Arabidopsis Dynamin-Like 2 That Binds Specifically to Phosphatidylinositol 4-Phosphate Assembles into a High-Molecular Weight Complex in Vivo and in Vitro

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Arabidopsis dynamin-like (ADL) 2, a member of the high-molecular weight (Mr) dynamin family found in Arabidopsis, has been shown to be targeted to the plastid. In the chloroplast, most of the ADL2 was present in the fraction containing the envelope membranes when analyzed by suborganellar fractionation. Sucrose gradient and gel filtration experiments showed that when associated with membranes, ADL2 existed as a high-Mr complex, whereas the soluble form existed as a monomer. The recombinant ADL2 expressed in Escherichia coli was present as a high-Mr form and showed higher GTPase activity at a low NaCl concentration, whereas ADL2 existed as a low-Mr form with a low level of GTPase activity at a high NaCl concentration. Electron microscopy studies revealed that the purified recombinant ADL2 formed spiral-coiled structures or rings. In the presence of guanosine-5′-O-(3-thio)triphosphate, these structures were transformed into a long rod structure. In contrast, in the presence of GDP, these structures disassembled into oligomers that were shown to be tetramer with 4-fold symmetry. Finally, a lipid-binding assay revealed that recombinant ADL2 purified from E. coli bound specifically to phosphatidylinositol 4-phosphate. Together, these results demonstrated that the biochemical properties of ADL2 were very similar to those of dynamin and other related proteins. Based on this similarity, we propose that ADL2 may be involved in vesicle formation at the chloroplast envelope membrane.

Since the discovery of dynamin I in the rat (Rattus norvegicus) brain, a large number of dynamin-like proteins have been isolated from various organisms and tissues and shown to be involved in diverse and seemingly unrelated biological processes (Obar et al., 1990; Rothman et al., 1990; Chen et al., 1991; Nakata et al., 1993; Cook et al., 1994; Dombrowski and Raikhel, 1995; Gammie et al., 1995; Gu and Verma, 1996; Kang et al., 1998; Park et al., 1998; Smirnova et al., 1998). The mechanism by which dynamin I is involved in endocytosis has been extensively studied and is now well understood at the molecular level (Gout et al., 1993; Herskovits et al., 1993; Damke et al., 1994; Hinshaw and Schmid, 1995; Takei et al., 1995; Sever et al., 1999, 2000). Dynamin I self-polymerizes into a ring or spiral structure around the neck of the invaginated membrane (Hinshaw and Schmid, 1995; Takei et al., 1995, 1998) and then undergoes a conformational change upon GTP hydrolysis, which results in the severing of the neck of the invaginated membrane to release a vesicle. The action of other dynamin-like proteins is not clearly understood, but several members of the dynamin family are associated with membranes (Gu and Verma, 1997; Park et al., 1997). The membrane association of dynamin I is mediated by the protein’s pleckstrin homology (PH) domain that has binding specificity for phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P2] (Lemmon et al., 1995; Salim et al., 1999). Another property that is shared by many members of the dynamin family proteins is the formation of a high-Mr complex, which is most likely a homopolymeric form of the protein (Hinshaw and Schmid, 1995; Tuma and Collins, 1995; Warnock et al., 1996; Gu and Verma, 1996; Park et al., 1997; Smirnova et al., 1999). The self-assembly domain is located between the PH domain and the Pro-rich domain (Okamoto et al., 1999; Smirnova et al., 1999) and is likely to be involved in homopolymeric complex formation. The self-polymerization of dynamin (and its related proteins) may increase the protein’s binding affinity to phospholipids because oligomerization of PH domains has been shown to play a role in high-affinity phosphoinositide binding (Klein et al., 1998). Also, phragmoplastin has been shown to self-assemble into a high-Mr complex both in vitro and in vivo (Gu and Verma, 1997; Zhang et al., 2000). It is interesting that phragmoplastin appears to have two independent assembly domains within the molecule, whereas Dnn1p/Vps1p-like protein (DVLP) has three inter-

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molecular interacting domains (Shin et al., 1999), suggesting that there may be more than one way for dynamin-like proteins to self-assemble.

