Plant Physiol. (1997) 114: 411–417

Importin α from *Arabidopsis thaliana* Is a Nuclear Import Receptor That Recognizes Three Classes of Import Signals[1]

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Protein import into the nucleus is a two-step process. In vitro import systems from vertebrate cell extracts have shown that several soluble factors are required. One of these factors is the receptor importin α, which binds to nuclear localization signals (NLS) in vitro. We previously cloned an importin α homolog from *Arabidopsis thaliana* (*At-IMPα*) and demonstrated that this protein was not depleted from tobacco (*Nicotiana tabacum*) protoplasts after permeabilization of the plasma membrane (Hicks et al., 1996). To determine if At-IMPα is functional, we used an in vitro NLS-binding assay. We found that At-IMPα binding is specific, and the receptor is able to recognize three classes of NLS identified in plants. Purified antibodies to At-IMPα were used to determine the in vivo location of importin α in tobacco protoplasts. Importin α is found in the cytoplasm and nucleus, and it is most highly concentrated at the nuclear envelope. The biochemical properties of nuclear importin α and localization studies using purified nuclei demonstrate that importin α is tightly associated with the plant nucleus. Moreover, these results suggest that a fraction of nuclear importin α interacts with the nuclear pore complex.

Protein import into the nucleus occurs through the NPC, which is a 124-MD proteinaceous complex embedded in the nuclear envelope that acts as a gateway for protein traffic in and out of the nucleus (for review, see Davis, 1995). This process is receptor-mediated and dependent upon targeting signals called NLS, which are found in most nuclear proteins (Dingwall and Laskey, 1991). Most NLS can be grouped into one of three classes (for reviews, see Boulikas, 1993, 1994; Hicks and Raikhel, 1995b). The SV40 large T-antigen-like and bipartite classes of NLS have been identified in animals, fungi, and plants, whereas the Mat α2-like NLS have been identified in fungi and plants. The NLS from the yeast protein Mat α2 functions in plants (Hicks et al., 1995) but not in mammals in vivo (Chelsky et al., 1989; Lanford et al., 1984).

Experimentally, protein import into the nucleus is divided into two distinct steps, docking and translocation. Docking is NLS-dependent and occurs when nuclear proteins dock at the cytoplasmic side of the NPC in an energy-independent fashion. Translocation through the NPC is an energy-dependent process (Newmeyer et al., 1986; Richardson et al., 1988). In vitro import systems using permeabilized vertebrate cells suggested that some of the factors that are necessary for import are soluble (Adam et al., 1990; Moore and Blobel, 1993). Subsequently, four soluble factors were identified that can mediate import in vitro (for reviews, see Hicks and Raikhel 1995b; Gorlich and Mattaj, 1996). The factors in vertebrates are known as importin α (Adam and Gerace, 1991; Gorlich et al., 1994, 1995a, 1995b; Enenkel et al., 1995; Imamoto et al., 1995a, 1995b; Moroianu et al., 1995; Radu et al., 1995a; Weis et al., 1995), importin β (Adam and Adam, 1994; Chi et al., 1995: Gorlich et al., 1995a; Imamoto et al., 1995a; Iovine et al., 1995; Radu et al., 1995b), Ran/TC4 (Melchior et al., 1993; Moore and Blobel, 1993), and p10 (Moore and Blobel, 1994; Paschal and Gerace, 1995). Mutations in some homologous import factors in yeast also block import in vivo (Loeb et al., 1995; Schlenstedt et al., 1995).

