffer appropriate for the experiment, using Bio-Rad P6.

Preparation of G-Actin and F-Actin

G-actin was prepared from rabbit muscle extracted to acetone powder (generously provided by John Sparrow, University of York, UK) similar to the method of Spudich and Watt (1971). G-actin was analyzed by SDS-PAGE and was found to consist of a single band at 42 kD (data not shown). F-actin was prepared by the polymerization of G-actin by the addition of 3 mM MgCl₂ and a 60-min incubation at 20°C. F-actin was recovered by a 3-hr centrifugation at 45,000 rpm in a Ti70.1 rotor (Beckman, High Wycombe, UK). The resulting pellet was resuspended in buffer (3 mM MgCl₂, 0.5 mM DTT, and 20 mM Tris-HCl, pH 8.0) and dialyzed overnight against 2 × 1 L of the same buffer.

Table 5. Effect of F-Actin on ATPase Activitya

<table>
<thead>
<tr>
<th>pH</th>
<th>Specific Activity (μmol Pi mg⁻¹ hr⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>p34, p35 Control</td>
</tr>
<tr>
<td>6</td>
<td>36.5 ± 4.2</td>
</tr>
<tr>
<td>7</td>
<td>28.5 ± 4.0</td>
</tr>
<tr>
<td>8</td>
<td>17.5 ± 2.1</td>
</tr>
</tbody>
</table>

a Before measuring ATPase hydrolytic activity, annexins were preincubated with F-actin for 30 min on ice in a reaction medium containing 1 mM Ca²⁺.
Table 6. Effect of Phospholipid on ATPase Activityx

<table>
<thead>
<tr>
<th>Phospholipid</th>
<th>ATPase Activity (μmol Pi mg⁻¹ hr⁻¹)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PS + EGTA</td>
<td>25.0 ± 2.8</td>
<td>0</td>
</tr>
<tr>
<td>PS + Ca²⁺</td>
<td>2.5 ± 0.7</td>
<td>90</td>
</tr>
<tr>
<td>BE + EGTA</td>
<td>23.2 ± 2.8</td>
<td>0</td>
</tr>
<tr>
<td>BE + Ca²⁺</td>
<td>7.0 ± 0.7</td>
<td>70</td>
</tr>
<tr>
<td>PC + EGTA</td>
<td>25.4 ± 1.4</td>
<td>0</td>
</tr>
<tr>
<td>PC + Ca²⁺</td>
<td>22.1 ± 2.5</td>
<td>13</td>
</tr>
</tbody>
</table>

x Annexins were preincubated with 50 μL of phospholipid-coated phenyl Sepharose (1 mg/mL brain extract VII, phosphatidylserine, or phosphatidylcholine) with 1 mM Ca²⁺ or EGTA. After 30 min on ice, 30 mM ATP was added to the reaction medium, and activity was measured as described. Phospholipids were removed from the assay medium prior to the determination of phosphate liberated. Percentage of inhibition is expressed as a value relative to the activity measured in the presence of EGTA.

Preparation of Polyclonal Antisera to p34 and p35

Purified annexins were exchanged into PBS buffer, lyophilized, and resuspended in a small volume of PBS (0.14 M NaCl, 80 mM Na₂HPO₄, 15 mM KH₂PO₄) for intramuscular injection into Wistar male rats, as a 1:1 mixture, with complete Freund's adjuvant for the initial inoculation and as a 1:1 mixture with incomplete Freund's adjuvant for the subsequent injections. After four immunizations over an 8-week period, the serum was collected. The antibody titer was determined by testing the immunoreactivity of serially diluted antisera. The antisera showed immunological reactivity against p34 and p35 on protein gel blots and dot blots and did not react with any other proteins on whole-cell extracts of tomato stems.

Preparation of Monoclonal Antibodies to Annexins p34 and p35

Hybridoma preparation and cloning by the limiting dilution procedure were performed essentially as described by Galfre and Milstein (1981). The myeloma cell line 1R983F was used for fusion with spleen cells. The cell line MRC5 (ICN-Flow, Costa Mesa, CA) was used as a feeder layer for the culture of fusion products and initial cloning steps. Culture supernatants from individual hybridoma wells were tested for immunoreactivity against immobilized annexins. Hybridomas that showed immunological reactivity against p34 and p35 were selected for further cloning and screening. The monoclonal DG12 was immunoreactive against a common epitope on p34 and p35.

Immunoprecipitation of Annexins

Annexins were mixed with anti-annexin antibody and incubated at 25°C with gentle shaking for 1 hr. Protein G Sepharose (Pharmacia) was then added, and the mixture was incubated for an additional hour under the same conditions. The protein G Sepharose, together with the antibody–annexin complex, was recovered by centrifugation at 2000g for 2 min.

Protein PAGE

Purified annexins (200 μL) were acetone precipitated at −20°C for 30 min, the protein was recovered by centrifugation for 10 min at 10,000g, and the pellet was resuspended in 30 μL of SDS-PAGE sample buffer (Laemmli, 1970). Samples were shaken for 45 min, followed by boiling for 5 min, and after centrifugation at 10,000g for 3 min, the final supernatants were loaded onto 0.75-mm-thick/10% SDS–polyacrylamide gels for electrophoresis (Laemmli, 1970). Native PAGE was performed in the same way but excluding SDS from all solutions.

