THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 35, Issue of August 30, pp. 31842–31849, 2002

Printed in U.S.A.

The Intermolecular Interaction between the PH Domain and the C-terminal Domain of Arabidopsis Dynamin-like 6 Determines Lipid Binding Specificity*

Received for publication, May 15, 2002, and in revised form, June 21, 2002
Published, JBC Papers in Press, June 24, 2002, DOI 10.1074/jbc.M204770200

Sung Hoon Lee, Jing Bo Jin, Jinhee Song, Myung Ki Min, Dae Sup Park, Yong-Woo Kim, and Inhwan Hwang‡

From the Center for Plant Intracellular Trafficking and the Division of Molecular and Life Sciences, Pohang University of Science and Technology, 790-784, Korea

Dynamin and its related proteins are a group of mechanochemical proteins involved in the modulation of lipid membranes in various biological processes. Here we investigate the nature of membrane binding of the *Arabidopsis* dynamin-like 6 (ADL6) involved in vesicle trafficking from the *trans*-Golgi network to the central vacuole. Fractionation experiments by continuous sucrose gradients and gel filtration revealed that the majority of ADL6 is associated with membranes *in vivo*. Amino acid sequence analysis revealed that ADL6 has a putative pleckstrin homology (PH) domain. *In vitro* lipid binding assays demonstrated that ADL6 showed high affinity binding to phosphatidylinositol 3-phosphate (PtdIns-3-P) and that the PH domain was responsible for this interaction. However, the PH domain alone binds equally well to both PtdIns-3-P and phosphatidylinositol 4-phosphate (PtdIns-4-P). Interestingly, the high affinity binding of the PH domain to PtdIns-3-P was restored by a protein-protein interaction between the PH domain and the C-terminal region. In addition, deletion of the inserted regions within the PH domain results in high affinity binding of the PH domain to PtdIns-3-P. These results suggest that ADL6 binds specifically to PtdIns-3-P and that the lipid binding specificity is determined by the interaction between the PH domain and the C-terminal domain of ADL6.

Dynamin, a high molecular weight GTP-binding protein originally found in rat brain tissue, has been shown to play an important role in vesicle formation during endocytosis. Since the discovery of dynamin I, numerous dynamin-related proteins have been identified from various organisms such as yeasts, plants, and humans (1–6). The mechanism by which dynamin I plays a role in endocytosis has been extensively studied (7–12). From these numerous studies, dynamin I has been shown to function as a mechanochemical enzyme that pinches off the neck of invaginated membranes, thereby releasing the budding membrane as a vesicle (10, 11, 13).

Unlike the dynamin I protein, which is involved in endocytosis in animal cells, other members of the dynamin family have been proposed to play roles in other biological processes, such as maintenance of mitochondrial morphology (14–16), cell plate formation in plant cells (5), thylakoid membrane biogenesis (17), and vacuolar trafficking of proteins at the *trans*-Golgi network (TGN)[1] (18). Although the exact mechanism of action of these proteins remains to be elucidated, they appear to be involved in various biological processes, including the modulation of membrane structures such as membrane fission (19). To modulate membrane structure, these proteins must be able to bind to membranes. The membrane association of dynamin I has been shown to be mediated by the PH domain of the protein that binds specifically to phosphatidylinositol 4,5-bisphosphate (PtdIns-4,5-P_2) (20, 21). Also, other members of the dynamin family, such as *Arabidopsis* dynamin-like 1 (ADL1), ADL2, and phragmoplastin also have been shown to bind to membranes *in vivo* (22–24). Among these, ADL2 has been shown to bind specifically to PtdIns-4-P *in vitro* (24). However, except for dynamin I, the nature of membrane association of dynamin-related proteins is unclear because the PH domain is apparently absent from certain members of the dynamin family, such as Vsp1p and ADL2 (2, 6, 24). Another important biochemical characteristic of these proteins for their role in membrane modulation is high molecular weight complex formation (10, 22, 24–26). These proteins have been shown to self-assemble into a homopolymeric form through the intermolecular interaction between self-assembly domains (25–27).

Previously, we have shown that ADL6 is localized to the TGN and involved in trafficking of cargo proteins from the TGN to the central vacuole in *Arabidopsis* (18). To further understand the role of ADL6 *in vivo*, we characterized the nature of its interaction with membranes.

In this study, we present evidence that ADL6 binds to phosphatidylinositol 3-phosphate with high affinity and that the lipid binding specificity of the PH domain is determined through an intermolecular interaction between the PH domain and the C-terminal domain (CTD).

EXPERIMENTAL PROCEDURES

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

Construction of Expression Plasmids—The PH domain of ADL6 (amino acid residues 558–759) was amplified by the polymerase chain reaction using two specific primers (GAGACGCCGGAGGTCTCTGG

* This work was supported by a grant from the Creative Research Initiatives Program of the Ministry of Science and Technology, Korea. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed. Tel.: 82-54-279-2128; Fax: 82-54-279-8159; E-mail: ihhwang@postech.ac.kr.

[1] The abbreviations used are: TGN, *trans*-Golgi network; ADL, *Arabidopsis* dynamin-like; PH, pleckstrin homology; CTD, C-terminal domain; MBP, maltose-binding protein; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PtdInd, phosphatidylinositol; PLC, phospholipase C; GED, GTPase effector domain.

and GGATCCGAACAACAGCCTTTGG). To generate the 1877 mutant containing the PH domain and the C-terminal domain (amino acid residues 558–914), a DNA fragment was amplified by two specific primers (GAGACGCCGGAGGTCTCTGG and ATACCTGTAAGCTGAACC). The CTD of ADL6 (amino acid residues 760–914) was PCR-amplified using two specific primers, TGTCAAGTAGAGAAAGCAAA and ATACCTGTAAGCTGAACC. To generate PHD(ΔI1), N- and C-terminal regions of the PH domain were amplified using two sets of primers: GAGACGCCGGAGGTCTCTGG and AATAGTGCATTCCTCC and AAGGACCAGGCCTTGT and GGATCCGAACAACAGCCTTTGG, respectively, and the resulting fragments were ligated. Similarly, the N- and C-terminal regions of PHD(ΔI2) were amplified using two sets of primers: GAGACGCCGGAGGTCTCTGG and AAGGGCATTGTGAGCTT and AACGAGTGGATTAATA and GGATCCGAACAACAGCCTTTGG, respectively. The N- and C-terminal regions of PHD(ΔI3) were PCR-amplified using two sets of primers: GAGACGCCGGAGGTCTCTGG and tccacgagcctggat and GGATCCGAACAACAGCCTTTGG and CCAGAAGAGGAGCTC, respectively. The N- and C-terminal fragments for PHD(ΔI1), PHD(ΔI2) and PHD(ΔI3) were then ligated. DNA fragments encoding all the deletion mutants and the full-length ADL6 were ligated in-frame with the maltose-binding protein (MBP) at the *Xba*I and *Eco*RI sites of pMAL-c2 (New England Biolabs, Beverly, MA). Also, DNA encoding these deletion mutants was ligated to pGEX-5X-1 (Amersham Biosciences) to generate glutathione *S*-transferase (GST) fusion proteins.

