Arabidopsis dynamin-related protein 1A polymers bind, but do not tubulate, liposomes

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The Arabidopsis dynamin-related protein 1A (AtDRP1A) is involved in endocytosis and cell plate maturation in Arabidopsis. Unlike dynamin, AtDRP1A does not have any recognized membrane binding or protein–protein interaction domains. We report that GTPase active AtDRP1A purified from Escherichia coli as a fusion to maltose binding protein forms homopolymers visible by negative staining electron microscopy. These polymers interact with protein-free liposomes whose lipid composition mimics that of the inner leaflet of the Arabidopsis plasma membrane, suggesting that lipid-binding may play a role in AtDRP1A function. However, AtDRP1A polymers do not appear to assemble and disassemble in a dynamic fashion and do not have the ability to tubulate liposomes in vitro, suggesting that additional factors or modifications are necessary for AtDRP1A’s in vivo function.

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Introduction

The Arabidopsis dynamin-related protein 1A (AtDRP1A) is a member of the dynamin superfamily of GTPases that plays a critical role in Arabidopsis development [1–5]. It is essential for proper maturation of the cell plate during cytokinesis [3,4,6], and recent studies have also suggested that it functions like dynamin in Clathrin-Mediated Endocytosis (CME) [5]. Dynamin is the founding and best characterized member of the dynamin superfamily, and plays both early regulatory and late mechanical roles in the formation and severing of clathrin-coated vesicles from the plasma membrane (PM) [7]. During CME, dynamin activity at endocytic buds is regulated by a combination of its pleckstrin homology (PH) domain, which binds the signaling phospholipid PI(4,5)P2, and its proline rich domain (PRD), which binds other endocytic proteins. Neither of these domains, nor any other recognized lipid- or protein-binding domains, are present in AtDRP1A, raising the question of how AtDRP1A is targeted and regulated during endocytosis and cytokinesis.

Subcellular fractionation studies have revealed that AtDRP1A is a peripheral membrane protein that is predominantly present as a high molecular weight protein complex [3,8]. However, it has not been determined whether AtDRP1A binds directly to membranes or indirectly via other protein partners, and whether the high molecular weight complexes are homopolymers of AtDRP1A or multi-protein complexes. The soybean homolog of AtDRP1A, GmDRP1 (Phragmoplastin), was reported to form a homopolymer when purified from E. coli as a glutathione-S-transferase (GST) fusion protein, and two self-interaction domains were identified by yeast-two-hybrid and in vitro binding studies [9]. However, GST-GmDRP1 was purified under denaturing conditions, and was not demonstrated to have GTPase activity, limiting its utility for biochemical characterization.

Here we present the in vitro characterization of GTPase active, E. coli expressed, AtDRP1A, including evidence of its inherent self-interaction and lipid-binding ability. Significantly, purified AtDRP1A behaves very differently than purified dynamin, and in ways that are difficult to reconcile with what is known of its in vivo activity, suggesting that additional factors or modifications are needed for AtDRP1A to function.

Materials and methods

General reagents. All reagents were purchased through Fisher Scientific (Pittsburg, PA) unless otherwise noted. SDS–PAGE and immunoblotting conditions and α-DRP1A antibodies are described in [3] with the exception that Supersignal West Pico (Pierce, USA) was used.
Rockford, IL) was used as the chemiluminesence substrate for detection of the HRP-labeled secondary antibodies. Rabbit α-MBP antibodies were purchased from Immunology Consultants Laboratories (Newberg, OR). Spotted lipid assays were performed as described by Dowler et al. [10]. All oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA) and PCR amplification was performed with PhuUltra (Stratagene, La Jolla, CA).

Generation of the His6-MBP-AtDRP1A expression clone. The AtDRP1A coding sequence (full length, including stop codon) was PCR amplified using primers 5'-ggggacaagtttgtacaaaaaagcaggctcaatg gaaaatctgatctctctggttaa-3' (forward) and 5'-ggggacactttggaaga aagtgtggtatctgacagcaaaagcaggctcaatg-3' (reverse), which introduced attB1/attB2 recombination sites at the ends of the gene. The PCR product was inserted into the plasmid pGEM-T-EASY (Promega, Madison WI) by TA cloning and then recombined into pDONR201 (Invitrogen, Carlsbad, CA) using standard Gateway cloning procedures (Invitrogen, Carlsbad, CA). A DNA sequence encoding the Tobacco-Etch Virus (TEV) cleavage site was inserted with shaking at 18°C (16 h) with shaking at 37°C for 16–24 h. Purity of the preparation and completeness of transformants were determined by addition of the color reagent (1 M HCl, 0.1% w/v Malachite Green, 1% w/v Ammonium Molybdate Tetrahydrate) and measurement of absorbance at 660 nm on a plate reader (Bio-Tek instruments EL311).

