The Plant Cell, Vol. 6, 1941–1953, December 1994

NPA Binding Activity Is Peripheral to the Plasma Membrane and Is Associated with the Cytoskeleton

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***N*-1-Naphthylphthalamic acid (NPA) binding activity is released into the supernatant when plasma membranes are subjected to high-salt treatment, indicating that this activity is peripherally associated with the membrane. Extraction of plasma membrane vesicles with Triton X-100 resulted in retention of NPA binding activity in the detergent-insoluble cytoskeletal pellet. Treatment of this pellet with KI released NPA binding activity, actin, and α-tubulin. Dialysis to remove KI led to the repolymerization of cytoskeletal elements and movement of NPA binding activity into an insoluble cytoskeletal pellet. NPA binding activity partitioned into the detergent-insoluble cytoskeletal pellet obtained from both zucchini and maize membranes and was released from these pellets by KI treatment. Treatment of a cytoskeletal pellet with cytochalasin B doubled NPA binding activity in the resulting supernatant. Together, these experiments indicate that NPA binding activity is peripherally associated with the plasma membrane and interacts with the cytoskeleton in vitro.**

INTRODUCTION

The auxins are a class of phytohormones that control plant growth, development, and response to the environment through regulation of cell division, differentiation, and tropic responses to light and gravity. Auxins, of which indole-3-acetic acid (IAA) is the predominant naturally occurring hormone, are delivered to plant cells by a unique polar transport mechanism (reviewed in Goldsmith, 1977; Lomax et al., 1995). Auxins are transported from their source in the apical meristem toward the base of coleoptiles and hypocotyls. Polar auxin transport depends upon the proton gradient across the plasma membrane of plant cells and has been described as chemiosmotic (Rubery and Sheldrake, 1974). The ionization states of IAA in the extracellular space and in the cytoplasm affect the membrane permeability of this phytohormone and lead to an accumulation of the anionic form of this molecule within the cytoplasm. Polar transport is believed to be mediated by an uptake IAA anion and proton symport carrier (Lomax et al., 1985; Sabater and Rubery, 1987) and an IAA anion efflux carrier (Rubery and Sheldrake, 1974). Basal localization of this IAA efflux carrier has been proposed to drive the polarity of IAA transport (Rubery and Sheldrake, 1974; Jacobs and Gilbert, 1983). One possible mechanism by which the localization of IAA efflux carriers within the basal plane of the plasma membrane could be controlled is through association of this protein complex with the cytoskeleton. In animal cells, membrane proteins have been shown to be localized in a single plane of the plasma membrane by interaction with actin filaments (Bloch and Resneck, 1986).

A number of synthetic inhibitors of auxin transport have been identified, with *N*-1-naphthylphthalamic acid (NPA) being the best characterized (Rubery, 1990). These auxin transport inhibitors, or phytotropins, specifically block auxin transport at the site of the efflux carrier (Rubery, 1990). Recently, naturally occurring flavonoid compounds have also been shown to act at the site of the efflux carrier (Jacobs and Rubery, 1988). These flavonoid compounds are intriguing candidates for natural ligands that may regulate auxin efflux in vivo.

The binding of radiolabeled inhibitors of auxin transport, predominantly ^{3}H-NPA, to membrane-associated proteins has now been extensively characterized (Rubery, 1990). Sussman and Gardner (1980) demonstrated that ^{3}H-NPA binds to a protein, because the ligand binding was heat and protease sensitive. ^{3}H-NPA binding activity, associated with plasma membrane vesicles isolated by aqueous two-phase partitioning, shows high affinity, with a dissociation constant (K_d) equal to 15 nM for this ligand (Brunn et al., 1992; Muday et al., 1993).

The specificity of auxin transport inhibitors is an important consideration. For these compounds to be useful in biochemical dissection of the auxin efflux carrier and exploration of the physiology of auxin transport, they must be specific for a single protein. Although the specificity of ^{3}H-NPA binding has been questioned by Michalke et al. (1992), Muday et al. (1993) have completed a detailed analysis of ^{3}H-NPA binding to zucchini microsomal and plasma membranes and report that only a single ^{3}H-NPA binding site can be resolved by Scatchard, Hill, and Lineweaver-Burke plots as well as through analysis with the computer program LIGAND (Munson and Rodbard, 1980). Data analysis using this program, which utilizes a nonweighted, nonlinear curve-fitting technique to determine the number of binding sites, also supports the presence of

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a single binding site. This analysis, which used two different filtration assays to measure ^{3}H-NPA binding, indicates that NPA binds not only with high affinity, but also with high specificity to a single site associated with the zucchini plasma membrane (Muday et al., 1993).

Identification and purification of the NPA binding protein (NBP) will provide additional information on the biochemical character of this protein and its function. Several approaches have been used to identify candidate proteins, but purified NBP has not yet been isolated. Jacobs and Gilbert (1983) prepared monoclonal antibodies that prevented NPA binding to both intact microsomal membrane vesicles and proteins solubilized from these vesicles. These antibodies were used to identify a 77-kD protein in fractionated extracts of pea membranes (Jacobs and Short, 1986). Zettl et al. (1992) have identified a 23-kD protein by photoaffinity labeling of maize coleoptile plasma membrane with 5′-azido-(3,6-$^3H_2$)-NPA. Binding of this azido–NPA derivative was reduced by adding high concentrations of auxin transport inhibitors or auxins (1 mM) to the labeling reaction. It is not yet clear why the two potential candidates for the NBP are so different in size or if either is involved in NPA regulation of auxin efflux.

Transport of molecules across membranes can be mediated either by single polypeptides or by complexes containing several polypeptides. It has been assumed that both the IAA transport activity and the NPA binding activity of the auxin efflux carrier reside on the same polypeptide. However, a recent examination of NPA binding activity suggests that this activity may be distinct from the IAA transport activity and that the auxin efflux carrier may consist of at least two polypeptides, with distinct catalytic (transport) activity and regulatory activity (Morris et al., 1991). We present data demonstrating that the NBP is peripheral to the membrane, further substantiating the role of the NBP as a distinct regulatory polypeptide. Furthermore, NPA binding activity appears to interact with the actin cytoskeleton in vitro, suggesting a mechanism by which the auxin efflux carrier may be localized to the basal plasma membrane.

RESULTS

Release of Peripheral Membrane Proteins with Salt Washes

To determine whether the NBP is peripheral to the membrane, salt solutions and chaotropic agents were used to release proteins that are peripherally associated with the membrane from zucchini plasma membrane vesicles isolated by aqueous two-phase partitioning. When intact plasma membrane vesicles in the presence of sucrose were incubated with 1 M NaCl, 2 M NaI, 8 M urea, 20 mM EDTA, or from 50 mM to 1 M KCl and subjected to ultracentrifugation, there was no increase in NPA binding activity in the supernatant samples (R.M. Greene and G.K. Muday, unpublished data). This could be attributable to a peripheral protein associated with the cytoplasmic face of the plasma membrane becoming dissociated but being trapped inside the intact vesicles.