Expression of Recombinant Proteins—To express MBP or GST fusion proteins, the expression constructs were introduced into JM109. Expression of recombinant proteins was induced with 0.3 mM isopropyl-D-thiogalactopyranoside for 4 h at 28 °C or for 1 h at 37 °C. The cultures were harvested by centrifugation at 5,000 × *g* for 5 min at 4 °C. The pellets were resuspended in ice-cold resuspension buffer (20 mM Tris-HCl, pH 7.4, 200 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride) containing protease inhibitors (1 μg/ml aprotinin, 1 μg/ml antipain) and sonicated with 30-s bursts at a maximal setting at 4 °C. Cell debris was then removed by centrifugation at 18,000 × *g* for 15 min at 4 °C.

For purification of recombinant proteins, cleared supernatant was incubated with 1/100 volume of pre-equilibrated glutathione-Sepharose beads (for GST fusions) or amylose resin (for MBP fusions) on an orbital shaker for 30 min at 4 °C. The beads were collected by centrifugation at 1,000 × *g* for 1 min and washed three times with ice-cold suspension buffer. The fusion proteins were eluted by adding 5 mM glutathione, 50 mM Tris-HCl, pH 8.0 (for GST fusions), or 10 mM maltose, 50 mM Tris-HCl, pH 8.0 (for MBP fusions).

Fat Western Blot Analysis—Various lipids, such as phosphatidylethanolamine (PE), phosphatidylcholine (PC), PtdIns, PtdIns-4-P, phosphatidylinositol 4,5-bisphosphate (PtdIns-4,5-P_2), PtdIns-3-P, phosphatidylinositol 3,4-bisphosphate (PtdIns-3,4-P_2), and phosphatidylinositol 3,4,5-trisphosphate (PtdIns-3,4,5-P_3), were used for lipid binding analysis. The lipid binding assays were done by Fat Western blot analysis (24, 28) using affinity-purified recombinant proteins. Briefly, 10-μl volumes of various concentrations of lipids dissolved in chloroform were applied to nitrocellulose membranes. The membranes were blocked with 10 ml of buffer containing 20 mM Tris-HCl, pH 7.5, 140 mM NaCl, and 0.1% Tween 20 (TTBS) overnight at 4 °C and then incubated with 0.5 μg/ml purified recombinant protein in 10 ml of TTBS containing 3% 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 Biosciences).

Sedimentation Assay—Purified proteins were dialyzed against HP buffer (10 mM Hepes, pH 7.5, 1 mM dithiothreitol, 1 mM $MgCl_2$, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride) containing 100 mM NaCl and then centrifuged at 18,000 × *g* for 15 min to remove aggregated proteins. Liposomes were prepared by mixing phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, PtdIns-3-P, and PtdIns-4-P at the ratio indicated in each experiment, drying the mixture under nitrogen, and resuspending to a final concentration of 2 mg of total phospholipid/ml buffer containing 50 mM Hepes-NaOH (pH 7.4), 100 mM NaCl, and 0.5 mM EDTA (29). The resuspended lipids were sonicated until a homogenous suspension was formed. Liposomes were collected by centrifugation at 16,000 × *g* for 10 min. Liposome (50 μl) was mixed with proteins (5 μg in 50 μl in the same buffer) and incubated for 15 min at room temperature. Proteins bound to the liposomes were sedimented by centrifugation at 16,000 × *g* for 30 min. Proteins present in the pellet and supernatant were fractionated by SDS-PAGE and the presence of MBP fusion proteins was detected by Western blot analysis using anti-MBP antibody.

Protein Pull-down Assay—For GST pull-down assays, cleared supernatant containing 10 μg of recombinant GST fusion proteins and cleared supernatant containing 20 μg of MBP fusion proteins were mixed in 10 ml of protein pull-down buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.2% Triton X-100, 0.1% Nonidet P-40) and incubated with agitation at 4 °C for 1 h with glutathione-Sepharose beads. The beads were then pelleted by centrifugation at 2,000 × *g* for 1 min at 4 °C and washed four times with protein pull-down buffer. The bound proteins were eluted, fractionated by 10% SDS-PAGE, and subjected to Western blot analysis using an anti-MBP antibody.

RESULTS

The Majority of ADL6 Is Associated with Membranes in Vivo—ADL6 is a homolog of the mechanochemical protein dynamin and has been shown to be involved in intracellular trafficking of cargo proteins from the *trans*-Golgi network to the central vacuole (18). As in the case of other members of the dynamin family (20, 22, 24), it is likely that ADL6 is associated with membranes. To enhance our understanding of the molecular mechanism by which ADL6 plays a role *in vivo*, we investigated the nature of membrane association of ADL6. First we investigated the subcellular distribution of ADL6 *in vivo* by ultracentrifugation through a sucrose gradient. As shown in Fig. 1, the majority of ADL6 was found in the region of 37–41% sucrose, indicating that ADL6 may be associated with membranes, as in the case of dynamin and its related proteins (20, 22, 24). To further confirm that ADL6 is associated with membranes, total protein extracts were treated with Triton X-100 and then fractionated in a continuous sucrose gradient by ultracentrifugation. The presence of ADL6 in these fractions was examined by Western blot analysis. As shown in Fig. 1*A*, ADL6 was detected in the region of 30–37% sucrose in the gradient after treatment with Triton X-100, compared with 37–41% sucrose in the gradient without Triton X-100 treatment. Interestingly, the behavior of ADL6 in the sucrose gradient was rather unusual. When protein extracts treated with Triton X-100, an agent that can solubilize membranes, are fractionated in a sucrose gradient by ultracentrifugation, membrane proteins are found at the top fractions (the soluble fractions) in the sucrose gradient. However, in contrast to this notion, ADL6 was detected in the region of 30–37% sucrose of the gradient after Triton X-100 treatment but not at the top of the gradient. The behavior of ADL6 was quite similar to ADL1 and ADL2 found in plant cells (22, 24, 30). The fact that ADL6 migrated at the low percentage of sucrose in the gradient strongly suggests that Triton X-100 may have removed membranes associated with ADL6. At the same time, the fact that it did not migrate at the top of the gradient (soluble fraction) after Triton X-100 treatment suggests that ADL6 may be present as a high molecular weight complex as in the case of dynamin and ADL isoforms (22, 24, 30). Previously, it was shown that ADL1 and other dynamin-related proteins are found as high molecular weight complexes (22, 24, 30). Thus, ADL6 may also be present as a high molecular weight complex. To examine this possibility, we performed a gel filtration assay using protein extracts obtained from leaf tissues. As shown in Fig. 1*B*, ADL6 was found in two positions. The first peak was eluted much earlier than the rubisco complex (560 kDa), whereas the other eluted at a position corresponding to an ADL6 dimer (200 kDa). When protein extracts were treated with Triton X-100, the first peak of ADL6 was eluted in later fractions, whereas the second peak was at the same dimer position, indicating that Triton X-100 treatment may have removed membranes associated with ADL6 present in the first peak. These results strongly support the notion that ADL6 may be