Fractionation. Purified His8-MBP-AtDRP1A or AtDRP1A was diluted to 200 nM in H(0.15)NG + 2 mM MgCl2 with or without 1 mM GTP and incubated 5 min 22°C. After 5 min, an additional 1 mM GTP was added to the + GTP sample, and 200 µl of each sample was transferred to a TLSA100.1 tube and pelleted for 30 min at 150,000g in a Beckman (Fullerton, CA) tabletop ultracentrifuge. The load and upper 80 µl of the reaction volume were analyzed by SDS–PAGE and immunoblotting against AtDRP1A and MBP. For sucrose gradient fractionation, 200 µl of 1.25 µM AtDRP1A was loaded on top of a 4.8 ml 5–50% (w/v) sucrose gradient in H(0.075)N (25 mM HEPES pH 7.5 m, 0.075 M NaCl, 2 mM MgCl2, and 10 mM β-ME) poured on a Beckman Ultracentrifuge gradient (Labconco, Kansas City, Kansas). Gradients were centrifuged 18 h at 4°C in a SW50.1 rotor at 150,000g. 200 µl fractions were collected using a gradient collector (model 640, Isco Inc., Lincoln, NE). Fractionation standards (75 µg BSA, 75 µg Catalase, 50 µg SDS PAGE followed by Coomassie staining (fractionation standards) or immunoblotting using α-AtDRP1A antibodies, and refractive index was used to compare fractions between gradients.

Liposome generation. DOPC, DOPE, DOPE, Soy PC and Ph(4,5)PS were purchased from Avanti Polar Lipids (Alabaster, AL). P(3/1), P(4/3) and P(5/5) were from Cayman Chemical (Ann Arbor, MI), and β-sitosterol was from Calbiochem (San Diego, CA). Dried lipids were resuspended in chloroform or 1:1 chloroform/methanol and mixed by vortexing in a 12 × 75 mm glass test tube. The lipid mixture was spiked with [3H]-DOPC (Perkin–Elmer) to 6 Ci/mmol and dried under a gentle stream of Argon until visibly dry (10–15 min), and then placed under house vacuum for an additional 30 min. The resulting film was resuspended to 330 mM total lipid in H(0.15NG) buffer and allowed to hydrate 15 min at RT before being vortexed for 5 min. The mixture was then subjected to five free-thaw cycles (liquid nitrogen → 37°C water bath) before being extruded through a 50 nm polycarbonate membrane (Avanti Polar Lipids, Alabaster, AL) and stored at −80°C under argon until use.

Liposome flotation assays. Liposome flotation assays were performed as described in [15]. Purified AtDRP1A protein (200 nM to 1 µM final concentration) was mixed with 50 nm liposomes (44 mM final concentration) and buffer H(0.15)NG to a final volume of 75 µl in a sialinized 0.6 ml ultracentrifuge tube and incubated 30 min at 22°C with occasional mixing. The binding reaction was diluted with an equal volume of ice-cold 80% (w/v) Accudenz (Accurate Chemical and Scientific Corporation, Westbury, NY) in H(0.015)NG, transferred to the bottom of a 5 × 41 mm Ultra-Clear centrifuge tube (Beckman–Coulter), and overlayed with 300 µl 30% (w/v) Accudenz followed by 100 µl H(0.15)NG then centrifuged 1 h or more at 243,000g at 4°C in an SW50.1 rotor with tube adapters. Fractions (80 µl) were collected from the top and analyzed for lipid content by scintillation counting and protein content by SDS–PAGE followed by Coomassie staining or immunoblotting.

