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By

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ABSTRACT

FUNCTIONAL ANALYSIS AND INTRACELLULAR TARGETING OF THE NUCLEAR LOCALIZATION SIGNAL RECEPTOR, IMPORTIN α, IN PLANTS

BY

Harley M. S. Smith

Macromolecular translocation into and out of the nucleus occurs through the nuclear pore complex (NPC) which is embedded in the nuclear envelope. A subset of proteins that are imported into the nucleus contain the classical nuclear localization signals (NLSs). The importin α/β heterodimer is required for NLS-protein import in vertebrates and yeast. Importin α is the NLS receptor that binds to the NLS-containing proteins in the cytoplasm and importin β interacts with the import machinery. To characterize the NLS-protein import pathway in plants, an importin α homologue (*At-IMPα*) was cloned from *Arabidopsis thaliana*. Antibodies generated against the recombinant expressed At-IMPα recognized a protein of the correct mass in *Arabidopsis* roots, stems, leaves and flowers as well as in tobacco suspension cells. In vitro binding studies demonstrated that At-IMPα specifically associated with three types of NLSs that function in plants. Immunolocalization of importin α in tobacco protoplasts demonstrated that this receptor was found in the cytoplasm and nucleus which is constant with its function as a nuclear shuttling NLS receptor. In contrast to yeast and vertebrate importin α subunits, At-IMPα mediated nuclear

import in the absence of an importin β subunit in a vertebrate import system, suggesting that plants may possess nuclear import pathways exclusively mediated by importin α subunits. These studies combined strongly suggest that At-IMPα is a functional NLS receptor in plants.

Prior to NLS-protein import, importin α/NLS-containing proteins complexes need to be somehow directed and transported to the NPC. Using antibodies to At-IMPα as a tool, we were interested in determining if the cytoskeleton could function in this transport process. 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. Subcellular localization studies indicated that a fraction of the cellular importin α cofractionated with the cytoskeleton and is extracted from this fraction under conditions similar to those that extract microtubule motor proteins. Lastly, 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.

FERTILE FIELDS
Sometimes those simple things won't turn the trick no more
And our self-important dreams lie shattered on the floor
Even the proletariat receives his royalty
And as the battle rages on and on I wish it wasn't me
And it seem so cruel
The last one breaking up
Until the winter finds its worth
As we glide upon the earth
Now the trees are swept aside by wind and sheets of rain
And the fertile fields once gilded have now withered and refrained
She (mother earth) who longs for comfort feels instead a savage thrust
And the ashen sky grows ever darker as dawn gives way to dust
As we set our dogs upon the earth
Feast on the dead until no life remains
Forward towards a pointless end we squander never gain

GOOD RIDDANCE

When I Get Old
What will it be like when I get old
Will I hop on my bike and ride around town
will I still want to be someone and not just sit around
I don't want to be like other adults cause they've already died
Cool condescending, fossilized
Will I be rich will I be poor will I still sleep on the floor
What will it be like when I get old
Will I still kiss my girlfriend and try to grab her ass
Will I still hate the cops and have no class
Will all my grown up friends say they've seen it all before
They say hey act your age and I'm immature
Will I do myself proud or only what's allowed
What will it be like when I get old
Will I sit around and talk about the old days
sit around and watch T.V.
I never want to go that way
Never burn out not fade away
As I travel through time will I like what I find
What will it be like when I get old

DESCENDENTS

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Chapter 1

Introduction

Nuclear Localization Signal Protein Import Pathway

1. The Nucleus

In eukaryotic cells, nuclear activities are compartmentalized from other biochemical processes by the double-membrane nuclear envelope. Macromolecular translocation into and out of the nucleus occurs through the nuclear pore complex (NPC), which is a 124 megaDalton proteinaceous complex that spans the nuclear envelope (for reviews see Davis, 1995; Pante and Aebi, 1996a). The NPC is a cylindrical structure with eight-fold symmetry that contains a cytoplasmic and nucleoplasmic ring. Both of the rings are 120 nm in diameter. These rings are connected to each other by 8 spoke-like structures that converge on a central gate where the pore is located. The pore has a diameter of 26-28 nm and contains a central plug, referred to as the transporter. It is hypothesized that the transporter is involved in the import and export processes (for reviews see Davis, 1995; Pante and Aebi, 1996a). Eight thin projections extend from the cytoplasmic ring, called "cytoplasmic fibrils," and they are distinct from the cytoskeleton. Connected to the nucleoplasmic ring is a structure called the nuclear basket that extends into the nucleoplasm. The distance from the cytoplasmic fibrils to the nuclear basket is ≈75 nm. At least 25 NPC proteins, called nucleoporins, have been identified in yeast and mammals (for reviews see Davis, 1995; Pante and Aebi, 1996). Small molecules, ions and metabolites can passively move through the 9 nm aqueous channels found in NPC, while

proteins and macromolecules larger than 40 kiloDaltons require targeting information for import into and export out of the nucleus (for reviews see Davis, 1995; Pante and Aebi, 1996a).

2. Nuclear Targeting Signals

Nuclear localization signals (NLSs) are targeting signals found in many nuclear proteins that required for import into the nucleus (Dingwall and Laskey, 1991). These signals can be grouped into one of three classes (Table 1.1; for reviews see Boulikas, 1993, 1994; Hicks and Raikhel, 1995b). The SV40 large T-antigen-like and bipartite classes have been identified in animals, fungi and plants, whereas the Mat α2-like NLSs have been found only in fungi and plants (Hicks and Raikhel, 1995b). Interestingly, the NLS from the yeast protein Mat α2 does not function in mammalian cells in vivo (Chelsky et al., 1989; Lanford et al., 1990). In each of these classes, there is no consensus sequence for these signals, however regions of basic amino acids are a common feature (Table 1.1; Hicks and Raikhel, 1995b).

Another group of targeting signals has been identified in mRNA binding proteins that shuttle into and out of the nucleus. The M9 signal, a 38 amino acid sequence found in the hnRNPA1 protein (Michael et al., 1995; Siomi and Dreyfuss, 1995; Weighardt et al., 1995), and the K signal, a 24 amino acid sequence found in hnRNPK (Michael, et al., 1997), function as nuclear shuttling signals and are distinct

Table 1.1
Three Classes of NLSs Indentified in Plants

Class	NLS Sequence	Example
1. Bipartite	RKRKESNRESARRSRYRK	02 (NLS B)
2. SV40-like	MSERKRREKL	R (NLS M)
3. Mat α2-like	MISEALRKAIGKR	R (NLS C)

The 02 NLS (NLSB) was identified in a maize transcription factor Opaque-2 (Varagona et al., 1992). The NLSs M and C were identified a maize regulatory protein called R (Shieh et al., 1993).

from NLSs (Michael et al., 1995; Siomi and Dreyfuss, 1995; Weighardt et al., 1995; Michael, et al., 1997). In addition, nuclear export signals (NES) have also been characterized in several proteins (for review see Gerace, 1995).

3. The NLS-Protein Import Pathway

Experimentally, NLS-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 and Forbes, 1988; Richardson et al., 1988). In vitro import systems using permeabilized vertebrate cells suggested that some of the factors necessary for import are soluble, since they are depleted from these cells during permeabilization (Adam et al., 1990; Moore and Blobel, 1993). Subsequently, three 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, Enenkel et al., 1995, Gorlich et al., 1994, 1995a, b; Imamoto et al., 1995a,b; 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) and Ran/TC4 (Melchior et al., 1993; Moore and Blobel, 1993). Mutations in homologous import

factors in yeast block import in vivo (Loeb et al., 1995; Schenstedt et al., 1995).

The following outline summarizes the roles of the NLS-import factors in the nuclear import process (shown schematically in Figure 1.1). Nuclear import occurs when a heterodimer of importins α and β bind 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,b; Weis et al., 1995). In yeast and vertebrates, importin α requires importin β for high affinity interaction with NLSs (Gorlich et al., 1995a; Rexach and Blobel, 1996; Efthymiadis et al., 1997; Hubner et al., 1997). After importin α binds to the NLS-containing protein, 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; Pante and Aebi, 1996; Radu et al., 1995a,b).

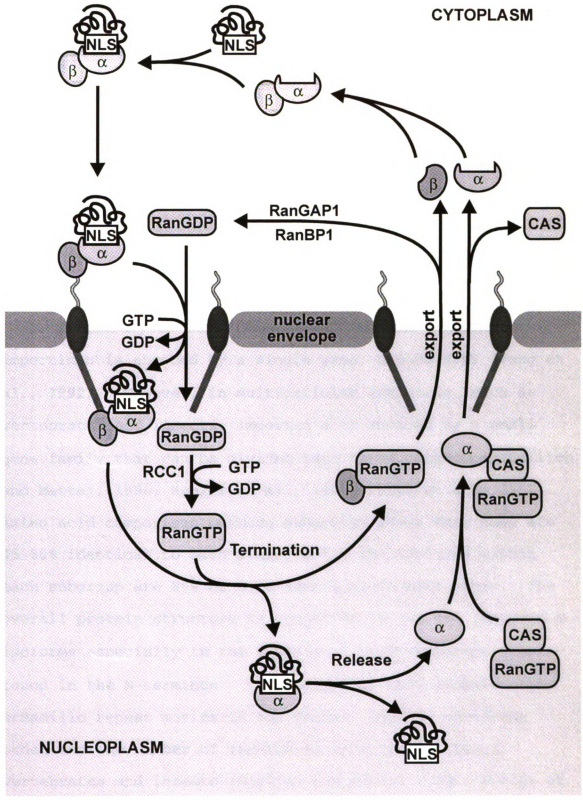
Translocation of the trimeric complex through the NPC requires free GTP (Gorlich et al., 1996c) and a small GTPase, Ran, (Melchior et al., 1993; Moore and Blobel, 1993; Gorlich et al., 1996c), in the GDP bound form (Gorlich et al., 1996c; Weis et al., 1996). Recently, it was shown that only the GDP bound form of Ran can bind to the NPC (Gorlich et al., 1996c), but the energy necessary for the translocation step requires GTP hydrolysis by Ran (Melchior et al., 1993; Moore and Blobel, 1993; Richards et al., 1997). Therefore, these

results suggest that RanGDP must targeted to the NPC and converted to RanGTP by a nucleotide exchange factor during the import process. The identity of the RanGDP receptor and the nucleotide exchange factor at the NPC have yet to be determined.

After translocation, the trimeric complex docks at the nuclear basket of the NPC where the termination step occurs (Kutay et al., 1997a). In vitro binding studies indicate that the binding of RanGTP to importin β terminates import by releasing the importin α/NLS-protein complex into the nucleoplasm (Rexach and Blobel, 1995; Chi et al., 1996; Gorlich et al., 1996a). Subsequently, importin α somehow dissociates from the NLS-containing protein and the importin α and β subunits are exported to the cytoplasm, where they can participate in another cycle of import (Gorlich et al., 1996a; Weis et al., 1996).

Importin β is probably exported to the cytoplasm in a complex with RanGTP (Bischoff and Gorlich, 1997; Floer et al., 1997). This complex is dissociated in the cytoplasm through the action of importin α and a set of RanGTP-activation proteins that are exclusively found in this compartment (Hopper et al., 1990; Melchior et al., 1993b; Bischoff et al., 1994; Coutavas et al., 1993; Beddow et al., 1995; Bischoff et al., 1995; Richards et al, 1996). Export of importin α is facilitated by a heterodimer consisting of an importin β homologue, called Cas, and RanGTP (Bischoff and Gorlich, 1997; Gorlich, 1997; Kutay et al., 1997b). Cas

Figure 1.1
Schematic diagram of the NLS-import pathway


CYTOPLASM
NLS
α
β
RanGDP
RanGAP1
RanBP1
CAS
GTP
GDP
nuclear
envelope
export
export
RCC1
RanGTP
Termination
Release
NUCLEOPLASM

binds to importin α in a RanGTP manner creating a trimeric complex that is exported to the cytoplasm (Kutay et al., 1997b) Complex dissociation occurs in the cytoplasm by the RanGTP activating proteins that are located in this compartment (Bischoff and Gorlich, 1997; Gorlich, 1997; Kutay et al., 1997b). Cas has a low binding affinity for importin α in the absence of RanGTP which allows the formation of the importin α/β heterodimer in the cytoplasm (Kutay et al., 1997b).

4. The Importin α Family

In *Saccharomyces cerevisiae*, a unicellular organism, importin α is encoded by a single gene, called Srp1 (Yano et al., 1992). However, in multicellular organisms, such as vertebrates and insects, importin α is encoded by a small gene family that can be divided into three subgroups (Gorlich and Mattaj, 1996; Kohler et al., 1997; Tsuji et al., 1997). Amino acid comparison between subgroups shows that they are 45-50% identical to each other, while the isoforms within each subgroup are 80% or more identical to each other. The overall protein structure is conserved in all the importin α isoforms especially in the importin β binding domain (IBB) found in the N-terminus. These isoforms also contain eight armadillo repeat motifs in the central domain, where the order and the number of repeats is conserved in fungi, vertebrates and insects (Gorlich and Mattaj, 1996; Kohler et al., 1997; Malik et al., 1997; Tsuji et al., 1997; Ryder et

al., 1998). Interestingly, these armadillo repeats are found in many cytoskeleton associated proteins (Barth et al., 1997). The C-terminal domain and a small region between the IBB domain and the first armadillo repeat are highly divergent between the different subgroups (Gorlich and Mattaj, 1996; Kohler et al., 1997; Malik et al., 1997; Tsuji et al., 1997; Ryder et al., 1998). These observations suggest that divergent regions allow the different isoforms to perform unique roles in nuclear import.

The presence of multiple importin α genes in higher eukaryotes suggest that these genes may recognize specific types of NLSs and/or be involved in nuclear import in certain tissues. Multiple isoforms of importin α also suggests that they are functionally redundant. In *Drosophila*, a mutation in an importin α isoform causes larvae to develop malignant brain tumors, suggesting that this isoform is involved in brain development (Kussel and Frasch, 1995; Torok et al., 1995). Northern and western blot analysis demonstrates that many of the importin α isoforms are differently expressed in mouse and humans (Prieve et al., 1996; Kohler et al., 1997; Tsuji et al., 1997; Ryder et al., 1998). Interestingly, up to 5 different isoforms are expressed in the testes of mouse suggesting that different NLS-protein import pathways may co-exist in the same tissue (Tsuji et al., 1997). A recently characterized importin α isoform from humans is highly expressed in skeletal muscle and represents more than 1% of the total protein in this tissue (Ryder et al., 1998).

Initial studies in humans demonstrate that two isoforms of importin α, NPI-1 and Rch1, are not only expressed in the same tissues (Gorlich and Mattaj, 1996), but they can import SV40 T-antigen and bipartite NLS substrates in permeabilized cells (Moroianu et al., 1995). However, recent results suggest that these isoforms are not fully redundant. First, in vitro NLS binding assays demonstrate that they associate differently with NLSs identified in a variety of nuclear proteins (Nadler et al., 1997). Second, only NPI-1 can bind and facilitate nuclear import of Stat1, a transcription factor involved in cytokine signaling. Furthermore, NPI-1 can associate with Stat1 and a SV40 T-antigen NLS-substrate at the same time, implying that NPI-1 can import different NLS-cargos at the same time (Sekimoto et al., 1997). This study suggests that different isoforms may play roles in specific signal transduction pathways. Unlike NPI-1 and Rch1, Qip1, a third importin α isoform characterized in humans, cannot recognize the SV40-like NLS peptides from helicase unless surrounding sequences are present (Miyamoto et al., 1997). In summary, it appears that NLS-protein import can be controlled through NLS-recognition by different importin α isoforms. In addition, many of these isoforms may also be involved in NLS-protein import in specific tissues that would contribute to the development of multicellular organisms.

5. The Importin β Family

Studies indicate that the importin β family of proteins are nuclear shuttle proteins that function as receptors for the import and export of most proteins and ribonucleoprotein (RNP) complexes through the NPC (Table 1.2). For example, importin β and transportin, specifically mediate nuclear import of NLS-containing proteins and M9-containing proteins respectively, while exportin and CAS specifically mediate the export of NES-containing proteins and importin α, respectively (for reviews see Gorlich, 1997; Ullman et al., 1997; Wozniak et al., 1998). A conserved feature of the importin β-like proteins is the RanGTP binding domain located in the N-terminus which allow these receptors to bind RanGTP, not RanGDP (Gorlich et al., 1997; Wozniak et al., 1998). Mutations that block the interaction of importin β with RanGTP also prevent the release and disassembly of the importin α/β/NLS-cargo complex from the nuclear basket of the NPC (Kutay et al., 1997a). Interestingly, this mutant not only blocks NLS-protein import, but it also blocks M9-protein import, as well as the export of mRNA, U small nuclear RNA (snRNA), and NES-containing proteins (Kutay et al., 1997a). This suggests that the receptors for these pathways share similar binding sites at the NPC. In addition, in vitro binding assays demonstrate that many of these receptors bind to the same subset of nucleoporins (Wozniak et al., 1998). Recent results suggest that importin β functions as the receptor for a subset of NLS-containing proteins that are not

Table 1.2
Importin β Family of Nuclear Import/Export Receptors

Name	Function	References
1. Importin β (Karyopherin β1, p97, and PATC97, Kap95) .	Involved in NLS-protein import in yeast and vertebrates. Forms a heterodimer with importin α and interacts with NPC during import. Also functions as a receptor for a subset of NLS-containing proteins. Binds to RanGTP and a subset of nucleoporins. In yeast, a null mutation is lethal.	(reviewed by Gorlich, 1997; Nigg, 1997)
2. Transportin (Karyopherin β2 Kap104)	Involved in M9-protein import in yeast and vertebrates. Binds to RanGTP. Associates with a subset of nucleoporins. Growth is severely perturbed in a yeast null mutation.	(reviewed by Nigg, 1997; Wozniak et al., 1998)
3. CAS (Cse1)	The export receptor for importin α in vertebrates. Binds to RanGTP. CAS binds to importin α in a RanGTP dependent manner. Associates with the NPC.	(reviewed by Gorlich, 1997; Nigg et al., 1997; Ullman et) al., 1997).

4. Exportin 1 (Crm1)	The export receptor for NESs in yeast and vetebrates. Binds to RanGTP. Exportin binds to NES-proteins in a RanGTP dependent manner.	(reviewed by Ullman eta l., 1997; Wozniak et al., 1998)
5. Exportin t (Los1)	The export receptor for tRNA in vertebrates. Binds to RanGTP and associates with tRNA in a RanGTP dependent manner. Associates with the NPC.	(reviewed by Wozniak et al., 1998; Arts et al., 1998; Kutay et al., 1998).
6. Pse1	In yeast, Pse1 is synthetically lethal with Yrb1. Null mutants combined with temperature sensitive mutants in Yrb block poly A^+ RNA export in yeast. Binds to RanGTP. Pse1 and Yrb1 may be functionally redundant in yeast. Associates with a subset of nucleoporins.	(reviewed by Ullman et al., 1997; Wozniak et al., 1998)
7. Yrb4	Mediates ribosomal protein import in yeast. Null mutants combined with temperature sensitive mutants in Yrb1 block poly A^+ RNA export in yeast. Binds to RanGTP. Pse1 and Yrb1 may be functionally redundant. Associates with a subset of nucleoporins.	(reviewed by Ullman et al., 1997; Wozniak et al., 1998)

8. Sxm1	Import receptor for several ribosomal proteins. Implicated in tRNA maturation. Suppressor of Pse1 mutation in yeast. Binds to RanGTP.	(reviewed by Ullman et al., 1997; Wozniak et al., 1998)
9. RanBP7 (RanBP8, Nmd5)	Forms a complex with importin β in vertebrates. Associates with RanGTP and the NPC.	(Gorlich et al., 1997)
10. Mtr10	Implicated in the export of the mRNA binding protein, Npl3, and mRNA in . yeast. Associates with a subset of nucleoporins. Binds to RanGTP.	(reviewed by Ullman et al., 1997; Wozniak et al., 1998)

recognized by importin α (Tiganis et al., 1997; Chan et al., 1998) including U snRNPs (Palacios, et al., 1997). Therefore, importin β appears to be a multifunctional protein that can serve as a import receptor and an NPC adapter protein for the NLS-protein import pathway.