In plant cells, many different isoforms of dynamin-like proteins have been identified. These include αG68/Arabidopsis dynamin-like (ADL) 1, ADL2, ADL3, ADL6 of Arabidopsis, and phragmoplastin of soybean (Glycine max; Dombrowski and Raikhel, 1995; Gu and Verma, 1996; Park et al., 1998; Kang et al., 1998, 2001; Mikami et al., 2000; Jin et al., 2001). These plant homologs can be grouped into several subfamilies, such as αG68/ADL1, ADL2, and ADL3 subfamilies based on their amino acid sequence similarity. In Arabidopsis, the ADL1 subfamily also consists of many highly homologous proteins (Kang et al., 2001). The ADL2 subfamily consists of its own subfamily. ADL3 and ADL6 are again highly homologous to each other and may belong to the same subfamily (Mikami et al., 2000; Jin et al., 2001). The biological roles of these dynamin-like proteins have been addressed recently with ADL1 and phragmoplastin (Gu and Verma, 1997; Park et al., 1998). From these studies, it has been shown that ADL1 and phragmoplastin may be involved in biogenesis of thylakoid membrane and cell division plate formation, respectively. However, a new study suggested that ADL1 may also be involved in embryogenesis and seedling development (Kang et al., 2001).

Although the presence of multiple dynamin-like proteins in plant cells is now evident from these studies, their biological roles are less clear. In this study, we characterized the biochemical properties of ADL2. We present evidence showing that a majority of ADL2 exists as a membrane-bound, high-M₉ complex in vivo and that the recombinant ADL2 can also form a high-M₉ complex having a spiral or ring structure in vitro. In addition, we demonstrate that ADL2 binds specifically to phosphatidylinositol 4-phosphate (PtdIns₄P).

RESULTS

The Majority of ADL2 Is Associated with Membranes

ADL2 is a dynamin-like protein from Arabidopsis (Kang et al., 1998) and is a member of the dynamin superfamily. The primary sequence of ADL2 revealed a highly conserved GTP binding domain at the N terminus. However, it was impossible to deduce the biological role of ADL2 solely from its amino acid sequence because dynamin-related proteins have been shown to be involved in diverse biological processes (Obar et al., 1990; Rothman et al., 1990; Gu and Verma, 1996; Kang et al., 1998; Park et al., 1998). Therefore, we initiated a biochemical characterization of ADL2 to understand its biological role in the cell. We raised a polyclonal antibody against a synthetic peptide corresponding to amino acid residues 555 through 569 of ADL2. As shown in Figure 1A, the polyclonal antibody recognized from whole cell extracts a protein species that migrated at approximately 100 kD, the expected size of ADL2 based on its calculated M₉. This antibody also recognized a protein band of similar size in protein extracts obtained from Arabidopsis seedlings (A) and from Escherichia coli harboring a pRSET vector or the ADL2 expression vector pRSET/ADL2 (B). ADL2 and Cont., Purified anti-ADL2 antibody and normal control serum, respectively. Vector and pRSET/ADL2, Protein extracts obtained from E. coli harboring a pRSET vector and the ADL2 expression vector pRSET/ADL2, respectively.

Figure 1. Specificity of the polyclonal anti-ADL2 peptide antibody. A synthetic peptide corresponding to amino acid positions 555 through 569 of ADL2 was chemically synthesized as a matrix-attached peptide and used to raise antibody in rabbits. The antibody was tested with protein extracts obtained from Arabidopsis seedlings (A) and from Escherichia coli harboring a pRSET vector or the ADL2 expression vector pRSET/ADL2 (B). ADL2 and Cont., Purified anti-ADL2 antibody and normal control serum, respectively. Vector and pRSET/ADL2, Protein extracts obtained from E. coli harboring a pRSET vector and the ADL2 expression vector pRSET/ADL2, respectively.
shown in Figure 2B, ADL2 was largely removed from membranes by treatment with 100 mM NaCO₃, pH 10.5, indicating that ADL2 is a peripheral membrane protein.

**ADL2 Is Present in the Membrane Fraction of Chloroplasts**

In a previous study, we showed by an in vivo targeting experiment that ADL2 is localized at the plastids (Kang et al., 1998). We therefore confirmed this further by using western-blot analysis. Chloroplasts were purified from gently lysed leaf protoplasts using a Percoll gradient and further fractionated into soluble, membrane, and thylakoid membrane fractions by differential centrifugation steps. The presence of ADL2 was then determined by western-blot analysis using the anti-ADL2 antibody. As shown in Figure 3, ADL2 was present only in the membrane fraction containing the envelope membranes. As controls for fractionation, light-harvesting complex protein 2 (LHCP) II, RbcL, and a subunit of the translocon of the inner envelope of chloroplasts of 110 kDa (tic110) were probed for thylakoid membrane, stromal proteins, and envelope membrane proteins using anti-LHCPII, anti-Rubisco complex, and anti-tic110 antibodies, respectively. As shown in Figure 3, there was no detectable level of cross contamination between these fractions. Thus, the suborganelar fractionation experiments suggested that ADL2 might be localized at the envelope membranes of the chloroplasts. This result is in contrast to that of ADL1, which has been shown to be tightly associated with thylakoid membranes (Park et al., 1998).