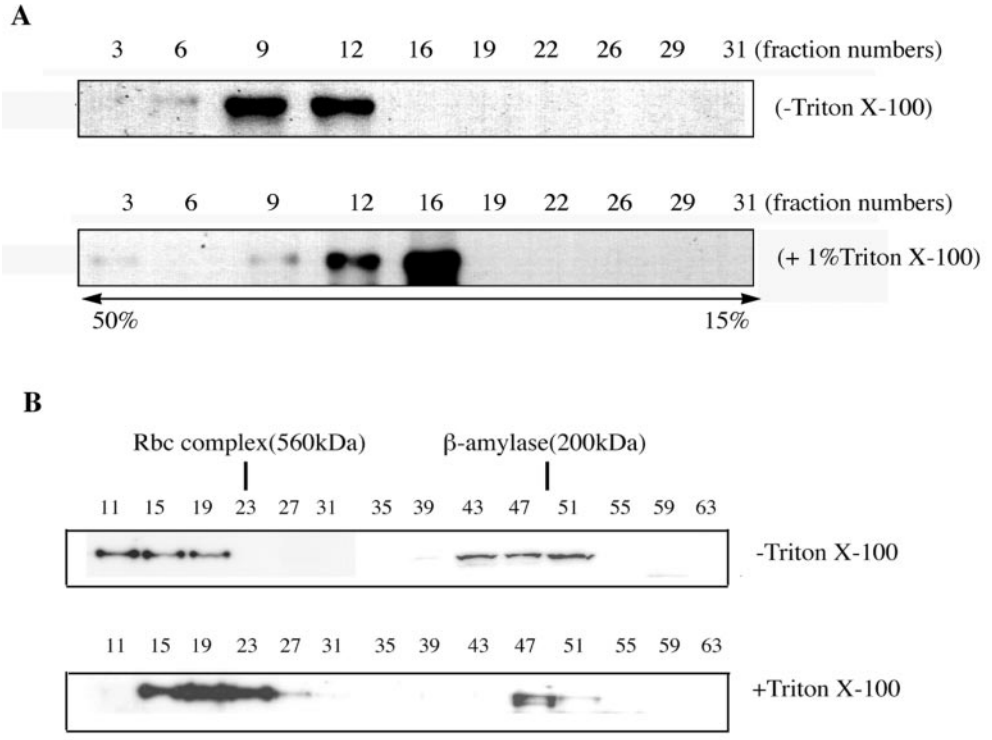


FIG. 1. **ADL6 is associated with membranes and forms a high molecular weight complex *in vivo*.** *A*, continuous sucrose gradient. Proteins from total leaf extracts were fractionated using linear sucrose gradients (15–50%) in the presence (*+1% Triton X-100*) or absence (*−Triton X-100*) of 1% Triton X-100. 1-ml fractions were collected. Proteins in 500 μl of each fraction were trichloroacetic acid-precipitated and separated by SDS-PAGE. The presence of ADL6 in these fractions was detected by immunoblot analysis with the anti-ADL6 antibody. The density of sucrose for each fraction was measured by a reflectometer. *B*, gel filtration column chromatography. Total leaf extracts (2–4 mg of protein) treated with 1% Triton X-100 (*+Triton X-100*) or without the treatment (*−Triton X-100*) were fractionated by gel filtration chromatography using Superose 300. The presence of ADL6 in these fractions was detected as described above. Also, the rubisco complex was detected by Western blot analysis using a polyclonal anti-rubisco complex antibody. The positions of β-amylase (200 kDa) and the Rubisco complex (560 kDa) are indicated.

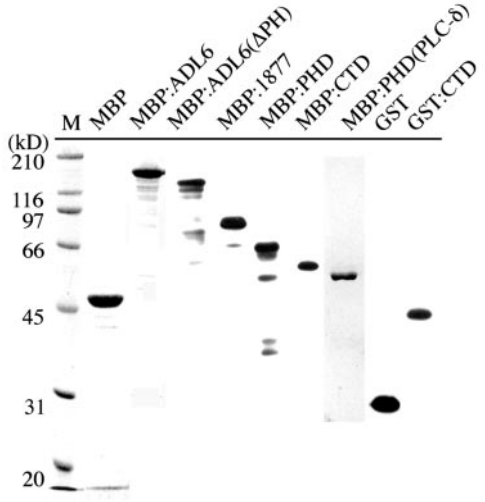


FIG. 2. **Expression and purification of recombinant proteins.** Various recombinants were expressed as MBP fusion proteins or GST fusion proteins. These recombinant proteins were expressed and purified as described under "Experimental Procedures." The purified proteins were fractionated by SDS-PAGE and stained with Coomassie Blue.

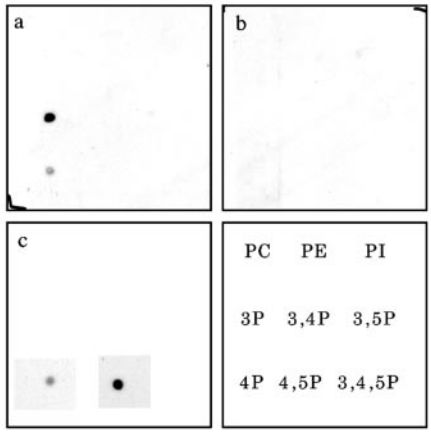
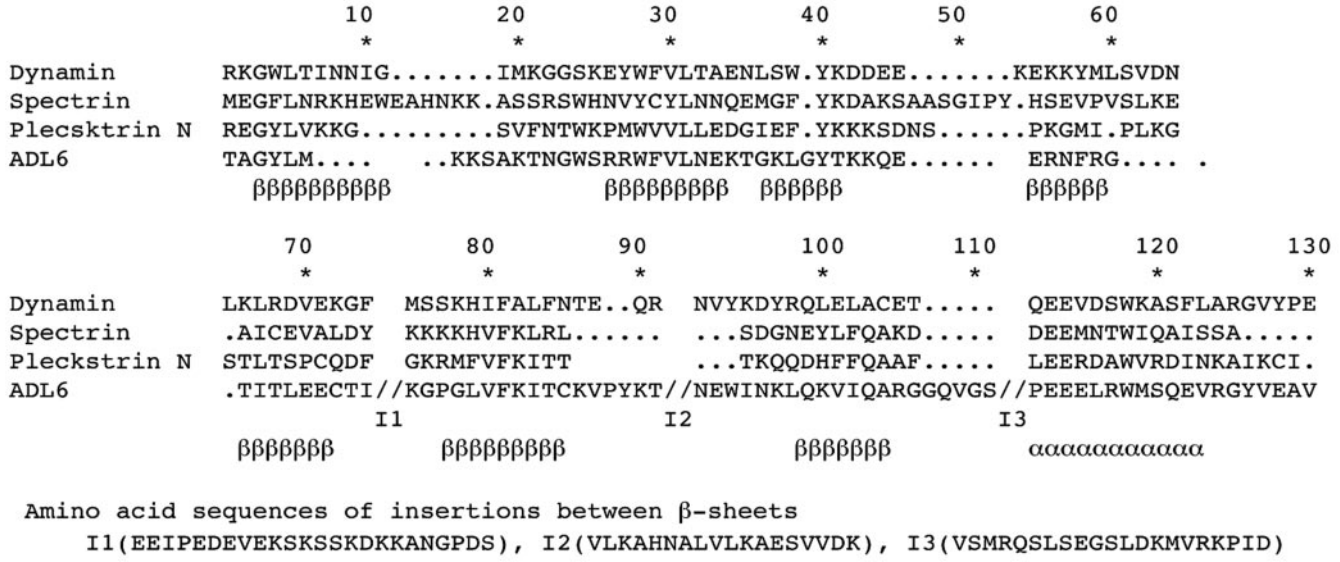


FIG. 3. **ADL6 shows a high affinity binding to PtdIns-3-P.** The phospholipid binding assay was carried out using the purified recombinant proteins, MBP·ADL6 (*a*), MBP (*b*), and MBP·PHD(PLC-δ) (*c*), as described under "Experimental Procedures." Binding of the recombinant proteins to the lipid was detected by Western blot analysis using the polyclonal anti-MBP antibody as the primary antibody. *PC*, phosphatidylcholine; *PE*, phosphatidylethanolamine; *PI*, phosphatidylinositol; *3P*, phosphatidylinositol 3-phosphate; *4P*, phosphatidylinositol 4-phosphate; *3,4P*, phosphatidylinositol 3,4-bisphosphate; *3,5P*, phosphatidylinositol 3,5-bisphosphate; *4,5P*, phosphatidylinositol 4,5-bisphosphate; *3,4,5P*, phosphatidylinositol 3,4,5-trisphosphate.

a high molecular weight complex *in vivo*, as in the case of dynamin, ADL1, and ADL2 (22, 24, 30).