6. The Ran System

Ran is a small GTPase that is required for the import and export of proteins and ribonucleoprotein complexes (Izaurralde et al., 1997). The nucleotide exchange factor for Ran is RCC1, a chromatin binding protein that generates RanGTP inside the nucleus (Ohtsubo et al., 19989; Bischoff and Postingl, 1991). The GTPase activating protein, RanGAP1 stimulates Ran GTPase activity (Hopper et al., 1990; Melchior et al., 1993b; Bischoff et al., 1994). RanGAP1 is found in the cytoplasm (Hopper et al., 1990; Melchior et al., 1993b; Bischoff et al., 1994) and the cytoplasmic face of the NPC (Matunis et al., 1996; Mahajan, et al., 1997). A Ran binding protein, RanBP1, is a cytoplasmic protein that induces GTP hydrolysis by Ran together with RanGAP1 (Coutavas et al., 1993; Beddow et al., 1995; Bischoff et al., 1995; Richards et al, 1996). Interestingly, a nucleoporin, RanBP2, found on the cytoplasmic fibrils of the NPC contains two RanGTP binding domains and it is implicated in Ran activation (Wu et al., 1995; Wilken et al., 1995; Yokoyama et al., 1995). Based on the compartmental localization of RCC1 and the RanGTP activating proteins, it is hypothesized that there is

a RanGTP gradient across the nuclear envelope, where the concentration of RanGTP is high in the nucleus and low in the cytoplasm (Gorlich, 1997). This gradient is crucial for nuclear transport, because microinjection of RanGAP1 into the nuclei of *Xenopus* oocytes, which should raise the RanGDP levels inside the nucleus, blocks NLS- and M9-protein import as well as the export of mRNA, U snRNA, tRNA, importin α, importin β, transportin, and NES-containing proteins.

Genetic studies also support this model in vivo. Mutations in the yeast Ran homologue, Gsp1, cause NLS-proteins to accumulate in the cytoplasm and poly(A)$^+$ RNA to accumulate inside the nucleus (Schlenstedt et al., 1995). Similar studies have also shown that Ran mutants block NLS-protein import in mammalian cells (Carey et al., 1996; Palacios et al., 1996). Mutations in the yeast RanGAP1 and RCC1, called Rna1 and Prp20, respectively, block RNA processing and export (Hartwell et al., 1967; Hopper et al., 1978; Amberg et al., 1990; Forrester et al., 1992; Amberg et al., 1993), and mutations in the yeast RanBP1, Yrb1, cause NLS-containing proteins to accumulate in the cytoplasm while poly (A)$^+$ RNA is retained in the nucleus (Schlenstedt et al., 1995). In mammalian cells, mutations that cause mislocalization of RanBP1 to the nucleus block NLS-protein import (Richards et al., 1996). Similarly, mutations in RCC1 also blocks mRNA processing and transport (Cheng et al., 1995) as well as NLS-protein import in mammalian cells (Tachibana et al., 1994). In summary, mutations in these proteins should lead to

disruption in the RanGTP gradient, which would result in a block in macromolecular transport into and out of the nucleus.

Why is the RanGTP gradient so important for nuclear translocation? In vitro binding studies show that RanGTP regulates the interaction between import/export receptors and their cargo (Bischoff and Gorlich, 1997; Floer et al., 1997; Gorlich 1997; Kutay et al., 1997a). This model predicts that import receptors, like importin β, interact with their cargo in the cytoplasm where the levels of RanGTP are low and release their cargo in the nucleus where RanGTP levels are high. On the other hand, export receptors bind and release their cargo in the opposite manner. Thus, the Ran gradient may provide a directionality for nuclear import/export so that cargo binding and release occurs in the appropriate cellular compartments (Gorlich, 1997; Nigg, 1997).

7. Mechanism of Translocation

Proteins destined for the nucleus must move ≈75 nm from the cytoplasmic fibrils, through the central gate, and to the nuclear basket (for reviews see Davis, 1995; Pante and Aebi, 1996a). Recently, electron microscopy studies have mapped two distinct binding regions on the cytoplasmic side of the NPC (Pante and Aebi, 1996b). The initial binding site is found near the ends of the cytoplasmic fibrils 50 nm from central plain of the NPC. The second binding site is about 10 nm from the central plane of the NPC, adjacent to the

central gate. Immunoelectron micrograph pictures displayed some fibrils containing NLS-substrates bent inward toward the central gate where the second binding site is located. Therefore, it was postulated the NLS-substrates may be transferred from the initial binding site to the second binding site by the inward bending of cytoplasmic fibrils (Pante and Aebi, 1996b).

A model based on in vitro binding studies predicts that the translocation of the NLS-protein import complex through the pore may occur by a series of binding steps mediated by importin β and release steps mediated RanGTP (Rexach and Blobel, 1995; Chi et al., 1996; Gorlich et al., 1996a). This model is referred to as the guided diffusion model (Rexach and Blobel, 1995). However, the mechanism that "guides" the NLS-protein import complex is not known. Clearly, a more defined structure of the NPC and the identification of more nucleoporins is required for better understanding of import as well as export. In addition, the localization of intermediates during the translocation process will also provide more information about this poorly defined process.

8. Regulatory Proteins

An import regulatory factor, p10, stimulates NLS-protein import in the presence of importin α, β, RanGDP, and free GTP in permeabilized cells (Moore and Blobel, 1994; Paschal and Gerace, 1995). Biochemical studies demonstrate that p10 can bind directly to RanGDP, importin β and to a subset of

nucleoporins suggesting that p10 may regulate the interaction of the import factors with the NPC transport machinery. (Nehrbass and Blobel, 1996; Paschal et al., 1996). Genetic studies in yeast demonstrate that this protein is an essential gene involved in NLS-protein import in vivo (Corbett and Silver, 1996; Nehrbass and Blobel, 1996; Paschal et al., 1996).

A significant step in the human immunodeficiency virus type 1 (HIV-1) infection process requires nuclear import of the pre-integration complex (Popov et al., 1998; Vodicka et al., 1998). The pre-integration complex contains the HIV-1 genome as well as some accessory proteins that contain NLSs. Nuclear import of this complex requires an HIV-1 encoded protein, viral protein R (Vpr), which stimulates the import of the pre-integration complex by enhancing the interaction of the viral NLS-proteins with importin α (Popov et al., 1998). In addition, Vpr mediates the import of importin α/NLS protein complex in the absence of importin β, suggesting that Vpr, which is not homologous to importin β, can interact with the import machinery during the import process (Vodicka et al., 1998). Interestingly, at high concentrations, 40 nM, Vpr blocks nuclear import indicating that the cytopathic effect observed in late stages of HIV-1 infection may be due to high concentrations of Vpr in the cell (Popov et al., 1998).

Lastly, 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 in exposing the NLSs to the import machinery.

9. Regulation of the Import Apparatus

Nuclear import of numerous transcription factors is regulated by various stimuli (environmental, chemical, or cell cycle progression) which can lead to changes in cell fate or metabolism indicating that this process is an important component in gene expression (Jans and Huebner, 1996; Mishra and Parnaik, 1995; Vandromme et al., 1996). For example, protein phosphorylation adjacent to NLSs can either stimulate or block the import of various transcription factors (Jans and Hubner, 1996). In addition, studies also indicate that the import apparatus itself is regulated by the cell (Mishra and Parnaik, 1995; Vandromme et al., 1996).

Proliferating and serum stimulated cells have a higher rate of NLS-protein import than quiescent and serum starved cells (Feldherr and Akin, 1990; 1993; Vriz et al., 1992). Comparisons between the NPCs found in these cells revealed that the diameter of the pore is two times smaller in quiescent cells than in proliferating cells (Feldherr and Akin, 1990). In serum starved cells, NLS-protein import can be enhanced by treating the cells with mitogens to raise the cAMP levels (Roux et al., 1990) or with protein kinase A (PKA) activators (Vandromme, et al., 1994; 1996) suggesting that the import apparatus may be regulated by a signal transduction pathway that utilizes cAMP and PKA

phosphorylation (Vandromme, et al., 1996). Interestingly, when permeabilized mammalian cells are treated with alkaline phosphatase to dephosphorylate proteins, NLS-protein import is blocked. However, import can be restored by incubating permeabilized cells with cytosol enriched with PKA or protein kinase C (PKC). Interestingly, two highly phosphorylated proteins in these cytosolic extracts are similar in mass as importin α (Mishra and Parnaik, 1995) suggesting that a PKA or PKC signal transduction pathway may regulate the function of the NLS-receptor.

10. Conclusion

Understanding of the NLS-protein import process has blossomed since the development of the permeabilize cell system that was used to identify proteins involved in this process. However, despite the recent advances in nuclear import and export field, the translocation process still remains a complete mystery. More attention needs to be focused in this area of research to understand the mechanism of translocation and how the import/export factors interact translocation machinery.

11. Thesis Scheme

Macromolecular traffic through the NPC, including NLS-protein import, is an essential process in all eukaryotes. When I started this project, the goal was to identify and characterize the NLS receptor in plants. At this time, the

NLS receptor had been purified in vertebrates (Adam and Gerace, 1991), but the gene had not been cloned and characterized.

In plants, an NLS binding site at the NPC and nuclear envelope had been characterized (Hicks and Raikhel, 1993). The SV40 large T-antigen and bipartite NLSs specifically and reversibly bound to and competed for this low-affinity site (Hicks and Raikhel, 1993). To identify the NLS binding site biochemically, a crosslinking approach was used and at least four NLS binding proteins (NBPs) were identified that specifically associated the bipartite NLS from Opaque-2 (Hicks and Raikhel, 1995a). The binding affinity and biochemical properties of the NBPs correlated closely with the NLS binding site. This evidence (Hicks and Raikhel, 1993; Hicks and Raikhel 1995a) indicated that at least one component of NLS recognition was located at the NPC and nuclear envelope in plants.

Shortly after we further characterized the NLS binding site (see Chapter 1), importin α was identified by Gorlich et al., (1994). The next step of my project was to identify and characterize an importin α homologue in plants. In addition, it was important to determine if importin α had similar properties as 50-60 kilodalton NBPs that are located at the NLS binding site in plants (Hicks and Raikhel, 1993; Hicks and Raikhel, 1995a) . Lastly, the plant importin α was used as a tool to address basic biological questions in NLS-protein import.

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Chapter 2

Three Classes of Nuclear Import Signals Bind to Plant Nuclei

This chapter was published in:

Hicks, G.R., Smith, H.M.S., Shieh, M. and Raikhel, N.V. (1995) Three classes of nuclear import signals bind to plant nuclei. Plant Physiol. 107, 1055-1058.

ABSTRACT

Three nuclear localization signals (NLS), including an unusual Mat α2-like NLS from maize (*Zea mays*) R, were found to compete for binding to plant nuclei. Our results, indicate that plants possess a site at the nuclear pore complex that recognizes the three known classes of NLSs.

INTRODUCTION

Since there are no strict consensus sequences for NLSs, a thorough study of each of the classes of signals is an essential step in identifying components of the import apparatus of plants. The initial event of import, binding at the NPC, has been examined with an in vitro nuclear binding assay using peptides to two classes of NLSs (Hicks and Raikhel, 1993). The bipartite NLS from Opaque-2 (02; Varagona, et al., 1992) and the SV40 large T-antigen NLS (Raikhel, 1992) specifically bind to and compete for a single low affinity site that is firmly associated with the nuclear envelope and NPC (Hicks and Raikhel, 1993). However, mutant O2 (Varagona and Raikhel, 1994) and mutant SV40 large T-antigen NLSs (Raikhel, 1992) that are impaired in import in vivo do not compete for binding (Hicks and Raikhel, 1993). In addition, a peptide to the SV40 large T-antigen that was synthsized in reverse order is an inefficent competitor compared to the the wild type SV40 large T-antigen NLS peptide. To determine if the third known class of NLSs, the Mat α2-like NLSs, could compete for binding to the same nuclear site, we examined the association of NLS C from the R protein with purified tobacco (*Nicatiana tabacum*) and maize (*Zea mays*) nuclei.

METHODS

Materials

The peptide to NLS C (CWT) was synthesized at the Peptide Synthesis Facility (Yale University, New Haven, CT). Synthetic peptides to the functional O2 bipartite NLS (O2WT), the functional SV40 large T-antigen NLS (SV40WT), and the peptide unrelated to NLSs (non-NLS) were previously described (Hicks and Raikhel, 1993). All peptides contained a Cys residue at the amino or carboxy terminus to facilitate radiolabeling with carbon-14. The peptides were as follows: (CWT) CYMISEALRK AIGKR; (O2WT) MPTEERVRKR KESNRESARR SRYRKAAHLK C; (SV40WT) CTPPKKKRKV; (non-NLS) CDGVFAGGG.

Nuclear binding assays

For binding assays, nuclei were prepared from protoplasts of *Nicotiana tabacum* or maize (*Zea mays*) Black Mexican Sweet suspension-cultured cells abbreviated protocol previously described (Hicks and Raikhel, 1993), except that DTT was omitted from the nuclei isolation buffer. The NLS peptide, CWT, was radiolabeled with carbon-14 by carboxymethylation of cysteine residues in the prescence of ^{14}C-iodoacetamide as described by Hicks and Raikehl, 1993. For NLS binding experiments to purified nuclei using the radiolabled [^{14}C]CWT NLS were performed as described by Hicks and Raikhel, 1993 except that 50 mM Tris-HCl, pH 7.3, was replaced by 50 mM Hepes-KOH, pH 7.3, in binding buffer. Briefly, 1 X 10^6 tobacco or maize nuclei were diluted to 70 ul with binding

buffer, and 200,000 cpm of [^{14}C]CWT (76 uCi/mmol; approximately 5 uM final concentration) in 30 ul of binding buffer was added. Binding was allowed to occur for 5 min on ice, after which time the nuclei were pelleted. The supernatant was removed and the cpm associated with the nuclei were quantitated by scintillation counting. For competitive displacement curves, unlabeled peptides were added from concentrated stocks made in binding buffer. Nonspecific backgrounds were estimated from the addition of 10 mM unlabeled CWT. Note: this assay measures relative binding of NLS peptides to purified nuclei. Assays points were the average of at least duplicate samples, and all experiments were done at least twice.

RESULTS

Three classes of NLSs compete for the same NLS binding site in purified nuclei

To determine whether NLS C could specifically bind to purified nuclei, CWT was synthesized that corresponded to the minimum region of R that functionally defined this signal in vivo (Sheih et al., 1993). In addition, the previously described (Hicks and Raikhel, 1993) O2WT and SV40WT peptides, and a peptide unrelated to NLSs (non-NLS), were used. The latter peptide, which corresponds to a defective vacuolar import signal (Dombrowski et al., 1993), served as a "non-NLS" control.

To examine the relative binding of NLS C, the CWT peptide was carbon-14 labeled and allowed to bind to purified tobacco nuclei. Displacement curves resulting from the addition of the CWT peptide as a competitor indicated that [^{14}C]CWT could specifically bind to plant nuclei at low affinity with an apparent dissociation constant of approximately 100 uM (Fig. 2.1 A). This is similar to the apparent dissociation constant for the binding of [^{14}C]O2WT and [^{14}C]SV40WT to tobacco nuclei (200 uM; Hicks and Raikhel, 1993). Displacement of [^{14}C]CWT by unlabeled SV40WT and O2WT indicated that the three peptides competed for binding to the same nuclear site (Fig. 2.1, B and C). The non-NLS control peptide was a poor competitor compared to the functional NLSs (Fig. 2.1 C). We also examined the binding of [^{14}C]CWT to nuclei from the distantly related monocot maize. Experiments

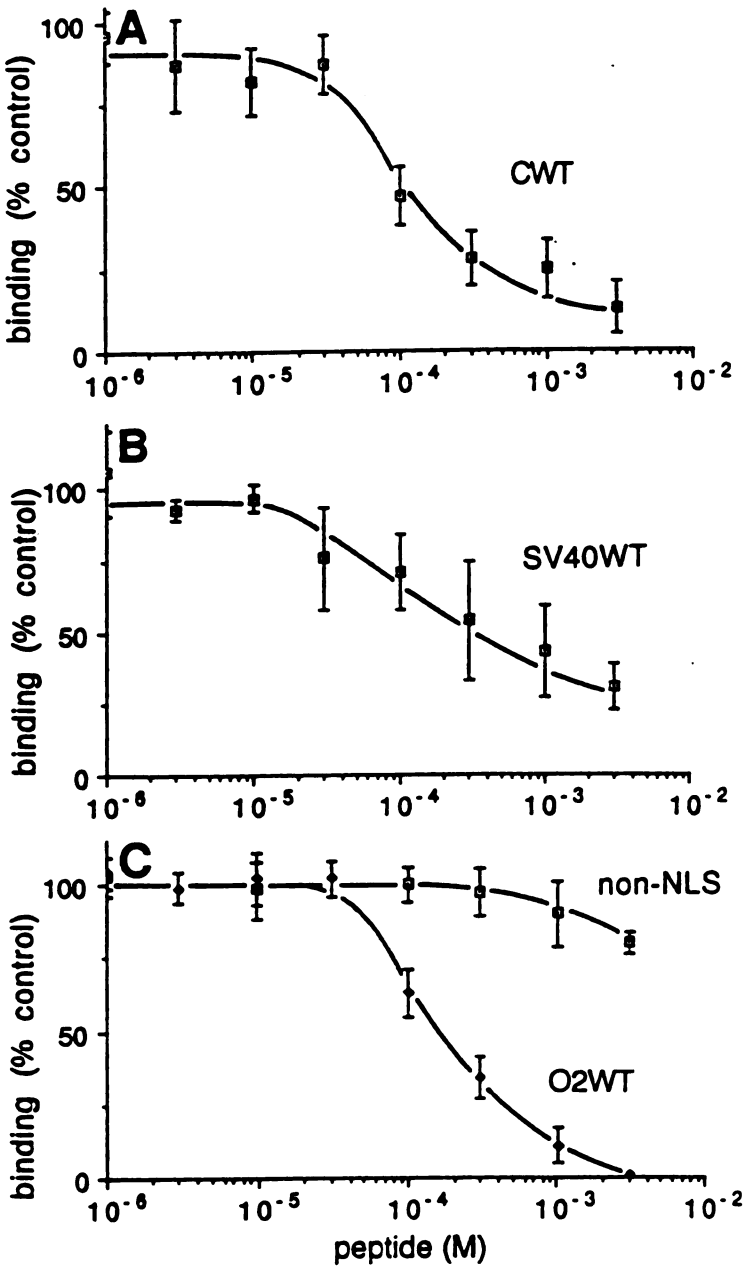

Figure 2.1
Specific binding of NLSs to purified tobacco nuclei. The [^{14}C]CWT peptide was incubated with nuclei in the presence of CWT (CYMISEALRKAIGKR) (A), SV40WT (CTPPKKKRKV) (B), or O2WT (MPTEERVRKRKESNRESARRSRYRKAAHLKC) or non-NLS (CDGNFAGGG) (C) competitor peptides. Results are reported as a percent binding of control (± SE) versus concentration (M) of added competitor. Average total binding and nonspecific association were, respectively, 16,900 and 11,000 cpm.

using purified nuclei from maize indicated that monocots possess a similar binding site to which [^{14}C]CWT could associate. Addition of CWT as a competitor demonstrated that binding was specific and of an affinity similar to that found with tobacco nuclei (Fig. 2.2 A). The SV40WT and O2WT peptides also competed effectively with [^{14}C]CWT for binding to the nuclear site (Fig. 2.2, B and C), whereas the non-NLS control peptide displayed no ability to compete (Fig. 2.2 C).

