The Actin Cytoskeleton May Control the Polar Distribution of an Auxin Transport Protein

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ABSTRACT

The gravitropic bending of plants has long been linked to the changes in the transport of the plant hormone auxin. To understand the mechanism by which gravity alters auxin movement, it is critical to know how polar auxin transport is initially established. In shoots, polar auxin transport is basipetal (i.e., from the shoot apex toward the base). It is driven by the basal localization of the auxin efflux carrier complex. One mechanism for localizing this efflux carrier complex to the basal membrane may be through attachment to the actin cytoskeleton. The efflux carrier protein complex is believed to consist of several polypeptides, including a regulatory subunit that binds auxin transport inhibitors, such as naphthylphthalamic acid (NPA). Several lines of experimentation have been used to determine if the NPA binding protein interacts with actin filaments. The NPA binding protein has been shown to partition with the actin cytoskeleton during detergent extraction. Agents that specifically alter the polymerization state of the actin cytoskeleton change the amount of NPA binding protein and actin recovered in these cytoskeletal pellets. Actin-affinity columns were prepared with polymers of actin purified from zucchini hypocotyl tissue. NPA binding activity was eluted in a single peak from the actin filament column. Cytochalasin D, which fragments the actin cytoskeleton, was shown to reduce the amount of NPA binding protein. The interaction of the NPA binding protein with the actin cytoskeleton may localize it in one plane of the plasma membrane, and thereby control the polarity of auxin transport.

INTRODUCTION

Auxins are a class of plant hormones that control elongation, development, and the response of plants to gravity and other environmental signals. Auxins, of which indole-3-acetic acid (IAA) is the predominant naturally occurring hormone, move through plants by a unique polar transport mechanism (as reviewed in Goldsmith, 1977; Lomax et al., 1995). This polar movement of auxin is from the shoot meristem towards the base of stems, and is a cell-to-cell movement. Polar auxin transport results in an auxin gradient down the length of the plant, with the highest auxin concentrations found in the regions of greatest elongation (Ortuno et al., 1990). Auxin is not transported at constant rates; rather its transport is regulated, and it changes during development and in response to environmental stimuli (Lomax et al., 1995). Several proteins are believed to control polar auxin transport. There are two protein complexes, the auxin uptake carrier and the auxin efflux carrier, that control auxin movement into and out of cells (Figure 1). IAA can move into cells both passively, since it is hydrophobic when protonated, and through an influx carrier (Lomax et al., 1995). The auxin efflux carrier is thought to control the amount and direction of polar auxin transport. It has also been proposed that the basal localization of an auxin efflux carrier determines the polarity of IAA transport in plant tissues (Rubery and Sheldrake, 1974; Jacobs and Gilbert, 1983; Muller et al., 1998).

In addition to moving by polar transport down the length of plant tissues, auxin can move laterally across gravity-stimulated shoots and roots. The Cholodny-Went hypothesis, originally proposed in 1937, suggests that the lateral transport of auxin across gravity-stimulated plant tissues drives differential gravitropic growth (Evans, 1991; Trewavas, 1992). Lateral redistribution of radiolabeled IAA has been measured in both shoots (Parker and Briggs, 1990) and roots (Young et al., 1990), and the redistribution of IAA has been shown to precede differential growth and the gravity response (Parker and Briggs, 1990). Applying auxin transport inhibitors to growing plants leads to an inhibition of the gravity response. These synthetic inhibitors inhibit auxin efflux (Rubery, 1990), and culturing plants on these compounds completely inhibits gravity response in the roots of a number of plant species, under conditions where growth still occurs (Katekar and Geissler, 1980; Muday and Haworth, 1994, Rashotte et al., 2000). The effect of the auxin transport inhibitor naphthylphthalamic acid (NPA) on gravity response is very rapid, with application at the time of gravitropic stimulation completely inhibiting gravitropic bending (Rashotte et al., 2000).