Negative staining electron microscopy. All electron microscopy was performed at the UW Madison Medical School EM Facility on a Phillips CM120 STEM. For visualization of AtDRP1A, purified protein was diluted to 1 µl in buffer H(0.075)N with or without 1 mM GTP, dried onto a polioform (Ted Pella, Redding, CA) coated copper grid, and stained with Nano-W® (Nanoprobes, Yaphank, NY), an organo-tungstate stain. Liposomes were diluted to 100 mM lipid in H(0.075)N, mixed with an equal volume 1% OsO4, then dried onto polioform coated grids and stained with Nano-W®. For visualization of AtDRP1A bound to liposomes, purified AtDRP1A was mixed with liposomes to a final concentration of 1 µM AtDRP1A and 100 mM lipid in H(0.075)N and incubated for 30 min 22°C with occasional mixing. The mixture was then stained with OsO4 and Nano-W® as for liposomes.
Results and discussion

Purification of GTPase active AtDRP1A

GTPase active AtDRP1A was expressed in *E. coli* as a translational fusion to a His8-MBP tag, purified using amylase affinity chromatography and treated with His8-TEV protease to remove the His8-MBP tag (Fig. 1A). The GTPase activity of the purified protein increased approximately twofold upon cleavage of the His8-MBP tag (Fig. 1B). In contrast, expression of other affinity tagged forms of AtDRP1A, including GST-AtDRP1A in *E. coli* and *S. cerevisiae* and AtDRP1A-His8 in *S. cerevisiae*, did not yield GTPase-active protein.

His8-MBP and His8-TEV protease could not be removed from the mixture by immobilized Ni affinity chromatography due to non-specific binding of AtDRP1A to the nickel resin. Therefore, in all subsequent experiments, AtDRP1A was assayed in the presence of cleaved His8-MBP and His8-TEV, with a mixture of His8-TEV and purified His8-MBP serving as a negative control.

The *k*<sub>cat</sub> and *k*<sub>M</sub> of GTP hydrolysis by AtDRP1A was measured by a colorimetric GTPase assay [13] in the presence of varying concentrations of GTP (Fig. 1C). *k*<sub>cat</sub> and *k*<sub>M</sub> values varied between preparations, as has been observed for dynamin [16]. The average *k*<sub>cat</sub> value was 28 ± 5.34/min, which falls between the *k*<sub>cat</sub> values reported for lipid-tubule stimulated (105 ± 47/min) and unstimulated dynamin (2.6 ± 0.98/min) [17]. The average *k*<sub>M</sub> was 99 ± 59 μM, which is lower than that reported for stimulated (37 ± 18 μM), but similar to that of unstimulated (102 ± 35 μM) dynamin [17].

**Purified AtDRP1A is polymeric**

As shown in Fig. 1A, His8-MBP-DRP1A, AtDRP1A, and His8-MBP migrated as 110 kD, 65 kD and 45 kD polypeptides, respectively, when analyzed by SDS–PAGE. However, when the AtDRP1A cleavage mixture, containing AtDRP1A, His8-MBP and His8-TEV, was subjected to centrifugation at 150,000 × g, AtDRP1A, but not His8-MBP, was fully depleted from the supernatant (Fig. 2A). His8-MBP-AtDRP1A likewise pelleted at 150,000 × g, indicating that both His8-MBP tagged and tag-free AtDRP1A form large homopolymers in the presence of 150 mM NaCl. This sedimentation behavior was not altered by incubation with 1 mM GTP (Fig. 2A). When subjected to velocity sedimentation gradient analysis in the presence of 75 mM NaCl, AtDRP1A sedimented beneath the 17S/550 kDa protein standard, CDC48 [14], further demonstrating the polymeric nature of *E. coli* expressed AtDRP1A (Fig. 2B). This behavior is distinctly different than that of purified dynamin, which is found both in polymeric and soluble, dimeric/tetrameric forms, with the soluble form favored in the presence of ≥ 25 mM NaCl or upon addition of GTP [18–20].

When purified dynamin is induced to polymerize by dilution into <25 mM NaCl buffers or by addition of GDP-BeF<sub>2</sub>, it forms rings and spirals with a constant diameter [21,22]. In contrast, the size and shape of tag-free AtDRP1A polymers visualized by negative staining electron microscopy (EM) was found to be highly heterogeneous, with no discernible regularity in structure (Fig. 2C). Addition of GTP to purified AtDRP1A did not result in a visible change in polymer size or structure (Supplementary Fig. 1).