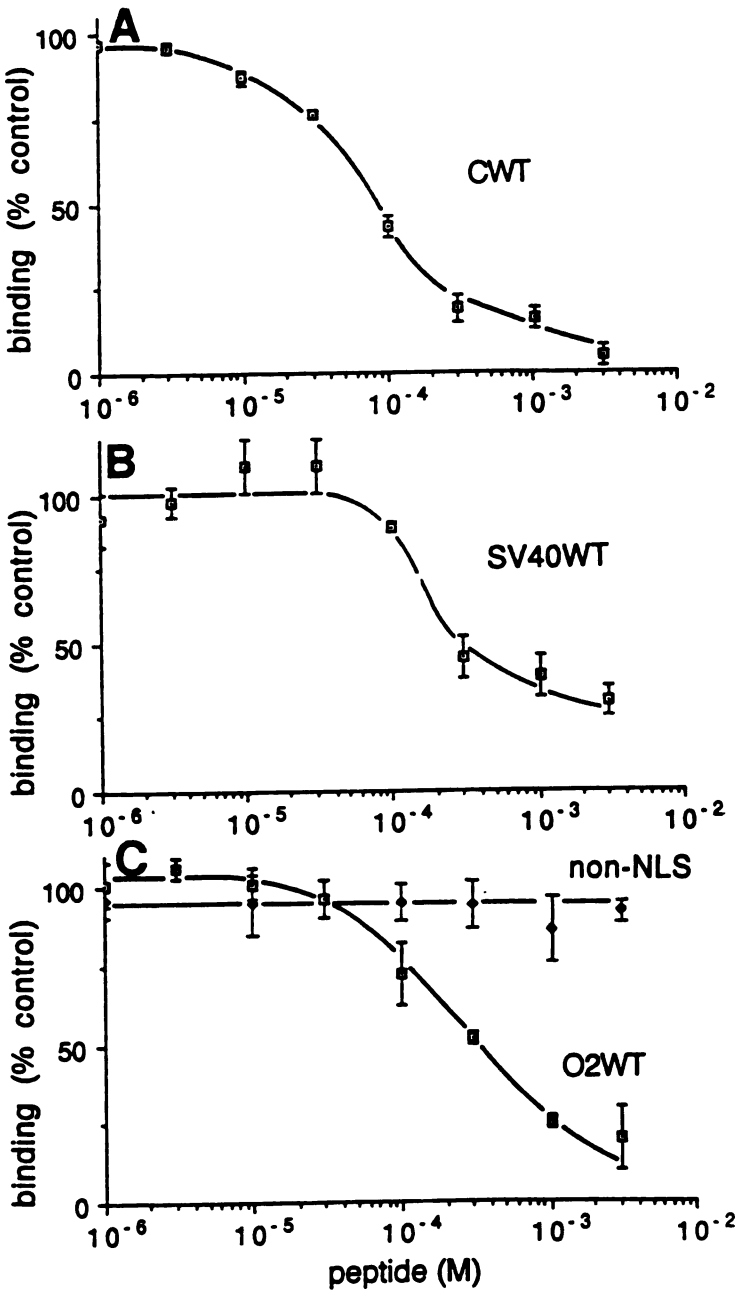

Figure 2.2
Specific binding of NLSs to purified maize nuclei. The [^{14}C]CWT peptide was incubated with nuclei in the presence of CWT (A), SV40WT (B), or 02WT or non-NLS (C) competitor peptides. Results are reported as percent binding of control (± SE) versus concentration (M) of added competitor. Average total binding and non-specific association were, respectively, 25,400 and 14,000 cpm.

DISCUSSION

A careful study of the binding of NLSs is an important step prior to identifying the corresponding NLS binding proteins. Although the binding of individual NLSs has been examined in animals and yeast (Forbes, 1992), a systematic approach using the three known classes of NLSs has not been reported. We previously demonstrated that higher plants possess a saturable low-affinity site at the NPC that can specifically bind to the SV40 large T-antigen NLS and the O2 bipartite NLS (Hicks and Raikhel, 1993). In this study, we examined the binding of the Mat α2-like NLSs, such as NLS C from the endogenous maize transcription factor R with purified nuclei isolated from tobacco and maize cell suspension cultures. In these experiments, we used 5 uM [^{14}C]CWT in our competition studies which is below the dissociation constant, Kd, for this site. Under these conditions the NLS binding sites are not saturated. Therefore, we expected to displace approximately half of the [^{14}C]CWT when the concentration of the competitor peptide, CWT, equals the dissociation constant. We found that this occurs at approximately 100 uM, which is similar to the apparent dissociation constant for the binding of [^{14}C]O2WT and [^{14}C]SV40WT to tobacco nuclei (Hicks and Raikehl, 1993). The SV40WT and O2WT peptides were also found to compete with [^{14}C]CWT for binding. Similar results were obtained using nuclei from maize, indicating that the binding site is probably a component that is common among divergent species.

It is generally accepted that animals require soluble factors for NLS recognition (Forbes, 1992; Moore and Blobel, 1994), and this may also be the case in yeast (Schenstedt et al., 1993). Our results indicate that at least some components of NLS recognition are located at the NPC of plants. We have recently extended our in vitro studies to include the addition of cross-linked reagents during NLS binding. Using this approach, several NLS binding proteins have been specifically radiolabeled with the peptides to the functional O2 bipartite NLS (Hicks and Raikhel, 1995). Future work is aimed at identifying and characterizing NLS-binding proteins to understand NLS-protein import proteins in plants.

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Chapter 3

Characterization of a Nuclear Localization Signal Receptor, Importin α, in Plants

This chapter was constructed from two published papers:

1. Hicks, G.R., Smith, H.M.S., Lobreaux, S. and Raikhel, N.V. (1996) Nuclear import in permeabilized protoplasts from higher plants has unique features. Plant Cell 8, 1337-1352.

2. Smith, H.M.S., Hicks, G.R., and Raikhel, N.V. (1997) Importin α from *Arabidopsis thaliana* is a nuclear import receptor that recognizes three classes of import signals. Plant Physiol. 114; 411-417.

In addition, the functional analysis of the *Arabidopsis* importin α protein in the mammalian in vitro import system was done in collaboration with Dr. David A. Jans's group at the Nuclear Signaling Laboratory, Division for Biochemistry and Molecular Biology, John Curtin School of Medical Research, Canberra, Australia.

ABSTRACT

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 (NLSs) in vitro. In this study, we cloned an importin α homologue (At-IMPα) from *Arabidopsis thaliana* and it was 36-48% identical to other importin α homologues identified in other organisms. Antibodies generated against the recombinant expressed At-IMPα specifically recognized the endogenous protein in *Arabidopsis* and tobacco. To determine if At-IMPα was a NLS receptor, we used an in vitro NLS binding assay. We found that NLS binding by At-IMPα was specific, and the receptor was able to recognize three classes of NLSs identified in plants. Purified antibodies to At-IMPα were used to determine the in vivo location of importin α in tobacco protoplasts. Importin α was found in the cytoplasm and nucleus, and it was highly concentrated at the nuclear envelope. The biochemical properties of nuclear importin α and localization studies using purified nuclei demonstrate that importin α was tightly associated with the plant nucleus. Moreover, these results suggest that a fraction of nuclear importin α interacts with the nuclear pore complex. Using purified subunits in a vertebrate in vitro import system, we showed that in the absence of exogenously added importin β subunit but in the

presence of RanGDP and NTF2, At-IMPα was able to mediate nuclear import to levels comparable to those mediated by the mouse importin α/β heterodimer. Neither mouse importin α nor β was able to mediate nuclear import in the absence of the other importin subunit. Therefore, At-IMPα's has unique properties that can fulfill the role of both mammalian importin subunits. In summary, our results taken together strongly suggest that At-IMPα is a functional NLS receptor in *Arabidopsis*.

INTRODUCTION

Protein import into the nucleus occurs through the nuclear pore complex (NPC), which is a 124 megadalton proteinaceous complex embedded in the nuclear envelope which acts as a gateway for protein traffic in and out of the nucleus (for reviews see Davis, 1995). For many nuclear localized proteins, this process is receptor mediated and dependent upon targeting signals called nuclear localization signals (NLSs; Dingwall and Laskey, 1991). Although there is no consensus sequence for these signals, NLSs can be grouped into three classes (for reviews see Boulikas, 1993, 1994; Hicks and Raikhel, 1995b). A NLS within the SV40 large T-antigen (Kalderon et al., 1984; Lanford and Butel, 1984) defines a class of signals, the SV40-like NLSs, that are composed of a single peptide region containing basic residues. The bipartite NLSs (Dingwall et al., 1988) are composed of two peptide regions containing basic residues separated by a spacer of variable length. Finally, Mat α2-like NLSs share similarity with the amino-terminal NLS of the yeast protein Mat α2 (Hall et al., 1984) and contain hydrophobic and basic residues.

Experimentally, NLS-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 driven process (Newmeyer and Forbes, 1988; Richardson

et al., 1988). In vitro import systems using permeabilized vertebrate cells suggested that some of the factors 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, Enenkel et al., 1995, Gorlich et al., 1994, 1995a, b; Imamoto et al., 1995a,b; 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 a., 1995; Radu et al., 1995b), and Ran/TC4 (Melchior et al., 1993; Moore and Blobel, 1993). Mutations in some homologous import factors in yeast also block import in vivo (Loeb et al., 1995; Schenstedt et al., 1995).

NLS-protein 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,b; Weis et al., 1995). Importin α requires importin β for high affinity interaction with NLSs in vertebrates and yeast (Gorlich et al., 1995a; Rexach and Blobel, 1996; Efthymiadis et al., 1997; Hubner et al., 1997). 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; Pante and Aebi, 1996; Radu et al., 1995a,b).

Translocation of the trimeric complex through the NPC requires free GTP (Gorlich et al., 1996c), 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 interacts with a subset of nucleoporins during the translocation process (Iovine et al., 1995; Pascal 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 leads to export of importin α into the cytoplasm, where it can participate in another cycle of import (Gorlich et al., 1996a; Weis et al., 1996). In addition, importin β functions as a NLS-receptor for a subset of proteins in the absence of importin α (Palacios, et al., 1997; Tiganis et al., 1997; Chan et al., 1998). 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 in exposing the NLSs to the import machinery. The protein import process can be regulated by phosphorylation (Jans and Huebner, 1996; Mishra and Parnaik, 1995; 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 NLSs 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 crosslinking approach was used and at least

four NLS binding proteins (NBPs) were identified using the bipartite NLS from a maize transcription factor, Opaque-2 (Hicks and Raikhel, 1995a). The binding affinity and biochemical properties of the NBPs correlate closely with the NLS binding site. This and other evidence (Hicks and Raikhel, 1993; Hicks and Raikhel 1995a) indicate that at least one component of NLS recognition is located at the NPC and nuclear envelope in plants. To get a better understanding of the NLS-protein import pathway in plants, we identified and characterized an *Arabidopsis* importin α homologue, At-IMPα.

METHODS

Materials

All chemicals were obtained from Sigma Chemical Co. unless otherwise noted. All NLS peptides were synthesized at the Peptide Synthesis Facility (Yale University, New Haven, CT).

Cloning and sequencing of At-IMPα

A 900-bp partial cDNA exhibiting significant homology with yeast SRP1 and animal importin α homologues was obtained from the Michigan State University-Department of Energy Plant Research Laboratory *Arabidopsis* Sequencing Project (Newman et al., 1994). The cDNA was used as a probe to screen the PRL2 *Arabidopsis* cDNA library (Newman et al., 1994) in λZiplox (Gibco BRL, Gaithersburg, MD). The library was made from mRNA of etiolated seedlings, roots, leaves, and flowers. The probe was synthesized with α-32-dATP (3000 Ci/mmol; NEN Research Products, Boston, MA) by Klenow (Boehringer Mannheim) and random hexanucleotide primers. Approximately 2 x 10^6 plaques were screened, and 12 additional cDNAs were purified by standard methods (Sambrook et al., 1989). The cDNAs were excised from λZiplox for sequencing, as described by Newman et al. (1994). A full length cDNA of 2.2 kb was obtained that hybridized to an mRNA of similar length (H.M.S. Smith and N.V. Raikhel, unpublished data), and 3′ and 5′ deletions were made (Henikoff, 1987) and sequenced using Sequenase 2.0 (United States Biochemical Corp., Cleveland,

OH). Deduced amino acid sequences were aligned by MegaAlign, DNASTAR Inc. (Nov. 1997 version).

Expression and purification of recombinant At-IMPα

The At-IMPα cDNA was cloned into pGEX5-2 (Pharmacia, Piscataway, NJ) by using EcoR1 and Not1 sites to produce an N-terminal glutathione-S-transferase (GST) fusion protein. The plasmid was transformed into DH5α and the production of fusion protein was induced overnight at 37°C, according to the manufacture's procedures. For protein purification, cells were chilled on ice for 20 min before centrifugation at 7500*g* and suspended in 20 ml of ice-cold PBS containing 1 mM PMSF. Cells were lysed by two treatments at 1100 p.s.i. in a French Press. Triton X-100 was added to 1%, and centrifuged at 1200*g*. Then, 40 ml of the supernatant was mixed with 2 ml of glutathione-agarose (Pharmacia) for 3 hr at 4°C. The beads were washed 10 times with 40 ml of ice-cold PBS containing 1 mM PMSF. The fusion protein was eluted with 5 ml of elution buffer (50 mM Tris-HCl, pH 8.0, 10 mM reduced glutathione) and concentrated using a Centricon-10 device (Amicon). The purified protein had a concentration of 2.5 mg/ml.

For antibody production, the fusion protein was purified further by 10% SDS-PAGE and prepared as follows: gel strips containing a total of ~6 mg of fusion protein were equilibrated in extraction buffer (50 mM ammonium bicarbonate, pH 7.8 0.05% SDS) for 5 min at 23°C, ground with mortal and pestle, and mixed overnight as a suspension in

extraction buffer at 37°C. The suspension was centrifuged twice at 1300*g*, filtered through a 45-um cellulose acetate filter, and washed by repeated dilution with 50 mM ammonium bicarbonate. pH 7.8, in a Centricon-30 device (Amicon). The sample was lyophilized twice and dissolved in 3.0 ml of PBS at a concentration of 0.9 mg/ml.

For expression as a His-tagged fusion protein, At-IMPα was cloned into pET14b (Novagen, Madison, WI) by using Nde1 and BamH1 sites. The plasmid was transformed into BL21 (DE3) and then induced and purified by affinity chromatography, according to the manufacturer's protocol. The final protein concentration was 1.6 mg/ml.

Antibody production and purification

Preimmune serum was collected; then one rabbit was immunized with 0.5 mg of purified GST-At-IMPα fusion protein in the presence of an adjuvant (TiterMax; Vaxel, Norcross, GA) for each of three injections over a 6-week period and a final injection of 1.0 mg. For affinity purification of At-IMPα antibodies, His-tagged At-IMPα was coupled to Affigel-10 (Bio-Rad Laboratories). Purified protein (3.5 mg) was mixed with 1 ml of Affigel-10 in 50 mM Hepes-KOH, pH 7.2 in a 3.5-ml column and rocked for 4 hr at 4°C. The column was washed with three column volumes of 50 mM Tris-HCl, pH 7.2, three volumes of 100 mM glycine pH 2.5 and finally equilibrated in 50 mM Hepes-KOH, pH 7.2, and filtered through a 45-uM cellulose acetate filter; specific antibodies were bound to

the At-IMPα affinity column by passage through the column three times. The column was then washed sequentially with 10 mM Tris-HCl, pH 7.2, 10 mM Tris-HCl, pH 7.2, plus 0.5 M NaCl, and 50 mM Hepes-KOH, pH 7.2. Specific At-IMPα antibodies were eluted with 100 mM glycine, pH 2.5, and 1-ml fractions were collected and neutralized with 0.6 ml of 1 M Hepes-KOH, pH 7.5, 2 mM magnesium acetate, 50 mM potassium acetate, and 0.225 M mannitiol.

Immunoblots

Ten percent of SDS-PAGE and blotting to nitrocellulose were performed by standard methods (Sambrook et al., 1989). Blots were blocked with nonfat dry milk and incubated overnight at 23°C with purified At-IMPα antibodies or preimmune sera (both stocks at 90 ug/ml) at a 1:3000 dilution. Blots were developed by using a 1:5000 dilution of goat anti-rabbit alkaline phosphatase-conjugated IgG (Kirkegaard and Perry Laboratories). Proteins were extracted from *Arabidopsis* roots, leaves, stems, and flowers by grinding in liquid nitrogen and suspended in extraction buffer (50 mM Tris-HCl, pH 6.8, 100 mM DTT, 2 % SDS) for SDS-PAGE (50 *u*g per lane). Tobacco nuclei were purified by the abbreviated procedure described by Hicks and Raikhel (1993). Nuclei (10^6 per gel lane) were diluted to 50 *u*l in 50 mM Tris-HCl, pH 7.3, 25 mM KCl, 2.5 mM $MgCl_2$, 3 mM $CaCl_2$, 20% glycerol and were incubated with 400 units per ml Dnase 1 (Boehringer Mannheim) at 23°C for 20 min. After centrifugation at 12,000*g* for 2 min, the

nuclear pellet was suspended in 25 ul of SDS sample buffer. Where indicated, 40 ug (0.53 x 10^5 cell equivalents per gel lane) of cytosol from evacuolated tobacco cells and 75 ng of His-tagged At-IMPα or mammalian importin α were loaded per gel lane.

In vitro binding/co-immunoprecipitation

For in vitro transcription and translation, full length *At-IMPα* was cloned into Bluescript (SK-; Strategene, Inc., La Jolla, CA) with EcoR1 (Boehringer and Mannheim, Indianapolis, IN) and BamH1 (Boehringer and Mannheim, Indianapolis, IN). The vector was linearized at the 3' end of the gene with BamH1 and transcribed with T7 RNA polymerase (Promega Biotech, Madison, WI). Two ug of mRNA was incubated in wheat germ extract (minus Met; Promega Biotech, Madison, WI) in the presence of 50 uCi [^{35}S] Met (New England Nuclear, Boston MA). Translation mix was diluted in Binding Buffer (50 mM Hepes pH 7.8, 25 mM KCl, 2.5 mM $MgCl_2$, 3 mM $CaCl_2$, 20% glycerol) and 250 ug of purified At-IMPα antibodies were added and mixed for 1 to 2 h at 4°C. Next, Protein A Sepharose was added to a final concentration of 0.05 %. Precipitated proteins were washed 4 times in Binding Buffer to remove any contaminants and suspended in SDS sample buffer (50 mM Tris pH 6.8, 100mM DTT, 2.0% SDS, 20% glycerol). To determine if the antibodies could also recognize the recombinant expressed At-IMPα, 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 24 h by autoradiography by standard methods (Sambrook et al., 1989).

