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Nuclear Localization Signal Receptor Importin α Associates with the Cytoskeleton

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Importin α is the nuclear localization signal (NLS) receptor that is involved in the nuclear import of proteins containing basic NLSs. Using importin α as a tool, we were interested in determining whether the cytoskeleton could function in the transport of NLS-containing proteins from the cytoplasm to the nucleus. Double-labeling immunofluorescence studies showed that most of the cytoplasmic importin α coaligned with microtubules and microfilaments in tobacco protoplasts. Treatment of tobacco protoplasts with microtubule- or microfilament-depolymerizing agents disrupted the strands of importin α in the cytoplasm, whereas a microtubule-stabilizing agent had no effect. Biochemical analysis showed that importin α associated with microtubules and microfilaments in vitro in an NLS-dependent manner. The interaction of importin α with the cytoskeleton could be an essential element of protein transport from the cytoplasm to the nucleus in vivo.

INTRODUCTION

Macromolecular translocation into and out of the nucleus occurs through the nuclear pore complex (NPC), which is embedded in the nuclear envelope. Import and export of most proteins and ribonucleoproteins through the NPC are receptor-mediated processes facilitated by the importin β–like proteins, in conjunction with a small GTPase, namely, Ran/TC4 (Gorlich et al., 1997; Izaurralde et al., 1997; Ullman et al., 1997; Wozniak et al., 1998).

The importin α/β heterodimer mediates the nuclear import of a subset of nuclear proteins that contain the classic nuclear localization signals (NLSs; Hicks and Raikhel, 1995; Jans and Hubner, 1996; Gorlich, 1997). The α subunit of the heterodimer specifically binds to basic NLSs in the cytoplasm, whereas the β subunit interacts with the NPC during the import process (Gorlich, 1997). Also, importin β can function alone as an import receptor for a subset of NLS-containing proteins (Palacios et al., 1997; Tiganis et al., 1997).

In plants, an importin α homolog (At-IMPα) identified in Arabidopsis specifically associates with three types of NLS that function in plant cells (Hicks et al., 1996; Smith et al., 1997). Interestingly, the At-IMPα subunit is a unique high-affinity NLS receptor that can facilitate NLS-mediated protein import in the absence of a β subunit in vertebrate import systems, indicating that plants may possess an additional NLS import pathway exclusively mediated by the α subunit alone (S. Hubner, H.M.S. Smith, N.V. Raikhel, and D.A. Jans, unpublished data).

Although import and export receptors have been identified, the transport mechanism that targets these import complexes from the cytoplasm to the NPC is poorly understood. Therefore, to study how importin α is targeted to the NPC and to identify cellular factors involved in this process, we were interested in analyzing whether importin α interacts with the cytoskeleton.

RESULTS

Importin α Colocalizes with Microtubules in the Cytoplasm

Previously, we showed that importin α is found in the cytoplasm and nucleus and that it is associated with the nuclear rim in fixed tobacco protoplasts (Smith et al., 1997). In addition, inside the nucleus, importin α is excluded from the nucleolus. The size of the nucleolus differs from cell to cell; as a consequence, the amount of importin α found in the nucleus is variable.

Because the localization pattern of importin α in the cytoplasm of tobacco protoplasts resembles the immunostaining pattern of cytoskeletal proteins (Smith et al., 1997), we were interested in examining whether importin α could interact with the cytoskeleton. Localization of importin α was examined to determine whether importin α colocalized with the cytoskeleton in fixed tobacco protoplasts.

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Double immunofluorescence experiments using monospecific antibodies generated against importin α and tubulin were visualized by confocal laser scanning microscopy (CLSM). Figure 1 shows tubulin staining visualized in green and importin α staining visualized in red; overlay images were used to show coalignment. Most of the microtubules extending from the nuclear envelope coaligned with cytoplasmic strands of importin α (Figure 1A). In addition, the localization pattern of importin α is also distinct from soluble and membrane proteins (data not shown).

Treatment of protoplasts with 10 μM oryzalin, a plant-specific microtubule depolymerizing agent, efficiently depolymerized the microtubules (Figure 1B, green). This treatment also abolished the cytoplasmic strands of importin α (Figure 1B, red). Similar results were obtained when protoplasts were treated with propyzamide, another plant microtubule depolymerizing agent (data not shown). When treated protoplasts were washed to remove the oryzalin, both microtubules and cytoplasmic strands of importin α reappeared and colocalized in the cytoplasm (Figure 2A). When protoplasts were incubated on ice, most of the cold-adapted microtubules found in the cytoplasm (Wallin and Stromberg, 1995) coaligned with the cytoplasmic strands of importin α (Figure 2B). Treatment of protoplasts with 10 μM taxol stabilized the microtubules and did not affect the colocalization of importin α and microtubules in the cytoplasm (Figure 3). These results suggest that the cytoplasmic strands of importin α associate with the microtubules.

