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A Dynamin-Like Protein, ADL1, Is Present in Membranes as a High-Molecular-Mass Complex in *Arabidopsis thaliana*[1]

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Dynamin, a GTP-binding protein, is involved in endocytosis in animal cells. We found that a dynamin-like protein, ADL1, is present in multiple forms in Arabidopsis leaf tissue. Subcellular fractionation experiments, together with gel-filtration and nondenaturing-gel electrophoresis revealed that most of ADL1 is present as a high-molecular-mass complex of 400 to 600 kD in the membrane or pellet fraction, whereas ADL1 is present in the soluble fraction as a monomer. The subcellular distribution of ADL1 is affected by various agents such as Ca^{2+}, cyclosporin A, GTP, and ATP. Ca^{2+} increases the amount of ADL1 present in the membrane fraction, whereas cyclosporin A inhibits the membrane association. Furthermore, Ca^{2+} and GTP change the migration pattern of ADL1 in nondenaturing polyacrylamide gels, indicating that these chemicals influence either the complex formation and/or the conformation of the ADL1 complex. Our results demonstrate that ADL1 has characteristics that are similar to Dynamin I, which is found in animal cells. Therefore, it is possible that ADL1 is also involved in biological processes that require vesicle formation.

Dynamin is a high-molecular-mass GTP-binding protein that is known to be required for endocytosis (van der Bliek and Meyerowitz, 1991; Herskovits et al., 1993a; Robinson et al., 1993; Damke et al., 1994). Many genes that belong to this family have been isolated and characterized in detail for various organisms ranging from yeasts to humans (Shpetner and Vallee, 1989; Horisberger et al., 1990; Obar et al., 1990; Rothman et al., 1990; Chen et al., 1991; van der Bliek and Meyerowitz, 1991; Yeh et al., 1991; Vater et al., 1992; Nakata et al., 1993; Wilsbach and Payne, 1993; Dombrowsky and Raikhel, 1995; Gu and Verma, 1996). The polypeptides of this family of proteins show a high degree of sequence similarity in their N-terminal regions, but only weak sequence similarity at their C terminus.

Analysis of the primary structure of Dynamin I shows that the protein is composed of multiple domains: a GTP-binding domain at the N terminus, a PH domain in the center, and an SH3-binding domain at the C terminus (Gout et al., 1993; Herskovits et al., 1993b; Seedorf et al., 1994). These domains are important for the regulation of GTPase activity and for the cellular distribution of dynamin in vivo. The GTPase activity of Dynamin I can be stimulated by phosphorylation through protein kinase C, by binding to microtubules and acidic phospholipids, and by interaction with a subset of SH3-containing proteins in vitro.

The importance of the intrinsic GTPase activity in the functioning of Dynamin I in endocytosis has been demonstrated by overexpressing a Dynamin I cDNA with mutations in the GTPase domain. The expressed mutant forms of Dynamin I disturb endocytosis in cultured cells (Herskovits et al., 1993b; van der Bliek et al., 1993; Damke et al., 1994). Another interesting feature of Dynamin I is its C-terminal PH domain. It has been suggested that PH domains are involved in the reversible anchoring of proteins to membranes via specific interaction with phospholipids (Hyvönen et al., 1995).

Lemmon et al. (1995) have shown that the PH domain of Dynamin I specifically interacts with PIP_2. The three-dimensional structure of this domain has been shown to be composed of seven β-sheets followed by an α-helix at the end (Ferguson et al., 1994). The cycle of phosphorylation and dephosphorylation of Dynamin I by protein kinase C and calcineurin, respectively, regulates the distribution of Dynamin I in vivo. The phosphorylated form appears to be soluble in the cytosol, whereas the dephosphorylated form is largely associated with membranes (Liu et al., 1994a, 1994b). Membrane association of Dynamin I must, therefore, be a critical step in endocytosis.

In situ cytohistochemistry has shown that, in vivo, Dynamin I is specifically localized at the plasma membrane, especially at the neck of invaginated membranes (Hinshaw and Schmid, 1995; Takei et al., 1995). Various experiments have shown that dynamin exists in high-order oligomeric forms that range from dimers to tetramers, depending on the conditions of the experiment (Hinshaw and Schmid, 1995; Tuma and Collins, 1995). Agents such as membrane vesicles and microtubules, which provide a multivalent surface, stimulate self-polymerization of Dynamin I, which may activate GTPase activity (Warnock et al., 1996). Other related proteins, Vps1p found in yeast and SDL found in

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Abbreviation: PH, Pleckstrin homology.

soybean (*Glycine max*), are also membrane associated (Rothman et al., 1990; Gu and Verma, 1996).

Recently, Gu and Verma (1997) have succeeded in cross-linking a high-molecular-mass complex of the soybean SDL. It is not yet clear whether these dynamin-like proteins share other characteristic features, such as the regulation of their GTPase activity by phosphorylation and the self-polymerization upon binding to membranes.

In this study we investigate the biochemical properties of ADL1, a dynamin-like protein from Arabidopsis. We show that ADL1 is present in different compartments, such as the cytosol and the membranes of cells. We also show that ADL1, when associated with membranes, exists in a high-molecular-mass protein complex.