For NLS binding substrates, 4 mg of Human Serum Albumin (HSA) was dissolved in PBS. The chemical crosslinker, Maleimidobenzoyl N-hydroxysuccinimide ester (MBS; Pierce, Rockford, IL), was added to a final concentration of 10 mM and incubated for 30 min at room temperature. Unbound crosslinker was removed by Gel filtration with a 2 ml G-25 column. NLS peptide was added to a final concentration of 1.2 uM 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 Centricon-10 microfiltration device (Amicon, Beverly, MA), and they were aliquoted and stored at -80°C. For co-immunoprecipitation, 2 ug of NLS-HSA substrate was mixed with 500 ng of recombinant At-IMPα in Binding Buffer for 2 to 4 h at 4°C. Then, 400 ng of purified At-IMPα antibodies were added and incubated for 1 to 2 h at 4°C. Protein A Sepharose (Pharmacia Biotech, Piscataway, NJ) was added to 0.05% and mixed at 4°C for 1 h. Samples were precipitated and washed 4 times in Binding Buffer to remove unbound substrates then suspended in SDS sample buffer. For competition experiments, NLS binding was performed as described above except the

Binding Buffer contained either 1 mM 02WT or 1 mM 02mut peptides. Separation of proteins by 10% SDS-PAGE and blotting were performed by standard methods (Sambrook et al., 1989). Blots were rinsed with TBST (TBS, O.05% Tween 20) then incubated overnight at room temperature with monoclonal antibodies raised against HSA (Sigma Chemicals Co., St. Louis, MO) at 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, Guithersburg, MD).

Immunolocalization

Nictotiana tabacum suspension-culture cells were maintained and protoplasts were prepared as described by Hicks and Raikhel, (1993). Protoplasts or purified nuclei were spun onto poly-lysine coated slides using a Cytospin 3 (Shandon Lipshaw, Pittsburg, PA), and immediatley fixed in Fix 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^{0}C for 24 h. Next, cells were dehydrated in cold methanol for 10 min, then washed in PBST (PBS, 0.5 % Tween 20). Affinity purified At-IMPα antibodies (100 ng/*u*l) were diluted 1:300 in PBST containing 60 *u*g 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 (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, San Diego, CA) and optically sectioned (0.5-1.0 um sections) using Confocal Laser Scanning Microscopy (Model 10 Carl Zeiss) equipped with 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 ul in Binding Buffer with 400 units of DNaseI (Boehringer Mannheim, Indianapolis, IN) at room temperature for 20 min. Nuclei were centrifuged at 12,000*g* for 2 min and suspended in 25 ul 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 6M urea. The samples were incubated for 15 min at 4°C, then centrifuged at 12,000*g* for 2 min. Ten ul of SDS sample buffer was added to the supernatant, and the pellet was resuspened in 25 ul of SDS sample buffer. Each sample was incubated at 65°C for 5 min before separation 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, Guithersburg, MD).

In Vitro Nuclear Transport

These experiments were done in collaboration with Dr. David A. Jans group at the Nuclear Signaling Laboratory, Division for Biochemistry and Molecular Biology, John Curtin School of Medical Research, Canberra, Australia

Analysis of the ability of At-IMPα to support nuclear import in vitro was performed by quantifying nuclear import kinetics at the single cell level using mechanically perforated HTC rat hepatoma cells in conjunction with confocal laser scanning microscopy (Jans et al., 1991; Xiao et al., 1997; Efthymiadis et al., 1997, 1998). Experiments were performed for 40 min at room temperature in a 5 ml volume containing 30 mg/ml BSA, 2 mM GTP, and an ATP regenerating system (0.125 mg/ml creatine kinase, 30 mM creatine-phosphate, 2 mM ATP), transport substrate (0.2 mg/ml IAF-labelled fusion protein) or a control to assess nuclear integrity (70 kDa FITC-labelled dextran; Sigma Chem. Co.). Where indicated, 4 mM RanGDP, 0.15 mM NTF2, 1 mM mouse-importin β (m-IMPβ), 1 mM mouse-importin α (m-IMPα) or 0.6 mM At-IMPα were added. Image analysis and curve-fitting was performed as described (Xiao et al., 1997; Efthymiadis et al., 1997, 1998); the level of accumulation at the nuclear envelope, relative to medium fluorescence, was measured using NIH Image 1.60 in line plot mode as previously (Piller et al., 1998). Experiments were repeated three times.

RESULTS

Cloning of the *Arabidopsis* importin α homologue

A partial 900 bp clone which had high homology to yeast and vertebrate importin α homologues was obtained from the MSU-DOE Plant Research Laboratory *Arabidopsis* Genome Sequencing Project (Newman et al., 1994). This clone was used as a probe to screen the *Arabidopsis* PRL2 cDNA library (Newman et al., 1994), and a full length clone (2.2 Kb) was identified that was similar in size to mRNA detected on *Arabidopsis* Northern blots (data not shown). The longest open reading frame encoded a polypeptide of 532 amino acids with an approximate molecular weight of 59 kD (Figure 3.1). This protein contains eight armadillo tandem repeats that are found in many proteins like armadillo, β-catenin, plakaglobin, a GTP exchange factor for small a Ras-GTPase (smgGDS), adenomatous polyposis coli tumor suppressor protein (APC) as well as importin α homologues (Figure 3.1). These repeats are 42 amino acids in length, highly hydrophobic and are believed to be involved in protein-protein interactions (Peifer et al., 1994). Interestingly, most of the basic residues in the N-terminus of importin α homologues are highly conserved and may function as an NLS (Figure 3.1). At the amino acid level, the *Arabidopsis* importin α homologue (At-IMPα) is 36-49% identical with other homologues found in *Saccharomyces cervisiae*, *Schizosaccharomyces pombe*, *Homo sapiens*, *Mus musculus*, *Xenpus laevis*, *Caenorhabditis elegans*, and *Drosophila melanogaster* (Table 3.1). Interestingly, the

Figure 3.1
Amino acid sequence of At-IMPα and other protein homologues. The sequences of At-IMPα was aligned with the other importin α sequences found in *Saccharomyces cervisiae* (Sc-IMP; GenBank accession number Q02821), *Schizosaccharomyces pombe* (Sp-IMP; Z98887), *Homo sapiens* (Hs-IMP; P52294), *Mus musculus* (Mm-IMP; Q60960), *Xenpus laevis* (Xl-IMP; P52170), *Caenorhabditis elegans* (Ce-IMP; AFO40995), *Drosophila melanogaster* (Dm-IMP; A57319), and *Selaginella ledpidophylla* (Sl-IMP; U96718).

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At-IMP MSLRPNAKT....TEVRRNRYKV..AVDAEEGRRRREDNMVEIRKSKREESLMKKRR.........EGMQALQGFPSASAASV
Sc-IMP MDNGTDSSTSKFVPEYRRTNFRNKGRFSADELRRRRDTQQVELRKAKRDEALAKRRNFIPPTDGADSDEEDESSVSADQQFYS
Sp-IMP M.....SASSRFIPEHRRQNYKGKGTFQADELRRRRETQQIEIRKQKREENLNKKRNLV....DVQEPAEETIPLEQDKENDL
Hs-IMP M...TTPGKENF....RLKSYKNKS.LNPDEMRRRREEEGLQLRKQKREEQLFKKRNVATAEEETEEEVMSDGGFHEAQISNM
Mm-IMP M...STPGKENF....RLKSYKNKS.LNPDEMRRRREEEGLQLRKQKREEQLFKKRNVATAEEETEEEVMSDGGFHEAQINNM
Xl-IMP MPTTNEADE.......RMRKFKNKGK.DTAELRRRRVEVSVELRKARKDEQILKKRNVCLPEELI.........LSPEKNAMQ
Dm-IMP MSKA DSNS.......RQGSYKANSI.NTQDSRMRRHEVTIELRKSKKEDQMFKKRNI...NDED.........LTSPLKELN
Ce-IMP MSS.....N.......RQAYYKNNAK.EQIGKEKRNEEV.VSIRKDKREEAISKKRNINTQIEDD.........SETSTTPPG
Sl-IMP MSLRPQDRT.....NLRKKIYKT..TVDADEARRKREDNMVEIRKAKREEGLMKKRR.........EGMQAVL.F...GSGLV

At-IMP DKKLD..SLKDMVAGVWSDDPALQLESTTQFRKLLSIERSPPIEEVISA.GVVPRFVEFLKKEDYPAIQFEAAWALTNIASGT
Sc-IMP QLQQE...LPQMTQQLNSDDMQEQLSATVKFRQILSREHRPPIDVVQSA.GVVPRLVEFMRENQPEMLQLEAAWALTNIASGT
Sp-IMP ELELQ...LPLLVKALYSDIEAQIQATAKQFRKALSKETNPPIQKVIDA.GVVPRFVEFLSHEN NLLKFEAAWALTNVASGS
Hs-IMP EMAPGGVITSDMIEMIFSKSPEQQLSATQKFRKLLSKEPNPPIDEVISTPGVVARFVEFLKRKENCSLQFESAWVLTNIASGN
Mm-IMP EMAPGGVTTSDMTDMIFSNSPEQQLSATQKFRKLLSKEPNPPIDEVINTPGVVARFVEFLKRKENCTLQFESAWVLTNIASGN
Xl-IMP SVQVPPLSLEEIVQGMNSGDPENELRCTQAARKMLSRERNPPLNDIIEA.VLIPKLVEFLSRHDNSTLQFEAAWALTNIASGT
Dm-IMP GQSPVQLSVDEIVAAMNSEDQERQFLGMQSARKMLSRERNPPIDLMIGH.GIVPICIRFLQNTNNSMLQFEAAWALTNIASGT
Ce-IMP PFDANLLRL..TVAAAQSSDPAEQLTAVQQARKMLSTDRNPPIDDLIGS.GILPVLVQCLKSSTYPNLQFEAAWALTNIASGT
Sl-IMP EKKLE..RLPAMVRGVWSEDSAAQLEATTQFRRMLSIERSPPIEEVIAA.GVVPRLVQFLQRTDYPQLQFEAAWALTNIASGT

At-IMP SDHTKVVIDHNAVPIFVQLLASPSDDVREQAVWALGNVAGDSPRCRKLVLGCGALLPLLNQLNEHAKLSMLRNATWTLSNFCR
Sc-IMP DAQTKVVVDADAVPLFIQLLYTGSVEVKEQAIWALGNVAGDSTDYRDYVLQCNAMEPILGLFNSNKP.SLIRTATWTLSNLCR
Sp-IMP DNQTHVVVEANAVPVFVSLLSSSEQDVREQAVWALGNIAGDSPMCRDHVLQCGVLEPLLNIIESNRRLSMLRNSTWTLSNMCR
Hs-IMP SLQTRIVIQARAVPIFIELLSSEFEDVQEQAVWALGNIAGDSTMCRDYVLDCNILPPLLQLFSKQNRLTMTRNAVWALSNLCR
Mm-IMP SLQTRNVIQAGAVPIFIELLSSEFEDVQEQAVWALGNIAGDSTMCRDYVLNCNILPPLLQLFSKQNRLTMTRNAVWALSNLCR
Xl-IMP SDQTKSVVDGGAIPAFISLISSPHLHISEQAVWALGNIAGDGPLYRDALINCNVIPPLLALVNPQTPLGYLRNITWMLSNLCR
Dm-IMP SDQTRCVIEHNAVPHFVALLQSKSMNLAEQAVWALGNIAGDGAAARDIVIHHNVIDGILPLINNETPLSFLRNIVWLMSNLCR
Ce-IMP SEQTQAVVNAGAVPLFLQLLSCGNLNVCEQSVWALGNIIGDGPHFRDYCLELGILQPLLQFINPEIPIGFLRNVTWVIVNLCR
Sl-IMP SDHTAVVIEQGAVPIFVQLLSSPSDDVREQAVWALGNVAGDSPKCRDFVLGHNAM
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At-IMP GK.PQPHFDQVKPALPALERLIHSDDEEVLTDACWALSYLSDGTNDKIQTVIQAGVVPKLVELLLHHSPSVLIPALRTVGNIV
Sc-IMP GKKPQPDWSVVSQALPTLAKLIYSMDTETLVDACWAISYLSDGPQEAIQAVIDVRIPKRLVELLSHESTLVQTPALRAVGNIV
Sp-IMP GKNPQPDWNSISQVIPVLSKLIYTLDEDVLVDALWAISYLSDGANEKIQAIIDAGIPRRLVELLMHPSAQVQTPALRSVGNIV
Hs-IMP GKSPPPEFAKVSPCLNVLSWLLFVSDTDVLADACWALSYLSDGPNDKIQAVIDAGVCRRLVELLMHNDYKVVSPALRAVGNIV
Mm-IMP GKSPPPEFAKVSPCLNVLSWLLFVSDTDVLADACWALSYLSDGPNDKIQAVIDAGVCRRLVELLMHNDYKVVSPALRAVGNIV
Xl-IMP NKNPYPPMSAVLQILPVLTQLMHHDDKDILSDTCWAMSYLTDGSNDRIDVVVKTGIVDRLIQLMYSPELSIVTPSLRTVGNIV
Dm-IMP NKNPSPPFDQVKRLLPVLSQLLLSQDIQVLADACWALSYVTDDDNTKIQAVVDSDAVPRLVKLLQMDEPSIIVPALRSVGNIV
Ce-IMP CKDPAPSPAVVRTILPALSLLIHHQDTNILIDTVWALSYLTDGGNEHIQMVIEAQVVTHLVPLLGHVDVKVQTAALRAVGNIV
Sl-IMP

At-IMP TGDDIQTQCVINSGALPCLANLLTQNHKKSIKKEACWTISNITAGNKDQIQTVVEANLISPLVSLLQNAEFDIKKEAAWAISN
Sc-IMP TGNDLQTQVVINAGVLPALRLLLS.SPKENIKKEACWTISNITAGNTEQIQAVIDANLIPPLVKLLEVAEYKTKKEACWAISN
Sp-IMP TGDDVQTQVIINCGALSALLSLLS.SPRDGVRKEACWTISNITAGNSSQIQYVIEANIIPPLIHLLTTADFKIQKEACWAISN
Hs-IMP TGDDIQTQVILNCSALQSLLHLLS.SPKESIKKEACWTISNITAGNRAQIQTVIDANIFPALISILQTAEFRTRKEAAWAITN
Mm-IMP TGDDIQTQVILNCSALQSLLHLLS.SPKESIKKEACWTISNITAGNRAQIQTVIDANMFPALISILQTAEFRTRKEAAWAITN
Xl-IMP TGTDKQTQAAIDAGVLSVLPQLLRHQ.KPSIQKEAAWAISNIAAGPAPQIQQMITCGLLSPLVDLLNKGDFKAQKEAVWAVTN
Dm-IMP TGTDQQTDVVIASGGLPRLGLLLQHN.KSNIVKEAAWTVSNITAGNQKQIQAVIQAGIFQQLRTVLEKGDFKAQKEAAWAVTN
Ce-IMP TGTDEQTQLVLDSGVLRFMPGLLAHY.KEKINKEAVWFVSNITAGNQQQVQDVFDAGIMPMIIHLLDRGDFPTQKEAAWAISN