ADL6 Binds Specifically to Phosphatidylinositol 3-Phosphate in Vitro—In the case of dynamin, the PH domain shows high affinity binding to phosphatidylinositol phosphates such as phosphatidylinositol 4,5-bisphosphate PtdIns-4,5-P_2 (20, 21). Also, ADL2 has been shown to bind to phosphatidylinositol 4-phosphate (PtdIns-4-P) (24). Binding to these phospholipids by the PH domain or similar lipid-binding domains is thought to allow the proteins with these domains to associate with membranes. Thus, to further understand the nature of the membrane association of ADL6, we wanted to investigate



Amino acid sequences of insertions between β-sheets
I1(EEIPEDEVEKSKSSKDKKANGPDS), I2(VLKAHNALVLKAESVVDK), I3(VSMRQSLSEGSLDKMVRKPID)

FIG. 4. **Amino acid sequence alignment of the putative PH domain.** Gaps were introduced to maximize the alignment. Also, three inserted regions were deleted from the sequence. The sequences of the three inserted regions are indicated by *I1*, *I2*, and *I3*. The secondary structure was predicted by the protein secondary structure prediction program from the ExPASy Molecular Biology Server. α and β indicate the α-helix and β-sheets, respectively.

whether ADL6 also binds to any of these phospholipids. To address this question, we prepared recombinant ADL6 protein from *Escherichia coli* as a MBP fusion protein. MBP:ADL6 was affinity-purified using amylose resin beads and used for lipid binding assays (Fig. 2) (24, 28). MBP:PHD(PLC-δ), which shows a high affinity binding to PtdIns-4,5-P_2 (31), was expressed and purified for use as a positive control for the lipid binding assays. In addition, MBP alone was included as a negative control. Among various phospholipid molecules examined, MBP:ADL6 showed high affinity binding to PtdIns-3-P by Fat Western blot analysis (Fig. 3). As expected, PHD(PLC-δ) showed a high affinity interaction with PtdIns-4,5-P_2 when used as a MBP fusion protein (31). In contrast, the negative control MBP did not show binding to any of these phospholipids. These results strongly suggest that ADL6 binds specifically to PtdIns-3-P.

The PH Domain of ADL6 Is Responsible for Binding of ADL6 to Phospholipids—To enhance our understanding of the lipid binding of ADL6, we wanted to investigate which region of ADL6 is responsible for binding to PtdIns-3-P. We first compared the amino acid sequence of ADL6 to other members of the dynamin family. Amino acid sequence analysis using Blastp from the NCBI server suggested that ADL6 has a PH domain (data not shown). In addition, as shown in Fig. 4, the region from amino acid residues 558–759 showed a significant degree of amino acid sequence homology to the PH domains of various proteins. Similar to the PH domains of other proteins, this region was predicted to consist of 7 β-sheets followed by an α-helix (20, 32) using the protein secondary structure prediction program of the ExPASy Molecular Biology Server. However, the PH domain of ADL6 was slightly larger than the PH domain found in dynamin I and phospholipase C-δ. Amino acid sequence alignment of these PH domains revealed that the PH domain of ADL6 has additional amino acids inserted between the β-sheet structures in the PH domain (Fig. 4).

With this information, several deletion mutants were generated, as shown in Fig. 5*A*, and expressed as MBP fusion proteins, as shown in Fig. 2. Using these recombinant proteins, the lipid binding assay was performed. As in the case of the full-length ADL6 protein, fusion proteins with the PH domain bound to PtdIns-3-P (Fig. 5*B*). However, interestingly, the fusion protein between MBP and the PH domain (MBP:PHD) showed a slightly different binding pattern than the full-length ADL6 protein (Fig. 6, *PHD*). The wild-type ADL6 and 1877 showed ~4-fold higher binding affinity to PtdIns-3-P than to PtdIns-4-P (Fig. 5*C*). In contrast, as shown in Fig. 5, *B* and *C*, the PH domain alone showed nearly equal binding affinity to both PtdIns-3-P and PtdIns-4-P. In addition, the binding affinity of MBP:PHD to these lipids was lower than that of the full-length ADL6. Thus, these results suggest two points: 1) the PH domain is responsible for the binding of ADL6 to phospholipids and 2) the lipid binding specificity of the PH domain is different from that of ADL6. This is quite unexpected because in most cases the PH domain alone is responsible for phospholipid binding (20). To further confirm the lipid binding of ADL6, we performed a liposome sedimentation assay using various lipid molecules. We used MBP:1877 because this protein showed lipid binding properties identical to the wild-type. Liposomes were mixed with MBP:1877 and precipitated by centrifugation. The pellet fractions were then probed for the presence of MBP:1877 by Western blot analysis using a polyclonal anti-MBP antibody. As shown in Fig. 5*D*, the liposome containing PtdIns-3-P was most effective in precipitating MBP:1877. Thus, these results further support the notion that ADL6 is associated with membranes through binding to PtdIns-3-P.

A Protein-Protein Interaction between the PH Domain and CTD Increases Binding Affinity to Phosphatidylinositol 3-Phosphate—As shown above, the PH domain showed a different lipid binding specificity from the full-length ADL6. To confirm this, we performed liposome-mediated sedimentation experiments with MBP:PHD. As shown in Fig. 6, liposomes containing PtdIns-4-P were nearly as effective as PtdIns-3-P in sedimenting MBP:PHD. This result strongly suggests that an additional region of ADL6 may contribute to its lipid binding specificity. Because MBP:1877 showed lipid binding properties identical to the full-length ADL6, it is likely that the C-terminal region of the PH domain may play a role in determining lipid binding specificity. To investigate how the C-terminal region contributes to the lipid binding of the PH domain, we examined possible interactions between the PH domain and the CTD of ADL6. We performed protein pull-down assays using recombinant proteins (MBP:1877,