**Figure 1. Schematic Model of the Chemiosmotic Hypothesis for Polar Auxin Transport.** Protonated IAA in the cell wall space can enter the cell either by diffusion or via an uptake carrier. Once in the more basic cytoplasm, the IAA dissociates and can exit only via the auxin efflux carrier. The names of the proteins that may constitute auxin transporters are in parentheses. (Reprinted from Muday, 2000, with kind permission from Kluwer Academic Publishers)
Although the validity of the Cholodny-Went hypothesis has been debated (Trewavas, 1992), recent molecular and genetic evidence has provided additional support (Chen et al., 1999). One powerful test has been through the construction of transgenic plants with an auxin-responsive promoter that drives the expression of acid-glucuronidase. Redistribution of auxin-induced gene expression across a gravity-stimulated shoot (Li et al., 1991) and root (Li et al., 1999) is consistent with changes in lateral auxin transport. This finding supports the Cholodny-Went hypothesis. The ability of auxin transport inhibitors to block both differential auxin-regulated gene expression and gravitropic bending suggests that lateral auxin transport, rather than a change in auxin sensitivity, is the mechanism leading to differential gene expression.

Another approach that has shown the dependence of gravity response on auxin transport has been to isolate plants with mutations in auxin transport proteins that result in an agravitropic phenotype. The aux1 and allelic eir1/agr1/pin2 mutants have agravitropic roots (Chen et al., 1999). The recent cloning of the genes that are mutated in these plants suggests that these genes are auxin transporters. It has been suggested that AUX1 may encode the auxin uptake carrier (Bennett et al., 1996), which transports auxin into cells, and that EIR1/AGR1/PIN2 and PIN1 may encode transmembrane protein of the auxin efflux carrier (Chen et al., 1998; Luschnig et al., 1998; Galweiler et al., 1998; Muller et al., 1998), which pumps auxin out of cells. That roots of agr1 accumulate more radiolabeled IAA than wild-type roots is consistent with an inhibition of auxin efflux (Chen et al., 1998). The recent development of assays to measure polar auxin movement in the roots of Arabidopsis has revealed a reduction in polar auxin transport in the roots of eir1 (Rashotte et al., 2000). Together, these results suggest that auxin transport plays an important role in controlling plant gravity response.

Although these results link lateral auxin transport to gravity response, there are some experimental results that do not easily fit the simple interpretation of the Cholodny-Went hypothesis. The growth characteristics of roots in response to gravitropic stimulation have been carefully examined through computerized image analysis, and the pattern of root growth is not as simple as initially predicted (Evans, 1991). The root over-responds to gravity, turning from horizontal to vertical to beyond vertical; then growth switches from one root side to the other, allowing reorientation to the vertical (Ishikawa et al., 1991). In addition, roots grown on high concentrations of auxin can still respond to gravity, even when growth is almost totally inhibited (Ishikawa and Evans, 1993; Muday and Haworth, 1994). These results, which appear contradictory to the Cholodny-Went hypothesis, indicate that the role of auxin in root growth and gravity response is complex, and that additional experimentation will be required to completely understand how gravitropic growth is controlled (Trewavas, 1992).

Identifying the proteins that transport auxin is the first step in understanding the molecular mechanisms that control both polar and gravity-induced lateral auxin transport. For example, it is not yet clear whether the same protein complex controls auxin efflux during both polar and lateral movement of IAA. If multiple auxin efflux carrier complexes are used, then gravity-induced lateral auxin transport could require expression of a gene that encodes another carrier, or it could be mediated by differential activation of one gene product. Alternatively, if a single efflux carrier complex controls both polar and lateral transport, we must alter either its localization or the activity of a subset of these carriers in order to change the directionality of auxin movement. Once we identify all the proteins that control auxin transport and obtain the molecular tools to study the position and activity of these proteins, we can elucidate the mechanisms by which auxin transport changes to allow gravity response.

**BIOCHEMICAL CHARACTER OF THE AUXIN EFFLUX CARRIER**

The biochemical dissection of the auxin efflux carrier will increase our understanding of how this protein complex is regulated and how its localization to the basal plasma membrane controls the polarity of auxin movement. The efflux carrier complex appears to be composed of more than one polypeptide. Composition includes:

- an integral membrane transporter encoded by a member of the PIN gene family;
- a NPA binding protein (NBP) that may act as a regulatory polypeptide;
- perhaps a third, rapidly turned-over protein that connects these two subunits (Morris et al., 1991).