Similar to animal dynamin [18] GST-GmDRP1 was reported to exist predominantly as monomers and dimers in the presence of 150 mM NaCl, only forming large polymers with a helical nature at 15 mM NaCl [9]. However, these results were based solely on EM analysis, and were not verified through other analytical methods. The smallest AtDRP1A particles we observed by EM (Fig. 2C, arrowheads) were similar in appearance to those interpreted as 68 kDa monomers or dimers of GST-GmDRP1 by Zhang et al. [9]; however by analytical sedimentation analysis (Fig. 2B) we estimate these structures to be comprised of more than 10 subunits (i.e., >680 kDa). The larger AtDRP1A structures we observed at 75 mM NaCl (Fig. 2C, arrows) are similar in appearance to the helical arrays of GST-GmDRP1 imaged by Zhang et al. [9] at 15 mM NaCl. However, the AtDRP1A structures (Fig. 2C) are heterogeneous in size and curvature, and resemble neither the regular polymers formed by purified dynamin [21] nor the 45 nm diameter AtDRP1A-containing rings observed encircling cell plate membrane tubules during syncytial endosperm cellularization [6].

**AtDRP1A interacts with PM-mimetic liposomes**

In interphase *Arabidopsis* cells, AtDRP1A-GFP localizes to endocytic sites at the PM [5], and fractionation studies of cell extracts have similarly shown AtDRP1A to be primarily associated with microsomal membranes [3,8]. Previous studies have demonstrated that dynamin assembles onto P(4,5)P<sub>2</sub>-containing liposomes via specific interactions between the P(4,5)P<sub>2</sub> headgroup and dynamin’s PH domain, and that this interaction is essential for dynamin’s function in CME [23,24]. However, the AtDRP1A amino acid sequence does not contain any predicted lipid-binding domains.
Therefore, we examined whether or not AtDRP1A polymers had any intrinsic affinity for PM phospholipids through binding studies to protein-free PM-mimetic (PMM) liposomes, whose lipid composition closely resembled that of the cytosolic face of the plant PM bilayer. Previous studies have determined the total lipid composition of Arabidopsis PM [26] and have shown that PS is restricted to the inner leaflet of plant cell PMs [27]. PMM liposomes were generated from a mixture of 40 mol% b-sitosterol, 25 mol% Soy PC, 20 mol% DOPE, 10 mol% DOPS and 5 mol% DOPG with trace amounts of H3-DOPC. Binding was assayed by liposome flotation followed by scintillation counting and immunoblotting. AtDRP1A, but not His8-MBP, showed robust binding to PMM liposomes (Fig. 3A).

The PMM liposomes have a net negative charge due to the presence of DOPS and DOPG, suggesting that the interaction with polymers of AtDRP1A, which is predicted to have a net positive charge (PI = 8.5), might be based on charge–charge interactions. Consistent with this, AtDRP1A did not show binding to uncharged liposomes lacking DOPS and DOPG (40 mol% b-sitosterol, 40 mol% Soy PC, 20 mol% DOPE) (Fig. 3B).

Interestingly, in spotted lipid overlay assays AtDRP1A did not show binding to DOPS, but instead showed specific binding to PI(3)P and PI(5)P, with less binding to PI(4)P, similar to what has been reported for AtDRP2A [28] (Supplementary Fig. 2A). However, in lipidosome flotation assays AtDRP1A showed similar binding to DOPC-based liposomes containing 20% DOPS or 10% PI(3)P, PI(4)P or PI(5)P, as well as liposomes containing as little as 2% DOPS (Supplementary Fig. 2B–C).