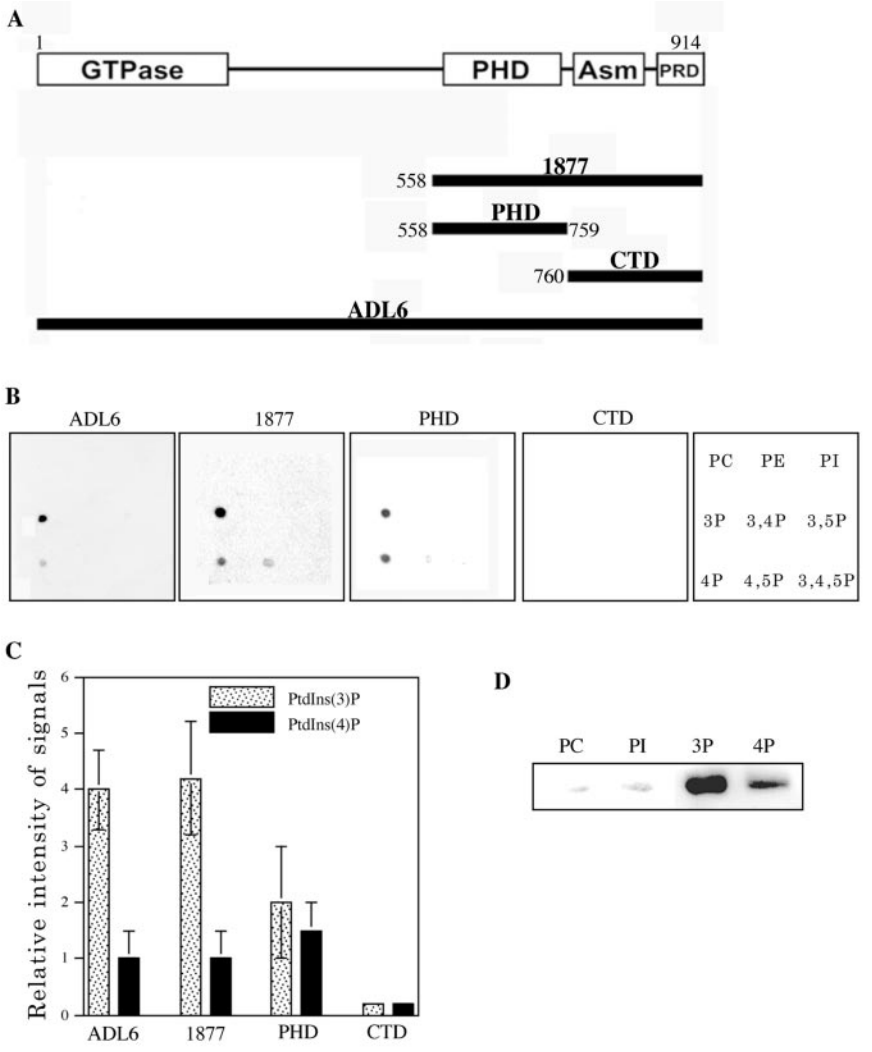


FIG. 5. **The PH domain of ADL6 is responsible for phospholipid binding.** *A*, *schemes* of various deletion mutants. The *numbers* indicate the positions of the amino acid residues. Domains of ADL6 showing homology to various domains found in dynamin I are depicted. *GTPase*, GTPase domain; *PHD*, PH domain; *Asm*, assembly domain; *PRD*, proline-rich domain. *CTD*, the C-terminal domain containing the assembly domain and proline-rich domain. *B*, Fat Western blot assay of lipid binding. The phospholipid-binding assay was carried out using the purified recombinant proteins indicated. *C*, quantification of lipid binding affinity. To quantify the lipid binding affinity, each spot was quantified by densitometry. The values are means ± S.D. with $n = 3$. *D*, liposome sedimentation assay. MBP:1877 bound to liposomes was sedimented by centrifugation at 16,000 × *g* for 30 min. MBP:1877 present in the pellet was detected by Western blot analysis using a polyclonal anti-MBP antibody. *PC*, liposome prepared with PC. *PI*, *3P*, and *4P* indicate liposome prepared with PC and PE at the ratio of 80:19 (wgt %) with 1% of PI, PtdIns-3-P, and PtdIns-4-P, respectively.

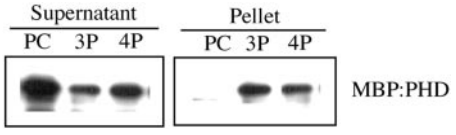


FIG. 6. **Liposome-mediated sedimentation of the PH domain.** MBP:PHD bound to liposomes was sedimented by centrifugation at 16,000 × *g* for 30 min. MBP:PHD present in the supernatant and pellet was detected by Western blot analysis using a polyclonal anti-MBP antibody. *PC*, liposome prepared with PC. *3P* and *4P* indicate liposomes prepared with PC and PE at the ratio of 80:19 (wgt %) with 1% of PtdIns-3-P and PtdIns-4-P, respectively.

GST·CTD, and MBP:PHD) expressed in *E. coli* (Fig. 2). As shown in Fig. 7, MBP:1877 strongly bound to GST:CTD. Furthermore, MBP:PHD was sufficient to bind to GST:CTD (Fig. 7). These results strongly suggest that the PH domain interacts with CTD. This is quite a contrast to the PH domain of dynamin because the PH domain from dynamin is known to bind to phospholipids but not involved in the protein-protein interaction (20, 21). Interestingly, the interaction between the PH domain and CTD was strongly dependent on the concentration of NaCl, indicating that ionic strength may play an important role in this interaction. With this information, we next examined whether the interaction between the PH domain and the CTD plays any role in determining the lipid binding specificity of the PH domain. To address this question, we again performed a lipid binding assay. This time, MBP:PHD was incubated with MBP:CTD and then used for the lipid binding assay. As shown in Fig. 8*A*, in the presence of CTD, the lipid binding specificity of the PH domain was nearly identical to that of MBP:1877 and MBP:ADL6. The binding affinity was ~4-fold higher when binding to PtdIns-3-P than to PtdIns-4-P (Fig. 8*B*). However, the binding affinity to PtdIns-4-P was nearly equal, indicating that the interaction between the PH domain and CTD increases the binding affinity of the PH domain to PtdIns-3-P. This result strongly suggests that the PH domain interacts with the CTD, which in turn determines the phospholipid binding specificity of ADL6.

The Inserted Regions Influence Lipid Binding Specificity of the PH Domain—The PH domain is an independently folded domain of ~100 amino acid residues and has been shown to consist of 7 β-sheets followed by an α-helix as determined by three-dimensional structure modeling (20, 32). However, the PH domain of ADL6 is unusually long and appears to have 3 extra inserted regions between the β-sheets (Fig. 4). Also, as shown above, unlike other PH domains, the lipid binding specificity of the PH domain is changed upon interaction of the PH domain with CTD. Thus, we asked whether this inserted region has any role in the unusual behavior of the PH domain of ADL6. To address this question, we generated several mutants without the inserted regions (Fig. 9*C*) and expressed them as MBP fusion proteins. These proteins were affinity-purified using amylose resin ((Fig. 9*B*) and used for lipid binding assays. As shown in Fig. 9*A*, the deletion of inserted region I1 slightly increased the binding affinity to PtdIns-3-P. In contrast, the

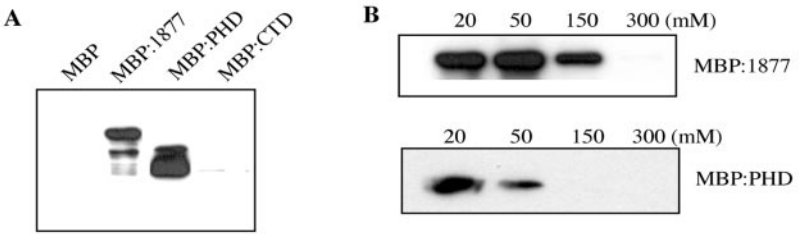


FIG. 7. **A protein-protein interaction between the PH domain and the C-terminal domain.** *A*, GST:CTD was mixed with MBP fusion proteins indicated at 50 mM NaCl concentration. Proteins bound to GST:CTD were precipitated by sedimentation of the glutathione-Sepharose beads. Pelleted proteins were detected by Western blot analysis using the anti-MBP antibody. *B*, protein pull-down assay was carried out at different NaCl concentrations as indicated.