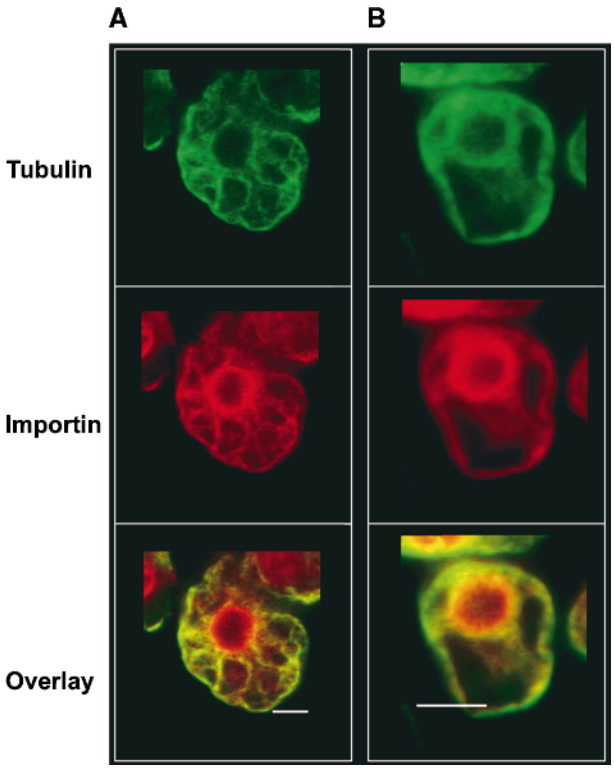

Figure 1. Importin α Colocalizes with Microtubules in the Cytoplasm of Tobacco Protoplasts.

(A) Most of the cytoplasmic importin α (Importin image) colocalized with microtubules (Tubulin image) in fixed tobacco protoplasts. The green image displays the microtubules, and the red image displays the cytoplasmic strands of importin α. Superimposing these two images demonstrates coalignment, which is shown in yellow/orange (Overlay image).

(B) Treatment of tobacco protoplasts with 10 μM oryzalin depolymerized the microtubules (Tubulin image). Oryzalin (10 μM) also disrupted the cytoplasmic strands of importin α (Importin image).

Bars in **(A)** and **(B)** (Overlay images) = 10 μm.

Importin α Colocalizes with Microfilaments in the Cytoplasm

Double immunofluorescence experiments with monospecific anti-actin antibodies and phalloidin were used to determine whether importin α colocalized with microfilaments by using CLSM (Figure 4). Immunostaining of fixed tobacco protoplasts showed that the cytoplasmic strands of importin α also coaligned with microfilaments (Figure 4A). When cells were treated with 50 μg/mL cytochalasin B, not only were the microfilaments depolymerized but some of the cellular actin accumulated inside the nucleus (Figure 4B, green image), which is consistent with recently published observations in plant cells (Jiang et al., 1997). Interestingly, depolymerization of microfilaments caused most of the cellular importin α to accumulate in the nucleus (Figure 4B, red image). When cells were washed to remove cytochalasin B, microfilaments and importin α cytoplasmic strands were restored in the cytoplasm (data not shown). Protoplasts treated with cytochalasin D also caused importin α to accumulate in the nucleus (data not shown). These results suggest that importin α may be retained in the cytoplasm by association with microfilaments.

Importin α Associates with Microtubules and Microfilaments

One of the intriguing features of the armadillo family of proteins is their ability to interact with the cytoskeleton. Some family members, such as spKAP115, APC, Pf16, and smgGDS, interact with microtubules, whereas Vac8 and the armadillo/catenin proteins interact with microfilaments (Smith and Lefebvre, 1996; Wedaman et al., 1996; Barth et al., 1997; Shimizu et al., 1998; Wang et al., 1998). Because the central domain of importin α contains eight armadillo repeat motifs (Peifer et al., 1994), we decided to test whether this receptor could interact with the cytoskeleton in vitro. A tobacco protoplast extract containing importin α was prepared, centrifuged at high speed to remove any insoluble