Nuclear import occurs when a heterodimer of importins α and β binds to an NLS-containing protein in the cytoplasm via the NLS-binding region of importin α (Adam 1995; Azuma et al., 1995; Gorlich et al., 1995a; Imamato et al., 1995a; Moroianu et al., 1995; Radu et al., 1995a, 1995b; Weis et al., 1995). Importin β mediates the docking of the trimeric complex to the cytoplasmic side of the NPC (Gorlich et al., 1995a; Imamato et al., 1995a; Moroianu et al., 1995; Radu et al., 1995a, 1995b; Pante and Aebi, 1996). Translocation of the trimeric complex through the NPC requires free GTP (Gorlich et al., 1996c), p10 (Moore and Blobel, 1994; Paschal and Gerace, 1995), and a small GTPase, Ran (Melchior et al., 1993; Moore and Blobel, 1993; Gorlich et al., 1996c). In vitro studies in vertebrates and yeast suggest that importin β, p10, and Ran interact with each other, as well as with a subset of NPC proteins, during the translocation process (Iovine et al., 1995; Paschal and Gerace, 1995; Gorlich et al., 1996c; Nehrbass and Blobel, 1996; Rexach and Blobel, 1996). After translocation, dissociation of importin α with the NLS-containing protein may lead to the export of importin α into the cytoplasm, where it can participate in another cycle of import (Gorlich et al., 1996a; Weis et al., 1996). Importin α may play a role in the export of spliceosomal small nuclear RNA (Gorlich et al., 1996b). In addition to Ran, another GTPase that is not

[1] This work was supported by grants from the Department of Energy (no. DE-FG02-90ER20021).

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Abbreviations: At-IMPα, *Arabidopsis thaliana* importin α; HSA, human serum albumin; NBP, NLS-binding protein(s); NLS, nuclear localization signal(s); NPC, nuclear pore complex; Srp1, suppressor of RNA polymerase I.

depleted from permeabilized cells is implicated in import (Sweet and Gerace, 1996). Hsp 70 is involved in the import of some proteins (Imamoto et al., 1992; Shi and Thomas, 1992; Shulga et al., 1996); however, its role may be to expose the NLS to the import machinery. The protein import process can also be regulated by phosphorylation (Mishra and Parnaik, 1995; Jans and Huebner, 1996; Vandromme et al., 1996).

An NLS-binding site at the NPC and nuclear envelope has been well characterized in plants (Hicks and Raikhel, 1993). Three classes of NLS specifically and reversibly bind to and compete for this low-affinity site (Hicks and Raikhel, 1993; Hicks et al., 1995). To identify the NLS-binding site biochemically, a cross-linking approach was used and at least four NBP were identified using the bipartite NLS from Opaque-2 (Hicks and Raikhel, 1995a). The binding affinity and biochemical properties of the NBP correlate closely with the NLS-binding site. This and other evidence (Hicks and Raikhel, 1993, 1995a) indicate that at least one component of NLS recognition is located at the NPC and nuclear envelope in plants.

Recently, an in vitro import system using permeabilized, evacuolated tobacco protoplasts was characterized (Hicks et al., 1996). Unlike vertebrate import systems, in vitro import in plants occurs in the absence of an added exogenous cytosolic fraction. To investigate whether plant import factors are retained in this system, *At-IMPα* was cloned from *Arabidopsis thaliana* (Hicks et al., 1996). At-IMPα is 45 to 56% identical to other importin α homologs found in vertebrates, fungi, and insects. Monospecific antibodies to the recombinant At-IMPα recognize specific proteins of the predicted mass in both Arabidopsis plants and tobacco protoplasts and also recognize human importin α (Hicks et al., 1996). Immunofluorescence studies in evacuolated tobacco protoplasts using At-IMPα monospecific antibodies demonstrated that importin α is not depleted from these protoplasts, but is found in the cytoplasm and nucleus after permeabilization (Hicks et al., 1996). We now provide evidence that At-IMPα is an NLS receptor in plants.

MATERIALS AND METHODS

Reagents

All chemicals were obtained from Sigma unless otherwise noted. All NLS peptides were synthesized at the Peptide Synthesis Facility (Yale University, New Haven, CT). Recombinant His-tagged At-IMPα was overexpressed in bacteria and purified by Ni^+-affinity chromatography, as described by Hicks et al. (1996). Antibodies to At-IMPα were purified by At-IMPα affinity chromatography, as described by Hicks et al. (1996).