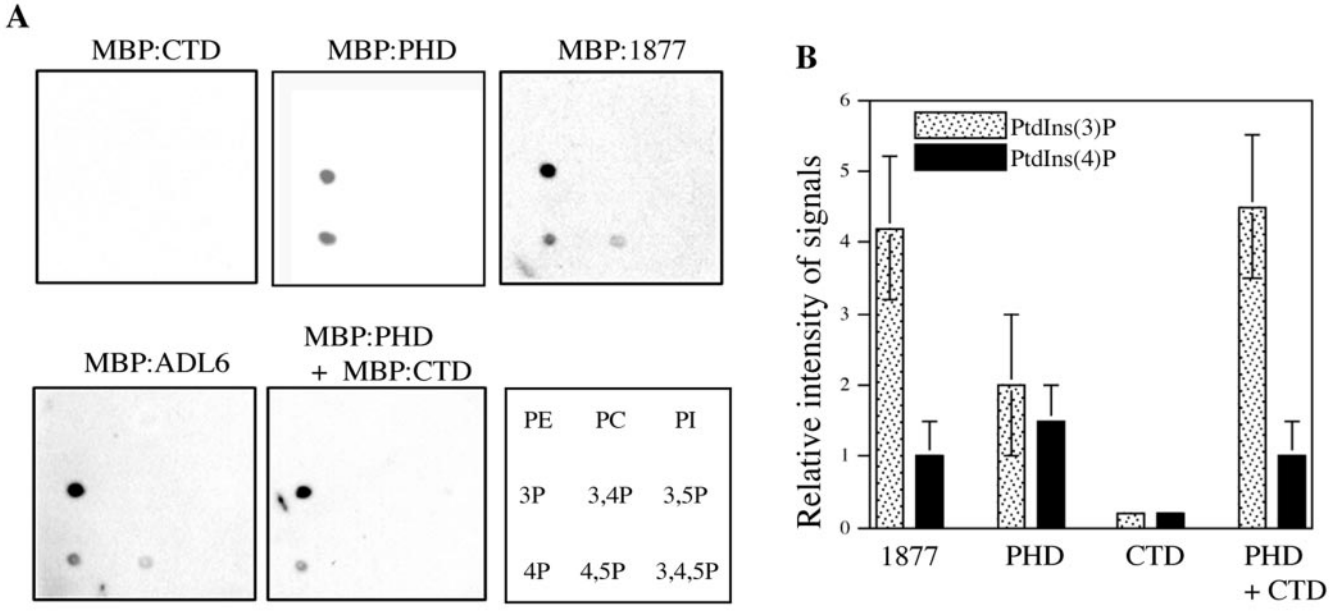


FIG. 8. **The C-terminal domain is involved in determining lipid binding specificity.** The phospholipid binding assay was carried out using the purified recombinant proteins indicated in a buffer containing 50 mM NaCl (*A*). The lipid binding affinity was quantified by densitometry (*B*). The values are means ± S.D. with $n = 3$.

deletion of I2 or I3 nearly completely eliminated binding to PtdIns-4-P. Thus, these results strongly suggest that the inserted regions may affect the lipid binding specificity of the PH domain.

DISCUSSION

A large number of proteins are known to bind to various phospholipids. In most cases, these proteins have a specific domain involved in phospholipid binding (33–35). The amino acid sequence analysis of ADL6 revealed that, among the various lipid binding domains known to exist, ADL6 has a PH domain as in the case of dynamins found in animal cells (20, 21, 36). This is in sharp contrast to other dynamin-like proteins found in plant cells (5, 24). Although these plant proteins have been shown to bind to membranes (22–24), it is not clear which domain is involved in membrane association, and, with the exception of ADL2, it is not known which specific phospholipids are bound (24).

Among the various lipids we examined by Fat Western blot analysis and liposome pull-down assays, ADL6 showed high affinity binding to PtdIns-3-P and weak binding to PtdIns-4-P. The PH domain of ADL6 was responsible for lipid binding. The lipid binding specificity of ADL6 was different from that of other PH domains. It has been shown that the PH domains of dynamin and PLC-δ show high affinity binding to PtdIns-4,5-P_2 (20, 21, 31). The PH domain of PtdIns 4-kinase β binds to PtdIns-4-P (28). Also, PH domains of Grp1 and PLC-β bind to PtdIns-3,4,5-P_3 (37, 38). However, when we examined the lipid binding of the PH domain of ADL6, it showed nearly equal binding affinity to both PtdIns-3-P and PtdIns-4-P, which is in contrast to the high affinity binding of ADL6 to PtdIns-3-P. This result was quite unexpected because, in most cases, the PH domain alone is sufficient to represent the lipid binding specificity of the whole protein (20, 21, 31). To maintain the lipid binding specificity of ADL6, the PH domain has to have the CTD, suggesting that the C-terminal domain of ADL6 affects the lipid binding. We examined the possibility of a protein-protein interaction between the PH domain and the CTD of ADL6 as a means by which the CTD could affect the lipid binding specificity of the PH domain. Recently it has been shown that the PH domain is also involved in protein-protein interactions (39, 40). Interestingly, there was a strong interaction between the PH domain and the CTD at low salt concentrations. Furthermore the interaction between the PH domain and the CTD restored lipid binding specificity to the PH domain so that it bound to PtdIns-3-P with high affinity.

When we examined the amino acid sequence of the CTD it has two motifs, a coiled-coil region and a proline-rich motif, that may be involved in protein-protein interactions. The coiled-coil region of the CTD has some degree of amino acid sequence homology to the GTPase effector domain (GED) (also known as the assembly domain) of dynamin I (27, 41, 42). The GED or assembly domain of dynamin I has been shown to be