MATERIALS AND METHODS

Growth of Plants and Preparation of Plant Extracts

Arabidopsis plants (*Arabidopsis thaliana* ecotype Columbia) were grown either on Murashige-Skoog plates at 20°C in a culture room or in a greenhouse under conditions of 70% RH and a 16-h/8-h light/dark cycle. Various parts of the plants were harvested and frozen immediately in liquid N_2.

Preparation of Membrane Fractions

Plant tissues of leaf, root, silique, and flower (5–10 g fresh weight) were ground in liquid N_2 and homogenized in 2 volumes (w/v) of homogenization buffer I (25 mM Hepes-Bis Tris propane, pH 7.5, 1.0 mM DTT, 1 mM $MgCl_2$, 250 mM Suc, 3 ng/mL pepstatin A, and 3 ng/mL leupeptin) using a polytron homogenizer at 4°C. The homogenates were fractionated according to the method of Hsieh et al. (1991). The total homogenate was centrifuged at 8,000*g* twice for 20 min each. The clarified supernatant was loaded onto a Suc step gradient (6 mL of 15%, 6 mL of 26%, and 8 mL of 35% Suc) containing 2.5 mM Hepes-BTP, pH 7.2, 1.0 mM DTT, and 1.0 mM $MgCl_2$. The sample was centrifuged at 70,000*g* for 2 h and the fractions, top, 26/35 interface, and pellet, were collected. The membrane fraction (26/35 interface) was diluted four times with buffer I, and centrifuged at 100,000*g* for 1 h. The pellet from this step (the membrane fraction) was then resuspended in buffer I. The pellet fraction of the step gradient was also resuspended in buffer I. In certain cases, the homogenate was fractionated on a linear Suc gradient (5–50%) in the presence or absence of Triton X-100. The presence of ADL1 in the fractions collected from this gradient was probed for with anti-ADL1 antibody.

To study the association between protein and membranes, an aliquot of the membrane fraction (the 26/35 interface) containing 50 μg of protein was incubated in the presence of either 0.5 or 1.0 M NaCl, or 0.1 M Na_2CO_3, pH 11.5, for 30 min on ice. To observe the effect of GTP and ATP on the membrane association of ADL1, the membrane fraction was incubated with various concentrations of GTP and ATP (Pharmacia) for 1 h on ice. Subsequently, proteins associated with membranes were pelleted by ultracentrifugation at 100,000*g* for 1 h. Pellet and supernatant fractions were collected separately, and the pellets were resuspended in their original volume in buffer L (2.5 mM Hepes-BTP, pH 7.2, 1.0 mM DTT, and 1.0 mM $MgCl_2$). The protein in the supernatant was precipitated with TCA and also redissolved in the original volume. Equal volumes of the samples were used for immunodetection of ADL1.

For treatment with Ca^{2+} and cyclosporin A, leaf tissue was homogenized in buffer I, and homogenates were incubated with various concentrations of Ca^{2+} or cyclosporin A at 4°C for 1 h and applied to a Suc step gradient to separate the membrane-bound ADL1 from the soluble form. Protein concentrations were determined by the Bradford method using the protein assay reagent from Bio-Rad (Bradford, 1976).

Western Analysis

For western analysis we prepared a polyclonal antibody against a truncated form of ADL1 (ADL1-N). ADL1-N was expressed in *Escherichia coli* as a recombinant protein using the expression vector pRSET-B (Invitrogen, San Diego, CA). An ADL1 cDNA fragment containing approximately 300 amino acid residues from the N-terminal region was isolated after digestion with the restriction endonuclease *Xba*I, and introduced into pRSET-B. Expression of the recombinant protein was induced with 0.4 mM isopropyl β-D-thiogalactopyranoside in BL21(DE3)LysS cells at 28°C for 5 h. The overexpressed protein was then purified by Ni^+-nitrilotriacetic acid affinity column chromatography (Qiagen, Chatsworth, CA) following the manufacturer's protocol.

The purified protein was injected into a rabbit to raise antibody according to a published protocol (Harlow and Lane, 1988). For western analysis 30 μg of protein was separated by 10% SDS-PAGE (Laemmli, 1970). The proteins were then blotted onto a PVDF membrane (Immobilon, Millipore) by the semidry blotting method (Harlow and Lane, 1988). Immunostaining was carried out according to published protocols (Harlow and Lane, 1988) using the polyclonal antibody raised against the N-terminal region of ADL1 (ADL1-N) as the primary antibody and horseradish peroxidase-conjugated anti-rabbit IgG antibody (Amersham) as the secondary antibody. The blots were developed with a detection system (ECL, Amersham) for western analysis.

Nondenaturing PAGE

Nondenaturing PAGE was carried out according to a published protocol (Chrambach et al., 1976) using a 7% polyacrylamide gel. The separated proteins were then blotted onto a nylon membrane and probed with the anti-ADL1 antibody described above. To test the effects of various agents on the mobility of ADL1, the homogenate was incubated in the presence of Ca^{2+} and GTP on ice for 1 h.