We treated intact plasma membrane vesicles with a 1 M Na_2CO_3 solution in Mg-cytoskeletal stabilization buffer (Mg-CSB) at pH 11.0 in the absence of sucrose. Na_2CO_3 washes at high pH have been shown to dissociate proteins that are peripherally associated with the membrane (Lai et al., 1988), and the absence of sucrose should osmotically shock membrane vesicles, causing their orientation to randomize. Additional membrane samples were treated with Mg-CSB, pH 7, also in the absence of sucrose. After ultracentrifugation, the pellet and supernatant fractions of both samples were collected and assayed for ^{3}H-NPA binding activity, as shown in Table 1. Eighty percent of ^{3}H-NPA binding activity was found in the supernatant obtained after treatment of intact plasma membrane vesicles with Na_2CO_3. In addition, treatment with 0.4 M KI without sucrose, which also disrupts peripheral protein interactions with the plasma membrane (Ward et al., 1992), resulted in the release of greater than 80% of NPA binding activity into the supernatant after centrifugation (Table 1). The distribution of ^{3}H-NPA binding activity after these treatments corresponded

Table 1. Effect of KI and Na_2CO_3 on the Membrane Association of NPA Binding Activity

	Total ^{3}H-NPA Binding				
		Pellet[a]		Supernatant[a]	
Sample	Total Binding (pmol)	pmol	% of Total	pmol	% of Total
Buffer treated[b]	0.25	0.20 ± 0.01	82	0.05 ± 0.01	18
Sodium carbonate[c]	0.23	0.05 ± 0.01	22	0.18 ± 0.01	78
Potassium iodide[d]	0.97	0.19 ± 0.01	20	0.78 ± 0.08	80

[a] Samples were treated as indicated and subjected to ultracentrifugation at 40,000*g* for 20 min.
[b] Mg-CSB, pH 7.0.
[c] 1 M Na_2CO_3, pH 11.0.
[d] 0.4 M KI in K-CSB, pH 7.0. Data are from a separate experiment with relative partitioning of NPA binding activity similar to that in the buffer control shown here but with a more active membrane preparation.

to locations expected for proteins associated peripherally with the plasma membrane. Throughout these experiments, we found that the NBP was completely active in the absence of membranes or detergent micelles, further suggesting that this protein is not integral to the plasma membrane.

Partitioning of NPA Binding Activity and the Cytoskeleton during Detergent Extraction

Zucchini plasma membrane vesicles were extracted with the nonionic detergent Triton X-100 at concentrations ranging from 0 to 2.5%. The extracted membranes were subjected to ultracentrifugation, which resulted in a supernatant containing solubilized proteins and a Triton X-100–insoluble pellet. ^{3}H-NPA binding was measured in these pellet and supernatant fractions, and the results are shown in Figure 1. In either the presence or absence of Triton X-100, the bulk of the NPA binding activity was found in the pellet fraction. Low levels of Triton X-100 (0.5%) resulted in a reduction in NPA binding activity in the pellet relative to samples not treated with detergent, although a concomitant increase in the supernatant was not observed. With increasing concentrations of Triton X-100, NPA binding activity in the pellet remained relatively constant and activity decreased in the supernatant. Although detergent solubilization released some NPA binding activity from the plasma membrane vesicle preparation, ~50 to 60% of the remaining NPA binding activity was associated with the Triton X-100–insoluble pellet. A detergent concentration of 2.5% Triton X-100 with a detergent-to-protein ratio of 12.5 mg/mg should have been sufficient to disrupt the membrane completely and to remove most integral membrane proteins. Previous experiments have reported the release of more NPA binding activity by Triton X-100 extraction of microsomal membranes from maize (Sussman and Gardner, 1980) and pea (Jacobs and Gilbert, 1983), but those detergent extractions were at lower pH. We have found that lower pH results in greater recovery of detergent-solubilized activity (data not shown). In addition, Sussman and Gardner (1980) report that greater than 50% of their starting activity remains in the detergent-insoluble sample after treatment with 1.0% Triton X-100.

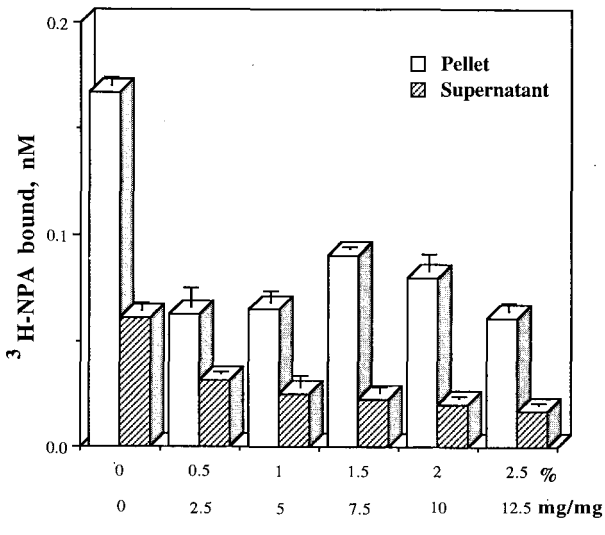

Figure 1. Triton X-100 Solubilization of ^{3}H-NPA Binding in Zucchini Plasma Membrane Vesicles.

Equal amounts of plasma membrane vesicles were extracted with 0 to 2.5% Triton X-100, and pellets and supernatants were obtained by ultracentrifugation. The concentration of ^{3}H-NPA binding with 5 nM ^{3}H-NPA is shown. The percentage of Triton X-100 and the detergent-to-protein ratio in milligrams per milligram are indicated.

Detergent-insoluble pellets from membrane preparations have previously been shown to be enriched in the cytoskeleton (Mescher et al., 1981; Tan and Boss, 1992; Xu et al., 1992). In the most parallel comparison, performed by Sonesson and Widell (1993), outside-out plasma membrane vesicles (from *Brassica oleracea*), isolated by aqueous two-phase partitioning, were shown to have retained the cortical actin cytoskeleton. Furthermore, Triton X-100 extraction resulted in insoluble cytoskeletal pellets enriched in actin. The presence of NPA binding activity in the detergent-insoluble pellet suggests that in addition to being peripheral to the membrane, the NBP may be fixed to the cytoskeletal network.

These membrane and cytoskeletal samples were also examined microscopically to verify the effectiveness of the detergent extraction, as shown in Figure 2. High-resolution differential interference contrast (DIC) microscopy was used to determine the size and shape of the plasma membrane vesicles. Vesicles were spherical in shape and ranged in size from 0.1 to 1 μm in diameter (Figure 2A). However, after 2.5% Triton X-100 extraction, the plasma membrane vesicles appeared clustered and less distinct in shape, but they clearly maintained their structure (Figure 2B). 1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate ($DiIC_{(18)}$), a plasma membrane–specific fluorescent probe, was used to monitor the ability of Triton X-100 to disrupt the plasma membrane. $DiIC_{(18)}$ staining of plasma membrane vesicles before and after Triton X-100 extraction suggests that Triton X-100 was effective in solubilization and removal of the plasma membrane from these vesicles, as evidenced by the reduced fluorescence after Triton X-100 extraction relative to intact plasma membrane vesicles (Figures 2C and 2D).

The presence of the cytoskeleton in the vesicle and detergent-insoluble samples was also verified by staining with rhodamine phalloidin, an F-actin–specific fluorescent probe. Fluorescence images of intact plasma membrane vesicles revealed little staining of F-actin by rhodamine phalloidin, which would be expected for intact vesicles, because this dye does not readily cross the plasma membrane (Figure 3B). For comparison, a DIC image of intact plasma membrane vesicles is shown in Figure 3A. After extraction with 2.5% Triton X-100, rhodamine phalloidin staining was detected with the silicon-intensified target (SIT) camera in the resulting Triton X-100–insoluble pellet, indicating that the actin filaments were both

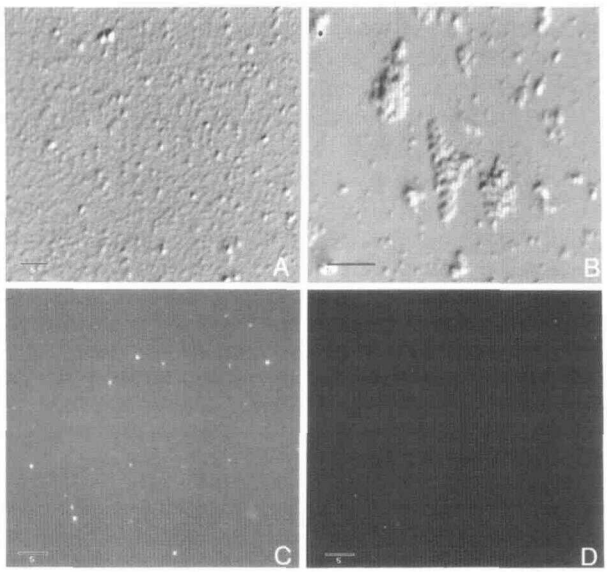

Figure 2. DIC and Fluorescence Micrographs of Zucchini Plasma Membrane Vesicles.

