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INSIGHT

An Emerging Model of Auxin Transport Regulation

The hormone auxin plays a critical role in the regulation of plant growth and development. Auxin is transported from cell to cell with strict directionality by uptake and efflux carrier proteins (reviewed by Muday and DeLong, 2001). The polarity of auxin transport is believed to be controlled by the localization of auxin transport proteins, with both putative efflux carriers and influx carriers having asymmetric distributions (Gälweiler et al., 1998; Muller et al., 1998; Swarup et al., 2001). This article highlights recent advances in identification of proteins that participate in auxin efflux and the mechanisms by which these proteins control the polar movement of auxin.

Several proteins have been implicated in the control of auxin efflux from cells. The best characterized are the PIN proteins, which are encoded by a large gene family in Arabidopsis (reviewed by Palme and Gälweiler, 1999). Other proteins that bind indoleacetic acid (IAA) efflux inhibitors, including a *multidrug resistance*–like protein, also may function in auxin transport, as discussed below. Defects in the genes encoding PIN proteins were identified first in mutants with shoot (*pin1*) and root (*agr1*/*eir1*/*pin2*/*wav6*) phenotypes consistent with reduced IAA transport (Chen et al., 1998; Gälweiler et al., 1998; Luschnig et al., 1998; Muller et al., 1998; Utsuno et al., 1998). Functional assays in yeast expressing *AGR1* or *EIR1* suggest carrier activity (Chen et al., 1998; Luschnig et al., 1998), although the ability of PIN proteins to mediate IAA transport has not been demonstrated directly. The most exciting aspect of these proteins is that they show asymmetric cellular localization consistent with a role in controlling the polar efflux of IAA from cells (Gälweiler et al., 1998; Muller et al., 1998). A very recent report presents evidence that both asymmetric localization and dynamic redistribution of PIN3 protein are involved in the lateral redistribution of auxin in response to tropic stimuli (Friml et al., 2002).

NEW EVIDENCE FOR ACTIN-DEPENDENT VESICULAR CYCLING OF PIN1

Recent immunocytochemical localization studies have shown that PIN1 cycles between the plasma membrane and an internal compartment. Inhibitors such as brefeldin A (BFA) or mutations in the GNOM gene encoding an ADP ribosylation factor–guanine nucleotide exchange factor (ARF-GEF), both of which block vesicle movement, destroy the normal asymmetric membrane localization of PIN1, and lead to its accumulation in internal cellular compartments (Steinmann et al., 1999). When BFA is removed, the normal localization is restored rapidly (Geldner et al., 2001), suggesting that this vesicle-dependent targeting is dynamic in character (Figure 1). Similarly, treatment with BFA blocks auxin efflux from tissue culture cells and reduces both seedling elongation and gravitropic bending, demonstrating that conditions that prevent PIN1 plasma membrane localization also reduce auxin efflux (reviewed by Morris, 2000; Geldner et al., 2001).

The localization of PIN1 also appears to depend on the actin cytoskeleton. Both PIN1 asymmetric localization and polar auxin transport are reduced in response to treatment with cytochalasin, a drug that fragments the actin cytoskeleton (Butler et al., 1998; Geldner et al., 2001). Cytochalasin treatment also prevents both the internalization of PIN1 in response to BFA treatment and the restoration of PIN1 localization upon the removal of BFA (Geldner et al., 2001). The role of the actin cytoskeleton in this process may be to provide tracks for vesicle movement and/or to fix the efflux carriers in an asymmetric distribution after delivery to the membrane surface (reviewed by Muday and DeLong, 2001). The involvement of the actin cytoskeleton in this process is consistent with results that indicate that a protein that binds auxin transport inhibitors, such as naphthylphthalamic acid (NPA), interacts with the actin cytoskeleton (Butler et al., 1998; Hu et al., 2000), and this NPA binding protein may provide a bridge between efflux carriers and the actin network used to transport and/or localize these complexes (Figures 1A and 1B). This rapid vesicle cycling of auxin efflux carriers may deliver these proteins to the appropriate membrane and could allow the redistribution of carriers to a new membrane when auxin transport polarity is changed by environmental stimuli such as light or gravity (reviewed by Muday, 2001; Friml et al., 2002).