Gel-Filtration Chromatography

The homogenate (2–4 mg of total protein) or the fractions obtained from the Suc step gradient (containing approxi-

mately 1 mg of protein in the membrane or pellet fraction and 1–2 mg of soluble protein in the top fraction) were applied to a Sepharose CL 6B column (120 × 1.5 cm) preequilibrated with a column buffer (10% glycerol, 50 mM Tris-Cl, pH 7.5, 2 mM EDTA, pH 8.0, 1 mM $MgCl_2$, 100 mM NaCl, 1 mM PMSF, and 1% Triton X-100). One-milliliter fractions were collected at a flow rate of 0.5 mL/min. The proteins contained in 500 μL of each fraction were concentrated by the cold TCA precipitation method, and then tested for the presence of ADL1 by western analysis. The Rubisco complex (560 kD), β-amylase (200 kD), and BSA (66 kD) were used as molecular mass standards (Sigma).

Alkaline Phosphatase Treatment

Alkaline phosphatase treatment was performed according to the method of Sarokin and Chua (1992), with minor modifications. Before carrying out the phosphatase treatment we separated the two protein species, monomer and multimer, using gel-filtration column chromatography. The homogenate was fractionated using the Sepharose CL 6B column, and the fractions were analyzed as described above. The fractions containing ADL1, which eluted around 68 kD, were pooled and the protein was concentrated. Fifty micrograms of protein was treated with calf intestinal alkaline phosphatase (50 or 100 units/mL; Sigma) in a 50-μL reaction volume for 10 min at room temperature in the presence or absence of 100 mM NaF. The proteins were separated by SDS-PAGE, and western analysis was carried out.

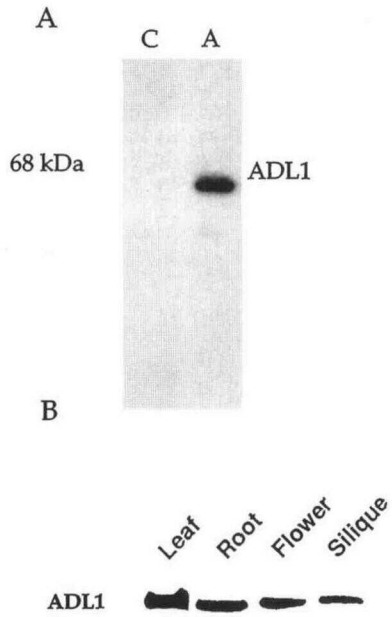

Figure 1. Levels of ADL1 in various tissues. A, Specificity of the polyclonal anti-ADL1 antibody. C, Control serum; A, anti-ADL1 antibody. The control serum was obtained from the same rabbit before immunization. The anti-ADL1 antibody was affinity-purified using the purified antigen (ADL1-N). Thirty micrograms of leaf tissue extracts was used for immunodetection with the anti-ADL1 antibody. B, Presence of ADL1 in various tissues. Thirty micrograms of total protein extracts was size-separated by SDS-PAGE, transferred onto a membrane, and immunostained for ADL1 using a polyclonal antibody raised against a truncated form of ADL1 (ADL1-N).

RESULTS

ADL1 Is Associated with Membranes

Dynamin is a GTPase required for endocytosis in animal cells. To further our understanding of the biological processes involved, we first set out to clone genes encoding polypeptides with sequence similarity to dynamin. By screening a λ-Zap II cDNA library constructed with poly(A^+) RNA prepared from Arabidopsis leaf tissues, we isolated a cDNA clone that we called *ADL1*. It turned out that the nucleotide sequence of the ADL1 cDNA was identical to the published sequence of the *aG68* cDNA of Arabidopsis (Dombrowski and Raikhel, 1995).

In an attempt to investigate the biological role of a dynamin-like protein in plants, we initiated the characterization of ADL1, the dynamin homolog in *A. thaliana*. Western analysis was carried out on protein extracts from Arabidopsis using an antibody against the N-terminal region (approximately 300 amino acid residues from the initiation codon). The truncated form of ADL1 was expressed in *E. coli* and used to raise antibody in rabbit. As shown in Figure 1A, the polyclonal antibody recognized specifically a protein species that migrated with an apparent molecular mass of 68 kD, the expected size based on the calculated molecular mass of ADL1 (Dombrowski and Raikhel, 1995). Also, the immunoreacting protein species migrated to the same position as the in vitro translated product of the ADL1 cDNA in wheat germ extract (data not shown). The control serum obtained from the same rabbit before immunization did not show any immunostaining. To detect the presence of this protein species in other tissues, we prepared protein extracts from root, siliques, and flowers, and probed them with the ADL1 antibody. As shown in Figure 1B, the same protein species was detected in each of these protein extracts, which indicates that the ADL1 gene may be ubiquitously expressed in Arabidopsis.

It is known that Dynamin I is associated with the plasma membrane in neuronal cells in the rat brain (Tuma et al., 1993). However, the yeast homolog Vps1p, which is involved in vacuolar protein sorting, is thought to be present in the Golgi membrane (Rothman et al., 1990). Therefore, we investigated the cellular distribution of ADL1 using leaf tissue. Homogenates prepared from leaf tissue were fractionated on a Suc step gradient into top (containing soluble proteins), membrane (26/35 interface), and pellet fractions (Hsieh et al., 1991). The presence of ADL1 in these fractions was examined by western analysis using anti-ADL1 antibody. Immunoreacting protein species were detected in all fractions, as seen in Figure 2. However, the intensity of the bands varied among these fractions. The membrane and pellet fractions had at least 10-fold stronger band intensities compared with the top fraction.