High-resolution DIC microscopy was used for **(A)** and **(B)**, and fluorescence microscopy of samples stained with the plasma membrane stain $DiIC_{(18)}$ was used for **(C)** and **(D)**.
(A) and **(C)** Intact plasma membrane vesicles.
(B) and **(D)** 2.5% Triton X-100–insoluble cytoskeletal pellets from plasma membranes.
Bars = 5 μm.

resistant to detergent extraction and more readily stained in the absence of the plasma membrane (Figure 3D). The paired DIC image is shown in Figure 3C to demonstrate the structure of the rhodamine phalloidin–stained cytoskeletal network. The SIT camera was used to record this paired DIC image; this accounts for the lower resolution when compared with the DIC image of Figure 3A, which was recorded with the higher resolution DIC camera.

Partitioning of NPA Binding Activity during Cytoskeletal Depolymerization and Repolymerization

To depolymerize cytoskeletal elements, the Triton X-100–insoluble pellet was treated with 0.4 M KI, which has been reported to depolymerize actin (Payrastre et al., 1991; Tan and Boss, 1992). Zucchini plasma membrane vesicles were extracted with 2.5% Triton X-100 as previously described, and the cytoskeletal (Triton X-100–insoluble) pellet was incubated in K-CSB containing KI. After this depolymerization, the sample was subjected to ultracentrifugation, and the supernatant containing the depolymerized cytoskeletal elements and associated proteins was separated from the pellet fraction. This chemical depolymerization resulted in a supernatant with a much higher specific activity of NPA binding, as shown in Figure 4. Furthermore, this treatment effectively released the bulk of NPA binding activity, with 70% of the total activity partitioning into the supernatant (16.5 pmol), as compared with the pellet (7 pmol).

To examine more thoroughly the association of the NBP with the cytoskeleton, we performed detergent extractions of the cytoskeleton followed by in vitro depolymerization and repolymerization of cytoskeletal elements, according to Payrastre et al. (1991) with the modifications of Tan and Boss (1992). The depolymerized supernatant was subjected to dialysis to remove KI, which resulted in the reformation of F-actin and microtubules. Ultracentrifugation of the dialyzed sample yielded pellet and supernatant samples. Under these conditions, Tan and Boss (1992) report that large actin filaments, actin bundles, and the associated actin binding proteins pellet; however, soluble proteins that do not bind actin, such as BSA, remain in the supernatant. These pellet and supernatant fractions were used to examine ^{3}H-NPA binding, and the results are shown in Figure 4. These data are reported in specific activity (picomoles per milligram of protein) to illustrate the purification throughout this procedure. The distribution of ^{3}H-NPA binding throughout this procedure corresponded to locations expected for cytoskeletal proteins. NPA binding activity was found associated with the newly formed cytoskeletal elements after the removal of KI, with a specific activity fourfold higher

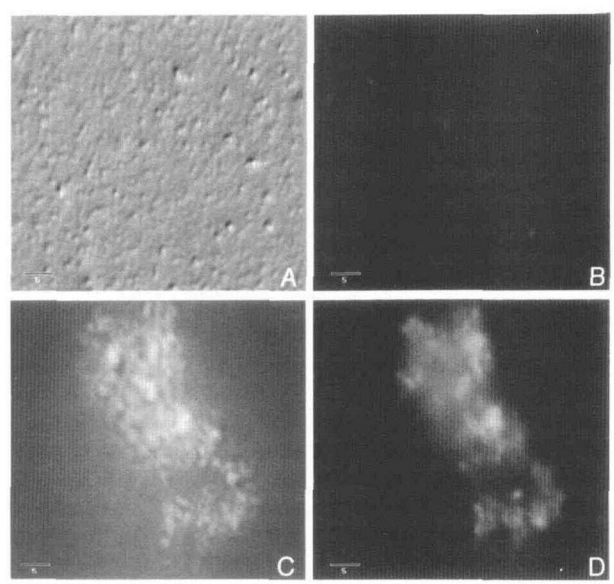

Figure 3. DIC Images and Rhodamine Phalloidin Staining of Intact and Triton X-100–Extracted Zucchini Plasma Membrane Vesicles.

DIC images are shown in **(A)** and **(C)**, and fluorescence images of rhodamine phalloidin–stained samples are shown in **(B)** and **(D)**.
(A) and **(B)** Intact plasma membrane vesicles.
(C) and **(D)** Paired DIC and fluorescence images of 2.5% Triton X-100–extracted plasma membrane vesicles.
Bars = 5 μm.

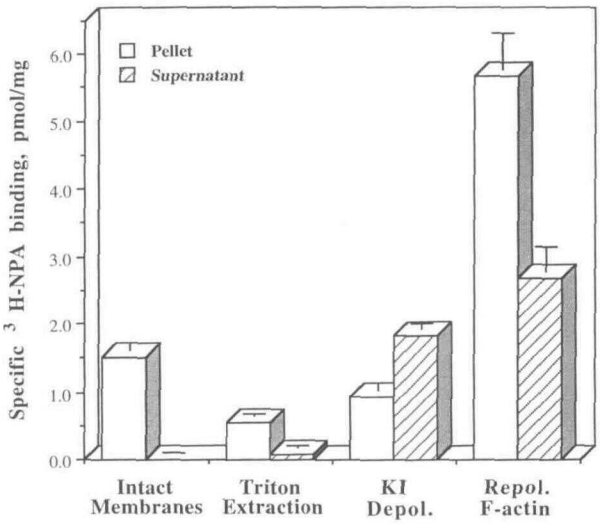

Figure 4. NPA Binding Activity during Cytoskeletal Depolymerization and Repolymerization.

Samples obtained sequentially through these procedures were assayed for NPA binding activity using 5 nM ^{3}H-NPA, and the specific binding activity is given in picomoles per milligram of protein. Plasma membrane vesicles were extracted with 2.5% Triton X-100, and pellet and supernatant samples were recovered by ultracentrifugation. The Triton X-100–insoluble pellet was treated with 0.4 M KI, and the resulting pellet and supernatants were obtained after ultracentrifugation. The KI supernatant was dialyzed to remove KI and repolymerized F-actin, and microtubules and associated proteins were recovered in the pellet after ultracentrifugation. Samples were assayed in quadruplicate, and the average and standard deviation are reported. Depol., depolymerized; Repol., repolymerized.

than that of any other samples. This result indicates that the procedure enhances the abundance of the NBP relative to other proteins in the repolymerized cytoskeletal pellet and suggests that the association of NPA binding activity with the cytoskeleton is not simply due to trapping of the protein within the cytoskeletal network.

The samples from the in vitro depolymerization and repolymerization experiment were also examined for content of actin and α-tubulin and formation of actin filaments. Rhodamine phalloidin was used to verify the presence of actin filaments in the repolymerized pellet, as shown in Figure 5. Immunoblot analysis of the proteins of the samples from each step of the procedure using both actin-specific and α-tubulin–specific monoclonal antibodies confirmed the presence of actin and α-tubulin in the repolymerized pellet, as shown in Figure 6. This gel was scanned with a densitometer to quantify the amount of actin present in each sample and to determine its distribution relative to the total amount of protein loaded. For most of the samples, the relative distribution of actin did not correlate directly with the distribution of NPA binding activity, with one clear exception. The amount of actin relative to total protein was 4.7 μg/mg for the KI supernatant and 20 μg/mg for the repolymerized pellet, indicating a fourfold enhancement in actin concentration by this procedure. In the same samples, the specific activity of NPA binding was 0.61 and 3.7 pmol/mg, respectively, indicating a sixfold enhancement in this activity. The relatively quantitative correlation between the amount of repolymerized actin and the amount of NPA binding activity associated with the actin pellet is suggestive of a specific interaction between F-actin and NPA binding activity.