Two recent reports have suggested that high concentrations of artificial IAA efflux inhibitors may reduce transport by perturbing the movement of vesicles containing auxin efflux carriers to and from the membrane surface. Geldner et al. (2001) have shown that the IAA efflux inhibitor triiodobenzoic acid (TIBA) can prevent both the internalization of PIN1 in response to BFA treatment and the recovery of PIN1 localization after BFA is removed (Figures 1C and 1D). An important caveat is that there is no detectable change in the localization of PIN1 when incubated with TIBA alone (Geldner et al., 2001). The most striking of these results is the effect of TIBA during BFA washout experiments. Under

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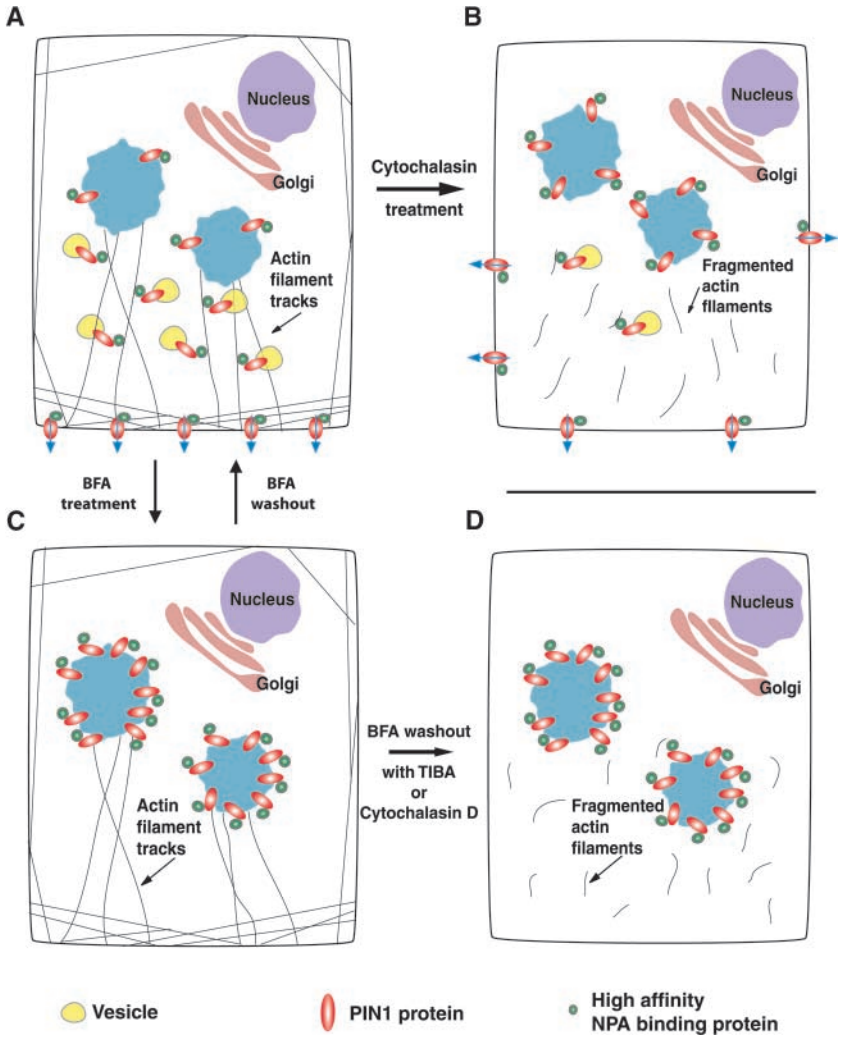

Figure 1. Model of Experiments That Have Examined the Mechanisms of the Control of PIN1 Protein Localization.