At-IMP ATSGGSHDQIKYLVEQGCIKYLVEQGCIKPLCDLLVCPDPRIITVCLEGLENILKVGEAEKNLGHTGDMNYYAQLIDDAEGLE
Sc-IMP ASSGGLQRPDI.......IRYLVSQGCIKPLCDLLEIADNRIIEVTLDALENILKMGEADKE.ARGLNINENADFIEKAGGME
Sp-IMP ATSGGARRPDQ.......IRYLVEQGAIKPLCNLLACQDNKIIQVALDGIENILRVGELDRA.NNPDKINLYAVYVEDAGGMD
Hs-IMP ATSGG..SAEQ.......IKYLVELGCIKPLCDLLTVMDSKIVQVALNGLENILRLGEQEAK.RNGTGINPYCALIEEAYGLD
Mm-IMP ATSGG..SAEQ.......IKYLVELGCIKPLCDLLTVMDAKIVQVALNGLENILRLGEQEAK.RNGSGINPYCALIEEAYGLD
Xl-IMP YTSGGTVEQVVQLVQ.........CGVLEPLLNLLTIKDSKTILVILDAISNIFLAAE...KLGEQEKL...CLLVEELGGLE
Dm-IMP TTTSGTPEQIVDLIEK........YKILKPFIDLLDTKDPRTIKVVQTGLSNLFALAE...KLGGTENL...CLMVEEMGGLD
Ce-IMP VTISGRPNQVEQMV.K........LGVLRPFCAMLSCTDSQIIQVVLDGINNILKMAG...E..AAEQV...TSEIEECGGLD
Sl-IMP
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At-IMP KIENLQSHDNNEIYEKAVKILETYWLEEEDDETQQPPGVDGSQAGFQFGGNQAPVPSGGFNFS
Sc-IMP KIFNCQQNENDKIYEKAYKIIETY.FGEEEDAVDETMAPQNAGNTFGFGSNVNQ....QFNFN
Sp-IMP LIHECQNSSNSEIYQKAYNIIEKF.FGEEDEI..EELEPETVGDTFTFGTTQEP.A.GDFQFSATNAEDMAM
Hs-IMP KIEFLQSHENQEIYQKAFDLIEHY.FGTEDEDSSIAPQVDLNQQQYIFQQCEAP.M..EGFQL
Mm-IMP KIEFLQSHENQEIYQKAFDLIEHY.FGTEDEDSSIAPQVDLSQQQYIFQQCEAP.M..EGFQL
Xl-IMP KIEALQTHDNHMVYHAALALIEKY.FS GEEADDIALEPEMGKDAYTFQV...PNMQKESFNF
Dm-IMP KLETLQQHENEEVYKKAYAIIDTY.FSNGDDEAEQELAPQEVNGALEFNATQ.PKAPEGGYTF
Ce-IMP KIENLQNHENEDIYKLAFEIIDNF.FSSDDETGNVE...GAQSSAFGGDVPPVPDAPNGGWNFGK
Sl-IMP
```

	Percent Identity								
Protein	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
At-IMP	-	48.1	43.3	48.5	48.1	37.7	36.4	37.0	72.2
Sc-IMP	-	-	54.1	46.3	46.5	39.1	35.8	37.5	47.0
Sp-IMP	-	-	-	48.0	47.8	39.7	38.5	38.7	39.9
Hs-IMP	-	-	-	-	97.2	38.3	37.0	39.7	38.4
Ms-IMP	-	-	-	-	-	38.5	39.7	39.5	38.9
Xl-IMP	-	-	-	-	-	-	41.4	47.5	41.9
Ce-IMP	-	-	-	-	-	-	-	42.2	39.9
Dm-IMP	-	-	-	-	-	-	-	-	39.9
Sl-IMP	-	-	-	-	-	-	-	-	-

Table 3.1
Comparison of amino acid identities of At-IMPα and other importin α homologues. For accession numbers, see Figure 3.1.

At-IMPα is 72% identical to an importin α homologue found in lower plants, *Selaginella lepidophylla* (Table 3.1). Because importin α is highly conserved throughout the different kingdoms, we propose that At-IMPα has a similar role in nuclear import of proteins in plants.

Characterization of At-IMPα antibodies

Antibodies were raised against a GST-At-IMPα fusion protein that was expressed in *E. coli* and purified from glutathione agarose beads. The fusion protein was further purified from SDS polyacrilaminde gels before injection. As a means to purify At-IMPα antibodies, His-tagged *Arabidopsis* importin α was expressed in *E coli*, purified from Ni-agarose beads, and cross-linked to Affigel 10 beads. Immune sera from the injected rabbit was passed through the Affigel His-tagged At-IMPα column, and the specificity of the purified antibodies were checked by immunoblot analysis. *Arabidopsis* protein isolated from roots, leaves, stems and flowers were separated by 10% SDS-PAGE, blotted to nitrocellulose and probed with At-IMPα antibodies. A 56 kD protein was detected in all tissues (Figure 3.2, right panel). Nuclei and cytosol were isolated from tobacco protoplasts for immunoblot analysis. At-IMPα antibodies also detected specific bands in isolated nuclei and cytosol fractions, that were similar in molecular weight as the recombinant His-tagged At-IMPα (Figure 3.2, right panel). Interestingly, a 57 kD band was detected in both nuclei and cytosol, however the cytosol contained a band

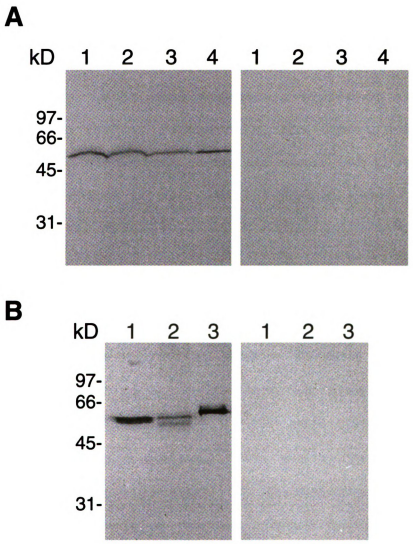


Figure 3.2
Purified antibodies to At-IMPα are specific in both *Arabidopsis* and tobacco. **(A)** Total protein (50 *ug*/gel lane) from *Arabidopsis* flowers (lanes 1), stems (lanes 2), leaves (lanes 3), or roots (lanes 4) was immunoblotted. Purified At-IMPα antibodies detected a protein of 56 kD in all tissues (left panel). The preimmune control blot is also shown (right panel). **(B)** Protein from tobacco cytosol (lanes 1) and nuclei (lanes 2) as well as purified His-tagged At-IMPα (lanes 3) was immunoblotted. Purified At-IMPα antibodies detected protein doublets of 56 kD in cytosol and nuclear extracts (left panel). The preimmune control blot is also shown (right panel)

at 56 kD whereas the nuclei contained a lower molecular weight band at 55 kD (Figure 3.2). The fact that importin α doublets were found in *Arabidopsis* and tobacco was not surprising since similar doublets have been found in bovine erthrocytes (Adam and Adam 1994), *Xenopus* cytosol (Gorlich et al., 1995; Moroianu et al., 1995), yeast (Belanger et al., 1994; Loeb et al., 1995), and *Drosophila* (Torok et al., 1995; Kussel and Frasch 1995). Furthermore, the importin α doublet in *Drosophila* has been shown to be the result of protein phosphorylation (Torok et al., 1995). We concluded that At-IMPα antibodies are specific in both *Arabidopsis* and tobacco plants.

At-IMPα binds to three classes of NLSs

To address the function of At-IMPα's, 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 mix was separated by SDS-PAGE, and analyzed by autoradiography (Figure 3.3, lane 1). Next, purified At-IMPα antibodies were added to the translation mix 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 (Figure 3.3, lane 2). The addition of 500

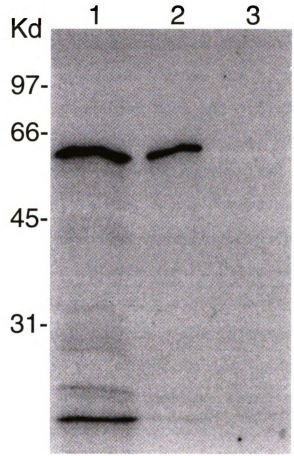


Figure 3.3
Immunoprecipitation of in vitro-translated At-IMPα. *At-IMPα* was in vitro translated with [^{35}S]Met (lane 1) and immunoprecipitated with purified At-IMPα antibodies (lane 2). Immunoprecipitation was also examined in the presence of 500 ng of recombinant At-IMPα (lane 3). The samples were analyzed by autoradiography after SDS-PAGE.

ng recombinant At-IMPα competed with the in vitro translated At-IMPα for immunoprecipitation (Figure 3.3, lane 3). Preimmune sera did not immunoprecipitate in vitro translated At-IMPα (data not shown).

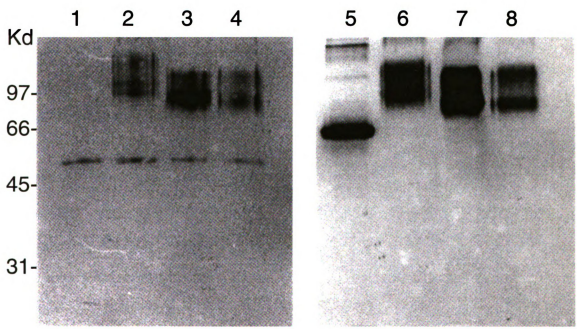
We next investigated whether At-IMPα could bind to NLSs in vitro. Representative NLS peptides from each of the three classes of NLSs were selected. The first NLS synthesized was the bipartite NLS, 02WT, identified in the maize transcription factor, Opaque-2 (Figure 3.4 A, Varagona et al., 1992). The SV40 T-antigen NLS, identified from the simian virus 40 large T-antigen (Kalderon et al., 1984; Lanford and Butel, 1984), was also synthesized (Figure 3.4 A). Lastly, a Mat α2-like NLS, NLSC, was synthesized which corresponds to one of the NLSs identified in the maize transcription factor, R (Figure 3.4 A, Shieh et al., 1993). These NLSs are functional in vivo (Varagona et al., 1992; Shieh et al., 1993), and they bind specifically to the NLS binding site in tobacco nuclei (Hicks and Raikhel, 1993; Hicks et al., 1995). A fourth peptide, 02mut (Figure 3.4 A), was also synthesized which corresponds to a mutant form of 02WT which 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). The peptides corresponding to the functional NLSs were chemically coupled to human serum albumin (HSA) and allowed to interact with recombinant At-IMPα. Then, At-IMPα antibodies were added to the binding assay, and the protein complexes were immunoprecipitated with

Figure 3.4
At-IMPα recognizes three classes of NLSs. **(A)** Amino acid sequences of peptides corresponding to the wild-type bipartite (02WT) and mutant (02mut) 02 NLS from Opaque-2 and the SV40 large T-antigen NLS (SV40) and the Mat α2-like NLS (NLSC) from the R protein are shown in a single-letter code. **(B)** HSA does not coimmunoprecipitate 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) coimmunoprecipitate 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-HSA (lane 8) were immunoblotted directly to nitrocellulose to determine the migration pattern of these cross-linked substrates after SDS-PAGE.

A

02WT	MPTEERVRKR KESNRESARRS RYRKAAHLK C
02mut	MPTEERVRTN KESNRESARRS NYRKAAHLK C
SV40	CTPPKKKRKV
NLSC	CYMISEALRK AIGKR

B



protein A Sepharose. The immunoprecipitated proteins were separated by SDS-PAGE and the NLS-HSA substrate that co-immunoprecipitated with At-IMPα was detected by Western blot analysis using monoclonal antibodies against HSA. Figure 3.4 B, lane 1 demonstrates that HSA alone was not co-immunoprecipitated with At-IMPα. However, co-immunoprecipitation occurred when HSA was coupled with peptides to the O2WT (Figure 3.4 B, lane 2), SV40 T-antigen (Figure 3.4 B, lane 3), or NLSC (Figure 3.4 B, lane 4). Figure 2B shows the mass of HSA (Figure 3.4 B, lane 8), 02WT-HSA (Figure 3.4 B, lane 7), SV40 T-antigen-HSA (Figure 3.4 B, lane 6), and NLSC-HSA (Figure 3.4 B, 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 due to variations in the number of peptides coupled per HSA molecule.

At-IMPα binding is specific for functional NLSs

To test the specificity of whether At-IMPα binding for functional NLSs, competition studies were set up using the 02WT and 02mut peptides (Figure 3.4 A). The functional substrate, 02WT-HSA, was co-immunoprecipitated with recombinant At-IMPα (Figure 3.5, lane 1). In the presence of 1 mM of 02WT peptide 02WT-HSA binding was greatly reduced (Figure 3.5, lane 2), whereas 1 mM 02mut peptide did not compete with 02WT-HSA for At-IMPα binding (Figure 3.5, lane 3). In addition, 02WT-HSA binding to At-IMPα can be competed

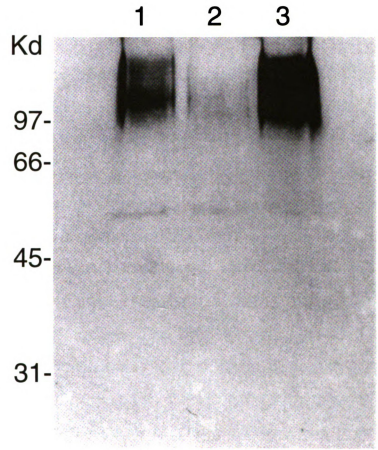


Figure 3.5
Specific interaction of At-IMPα with a functional NLS substrate; 02WT-HSA is coimmunoprecipitated with At-IMPα (lane 1). Specificity of At-IMPα binding was determined by coimmunoprecipitation of 02WT-HSA in the prescence of 02WT (lane 2) or 02mut (lane 3) peptides. The secondary goat anti-mouse antibodies cross-react with the IgG heavy chain (50 kD protein band) of the rabbit importin α antibodies.

with 200 um of 02WT peptide (H.M.S. Smith and N.V. Raikhel, unpublished). Specific NLS binding indicates that At-IMPα is likely to be a functional NLS-receptor in plants. Furthermore, this single receptor recognizes the three classes of NLSs found in plants.

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 um optical section through the protoplasts indicated that importin α was located in the nucleus and cytoplasm (Figure 3.6, A). 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). It also appears that importin α is excluded from the nucleolus in protoplasts and isolated nuclei (Figure 3.6; unstained region in the nucleus). Intense immunofluorescence at the nuclear envelope demonstrated that importin α was highly concentrated at the nuclear envelope (Figure 3.6, A; yellow region). A 1.0 um optical section displays similar fluorescence at the nuclear envelope when using tobacco nuclei purified in the presence

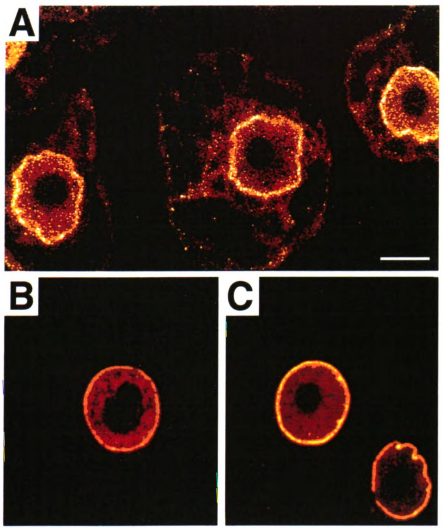


Figure 3.6
Immunolocalization of importin α in fixed tobacco protoplasts **(A)** or purified nuclei **(B and C)** were visualized by confocal laser scanning microscopy using affinity-purified At-IMPα antibodies followed by CY3-labeled secondary antibodies. Bar = 10 *u*m.

of 0.6% Triton X-100 (Figure 3.6, 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), however in yeast it is associated with the NPC (Yano et al., 1992; Belanger et al., 1994, Aitchison et al., 1996). In plants, previous studies demonstrate that importin α is strongly associated with cellular structures in tobacco permeabilized cells, even in the presence of 0.1% Triton X-100 (Hicks et al., 1996). In order 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 minutes with 1% Triton X-100, 0.25 M NaCl, 1% Triton X-100 plus 0.25 M NaCl, or 6M urea. Samples were centrifuged and the nuclear pellet (P) and supernatant (S) were examined by immunoblot analysis using At-IMPα antibodies. Most of importin α was resistant to extraction by 1% Trition X-100 (Figure 3.7, lanes 1), 0.25 M NaCl (Figure 3.7, lanes 2) and 1% Triton X-100 plus 0.25 M NaCl (Figure 3.7, lanes 3); however, treatment of nuclei with 6 M Urea extracted some of the importin α (Figure 3.7, lanes 4). Importin α is also partially extracted with 0.5 M NaCl (H.M.S. Smith and N.V. Raikhel, unpublished). These biochemical properties indicate that importin α is tightly associated with the plant nucleus. In addition, the

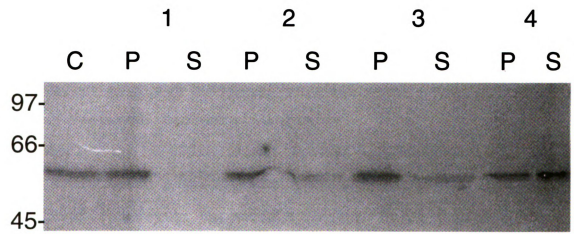


Figure 3.7
Biochemical properties of nuclear importin α. Tobacco nuclei were treated with 1% Triton X-100 (lanes1), 0.25 M NaCl (lanes 2), 1% Triton X-100 plus 0.25 M NaCl (lanes 3), or 6 M urea (lanes 4). After treatment the samples were centrifuged and the supernatant (S) and nuclear pellet (P) were extracted with 2% SDS-PAGE sample buffer. Untreated nuclei were extracted with 2% SDS-PAGE sample buffer (C). The samples were separated by 10% SDS-PAGE, and importin α was detected with affinity-purified At-IMPα antibodies.

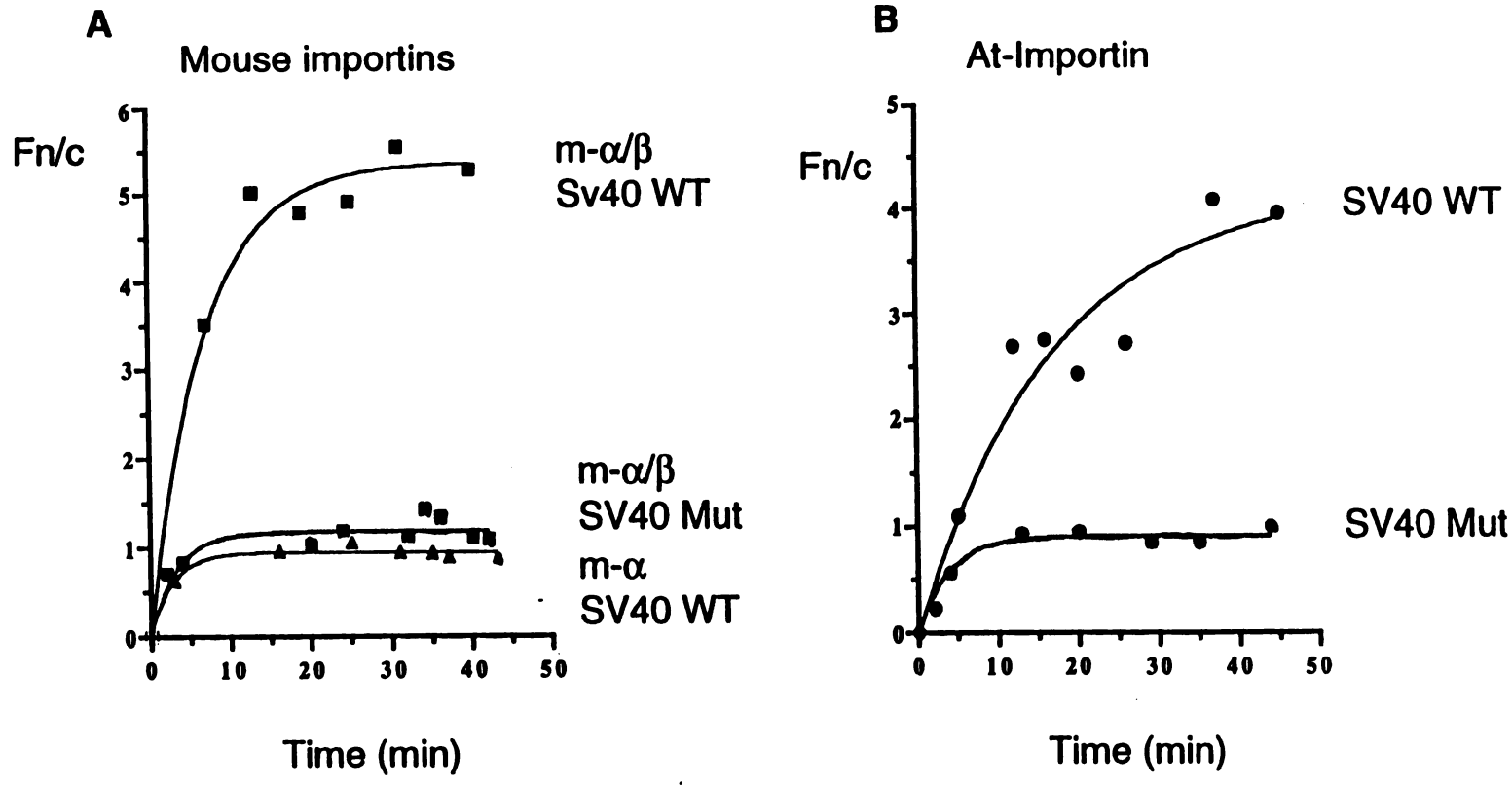
biochemical properties of nuclear importin α and NPC proteins (Heese-Peck et al., 1995) correlate closely, indicating that a fraction of nuclear importin α is probably associated with the NPC.

At-IMPα can mediate nuclear protein import independent of importin β

These experiments were done in collaboration with Dr. David A. Jans's group at the Nuclear Signaling Laboratory, Division for Biochemistry and Molecular Biology, John Curtin School of Medical Research, Canberra, Australia.

Recently, an in vitro import system was developed in plants using evacuolated permeabilized tobacco protoplasts (Hicks et al., 1996). However, importin α is not depleted from these permeabilized cells and its addition does not stimulate import (Hicks et al., 1996; H.M.S. Smith, G.R. Hicks and N.V. Raikhel, unpublished data). Therefore, we analyzed the function of At-IMPα in permeabilized vertebrate cells. We compared the ability of At-IMPα to that of the mouse importins (m-IMP) to mediate nuclear import in a reconstituted in vitro system. In the presence of RanGDP and p10, m-IMPα or β alone could not mediate nuclear import of SV40 T-antigen NLS substrate (data not shown; Table 3.2), levels of nuclear accumulation being similar to those in the absence of importin subunits (data not shown). This was in contrast to the combination of m-IMPα/β where maximal nuclear accumulation relative to that in the cytoplasm (Fn/c_{max}) was

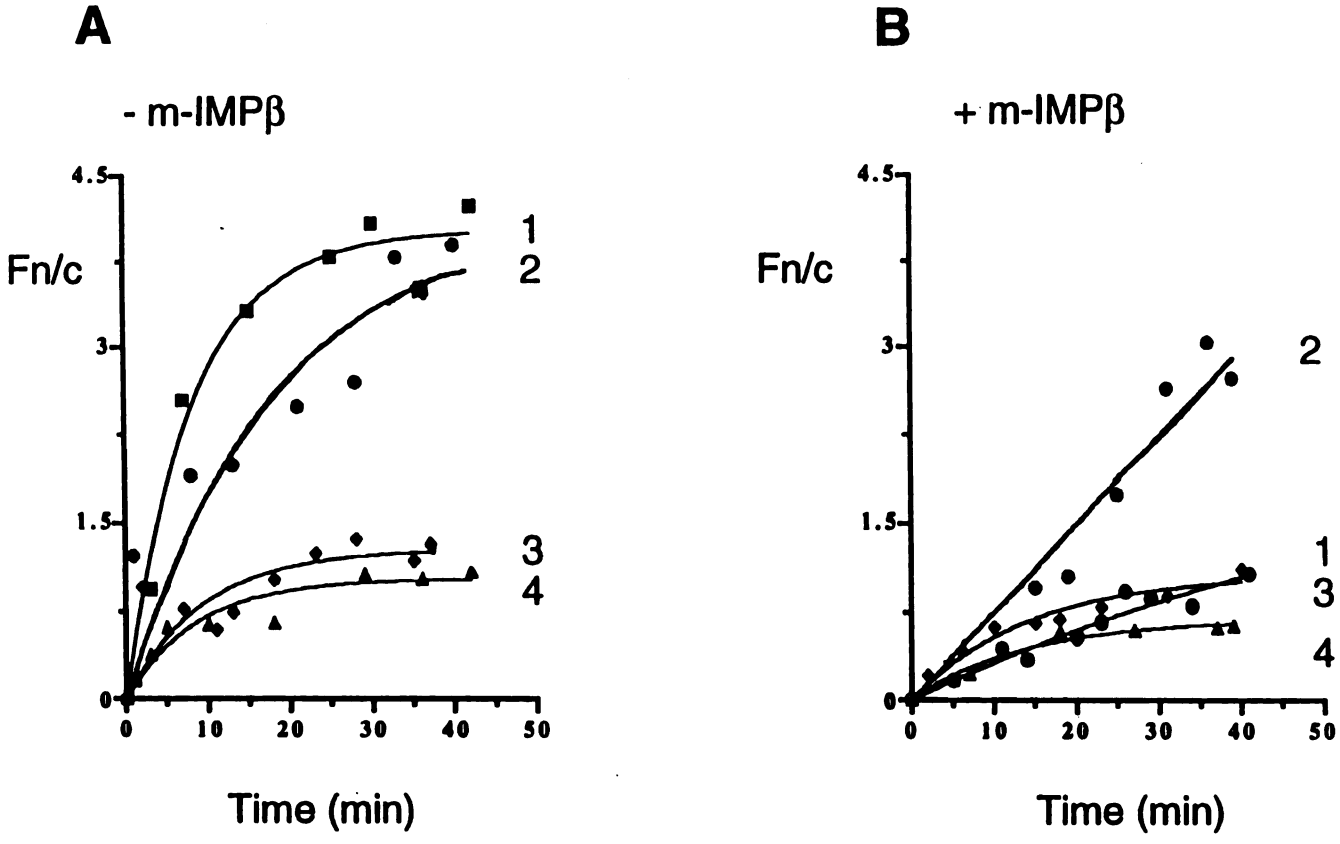
Figure 3.8
Ability of At-IMPα to mediate nuclear protein import reconstituted in vitro using purified components in the absence of exogenously added importin β subunit. Nuclear import was reconstituted in mechanically perforated rat HTC cells in the presence of an ATP-regenerating system containing GTP/GDP and using p10 and GDP-loaded Ran as described in the Methods. Quantitative results for nuclear import kinetics of the fusion proteins SV40WT-β-Galactosidase and SV40mut-β-Galactosidase mediated by mouse importin subunits **(A)** or At-IMPα **(B)** in the presence of Ran and p10. Results shown are from a single typical experiment where each data point represents at least 5 separate measurements of nuclear fluorescence (Fn), cytoplasmic fluorescence (Fc) and background fluorescence (see methods). Data was fitted for the function $Fn/c = Fn/c_{max} (1-e^{-kt})$, where Fn/c is the nuclear/cytoplasmic ratio, k is the rate constant, and t is the time in minutes. Pooled data are presented in Table 3.2.


A
Mouse importins
Fn/c
m-α/β
Sv40 WT
m-α/β
SV40 Mut
m-α
SV40 WT
Time (min)
B
At-Importin
Fn/c
SV40 WT
SV40 Mut
Time (min)

over 5-fold (Figure 3.8 A; Table 3.2; half-maximal within 2.6 min). Significantly, At-IMPα, in the absence of exogenously added importin β subunit, was able to mediate nuclear import to comparable levels (Figure 3.8 B and 3.9 A; Fn/c_{max} of 3.7 - half-maximal within 8 min). The specificity of transport in all cases was demonstrated by the fact that the mutant SV40 T-antigen NLS substrate did not accumulate in the nuclei to any significant extent (Figure 3.8 A and B; Table 4). The results thus demonstrated that At-IMPα could mediate nuclear import of an NLS-containing transport substrate independent of an importin β subunit. Nuclear import mediated by At-IMPα was enhanced by p10, especially in terms of the rate of import. Nuclear import did not absolutely appear to require p10, since maximal import levels of 3.2 were observed in its absence (Figure 3.9; Table 3.2).

In vitro binding studies showed that At-IMPα can bind to yeast and mouse importin β (Hubner et al., submitted). Nuclear import mediated by At-IMPα was tested in the presence of the m-IMPβ subunit (Figure 3.9 B). Interestingly, nuclear import was reduced by more than 50%, compared to in the absence of m-IMPβ, in either the absence or presence of p10 (Figure 3.9 B; Table 3.2). This inhibition of At-IMPα-mediated transport by m-IMPβ is unlikely to be due to its ability to bind At-IMPα since At-IMPα's affinity for SV40 T-antigen NLS is over two times higher than that for m-IMPβ (Hubner et al., submitted). Several studies (eg. Fagotto et

Figure 3.9
Nuclear import reconstituted in vitro using purified components mediated by At-IMPα and m-IMPα in the absence **(A)** and presence **(B)** of exogenously added m-IMPβ. **(A)** Nuclear import kinetics of the fusion protein SV40WT-β-Galactosidase mediated (1) At-IMPα plus Ran plus p10, (2) At-IMPα plus Ran, (3) m-IMPα plus Ran plus p10, or (4) Ran plus p10. **(B)** Nuclear import kinetics of the fusion protein SV40WT-β-Galactosidase mediated (1) At-IMPα plus Ran, (2) At-IMPα plus Ran plus p10, (3) At-IMPα, or (4) Ran plus p10. Import assays were measured in the presence of an ATP-regenerating system containing GTP/GDP. Experiments for transport were performed as described in the legend to Figure 3.8. Results shown are from a single typical experiment where each data point represents at least 4 separate measurements of Fn, Fc, and background fluorescence. Pooled data are presented in Table 3.2.