To assess the purity of each fraction, we probed them with antibodies for soluble and membrane proteins. An anti-SPK4 antibody raised against a soybean protein ki-

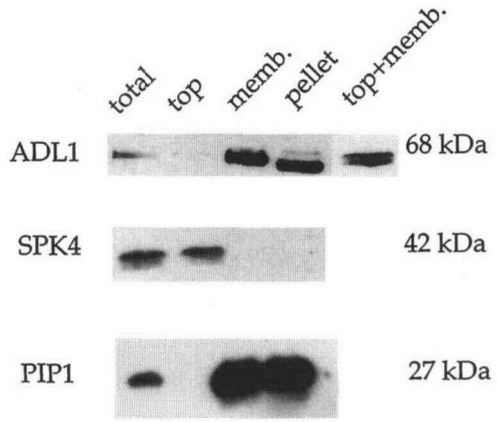

Figure 2. Cellular distribution of ADL1. Leaf extracts were fractionated into top (soluble), membrane (26/35 interface), and pellet fractions using a Suc step gradient, as described in "Materials and Methods." The proteins (30 μg for the total extract and top fraction, and 15 μg for the membrane [memb.] and pellet fractions) were separated by 10% SDS-PAGE and subjected to immunodetection using anti-ADL1 antibody. total, Leaf extract before fractionation on the Suc step gradient; top, 26/35%, and pellet, fractions obtained from the gradient. To clarify the difference in the migration pattern between the protein in the top fraction and the membrane fraction, an aliquot of the top fraction was mixed with an aliquot of the membrane fraction and then separated by 10% SDS-PAGE (top+memb.). The anti-PIP1 antibody raised against PIP1, a plasma membrane small-channel protein, and anti-SPK4 antibody raised against SPK4, a soluble soybean protein kinase, were used as efficiency controls for the fractionation by the Suc step gradient.

nase, SPK4 (Yoon et al., 1997), was used as a marker for soluble proteins. Also, a polyclonal anti-PIP1 antibody (a gift of Dr. Schaffner, Ludwig-Maximilians-Universität) raised against a plasma membrane small-channel protein, PIP1 (Kammerloher et al., 1994), was used as a marker for membrane proteins. When these fractions were examined with the anti-SPK4 antibody, a cross-reacting protein was detected only in the total cell extract and the top fraction, as shown in Figure 2. The membrane protein PIP1a was highly enriched in the membrane fraction as well as the pellet fraction, but there was no detectable level of PIP1 in the top fraction. Therefore, we must assume that the pellet fraction may also contain plasma membranes associated with certain organelles.

In addition to the differential cellular distribution of ADL1, the position of the protein band in the lane for the top fraction was slightly different from the corresponding ones in the membrane and the pellet lanes (Fig. 2). To make the difference in the migration pattern of the bands more discernible, an aliquot of the top fraction was mixed with an aliquot of the membrane fraction, and the migration of the proteins were compared in one lane. As seen in Figure 2, the mixture clearly resolves two protein species that are detected by the antibody.

In animal cells Dynamin I exists in a soluble form and a membrane-bound form. The soluble form of Dynamin I becomes phosphorylated in the C-terminal region by protein kinase C, whereas the membrane-bound form remains unphosphorylated (Liu et al., 1994a). Therefore, it seemed possible that the two immunoreacting protein species in Arabidopsis leaf extracts were two forms of ADL1, one of which was posttranslationally modified. As in the case of dynamin, one possible modification is phosphorylation. To investigate this possibility, we attempted to detect a change in the mobility of the protein in an SDS gel after removal of one or more anticipated phosphate groups by treatment of the slower-migrating protein species with phosphatase.

We separated the slower-migrating protein from the faster-migrating protein using Sepharose column chromatography. The slower-migrating protein species eluted around 68 kD from the Sepharose CL 6B column, whereas the faster-migrating one eluted as a high-molecular-mass protein complex (see below for details). The proteins in the fractions containing the 68-kD proteins were concentrated and treated with alkaline phosphatase. As shown in Figure 3, the alkaline phosphatase treatment resulted in a mobility shift of the immunoreacting protein, which now migrated at nearly the same position as the faster-migrating protein species. In the control sample, in which NaF, an inhibitor of alkaline phosphatase, was included in the reaction mixture, there was no change in the migration pattern. Phosphorylation of a protein has been shown to retard migration of the protein in SDS-PAGE (Bai and Weigel, 1996), and when phosphate groups are removed from proteins by phosphatase, the proteins reveal the change in phosphorylation in their electrophoretic mobility in SDS gels.

Since the hydropathy profile of the primary amino acid sequence of ADL1 did not reveal any membrane-spanning regions, we examined the nature of the membrane association of ADL1 by treating the membrane fraction (26/35 interface) with 0.5 or 1.0 M NaCl, or 0.1 M Na_2CO_3, pH 11.5. The proteins solubilized under these conditions were removed by ultracentrifugation, and the pellet was washed once with suspension buffer. The pellet and supernatant were then analyzed for the presence of ADL1 by western analysis. As seen in Figure 4, the majority of ADL1 washed away with 0.1 M Na_2CO_3, pH 11.5, indicating that ADL1 is a peripheral protein.