(A) Vesicle-dependent transport of the PIN1 protein to the basal membrane appears to be along actin tracks. A high-affinity NPA binding protein has been found to interact with actin and may act as a bridge between these transport vesicles and the actin tracks and/or may serve to localize PIN1-containing IAA efflux complexes to the basal membrane.
(B) Treatment with cytochalasin leads to a random PIN1 protein distribution and reduces polar auxin transport.
(C) Treatment with BFA leads to a loss of PIN1 protein on the membrane and an accumulation of PIN1 protein at two undefined internal membrane structures. The effect is reversible upon removal of the BFA.
(D) Treatment with either cytochalasin or the IAA efflux inhibitor TIBA during the removal of BFA prevents the restoration of asymmetric PIN1 distribution. Treatment with either cytochalasin or TIBA before BFA treatment prevents the BFA-induced PIN1 internalization, and treatment with TIBA alone has no effect on PIN1 localization (data not shown).
(*This figure was modified from Muday and DeLong [2001]*).

these conditions, it is apparent that TIBA inhibits the movement of PIN1 to the plasma membrane, but it is not yet clear whether TIBA or other IAA efflux inhibitors function similarly in the absence of BFA pretreatment.

A similar result was described by Gil et al. (2001), who examined the localization of PIN1 in the *tir3*/*doc1* mutant. The *tir3* mutant was isolated in a screen for reduced sensitivity to auxin transport inhibitors and has reduced auxin transport in the inflorescence (Ruegger et al., 1997). The mutant gene has been cloned, and the resulting protein has been renamed BIG, because of its large size. The BIG protein has sequence similarity to the calossin family of integral membrane proteins, which are essential for secretory vesicle formation (Gil et al., 2001). In the NPA-treated *tir3*/*doc1* mutant, PIN1 was distributed more randomly than in the NPA-treated wild type. Although the authors indicate that this is an NPA-dependent effect (Gil et al., 2001), the absence of micrographs showing PIN1 localization in *tir3* and the wild type in the absence of NPA treatment makes it difficult to assess the direct effect of this mutation on PIN1 localization. The hypersensitivity of the *tir3*/*doc1* mutant to NPA-induced delocalization appears to be inconsistent with its decreased sensitivity to growth inhibition by IAA efflux inhibitors and reduced auxin transport in the inflorescence (Ruegger et al., 1997). Additionally, the alterations in gene expression that led to the isolation of the *doc1* mutant, which is allelic to *tir3* (Gil et al., 2001), are not yet possible to reconcile with the function of the BIG protein.

NEWLY IDENTIFIED NPA BINDING PROTEINS MAY FUNCTION IN THE VESICULAR CYCLING OF EFFLUX CARRIERS

Although high-affinity binding of IAA efflux inhibitors correlates with the specific inhibition of auxin transport,

the high concentrations of inhibitors required for the delocalization of PIN1 from the plasma membrane in Arabidopsis (Geldner et al., 2001; Gil et al., 2001) suggests that IAA efflux inhibitors also may act at low-affinity sites involved in more global regulatory pathways. Therefore, direct analysis of the proteins that bind IAA efflux inhibitors, and examination of endogenous molecules, such as flavonoids, that may regulate IAA efflux in vivo (Murphy et al., 2000; Brown et al., 2001), are crucial to understanding the action of these compounds.

Radiolabeled NPA often is used to follow proteins thought to be targets of IAA efflux inhibitors. Early studies focused on a single high-affinity plasma membrane NPA binding site isolated from etiolated zucchini hypocotyls and red light–grown maize coleoptiles (reviewed by Lomax et al., 1995; Muday, 2000). The binding of NPA and other IAA efflux inhibitors to this site has been well characterized and correlates well with the activity of these inhibitors in auxin transport and growth assays conducted in the dark. High-affinity NPA binding proteins include the protein that interacts with the actin cytoskeleton described above (Butler et al., 1998; Hu et al., 2000; reviewed by Muday, 2000).

However, additional lower affinity NPA binding and growth inhibition activities are found in light-grown Arabidopsis seedlings (Jensen et al., 1998; Murphy and Taiz, 1999; Murphy et al., 2000). Several proteins now have been purified by NPA affinity chromatography from membrane fractions derived from light-grown Arabidopsis seedlings (Murphy et al., 2000, 2002). The identified proteins fall into the lower affinity category, because the NPA affinity matrix used in these experiments contained NPA that was coupled through the carboxylic group, which is required for both auxin transport inhibition and high-affinity binding activity. NPA affinity fractions contained two novel aminopeptidases with NPA amidase activity as well as the secretory dynamin ADL1A (Kang et al., 2001) and orthologs of proteins involved in mammalian actin-dependent vesicular cycling (β-adaptin, cyclophilin 5, and protein disulfide isomerase), which, by analogy, also might play a role in the regulation of the actin-dependent vesicular cycling of the PIN protein in Arabidopsis.