Several members of the PIN gene family in Arabidopsis have been identified (Galweiler et al., 1998; Muller et al., 1998), indicating that there are multiple auxin efflux carriers with distinct expression patterns. Plants with mutations in two genes of this family have phenotypes consistent with tissue-specific alterations in auxin transport (Okada et al., 1991; Galweiler et al., 1998; Muller et al., 1998), and alterations in auxin transport occur in the affected tissues (Okada et al., 1991; Chen et al., 1998; Rashotte et al., 2000). PIN genes encode proteins with ten membrane-spanning domains that are similar to other membrane transport proteins (Chen et al., 1999). The protein products of these genes show an asymmetric localization in the plasma membrane that is consistent with controlling the polarity of auxin movement (Galweiler et al., 1998; Muller et al., 1998).

Therefore, it has been suggested that the PIN genes encode one polypeptide of the auxin efflux carrier.

Until the PIN proteins were identified, most studies of the auxin efflux carrier focused on the NPA binding protein. The activity of this protein can be followed using a binding assay with [3H]-NPA: radiolabeled NPA is incubated with membrane vesicles or solubilized protein, and the protein and ligand complexes are recovered by
filtered or centrifugation. The NPA ligand binds with high affinity to a single class of NPA binding proteins associated with the zucchini plasma membrane (Muday et al., 1993). Therefore, this assay has allowed extensive biochemical characterization of the NPA binding protein.

Several lines of evidence suggest that the protein that binds inhibitors of auxin efflux is distinct from the PIN gene products. Treatments with inhibitors of protein translation and protein processing in the Golgi reduce the regulation of auxin transport by NPA without altering the amount of NPA binding activity. (Morris et al., 1991; Wilkinson and Morris, 1994; Morris and Robinson, 1998). These results (1) suggest that the NPA binding and auxin efflux activities are on separate proteins, and (2) support the idea that a third protein may connect them (Morris et al., 1991).

It also appears that the NBP is peripherally associated with the plasma membrane. Treatment of plasma membrane vesicles with potassium iodide (KI) or sodium bicarbonate released the NPA binding protein into the supernatant after ultracentrifugation, suggesting that NPA binds to a peripheral protein (Cox and Muday, 1994). Furthermore, the NBP is still active in detergent-insoluble pellets. These pellets should be almost free of lipids, yet the majority of NPA binding activity was recovered, suggesting that the NBP does not require a lipophilic environment for activity (Cox and Muday, 1994; Butler et al., 1998). Most integral membrane proteins would lose activity under these conditions. Therefore, in our current model, the NBP is a peripheral membrane regulatory protein. This model is consistent with the results of Morris et al. (1991) and Wilkinson and Morris (1994), which indicate that NPA binding activity and auxin efflux activity are on two distinct polypeptides.

Biochemical evidence suggests that NPA binding activity is localized to the cytoplasmic face of the plasma membrane. Several investigators have examined the protease sensitivity of NPA binding activity in plasma membranes isolated from zucchini hypocotyls. Consistent with a cytoplasmic localization, treatment of intact right-side-out vesicles with protease does not lead to loss of NPA binding activity (Dixon et al., 1996; Bernasconi et al., 1996). In contrast, disruption of membranes by detergent, followed by protease treatment, results in a total loss of NPA binding activity (Bernasconi et al., 1996). Furthermore, plasma membrane vesicles have been subjected to several different treatments that should have converted them to inside-out orientation (Hertel et al., 1983; Dixon et al., 1996; Bernasconi et al., 1996). The effectiveness of these treatments, however, was only verified by analysis of marker enzymes in one case (Dixon et al., 1996). When the ability of treatments to convert vesicles to an inside-out orientation was verified, both NPA binding activity and the protease sensitivity of that activity had increased in inside-out vesicles (Dixon et al., 1996). Therefore, the NPA binding site appears to be localized to the cytoplasmic face of the membrane and positioned for interaction with the cytoskeleton.

NPA-binding proteins partitions with the actin cytoskeleton during detergent extraction

To purify integral membrane proteins, the first step is to treat them with detergent, which solubilizes the protein and releases it from the membrane. As it was initially assumed that the NPA binding activity and the auxin efflux carrier activity were localized on the same polypeptide, most investigators initiated experiments using detergent solubilization with the goal of releasing NPA binding activity. Although several reports in the literature indicate that NPA binding activity can be released from the membrane by detergent treatment, all of these procedures resulted in very low yields of soluble NPA binding activity (Sussman and Gardner, 1980; Jacobs and Gilbert, 1983; Cox and Muday, 1994; Bernasconi et al., 1996). In two of these reports, the amount of NPA binding activity in the detergent-insoluble pellet was quantified, and in both cases, the majority of the activity was in the detergent-insoluble pellet (Sussman and Gardner, 1980; Cox and Muday, 1994). The insolubility of the NPA binding protein during detergent extraction may be due to interaction with the cytoskeleton, as proteins associated with the cytoskeleton show this behavior (Carraway, 1992).