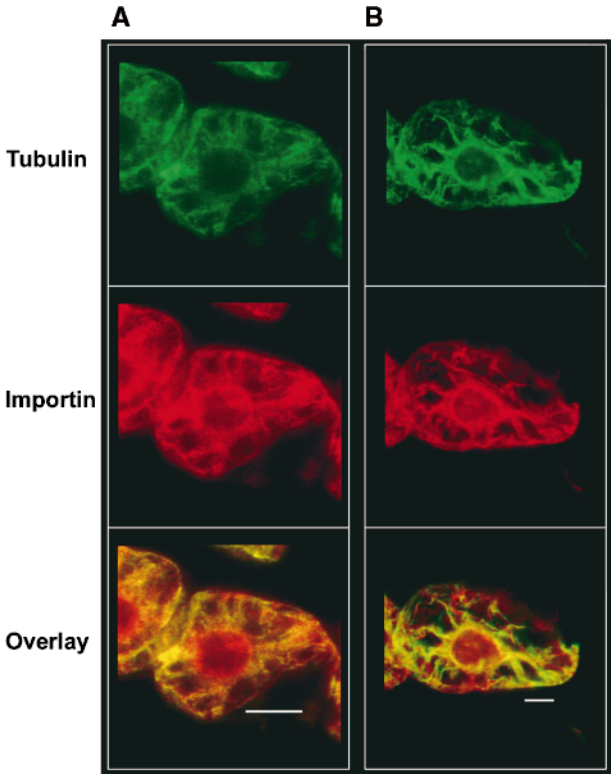

Figure 2. Colocalization of Importin α with the Microtubules Was Detected When Oryzalin Was Washed from the Protoplasts and When Untreated Protoplasts Were Chilled on Ice.

(A) Tobacco protoplasts treated with 10 μM oryzalin were washed and incubated for 3 hr before the double immunofluorescence experiments. The effect of oryzalin was reversible because microtubules reappeared in the cytoplasm when the oryzalin was washed from the protoplasts (Tubulin image). Removal of the oryzalin also restored the cytoplasmic strands of importin α (Importin image). Superimposing the tubulin and importin α images shows that most of the importin α colocalized with the microtubules (Overlay image).
(B) Protoplasts were incubated on ice for 1 hr to determine whether this condition would disrupt the colocalization of importin α with the microtubules. Microtubules were detected in the cytoplasm of protoplasts chilled on ice for 1 hr (Tubulin image). Under these conditions, strands of importin α were also detected in the cytoplasm (Importin image). Furthermore, most of the cytoplasmic strands of importin α colocalized with microtubules when the protoplasts were chilled on ice (Overlay image).
Bars in **(A)** and **(B)** (Overlay images) = 10 μm.

material, and used to determine whether importin α could interact with microtubules and microfilaments in vitro.

Microtubules were polymerized in the presence of taxol and then mixed with the tobacco protoplast extract in the absence of NLS peptides or in the presence of a functional NLS peptide, Opaque2 wild type (O2WT), or a mutated form of this NLS peptide, Opaque2 mutant (O2MUT) (Varagona et al., 1992; Varagona and Raikhel, 1994). After a 30-min incubation, the samples were loaded on a 40% sucrose cushion containing taxol and centrifuged to pellet the microtubules. Microtubule pellets were resuspended in high-salt buffer to solubilize protein complexes and then separated by SDS-PAGE and probed for importin α by using immunoblot analysis.

When the tobacco protoplast extract was incubated with microtubules in the presence of O2WT, we detected an association of importin α with these structures (Figure 5, lane 2). In the absence of a functional NLS or in the presence of O2MUT peptides, this association was substantially reduced (Figure 5, lanes 1 and 3, respectively). Control experiments prepared under the same conditions without added microtubules showed that importin α does not pellet in the absence or presence of functional or mutated NLS peptides (Figure 5, lanes 4 to 6, respectively). These results indicate

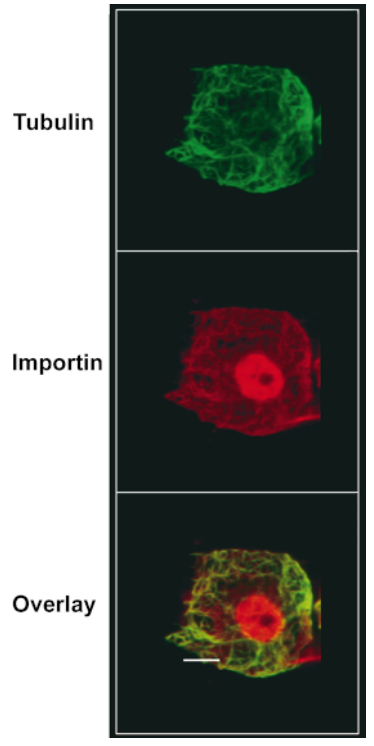

Figure 3. Stabilization of the Microtubules with Taxol Does Not Affect the Coalignment of Importin α with the Microtubules.

Protoplasts were treated with 10 μM taxol to stabilize the microtubules. Under these conditions, a microtubule network was detected in the cytoplasm of treated protoplasts (Tubulin image) and strands of importin α could be detected throughout the cytoplasm (Importin image). When these two images were superimposed, most of the importin α colocalized with the microtubules (Overlay image).
Bar = 10 μm.