A MODEL FOR TARGETING OF AUXIN EFFLUX PROTEINS TO SPECIFIC MEMBRANE DOMAINS

A comparison of the proteins identified with a role in IAA efflux described above with the proteins that mediate the inducible asymmetric vesicular cycling of glucose transporters in mammalian insulin-responsive tissues reveals striking similarities. When blood glucose levels increase, an insulin-induced, calcium-dependent, phosphoinositol/protein kinase B-C signaling cascade causes endomembrane vesicles containing the mammalian GLUT4 glucose transporter to be dispatched asymmetrically to the plasma membrane via an actin-dependent mechanism, as summarized in Figure 2 (Baumann and Saltiel, 2001; Simpson et al., 2001). Phosphorylation of protein components of GLUT4 secretory vesicles (GSVs) and recruitment of the general receptor for phosphoinositides (GRP1) ARF-GEF trafficking inhibitor away from GSVs results in actin-dependent movement to the plasma membrane. Essential to this trafficking are the phosphorylation by protein kinase C of the GSV insulin-responsive aminopeptidase (IRAP) and the v-SNARE Vamp2, which mediates docking with the Munc13c plasma membrane docking complex via an interaction with the t-SNARE syntaxin 4 (Baumann and Saltiel, 2001; Simpson et al., 2001). GTP binding dynamins, which function in calcium-dependent Golgi vesicle budding and clathrin-coated vesicle formation, appear to be required for both GLUT4 secretion and endocytosis (Al-Hasani et al., 1998).

Many of the components of the mammalian GLUT4 signal transduction and vesicle secretion mechanism have orthologs in Arabidopsis, a number of which have been implicated directly or indirectly in the regulation of auxin transport and/or the asymmetric distribution of the PIN1 protein (Figure 2). First, the possibility that kinase-derived signals modulate auxin transport has been tested using both mutants and transgenic mutations in expression of genes encoding kinases or phosphatases, leading to consistent evidence that protein phosphorylation positively regulates auxin transport, as predicted by this model. A mutation in *PINOID* (*PID*), which encodes a protein kinase C–like protein, leads to reduced auxin transport and *PIN*-like phenotypes (Bennett et al., 1995; Christensen et al., 2000), whereas overexpression of the *PID* gene leads to growth effects consistent with increased auxin transport (Christensen et al., 2000; Benjamins et al., 2001). In contrast, the *rcn1* mutation in a protein phosphatase 2A regulatory subunit gene results in reduced phosphatase 2A activity (Deruère et al., 1999), increased root basipetal IAA transport, and gravitropic defects (Rashotte et al., 2001). Attempts to identify the target of RCN1 action find no evidence that this regulation is at the level of the putative auxin carriers (Rashotte et al., 2001) and is consistent with an action upstream.

A second important player in GLUT4 vesicle cycling is the GRP1 ARF-GEF protein, which has been suggested to be a direct target of BFA. The ARF-GEF ortholog in Arabidopsis is the protein GNOM, which has been shown to be essential for the proper localization of PIN1 as well as for proper embryonic polarity formation (Steinmann et al., 1999). A third set of Arabidopsis proteins includes KEULE and KNOLLE, which are orthologs of the mammalian Munc18c and Syntaxin4 components of the GLUT4 plasma membrane vesicle