Cox and Muday (1994) reported the first study that addressed whether the detergent insolubility of the NPA binding was due to association with the cytoskeleton. After purified zucchini plasma membranes were treated with Triton X-100, NPA binding activity and both actin and tubulin polypeptides were examined in the pellet and supernatant fractions after ultracentrifugation (Cox and Muday, 1994). Actin, tubulin, and NPA binding activity all partitioned preferentially into the detergent-insoluble pellet. Treatment of the detergent-insoluble or cytoskeletal pellet with cytochalasin B, a drug that fragments the filamentous form of actin (F-actin), released [3H]-NPA binding activity into the supernatant after ultracentrifugation. Use of this drug in vitro caused the release of both actin and tubulin cytoskeletal fragments, so these experiments could not differentiate between association with actin and association with tubulin (Cox and Muday, 1994).

Although this initial study supported the argument that the NPA binding protein interacts with the cytoskeleton, the use of purified plasma membranes with treatments designed to alter actin polymerization was not optimal. Rather, the use of fresh and relatively crude extracts of zucchini hypocotyl proteins proved to be a better method for determining whether the NPA binding protein was interacting with the cytoskeleton, and for determining which cytoskeletal polymer was the site of interaction (Butler et al., 1998). Butler et al. (1998) also found that NPA binding activity and actin partitioned into the cytoskeleton pellet after detergent extraction of fresh extracts. There was very little tubulin polypeptide found in these extracts, suggesting an interaction between the NPA binding protein and actin (Butler et al., 1998).
To more directly test for an interaction between the NPA binding protein and actin, intact zucchini hypocotyls were treated in vivo with one of three drugs known to alter cytoskeletal organization—phallloidin, cytochalasin D, or taxol (Butler et al., 1998). After drug treatment, extracts were prepared and treated with detergent, and the amount of NPA binding activity in the detergent-insoluble cytoskeletal pellets was measured. Phallloidin and cytochalasin D act on the actin cytoskeleton to stabilize polymers or to fragment polymers, respectively. Treatment with phallloidin increased both the amount of pelletable actin and NPA binding activity, while treatment with cytochalasin D decreased both pelletable actin polypeptide and NPA binding activity. In contrast, taxol treatment stabilized microtubules, resulting in increased pelletable tubulin after detergent solubilization, but not increased pelletable NPA binding activity (Butler et al., 1998).

Butler et al. (1998) used one additional treatment to depolymerize actin. The buffer Tris has been previously reported to lead to actin depolymerization (Pinder et al., 1995). Butler and colleagues (1998) found that using Tris to treat detergent-insoluble pellets (from either fresh extracts or plasma membranes isolated from zucchini hypocotyls) led to a dose-dependent decrease in pelletable actin and NPA binding activity (Butler et al., 1998). Initial experiments using Tris did not result in a concomitant increase in NPA binding activity in the detergent supernatant, so it was not clear whether Tris was releasing NPA binding activity or denaturing the NPA binding activity. To stabilize the NPA binding protein during its release, the NPA ligand was included during the detergent extraction and Tris treatment. The Tris was removed, and the pH was lowered to a proton concentration more optimal for NPA binding, resulting in the recovery of NPA binding activity in the supernatant (Butler et al., 1998). Therefore, it appeared that Tris released NPA binding activity, and did not denature the protein (Butler et al., 1998). Together, these results were consistent with an association of the NPA binding protein with the actin cytoskeleton, although the interactions were only indirectly demonstrated. The next step to demonstrate this interaction was to show that the NPA binding protein could bind in vitro to homogenous and purified actin filaments.