In Vitro Binding/Co-Immunoprecipitation

For in vitro transcription and translation, full-length *At-IMPα* was cloned into Bluescript (SK-, Strategene) with *Eco*RI (Boehringer Mannheim) and *Bam*HI (Boehringer Mannheim). The vector was linearized at the 3′ end of the gene with *Bam*HI and transcribed with T7 RNA polymerase (Promega). Two micrograms of mRNA was incubated in wheat germ extract (minus Met; Promega) in the presence of 50 μCi [^{35}S]Met (New England Nuclear). A translation mixture was diluted in a binding buffer (50 mM Hepes, pH 7.8, 25 mM KCl, 2.5 mM $MgCl_2$, 3 mM $CaCl_2$, and 20% glycerol) and 250 μg of purified At-IMPα antibodies was added and mixed for 1 to 2 h at 4°C. Next, protein A Sepharose (Pharmacia) was added to a final concentration of 0.05%. Precipitated proteins were washed four times in the binding buffer to remove any contaminants and suspended in an SDS sample buffer (50 mM Tris, pH 6.8, 100 mM DTT, 2.0% SDS, and 20% glycerol). Specificity of immunoprecipitation was checked by adding 500 ng of His-tagged At-IMPα to the immunoprecipitation reaction. Samples were separated by 10% SDS-PAGE and the gels were developed for 24 h by autoradiography using standard methods (Sambrook et al., 1989).

For NLS-binding substrates, 4 mg of HSA was dissolved in PBS. The chemical cross-linker maleimidobenzoyl *N*-hydroxysuccinimide ester (Pierce) was added to a final concentration of 10 mM and incubated for 30 min at room temperature. The unbound cross-linker was removed by gel filtration with a 2-mL G-25 column. NLS peptide was added to a final concentration of 1.2 μM to the flow-through and incubated for 3 h at room temperature. Next, free peptide was removed by gel filtration through a 2-mL G-25 column and substrates were washed extensively in PBS. NLS substrates were concentrated in a microfiltration device (Centricon-10, Amicon, Beverly, MA) and were aliquoted and stored at −80°C. For coimmunoprecipitation, 2 μg of the NLS-HSA substrate was mixed with 500 ng of recombinant At-IMPα in the binding buffer for 2 to 4 h at 4°C. Then, 400 ng of purified At-IMPα antibodies was added and incubated for 1 to 2 h at 4°C. Protein A Sepharose was added to 0.05% and mixed at 4°C for 1 h. Samples were precipitated and washed four times in the binding buffer to remove unbound substrates, and were then suspended in SDS sample buffer. For competition experiments, NLS binding was performed as described above, except that the binding buffer contained either 1 mM 02WT or 1 mM 02mut peptides (see "Results"). Separation of proteins by 10% SDS-PAGE and blotting was by standard methods (Sambrook et al., 1989). Blots were rinsed with TBST (TBS and 0.05% Tween 20) and then incubated overnight at room temperature with monoclonal antibodies raised against HSA (Sigma) at a 1:2000 dilution in TBST. Blots were developed by using a 1:5000 dilution of goat anti-mouse alkaline phosphatase-conjugated IgG (Kirkegaard and Perry Laboratories, Gaithersburg, MD).

Immunolocalization

Tobacco suspension-cultured cells were maintained and protoplasts were prepared as described by Hicks and Raikhel (1993). Protoplasts or purified nuclei were spun (Cytospin 3, Shandon Lipshaw, Pittsburg, PA) onto poly-Lys-coated slides and immediately fixed in a fixing buffer (3% paraformaldehyde in 50 mM potassium phosphate, pH 7.2) for 30 min at room temperature. Fixed cells were dried

at room temperature and stored at 4°C for 24 h. Cells were then dehydrated in cold methanol for 10 min and washed in PBST (PBS and 0.5% Tween 20). Affinity-purified At-IMPα antibodies (100 ng/μL) were diluted 1:300 in PBST containing 60 μg of HSA and incubated on the cells at room temperature for 1 h in a moist chamber. After washing the cells in PBST, CY3-labeled goat anti-rabbit antibody (Molecular Probes, Inc., Eugene, OR) was diluted 1:50 in PBST and incubated on the cells for 1 h in a moist, dark chamber. After washing in PBST, cells were mounted in MOWIOL (Calbiochem) and optically sectioned (0.5–1.0-μm sections) using confocal laser scanning microscopy (model 10, Zeiss) equipped with a 514-nm argon laser. Micrographs were produced with Kodacolor Gold 100 film (Kodak).