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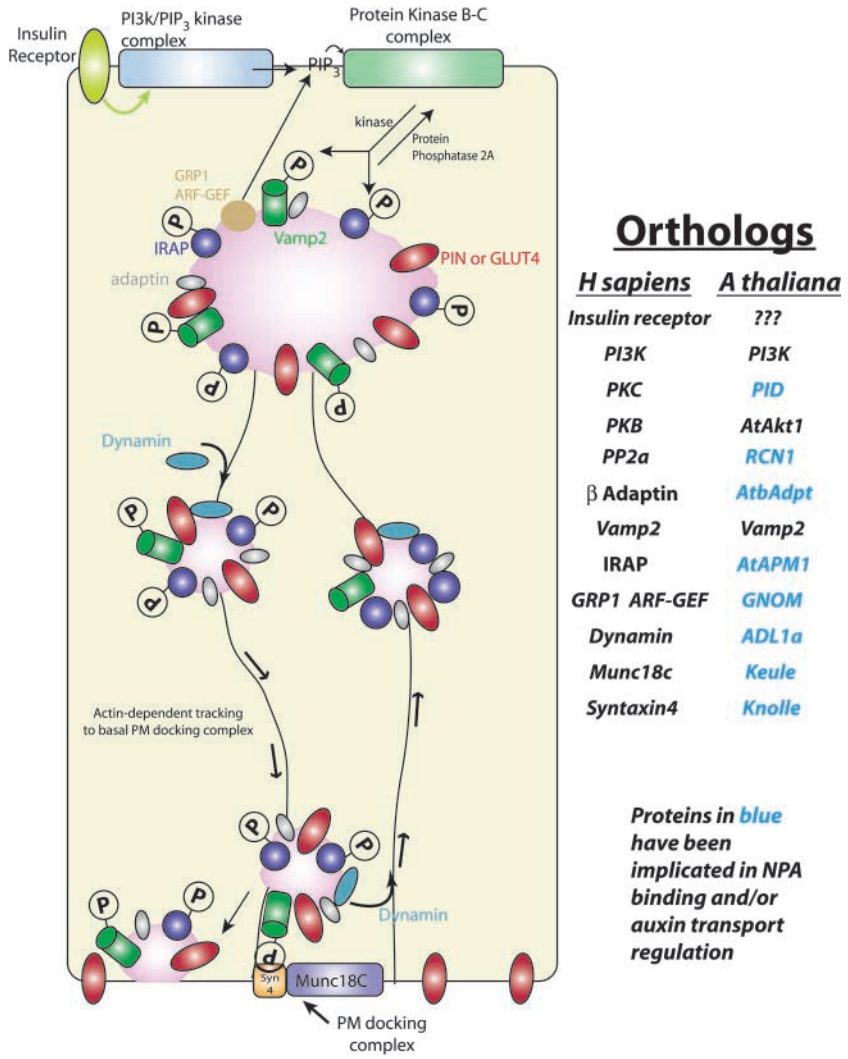

Figure 2. The Mammalian GLUT4 Asymmetric Vesicular Targeting Mechanism as a Model for the Localization of the Auxin Efflux Carrier.

A vesicular cycling mechanism similar to the mammalian insulin-inducible GLUT4 glucose transporter trafficking system is suggested by recent studies of PIN protein localization and protein interactions with auxin transport inhibitors. Sequence homologies and analogous functions of many of the protein components of the two systems further suggest parallel mechanisms. An external signal (hormone binding) triggers a phosphatidylinositol/phosphorylation cascade that activates asymmetric vesicular trafficking by (1) causing relocation of an inhibitory ARF-GEF protein (GRP1 or GNOM) from an endomembrane compartment to phosphatidylinositol triphosphate-enriched plasma membranes and (2) phosphorylating both a vesicular aminopeptidase (IRAP or AtAPM1) and the Vamp2 adaptor protein. Vesicles then traffic on actin filaments to a plasma membrane docking site, where Vamp2 interacts with Syntaxin 4/Knolle to initiate docking with Munc18c/Keule and subsequent vesicle fusion. Endocytotic vesicles enriched in dynamin and β-adaptin traffic back to the endosomal compartment in a similar actin-dependent manner. AtAPM1, *Arabidopsis thaliana* microsomal aminopeptidase; GRP1 ARF-GEF, general receptor for phosphoinositides ADP ribosylation factor guanine nucleotide exchange factor; IRAP, insulin-responsive aminopeptidase; Munc18c, mammalian homolog of unc18c; PID, PINOID; PI3K, phosphatidylinositol 3-kinase; PKB, protein kinase B/KT; PKC, protein kinase C; PP2a, phosphatase 2a; RCN1, root curling in NPA-1 PP2a; Vamp2, vesicle-associated membrane protein 2 (v-SNARE2).

docking complex and are required for the vesicle-mediated fusion of asymmetrically distributed plasma membrane proteins (Assaad et al., 2001; Heese et al., 2001). Finally, the NPA binding aminopeptidase AtAPM1 is a homolog of the mammalian IRAP, which is one of the two known phosphorylation targets of the insulin-activated protein kinase C complex and is essential for GSV trafficking. IRAP and AtAPM1 have a high degree of sequence similarity, have similar membrane orientations and enzymatic activities, and undergo unique processing of their C-terminal domains when secreted to the plasma membrane (Murphy et al., 2002).