A
- m-IMPβ
Fn/c
4.5
3
1.5
0
0
10
20
30
40
50
Time (min)
1
2
3
4
B
+ m-IMPβ
Fn/c
4.5
3
1.5
0
0
10
20
30
40
50
Time (min)
2
1
3
4

al., 1998) have shown that importin β can inhibit nuclear import through effects not directly related to substrate recognition, but rather to binding to NPC components, implying that the effect observed in Figure 3.9 B may be similarly mediated.

Nuclear import kinetic measurements were also performed in vitro for the first time using the bipartite NLS identified in the *Xenopus* histone binding protein NIN2 (Kleinschmidt and Seiter, 1988). Peptides to this NLS were fused to β-galactosidase (N1N2-β-Gal) and produced results similar to those for SV40 T-antigen NLS substrates when they were added to the in vitro import system (Table 3.2 and data not shown). Maximal levels of At-IMPα-mediated import (Fn/c_{max} of about 3.4; half-maximal within 15.5 min) were obtained in the presence of RanGDP; the rate of import was almost twice as fast in the presence of p10 ($t_{1/2}$ of c. 9 min; Table 3.2). In the presence of m-IMPβ, accumulation was reduced by c. 40 and 60% in the presence and absence of p10 respectively.

Table 3.2
Kinetics of nuclear import reconsituted in vitro using purified subunits.

	Conditions			Nuclear Import	
parameters+ Receptor	Substrate	Ran	p10	Fn/c_{max}	$t_{1/2}$ (min)
At-IMPα	SV40WT-β-Gal	+	+	3.73 ± 0.66	08.1 ± 2.8
	SV40WT-β-Gal	+	-	3.21 ± 1.1	14.3 ± 0.8
	SV40WT-β-Gal	-	-	1.30 ± 0.07	N D
	SV40mut-β-Gal	+	+	0.92 ± 0.11	N D
	NIN-β-Gal	+	+	3.31 ± 0.70	09.0 ± 4.7
	NIN-β-Gal	+	-	3.39 ± 0.21	15.5 ± 0.5
At-IMPα/m-IMP β	SV40WT-β -Gal		+	+ 1.80 ± 0.10	35.5 ± 3.3
	SV40WT-β -Gal	+	-	1.78 ± 0.33	15.4 ± 0.4
	SV40WT-β -Gal	-	-	1.39 ± 0.47	N D
	NIN-β-Gal	+	+	1.91 ± 0.85	07.9 ± 1.0
	NIN-β-Gal	+	-	1.24 ± 0.34	N D
	NIN-β-Gal	-	-	1.13 ± 0.34	N D
m-IMPα	SV40WT-β-Gal	+	+	1.46 ± 0.28	N D
	SV40WT-β-Gal	-	-	1.20 ± 0.07	N D
	SV40mut-β-Gal	+	+	0.98 ± 0.08	N D
	NIN-β-Gal	+	+	1.25 ± 0.27	N D

m-IMPα/β	SV40WT-β-Gal	+	+	5.12 ± 0.54	2.6 ± 1.1
	SV40WT-β-Gal	+	-	3.76 ± 0.85	5.3 ± 2.2
	SV40WT-β-Gal	-	-	01.0 ± 0.04	N D
	SV40mut-β-Gal	+	+	1.37 ± 0.18	N D
	NIN-β-Gal	+	+	4.48 ± 0.97	13.9 ± 6.1
m-IMPβ	SV40WT-β-Gal	+	+	1.15 ± 0.20	N D
	NIN-β-Gal	+	+	1.42 ± 0.24	N D
none	SV40WT-β-Gal		+	+ 0.88 ± 0.28	N D
	SV40WT-β-Gal	-	-	0.84 ± 0.06	N D
	NIN2-β-Gal	+	+	1.13 ± 0.09	N D

+Mean ± SEM for Fn/c_{max} and $t_{1/2}$ from data (see Figures 3.8 and 3.9) fitted to the function $Fn/c(t)=Fn/c_{max}*(1-e^{-kt})$, where Fn/c_{max} is the maximal level of nuclear accumulation,
k is the rate constant, and t is time in minutes.
*SE from curve fit.
The abbreviations used are: SV40WT-β-Gal, SV40WT large T-antigen NLS fused to β-galactosidase; SV40mut-β-Gal, SV40mut large T-antigen NLS fused to β-galactosidase; NIN2-β-Gal, NIN2 bipartite NLS fused to β-galactosidase; N D, not determined.

DISCUSSION

Importin α is a cytosolic NLS receptor identified in vertebrates which mediates import in vertebrate 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: it facilitates NLS binding to the NPC in permeabilized cell systems (Adam and Adam, 1994; Chi et al., 1995; Gorlich et al., 1995a,b; Imamato et al., 1995b; Moroianu et al., 1995; Radu et al., 1995a,b); it specifically binds to functional NLSs 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 mutations in the yeast importin α, SRP1, block NLS-protein import in vivo (Loeb et al., 1995).

Our study was aimed at determining for the first time whether or not At-IMPα functions as an NLS-receptor in plants. As a means to determine the function of importin α, we used an in vitro binding/co-immunoprecipitation assay. We demonstrated that At-IMPα binds specifically to NLSs. Moreover, it is capable of recognizing three classes of NLSs, suggesting that a single receptor recognizes the different classes of NLSs. In humans, two importin α isoforms bind specifically to the SV40 T-antigen and bipartite NLSs, and the yeast Srp1 has been shown to bind to the SV40 T-antigen NLS (Moroianu et al., 1995; Enenkel, et al., 1995). Because Srp1 is a single gene in yeast (Yano et al., 1992), it should

be able to interact with multiple types of NLSs, similar to At-IMPα. In vertebrates and yeast, importin α's NLS binding affinity is enhanced when its associated with importin β (Gorlich et al., 1995a; Rexach and Blobel, 1995; Efthymiadis et al., 1997; Hubner et al., 1997). However, we found that At-IMPα, from plants, binds NLSs with high affinity in the absence of a β subunit, and that this NLS binding affinity is similar to that possessed by yeast and mammalian importin α/β heterodimers (S. Hubner, H.M.S. Smith, W. Hu, C.K. Chan, H-P. Rihs, B.M. Paschal, N.V. Raikhel, and D.A. Jans unpublished data). Further, although At-IMPα is able to bind the importin β subunits from yeast or mouse, the NLS binding affinity was unaffected. In addition, importin β functions as a receptor for a subset of NLS-containing proteins including the yeast Gal4 transcription factor (Palacios, et al., 1997; Tiganis et al., 1997; Chan et al., 1998). In contrast to the mouse importin α, At-IMPα associates with the bipartite NLS found in GAL4, indicating that At-IMPα can also mediate the import of the importin β NLS-cargo (S. Hubner, H.M.S. Smith, W. Hu, C.K. Chan, H-P. Rihs, B.M. Paschal, N.V. Raikhel, and D.A. Jans unpublished data). To date, it is not known if an importin β subunit exists in plants. If a β subunit exists in plants, it would be interesting to know if functions in similar manner as the mammalian and yeast β subunits.

Importin α is encoded by a multigene family in *Arabidopsis* (H.M.S. Smith and N.V. Raikhel, unpublished), vertebrates (Gorlich et al., 1994; Morianou et al., 1995; Gorlich and Mattaj, 1996), and *Drosophila* (Gorlich and Mattaj, 1996), but a single gene is found in yeast (Yano et al., 1992). Studies in vertebrates show that many of the importin α isoforms are expressed in a tissue specific manner, indicating that different isoforms of receptors may be involved organogenesis (Prieve et al., 1996; Kohler et al., 1997; Tsuji et al., 1997; Ryder et al., 1998). In addition, import is also controlled at the level of NLS recognition since some isoforms also recognize specific types of NLSs (Miyamoto et al., 1997; Nadler et al., 1997; Sekimoto et al., 1997). At-IMPα may represent a general NLS-receptor plants, because it recognizes three classes of NLSs and it is expressed in roots, leaves, stems and flowers (H.M.S. Smith and N.V. Raikhel, unpublished). Further characterization of the entire importin α gene family in *Arabidopsis* is required to reveal if other isoforms have unique features in NLS-protein import in plants.

In vertebrates, it has been demonstrated that importin α is a nuclear shuttling receptor (Gorlich et al., 1996a, Weis et al., 1996). Our immunolocalization studies of importin α in tobacco protoplasts demonstrate that it is found in the nucleus and cytoplasm. Therefore, importin α is probably a nuclear shuttling receptor in plants. 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 find that importin α is highly concentrated at the nuclear envelope in vivo and tightly associated with purified nuclei, 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), co-fractionates with NPC proteins (Aitchison 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 it cannot associate with the NPC unless it forms a heterodimer with importin β.

Nuclear import of NLS-containing proteins recognized by importin α is dependent on importin β for the NPC docking and translocation steps in yeast and vertebrates (Enenkel et al., 1995; Görlich et al., 1995; Radu et al., 1995; see Jans and Hübner, 1996). We found that At-IMPα mediates NPC docking (data not shown) and import in the absence of exogenously added importin β subunit demonstrating that At-IMPα can fulfill the NLS-binding and NPC docking/import roles of the importin α/β heterodimer. These results suggest that plants possess a NLS-protein import pathway exclusively mediated by importin α subunits. Future work should be directed to determine if a β subunit exists in plants. If so, it would

be possible to determine if the "classical" NLS-protein import pathway mediated by the importin α/β heterodimer exists in plants.

Previously, we have characterized an NLS binding site at the nuclear envelope and the NPC in plants (Hicks and Raikhel, 1993; Hicks et al., 1995; see Chapter 1). In addition, two NLS binding proteins (NBPs) between 50-60 kD were identified biochemically and are similar in mass as importin α (Hicks and Raikhel, 1995a). Although we were unsuccessful in immunoprecipitating these NBPs (Hicks and Raikhel, 1995a) using purified At-IMPα antibodies (H.M.S. Smith and N.V. Raikhel, not shown), several pieces of evidence suggest that importin α is a component of the NLS binding site; 1) both are located at the nuclear envelope and NPCs in purified nuclei from tobacco, 2) the biochemical properties of nuclear importin α are similar to the ones of the NLS binding site and NBPs, 3) the NLS binding site and At-IMPα interact specifically with NLSs, and 4) both recognize three classes of NLSs. All and all, 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.

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Chapter 4

Association of Importin α with the Cytoskeleton

This paper was submitted for publication:

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Abstract

Importin α is the nuclear localization signal (NLS) receptor that is involved in NLS-protein import into the nucleus. Using importin α as a tool, we were interested in determining if 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. Subcellular localization studies indicated that a fraction of the cellular importin α cofractionated with the cytoskeleton and is extracted from this fraction under conditions similar to those that extract microtubule motor proteins. Lastly, 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 is a receptor mediated process facilitated by the importin β-like proteins, in conjunction with a small GTPase, 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 which contain the classical nuclear localization signals (NLSs; Hicks and Raikhel, 1995; Jans and Hubner, 1996; Gorlich, 1997). The α subunit of the heterodimer specifically binds to NLSs in the cytoplasm, while 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-proteins (Palacios et al., 1997; Tiganis, et al., 1997). Interestingly, the importin α homologue (At-IMPα) from *Arabidopsis thaliana* specifically interacts with three types of NLSs (Smith, et al., 1997; Hicks, et al., 1996) and can facilitate NLS-protein import in the absence of a β subunit (S. Hubner, H.M.S. Smith, W. Hu, C.K. Chan, H-P. Rihs, B.M. Paschal, N.V. Raikhel, and D.A. Jans unpublished data). Thus, variations within the NLS-protein import pathway exist, where the individual α and β subunits can also function independently of each other. Although many 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 understand how importin α is targeted to the NPC and to identify cellular factors involved in this process, we were interested in analyzing if importin α interacts with the cytoskeleton.

METHODS

Materials

All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise noted. NLS peptides were synthesized at the Peptide Synthesis Facility (Yale University, New Haven, CT). Antibodies to 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). Secondary antibodies, BODIPY labeled goat anti-mouse and BODIPY labeled pallodian were purchases from Molecular Probes Inc. (Eugene, OR). Secondary antibodies to importin α, Goat anti-rabbit CY3 conjugated antibodies, were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Nucleotides were purchased from Boehringer Mannheim (Indianapolis, IN). Cytochalasin 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). *Nictotiana tabacum* cell suspension culture transformed with *β-glucuronidase, GUS,* was a generous supplied gift from Dr. P.J. Green and monospecific antibodies to GUS were generous supplied gift from Dr. G.R. Hicks.

Immunolocalization in tobacco protoplasts

Nictotiana tabacum suspension-culture cells were maintained and protoplasts were prepared as described by (Hicks and

Raikhel, 1993). Fixation and immunolocalization of importin α were performed as described (Smith et al., 1997). Briefly, fixed protoplasts were dehydrated in cold methanol for 10 min, then washed in PBST (PBS, 0.5 % Tween 20) for 30 min at 23°C, then blocked for 20 min in PBST plus 5% BSA at room temperature. For single labeling experiments, 150 ng of affinity purified At-IMPα rabbit antibodies and tubulin monoclonal antibodies were mixed with 50 ul of antibody buffer (AB buffer; PBST plus 1% BSA), then applied to the fixed samples. At-ELP immune or preimmune sera (Ahmed et al., 1997) was diluted 1:75 in AB buffer and added to fixed samples. Protoplasts prepared from these *GUS* expressing cell cultures were fixed and incubated with monospecific antibodies to GUS, diluted 1:50 in AB buffer. Fixed protoplasts, not transformed with *GUS*, were used as controls to determine the non-specific staining of the GUS antibodies. Each of the individually stained samples were incubated at 23°C for 1 hr in a moist chamber, then washed in PBST for 30 min. The secondary antibodies, CY3-labeled goat anti-rabbit antibodies and BODIPY-conjugated goat anti-mouse antibodies, were diluted 1:50 in AB buffer and applied to the appropriate samples. Samples were incubated for 1 hr in a moist dark chamber, then washed in PBST for 30 min at 23°C. Samples were mounted with a 50/50 mixture of Slow Fade and Permafluor.

In double labeling experiments, protoplasts were fixed, dehydrated, and washed in PBST as described above. 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. For colocalization experiments with importin α and actin, 150 ng of At-IMPα rabbit antibodies and BODIPY conjugated phalloidin (final concentration was 1 uM) were mixed together in 50 ul of AB buffer. To detect actin in protoplasts treated with cytochalasin B or D, 150 ng of At-IMPα rabbit antibodies and actin monoclonal antibodies were mixed together in AB buffer. The three immuno-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, 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 incubated for 1 hr in a moist dark chamber. The samples were washed and mounted as described above. No cross reactivity was detected in samples incubates 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, protoplasts were treated with 10 um oryzalin or 25 um proyzamide (data not shown) for 1 hr to disrupt microtubules, prior to fixation. Microfilaments were depolymerized with either 50 ug/ml of cytochalasin B or 50-100 um cytochalasin D (data not shown) for 1 hr, prior to 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, 30g/L sucrose adjusted pH 5.7) to remove the depolymerizing agents. Protoplasts were incubated for 2-3 hr at 23°C before fixation and immunolocalization (data not shown).

Confocal Laser Scanning Microscopy

Confocal laser scanning microscopy was performed with a Zeiss Axioskop (Carl Zeiss) slit scanning microscope equipped with the Meridan InSIGHT PLUS™ imaging system (Genomics Solutions Lansing Div., Okemos, MI). Excitation of the BODIPY and CY3 chromophores were performed with a 488-nm laser using a 505-nm dichroic mirror and a 515 barrier filter. Samples were viewed with a 63X oil immersion objective and the BODIPY and CY3 fluorescence was detected with the 515-545 nm and 580-600 nm filters, respectively. Images were collected with cooled DNC 1000 cooled and intensified CCD camera and viewed with the InSIGHT™ imaging system. For double immunofluorescence studies, fluorescence from BODIPY and CY3 were collected simultaneously then separated with the RGB program. There was no fluorescence crossover detected with single labeled controls (data not shown). Figures were composed with photoshop 3.0 (Adobe Systems, Mountain View, CA). Optical sections for these studies were 1 um thick.

Isolation of cytoskeleton fractions from tobacco protoplasts

For cell fractionation studies, protoplasts were resuspened and incubated in cold PM buffer plus protease inhibitors (50 mM Pipes, 1 mM $MgSO_4$, 1 mM EGTA, pH 6.8 plus 2 mM phenylmethylsulfonyl fluoride, and 5 ug/mL of caproic acid, pepstatin, aprotinin, and leupeptin) and lysed with a french press at 450 p.s.i. This lysate was centrifuged twice at 5,000*g* for 30 min at 4°C to pellet unbroken cells, nuclei and heavy organelles. The 5,000*g* supernantant was centrifuged at 27,000*g* for 90 min at 4°C to pellet the cyotskeleton (Marc et al., 1996). Finally, the 27,000*g* supernatant was centrifuged at 100,000*g* for 90 min at 4°C. The pellets were resuspended in PM buffer plus protease inhibitors and the protein concentration was determined for all the fractions. 10 ug or 50 ug of total protein was used to detect importin α and the cytoskeleton markers, respectively, by immunoblot analysis as described by Hicks et al., 1996.

Biochemical properties of importin α in the cytoskeleton fraction

To determine the biochemical properties of importin α in the cytoskeleton fraction, 27,000*g* pellets were isolated and resuspended in cold PM buffer plus protease inhibitors containing either 10 mM CHAPS (3[{3-Cholamidopropyl}-dimethylammonio]-1-propane-sulfonate), 1.0% Triton X-100, 0.5 M NaCl plus 5 mM ATP/GTP, 8 M urea or 0.5 M NaCl. The