**ADL2 Exists as a Large Molecular Mass Complex**

Dynamin and its related proteins are known to form high-\( M_r \) complexes (Hinshaw and Schmid, 1995; Shin et al., 1999). To address whether ADL2 is also present as a high-\( M_r \) complex, whole cell extracts were fractionated on a linear Suc gradient (5%–50% [w/v] Suc) in the presence and absence of Triton X-100, and fractions were analyzed for the presence of ADL2 by western-blot analysis using the anti-ADL2 antibody. As shown in Figure 4, ADL2 was detected in two positions in the absence of Triton X-100: one major peak at a Suc concentration of 30% to 40% and a minor peak at a concentration of 5% to 18%. In contrast, in the presence of Triton X-100, the majority of ADL2 was detected in the 18% to 28% (w/v) Suc layer of the gradient with only minor amounts in the 5% to 18% (w/v) gradient (though not clearly shown in the figure, with longer exposure weak bands were observed between 5% and 18%). The fact that the majority of ADL2 did not move to the top of the gradient even in the presence of Triton X-100 suggested that ADL2 may exist as a high-\( M_r \) complex in vivo, as found in dynamin and other related proteins (Hinshaw and Schmid, 1995; Tuma and Collins, 1995; Gu and Verma, 1997; Park et al., 1997). To investigate this possibility further, we carried out gel filtration column chromatography using the whole cell extracts or fractions obtained from a
been shown that dynamin has a very low level of intrinsic GTPase activity that can be modulated by various means such as salt concentration, phospholipids, and interacting proteins (Gout et al., 1993; Warnock et al., 1996; Klein et al., 1998). Thus, to examine the GTPase activity of ADL2, we expressed a recombinant ADL2 protein in *E. coli* and purified to near homogeneity using Ni$^{2+}$-nitrotroacetate acid agarose (NTA) affinity column chromatography (Fig. 6A). We performed GTPase activity assay using the purified recombinant ADL2 at various NaCl concentrations. As shown in Figure 6B, GTPase activity of ADL2 showed a very low level of GTPase activity at 200 mM NaCl concentration. However, the GTPase activity was 3- and 5-fold higher in 100 and 20 mM NaCl concentration, respectively, than in 200 mM NaCl concentration. The behavior of ADL2 was quite similar to that of dynamin I of animal cells (Warnock et al., 1996). The higher level of GTPase activity of dynamin I at low NaCl concentration has been shown to result from formation of a high-$M_r$ complex at the low NaCl condition (Warnock et al., 1996). Thus, as in the case of dynamin I, low NaCl concentration may allow ADL2 to form a high-$M_r$ complex, which may be necessary to get higher GTPase activity of ADL2. To confirm this possibility, next we wanted to examine whether ADL2 can self-assemble into a high-$M_r$ complex at a low NaCl concentration. To address this question, purified ADL2 was first dialyzed against various NaCl concentrations and then fractionated into the supernatant and pellet by ultracentrifugation. The amounts of ADL2 present in the supernatant and pellet fractions were then examined by western-blot analysis using the anti-ADL2 antibody. As shown in Figure 6C, ADL2 was present in the pellet and nearly undetectable in the supernatant in 20 mM NaCl concentration. In 100 mM NaCl concentration, approximately 70% of ADL2 was recovered in the pellet and 30% remained in the supernatant. In contrast, the majority of ADL2 was present in the supernatant with only a minor fraction in the pellet in a 200 mM NaCl concentration. These results clearly indicated that different forms of ADL2 may exist in different NaCl concentrations. One possible explana-
tion for the precipitated form of ADL2 in 20 mM NaCl is that ADL2 may assemble into a high-Mr complex at low NaCl concentrations, which may in turn result in the higher level of GTPase activity of ADL2 as observed with dynamin I (Warnock et al., 1996).