The depolymerization and subsequent repolymerization of the cytoskeletal network led to purification of the NBP. The release of NPA binding activity by KI treatment of the 2.5% Triton X-100 pellet also led to a membrane-dissociated and detergent-free sample that would be easier to manipulate in future analyses. Binding constants describing the affinity (K_d) and abundance (maximum binding; B_{max}) of ^{3}H-NPA activity in the KI supernatant were determined. A supernatant sample was prepared by depolymerization of actin by KI treatment of the 2.5% Triton X-100 pellet, and this sample was examined for NPA binding activity. The B_{max} was 69.5 pmol/mg, which is 10-fold higher that the B_{max} of 6.5 pmol/mg for intact membranes (Muday et al., 1993). This reflects an increase in the total number of NPA binding sites relative to other proteins. In the actin depolymerization supernatant, the K_d is 43 nM, which is 2.5-fold higher than the dissociation constant for intact membranes (Muday et al., 1993). This reflects a slight and perhaps insignificant decrease in affinity.

Partitioning of NPA Binding Activity from Maize and Zucchini Microsomal Membrane Preparations during Detergent Extraction and KI Treatment

Although NPA binding activity from zucchini appears to be associated with the cytoskeleton in vitro, an important question

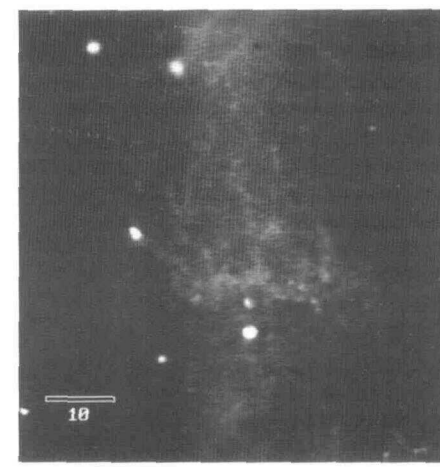

Figure 5. Rhodamine Phalloidin Staining of Repolymerized F-actin.

The presence of F-actin in the repolymerization pellet was verified by rhodamine phalloidin staining and visualization by fluorescence microscopy using the SIT camera. Bar = 10 μm.

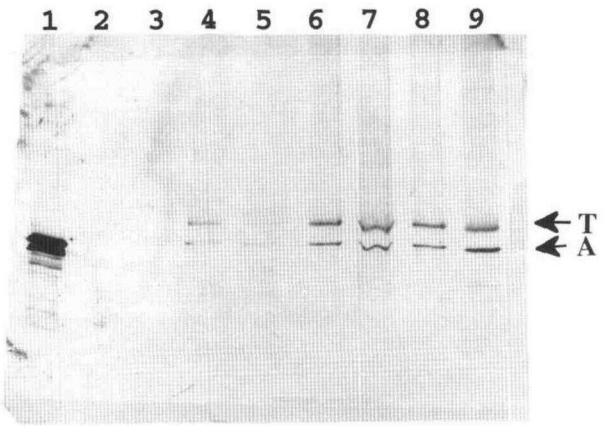

Figure 6. Immunoblot of Samples from the Cytoskeleton Depolymerization and Repolymerization Procedures.

Actin and α-tubulin were detected in samples with monoclonal antibodies. Lane 1 contains chicken muscle actin, 5 μg; lanes 2 and 3, supernatant remaining after repolymerization and recovery by ultracentrifugation, 5 μg; lane 4, repolymerized pellet, 6 μg; lane 5, KI supernatant, 15 μg; lane 6, pellet remaining after KI treatment, 25 μg; lane 7, 2.5% Triton X-100 supernatant, 25 μg; lane 8, 2.5% Triton X-100 pellet, 25 μg; lane 9, intact zucchini plasma membrane, 25 μg. Actin and α-tubulin bands are indicated by arrows and labeled A and T, respectively.

is whether this association can be demonstrated in membrane preparations from other plants. Microsomal membrane preparations were prepared from etiolated maize and zucchini tissues, and the partitioning of NPA binding activity during detergent extraction and subsequent KI treatment was compared. The binding activity of these samples is shown in Table 2. Analysis of NPA binding activity with microsomal membrane–derived samples leads to higher background, so heat treatments were used to determine the amount of NPA binding activity associated with the native protein. It is apparent that in microsomal membranes from both species, the majority of NPA binding activity partitions into the cytoskeletal pellet and is released during cytoskeletal depolymerization by KI.

Partitioning of NPA Binding Activity during Cytochalasin B Treatments

To determine whether the NBP interacts with the actin microfilaments in the cytoskeleton, the cytoskeletal pellet was incubated with several concentrations of cytochalasin B, an actin binding drug that reduces the polymerization state of F-actin (Pollard, 1990). The samples were then subjected to ultracentrifugation at 50,000*g*, and ^{3}H-NPA binding was determined in the supernatant and pellet samples, as shown in Figure 7. At 0 μM cytochalasin B, there was approximately twofold more ^{3}H-NPA binding in the pellet fraction versus the supernatant fraction. Over the concentration range of 0 to 50 μM cytochalasin B, there was a gradual decrease in ^{3}H-NPA binding in the pellet fraction with increasing drug concentration and a gradual increase in ^{3}H-NPA binding in the supernatant fraction, with a maximum at a cytochalasin B concentration of 20 μM. Incubation with 20 μM cytochalasin B increased the total amount of ^{3}H-NPA binding activity in the supernatant fraction twofold and decreased binding in the cytoskeletal pellet fraction twofold relative to the untreated samples. Treatment with cytochalasin D yielded similar results (data not shown).

An immunological method was used to verify the release of actin monomers in response to cytochalasin B treatment. Triton X-100–insoluble pellets were treated with 20 μM cytochalasin B or buffer, and the supernatant and pellet obtained after ultracentrifugation were examined by immunoblot with an actin-specific monoclonal antibody, as shown in Figure 8. The presence of an actin band in the cytochalasin B–treated supernatant (lane 2) can be compared with a buffer-treated supernatant (lane 4). Actin from zucchini plasma membrane

Table 2. NPA Binding Activity in Maize and Zucchini Microsomal Membranes after Triton X-100 and KI Treatments

	Total ^{3}H-NPA Binding					
	Maize[a]			Zucchini[b]		
Sample[c]	– Heat[d] (pmol)	+ Heat[e] (pmol)	Heat-Labile Activity (pmol)	– Heat[d] (pmol)	+ Heat[e] (pmol)	Heat-Labile Activity (pmol)
Triton X-100 pellet	0.51	0.21	0.30	0.62	0.20	0.42
Triton X-100 supernatant	0.085	0.12	<0	0.099	0.020	0.079
KI pellet	0.12	0.069	0.046	0.046	0.058	<0
KI supernatant	0.89	0.41	0.48	0.33	0.044	0.29

[a] Standard deviations were less than 15% of total binding.
[b] Standard deviations were less than 10% of total binding.
[c] Samples were treated with 2.5% Triton X-100 or 0.4 M KI and subjected to ultracentrifugation at 40,000*g* for 20 min.
[d] No heat treatment.
[e] Heat treatment was for 20 min at 95°C.