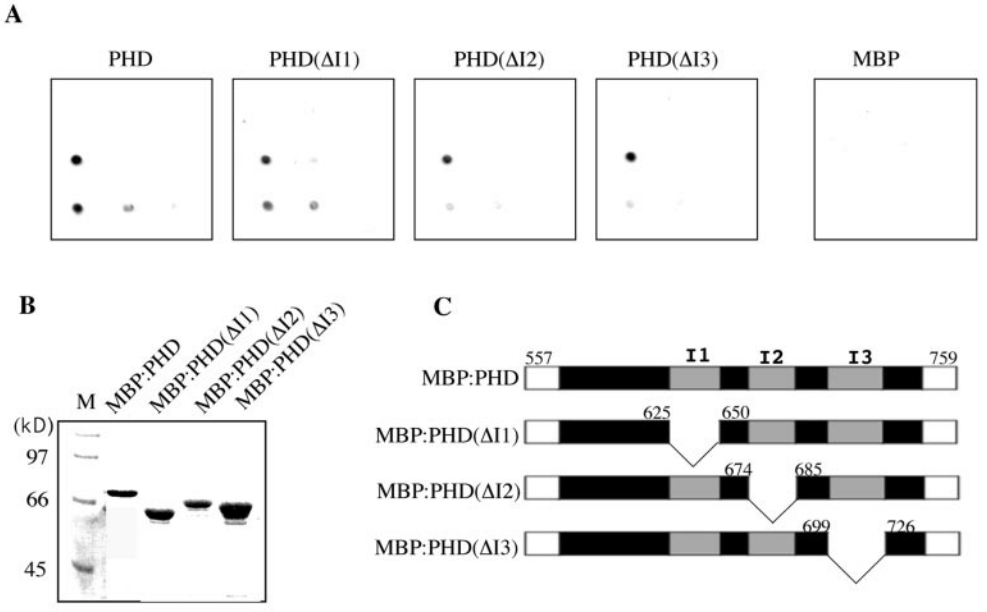


FIG. 9. **Deletion of inserted regions from the PH domain result in specific binding to PtdIns-3-P.** *A*, binding of deletion mutants to phospholipids. Fat Western blot analysis was carried out using the purified recombinant proteins indicated. *B*, expression and purification of deletion mutants. These deletion mutants were expressed and purified as described under "Experimental Procedures." Affinity-purified proteins were fractionated by SDS-PAGE and stained with Coomassie Blue. *C*, *schemes* of deletion mutants. The *numbers* indicate amino acid positions.

involved in interaction between dynamin molecules. In contrast, the proline-rich motif (P*XX*P) found in dynamin I has been shown to be involved in interactions with the SH3 domain of various proteins involved in intracellular signaling (7). Thus, it is likely that the coiled-coil region with homology to the assembly domain of dynamin I may be involved in protein-protein interaction with the PH domain. In the case of dynamin I, the GED located downstream of the PH domain was shown to be involved in intermolecular interactions with another domain located at the N terminus, resulting in homopolymeric assembly of dynamin I (27, 41, 42). Similar intermolecular interactions have also been shown to occur in other dynamin-related proteins (15, 25, 26). The CTD also interacts with other domains of ADL6.[2] However, the CTD interacts most strongly with the PH domain. These results strongly suggest that the CTD interacts mainly with the PH domain. If the intermolecular interaction between the CTD (or coiled-coil region with homology to the assemble domain) and the PH domain of ADL6 occurs in an orientation of tail to head it could result in homopolymeric self-assembly of ADL6, as in the case of dynamin and other dynamin-like proteins (25, 27, 41, 42). In fact, *in vivo* the majority of ADL6 was present as a high molecular weight complex with only a minor portion as the dimeric form. Thus, it is possible that the CTD may interact with the PH domain for self-assembly of ADL6 into a homopolymeric form. In fact, when MBP:1877 containing both the PH domain and the CTD was fractionated by gel filtration chromatography, it was eluted as a high molecular weight form,[2] which strongly supports the notion that the interaction between the CTD and PH domain is sufficient to self-assemble the protein into a high molecular weight complex. Interestingly, the interaction between the CTD and the PH domain was favored at low NaCl concentration. At the moment it is not clear about the dependence of interaction on ionic strength. It is possible that the PH domain and/or the CTD may undergo conformational change upon ionic strength *in vitro*. However, *in vivo* it is likely that the conformational change of these domains, if there is any, may be caused by other means such as phosphorylation.

It is well understood that dynamin localized at the plasma membrane is critical for endocytosis (8, 11, 12). In contrast, ADL6 has been suggested to be localized at the TGN and involved in vacuolar trafficking of cargo proteins (18). This indicates that although dynamin and ADL6 are involved in very similar processes (such as severing the neck of an invaginated bud as a vesicle from the donor compartment) they must be targeted to different compartments. One possible mechanism for targeting similar proteins to different compartments is to use different lipid binding specificity. In *Arabidopsis* it has been shown that PtdIns-3-P is transported from the TGN to the central vacuole through the prevacuolar compartment (43), thus raising the possibility that PtdIns-3-P concentration is high at the TGN. In contrast, PtdIns-4-P and PtdIns-4,5-P_2 appear to be rich in the plasma membrane of plant cells because the PH domain of PLC-δ in rats, which is known to bind specifically to PtdIns-4,5-P_2 (31), was shown to be targeted to the plasma membrane in pollen (44) and *Arabidopsis* leaf protoplasts.[3] In addition, a PH domain known as phosphatidylinositol four-phosphate adaptor protein (45) is also primarily targeted to the plasma membrane when expressed as a GFP fusion protein in *Arabidopsis* protoplasts.[3] If this hypothesis is true, it is quite reasonable for ADL6 to have high binding affinity for PtdIns-3-P instead of PtdIns-4-P or PtdIns-4,5-P_2 in order to target ADL6 to the TGN. In fact, depletion of PtdIns-3-P caused inhibition of vacuolar trafficking at the TGN (43), thus raising the possibility that the inhibition may be caused by the lack of PtdIns-3-P, which may play an important role at the TGN such as recruitment of ADL6 to the TGN.

Interestingly, the PH domain of ADL6 is larger than other PH domains. Amino acid sequence analysis revealed that it appears to have three extra inserted regions between the β-sheets. The deletion of the inserted region allows the PH domain alone to bind PtdIns-3-P with high affinity. This result raises the possibility that the high lipid binding affinity of ADL6 to PtdIns-3-P must be achieved as a result of an interaction between the PH domain and the CTD. Previously, it was shown that dynamin is cycled between the low molecular

[2] S. H. Lee and I. Hwang, unpublished data.

[3] Y.-W. Kim and I. Hwang, unpublished data.

weight soluble form and a membrane-bound high molecular weight complex (46–47). In addition, the lipid binding of dynamin promotes self-assembly into the homopolymeric form. Furthermore, oligomerization of the PH domain of dynamin isoforms increases binding affinity to phospholipids (48). Thus, the high affinity lipid binding to PtdIns-3-P and self-assembly into a homopolymeric form is a coupled process in ADL6, which in turn could facilitate a transition from the soluble dimeric form to the membrane-associated polymeric form during vesicle formation.

At the moment, it is not clearly understood how the protein-protein interaction between the PH domain and CTD changes the lipid binding specificity. Sequence alignment and modeling of the putative PH domain might give a clue to the understanding of the observed change in specificity by protein interactions and loop insertions. However, this is beyond our expertise.