Membrane Association of ADL1 Is Affected by Ca^{2+}, Cyclosporin A, GTP, and ATP

It has been shown that phosphorylation is an important regulatory mechanism for the cellular distribution of Dynamin I. Agents such as Ca^{2+}, GTP, and ATP also influence the membrane association of Dynamin I (Robinson et al., 1993; Liu et al., 1994a; Liu et al., 1994b). To investigate whether ADL1 has similar characteristics, we examined the membrane association of ADL1 in vitro in the presence of Ca^{2+} and cyclosporin A (an inhibitor of calcineurin). Cell extracts were incubated with these agents on ice or at room temperature and then fractionated on a Suc step gradient. The membrane fraction was collected and assayed for the presence of ADL1 using western analysis. As shown in Figure 5, the amount of ADL1 in the membrane fraction increased in the presence of Ca^{2+} in a concentration-dependent manner, whereas cyclosporin A, an inhibitor of calcineurin, inhibited the membrane association of ADL1. These results are similar to the behavior of dynamin in

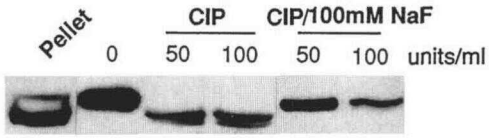

Figure 3. Alkaline phosphatase treatment affects the mobility of ADL1. Protein fractions containing only the slower-migrating protein species were obtained by gel filtration. The protein sample was treated with calf intestinal alkaline phosphatase (CIP) in the presence or absence of 100 mM NaF and immunoanalyzed using the anti-ADL1 antibody. The numbers above each lane indicate the amount of calf intestinal alkaline phosphatase used in units per milliliter.

animal cells (Robinson et al., 1993; Liu et al., 1994a; Liu et al., 1994b). In the case of Dynamin I, it has been shown that calcineurin is responsible for dephosphorylation of phosphorylated Dynamin I.

We also investigated the effect of GTP and ATP on the membrane association. The membrane fraction was treated with GTP and ATP on ice and proteins associated with membranes were pelleted by ultracentrifugation. As shown in Figure 6, the amount of ADL1 present in the membrane fraction was significantly reduced, whereas the amount of ADL1 in the supernatant had increased, indicat-

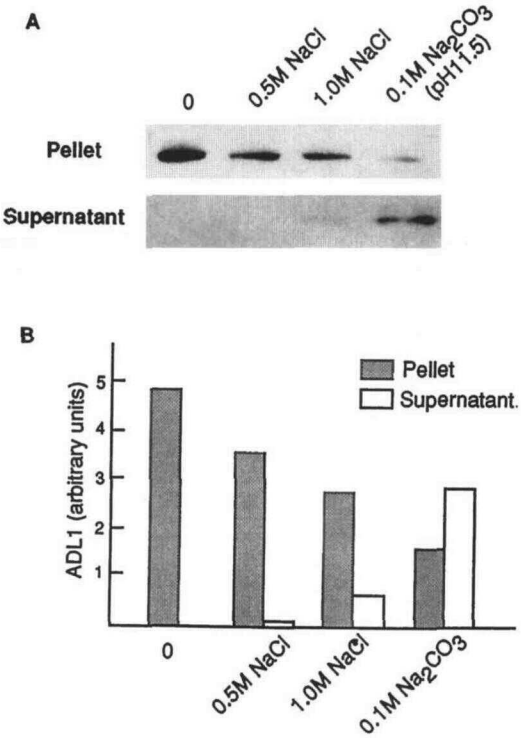

Figure 4. Membrane association of ADL1. The membrane fraction obtained at the 26/35% interface of the Suc step gradient was incubated in the presence of the indicated reagents at 4°C with gentle agitation for 1 h, and then subjected to ultracentrifugation to separate soluble and membrane-bound proteins. The pellets were resuspended in the original volume. Protein in the supernatant was concentrated by TCA precipitation and redissolved in the original volume. Equal volumes of the samples were loaded on a 10% SDS/PAGE. A, Autoradiogram of the western analysis. B, Densitometric measurement of the band intensity.

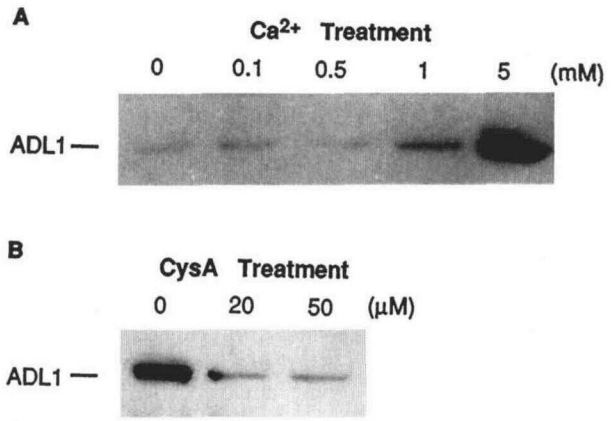

Figure 5. The effect of Ca^{2+} and cyclosporin A on the membrane association of ADL1. Cell extracts were prepared in the presence of various concentrations of $CaCl_2$ (A) and cyclosporin A (CysA) (B) and incubated on ice for 1 h. The membrane-bound and soluble proteins were separated on a Suc step gradient. Equal amounts of protein derived from the membrane fraction (15 μg/lane) were separated and subjected to immunodetection with anti-ADL1 antibody.