Extraction of Importin α from Purified Nuclei

Nuclei were prepared as described by Hicks and Raikhel (1993). One million nuclei were diluted to 50 μL in the binding buffer with 400 units of DNaseI (Boehringer Mannheim) at room temperature for 20 min. Nuclei were centrifuged at 12,000*g* for 2 min and suspended in 25 μL of cold binding buffer containing either 1.0% Triton X-100, 0.25 M NaCl, 1.0% Triton X-100 and 0.25 M NaCl, or 6 M urea. The samples were incubated for 15 min at 4°C, then centrifuged at 12,000*g* for 2 min. Ten microliters of SDS sample buffer was added to the supernatant, and the pellet was resuspended in 25 μL of SDS sample buffer. Each sample was incubated at 65°C for 5 min before being separated by 10% SDS-PAGE and blotted to nitrocellulose by standard protocols (Sambrock et al., 1989). Purified At-IMPα antibodies were diluted 1:2000 in TBST and blots were developed by using a 1:5000 dilution of goat anti-rabbit alkaline phosphatase-conjugated IgG (Kirkegaard and Perry Laboratories).

RESULTS

At-IMPα Binds to Three Classes of NLS

To address the function of At-IMPα, we used an in vitro-binding/co-immunoprecipitation approach (Weis et al., 1995). Affinity-purified antibodies to At-IMPα were characterized to determine if in vitro translated [^{35}S]Met At-IMPα could be specifically immunoprecipitated. To demonstrate that At-IMPα was translated, a sample of the translation mixture was separated by SDS-PAGE and analyzed by autoradiography (Fig. 1, lane 1). Next, purified At-IMPα antibodies were added to the translation mixture, followed by protein A Sepharose for immunoprecipitation. When immunoprecipitates were separated by SDS-PAGE and visualized by autoradiography, [^{35}S]Met At-IMPα was apparent (Fig. 1, lane 2). The addition of 500 ng of recombinant At-IMPα competed with the in vitro-translated At-IMPα for immunoprecipitation (Fig. 1, lane 3). Preimmune sera did not immunoprecipitate in vitro-translated At-IMPα (data not shown).

We next investigated whether At-IMPα could bind to NLS in vitro. Representative NLS peptides from each of three classes of NLS were selected. The first NLS synthesized was the bipartite NLS 02WT, identified in the maize

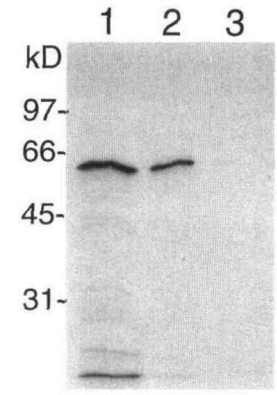

Figure 1. Immunoprecipitation of in vitro-translated At-IMPα. At-IMPα was in vitro translated with [^{35}S]Met (lane 1) and immunoprecipitated with purified At-IMPα antibodies (lane 2). Immunoprecipitation of [^{35}S]Met At-IMPα was also examined in the presence of 500 ng of recombinant At-IMPα (lane 3). The samples were analyzed by autoradiography after SDS-PAGE.

transcription factor Opaque-2 (Fig. 2A; Varagona et al., 1992). The SV40 T-antigen NLS, identified from the simian virus 40 large T-antigen (Kalderon et al., 1984; Lanford and Butel, 1984), was also synthesized (Fig. 2A). Last, a Mat α2-like NLS, NLSC, which corresponds to one of the NLS identified in the maize transcription factor R (Fig. 2A, Shieh et al., 1993), was synthesized. These NLS are functional in vivo (Varagona et al., 1992; Shieh et al., 1993) and bind specifically to the NLS-binding site in tobacco nuclei (Hicks and Raikhel, 1993; Hicks et al., 1995). A fourth peptide, 02mut (Fig. 2A), which corresponds to a mutant form of 02WT that does not function in vivo (Varagona and Raikhel, 1994) or compete with 02WT for binding to the site in tobacco nuclei (Hicks and Raikhel, 1993), was also synthesized.