**INTERACTION OF THE NPA BINDING PROTEIN WITH ACTIN FILAMENTS IN VITRO**

The first approach to demonstrate the interaction of the NBP with actin filaments was to subject detergent-insoluble cytoskeletal pellets to rounds of polymerization and depolymerization. Throughout these cycles of actin polymerization, the location of both the actin polypeptide and the NPA binding activity were followed. Initially, actin and NPA binding activity were recovered in the detergent-insoluble pellet. Upon treatment with potassium iodide (KI), both actin and NPA binding activity moved into the supernatant as filaments were depolymerized (Cox and Muday, 1994). The KI was removed to allow actin filaments to reform, and the resulting sample was subjected to centrifugation. After this treatment, the pellet and supernatant were collected, and both actin filaments and NPA binding activity were found predominantly in the pellet. Although NPA binding activity was preferentially partitioned into the samples that were enriched in actin, there was very low recovery. Furthermore, both actin and tubulin were enriched in these samples, suggesting that this procedure was not a very specific way to recover actin polymers (Cox and Muday, 1994). Therefore, an alternative approach was developed to more directly examine actin interactions.

To directly test the interaction of the NPA binding protein with purified actin filaments, F-actin affinity columns were prepared and their ability to retain binding activity was examined. There are a number of actin genes in plants, encoding as many as 20 different actin polypeptides (Kandasamy et al., 1999), for which there are specific tissue-specific expression patterns. It was therefore critical to obtain actin isoforms from the tissues that are known to transport auxin and to possess NPA binding activity.

There are no procedures in the literature for purifying actin that is competent for polymerization from plant vegetative tissues, although procedures to purify maize pollen actin have been published (Liu and Yen, 1992; Ren et al., 1997). However, there are numerous reports in the literature of purifying animal actin using affinity columns prepared with the enzyme DNase I coupled to a solid support (Sheterline et al., 1998; Zechel, 1980), so this approach was chosen to purify zucchini hypocotyl actin. DNase I binds G-actin with a 1:1 ratio with high affinity. It is also commercially available, and it has previously been used to partially purify actin from pea roots (Andersland et al., 1992).

Using DNase I chromatography followed by ultracentrifugation, Hu et al. (in review) purified actin from zucchini hypocotyls to electrophoretic homogeneity. Since actin was eluted from the DNase I resin with formamide, which can denature proteins, it was particularly critical to demonstrate that this actin was native. First, the profilin binding activity of purified zucchini hypocotyl actin was compared to purified and native maize pollen actin that had been isolated according to Ren et al. (1997). The profilin binding ability was compared using two isoforms of maize profilin, one expressed in pollen (ZmPRO1) and the other expressed predominantly in vegetative tissues (ZmPRO5). The resulting $K_d$ values for these two actin pools were not statistically different under these conditions (Hu et al., in review). The native structure of the actin was also confirmed by the ability of the purified actin to bind and inhibit DNase I activity (Hu et al., in review).

The ability of purified zuchinni hypocotyl actin to form filaments was demonstrated by sedimentation of F-actin during ultracentrifugation, by decreased mobility of F-actin on native gels, and by examination of actin filaments with electron microscopy (Hu et al., in review).
Ultrastructural examination of in vitro polymerized actin showed helical filaments with a width of 6.8 nm (Hu et al., in review), consistent with the conformation and size of maize actin filaments assembled in vitro (Ren et al., 1997). Together, these results demonstrated that purified zucchini hypocotyl actin was native and competent for polymerization.

Purified, native zucchini hypocotyl actin was then used to prepare both G- and F-actin columns. BSA was used to create a third affinity matrix to test for nonspecific protein interactions (Hu et al., in review). Examining the binding of vertebrate α-actinin to the F-actin column confirmed the selectivity of the F-actin column. Purified α-actinin was shown to bind tightly to the F-actin, but weakly to the G-actin column (Hu et al., in review).

Since the NPA binding protein is associated with the plasma membrane, isolated plasma membranes were used as the starting sample for chromatography on the actin columns. Treating plasma membranes with Triton X-100 and Tris resulted in the recovery of NPA binding activity in the supernatant after ultracentrifugation. This soluble sample was applied to the actin or BSA columns. Eluted protein samples were analyzed for NPA binding activity, and were examined by silver stain after SDS-PAGE. NPA binding activity was retained by the F-actin column and reproducibly eluted with high salt concentrations. In five separate experiments, NPA binding activity was localized to one or two fractions eluted from the F-actin column. This activity was significantly greater than the activity eluted from a BSA column or an F-actin column to which no solubilized proteins had been applied (Hu et al., in review).