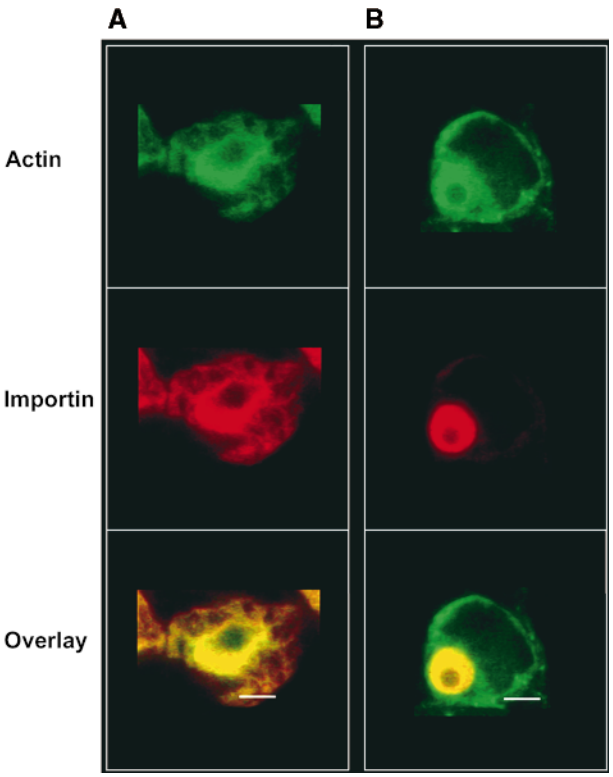

Figure 4. Importin α Colocalizes with Microfilaments in the Cytoplasm of Tobacco Protoplasts.

(A) Double immunofluorescence experiments were performed to determine whether importin α colocalized with microfilaments. BODIPY-labeled phalloidin was used to visualize the microfilaments (Actin image). Most of the cytoplasmic strands of importin α (Importin image) colocalized with microfilaments in fixed tobacco protoplasts (Overlay image).

(B) Protoplasts were treated with 50 μg/mL cytochalasin B to determine whether these conditions would disrupt the cytoplasmic strands of importin α. Under these conditions, anti-actin antibodies were used to detect the actin in these samples. Most of the microfilaments were depolymerized in the presence of cytochalasin B (Actin image). Importin α accumulated inside the nucleus when the microfilaments were depolymerized (Importin image). When untreated protoplasts were stained for microfilaments by using BODIPY-labeled phalloidin, fluorescence was always detected in the nuclei and cytoplasm of these samples (**[A]**, Actin image). However, nuclear fluorescence was not detected when protoplasts were immunostained using purified antibodies directed against actin (data not shown). In addition, nuclear forms of actin have been detected when animal cells are stained with fluorescently labeled phalloidin and not anti-actin antibodies (Clubb and Locke, 1998).

Bars in **(A)** and **(B)** (Overlay images) = 10 μm.

that importin α associates with microtubules in an NLS-dependent manner in vitro.

We next analyzed whether importin α associated with actin microfilaments in vitro. Actin microfilaments were polymerized and mixed with the tobacco protoplast extract described above, in the absence or presence of O2WT or O2MUT peptides. After incubation, the samples were loaded on a 10% glycerol cushion and centrifuged to pellet actin microfilaments. Actin pellets were resuspended in high-salt buffer to solubilize the protein complexes, separated by SDS-PAGE, and analyzed by immunoblotting to detect association of importin α.

Importin α pelleted with the microfilaments in the presence of O2WT peptides (Figure 6, lane 2); however, in the absence or presence of O2MUT peptides, we detected little or no association (Figure 6, lanes 1 and 3, respectively). Control experiments performed under the same conditions showed that importin α did not pellet when microfilaments were omitted from the association assay (Figure 6, lanes 4 to 6). These results indicate that importin α associates with microfilaments in an NLS-dependent manner in vitro.

DISCUSSION

Intracellular transport of organelles (Hirokawa, 1998; Mermall et al., 1998), viruses (Greber et al., 1997; Sodeik et al., 1997), and mRNA–protein complexes (Hovland et al., 1996; Bassel

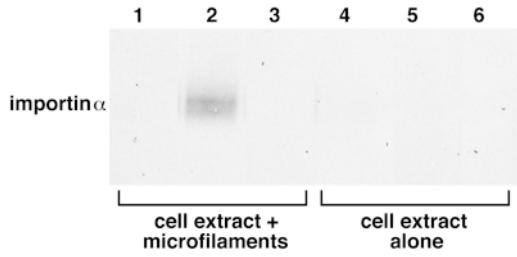

Figure 5. Association of Importin α with Microtubules Was Dependent on Functional NLSs.