The peptides corresponding to the functional NLS were chemically coupled to HSA and allowed to interact with recombinant At-IMPα. At-IMPα antibodies were then added to the binding assay, and the protein complexes were immunoprecipitated with protein A Sepharose. The immunoprecipitated proteins were separated by SDS-PAGE; the NLS-HSA substrate that co-immunoprecipitated with At-IMPα was detected by western analysis using monoclonal antibodies against HSA. HSA alone was not co-immunoprecipitated with At-IMPα (Fig. 2B, lane 1). However, co-immunoprecipitation occurred when HSA was coupled with peptides to O2WT (Fig. 2B, lane 2), SV40 T-antigen (Fig. 2B, lane 3), or NLSC (Fig. 2B, lane 4). Figure 2B shows the mass of HSA (Fig. 2B, lane 8), 02WT-HSA (Fig. 2B, lane 7), SV40 T-antigen-HSA (Fig. 2B, lane 6), and NLSC-HSA (Fig. 2B, lane 5) when they are directly blotted to nitrocellulose after separation by SDS-PAGE. Note that NLS-HSA substrates do not migrate as distinct bands because of variations in the number of peptides coupled per HSA molecule.

At-IMPα Binding Is Specific for Functional NLS

To test the specificity of At-IMPα binding for functional NLS, competition studies were set up using the 02WT and

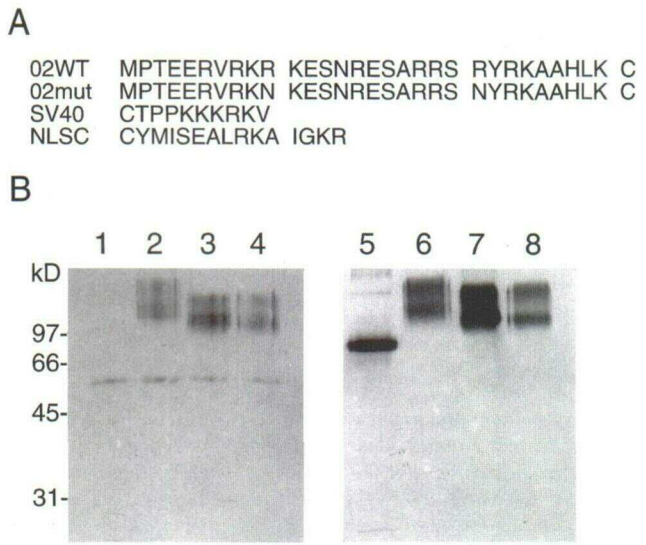

Figure 2. At-IMPα recognizes three classes of NLS. A, Amino acid sequences of peptides corresponding to the wild-type bipartite (02WT) and mutant (02mut) 02 NLS and the SV40 large T-antigen NLS (SV40) and the Mat α2-like NLS (NLSC) from R are shown in a single-letter code. B, HSA does not co-immunoprecipitate with At-IMPα (lane 1). Functional NLS substrates such as 02WT-HSA (lane 2), SV40 T-antigen-HSA (lane 3), or NLSC-HSA (lane 4) co-immunoprecipitate with At-IMPα. The secondary goat anti-mouse antibodies cross-react with the IgG heavy chain (50-kD protein band) of the rabbit importin α antibodies. HSA (lane 5), 02WT-HSA (lane 6), SV40 T-antigen-HSA (lane 7), and NLSC (lane 8) were immunoblotted directly to nitrocellulose to determine the migration pattern of these cross-linked substrates after SDS-PAGE.

02mut peptides (Fig. 2A). The functional substrate 02WT-HSA was co-immunoprecipitated with recombinant At-IMPα (Fig. 3, lane 1). In the prescence of 1 mM 02WT peptide, 02WT-HSA binding was greatly reduced (Fig. 3, lane 2), whereas 1 mM 02mut peptide did not compete with 02WT-HSA for At-IMPα binding (Fig. 3, lane 3). In addition, 02WT-HSA binding to At-IMPα can be competed with 200 μm of 02WT peptide (H.M.S. Smith and N.V. Raikhel, unpublished data). AtIMPα specifically interacts with SV40 and NLSC, as well (H.M.S. Smith and N.V. Raikhel, unpublished data). Specific NLS binding indicates that At-IMPα is likely to be an NLS receptor in plants. Furthermore, this single receptor specifically recognizes the three classes of NLS found in plants.