To date, the elution of NPA binding activity from the F-actin column is the strongest evidence to indicate association of the NPA binding protein with the actin cytoskeleton. The next question was whether these columns would retain sufficient quantities of the NPA binding protein to allow the protein’s isolation for amino acid sequence analysis. The pattern of proteins retained by the column was examined by subjecting samples to SDS-PAGE, followed by silver staining. There was no consistent band found in samples that contained NPA binding activity (Hu and Muday, unpublished result). In contrast, two proteins were routinely eluted from the F-actin column with high salt concentrations (higher than those required to elute NPA binding activity) (Hu et al., in review). These two proteins of 30-35 kDa were recognized by annexin antisera (Hu et al., in review). As annexins are plasma membrane proteins that have been shown to interact with the actin cytoskeleton (Calvert et al., 1996), this result provides further evidence that these columns can retain F-actin binding proteins in a specific fashion.

**DRUGS THAT FRAGMENT THE ACTIN CYTOSKELETON REDUCE POLAR AUXIN TRANSPORT**

The interaction of the NPA binding protein with the actin cytoskeleton may be necessary either for movement of auxin across the membrane or for the polar localization of the efflux carrier complex. If either of these hypotheses is correct, then disruption of the actin cytoskeleton would be predicted to reduce polar auxin transport. Treatment of either corn coleoptiles (Cande et al., 1973) or zucchini hypocotyls (Butler et al., 1998) with cytochalasins, drugs which fragment the actin cytoskeleton, have led to the reduction of auxin transport.

The effect of cytochalasin D on auxin transport in zucchini hypocotyls was measured using a modification of previously published assays (summarized in Figure 2). Zucchini hypocotyl segments treated with and without cytochalasin D were simultaneously loaded with [3H]-IAA and [14C]-benzoic acid, and the amount of radioactivity transported out of each end of the segment was recovered in agar blocks. When zucchini hypocotyls were treated with cytochalasin D, there was a statistically significant reduction in basipetal auxin transport, as shown in Table I (data from Butler et al., 1998). This reduction in transport is not at the level of diffusion, as there are no changes in the amount of either basipetal benzoic acid movement or acropetal auxin transport. Because this assay measures passive diffusion from the segment as well as polar transport, each measurement contains some background diffusion. The level of background diffusion can be assessed by examining the percentage of acropetal auxin movement or the percentage of benzoic acid diffusion. Basipetal IAA transport can be normalized by subtracting the amount of diffusion (calculated by averaging the percentage of BA diffusion, both acropetal and basipetal, and the percentage of acropetal IAA diffusion) from the basipetal auxin transport. The magnitude of the effect of cytochalasin D treatment increases to two-fold when the normalized values are compared.
AN AUXIN TRANSPORT PROTEIN INTERACTS WITH ACTIN FILAMENTS

Table I. Cytochalasin D Reduces Polar Auxin Transport

<table>
<thead>
<tr>
<th></th>
<th>-Cytochalasin D</th>
<th>+Cytochalasin D</th>
<th>p value^b</th>
</tr>
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<tbody>
<tr>
<td>Basipetal IAA</td>
<td>19.0 ± 1.2</td>
<td>13.3 ± 1.1</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>Acropetal IAA</td>
<td>8.0 ± 0.4</td>
<td>8.8 ± 0.8</td>
<td>&gt;0.2</td>
</tr>
<tr>
<td>Basipetal BA</td>
<td>7.0 ± 0.9</td>
<td>7.0 ± 0.8</td>
<td>&gt;0.2</td>
</tr>
<tr>
<td>Normalized Basipetal IAA^c</td>
<td>11.3</td>
<td>5.6</td>
<td></td>
</tr>
</tbody>
</table>

^a The % transport is the average and standard error of 12 separate experiments.
^b The % transport in the absence and presence of 200 μM cytochalasin D is compared by student t-test.
^c Normalized basipetal IAA transport was calculated by subtracting the background diffusion (the average of the % of acropetal IAA and % basipetal BA transport) from the % basipetal IAA transport.

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If an intact actin cytoskeleton is required for localization of the auxin efflux carrier complex, should fragmentation of actin filaments with cytochalasin lead to a total loss of auxin transport? This question can be considered by examining the model in Figure 3 (opposite page). If the efflux carriers are totally randomized, then transport should be at the level of diffusion. Basipetal IAA transport is not reduced to the level of diffusion by cytochalasin. However, the cytochalasin D treatment was for only one hour, perhaps not enough time to allow all the efflux carriers to randomize. Also, there may have been partial recovery of polar auxin transport capacity during the 1.5-hour transport period. These results are consistent with the model shown in Figure 3, although more complex possibilities cannot yet be eliminated. Finally, it should be noted that the treatment of roots does not completely abolish root gravitropism, suggesting that lateral auxin transport may not depend on the actin cytoskeleton (Blandoflor and Hasenstein, 1997; Staves et al., 1997).