samples were incubated for 20 min at 4°C, then centrifuged at 100,000*g* for 30 min at 4°C. Equal volumes, 10 *u*l, of the supernatant was mixed with SDS-sample buffer, separated by SDS-PAGE and importin α was detected by immunoblot analysis as described by Hicks et al., 1996.

Preparation of the plant cell extract

A plant cell extract was prepared by isolating 27,000*g* pellets and extracting it with cold PM buffer plus protease inhibitors containing 0.5 M NaCl and 5 mM ATP/GTP for 20 min. For microtubule association assays, the extracts were pelleted at 100,000*g* for 2 hrs at 4°C then dialyzed in cold PM buffer plus 1 mM DTT to remove the NaCl and nucleotides. The desalted extract was concentrated with a concentration device (Millipore, Bedford, MA) and 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 the following; 1) all centrifugation steps were performed at 150,000*g*, 2) all fractions were dialyzed against cold actin stabilization buffer (ASB; 5 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$, and 5 mM KCl) to remove NaCl and nucleotides. The protein concentration of these plant cell extracts was 3-5 mg/mL. Aliquots were frozen at -80°C and used in the cytoskeleton pelleting assays.

Microtubule association assay

For microtubule association assays, 100 *u*M of bovine brain tubulin (Cytoskeleton, Denver, CO) was polymerized in PM buffer plus protease inhibitors, 100 *u*M taxol, and 1mM GTP at 4°C for 30 min, followed by an incubation at 23°C for 30 min, then transfered to 37°C for 30 min. Polymerized microtubules were visualized by immunofluorescence (data not shown). To determine if importin α associates with microtubules, polymerized tubulin was diluted to 10-20 *u*M in 50-100 *u*l of the plant cell extract at 23°C. The functional NLS peptide 02WT or the mutated NLS 02MUT were added to a final concentration of 200 *u*m to the association assays. After the 30 min incubation period, samples were loaded onto a 40% sucrose in PM buffer containing 10 *u*M taxol and pelleted at 100,000*g* for 1 hr at 23°C. Microtubule pellets were resuspended in 0.5 M NaCl in PM plus protease inhibitors for 20 min then pelleted at 12,000*g* for 10 min to remove any insoluble material. Association of importin α with the microtubule pellets were detected by immunoblot analysis as described by Hicks et al., 1996. Control experiments were performed without added microtubules to determine if importin α could pellet through the 40% sucrose cushion under the same conditions.

Microfilament association assay

The actin binding protein biochemical kit BK001 (Cytoskeleton, Denver, CO) was used to determine if importin

α could associate with microfilaments. Actin polymerization, association and sedimentation experiments were performed according to the manufacturer's protocol. The functional NLS peptide 02WT or the mutated NLS 02MUT were added to a final concentration of 200 um in the microfilament association assays. 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 if importin α could pellet through the 10% glycerol cushion under the same conditions.

RESULTS

The cytoplasmic localization of importin α is distinct from the immunostaining patterns of soluble and membrane proteins

Immunofluorescence confocal laser scanning microscopy (CLSM) was used to compare the cytoplasmic staining pattern of four different proteins in fixed tobacco protoplasts; importin α, tubulin, β-glucuronidase (GUS), and a putative vacuolar receptor like protein, ELP. When protoplasts were stained for importin α only, it was found in the cytoplasm and nucleus (Figure 4.1A; Smith et al., 1997). In the cytoplasm, importin α localized to strands extending from the nucleus to the plasma membrane (Figure 4.1A). The strands were similar to those detected in protoplasts immunostained for microtubules (Figure 4.1B). Immunolocalization of ELP, which is found on the membranes of the Golgi and prevacuolar compartment in *Arabidopsis* roots (Ahmed, et al., 1997), displays a diffuse membranous like staining pattern throughout the cytoplasm which is absent from the nucleus (Figure 4.1C). Preimmune sera for ELP gave no specific staining in these protoplasts (data not shown). Lastly, tobacco suspension cells transformed with *GUS*, were used to determine the immunostaining pattern of a soluble protein. In these experiments, a diffuse staining pattern throughout the cytoplasm was observed (Figure 4.1D). In untransformed cells, the antibodies against GUS cross-react with protein(s) in the nucleus (data not shown), therefore the nuclear

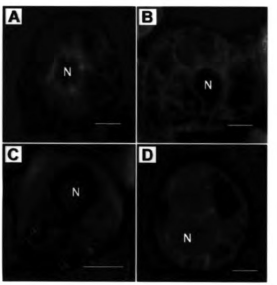


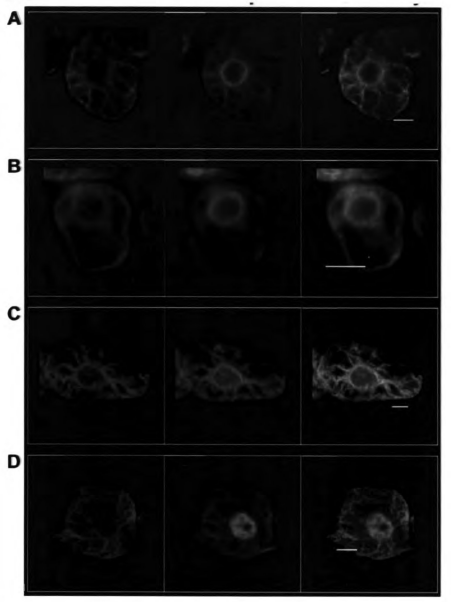
Figure 4.1
Immunolocalization of (**A**) importin α, (**B**) tubulin, (**C**) ELP and (**D**) GUS in fixed tobacco protoplasts. Protoplasts were individually stained for each of these proteins. The cytoplasmic staining of importin α (**A**) resembled protoplasts stained for tubulin (**B**) and this was distinct from protoplasts stained for a membrane protein (**C**), or a soluble protein (**D**). "N" denoted the nucleus in each of these optical sections. Bar = 10 *u*m.

staining observed in GUS expressing cells is non-specific (Figure 4.1D). Thus, the pattern of immunostaining of the soluble and membrane proteins, GUS and ELP respectively, were distinct from the cellular staining of importin α in tobacco protoplasts.

The cytoplasmic localization of importin α colocalizes with microtubules

Localization of importin α was examined further to determine if importin α colocalized with the cytoskeleton in fixed tobacco protoplasts. Double immunofluorescence experiments using monospecific antibodies to importin α and tubulin were visualized by CLSM. In Figure 4.2, tubulin staining was visualized in green and importin α staining 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 4.2A). In GUS expressing protoplasts, GUS did not colocalize with the microtubules (data not shown). Treatment of protoplasts with 10 *u*M of oryzalin, a plant specific microtubule depolymerizing agent, efficiently depolymerized the microtubules (Figure 4.2B, green) which also abolished importin α's cytoplasmic strands (Figure 4.2B, red). When treated protoplasts were washed to remove the oryzalin, both microtubules and cytoplasmic strands of importin α reappeared (data not shown). Similar results were obtained when protoplasts were treated with propyzamide, another plant

Figure 4.2
Importin α colocalized with microtubules in fixed tobacco protoplasts (A; overlay image). Depolymerization of microtubules with 5-10 *u*M oryzalin (B; tubulin image) disrupted the cytoplasmic strands of importin α (B; importin image). The cytoplasmic strands of importin α colocalized with microtubules when protoplasts were incubated on ice for 1 hr prior to fixation (C; overlay image). Stabilization of microtubules with taxol also did not effect this colocalization (D; overlay image). Bar = 10 *u*m.


A
B
C
D

microtubule depolymerizing agent (data not shown). However, when protoplasts were incubated on ice to stabilize the microtubules (Wallin and Stromberg,1995), these structures were detected in the cytoplasm and they coaligned with the cytoplasmic strands of importin α (Figure 4.2C). Treatment of protoplasts with 10 uM taxol stabilized the microtubules and did not effect the colocalization of importin α and microtubules in the cytoplasm (Figure 4.2D). These results suggest that the cytoplasmic strands of importin α associate with the microtubules.

The cytoplasmic localization of importin α colocalizes with microfilaments

Double immunofluorescence experiments using monospecific anti-actin antibodies and phalloidin were used to determine if importin α colocalized with microfilaments using CLSM (Figure 4.3). Immunostaining of fixed tobacco protoplasts showed importin α's cytoplasmic strands also coaligned with microfilaments (Figure 4.3A). When cells were treated with 50 ug/mL cytochalasin B, not only were the microfilaments depolymerized but some of the cellular actin accumulated inside the nucleus (Figure 4.3B, 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 4.3B, red image). When cells were washed to remove the cytochalasin B,

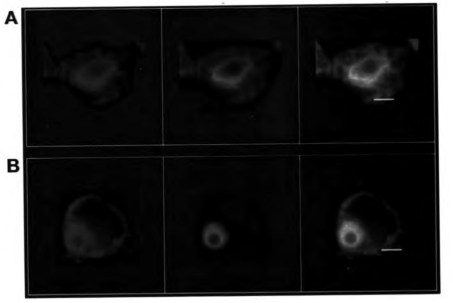


Figure 4.3
The cytoplasmic strands of importin α colocalized with microfilaments in fixed tobacco protoplasts (A; overlay image; actin was visualized with BODIPY-phalloidin). Importin α accumulated inside the nucleus when the microfilaments were deploymerized with 50 *u*g/mL of cytochalasin B (B; actin image; actin was visualized with monospecific anti-actin antibodies). Bar = 10 *u*m.

microfilaments and importin α cytoplasmic strands were restored in the cytoplasm (data not shown). Protoplasts treated with cytochalasin D to depolymerize the microfilaments 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 a cytoskeleton fraction

Cell fractionation studies were used to determine if importin α cofractionated with the cytoskeleton. Protoplasts were lysed and subjected to several differential centrifugation steps to isolate cytoskeleton fractions (Marc, et al., 1996). Equal amounts of protein from each of the fractions were separated by SDS-PAGE and probed using monospecific antibodies to At-IMPα, tubulin and actin by immunoblot analysis. As previously reported (Marc, et al., 1996), the cytoskeleton markers were enriched in the 27,000*g* pellet (Figure 4.4, lane P27) and the 100,000*g* pellet (Figure 4.4, lane P100). Immunoblot analysis showed that importin α was found in all fractions including the cytoskeleton fractions (Figure 4.4, lanes P27 and P100). Because importin α is a nuclear shuttle protein (Gorlich, et al., 1996; Weis, et al., 1996; Kutay, et al., 1997) and part of it is localized to the nucleus in tobacco protoplasts (Hicks et al., 1996; Smith et al., 1997), it was not surprising to find this protein in the 5,000*g* pellet which contains the nuclei

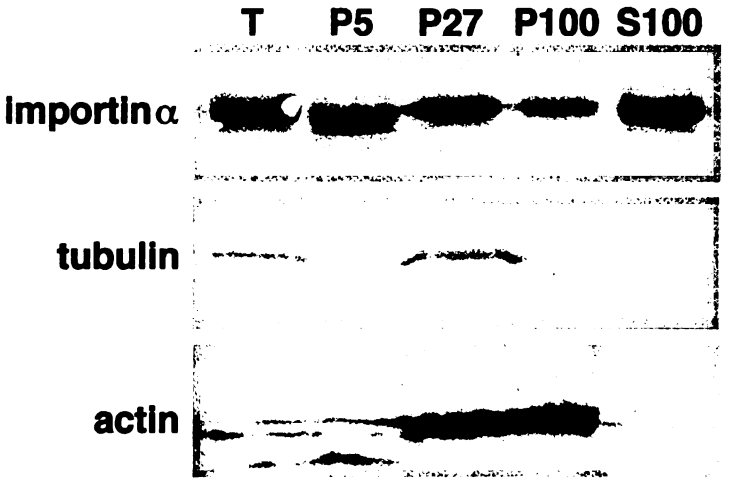


Figure 4.4
Subcellular localization of importin α. Tobacco protoplasts were lysed and differential centrifugation was used to isolate cytoskeleton enriched fractions (P27 and P100). Equal amounts of protein from each fraction separated by 10% SDS-PAGE and blotted to nitrocellulose. Monospecific antibodies were used as probes to detect importin α, tubulin, and actin in each of these fractions. Importin α was found in all the fractions including the cytoskeleton pellets (P27 and P100). Lane T = Total lysate, Lane P5 = 5,000*g* pellet, Lane P27 = 27,000*g* pellet, Lane P100 = 100,000*g* pellet, and Lane S100 = 100,000*g* supernatant.

as well as unbroken cells (Figure 4.4, lane P5). Importin α in the soluble fraction, (S100,000*g*) suggests that a cellular form of this receptor may be soluble (Figure 4.4, lane S100).

To characterize the association of importin α with the cytoskeleton fractions, we examined the biochemical properties of this protein with the 27,000*g* pellet. Pellets were resuspended under various conditions (see below) then centrifuged at high speed to pellet any insoluble material. Equal volumes from each extract were separated by SDS-PAGE, then analyzed by immunoblot analysis using monospecific At-IMPα antibodies. Suspension of pellets in buffer alone was not sufficient to solubilize importin α (Figure 4.5, lane 1), and detergents such as CHAPS or Trition X-100 solubilized importin α poorly (Figure 4.5, lanes 2 and 3, respectively), indicating that this protein was not membrane associated. Solubilization of importin α occurred when pellets were resuspended with buffer containing 8 M urea (Figure 4.5, lane 5), 0.5 M NaCl plus nucleotide triphosphates (Figure 4.5, lane 4) or 0.5 M NaCl (data not shown). The experiment indicates that a portion of the cellular importin α fractionated with the cytoskeleton and was only extracted from these pellets under high ionic strength, indicating that the NLS receptor was associated with large proteinaceous structures which may represent the cytoskeleton.

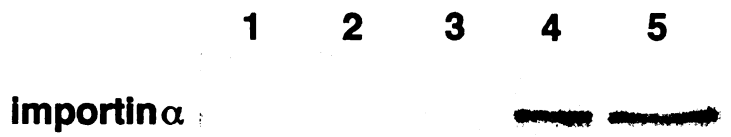


Figure 4.5.
The biochemical properties of importin α were analyzed in the P27 pellet. These pellets were resuspended in PM buffer (lane 1) or PM buffer containing 10 mM CHAPS (lane 2), 1% Trition X-100 (lane 3), 8 M urea (lane 4), or 0.5 M NaCl plus 5 mM ATP and GTP (lane 5) for 20 min on ice. Samples were pelleted at 100,000*g* and equal amounts of the supernatant were separated by SDS-PAGE, blotted to nitrocellulose, and probed for importin α using At-IMPα antibodies.

Importin α associates with microtubules and microfilaments

One of the intriguing feature 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 while 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). Since the central domain of importin α contains 8 armadillo repeat motifs (Hicks et al., 1996; Smith et al., 1996), we decided to test if this receptor could interact with the cytoskeleton in vitro. A plant cell extract containing importin α was prepared, centrifuged at high speed to remove any insoluble material and used to determined whether or not importin α could interact with microtubules and microfilaments in vitro.

Microtubules were polymerized in the presence of taxol then mixed with the plant cell extract in the absence of NLS peptides or in the presence of a functional NLS peptide, Opaque-2 Wild type (02WT) or a mutated form of this NLS peptide, Opaque-2 mutant (02MUT; 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, then separated by SDS-PAGE and probed for importin α by immunoblot analysis.

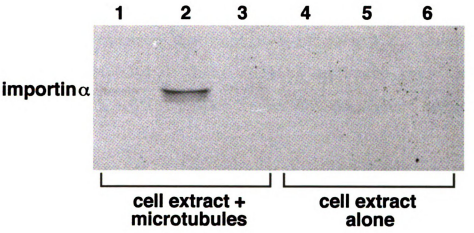


Figure 4.6.
Association of importin α with microtubules was dependent on functional NLSs. Taxol-stabilized microtubules were mixed with a plant cell extract alone (lane 1), or with 200 *u*M 02WT NLS peptides (lane 2) or 200 *u*M 02MUT NLS peptides (lane 3). Association was determined by the ability of importin α to pellet with the microtubules through a 40% sucrose cushion. Microtubule pellets were solubilized, separated by 10% SDS-PAGE, blotted to nitrocellulose and probed using At-IMPα antibodies to detect importin α. In control experiments, the plant cell extract was incubated alone (lane 4) or with 200 *u*M 02WT NLS peptides (lane 5) or 200 *u*M 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.

When the plant cell extract was incubated with microtubules in the presence of O2WT, we detected association of importin α with these structures (Figure 4.6, lane 2). In the absence of a functional NLS or in the presence of O2MUT peptides, this association was substantially reduced (Figure 4.6, 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 4.6, lanes 4, 5, and 6, respectively). These results indicate that importin α associates with microtubules in an NLS-dependent manner in vitro.

We next analyzed if importin α associated with actin microfilaments in vitro. Actin microfilaments were polymerized and mixed with the plant cell 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 immunoblot analysis to detect association of importin α.

Importin α pelleted with the microfilaments in the presence of O2WT peptides (Figure 4.7, lane 2), but in the absence or presence of O2MUT peptides, we detected little association (Figure 4.7, lane 1 and 3, respectively). Control experiments performed under the same conditions showed that

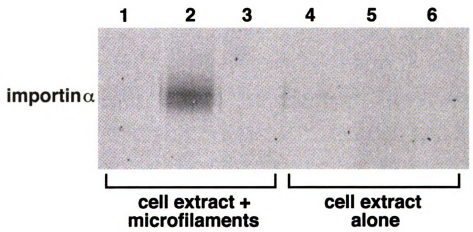


Figure 4.7
Importin α associates with microfilaments in an NLS dependent manner. Polymerized microfilaments were mixed with a plant cell extract in the absence of NLS peptides (lane 1) or the presence of 02WT peptides (lane 2) or 02MUT peptides (lane 3). The association was determined by the ability of importin α to pellet through a 10% glycerol cushion with the microfilaments. Solubilized pellets were subjected to immunoblot analysis using 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 peptides (lane 5) or 02MUT peptides (lane 6) and importin α did not pellet through the 10% glycerol gradient when microfilaments were omitted from the association assay. The amino acid sequence of 02WT and 02MUT peptides was described by Smith et al., 1997.

importin α did not pellet when microfilaments were omitted from the association assay (Figure 4.7, lanes 4, 5, and 6). These results indicate that importin α associated with microfilaments in an NLS-dependent manner in vitro.

Discussion

Intracellular transport of organelles (Hirokawa, 1998; Mermall, et al., 1998), viruses (Heinlein, et al., 1995; McLean, et al., 1995), and mRNA-protein complexes (Hovland, et al., 1996; Bassel, and Singer, 1997) are 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, we found that the cytoplasmic localization of importin α was distinct from protoplasts immunostained for soluble and membrane proteins. The cytoplasmic strands of importin α had a similar distribution as the microtubules and microfilaments in the cytoplasm of fixed tobacco protoplasts. However, the colocalization of importin α with the cytoskeleton was not 1:1 ratio in the cytoplasm, demonstrating that a fraction of importin α's localization is independent of the cytoskeleton. Other proteins such as tobacco mosaic virus (TMV) movement protein (Heinlein et al., 1995; McLean et al., 1995) and elongation factor-1α (for a review see Durso and Cyr, 1994; Clore et al., 1996) also colocalize with both cytoskeletal structures. In fact, microtubules and microfilaments