Insulin signaling is a key component of vesicle targeting in the GLUT4 localization system. For vesicle-mediated targeting of IAA transport proteins to be truly parallel to the GLUT4 model, it is necessary to ask what signal(s) might control the localization of auxin transport proteins. The simplest possibility is that auxin acts as the signal to stimulate its own transport. IAA has been reported to stimulate IAA transport (Rayle et al., 1969), and auxin generally is thought to be required for the establishment of embryonic polarity (Geldner et al., 2000). Additionally, auxin has been suggested to stimulate the formation of auxin transport pathways (reviewed by Berleth and Sachs, 2001). Additional immunocytochemical studies of PIN carrier localization in Arabidopsis mutants lacking all of the orthologs of the mammalian GLUT4/IRAP cycling mechanism should determine if this model is appropriate for auxin transport in Arabidopsis.

MULTIDRUG RESISTANCE–LIKE PROTEINS MAY PARTICIPATE IN AUXIN TRANSPORT

A role for a different class of membrane transporters in the regulation of auxin transport was identified recently by a combination of genetic and biochemical approaches (Noh et al., 2001). The NPA affinity chromatography described above also produced a fraction containing three *multidrug resistance*–like, integral membrane p-glycoproteins, designated AtMDR1, AtPGP1, and AtPGP2. In mammalian cells, MDR genes encode proteins that enhance the export of chemotherapeutic agents (Roepe, 2000). Knockout mutants in *AtMDR1* and *AtPGP1* genes have phenotypes consistent with altered auxin transport, including epinastic cotyledons and reduced apical dominance (Noh et al., 2001). Additionally, auxin transport is reduced substantially in *atmdr1* inflorescences and seedlings (Noh et al., 2001). Several lines of evidence suggest that the MDR proteins may be one site of action of IAA efflux inhibitors. First, microsomal membranes isolated from the *atmdr1* mutant have reduced NPA binding activity compared with that of the wild type. Second, expression of the *AtMDR1* gene in yeast results in increased specific NPA binding or retention in nonpermeabilized whole yeast cells. Third, AtMDR1 and two homologs were isolated by NPA affinity chromatography and subsequently bound radiolabeled NPA in vitro (Noh et al., 2001; Murphy et al., 2002).

However, AtMDR1 and its homologs are not necessarily the exclusive sites of NPA's action on auxin transport regulation. NPA still reduces auxin transport to background levels in the *atmdr1* mutant, and there are significant amounts of residual NPA binding (64% of wild-type levels) in light-grown *atmdr1* microsomal membranes (Noh et al., 2001). These disparities could be the result of the observed tissue-specific expression patterns of *AtMDR1* and the other related *AtPGP* genes, but they also could represent additional non-MDR binding sites. The reduction of auxin transport in *atmdr1* mutants and the even greater reduction in *atmdr1 atpgp1* double mutants suggest that the MDR proteins play a role in the transport of auxin or auxin conjugates, particularly in regions of high IAA accumulation or near sites of IAA synthesis.

CONCLUSIONS

Recent results have implicated a number of new proteins in IAA efflux and have suggested that large protein complexes may be necessary for the movement of auxin and the dynamic localization of efflux carriers to the proper membranes. Actin-dependent vesicle sorting of carrier proteins to specific membrane surfaces appears to play a critical role in establishing auxin transport polarity and might play a role in the redirection of auxin flow in response to environmental stimuli and the resultant differential growth. In addition, the ATP dependence of both vesicular trafficking and MDR activity suggests that the energetics and chemiosmotic models of auxin transport require reexamination. A combination of genetic, cell biological, and biochemical approaches will be necessary to elucidate the complexities of this process and to understand the interactions between the proteins that mediate auxin transport.

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