Taxol-stabilized microtubules were mixed with a plant cell extract alone (lane 1) or with 200 μM 02WT NLS (lane 2) or 200 μM 02MUT NLS (lane 3) peptides. Association was determined by the ability of importin α to pellet with the microtubules through a 40% sucrose cushion. Microtubule pellets were solubilized, separated by 10% SDS-PAGE, blotted to a nitrocellulose membrane, and probed using anti–At-IMPα antibodies to detect importin α. In control experiments, the plant cell extracts were incubated alone (lane 4) or with 200 μM 02WT NLS peptides (lane 5) or with 200 μM 02MUT NLS peptides (lane 6) under the same conditions as described above. Immunoblot analysis showed that importin α did not pellet in the absence of microtubules.

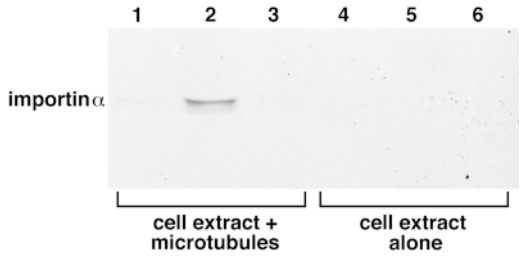

Figure 6. Importin α Associates with Microfilaments in an NLS-Dependent Manner.

Polymerized microfilaments were mixed with a plant cell extract in the absence of NLS peptides (lane 1) or the presence of 02WT (lane 2) or 02MUT (lane 3) peptides. The association was determined by the ability of importin α to pellet through a 10% glycerol cushion with the microfilaments. Solubilized pellets were subjected to immunoblot analysis using anti–At-IMPα antibodies to detect the association of importin α with the microfilaments. In control experiments, plant cell extracts were incubated alone (lane 4) or with 02WT (lane 5) or 02MUT (lane 6) peptides, and importin α did not pellet through the 10% glycerol gradient when microfilaments were omitted from the association assay.

and Singer, 1997) is mediated by the cytoskeleton. A fundamental question in nuclear transport is how cytoplasmically synthesized proteins are targeted and directed to the NPC. Attempts to block NLS protein import in vitro with cytoskeleton depolymerizing agents have not been successful in plants and in other systems (Schmalz et al., 1996; H.M.S. Smith and N.V. Raikhel, unpublished data). Therefore, it appears that the cytoskeleton is not involved in the binding and translocation steps of import.

In immunofluorescence studies in protoplasts, we found that the cytoplasmic localization of importin α was distinct from that of immunostained soluble and membrane proteins. The cytoplasmic strands of importin α had a distribution similar to that of microtubules and microfilaments in the cytoplasm of fixed tobacco protoplasts. However, the colocalization of importin α with the cytoskeleton was not in a 1:1 ratio in the cytoplasm, demonstrating that a fraction of importin α's localization is independent of the cytoskeleton. The portion of importin α that did not coalign with the cytoskeleton may represent a soluble form of the NLS receptor.

Other proteins, such as the tobacco mosaic virus movement protein (Heinlein et al., 1995; McLean et al., 1995) and elongation factor–1α (reviewed in Durso and Cyr, 1994; Clore et al., 1996), also colocalize with both cytoskeletal structures. In fact, immunofluorescence and immunoelectron analyses demonstrate that microtubules and microfilaments colocalize in plant cells, suggesting that these structural elements interact with each other (reviewed in Lancelle et al., 1987; Staiger and Lloyd, 1991). In addition, microtubules and microfilaments are integrated structures that make up the preprophase band, mitotic spindle, and phragmoplast in plant cells.

As a means to characterize the colocalization of importin α with the cytoskeleton, we treated tobacco protoplasts with oryzalin and cytochalasin B to depolymerize the microtubules and microfilaments, respectively. Treating protoplasts with oryzalin disrupted the cytoplasmic strands of importin α, suggesting that importin α may interact with microtubules in vivo. Like importin α, the tobacco mosaic virus movement protein that colocalizes with microtubules and microfilaments also becomes diffuse in the cytoplasm when the microtubules in tobacco protoplasts are depolymerized (Heinlein et al., 1995; McLean et al., 1995). Interestingly, depolymerization of microtubules also disrupts the microfilaments in the plant cells (Panteris et al., 1992; McLean et al., 1995), and mutations in maize that disrupt microtubule arrays formed during meiosis also alter the microfilament structures (Staiger and Cande, 1991). These observations indicate that the integrity of the plant cytoskeleton is dependent on the interaction of microtubules and microfilaments. Therefore, depolymerization of microtubules might also disrupt the interaction of importin α with the microfilaments in vivo, which explains why all of the importin α becomes dispersed throughout the cytoplasm in these experiments.