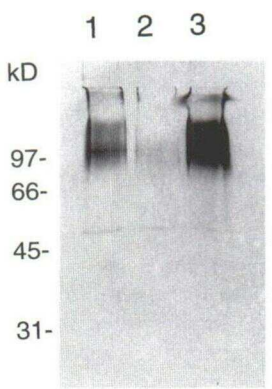

Figure 3. Specific interaction of At-IMPα with a functional NLS substrate; 02WT-HSA is co-immunoprecipitated with At-IMPα (lane 1). Specificity of At-IMPα binding was determined by co-immunoprecipitation of 02WT-HSA in the prescence of 02WT (lane-2) or 02mut (lane 3) peptides. The secondary goat anti-mouse antibodies cross-react with the IgG heavy chain (50-kD protein band) of the rabbit importin α antibodies.

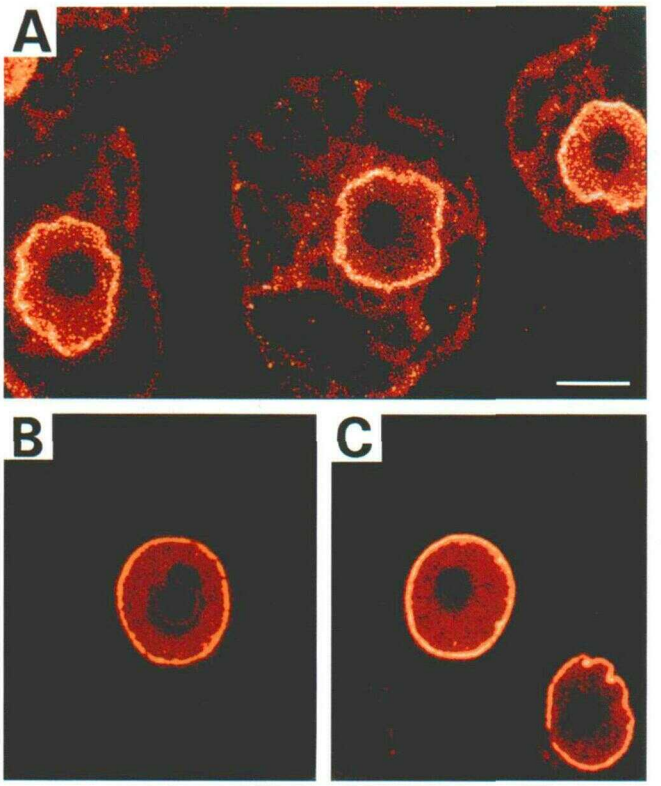

Figure 4. Location of importin α in tobacco protoplasts (A) or purified nuclei (B and C) that were fixed, and importin α was visualized by confocal laser scanning microscopy using affinity-purified At-IMPα antibodies followed by CY3-labeled secondary antibodies. Bar = 10 μm.

Localization of Importin α in Tobacco Protoplasts and Nuclei

To investigate the intracellular location of importin α, tobacco protoplasts were fixed and incubated with affinity-purified antibodies to At-IMPα, followed by CY3-labeled secondary antibodies. Immunofluorescence was detected in optical sections by confocal laser scanning microscopy. CY3 fluorescence from a 0.5-μm optical section through the protoplasts indicated that importin α was located in the nucleus and cytoplasm (Fig. 4A), which is consistent with its role as a nuclear/cytoplasmic shuttle protein in vertebrates (Gorlich et al., 1996a; Weis et al., 1996). This also supports previous cell fractionation studies indicating that importin α is found in cytoplasmic and nuclear fractions from tobacco protoplasts (Hicks et al., 1996). Intense immunofluorescence at the nuclear envelope demonstrated that importin α was highly concentrated at the nuclear

envelope (Fig. 4A, yellow region). A 1.0-μm optical section displays similar fluorescence at the nuclear envelope using tobacco nuclei purified in the presence of 0.6% Triton X-100 (Fig. 4, B and C), indicating that importin α is tightly associated with the nuclear envelope.