CONCLUSIONS

The results from these studies indicate that a regulatory subunit of the auxin efflux carrier, the NPA binding protein, binds directly to actin filaments. The actin association of the NPA binding protein may localize the auxin efflux carrier complex in one plane of the plasma membrane and thereby control the polarity of auxin transport. Galweiler et al. (1998) and Muller et al. (1998) have used antibodies that recognize two different isoforms of an integral membrane protein of the auxin efflux carrier to show that these proteins are localized to one plane of the plasma membrane. Butler et al. (1998) and Cande et al. (1973) have also shown that cytochalasin-D-treatment of zucchini hypocotyls and corn coleoptiles, respectively, reduces polar auxin transport, a result that is consistent with the actin cytoskeleton’s role in maintaining the polar distribution of auxin transport proteins. Our studies have shown that a key regulatory subunit of the auxin efflux carrier, the NPA binding protein, binds actin filaments (Hu et al., in review).

Other studies, in both plants and animals, have demonstrated the importance of the actin cytoskeleton in establishing and maintaining cell polarity. In yeast and the brown alga, *Fucus*, initial establishment of cell polarity requires an intact actin cytoskeleton (Goodner and Quantrano, 1993; Li et al., 1995) and is preceded by changes in the organization of the actin cytoskeleton (Ayscough and Drubin, 1996; Kropf et al., 1989; Alessa and Kropf, 1999; Chant, 1999). To preserve cellular polarity, a number of plasma membrane proteins with asymmetric localization maintain their distribution by attaching themselves to the actin cytoskeleton. In the developing zygotes of *Fucus*, the dihydropyridine

![Figure 3. Model for the Effect of Cytochalasin D on Polar Auxin Transport. A file of untreated cells (left) compared to those treated with cytochalasin D (right). The fragmentation of actin filaments by cytochalasin D is shown, as well as the randomization of the auxin efflux carrier complex as a result of the loss of actin structure, which may serve to localize this protein. (Reprinted from Muday, 2000, with kind permission from Kluwer Academic Publishers)
receptor has been shown to develop asymmetry that also requires an intact actin cytoskeleton (Shaw and Quatrano, 1996). Both the acetylcholine receptor of neurons and the Na+, K+-ATPase of epithelial cells have polar distributions that are required for their function (Froehner, 1993; Apel and Merlie, 1995). Evidence for both of these protein complexes indicates that attachment to the actin cytoskeleton controls their localization (Nelson and Hammerton, 1989; Froehner, 1993). The acetylcholine receptor is a particularly interesting example, because one of the proteins in the complex is a 43 kDa peripheral membrane protein (rapsyn), which is associated with the actin cytoskeleton. In mice that are deficient in this protein, the acetylcholine receptor fails to properly localize (Gautam et al., 1995). The NPA binding protein may function in a similar way to localize the efflux carrier.

Differential actin association of the NPA binding protein may also provide a level of auxin transport regulation. Morris and Johnson (1990) suggest that, in tissues that have reduced polar auxin transport, the randomized location of the efflux carrier—rather than changes in its abundance—may cause transport reduction. This could be mediated by a decrease in the actin association of the NPA binding protein. Additionally, auxin transport is reduced in response to ethylene treatment (Suttle, 1988), and this reduction may be controlled by changes in the cytoskeletal attachment of the NBP (Ebenezer, 1997). The ethylene-induced reduction in auxin transport is accompanied by a statistically significant (p<0.01) decrease in the amount of NPA binding activity in detergent-insoluble cytoskeletal pellets, although the abundance of the protein in crude extracts is constant (Ebenezer, 1997). Consequently, it may be that alteration in the cytoskeletal association of the NPA binding protein acts to control the amount of polar auxin transport.

Together, these results suggest that the NPA binding protein is associated with the actin cytoskeleton in vitro, and that this association is required for maximal auxin transport and its regulation by NPA in vivo. Although the significance of this association is not yet clear, it may be that attachment of the NBP to the actin cytoskeleton serves to localize this protein in one plane of the plasma membrane and thereby to control the polarity of auxin transport.

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