Protein Immunoblotting

After separation, proteins were transferred to nitrocellulose membrane filters, using the method of Towbin et al. (1979). Actin was immobilized onto nitrocellulose using a Biodot apparatus (Bio-Rad), according to the manufacturer's instructions. The membrane used for immobilization was nitrocellulose with a 0.45-μm pore diameter, purchased from Whatman (Maidstone, UK). After adsorption of actin onto the membrane (final concentration of 3 μg per dot), the membrane was washed under vacuum with TBS (Tris-buffered saline; 20 mM Tris-HCl, pH 7.5, containing 0.5 M NaCl). The nitrocellulose filters were incubated for at least 2 hr at room temperature in blocking buffer (2% [w/v] BSA prepared in TBS containing 0.05% [w/v] Na₂SO₄). The blotted membrane was probed with annexin antisera in TBS for 2 hr at room temperature. After a 30-min wash in TBS, horsedardish peroxidase–conjugated anti-rat antibody (Sigma) was used to detect the primary antibodies. After 1 hr, the color reaction was developed using 4-chloronaphthol as the substrate.

Coupling of Actin to CNBr-Activated Sepharose

CNBr-activated Sepharose 4B was purchased from Pharmacia Biotech (St. Albans, UK) and was used in accordance with the manufacturer's instructions. Actin was diluted into coupling buffer (0.1 M NaHCO₃, pH 8.3, containing 0.5 M NaCl); 3 mM MgCl₂ was included in all buffers containing F-actin. Actin (8 mg) was coupled to 3 mL of Sepharose gel at 4°C, for 12 hr on a rocking platform. Excess ligand (generally <0.7 mg protein) was then washed away with 10 volumes of coupling buffer; the coupling efficiencies of F-actin and G-actin were approximately the same. Any remaining active groups on the Sepharose gel were blocked with 0.1 M Tris-HCl, pH 8.0, for 16 hr at 4°C. The final gel product was then washed three times in alternating cycles of 15 mL 0.1 M acetate buffer, pH 4.0, containing 0.5 M NaCl and 15 mL of 0.1 M Tris-HCl buffer, pH 8.0, containing 0.5 M NaCl.

Actin–Sepharose Binding Assays

Actin–Sepharose gel was packed into Poly-Prep columns (Bio-Rad) to a bed height of 0.6 mL, and equilibrated with an appropriate buffer. To determine whether annexins bound to G-actin, the matrix was equilibrated in 20 bed volumes of 5 mM bis-tris propane–2-(N-morpholino)ethanesulfonic acid buffer, pH 7.0, 0.5 mM CaCl₂, and 10 mM NaCl. For F-actin matrices, 3 mM MgCl₂ was added to the buffer. The annexin preparation (30 μg) was loaded, and the effluent was collected; elution with 2 mM EGTA followed. The matrices were developed at room temperature over 15 to 20 min. The fractions (200 μL) were acetone precipitated, resuspended in 25 μL of SDS–polyacrylamide sample buffer, and loaded onto 10% SDS–polyacrylamide gels. The gels were
then stained or protein gel blotted. Antibody detection of p34 and p35 was used to determine their elution pattern. Alternatively, the gel was used in 20-μL aliquots for low-speed cosedimentation assays. The binding assay was initiated by the addition of 10 μg of annexin, tubes were shaken for 20 min at room temperature, and the Sepharose was pelleted at 4000g for 2 min. Each pellet was then washed in 200 μL of the same buffer in which binding took place. The pellets were resuspended in 40 μL of SDS sample buffer, shaken for 20 min at room temperature, boiled for 4 min, and loaded directly onto a 10% SDS-polyacrylamide gel. The results shown are representative of many experiments.

**Measurement of ATPase Activity**

ATPase activity was measured by estimating phosphate liberation according to the method of Ames (1966). The reaction medium (total volume 200 μL) contained 3.0 mM MgCl₂, 50 mM KCl, 5 mM bis-tris propane–2-(N-morpholino)ethanesulfonic acid buffer at the selected pH and 10 μg of purified annexins; inhibitors were added to the reaction medium. The reaction was initiated by the addition of 30 mM ATP, mixed rapidly, and incubated at 37°C for 30 to 60 min. The reaction was stopped by the addition of 0.6 mL of ice cold Ames reagent (six parts 0.42% [w/v] ammonium molybdate prepared in 0.5 M H₂SO₄ to one part 10% [w/v] ascorbic acid). The color reaction was allowed to develop at room temperature for 30 min, and then absorbance was measured at 820 nm. Purity of each annexin preparation was always confirmed by SDS-PAGE analysis before use of the proteins in the assay.

To assess the effect of F-actin on annexin ATPase activity, the assay was performed as described, but with the inclusion of a preincubation step in which annexin was mixed with actin in a Ca²⁺-containing buffer and left on ice for 20 to 30 min. ATP was then added to the reaction medium to initiate the ATPase, and liberation of Pi was measured with Ames reagent. Before reading the absorbance at 820 nm, all tubes were centrifuged at 8000g for 1 min.

The effect of phospholipid on annexin ATPase activity was investigated by the addition of phospholipid-coated phenyl Sepharose with 1 mM Ca²⁺ to the reaction medium. After a 30-min incubation on ice (to allow the annexin to bind to the phospholipid), an ATPase assay was performed as described previously. At the end of the reaction time, the annexin–phospholipid–phenyl Sepharose complex was removed from the reaction medium by centrifugation at 2000g for 1 min, and 0.6 mL of Ames reagent was added to 200 μL of the supernatant. Blanks containing all of the assay constituents except ATP were performed for all experiments to assess the amount of endogenous Pi. ATP was added to the Pi calibration tubes to account for nonenzymatic breakdown of ATP. Results were adjusted to account for endogenous phosphate in the samples.

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