colocalize in plant cells by immunoflourescence and immunoelectron studies suggesting that these structural elements interact with each other (for a review see Staiger and Lloyd, 1991; Lancelle, et al., 1987). In addition, microtubules and microfilaments are integrated structures that make up the preprophase band, mitotic spindle, and phragmoplast.

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. Protoplasts treated with oryzalin disrupted the cytoplasmic strands of importin α, suggesting that importin α may interact with microtubules in vivo. Like importin α, the TMV 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 upon 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 importin α is dispersed throughout the cytoplasm.

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 (data not shown). 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 is 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.

Cell fractionation studies showed that importin α was found in the cytoskeleton and soluble fractions. Microtubule and actin binding proteins are also found in soluble fractions isolated from plant cells (Chan, *et al.*, 1996; Marc et al., 1996; Staiger et al., 1997; Yokota, et al., 1998), similar as importin α, suggesting that these proteins are displaced from the cytoskeleton during cell lysis. In tobacco cell fractionation studies, 10 mM CHAPS removed a 90 kD

microtubule binding protein from 27,000*g* pellets that may be involved in linking cortical microtubules to the plasma membrane (Marc et al., 1996), whereas cytoskeletal fractions extracted with high salt buffers with or without nucleotide triphosphates solubilzed microtubule motor proteins (Asada and Shibaoka, 1994; Asada, et al., 1997). Thus, the 27,000*g* pellet form of importin α has similar biochemical properties as microtubule motor proteins.

Lastly, in vitro pelleting studies suggested that importin α associates with microtubules and microfilaments in an NLS dependent manner. Since a system to study cytoskeleton transport in vitro does not exist in plants, it is not possible to determine if importin α moves along these structures during transport, prior to import. However, based on our results we found that importin α interacted with the cytoskeleton, possibly through the armadillo repeats (Hicks et al., 1996; Smith et al., 1997).

The role of the cytoskeleton in the transport of NLS-containing proteins from the cytoplasm to the NPC is illustrated in figure 4.8. We propose that the microtubules are involved in the transport of NLS-proteins from the cytoplasm to the NPC. Interestingly, it has been shown that retrograde transport of fluorescently labeled NLS-containing proteins towards 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

requires NLS-cargo. Furthermore, transport is probably facilitated by a microtubule motor protein since importin α is not homologous to microtubule motor proteins. Since importin α accumulated inside the nucleus when the microfilaments were depolymerized, this indicates 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 similar distribution as 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 where nuclear proteins are synthesized, so that it can be immediately assembled with these proteins. After assembly, these import complexes can be loaded onto microtubules tracks for NLS-protein transport to the nucleus.

In summary, using various approaches, we found that importin α associated with the cytoskeleton. These studies are significant because they provide a link between the cytoskeleton and a import factor, importin α, suggesting that these cellular arrays are essential structures involved in the intracellular transport of a nuclear shuttling NLS

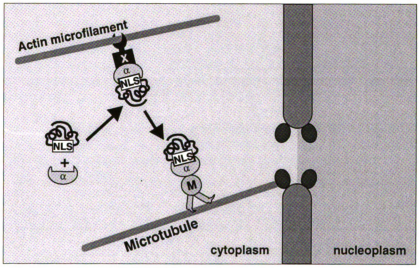


Figure 4.8
Schematic diagram of NLS protein transport from the cytoplasm to the NPC.

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 expression in eukaryotes. All and all, this studies support the idea that the cytoskeleton is a dynamic structure involved in many intracellular transport pathways as well as maintaining the shape and structure of the cell.

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Chapter 5

Conclusions and Future Directions

NLS-protein import is an essential process in all eukaryotes. My thesis has involved the cloning and characterization of an *Arabidopsis* importin α homologue, At-IMPα. At-IMPα is 36-48% identical to other importin α homologues found in vertebrates, fungi, worms, and insects (Hicks et al., 1996). Antibodies raised against the recombinant expressed At-IMPα (Hicks et al., 1996) were used as a tool to study the function of this protein in plants. Immunoblot analysis has demonstrated that At-IMPα is found in the roots, stems, leaves and flowers of *Arabidopsis*. In addition, these antibodies specifically recognize an importin α homologue in tobacco protoplasts (Hicks et al., 1996). This was significant because tobacco protoplasts are an extremely useful system in plant cell biology and I have used these cells as an experimental system.

The function of At-IMPα was addressed by two different approaches. First, NLS binding assays were used to show that At-IMPα specifically recognizes three types of NLSs that function in plants (Smith et al., 1997). Second, At-IMPα was shown to mediate NLS-protein import in the mammalian in vitro import system when the vertebrate import factors Ran and p10 were added. In contrast to the yeast and mammalian importin α homologues, At-IMPα was found to mediate import in the absence of an importin β subunit (Hubner et al., submitted), indicating that plants contain a unique NLS-protein import pathway mediated by a class of multifunctional importin α subunits.

Immmunolocalization of importin α in tobacco protoplasts has shown that the receptor is found in the cytoplasm and nucleus. Importin α is also concentrated at the rim of the nucleus in protoplasts, as well as nuclei purified in the presence of 1% Trition X-100. These localization studies indicate that importin α is associated with the NPC, that is consistent with its function as a nuclear shuttling NLS receptor (Smith et al., 1997).

I was also interested in determining whether or not importin α associated with the cytoskeleton. The cytoskeleton is involved in intracellular transport of viruses, mRNA/protein complexes, vesicles, and organelles in the cytoplasm (Heinlein, et al., 1995; McLean, et al., 1995; Hovland, et al., 1996; Bassel, and Singer, 1997; Hirokawa, 1998; Mermall, et al., 1998). Therefore, the cytoskeleton could also mediate the transport of NLS-containing proteins from the cytoplasm to the NPC, prior to NLS-protein import. Several observations have suggested that importin α associates with the cytoskeleton. Immunolocalization of importin α in tobacco protoplasts has shown a radial cytoplasmic staining extending from the nucleus to the plasma membrane (Smith et al., 1997). This pattern is similar to that seen when plant cells are immunostained for cytoskeletal proteins (Hepler et al., 1993). Importin α is not depleted from tobacco protoplasts after permeabilization, indicating that this receptor is associated with structures in the cytoplasm and nucleus (Hicks et al., 1996). The presence of

armadillo repeats in importin α (Hicks et al., 1996) also suggests that it could interact with the cytoskeleton, since other armadillo repeat containing proteins such as the armadillo/catenin family of proteins (Barth et al., 1997), Vac8 (Wang et al., 1998),smgGDS (Shimizu et al., 1998), spKAP115 (Wedaman et al., 1996) APC (Barth et al., 1997) and Pf16 (Smith et al., 1996) interact with the cytoskeleton.

To investigate whether or not importin α associated with the cytoskeleton, I first showed that this receptor colocalizes with the cytoskeleton by immunofluorescence confocal laser scanning microscopy in tobacco protoplasts. Depolymerization of the cytoskeleton disrupts importin α's cytoskeleton-like pattern in the cytoplasm of tobacco protoplasts. Interestingly, when the microtubules are depolymerized, importin α staining in the cytoplasm was diffuse. However, depolymerization of microfilaments causes importin α to accumulate inside the nucleus, indicating that the microfilaments are involved in retaining this receptor in the cytoplasm. Using a biochemical approach, a fraction of importin α is tightly associated with a cytoskeleton fraction isolated from tobacco protoplasts. In addition, these studies have also indicated that a fraction of importin α is soluble. Using in vitro cytoskeleton binding assays, importin α associated with microtubules and microfilaments in an NLS dependent manner. In summary, these observations show that importin α associates with the cytoskeleton.

A working model for the role of the cytoskeleton in NLS-protein transport can be developed from these observations, as well as studies from other systems. In this model, 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 towards the nucleus in neurons is microtubule dependent (Ambron et al., 1992). This transport mechanism is probably facilitated by a microtubule motor protein, since importin α is not homologous to kinesin or dynein proteins. Since importin α accumulated inside the nucleus when the microfilaments were depolymerized, this strongly suggests that these structures are involved in retaining this receptor in the cytoplasm. In yeast, importin α directly binds to Act2, an actin related protein (Yan et al., 1997). Act2 has a similar distribution as the microfilaments in the cytoplasm of yeast cells (Yan et al., 1998), therefore it is tempting to speculate that Act2 could be involved in retaining importin α in the cytoplasm. This retention mechanism may function to assemble the importin α/NLS-containing protein complexes, since the translation of many mRNA's, which encode nuclear proteins, occurs on microfilaments (Hovland et al., 1996; Bassel and Singer, 1997). Once the importin α/NLS-cargo complexes are assembled they would be loaded and transported on microtubule railways that are connected to the NPC.

Future work should be directed to understand the molecular mechanisms involved in the intracellular transport of importin α/NLS-containing protein complexes in the cytoplasm of the cell. First, a NLS-protein transport system should be developed to characterize the movement of NLS-containing proteins along microtubules. Second, cytoskeleton binding factors that mediate importin α's interaction with the microtubules and microfilaments should also be identified. The development of a cytoskeleton transport system could ultimately be used to test the function of these cytoskeleton binding proteins in the intracellular transport of NLS-containing proteins.

In order to study NLS-protein transport, a cell type must be selected that would allow one to directly measure this process. Therefore, elongated cell types that have their nuclei positioned at one end of the cell could be used as a model system to study the transport of NLS-containing proteins across the cell. In plants, pollen tubes, root hairs and trichomes represent cell types that could be amenable for these studies. Unfortunately, molecular mechanisms that govern the movement vesicles and organelles along the plant cytoskeleton is purely descriptive. To date only 5 microtubule motor proteins, kinesins, have been identified in plants, and only one localizes to pollen tubes (Asada and Collings, 1997). Therefore, the tools and systems needed to study cytoskeletal dynamics in plants have not been developed.

In animals, neurons are excellent model systems to study the intracellular transport of vesicles and organelles (Hirokawa, 1996). Neurons are composed of a cell body, dendrites and a long axon. The cytoskeletal apparatus in the axons of neurons, called the axoplasm, can be extruded from the neurons to characterize vesicle and organelle transport in vitro (Hirokawa, 1996). The movement of vesicles and organelles along the cytoskeleton can be visualized by differential interference contrast (DIC) light microscopy (Salmon, 1995). This system could be put to use to study NLS-protein transport, since microtubules are involved in this process in the axons of neurons (Ambron et al., 1992). To visualize the movement of NLS-containing proteins along the cytoskeleton, functional NLS-peptides can be coupled to latex beads. These NLS-peptide beads can be added to the extruded axoplasm to characterize the movement of these substrates along the cytoskeleton by DIC light microscopy. Similar studies were used to characterize the movement of kinesins in extruded axoplasms (see Salmon, 1995). Beads coupled with mutant NLS-peptides or beads alone can be used as a controls in these experimental approaches. In this system, I would expect the beads coated with functional NLSs to move in a retrograde manner, toward the cell body. This process should be energy dependent and move at a rate similar to motor proteins that move in a retrograde manner. Lastly, movement of the beads should be specific to the beads coated with functional NLSs.

According to the transport model described above, two types of cytoskeleton binding proteins could link importin α to the cytoskeleton. Proteins that link importin α to microfilaments could be involved in retaining this import receptor in the cytoplasm. On the other hand, proteins that bridge importin α to the microtubules could be involved in the transport of importin α/NLS-cargo complexes from the cytoplasm to the NPC. Several potential candidate proteins that could link importin α to the cytoskeleton have been identified in animals and yeast. Therefore, one approach to identify proteins that may bridge importin α to the cytoskeleton is to determine if any of these candidate proteins bind to importin α. In these experiments, I would use the vertebrate importin α subunits that are expressed in neurons and a verebrate importin β subunit to identify these cytoskeletal linker proteins.

For example, in the transport model described above, Act2 could be involved in retaining importin α in the cytoplasm. To test this hypothesis, the vertebrate Act2 homologue (Mullins et al., 1996) would be used to examine whether or not it links importin α to microfilaments in vitro. The identification of proteins that link importin α to microfilaments would be essential tools to study the retention mechanism that anchors importin α in the cytoplasm.

A potential candidate that could link importin α to the microtubules are dynein motor proteins. Dyneins are large

multisubunit proteinaceous complexes that transport vesicles and organelles in a retrograde manner (Hirokawa, 1998). Interestingly, the cargo binding complex, called dynactin, contains a 37 nm filament composed of the actin related protein, Arp1. The Arp1 filament is believed to be the structure that directly interacts with the cargo (Mullins, et al., 1996; Schroer et al., 1996; Hirokawa, 1998). Since the yeast importin α interacts with the actin related protein, Act2, it tempting to speculate that importin α could also interact with Arp1 filaments. Therefore, in vitro binding experiments can be performed to examine whether or not importin α associates with Arp1 filaments. If importin α associates with the Arp1 filament, then the function of dynein complexes can be analyzed in a NLS-protein transport system.

In conjunction with these experiments, the yeast two-hybrid system could also be used to identify proteins that interact with the importin α. Importin α is composed of three protein domains (Gorlich and Mattaj, 1996; Smith et al., 1997), where the N-terminal domain interacts with importin β (Gorlich et al., 1996; Weis et al., 1996), and the C-terminal domain interacts with NLSs (Moroianu, et al., 1996). Therefore, the central domain importin α that contains the armadillo repeat motifs could be used in the yeast two-hybrid system to identify factors that may link this receptor to the cytoskeleton.

Biochemical approaches, could also be used to identify factors that may bridge importin α to the cytoskeleton. Based upon the transport model described above, the soluble form of importin α cannot interact with the cytoskeleton, unless it is bound to NLS-containing proteins. Therefore, NLSs peptides or proteins can be added to a soluble cell extracts to determine if importin α forms large complexes. If large complexes are formed in the presence of functional NLSs, they can be isolated by immunoprecipitation to identify the proteins that make up this complex.

Importin α-binding proteins that are identified by the yeast two-hybrid and/or biochemical methods, could be cloned and sequenced, and compared to other known genes in the GeneBank data base to determine the possible functions of these proteins. For example, if a putative microtubule motor protein is identified, cytoskeleton motility assays can be used to characterize the function of this type of protein. If proteins of unknown function are identified, they will be characterized to determine if they interact with the cytoskeleton. If so, additional studies can analyze the ability of these proteins to link importin α to the microtubules or microfilaments in the absence and presence of NLSs peptides. The ultimate goal to address the function of the cytoskeleton binding proteins will be to reconstitute a NLS-protein transport system with purified components in vitro. All and all, these studies could contribute to the idea that the cytoskeleton is a dynamic structure involved in

many intracellular transport pathways as well as maintaining the shape and structure of the cell.

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