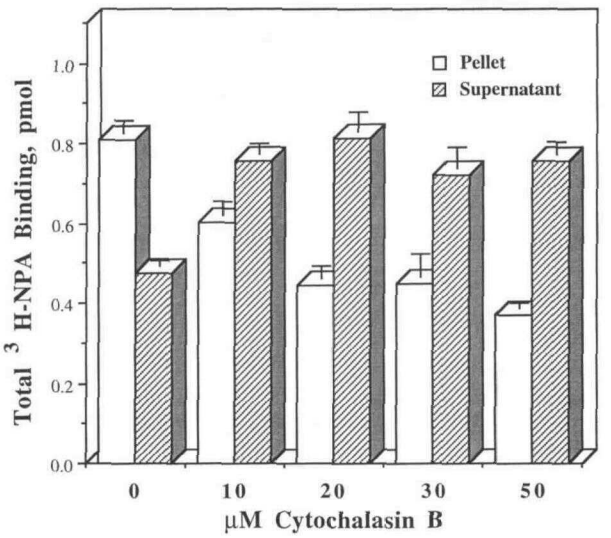

Figure 7. *Effects of Cytochalasin B on Dissociation of* ^{3}H-NPA Binding from the Cytoskeleton.

Triton X-100–extracted plasma membrane vesicles were incubated with the indicated concentrations of cytochalasin B for 30 min. The pellet and supernatant fractions were separated by ultracentrifugation and assayed for ^{3}H-NPA binding using 5 nM ^{3}H-NPA. Total ^{3}H-NPA binding activity is given in picomoles to indicate the amount of activity released by these treatments.

samples migrated with the same mobility as chicken muscle actin, with a molecular mass of 43 kD. This cytochalasin B treatment resulted in partial release of G-actin monomers from the cytoskeletal pellet into the supernatant but not complete depolymerization. The amount of actin released by cytochalasin B treatment was determined by scanning densitometry of the immunoblot. When the actin released was normalized for the amount of protein loaded, it became apparent that less than 10% of the actin was released by this treatment. In contrast, greater than 65% of NPA binding activity was released by this treatment. This lack of quantitative correlation is not surprising due to the complex mechanism of action of cytochalasins. Although cytochalasins alter the actin microfilament systems in cells, their mechanism of action is a subject of controversy in both animal and plant cells (Vaughan and Vaughn, 1987). In vitro, it is generally agreed that the cytochalasins slow the rate of filament polymerization by inhibiting the rate of elongation (Brown and Spudich, 1979; Bonder and Mooseker, 1986; Cooper, 1987; and Sampath and Pollard, 1991). It has also been suggested that in cultured animal cells, cytochalasins may interfere with the anchoring of actin to the plasma membrane (Weber et al., 1976). In tobacco pollen tubes, cytochalasins B and D have been shown to cause microfilaments to form massive bundles throughout the cytoplasm (Lancelle and Hepler, 1988), and in maize root cells, cytochalasin causes a reorganization of microfilaments into abnormal crossed and highly condensed masses (Vaughan and Vaughn, 1987). Clearly, cytochalasins do alter actin dynamics, and it is possible that these changes more completely release actin-associated proteins than actin. In other studies, cytochalasins have been found to release significant proportions of actin binding proteins, without a parallel release of actin monomers from detergent-insoluble pellets (Morris and Tannenbaum, 1980; Lacy and Underhill, 1987). Therefore, the lack of quantitative correlation between the release of actin and the release of NPA binding activity does not appear to be an unusual result.

Although the mechanism by which cytochalasins alter actin dynamics is not completely clear, it is generally assumed that in vivo, these compounds alter only actin dynamics, not microtubules. To determine whether this action is specific in vitro, we reprobed the blot in Figure 8 with an antibody against α-tubulin. We found that upon release of actin, α-tubulin was also released. There are two possible conclusions for this experiment. Either cytochalasins are not specific for microfilaments, or there is a connection between the actin filaments and the microtubules that leads to the release of tubulin when actin dynamics are altered. We favor the latter hypothesis because several investigators have demonstrated this connection, both in vivo and in vitro (Griffith and Pollard, 1982; Pollard et al., 1984; Euteneuer and Schliwa, 1985; Schmit and Lambert, 1988; Abe et al., 1992). As a result, it is not yet possible to determine whether NPA binding activity is interacting with actin or with microtubules. We have attempted a parallel approach using colchicine to treat intact plasma membrane vesicles and detergent-insoluble pellets to depolymerize tubulin. This

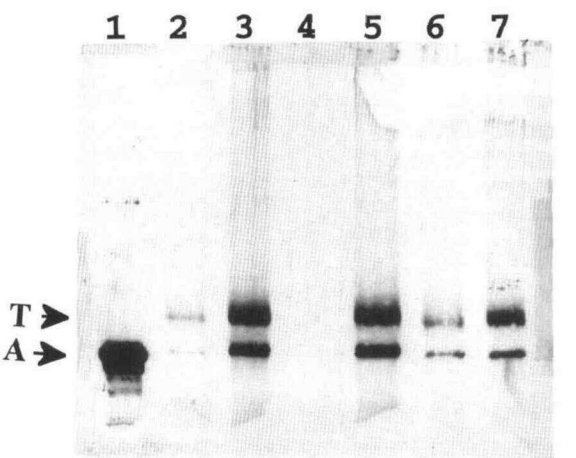

Figure 8. Immunoblot Analysis of Cytochalasin B–Treated Samples.

Samples were analyzed on an immunoblot with monoclonal antibodies against actin and α-tubulin. Lane 1 contains chicken muscle actin, 10 μg; lane 2, 20 μM cytochalasin B supernatant, 17 μg; lane 3, the cytochalasin pellet, 26 μg. Lane 4 contains the buffer-treated supernatant, 16 μg; lane 5, buffer pellet, 26 μg; lane 6, 2.5% Triton X-100 supernatant, 11 μg; lane 7, 2.5% Triton X-100 pellet, 28 μg. Actin and α-tubulin bands are indicated by arrows and are labeled A and T, respectively.

procedure has not been successful in releasing either tubulin monomers or NPA binding activity (M.W. Dixon and G.K. Muday, unpublished data).

Analysis of the Ability of Actin to Bind ^{3}H-NPA

Because NPA binding activity partitions with samples enriched in actin, it may be possible that ^{3}H-NPA is directly interacting with actin. To address this possibility, purified actin was used in an ^{3}H-NPA binding assay. The only commercially available source of pure actin is from chicken muscle. This chicken actin was substituted in the assay for the membrane protein samples in the concentration range of 4 μg/mL (0.8 μg total) to 50 μg/mL (10 μg total). This range encompasses the actin concentrations that were in samples assayed for NPA binding previously described. Binding was measured in samples containing native and heat-denatured actin. The binding activity was identical in both denatured and native samples (data not shown), suggesting that chicken actin does not directly bind NPA. This result cannot rule out the possibility that NPA interacts with plant actin. Quantitative analysis of the amounts of actin in samples obtained from cytochalasin B, Triton X-100, or KI treatment using a densitometer to scan immunoblots indicates that there is not a direct relationship between the amount of actin in most samples and the amount of NPA binding activity. This result suggests that zucchini actin does not bind ^{3}H-NPA directly.

DISCUSSION

To understand the mechanisms by which the auxin transport inhibitor or NPA binding protein regulates auxin transport, the biochemical character of this protein needs to be elucidated. To explore the membrane interaction of the NBP, we treated intact plasma membrane vesicles with 1 M Na_2CO_3, pH 11.0, in the absence of sucrose. This treatment released NPA binding activity from intact plasma membrane vesicles. Furthermore, treatment with 0.4 M KI in the absence of sucrose was able to release NPA binding activity into the supernatant. Both treatments indicate that the NBP is peripherally associated with the plasma membrane. Furthermore, after these treatments, we found that NPA binding activity was completely active in the absence of membranes and detergent, which indicates that this protein does not require a lipophilic environment for activity.