Biochemical Properties of Nuclear Importin α

In vertebrates importin α is soluble (Adam and Gerace, 1991), but in yeast it is associated with the NPC (Yano et al., 1992; Belanger et al., 1994; Atchison et al., 1996). Previous studies in plants demonstrated that importin α is strongly associated with cellular structures in permeabilized tobacco cells, even in the presence of 0.1% Triton X-100 (Hicks et al., 1996). To characterize the association of importin α with the nuclear envelope, we examined the biochemical properties of importin α in purified nuclei. Nuclei were purified from tobacco protoplasts and treated on ice for 15 min with 1% Triton X-100, 0.25 M NaCl, 1% Triton X-100 plus 0.25 M NaCl, or 6 M urea. Samples were centrifuged and the nuclear pellet and supernatant were examined by western analysis using At-IMPα antibodies. Most of importin α was resistant to extraction by 1% Triton X-100 (Fig. 5, lanes 1), 0.25 M NaCl (Fig. 5, lanes 2), and 1% Triton X-100 plus 0.25 M NaCl (Fig. 5, lanes 3); however, treatment of nuclei with 6 M urea extracted some of the importin α (Fig. 5, lanes 4). Importin α is also partially extracted with 0.5 M NaCl (H.M.S. Smith and N.V. Raikhel, unpublished data). These biochemical properties indicate that importin α is tightly associated with the plant nucleus. In addition, the biochemical properties of nuclear importin α and NPC proteins (Heese-Peck et al., 1995) correlate closely, indicating that a fraction of nuclear importin α is probably associated with the NPC.

DISCUSSION

Importin α is a cytosolic NLS receptor identified in vertebrates that mediates import in permeabilized cells (Adam and Gerace, 1991; Gorlich et al., 1994; Imamato et al., 1995b; Radu et al., 1995a). The function of importin α has been demonstrated in several ways: (a) it facilitates NLS binding to the NPC in permeabilized cell systems (Adam and Adam, 1994; Chi et al., 1995; Gorlich et al., 1995a, 1995b; Imamato et al., 1995b; Moroianu et al., 1995; Radu et al., 1995a, 1995b); (b) it specifically binds to functional NLS in the yeast two-hybrid system and in NLS-binding assays in vitro (Cortes et al., 1994; Cuomo et al., 1994; Adam 1995; Azuma et al., 1995; Gorlich et al., 1995a; Moroianu et al., 1995; Weis et al., 1995); and (c) mutations in the yeast importin α, SRP1, block protein import in vivo (Loeb et al., 1995). Our study was aimed at determining whether At-IMPα functions as an NLS receptor in plants.

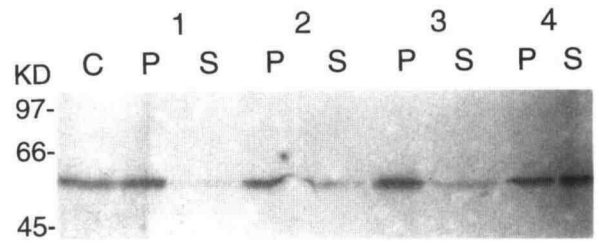

Figure 5. Biochemical properties of nuclear importin α. Tobacco nuclei were treated with 1% Triton X-100 (lanes 1), 0.25 M NaCl (lanes 2), 1% Triton X-100 plus 0.25 M NaCl (lanes 3), or 6 M urea (lanes 4). After treatment the samples were centrifuged and the supernatant (S) and nuclear pellet (P) were extracted with 2% SDS sample buffer (C). Untreated nuclei were extracted with 2% SDS sample buffer. The samples were separated by 10% SDS-PAGE, and importin α was detected with affinity-purified At-IMPα antibodies.