REFERENCES

1. Obar, R. A., Collins, C. A., Hammarback, J. A., Shpetner, H. S., and Vallee, R. B. (1990) *Nature* **347,** 256–261
2. Rothman, J. H., Raymond, C. K., Gilbert, T., O'Hara, P. J., and Stevens, T. H. (1990) *Cell* **61,** 1063–1074
3. Chen, M. S., Obar, R. A., Schroeder, C. C., Austin, T. W., Poodry, C. A., Wadsworth, S. C., and Vallee, R. B. (1991) *Nature* **351,** 583–586
4. Dombrowski, J. E., and Raikhel, N. V. (1995) *Plant Mol. Biol.* **28,** 1121–1126
5. Gu, X., and Verma, D. P. S. (1996) *EMBO J.* **15,** 695–704
6. Kang, S. G., Jin, J. B., Piao, H. L., Pih, K. T., Jang, H. J., Lim, J. H., and Hwang, I. (1998) *Plant Mol. Biol.* **38,** 437–447
7. Gout, I., Dhand, R., Hiles, I. D., Fry, M. J., Panayotou, G., Das, P., Truong, O., Totty, N. F., Husan, J., Booker, G. W., Campbell, I. D., and Waterfield, M. D. (1993) *Cell* **75,** 25–36
8. Herskovits, J. S., Burgess, C. C., Obar, R. A., and Vallee, R. B. (1993) *J. Cell Biol.* **122,** 565–578
9. Damke, H., Baba, T., Warnock, D. E., and Schmid, S. L. (1994) *J. Cell Biol.* **127,** 915–934
10. Hinshaw, J. E., and Schmid, S. L. (1995) *Nature* **374,** 190–192
11. Takei, K., McPherson, P. S., Schmid, S. L., and De Camilli, P. (1995) *Nature* **374,** 186–190
12. Sever, S., Damke, H., and Schmid, S. L. (2000) *J. Cell Biol.* **150,** 1137–1148
13. Takei, K., Haucke, V., Slepnev, V., Farsad, K., Salazar, M., Chen, H., and De Camilli, P. (1998) *Cell* **94,** 131–141
14. Mozdy, A. D., McCaffery, J. M., and Shaw, J. M. (2000) *J. Cell Biol.* **151,** 367–380
15. Fukushima, N. H., Brisch, E., Keegan, B. R., Bleazard, W., and Shaw, J. M. (2001) *Mol. Biol. Cell* **12,** 2756–2766
16. Arimura Si, S., and Tsutsumi, N. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99,** 5727–5731
17. Park, J. M., Cho, J. H., Kang, S. G., Jang, H. J., Pih, K. T., Piao, H. L., Cho, M. J., and Hwang, I. (1998) *EMBO J.* **17,** 859–867
18. Jin, J. B., Kim, Y. A, Kim, S. J., Lee, S. H., Kim, D. H., Cheong, G. W., and Hwang, I. (2001) *Plant Cell* **13,** 1511–1526
19. Yoon, Y., Pitts, K. R., and McNiven, M. A. (2001) *Mol. Biol. Cell* **12,** 2894–2905
20. Salim, K., Bottomley, M. J, Querfurth, E., Zvelebil, M. J., Gout, I., Scaife, R., Margolis, R. L., Gigg, R., Smith, C. I., Driscoll, P. C., Waterfield, M. D., and Panayotou, G. (1996) *EMBO J.* **15,** 6241–6250
21. Achiriloaie, M., Barylko, B., and Albanesi, J. P. (1999) *Mol. Cell. Biol.* **19,** 1410–1415
22. Park, J. M., Kang, S. G., Pih, K. T., Jang, H. J., Piao, H. L., Yoon, H. W., Cho, M. J., and Hwang, I. (1997) *Plant Physiol.* **115,** 763–771
23. Gu, X., and Verma, D. P. (1997) *Plant Cell* **9,** 157–169
24. Kim, Y. W., Park, D. S., Park, S. C., Kim, S. H., Cheong, G. W., and Hwang, I. (2001) *Plant Physiol.* **127,** 1243–1255
25. Shin, H. W., Takatsu, H., Mukai, H., Munekata, E., Murakami, K., and Nakayama, K. (1999) *J. Biol. Chem.* **274,** 2780–2785
26. Zhang, Z., Hong, Z., Verma, D. P. (2000) *J. Biol. Chem.* **275,** 8779–8784
27. Smirnova, E., Shurland, D. L., Newman-Smith, E. D., Pishvaee, B., and van der Bliek, A. M. (1999) *J. Biol. Chem.* **274,** 14942–14947
28. Stevenson, J. M., Perera, I. Y., and Boss, W. F. (1998) *J. Biol. Chem.* **273,** 22761–22767
29. Patki, V., Virbasius, J., Lane, W. S., Toh, B. H., Shpetner, H. S., and Corvera, S. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 7326–7330
30. Warnock, D. E., Hinshaw, J. E., and Schmid, S. L. (1996) *J. Biol. Chem.* **271,** 22310–22314
31. Garcia, P., Gupta, R., Shah, S., Morris, A. J., Rudge, S. A., Scarlata, S., Petrova, V., McLaughlin, S., and Rebecchi, M. J. (1995) *Biochemistry* **34,** 16228–16234
32. Soisson, S. M., Nimnual, A. S., Uy, M., Bar-Sagi, D., and Kuriyan, J. (1988) *Cell* 16, **95,** 259–268
33. Stenmark, H., and Aasland, R. (1999) *J. Cell Sci.* **112,** 4175–4183
34. Lemmon, M. A., and Ferguson, K. M. (2001) *Biochem. Soc. Trans.* **29,** 377–384
35. Xu, Y., Seet, L. F., Hanson, B., and Hong, W. (2001) *Biochem. J.* **360,** 513–530
36. Lemmon, M. A., Ferguson, K. M., Sigler, P. B., and Schlessinger, J. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 10472–10476
37. Lietzke, S. E., Bose, S., Cronin, T., Klarlund, J., Chawla, A., Czech, M. P., and Lambright, D. G. (2000) *Mol. Cell* **6,** 385–394
38. Razzini, G., Ingrosso, A., Brancaccio, A., Sciacchitano, S., Esposito, D. L., and Falasca, M. (2000) *Mol. Endocrinol.* **14,** 823–836
39. Chang, J. S., Seok, H., Kwon, T. K., Min, D. S., Ahn, B. H., Lee, Y. H., Suh, J. W., Kim, J. W., Iwashita, S., Omori, A., Ichinose, S., Numata, O., Seo, J. K., Oh, Y. S., and Suh, P. G. (2002) *J. Biol. Chem.* **277**, 19697–19702
40. Kim, J. H., Lee, S., Kim, J. H., Lee, T. G., Hirata, M., Suh, P. G., and Ryu, S. H. (2002) *Biochemistry* **41,** 3414–3421
41. Muhlberg, A. B., Warnock, D. E., and Schmid, S. L. (1997) *EMBO J.* **16,** 6676–6683
42. Okamoto, P. M., Tripet, B., Litowski, J., Hodges, R. S., Vallee, R. B. (1999) *J. Biol. Chem.* **274,** 10277–10786
43. Kim, D. H., Eu, Y. J., Yoo, C. M., Kim, Y. W., Pih, K. T., Jin, J. B., Kim, S. J., Stenmark, H., and Hwang, I. (2001) *Plant Cell* **13,** 287–301
44. Kost, B., Lemichez, E., Spielhofer, P., Hong, Y., Tolias, K., Carpenter, C., and Chua, N. H. (1999) *J. Cell Biol.* **145,** 317–330
45. Dowler, S., Currie, R. A., Campbell, D. G., Deak, M., Kular, G., Downes, C. P., and Alessi, D. R. (2000) *Biochem. J.* **351,** 19–31
46. Tuma, P. L., and Collins, C. A. (1995) *J. Biol. Chem.* **270,** 26707–26714
47. Carr, J. F., and Hinshaw, J. E. (1997) *J. Biol. Chem.* **272,** 28030–28035
48. Klein, D. E., Lee, A., Frank, D. W., Marks, M. S., and Lemmon, M. A. (1998) *J. Biol. Chem.* **273,** 27725–27733