High-Mr Complex Formation of ADL2 in Vitro

To further enhance our understanding on the high-Mr complex formation of ADL2, we wanted to examine the structure of the high-Mr complex of ADL2 in vitro. The purified recombinant ADL2 protein was observed by negative-stain electron microscopy. As shown in Figure 7A, we observed two different forms of ADL2, a ring form and a spiral-coiled structure (Fig. 7A, a). These structures were quite similar to those observed from dynamin and phragmo- plastin (Hinshaw and Schmid, 1995; Zhang et al., 2000). It is interesting that when the purified protein was treated with guanosine-5’-O-(3-thio)triphosphate (GTPγS), a non-hydrolyzable analog of GTP, the spiral-coiled structure or ring form was transformed into a long rod structure (Fig. 7A, b). Close examination of these structures revealed that the presence of GTPγS decreased the width of the high-Mr structure, suggesting that GTPγS-bound ADL2 may exist in a tighter conformation. In contrast, treatment with GDP caused disassembly of the spiral or ring structures into much smaller oligomers (Fig. 7A, c). The electron micrograph of the negatively stained ADL2 oligomer in the presence of GDP showed a square plane. Therefore, we further analyzed the structure of this ADL2 oligomer by the imaging processing method. A total of 191 well-stained particles were translationally and rotationally aligned and analyzed on the basis of eigenvector-eigenvalue and correlation average (van Heel and Frank, 1981). For classification, we selected the 10 most significant eigenvectors for elimination of the less significant information represented by the other eigenvectors. By this approach, three classes (Fig. 7B, 1–3) were discriminated according to the similarity of features. The unsymmetrized class average of ADL2 oligomer treated with GDP revealed the 4-fold symmetry (Fig. 7B, 4). Moreover, the angular correlation coefficients obtained by cross correlation of the average showed almost equal height with an angular increment of 90° (data not shown), again indicating that the ADL2 oligomer showed 4-fold symmetry. No other statistically significant symmetry was detected. The averaged image obtained after alignment showed four centers of mass arranged on a square plane with heavy stain accumulation in its center (Fig. 7B, 4). The diameter of the square plane and the central hole were approximately 9 and 2 nm, respectively. Taken together, these results suggest that the oligomer is tetramer. Similarly, dynamin I and DVLP have been shown to be tetramers at the physiological salt concentration, and the tetramer can be induced into a spiral or ring structure by reducing the salt concentration (Carr and Hinshaw, 1997; Sweitzer and Hinshaw, 1998; Stowell et al., 1999).

ADL2 Is Specifically Bound to PtdIns4P

The membrane association of dynamin I is thought to be mediated by the PH domain (Klein et al., 1998).
that has a high binding affinity to PtdIns(4,5)P$_2$ (Lemmon et al., 1995; Salim et al., 1996). However, amino acid sequence analysis of ADL2 did not reveal any region that showed a high degree of sequence similarity to known PH domains of proteins found in both animal and plant cells (Fig. 8). The members of the dynamin family can be divided into two groups: one with the PH domain and the other without the PH domain. The members of both groups are found in the same organisms including Arabidopsis. Among the large numbers of dynamin-like proteins found in Arabidopsis (Dombrowski and Raikhel, 1995; Kang et al., 1998; Mikami et al., 2000; Kang et al., 2001; Jin et al., 2001), ADL2 appeared to belong to the group without the PH domain. Usually, the PH domain is located between the conserved central domain and the GTPase effector domain. Thus, we compared the amino acid sequence of this region with those of the PtdIns4P-specific PH domain of phosphatidylinositol-4-phosphate adaptor protein-1 of mouse (Mus musculus; Dowler et al., 2000) or the PH domain of Arabidopsis phosphatidylinositol 4-kinase with a higher binding affinity to PtdIns4P (Stevenson et al., 1998). However, the PH domain of ADL2 did not show any significant amino acid sequence similarity to either of the PH domains (data not shown). To understand the membrane association of ADL2, we examined whether ADL2 also binds to phosphatidylinositol phosphates as in the case of dynamin. To address this question, we used recombinant ADL2 expressed in and purified from *E. coli*. The lipid binding properties of ADL2 were assayed by Fat western-blot analysis as described previously (Stevenson et al., 1998). As shown in Figure 9, the recombinant ADL2 bound specifically to PtdIns4P but not to other phosphatidylinositol phosphates (Fig. 9, ADL2). As negative and positive controls, we used his-tagged binding protein (BiP) and the glutathione S-transferase (GST)-tagged PH domain of rat phospholipase C (PLC)-81 (GST:PLC-PHD), respectively, that were expressed in *E. coli*. The negative control did not show any binding to phosphatidylinositol phosphates (Fig. 9, BiP). In contrast, as shown in Figure 9, the PH domain of PLC-81, GST: PLC-PHD, was specifically bound to PtdIns(4,5)P$_2$ (Fig. 9, GST:PLC-PHD), as has been observed previously (Garcia et al., 1995), although GST alone did not show any binding to these phospholipid molecules (Fig. 9, GST). Thus, these results suggest that although ADL2 does not have a PH domain based on sequence homology, it is a PtdIns4P-specific binding protein. The region between the conserved central

![Figure 6](image_url)