To determine the function of importin α we used an in vitro-binding/co-immunoprecipitation assay. We demonstrated that At-IMPα binds specifically to NLS. Moreover, it is capable of recognizing three classes of NLS, suggesting that a single receptor recognizes the different classes of NLS. Although human importin α binds specifically to the SV40 T-antigen and bipartite NLS, and yeast SRP1 has been shown to bind to the SV40 T-antigen NLS, it has not been determined if yeast SRP1 can bind to multiple classes of NLS. In vertebrates the NLS-binding affinity of importin α is enhanced when it is associated with importin β (Gorlich et al., 1995a; Rexach and Blobel, 1996). Therefore, since we have not cloned and characterized an importin β homolog from plants, we did not address the binding affinity of At-IMPα. Since importin α is not depleted from permeabilized plant cells, its addition does not stimulate import (H.M.S. Smith, G.R. Hicks, and N.V. Raikhel, unpublished data).

Importin α is encoded by a multigene family in Arabidopsis (H.M.S. Smith and N.V. Raikhel, unpublished data), *Xenopus* (Gorlich et al., 1994), humans (Morianou et al., 1995), and *Drosophila* (Gorlich and Mattaj, 1996), but a single gene is found in yeast (Yano et al., 1992). Why is there a multigene family to encode importin α? It seems unlikely that different isoforms of importin α recognize distinct classes of NLS. Neither our results nor results from human importin α-binding studies support this type of model. In addition, the two isoforms of human importin α can import SV40 T-antigen NLS substrates in permeabilized mammalian cells (Moroianu et al., 1995), and these two isoforms are expressed in the same tissues (Gorlich and Mattaj, 1996). However, the different isoforms of importin α are not fully redundant. In *Drosophila* a mutation in one of the importin α homologs, oho-31, causes larvae to develop malignant tumors that lead to death (Kussel and Frasch, 1995; Torok et al., 1995). This indicates that different isoforms of importin α are important not only in nuclear targeting, but also have roles that remain to be determined.

Our localization studies of importin α in tobacco cells demonstrate that it is found in the nucleus and cytoplasm, which is consistent with the idea that importin α is a shuttle protein in plants as well as vertebrates (Gorlich et al., 1996a; Weis et al., 1996). Immunofluorescence studies in permeabilized tobacco protoplasts demonstrate that nucleoplasmic and cytoplasmic importin α is not readily extracted from the protoplasts (Hicks et al., 1996). We also found that importin α is highly concentrated at the nuclear envelope in vivo and tightly associated with purified nu-

clei, suggesting that at least a fraction of nuclear importin α is a component of the NPC. In yeast importin α is localized to the NPC (Yano et al., 1992), cofractionates with NPC proteins (Atchison et al., 1996), and directly interacts with the two NPC proteins Nup1 and Nup2 (Belanger et al., 1994). However, in vertebrates importin α is soluble (Adam and Gerace, 1991). In fact, during permeabilization of mammalian cells importin α is depleted (Gorlich et al., 1995b) and cannot associate with the NPC unless it forms a heterodimer with importin β. The differences in importin α localization between plants, fungi, and animals suggest that there may be unique or adaptative nuclear import features in the different kingdoms.

Although we were unsuccessful in immunoprecipitating the previously identified 50 to 60 cross-linked NBP (Hicks and Raikhel, 1995a) using purified At-IMPα antibodies (H.M.S. Smith and N.V. Raikhel, unpublished data), several pieces of evidence suggest that importin α is a component of the NLS-binding site: (a) both are located at the nuclear envelope and NPC in purified nuclei from tobacco; (b) the biochemical properties of nuclear importin α are similar to the ones of the NLS-binding site and NBP; (c) the NLS-binding site and At-IMPα interact specifically with NLS; and (d) both recognize three classes of NLS. The localization and biochemical properties combined suggest that NLS recognition can occur directly at the NPC in plants (Hicks and Raikhel, 1993), and this recognition probably occurs via importin α. Our localization and analysis of At-IMPα function in vitro are essential steps toward functional studies in vivo. These combined approaches should identify the role(s) of importin α in plant development.

ACKNOWLEDGMENTS

The authors are grateful to Kurt Stepnitz for photography and to Marlene Cameron for graphics. We also thank other members of the laboratory for their thoughtful discussions related to this work.

Received February 28, 1997; accepted March 18, 1997.

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