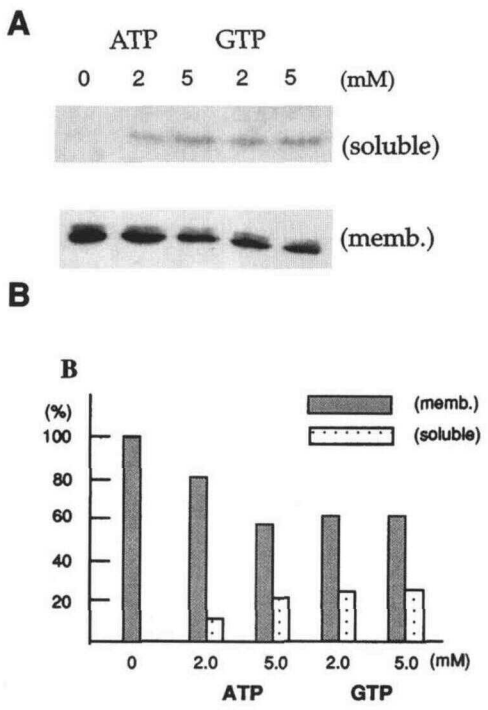

Figure 6. Effects of ATP and GTP on the membrane association of ADL1. The membrane fraction obtained from the Suc step gradient was incubated for 1 h on ice with various concentrations of ATP or GTP, and then fractionated into soluble and membrane-bound proteins by ultracentrifugation at 100,000*g* for 1 h. The presence of ADL1 in the supernatant and pellet was analyzed by immunodetection using anti-ADL1 antibody. The proteins in one-half of the supernatant were TCA-precipitated and used for immunodectection. A, Autoradiogram of the supernatant (containing solublilized proteins) and the pellet fraction (memb.; containing membrane-bound proteins). B, Densitometric measurement of the band intensities.

ing that membrane-bound ADL1 became solubilized by the treatment.

Formation of a High-Molecular-Mass Complex

Dynamin I has been shown to form an aggregate (Hinshaw and Schmid, 1995; Takei et al., 1995). The formation of the high-molecular-mass aggregate has been proposed as a mechanism for the regulation of its intrinsic GTPase activity (Carter et al., 1993; Hinshaw and Schmid 1995; Takei et al., 1995). We examined the possibility of complex formation of ADL1 by fractionating cell extracts on a Suc density gradient. Triton X-100 was added to the gradient to eliminate the effect of membranes on the migration of ADL1. Fractions of the gradient were then assayed for the presence of ADL1 by western analysis. As shown in Figure 7, ADL1 migrated to a narrow region between approximately 15% (fraction number 32) and 20% (fraction number 22) Suc in the 5 to 50% continuous Suc gradient. As a control we ran the same Suc gradient without Triton X-100.

As seen in Figure 7, in the absence of Triton X-100, ADL1 was distributed over a broad region ranging from approximately 15% (fraction number 32) to 40% (fraction number 6) Suc. The difference in the migration pattern of ADL1 between the two gradients was likely due to the association of ADL1 with membranes. However, it is interesting that even in the presence of Triton X-100 the majority of the protein migrated to the position corresponding to approximately 15 to 20% Suc in the gradient. Only a minor portion of the ADL1 pool was found in the top fraction. This suggests that ADL1 may continue to exist in a high-molecular-mass complex even after solubilization of membranes with Triton X-100. To confirm this, we employed nondenaturing PAGE. Proteins were separated in a 7%

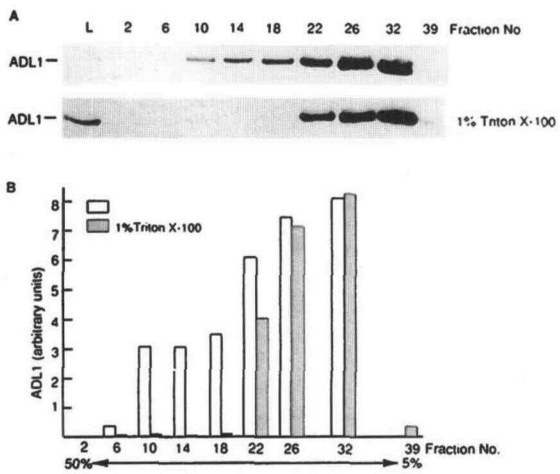

Figure 7. Fractionation of ADL1 on continuous Suc gradients. Proteins of total leaf extracts were fractionated using linear Suc gradients (5–50%) in the presence or absence of 1% Triton X-100. One-milliliter fractions were collected. The protein in 500 μL of each fraction was TCA-precipitated and separated by SDS-PAGE. The presence of ADL1 in these fractions was immunodetected with anti-ADL1 antibody. A, Autoradiogram of the western analysis. B, Densitometric measurement of the band intensity.