**Figure 6.** The NaCl concentration affects ADL2 GTPase activity and ADL2 assembly into high-$M_r$ complexes. A, Purification of His-tagged ADL2 protein. After being passed through an Ni$^{2+}$-NTA column, the protein was separated by SDS-PAGE and stained with Coomassie Brilliant Blue (2) or probed with anti-ADL2 antibody (3). Lane 1 shows the total *E. coli* extracts. M, Standard markers. B, GTPase activity of His-tagged ADL2 protein. ADL2 protein was dialyzed against buffer containing 20, 100, and 200 mM NaCl and then incubated with GTP. The amount of monophosphates released was measured by spectrophotometry at 660 nm. The values are means with SD ($n = 3$). C, Sedimentation assay of ADL2 in various salt concentrations. ADL2 protein was dialyzed against buffer containing 20, 100, or 200 mM NaCl and then sedimented by ultracentrifugation. The pellet was redissolved in the original volume. Equal volumes of the supernatant and pellet fractions were separated by SDS-PAGE and probed with the polyclonal anti-ADL2 antibody. Supernatant and pellet are indicated.
domain and the GTPase effector domain of ADL2 may also be responsible for binding to PtdIns4P as in the case of dynamin and other dynamin-like proteins. However, we did not address further which region of the ADL2 molecule is responsible for the lipid binding.

DISCUSSION

ADL2 Forms a High-\(M_r\) Complex in Vivo and in Vitro

ADL2 is a member of the dynamin family isolated from Arabidopsis (Kang et al., 1998). The biochemical properties of ADL2 were studied by Suc gradient centrifugation and gel filtration experiments. These experiments demonstrated that ADL2 was present in multiple forms in vivo. Most of ADL2 was associated with membranes as a high-\(M_r\) complex, although a small amount of ADL2 was also present in a soluble form with an \(M_r\) of 100 kD. Similarly, the high-\(M_r\) complex formation has been observed with dynamin and other dynamin-related proteins such as ADL1, phragmoplastin, and DVLP (Tuma and Collins, 1995; Park et al., 1997; Gu and Verma, 1997; Shin et al., 1999). The high-\(M_r\) complex formation was further studied using recombinant ADL2 expressed in \(E. coli\). This recombinant ADL2 also existed as a high-\(M_r\) complex in vitro at a low NaCl concentration, as evidenced by the fact that the majority of ADL2 was present at the pellet fraction of ultracentrifugation. However, at a high NaCl concentration, the majority of ADL2 was present in the supernatant and showed a low level of GTPase activity. It has been shown that dynamin I assembles into a high-\(M_r\) complex at a low NaCl concentration, which in turn shows a high GTPase activity (Warnock et al., 1996). Thus, the correlation between the high-\(M_r\) complex formation and the higher GTPase activity at a low NaCl concentration strongly suggests that the assembled ADL2 may have higher GTPase activity as in the case of dynamin I (Warnock et al., 1996).

To investigate the nature of the high-\(M_r\) complex, we examined purified ADL2 by negative stain electron microscopy. As shown in Figure 7, it existed as spiral-coiled or ring structures at the condition where ADL2 was purified from \(E. coli\) extract. In the presence of GTP\(\gamma\)S, a non-hydrolyzable GTP analog,
these structures were transformed into a long rod structure, which was much more compact in width compared with the spiral-coiled or ring structures. Similarly, dynamin I and phragmoplastin have also been shown to assemble into ring or spiral structures in vitro, and treatment with GTP\(^{\gamma}S\) transformed them into more compact structures (Carr and Hinshaw, 1997; Sweitzer and Hinshaw, 1998; Stowell et al., 1999). However, at the moment, the molecular mechanism of such transformation of ADL2 in the presence of GTP\(^{\gamma}S\) is not clearly understood. It is interesting that in the presence of GDP, the spiral and ring structures of ADL2 disassembled into smaller oligomers. The effect of GDP on the high molecular structure appeared to be similar to dynamin and other dynamin-related proteins. When self-assembled dynamin was treated with GDP, dynamin disassembled into a form that was not sedimentable by ultracentrifugation (Carr and Hinshaw, 1997). It is interesting that the ADL2 oligomer in the presence of GDP had 4-fold symmetry when analyzed by the imaging processing method, suggesting that the oligomer is tetramer. The tetramer has also been observed with dynamin I and DVLP. It has been proposed that dynamin and DVLP are present as tetramers at physiological salt concentrations (Muhlberg et al., 1997; Shin et al., 1999; Smirnova et al., 1999). Shin et al. (1999) proposed a possible model for the tetramer of DVLP: a tetramer consisting of four monomeric subunits interacting with each other by a sequential interaction. The ADL2 tetramer can also be explained by a similar model (Carr and Hinshaw, 1997; Shin et al., 1999; Zhang et al., 2000). Studies on the molecular mechanism of self-assembly of dynamin have shown that formation of spiral and ring structures is largely dependent on three interacting domains (Smirnova et al., 1999), although the membrane association of dynamin has been shown to facilitate the self-assembly (Tuma and Collins, 1995). In addition, three interacting domains required for self-assembly of DVLP have been identified (Shin et al., 1999), whereas phragmoplastin has two interacting domains for self-assembly (Zhang et al., 2000). Of these assembly domains, the one at the N terminus appears to be highly conserved. ADL2 also has the conserved N-terminal domain (data not shown). However, the other interacting domain of ADL2 still needs to be elucidated.