Treatment of tobacco protoplasts with cytochalasin B caused most of the cellular importin α to accumulate inside the nucleus. Similar results were obtained when protoplasts were treated with cytochalasin D. These observations suggest that microfilaments could be involved in retaining importin α in the cytoplasm. In addition, some of the cellular actin also accumulated inside the nucleus when the microfilaments were depolymerized. Treatments of maize root tips with cytochalasin D caused the intranuclear accumulation of short actin filament/aggregate-like structures and the actin depolymerization factor ZmADF3 (Jiang et al., 1997). However, the translocation and accumulation of actin and ZmADF3 in the nucleus are not understood. Because oryzalin and cytochalasin B had different effects on importin α's subcellular location, our results suggest that microtubules and microfilaments could play different roles in NLS protein transport.

In vitro pelleting studies suggested that importin α associates with microtubules and microfilaments in an NLS-dependent manner. This association could be essential for the transport of NLS-containing proteins in the cytoplasm of the cell. Because a system to study cytoskeleton transport in vitro does not exist in plants, it is not possible to determine whether importin α moves along these structures during transport before import.

NLS-dependent association of importin α with the cytoskeleton could also be important for anchoring the NLS receptor in the cytoplasm. Gene expression can be controlled by regulating the nuclear import of transcription factors and regulatory proteins (Jans and Hubner, 1996). Many of these nuclear regulatory proteins, such as the NF-κB class of transcription factors, could be synthesized and tethered to the

cytoskeleton (Blank et al., 1992). Importin α could be attached to the cytoskeleton through the interaction of the nuclear regulatory proteins anchored to the cytoskeleton. Thus, when the appropriate environmental stimulus is given to the cells, importin α and the regulatory proteins could be released from the anchor mechanism for nuclear import. In plants, nuclear import of regulatory proteins, such as CONSTITUTIVE PHOTOMORPHOGENIC1, phytochrome B, and the transcription factor G-box binding factor, are mediated by light (Harter et al., 1994; von Armin and Deng, 1994; Sakamoto and Nagatani, 1996). Therefore, importin α could be an essential factor in regulating the light-dependent import of these proteins.

We propose that the microtubules are involved in the transport of NLS-containing proteins from the cytoplasm to the NPC. Interestingly, it has been shown that retrograde transport of fluorescently labeled NLS-containing proteins toward the nucleus in neuron cells is microtubule dependent (Ambron et al., 1992). The fact that importin α associated with microtubules in the presence of NLSs suggests that targeting and transport of importin α to the NPC require NLS-containing cargo. Furthermore, transport must be facilitated by a microtubule motor protein because importin α is not homologous to microtubule motor proteins. Also, importin α accumulated inside the nucleus when the microfilaments were depolymerized, thus indicating that these structures are involved in retaining this receptor in the cytoplasm. In yeast, importin α associates with an isoform of actin, Act2 (Yan et al., 1997). Because Act2 has a distribution similar to the microfilaments in the cytoplasm, it is tempting to speculate that Act2 could be involved in retaining importin α in the cytoplasm.

Interestingly, many mRNAs found in a subset of cellular polysomes anchored on microfilaments encode nuclear proteins, indicating that this interaction is necessary for targeting the newly synthesized proteins to the nucleus (Abe and Davies, 1995; Hovland et al., 1996; Bassel and Singer, 1997). Therefore, we propose that importin α associates with microfilaments at sites at which nuclear proteins are synthesized, so that it can be immediately assembled with these proteins. After assembly, these import complexes can be loaded onto microtubule tracks for NLS protein transport to the nucleus.

In summary, using various approaches, we found that importin α associates with the cytoskeleton. These studies are significant because they provide a link between the cytoskeleton and an import factor, importin α, suggesting that these cellular arrays are essential structures involved in the intracellular transport of a nuclear shuttling NLS receptor in the cytoplasm. In addition, the association of importin α cargo complexes with the cytoskeleton could be a highly regulated process, adding another level of gene regulation in eukaryotes. Taken together, the results of this study support the idea that the cytoskeleton is a dynamic structure involved in many intracellular transport pathways as well as in maintaining the shape and structure of the cell.