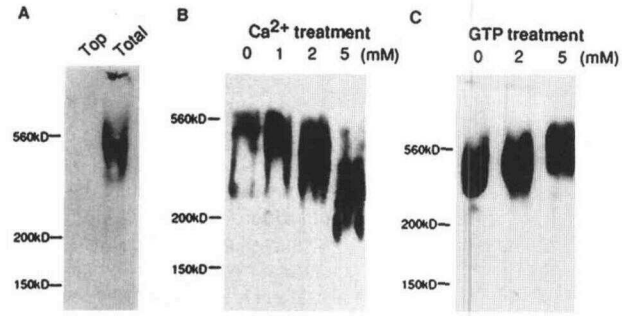

Figure 8. Formation of a higher-molecular-mass complex of ADL1. Proteins of total leaf extract (20 μg of protein) and proteins in the top fraction (20 μg of protein) of the Suc gradient were separated by a 7% nondenaturing PAGE and probed for the presence of ADL1 with anti-ADL1 antibody (A). The total leaf extract was incubated in the presence of various concentrations of Ca^{2+} (B) or GTP (C), separated by nondenaturing PAGE, and probed with anti-ADL1 antibody. The size markers used for the gel were the Rubisco complex (560 kD), β-amylase (200 kD), and alcohol dehydrogenase (150 kD).

polyacrylamide gel under nondenaturing conditions. As shown in Figure 8A, the immunostaining protein species in the cell extract migrated with a size of 400 to 600 kD, confirming the notion of complex formation.

In an attempt to characterize the complex formation we incubated the protein extract in the presence of agents that have a known effect on the membrane association of ADL1. As seen in Figure 8B, ADL1 in the Ca^{2+}-treated sample migrated faster than in the control sample and in a concentration-dependent manner. The effect of GTP on the mobility of the protein complex was also examined. As seen in Figure 8C, GTP treatment resulted in retardation of the complex migration under nondenaturing conditions. These results clearly indicate that ADL1 is present as a high-molecular-mass complex and that the complex formation or the conformation of the complex is influenced by agents such as Ca^{2+} and GTP. However, we do not know whether the protein complex is a homocomplex or a heterocomplex. In the case of Dynamin I, the purified protein has a tendency to form high-molecular-mass complexes in vitro that result in the activation of the intrinsic GTPase activity (Warnock et al., 1996). We can, therefore, anticipate that ADL1 may form a homocomplex.

ADL1 Is Present in a High-Molecular-Mass Complex

Data from the Suc gradient and native gel electrophoresis suggest that ADL1 exists in a high-molecular-mass complex in vivo. We investigated whether there is any correlation between cellular distribution and high-molecular-mass complex formation. Proteins from the top of the Suc step gradient, the membrane, the pellet fractions of the gradient, and the cell extracts were separated by gel-filtration column chromatography using Sepharose CL 6B. To remove the effect of membranes associated with ADL1 on the elution profile, membrane and pellet fractions were treated with 1% Triton X-100 before loading onto the column. Also, the column was preequilibrated with 1% Triton X-100. Each fraction eluted from the column was then

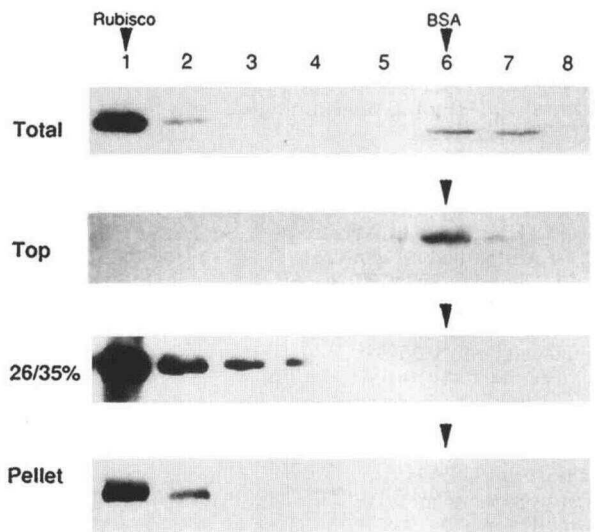

Figure 9. Gel-filtration column chromatography separates two forms of ADL1. Total leaf extract (2–4 mg of protein) and fractionated extracts (equivalent to approximately 1 mg of protein for the membrane and pellet fractions and 2 to 4 mg of protein for the top fraction) obtained from the Suc step gradient were size-fractionated by gel-filtration chromatography using Sepharose CL 6B. One-milliliter fractions were collected, and the proteins in 500 μL of each fraction were TCA-precipitated, separated by SDS-PAGE, and then analyzed for the presence of ADL1 by immunostaining with anti-ADL1 antibody. The immunoblots with the total extract and the membrane and pellet fractions were exposed to film for 5 min; the immunoblot containing the top fraction was exposed for 30 min. The positions of BSA (66 kD) and the Rubisco complex (560 kD) are indicated.

analyzed by western blotting and by probing for the presence of ADL1.