**AtDRP1A-induced liposome clustering**

Both dynamin and the yeast dynamin-related protein ScDMN1 (involved in mitochondrial fission) have been shown to assemble onto the outer surface of liposomes in vitro, and cause the deformation of those liposomes into tubules [29–34]. To determine whether AtDRP1A polymers similarly affect liposome structure, protein-free PMM liposomes (Fig. 3B) and PMM liposomes preincubated with AtDRP1A (Fig. 3C) were stained and visualized by EM. Liposomes bound to AtDRP1A appeared as darkly staining clusters, which were not observed in protein-free liposome samples. The addition of GTP to these AtDRP1A-liposome complexes resulted...
Soy PC, 20% DOPE, 10%DOPS and 5% DOPG) or (B) neutrally charged liposomes ("Neutral": 40% b-sisterol, 20% Soy PC, 20% DOPE, 10%DOPS and 5% DOPG) spiked with trace H3-DOPC were generated by extrusion through a 50 nm membrane. These liposomes (44 mM) were incubated with purified AtDP1A (250 nM) and separated from the load by flotation on a 40–30–0% (w/v) Accudenz step-gradient. The two top (1, 2) and the two bottom (6, 7) fractions of the gradient were analyzed by scintillation counting and immunoblotting with antibodies against AtDP1A and MBP. (C) PMM liposomes were stained with OsO4 followed by Nano-W® and visualized by electron microscopy. (D) PMM liposomes were incubated with AtDP1A then stained and visualized as in (C). Arrows indicate clusters of liposomes induced by binding of AtDP1A. Scale bars in (C–D) = 100 nm.

Fig. 3. AtDP1A binds and clusters protein-free liposomes. (A–B) Liposome flotation assay. (A) Arabidopsis Plasma Membrane Mimetic liposomes ("PMM": 40% b-sisterol, 25% Soy PC, 20% DOPE, 10%DOPS and 5% DOPG) or (B) neutrally charged liposomes ("Neutral": 40% b-sisterol, 40% Soy PC, 20% DOPE) spiked with trace H3-DOPC were generated by extrusion through a 50 nm membrane. These liposomes (44 mM) were incubated with purified AtDP1A (250 nM) and separated from the load by flotation on a 40–30–0% (w/v) Accudenz step-gradient. The two top (1, 2) and the two bottom (6, 7) fractions of the gradient were analyzed by scintillation counting and immunoblotting with antibodies against AtDP1A and MBP. (C) PMM liposomes were stained with OsO4 followed by Nano-W® and visualized by electron microscopy. (D) PMM liposomes were incubated with AtDP1A then stained and visualized as in (C). Arrows indicate clusters of liposomes induced by binding of AtDP1A. Scale bars in (C–D) = 100 nm.

in no discernible change in structure (Supplementary Fig. 3). This clustering of liposomes onto AtDP1A polymers is very different than what has been observed for dynamin or ScDMN1 in vitro, and is also distinct from the AtDP1A-containing rings observed encircling cell plate membrane tubules in vivo during syncytial endosperm cellularization [6].

Conclusions

Our analysis of the in vitro structure and membrane lipid interaction of purified, bacterially-expressed AtDP1A suggest that the plant-specific DRP1 family has distinct characteristics from animal dynamin, even though previous studies have demonstrated that AtDP1A, like dynamin, functions in CME [5]. Likewise, the propensity of purified AtDP1A to form stable, GTP-insensitive, heterogeneous polymers that promote liposome clustering contrasts with the in vivo observation that AtDP1A-GFP exists in a cytoplasmic (presumably soluble) pool [4], and that AtDP1A can polymerize around membrane tubules during cell plate formation [6]. This suggests that E. coli expressed AtDP1A, while GTPase active, is lacking one or more in vivo factors necessary for modulating the polymeric state of individual AtDP1A subunits, and thereby polymerizes inappropriately into a form that does not retain full functionality.

One possibility is that the activity and polymeric structure of AtDP1A is regulated by post-translational modification, such as phosphorylation. Park et al. [8] found approximately 10% of cellular AtDP1A to be soluble upon cell disruption, and reported that this soluble form migrated slightly slower on SDS–PAGE gels. This slower migration could be reversed by alkaline-phosphatase treatment, suggesting that the soluble form of AtDP1A is phosphorylated. These results, together with our observations that E. coli expressed AtDP1A, which lacks phosphorylation, forms stable polymers, point to the need for further study of native AtDP1A, in particular the identification of post-translational modifications and/or relevant interacting proteins. The ability of these putative modifications or interacting proteins to modulate the polymeric and membrane binding characteristics of purified AtDP1A will be a key step in understanding the targeting and regulation of the plant-specific DRP1 family.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.02.070.

References