METHODS

All chemicals were obtained from Sigma, unless otherwise noted. Nuclear localization signal (NLS) peptides were synthesized at the Peptide Synthesis Facility (Yale University, New Haven, CT). NLS peptides had the following sequences: functional Opaque2 wild-type NLS (O2WT), MPTEERVRKRKESNRESARRSRYRKAAHLKC; and mutant Opaque2 NLS (O2MUT), MPTEERVRTNKESNRESARRSNYRKAAHKLC. Antibodies to *Arabidopsis thaliana* importin α (At-IMPα) were purified by At-IMPα affinity chromatography, as described by Hicks et al. (1996). Monoclonal antibodies to tubulin and actin were obtained from Amersham, Inc. (Arlington Heights, IL). The secondary antibodies BODIPY-labeled goat anti-mouse and BODIPY-labeled phalloidin were purchased from Molecular Probes Inc. (Eugene, OR). Secondary antibodies generated to importin α and goat anti-rabbit CY3–conjugated antibodies were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Nucleotides were purchased from Boehringer Mannheim. Cytochalasins B and D were obtained from Calbiochem (La Jolla, CA). Oryzalin was purchased from DowElanco (Indianapolis, IN), and propyzamide was purchased from Chem Service (West Chester, PA). Cytoskeleton depolymerizing agent stock solutions were dissolved in DMSO such that the final concentration of DMSO added to the protoplasts did not exceed 1%.

Immunolocalization in Tobacco Protoplasts

Tobacco suspension–cultured cells were maintained and protoplasts were prepared as described by Hicks and Raikhel (1993). Fixation and immunolocalization of importin α were performed as described by Smith et al. (1997), except that protoplasts were fixed with 4% paraformaldehyde instead of 3.5%. Briefly, paraformaldehyde-fixed protoplasts were dehydrated in cold methanol for 10 min, washed in PBST (PBS and 0.5% Tween 20) for 30 min at 23°C, and then blocked for 20 min in PBST plus 5% BSA at room temperature.

For colocalization experiments with importin α and tubulin, 150 ng of At-IMPα rabbit antibodies and tubulin monoclonal antibodies were mixed together in AB buffer (PBST and 1% BSA). For colocalization experiments with importin α and actin, 150 ng of At-IMPα rabbit antibodies and BODIPY-conjugated phalloidin (final concentration was 1 μM) were mixed together in 50 μL of AB buffer. To detect actin in protoplasts treated with cytochalasin B or D, we mixed together 150 ng of At-IMPα rabbit antibodies and actin monoclonal antibodies in AB buffer. The primary antibody solutions were added to separate samples of fixed protoplasts and incubated for 1 hr at 23°C in a moist chamber.

After incubation, the samples were washed in PBST for 30 min at 23°C. The secondary antibodies generated to CY3-labeled goat anti-rabbit and BODIPY-conjugated goat anti-mouse antibodies were diluted 1:50 in AB buffer and applied to the samples; then they were incubated for 1 hr in a moist dark chamber. Samples were washed in PBST for 30 min at 23°C and then mounted with a 50/50 mixture of Slow Fade (Molecular Probes Inc.) and Permafluor (Immunon, Pittsburgh, PA). No cross-reactivity was detected in samples incubated with CY3-labeled goat anti-rabbit and the monoclonal tubulin or actin antibodies (data not shown). Similar results were observed when samples were stained with BODIPY-labeled goat anti-mouse and importin α antibodies (data not shown).

For cytoskeleton depolymerization experiments, we treated protoplasts with 10 μM oryzalin or 25 μM propyzamide (data not shown)

for 1 hr to disrupt microtubules before fixation. Microfilaments were depolymerized with either 50 μg/mL cytochalasin B or 50 to 100 μM cytochalasin D (data not shown) for 1 hr before fixation. Restoration of microtubules or microfilaments was achieved by washing the treated protoplasts in NT buffer (4.3 g/L Murashige and Skoog salts [Gibco BRL], 1 mg/L thiamine, 0.1 g/L myoinositol, 0.18 g/L KH_2PO_4, and 30 g/L sucrose, adjusted to pH 5.7) to remove the depolymerizing agents. Protoplasts were incubated for 2 to 3 hr at 23°C before fixation and immunolocalization (data not shown).

Confocal Laser Scanning Microscopy

Confocal laser scanning microscopy (CLSM) was performed with a Zeiss Axioskop (Carl Zeiss, Oberkochen, Germany) slit scanning microscope equipped with the Meridan InSIGHT PLUS imaging system (Genomics Solutions Lansing Division, Okemos, MI). Excitation of the BODIPY and CY3 chromophores was performed with a 488-nm laser by using a 505-nm dichroic mirror and a 515-nm barrier filter. Samples were viewed with a 63× oil immersion objective, and BODIPY and CY3 fluorescence were detected with the 515- to 545-nm and 580- to 600-nm filters, respectively. Images were collected with a cooled DNC 1000 intensified CCD camera (Genomics Solutions Lansing Division) and viewed with the InSIGHT imaging system. For double immunofluorescence studies, we collected fluorescence from BODIPY and CY3 simultaneously and then separated them with the InSIGHT RGB program. There was no fluorescence crossover detected with single-labeled controls (data not shown). Figures were composed with Photoshop 5.0 (Adobe Systems, Mountain View, CA). Optical sections for these studies were 1 μm thick.