As shown in Figure 9, the major portion of the ADL1 protein pool in the total protein extract eluted right after the void volume at nearly the same position as the Rubisco complex, the molecular mass of which is known to be approximately 560 kD. Only a minor amount of ADL1 eluted at a position close to BSA, a position expected based on the calculated molecular mass of ADL1. ADL1 in the membrane and pellet fractions eluted at the position of the Rubisco complex. However, ADL1 in the top fraction of the gradient eluted at the position of BSA, i.e. as an ADL1 monomer. These results clearly indicate that ADL1 in the membrane and pellet fractions is part of a high-order complex, whereas ADL1 is present in the soluble fraction as a monomer.

DISCUSSION

We studied the biochemistry of a dynamin-like protein of Arabidopsis, ADL1, using western analyses. Subcellular fractionation studies revealed that a major portion of ADL1 was present in insoluble fractions, such as the membrane and pellet fraction of a Suc step gradient. However, there was still a small amount of ADL1 present in the soluble fraction. ADL1 present in the insoluble and the soluble fraction exhibited different migration rates in SDS-PAGE, suggesting the possibility of posttranslational modification. When the slower-migrating protein species of the soluble fraction was treated with alkaline phosphatase, it migrated to nearly the same position as the protein species in the pellet fraction. One possible explanation for the change in mobility would be the removal of one or more phosphate groups from ADL1 by the alkaline phosphatase treatment. At a minimum, our results suggest the possibility of phosphorylation of ADL1, as is the case with Dynamin I from rat.

It has been suggested that the membrane association of Dynamin I is mediated by the PH domain. Thus, the membrane association of ADL1 hints at the possibility that ADL1 may also have a domain similar to the PH domain. It is very difficult to define the PH domain from the primary structure because of the low level of amino acid sequence conservation among PH domains (Gibson et al., 1994). Ca^{2+} and cyclosporin A, which represent an activator and an inhibitor of calcineurin, respectively, had opposite effects on the membrane association of ADL1. Ca^{2+} stimulated membrane association, whereas cyclosporin A inhibited it. These results imply that a calcineurin-like phosphatase may be involved in the regulation of the cellular distribution of ADL1 in Arabidopsis, as is the case with animal dynamin. Furthermore, nucleotides such as GTP and ATP decreased the amount of ADL associated with the membrane.

In addition to membrane association, ADL1 displayed another characteristic feature of Dynamin I, the formation of a higher-order complex (Hinshow and Schmid, 1995). The self-assembly of Dynamin I into a high-molecular-mass complex is promoted by the presence of lipid membranes. However, purified dynamin has a tendency to form aggregates in vitro, even in the absence of membranes, when it is at a high concentration.

It has recently been shown that the soybean homolog of dynamin, phragmoplastin, can be cross-linked in the form of a high-molecular-mass complex (Gu and Verma, 1997). In this study we demonstrated similar characteristic features for ADL1 by taking three independent approaches: Suc-density gradient, native PAGE, and gel-filtration chromatography. The nondenaturing acrylamide gel electrophoresis showed that ADL1 migrates with a molecular mass of 400 to 600 kD. The mobility of ADL1 in the native gel changes in the presence of Ca^{2+} or GTP. The ADL1 complex migrates faster in the presence of Ca^{2+}. However, the same complex migrates slower in the presence of GTP. In the case of Dynamin I, it has been suggested that the protein self-polymerizes into a high-molecular-mass complex on the surface of lipid membranes. The polymerized complex then undergoes conformational changes upon binding and hydrolysis of GTP, and finally dissociates from the membrane to become the soluble form (Tuma and Collins, 1995). In ADL1, GTP may have the same effect on the conformation of the complex and its dissociation from the membrane.

We also examined the relationship between complex formation and cellular distribution. When we examined the gel-filtration pattern of ADL1 in the membrane or pellet fraction of the Suc gradient, the majority eluted at a position similar to the Rubisco complex, the size of which is known to be approximately 560 kD. In contrast, the soluble

ADL1 present in the top fraction of the Suc gradient eluted at the position of BSA, which coincides with the expected position of ADL1 as a monomer. Taken together with the results of the alkaline phosphatase treatment, these data further support the possibility that ADL1 in the soluble fraction is phosphorylated and exists as a monomer, whereas the membrane-bound form may be unphosphorylated and present in a high-molecular-mass complex.

It has been suggested that dynamin plays a role in the severing of the neck of invaginated plasma membranes during the vesicle-formation process (Hinshow and Schmid, 1995; Takei et al., 1995). During this process dynamin accumulates at the invaginated membranes, which leads to the formation of a high-molecular-mass complex. Self-assembly of dynamin activates its intrinsic GTPase activity, which is critical for its function in vivo. Thus, the activation of the GTPase activity of dynamin may be an important step toward severing the neck of invaginated membranes.

Although in this study we did not investigate the relationship between GTPase activity and high-molecular-mass complex formation in ADL1, the similarity between ADL1 and dynamin with regard to membrane association and the formation of high-molecular-mass complexes suggests that ADL1 may also be involved in similar biological processes in plants. However, the existence of endocytosis in plants has not yet been conclusively established, although the presence of many protein components involved in endocytosis has been demonstrated at the molecular level. It has been proposed that the dynamin-like protein found in soybeans, SDL, is involved in the transport of materials at the early stage of plate formation during cell division, a process different from endocytosis (Gu and Verma, 1996, 1997). It is possible that ADL1 may also play a role in the process of vesicle formation.

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