**ADL2 Binds Specifically to PtdIns4P**

The membrane association of dynamin I is thought to be mediated by the PH domain located in the central region of the protein (Salim et al., 1996; Klein et al., 1998). PH domains display specific binding to phosphoinositides such as PtdIns4P (Stevenson et al., 1998) and PtdIns(4,5)P\(_2\) (Salim et al., 1996; Klein et al., 1998). However, sequence analysis of ADL2 did not reveal any region that showed a high degree of homology to known PH domains (Kang et al., 1998). Similarly, it has been thought that yeast Vps1p also lacks the PH domain. The lipid binding property of ADL2 was investigated by Fat western-blot analysis. Recombinant ADL2 expressed in and purified from *E. coli* showed specific binding to PtdIns4P. These results raise the possibility that certain members of the dynamin family may have a domain, other than known PH domains, that is involved in protein-lipid interaction. The undefined membrane-binding do-

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**Figure 8.** Various conserved domains of dynamin-like proteins. Domains conserved in the members of the dynamin family were compared using BLASTp of the National Center for Biotechnology Information server. ADL2 (Arabidopsis, accession no. AAC61784), αG68/ADL1 (Arabidopsis, accession no. S59558), DVLP (human, accession no. JC5695), Shi (Shibire; *Drosophila melanogaster*, accession no. AAF48536), dynamin I (Dyn I; human, accession no. NP 004399), dynamin I-like (Dy I-like; human, accession no. NP 0036193), ADL6 (Arabidopsis, accession no. AAF22291).
Main of ADL2 may play a similar role to the PH domain of dynamin. However, at the moment, it is not known what domain of ADL2 is responsible for binding to PtdIns4P.

ADL2 Is Localized at the Envelope Membranes of Chloroplasts

In a previous study, we demonstrated that ADL2 is localized at the plastids by using an in vivo targeting approach with ADL2:GFP fusion constructs (Kang et al., 1998). Here, we further demonstrated by organelle and suborganellar fractionation experiments that ADL2 is associated with membranes, possibly chloroplast envelope membranes of the chloroplasts, but not with thylakoid membranes. In contrast to ADL2, another ADL protein, ADL1, has been shown to be tightly associated with thylakoid membranes (Park et al., 1998). Thus, these data indicate that the two ADL proteins are localized at different locations within the chloroplast. The fact that biochemical properties of ADL2 were very similar to those of dynamin and that ADL2 was localized at the chloroplast envelope membrane strongly suggested that ADL2 may be involved in vesicle formation from the inner membrane of chloroplasts. However, other roles for ADL2 within the chloroplast cannot be ruled out. The presence of vesicles in the chloroplast has previously been demonstrated in several different plant species by electron microscopy (Carde et al., 1982; Morré et al., 1991). The electron micrographs showed processes that could be interpreted as vesicles forming from the inner membrane of the chloroplast envelope or as vesicles fusing with the thylakoid membranes. This finding raises the interesting possibility that the chloroplast, which is thought to have been originated by symbiotic engulfing of a prokaryote, may have acquired a vesicle trafficking process from the cytosol—a process that is absent from prokaryotic cells. However, additional evidence will be necessary to validate such a hypothesis.

MATERIALS AND METHODS

Growth of Plants

Arabidopsis (ecotype Columbia) was grown in a greenhouse under a 16-h-dark/8-h-light cycle at a temperature of 20°C and relative humidity of 70%. Some plants were grown on Murashige and Skoog plates in a growth chamber at 20°C with a 16-h-dark/8-h-light cycle.

Preparation of Membrane Fractions

Protein extracts from plant tissues were prepared and fractionated according to the method of Park et al. (1997). A polyclonal anti-ADL2 antibody was used to probe for ADL2 in fractions collected from the gradient. Protein/membrane associations were studied as described previously (Park et al., 1997).