We found that NPA binding activity from zucchini hypocotyls is associated with the cytoskeleton in vitro. The cytoskeleton of eukaryotic cells is a network of cytoplasmic filaments that drives a number of mechanical processes, such as locomotion, cytokinesis, polar growth, and perhaps gravity response (Lloyd, 1991). We have used a number of approaches to examine the interaction of NPA binding activity with the cytoskeleton. During extraction with detergents, the majority of NPA binding activity partitions with the cytoskeleton into a detergent-insoluble pellet. The most convincing result to support the cytoskeletal association of the NBP is the partitioning of NPA binding activity with cytoskeletal elements through a complete chemical depolymerization and subsequent repolymerization of cytoskeletal filaments. The cytoskeletal (Triton X-100–insoluble) pellet is treated with KI to depolymerize the cytoskeletal network, and the supernatant (enriched in cytoskeletal monomers and associated proteins) is separated from the pellet fraction by ultracentrifugation. The supernatant sample is dialyzed to remove the KI, leading to repolymerization of actin filaments and microtubules, which are then recovered in the pellet after another cycle of ultracentrifugation.

Under these conditions, the only proteins in the pellet should be large actin filaments, actin bundles, microtubules, and associated proteins. The presence of actin and α-tubulin in the appropriate samples was verified by immunoblot analysis. In addition, formation of actin filaments in the polymerized pellet was demonstrated by staining with rhodamine phalloidin. This procedure is quite rigorous because cytoskeletal and associated proteins move from a pellet fraction to a supernatant fraction and back to a pellet fraction. NPA binding activity is found in the pellet after cytoskeletal repolymerization, suggesting that the NBP either stays bound to cytoskeletal monomers throughout the procedure or reassociates with the polymers during repolymerization. This procedure has been used to conclude that other proteins bind specifically to actin (Payrastre et al., 1991; Tan and Boss, 1992). In contrast, we find that this procedure is not specific for actin and actin binding proteins. Animal microtubules appear to be more cold sensitive and are therefore depolymerized in early steps of the procedure used by Payrastre et al. (1991). Plant microtubules apparently survive such rigorous treatments as freezing in liquid nitrogen and extended periods of exposure to 4°C, as demonstrated by their presence in the plasma membrane vesicles used as starting material in this study.

The apparent cytoskeletal association of the NBP is not unique to this protein derived from zucchini plasma membranes. When zucchini microsomal membranes are used as starting material, NPA binding activity partitions with the detergent-insoluble cytoskeleton and is released from the cytoskeletal network by KI treatment. Maize microsomal membrane–derived proteins partition in a similar fashion, indicating that the cytoskeletal association of the NBP may be a general phenomena.

Treatment of the cytoskeletal pellet with cytochalasin B, a drug that leads to net depolymerization of actin filaments, causes a release of NPA binding activity from the cytoskeleton. Furthermore, cytochalasin B has previously been shown to reduce auxin transport and auxin-dependent growth by greater than 40% in maize coleoptiles (Cande et al., 1973). The ability of this drug to release actin monomers in vitro was verified by an immunoblot with an actin-specific monoclonal antibody. The specificity of the treatment was also examined

by immunoblot with an α-tubulin–specific monoclonal antibody. Cytochalasin B treatments were also shown to release α-tubulin monomers. This result does not necessarily indicate that cytochalasin B acts on microtubules, rather it may indicate that there are connections between cytoskeletal elements that result in the release of components of both networks when one is disrupted. There is evidence in both animals and plants supporting this latter possibility. The interactions between microtubules and actin have been examined in plant and animal systems using both in vivo and in vitro approaches (Griffith and Pollard, 1982; Pollard et al., 1984; Euteneuer and Schliwa, 1985; Schmit and Lambert, 1988; Abe et al., 1992).

The ability of the NBP to associate with the cytoskeleton in vitro not only provides information about the biochemical behavior of this protein, but also serves as a method for purification of this protein. The depolymerization and repolymerization procedure purifies NPA binding activity by between four- and 12-fold and with a high recovery of activity (verified in four separate experiments). The fold purification is greatest in the actin repolymerization pellet, indicating the tight association of this protein with the cytoskeleton. Furthermore, the recovery of NPA binding activity in the depolymerization or KI supernatant is close to 100% of the starting activity from intact vesicles. The binding constants for ^{3}H-NPA binding activity in the actin depolymerization supernatant sample were determined. The number of NPA binding sites, as assessed by the B_{max}, was increased by 10-fold, as expected with the increase in purity. The dissociation constant in the soluble sample was less than threefold different from the dissociation constant of intact membranes, indicating that affinity for NPA is not significantly different as a result of this manipulation.

The association of the NBP with the cytoskeleton suggests that this protein is on the cytoplasmic face of the plasma membrane. Several other lines of experimental evidence have also suggested that this protein functions on the cytoplasmic face of the membrane. Michalke (1982) isolated two membrane populations that were proposed to differ in orientation of the vesicles. The population suggested to be outside-out was unable to bind the NPA anion but bound the membrane-permeant, uncharged NPA species. The inside-out population was able to bind NPA in either ionization state. Furthermore, NPA binding activity increased as outside-out zucchini vesicles were converted to inside-out or open vesicles by multiple freeze-thaw cycles or by removal of sucrose in the buffer (Hertel et al., 1983). This further suggested that the site of binding of NPA and other phytotropins is on the internal face of the membrane.

Although it has been generally assumed that NPA binding activity is on the same polypeptide as the activity that transports the IAA anion, recent evidence suggests that this simple model of the efflux carrier as a single polypeptide with both regulatory and catalytic activities may not be correct. Morris et al. (1991) treated zucchini hypocotyl sections with inhibitors of protein synthesis. They found that this treatment did not significantly affect either the ability of hypocotyl segments or isolated membrane vesicles to accumulate radiolabeled IAA or the ability of membrane vesicles to bind ^{3}H-NPA. Yet, these treatments significantly reduced the ability of NPA to elevate auxin accumulation, through inhibition of efflux, both in segments and in vesicles. Morris et al. (1991) concluded that the auxin transport site on the efflux carrier system and the NPA binding site may reside on separate proteins linked by a third, rapidly turned over protein. The uncoupling of auxin movement and its NPA sensitivity have been shown to occur in vivo by Allan and Rubery (1991), who report that calcium deficiency in zucchini hypocotyls leads to a reduction in polar auxin transport, although both IAA uptake and NPA binding are not affected by this treatment. They also report that NPA sensitivity of uptake is diminished. These results and the conclusions of Morris et al. (1991) suggest that the regulatory and catalytic activities of the auxin efflux carrier can be uncoupled.

Our results suggest that a cytoskeletal protein may be the rapidly turned over protein that Morris et al. (1991) suggested might link the NBP to the auxin efflux carrier. The significance of the cytoskeletal association of the NBP is not yet clear, but several hypotheses can be proposed. Thimann et al. (1992) have shown that an intact actin cytoskeleton is required for auxin-mediated growth. It may be that the relationship between these two processes is indirect. Auxin transport may require an intact cytoskeleton, and disruption of the cytoskeleton may abolish transport and lead to an inhibition of auxin-induced growth. This possibility is supported by the experiments of Cande et al. (1973), who demonstrated that cytochalasin B reduced both auxin transport and auxin-induced growth. Another possibility is that the NBP utilizes the cytoskeletal network for movement to allow spatial reorientation of the auxin transport stream during phototropism and gravitropism. Morris and Johnson (1990) suggest that the loss of an auxin source leads to a randomization of the distribution of the auxin efflux carrier in the plane of the plasma membrane. Their study does not differentiate between changes in the distribution of the catalytic or transport component of the efflux carrier versus the NBP or regulatory subunit. Finally, Nick et al. (1990) demonstrated that auxin can cause a change in microtubule orientation, either by exogenous application to cut coleoptiles or during apparent redistribution during the phototropism or gravitropism. It may be that this change in microtubule orientation controls or is controlled by a change in the distribution of the NPA binding subunit of the auxin efflux carrier.

It is also possible that the peripheral membrane and cytoskeletal associations of the NBP serve to organize the efflux carrier complex in one plane of the membrane. An example in which an actin-associated and peripheral membrane protein can act to localize membrane components is found in the clustering of acetylcholine receptors in vertebrate neuromuscular junctions (Bloch and Resneck, 1986; Yoshihara and Hall, 1993). The receptor clusters are positioned opposite to synaptic sites of neurotransmitter release, where their density is essential for efficient signal propagation (Yoshihara and Hall, 1993).