Preparation of the Tobacco Protoplast Extract

In cell fractionation studies using differential centrifugation to isolate cytoskeleton fractions (Marc et al., 1996), we found that a portion of importin α cofractionated with the 27,000*g* pellets enriched with tubulin and actin (H.M.S. Smith and N.V. Raikhel, unpublished data). In addition, importin α was also detected in the soluble fraction, indicating that a portion of NLS receptor is soluble in the tobacco protoplasts. A tobacco protoplast extract containing importin α was prepared to examine whether importin α could associate with microtubules and microfilaments in vitro. This extract contained proteins solubilized from the 27,000*g* pellet mixed with the soluble fraction isolated from tobacco protoplasts.

Briefly, tobacco protoplast extract was prepared by isolating 27,000*g* pellets and extracting it with cold PM buffer (50 mM Pipes, pH 6.8, 1 mM $MgSO_4$, 1 mM EGTA) plus protease inhibitors containing 0.5 M NaCl and 5 mM ATP/GTP for 20 min. For microtubule association assays, we pelleted the extracts at 100,000*g* for 2 hr at 4°C and then dialyzed them in cold PM buffer plus 1 mM DTT to remove the NaCl and nucleotides. The desalted extract was concentrated with the Ultrafree-4 Centrifugal Filter Unit used to concentrate protein samples (Millipore, Bedford, MA) and then mixed 50/50 with the 100,000*g* soluble fraction isolated from lysed protoplasts.

For microfilament association assays, a similar procedure was used to isolate a plant cell extract except for the following: (1) all centrifugation steps were performed at 150,000*g*, and (2) all fractions were dialyzed against cold actin stabilization buffer (ASB; 5 mM Tris-HCl, pH 8.0, 2 mM $MgCl_2$, and 50 mM KCl) to remove NaCl and nucleotides. The protein concentration of these plant cell extracts was 3 to 5 mg/mL. Aliquots were frozen at −80°C and used in the cytoskeleton pelleting assays.

Microtubule Association Assay

For microtubule association assays, 100 μM bovine brain tubulin (Cytoskeleton, Denver, CO) was polymerized in PM buffer plus protease inhibitors, 100 μM taxol, and 1 mM GTP at 4°C for 30 min, followed by incubation at 23°C for 30 min and transfer to 37°C for 30 min. Polymerized microtubules were visualized by immunofluorescence (data not shown).

To determine whether importin α associates with microtubules, we diluted polymerized tubulin to 10 to 20 μM in 50 to 100 μL of the plant cell extract at 23°C. The functional NLS peptide O2WT or the mutated NLS O2MUT was added to a final concentration of 200 μM to the association assays. After the 30-min incubation period, samples were loaded onto a 40% sucrose in PM buffer containing 10 μM taxol and pelleted at 100,000*g* for 1 hr at 23°C. Microtubule pellets were resuspended in 0.5 M NaCl in PM buffer plus protease inhibitors for 20 min and then pelleted at 12,000*g* for 10 min to remove any insoluble material. The association of importin α with the microtubule pellets was detected by immunoblot analysis as described by Hicks et al. (1996). Control experiments were performed without added microtubules to determine whether importin α could pellet through the 40% sucrose cushion under the same conditions.

Microfilament Association Assay

The actin binding protein biochemical kit BK001 (Cytoskeleton) was used to determine whether importin α could associate with microfilaments. Briefly, a stock of 1 mg/mL actin was polymerized in the presence of 2 mM $MgCl_2$, 50 mM KCl, and 1 mM ATP at 23°C for 1 hr. The final concentration of F-actin was 23 μM. F-actin was diluted in the tobacco protoplast extract to 15 μM for 30 min at 23°C. The functional NLS peptide O2WT or the mutated NLS peptide O2MUT was added to a final concentration of 200 μM in the microfilament association assays. After incubation, samples were loaded onto 10% glycerol cushions in ASB and pelleted at 150,000*g* for 1.5 hr at 4°C. F-actin pellets were resuspended in 0.5 M NaCl in ASB plus protease inhibitors for 20 min and then pelleted at 12,000*g* for 10 min to remove any insoluble material. The association of importin α with the microfilament pellets was detected by immunoblot analysis as described by Hicks et al. (1996). Control experiments were performed without added microfilaments to determine whether importin α could pellet through the 10% glycerol cushion under the same conditions.

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