Expression and Generation of Antibodies against Recombinant Proteins

BiP (accession no. D82817) cDNA was ligated in-frame to the expression vector pRSET-B (Invitrogen, Carlsbad, CA). A DNA fragment encoding the central region of the soybean (Glycine max) clathrin heavy chain (amino acid residues 1025–1497; accession no. U42608) was PCR amplified from a soybean cDNA library using specific primers (5'H11032-TGTGCTTCAGAATTCTGCAT-3' and 5'H11032-TCATTCAGGTGAATTGGTTCATG-3'). The clathrin heavy chain cDNA fragment was also ligated in-frame to the expression vector, pRSET-B. Following transformation into Escherichia coli, BL21(DE3) LysS protein expression was induced by isopropylthio-/H9252-galactoside. The soluble BiP and clathrin heavy chain fragment proteins were purified using Ni/H11001-NTA affinity columns according to the manufacturer's instructions (Invitrogen). These recombinant proteins were then used to raise antibodies in rabbits. Also, a polyclonal antibody for ADL2 was raised in rabbits against the synthetic peptide ADL2–1 (555RPKDTVEPDRTSST569) according to the published protocol (Harlow and Lane, 1988).

Figure 9. ADL2 binds specifically to PtdIns4P. The phospholipid binding assay was carried out using the purified recombinant proteins (ADL2, BiP, GST:PLC-PHD, and GST) as described in “Materials and Methods.” Binding of the recombinant proteins was detected by western-blot analysis using the polyclonal anti-ADL2 (ADL2), anti-BiP (BiP), and anti-GST (GST:PLC-PHD and GST) antibodies as the primary antibody. Anti-rabbit IgG antibody was used as the secondary antibody. The blot was detected using an ECL kit for western blot (Amersham Pharmacia Biotech, Piscataway, NJ). The amount of lipids applied on the membrane was indicated.
These antibodies were affinity purified using the corresponding antigens as ligands and used for western-blot analysis as described previously (Park et al., 1997). The polyclonal anti-Rubisco complex antibody and anti-LHCP II antibody were obtained from Jeong Hee Kim (Kyeonghee University, Seoul, Korea). The anti-tic110 antibody was a gift of Felix Kessler (Swiss Federal Institute of Technology, Zurich).

Suborganelle Fractionation of the Chloroplast Extract

Plants were grown on Murashige and Skoog plates at 20°C for 2 weeks. Homogenates were prepared and applied to Percoll step gradients, and the intact chloroplast fractions were collected (Robinson and Barnett, 1988). The intact chloroplasts were lysed and further fractionated into supernatant and pellet fractions by low-speed centrifugation (Li et al., 1991). The supernatant was further fractionated into soluble and membrane fractions by ultracentrifugation using a Suc step gradient as described above. The presence of ADL2 was confirmed using a polyclonal anti-ADL2 antibody.

Gel Filtration Chromatography

Gel filtration experiments were carried out as described previously (Park et al., 1997). In brief, protein samples were applied to a Sephacryl S-300 HR gel filtration column (120 x 1.5 cm) pre-equilibrated with column buffer (10% [v/v] glycerol, 50 mM Tris-HCl, pH 7.5, 2 mM EDTA, pH 8.0, 1 mM MgCl2, 100 mM NaCl, and 1 mM phenylmethylsulfonyl fluoride). Fractions (1.0 mL) were collected at a flow rate of 0.5 mL min⁻¹. The proteins contained in a 500-μL aliquot of each fraction were concentrated by cold TCA precipitation and then analyzed for the presence of ADL2 by western-blot analysis. β-Amylase (200 kD) and bovine serum albumin (66 kD) were used as M₀ standards (Sigma, St. Louis). The clathrin complex and the Rubisco complex were probed with polyclonal anticlathrin heavy chain antibody and anti-Rubisco complex antibody, respectively.

Expression and Purification of Recombinant ADL2

Full-length ADL2 cDNA was ligated in-frame to an expression vector, pRSET-B (Invitrogen). The pRSET expression vectors were introduced into E. coli BL21(DE3) LysS. The PH domain of rat (Rattus norvegicus) PLC-1 (accession no. NP058731; Suh et al., 1988) was ligated in-frame to pGEX-5X-1 (Amersham Pharmacia Biotech) to give GST: PLC-PHD, and resulting plasmid was introduced into E. coli strain XL1 Blue. The cells were grown in Luria-Bertani medium until the absorbance reached 0.5 of A₆₀₀ at 30°C. Expression of protein was induced by 0.1 mM isopropylthio-β-galactoside for 2 h. The cells were harvested and resuspended in 1/50 original culture volume of lysis buffer containing 50 mM Na₂HPO₄ (pH 8.0), 300 mM NaCl, 10 mM imidazole, and the complete protease inhibitor cocktail (EDTA free; Roche Molecular Biochemicals, Indianapolis). The cell suspension was sonicated and centrifuged at 12,000g for 20 min, and the supernatants for ADL2 and BiP were then applied to an Ni²⁺-NTA agarose column according to the manufacturer’s protocol. After washing the column with the lysis buffer, proteins were eluted with a stepwise gradient consisting of 50, 100, 150, and 250 mM imidazole. GST:PHD and GST were purified using glutathione affinity column chromatography according to the manufacturer’s protocol.