The results presented in this study provide a basis for two conclusions. First, the NBP is peripherally associated with the plasma membrane. Second, these data support the hypothesis that the NBP is associated with the cytoskeleton in vitro. These results may now provide a context for future investigations into the regulation of auxin transport by interactions between the cytoskeleton and the NBP, the putative regulatory subunit of the auxin efflux carrier. The understanding that this is a peripheral membrane protein will facilitate development of purification approaches. Purified NBP will facilitate the preparation of molecular probes to explore the mechanisms by which auxin transport is regulated in much greater depth.

METHODS

Chemicals and Radiochemicals

(2,3,4,5-[*N*]-^{3}H)-*N*-1-Naphthylphthalamic acid (^{3}H-NPA) (49.0 Ci/mmol) was obtained from Research Products International Corp. (Mount Prospect, IL) and (2,3,4,5-[*N*]-^{3}H)-NPA (58.0 Ci/mmol) was obtained from American Radiolabeled Chemicals Inc. (Saint Louis, MO). The bicinchoninic acid (BCA) protein assay kit, Micro-BCA protein assay kit, and Triton X-100 detergent were from Pierce. Trichloroacetic acid/deoxycholate protein precipitation was performed using a protein assay kit purchased from Sigma. NPA was purchased from Chemical Services (West Chester, PA). Cytochalasin B was purchased from Aldrich Chemical Company and was dissolved in DMSO. Monoclonal anti-actin antibody (clone C4) was purchased from ICN (Costa Mesa, CA), and monoclonal anti–α-tubulin antibody was from Amersham. Anti–mouse IgG_1 antibody conjugated with alkaline phosphatase and Scintiverse scintillation fluid were purchased from Fisher Scientific. Alkaline phosphatase substrates 5-bromo-4-chloro-3-indolyl phosphate (X-phosphate, toluidine salt) and 4-nitro blue tetrazolium chloride and the protease inhibitor leupeptin were purchased from Boehringer Mannheim. Density gradient gels (4 to 20%) were from Jule Inc. (New Haven, CT). Silver stain standards (low range) and biotinylated SDS-PAGE standards (low range) were purchased from Bio-Rad. Streptavidin conjugated with alkaline phosphatase was purchased from United States Biochemical Corp. Rhodamine phalloidin fluorescent probe and 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate ($DiIC_{(18)}$) were purchased from Molecular Probes (Eugene, OR). All other chemicals were purchased from Sigma and were of the highest grade available.

Plant Material and Plasma Membrane Preparation

Zucchini (*Cucurbita pepo* cv Burpee Fordhook) seedlings were grown for 5 days in the dark in vermiculite at 30°C. The upper thirds of the etiolated hypocotyls (~4 cm) were harvested and homogenized with an equal ratio of weight per volume of buffer A (0.25 mM Tris, pH 7.2, 3.0 mM EDTA, 250 mM sucrose, 0.25 mM phenylmethylsufonyl fluoride [PMSF], 1 mM DTT). After filtration through nylon cloth, cellular debris was removed by centrifugation at 6700*g* for 10 min. The supernatant was then subjected to centrifugation at 100,000*g* for 30 min, and the pellet was resuspended in buffer B (5.0 mM K_2HPO_4, pH 7.8, 250 mM sucrose). The membranes were then subjected to two-phase aqueous partitioning according to the procedure of Widell and Larsson (1981) with 6.3% dextran and 6.3% polyethylene glycol, as previously described (Muday et al., 1993). Membranes were resuspended in low ionic strength Mg-cytoskeleton stabilization buffer (Mg-CSB) as described by Abe and Davies (1991) with minor modifications described by Tan and Boss (1992). Mg-CSB (5 mM Hepes, pH 7.0, 10 mM $MgCl_2$, 2 mM EGTA, 1 mM PMSF, 250 mM sucrose, 1 mg leupeptin per 100 mL, 0.1 mM sodium vanadate) was added at a ratio of 1 mL for every 33 g of hypocotyl fresh weight, which yielded a final protein concentration of ~5 mg/mL, as determined by the BCA assay procedure (Smith et al., 1985). Membranes were frozen in liquid nitrogen and stored at −70°C until use.

^{3}H-NPA Binding Studies

^{3}H-NPA binding assays were performed in 200-μL volumes of NPA binding buffer (NBB; 20 mM sodium citrate, pH 5.3, 1 mM $MgCl_2$, 250 mM sucrose) in Multiscreen HA (mixed esters of cellulose) filtration plates (Millipore, Bedford, MA) pretreated with 0.3% polyethylenimine (Bruns et al., 1983). Samples had a final protein concentration of 0.2 mg/mL for plasma membranes with a ^{3}H-NPA concentration of 5.0 nM or in the range of 1 nM to 15 nM, as described by Muday et al. (1993). Incubations were for 45 min at 4°C. Background binding was determined by addition of 10^{-5} M nonradioactive NPA to each ^{3}H-NPA concentration and was shown to be equivalent to nonspecific binding of ^{3}H-NPA in the absence of protein. Samples were filtered and washed with 200 μL of NBB and then dried at 50°C for 15 min. The filters were punched out and soaked for 30 min in 250 μL of water prior to the addition of 2.5 mL of scintillation fluid and counting in a Tm Analytic (Elk Grove Park, IL) liquid scintillation counter for 2 min. Binding is reported in picomoles of ^{3}H-NPA bound per milligram of protein in the assay (specific activity), in nanomolar concentrations of ^{3}H-NPA bound, or in picomoles bound (total binding). Each value represents the average of three to four different replicates. The standard deviation is reported whenever appropriate.

Membrane Association of the NPA Binding Protein

Intact membrane vesicles were treated with 1 M Na_2CO_3, pH 11.0, in Mg-CSB without sucrose to disrupt peripheral protein interactions with the plasma membrane. The intact plasma membranes are osmotically sensitive; therefore, the absence of sucrose is essential because it allows the membrane vesicles to rupture and reform with random orientation. Untreated membrane vesicles in Mg-CSB, pH 7, were run in parallel with the Na_2CO_3 treatment. The membranes were gently rotated at 4°C for 1 hr and then subjected to ultracentrifugation at 40,000*g* for 20 min. Both the supernatant and the resuspended pellet fractions were diluted fivefold with NBB, and these samples were assayed for ^{3}H-NPA binding. The final concentration of Na_2CO_3 in the binding assay was 0.2 M.

In a second set of experiments, intact membrane vesicles were treated with 0.4 M KI in K-CSB (5 mM Hepes, pH 7.0, 100 mM KCl, 2 mM EGTA, 1 mM PMSF, 0.1 mM sodium vanadate, 1 mg of leupeptin per 100 mL) without sucrose to depolymerize actin filaments and rupture membrane vesicles. This allows for greater accessibility of KI to the actin cytoskeleton and for proteins released from the inside of the plasma membrane vesicles to leave the sealed vesicles. The membranes were gently rotated at 4°C for 1 hr and then subjected to ultracentrifugation at 40,000*g* for 20 min. All pellet samples were resuspended in equal volumes in K-CSB without sucrose. The supernatant and a fraction of the resuspended pellet were diluted fivefold with NBB, and these samples were used to measure ^{3}H-NPA binding activity. The final concentration of KI in the binding assay was 0.08 M.

Preparation of Cytoskeleton and Cytochalasin B Treatments

Detergent-insoluble cytoskeletal protein preparations were prepared in the following manner. Plasma membranes were resuspended in Mg-CSB containing 2.5% (w/v) Triton X-100 to a protein concentration of 2 mg/mL, such that the detergent-to-protein ratio was 12.5 mg/mg. Plasma membranes were gently rocked at 4°C for 20 min. The cytoskeleton (the Triton X-100–insoluble fraction) was separated from solubilized plasma membrane proteins by centrifugation at 50,000*g* for 20 min. The cytochalasin B assays were performed using the cytoskeletal pellet fractions, which were resuspended in cytochalasin B at the indicated concentrations in Mg-CSB and gently rocked at 4°C for 30 min. The samples were then subjected to centrifugation at 50,000*g* for 30 min. The pellet was resuspended in NBB. Both the resuspended pellet and the supernatant were diluted fivefold with NBB, and these samples were assayed for ^{3}H-NPA binding.

Actin Depolymerization and Repolymerization

The F-actin fraction, which contained actin filaments and actin binding proteins, was prepared as described by Payrastre et al. (1991) with some modifications to the buffers as described by Tan and Boss (1992). The cytoskeletal fraction was treated with K-CSB, containing KI, resulting in a final KI concentration of 0.4 M. After 20 min at 4°C with gentle shaking, the solution was centrifuged at 40,000*g* for 20 min. The supernatant was dialyzed two times against 2 L of K-CSB containing 5 mM benzamidine, instead of leupeptin, and centrifuged at 12,000*g* for 5 min. The resultant pellet (denoted the F-actin fraction) was resuspended in 0.3 mL of polymerization buffer (Mg-CSB with 5 mM benzamidine). Protein concentrations were determined using the Micro-BCA procedure according to Smith et al. (1985) with BSA as a standard. Actin content in the samples obtained through this procedure was monitored by immunoblot analysis and fluorescence microscopy using rhodamine phalloidin–monitored F-actin integrity.

SDS-PAGE and Immunoblotting

Proteins were trichloroacetic acid/deoxycholate precipitated according to Ozols (1990). The precipitated proteins were solubilized in SDS-PAGE sample buffer, boiled at 95°C for 5 min, and separated on a 12% SDS-polyacrylamide gel (according to the procedure of Laemmli, 1970) for the cytochalasin B samples; the F-actin samples were separated on a 4 to 20% gradient gel. Two sets of identical samples were subjected to electrophoresis: one gel was electrophoretically transferred onto supported nitrocellulose BA-S 85 (Schleicher & Schuell) for immunoblotting by the method of Burnette (1981), and one gel was used for silver staining (data not shown). Immunoblot analysis with the anti-actin monoclonal antibody was performed using Blotto plus 0.02% Tween 20 as the blocking agent according to Johnson et al. (1984). For detection with the anti–α-tubulin antibody, the actin immunoblots were reprobed by blocking and incubation in 3% BSA in PBS. Anti–mouse IgG_1 conjugated with alkaline phosphatase was the secondary antibody. Primary antibody incubation time was overnight at a dilution of 1:1000, and the secondary antibody incubation time was 2 hr at a dilution of 1:2000. The biotinylated SDS-PAGE standards were detected using 4-nitro blue tetrazolium and 5-bromo-4-chloro-indolyl phosphate according to the manufacturer's recommendations, using streptavidin conjugated with alkaline phosphatase.

The amount of actin in each lane of the immunoblot was quantified with a Fisher FB910 scanning densitometer using a 570-nm dielectric filter. The intensity of the bands was measured after the blots were moistened with 50% glycerol. These data were collected with the data acquisition hardware MP100 and analyzed using the program Acqknowledge 3.0 for Macintosh, both from BIOPAC Systems, Inc. (Goleta, CA). The intensity of each band is reported in a scan, from which the peak area corresponding to each band can be quantified. Because each gel contained a control of chicken actin at a defined concentration, the area of each peak could be converted to an amount of actin. Finally, the amount of actin in each peak could be normalized by the amount of protein loaded to determine the amount of actin in micrograms per milligram of protein loaded.

Differential Interference Contrast and Fluorescence Microscopy

Differential interference contrast (DIC) and fluorescence imaging of plasma membrane vesicles was performed using both DIC and low-light level video fluorescence microscopy (Hamamatsu [Waltham, MA] DIC and silicon-intensified target [SIT] cameras, respectively) and a Universal Imaging system on a Zeiss (Thornwood, NJ) Axiophot microscope. Images were either recorded on a Panasonic optical memory disc recording system or saved directly to the Image-1 system software (Universal Imaging, West Chester, PA). DIC images of plasma membrane vesicles were recorded at a magnification of ×63 with a 1.25 zoom lens using the DIC camera. DIC images of Triton X-100–extracted plasma membrane vesicles were recorded with a magnification of ×63 with a 1.6 zoom lens using the DIC camera. $DiIC_{(18)}$ staining of intact and Triton X-100–extracted plasma membrane vesicles was recorded at ×100 oil magnification with a 1.25 zoom lens using the SIT camera. The DIC image of rhodamine phalloidin staining of intact plasma membrane vesicles was recorded at ×100 oil magnification with a 1.25 zoom lens using the high-resolution DIC camera and was representative of intact plasma membrane vesicles stained with rhodamine phalloidin. Fluorescence images of rhodamine phalloidin staining of intact plasma membrane vesicles were recorded at ×100 oil magnification with a 1.25 zoom lens using the SIT camera. Paired DIC and fluorescence images of Triton X-100–extracted plasma membrane vesicles were recorded at ×100 oil magnification with a 1.25 zoom lens using the SIT camera. Rhodamine phalloidin staining of repolymerized F-actin was recorded at a magnification of ×63 with a 2.0 zoom lens using the SIT camera. All images were processed and enhanced using Image-1 software. DIC images were recorded to detect vesicle shape and size. For size determination the optical distance was calibrated to the magnification used. $DiIC_{(18)}$ was used to stain for plasma membrane lipids in intact and Triton X-100– extracted plasma membrane vesicles. Rhodamine phalloidin was used to stain for F-actin in intact plasma membrane vesicles and cytoskeletal fractions (Triton X-100–insoluble fractions). In addition, the F-actin fraction collected from actin depolymerization and repolymerization experiments was stained with rhodamine phalloidin to confirm the presence of F-actin in this fraction.

Microsomal Membrane Preparation and Analysis of Partitioning of NPA Binding Activity

Zucchini microsomal membranes were prepared as described for plasma membranes, but after the first ultracentrifugation, the pellets were resuspended in Mg-CSB. Maize microsomal membranes were similarly prepared, with etiolated coleoptiles grown for 5 days at 30°C. Both zucchini and maize microsomal membranes were diluted in Mg-CSB containing 2.5% (w/v) Triton X-100 to a protein concentration of

2 mg/mL. The microsomal membranes were gently rocked at 4°C for 20 min. The cytoskeleton (Triton X-100–insoluble fraction) was separated from solubilized microsomal membrane proteins by ultracentrifugation at 50,000*g* for 30 min. The Triton X-100–insoluble pellet was resuspended in Mg-CSB without sucrose and treated with K-CSB containing KI, bringing the final concentration to 0.4 M KI. After 30 min at 4°C with gentle shaking, the solution was subjected to ultracentrifugation at 40,000*g* for 30 min. The pellet was resuspended in Mg-CSB without sucrose. Both the supernatant and the resuspended pellet fractions were diluted fivefold with NBB, and these samples were assayed for ^{3}H-NPA binding activity. Background was assessed by heating the samples for 20 min at 95°C to heat inactivate the protein components. Binding activity is reported in saturable binding, with and without heat treatment. Heat-labile activity is the difference between these two activities and represents the binding that depends upon active protein.

ACKNOWLEDGMENTS

We thank Wendy F. Boss and Zhang Tan for helpful discussions throughout the course of this work. We also thank Ryan M. Greene and Michael W. Dixon for their communication of unpublished results. We especially thank Nina S. Allen for use of her Zeiss Axiophot microscope and Marty N. Bennett for his technical expertise in microscopy. This work was supported by grants from Sandoz Agro, Inc., Palo Alto, CA, and the National Science Foundation Grant No. IBN-9318250.

Received July 5, 1994; accepted October 20, 1994.

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