Assay for ADL2 GTPase Activity

ADL2 GTPase activity was measured by the colorimetric assay (Lanzetta et al., 1972), with some modifications. In brief, malachite green hydrochloride (0.045%, w/v) was completely mixed with 4.2% (w/v) ammonium molybdate dissolved in 4 N HCl at a ratio of 3:1. After addition of Triton X-100 (1%, w/v; instead of Sterox), 800 μL of green polyniobylate was added to samples. The reaction was terminated by heating at 100°C for 5 min. Immediately after heating, 100 μL of sodium citrate (34%, w/v) was added. The monophosphates released by GTP hydrolysis by ADL2 were measured at 660 nm using a spectrophotometer. KH₂PO₄ (10 mM) was used to provide a standard curve.

Sedimentation Assay

Purified proteins were dialyzed against HP buffer (10 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], pH 7.5, 1 mM dithiothreitol, 1 mM MgCl₂, 1 mM EGTA, and 1 mM phenylmethylsulfonyl fluoride) containing 20, 100, or 200 mM NaCl and then centrifuged at 18,000g for 15 min to remove aggregated protein debris prior to ultracentrifugation at 100,000g for 30 min. The pellet was resuspended in the original volume, and equal volumes of supernatant and pellet fractions were separated by SDS-PAGE. The presence of ADL2 protein in the supernatant and pellet fractions was then assayed by western-blot analysis using the anti-ADL2 antibody as the primary antibody.

Fat Western-Blot Analysis

Various lipids, PtdIns, PtdIns4P, PtdIns(4,5)P₂, phosphatidylinositol3-phosphate, phosphatidylinositol(3,4)-bisphosphate, and phosphatidylinositol(3,4,5)-trisphosphate were used for lipid binding analysis. The lipid binding assays were done by Fat western-blot analysis (Stevenson et al., 1998) using purified recombinant ADL2. BiP, GST:PHD, and GST were used as controls for the Fat western-blot analysis. In brief, 10-μL volumes of various concentrations (0.5, 1.0, and 5.0 μg) of lipids dissolved in chloroform were applied to nitrocellulose membranes. The membranes were blocked with a 10-mL buffer containing 20 mM Tris-HCl, pH 7.5, 140 mM NaCl, and 0.1% (v/v) Tween 20 (TTBS) overnight at 4°C and then incubated with 0.5 μg mL⁻¹ of purified recombinant protein in 10 mL of TTBS containing 3% (w/v) fatty acid-free bovine serum albumin for 1 h at room temperature. After washing three times with TTBS, the blot was incubated...
with the primary antibody for 1 h at room temperature and washed three times for 20 min each time. A secondary antibody was then incubated and washed under the same conditions as the primary antibody. The ECL detection system was used for visualization (Amersham Pharmacia Biotech).

Electron Microscopy and Image Processing

The purified ADL2 recombinant protein (0.1 µg µL⁻¹) was dialyzed in a buffer containing 50 mM Tris, pH 7.8, and 100 mM NaCl and applied to a glow-discharged carbon-coated copper grid for 2 min. The grid was rinsed on droplets of deionized water and stained with 1% (w/v) uranyl acetate. Specimens were examined in the TECNAI 12 at an accelerating voltage of 120 kV using a low-dose unit. Electron micrographs were recorded on a Kodak film (SO163; Eastman Kodak, Rochester, NY) at a nominal magnification of 46,000×.

Light-optical diffractograms were used to select the micrographs, to examine the defocus, and to verify that no drift or astigmatism was present. Suitable areas were digitized as arrays of 1,024 × 1,024 pixels with Leaf Scan 45 (Scitex, Herzlia, Israel) at a pixel size of 20 µm, corresponding to 0.44 nm at the specimen level. For image processing, the SEMPER (Saxton et al., 1979) and EM (Hegerl, 1996) software packages were used. From digitized micrographs, smaller frames of 64 × 64 pixels containing individual particles were extracted interactively. These images were aligned translationally and rotationally using standard correlation methods (Baumeister et al., 1988; Kim et al., 2000). An arbitrarily chosen reference was used for the first cycle of alignment and averaging, and the resulting average was used as a reference in a second refinement cycle. The aligned images were classified based on eigenvector-eigenvalue data analysis. The average was finally symmetrized based on angular correlation coefficients (Dürr, 1991).

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