Functional Characterization of a Plant Importin α Homologue

NUCLEAR LOCALIZATION SIGNAL (NLS)-SELECTIVE BINDING AND MEDIATION OF NUCLEAR IMPORT OF NLS PROTEINS IN VITRO∗

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Nuclear import of most nuclear proteins is initiated by recognition of the nuclear localization signal (NLS) by importin α. We recently isolated an importin α homologue from rice (rice importin α1) and demonstrated that transcription of the gene is down-regulated by light in rice leaves. To address the function of rice importin α1 in the process of nuclear import of proteins, we performed in vitro binding and nuclear import assays. The rice importin α1 showed specific binding to fusion proteins containing either monopartite or bipartite NLSs, but not to a fusion protein containing a Mato-2-type NLS, suggesting that there exists selective binding of rice importin α1 to different plant NLSs. The rice importin α1 is also capable of forming a complex with mouse importin β and NLS protein in vitro. An in vitro nuclear import assay using permeabilized HeLa cells revealed that rice importin α1, in conjunction with other vertebrate transport factors, mediates the nuclear envelope docking of NLS proteins and their subsequent translocation into the nucleus. These data provide strong, direct evidence suggesting that rice importin α1 functions as a component of the NLS receptor in plant cells.

Due to the existence of a nuclear envelope that sequesters genomic DNA and separates nuclear and cytoplasmic activities, eukaryotic cells have established an active and strictly regulated, bi-directional nucleocytoplasmic transport system, by which macromolecules larger than 40 kDa in granular size enter and exit the nucleus through the nuclear pore complex (NPC) (1–5). A growing body of recent experimental evidence has revealed the existence of multiple pathways in this transport system (6–9). Among them, the best characterized pathway is nuclear import of proteins bearing basic nuclear localization signals (NLSs). NLSs consist of either a short stretch of 3–5 basic amino acids or two basic domains separated by a spacer, referred to as monopartite and bipartite NLSs, respectively. Using an in vitro nuclear import assay with digitonin-permeabilized vertebrate cells, in conjunction with other biochemical techniques, a set of transport factors required for the NLS-mediated nuclear import of proteins has been identified and extensively characterized. NLS-mediated import has been described as a multistep process: NLS recognition and pore docking, translocation through the NPC, and release of the cargo from the inner side of the pore (10–12). The NLS receptor consists of importin α and importin β, which form a heterodimer. Importin α recognizes and binds to the NLS of a nuclear protein, forming a stable pore-targeting complex (PTAC) in the cytoplasm (13–15), whereas importin β interacts directly with nucleoporins that contain FXFG or GLFG repeats, docking the PTAC to the cytoplasmic face of the NPC (16–18). Translocation of the docked PTAC into the nucleus is an energy-dependent process, mediated by the small GTPase, Ran, along with a homodimeric factor known as p10 or NTF2 (19–22). Ran's nucleotide-bound state is regulated by two different proteins, the chromatin-bound exchange factor, RCC1, which generates Ran-GTP in the nucleus (23, 24), and the cytoplasmic GTPase-activating protein, RanGAP1, which dephosphorylates Ran-GTP (from the cytoplasm) (19, 25–28). Ran-GTP binding to importin β has been shown to release importin α-substrate complex into the nucleus (11, 29, 30).

In higher plants, although our knowledge about the nuclear import of proteins is much more limited, a variety of highly conserved NLSs and transport factors have been identified (31). Plant NLSs have been classified into three major types: the monopartite NLS, the bipartite NLS, and the NLS of the yeast mating factor Mato 2 (Mato-2 NLS), which consists of basic and hydrophobic amino acid residues and has also been shown to be functional in plants (32, 33). Utilizing both homology search and a biochemical approach, cDNAs encoding importin α-like proteins have been isolated from Arabidopsis and rice, and their recombinant proteins have been shown to specifically bind to NLS-conjugated BSA in vitro (33–36). Furthermore, cDNAs for Ran homologues from Arabidopsis, Vicia faba, tobacco and tomato (37–39), and Ran-binding protein (RanBP1) homologues from Arabidopsis have also been identified (40). However, there is currently no direct experimental evidence demonstrating the involvement of these plant homologues in the process of nuclear import of proteins. This is due mainly to the lack of an appropriate plant experimental system in which a specific transport factor can be characterized. Recently, two plant in vitro assays for nuclear import of proteins have been developed using permeabilized, evacuated protoplasts from tobacco BY-2 cells (34, 41). In these assays, however, in vitro
import did not require exogenous cytosol and ATP, suggesting that all necessary factors are retained within the permeabilized protoplasts in sufficient amounts for efficient nuclear import. More recently, Broder et al. (42) have demonstrated that plant cell extract can support the transport of NLS-BSA into nuclei of digitonin-permeabilized HeLa cells, presenting an heterologous experimental system in which to study specific plant factors putatively involved in the nuclear import of proteins.

We previously identified a rice cDNA encoding an importin α homologue, tentatively named rice importin α1, and demonstrated that transcription of the gene is down-regulation by light in rice leaves (36). In this paper, we show that rice importin α1 selectively binds to different types of plant NLSs and mediates the nuclear import of NLS substrates in digitonin-permeabilized HeLa cells.

MATERIALS AND METHODS

DNA Constructions—To generate a GST-NLS-GFP fusion protein, an oligonucleotide encoding an appropriate NLS peptide was coupled to the 5 ’-end of the GFP gene by polymerase chain reaction (PCR) using pSG5T-C1 (CLONTECH) as template DNA. The 5 ’-end primer used for the PCR incorporates an EcoRI site at the 5 ’-end followed in frame by the NLS-encoding nucleotide sequence in the middle. The 3 ’-end primer incorporates an XhoI site at its 5 ’-end. The PCR product was digested with EcoRI and XhoI restriction enzymes and cloned in frame in the 3 ’-end of the GST in pGEX-4T-1 (Amersham Pharmacia Biotech). The NLSs and the corresponding 5 ’-end oligonucleotide primers used for the PCR are as follows: 1) SV40 T-NLS (T-NLS, monopartite type): CTPPKKKRKV/5 ’-ACGCGGCGGCGGAAAGACGCGCGCTGGAACGGCGACCCCGGAAAGCCGTGAATGATGTTAAAGGAAAGAACACTTCTTCTGAGA-3 ’; 2) SV40 T-NLS mutant (TM-NLS): CTPPKKKRKV/5 ’-ACCGAAATTCGTCACTCCCGGCAACCGGAAAGACGCGCGCTGGAACGGCGACCCCGGAAAGCCGTGAATGATGTTAAAGGAAAGAACACTTCTTCTGAGA-3 ’; 3) NLS of the maize Opaque-2 transcription factor (O2-NLS, bipartite type): MPTEERVRKRKESNRESAGCGAGCGAAACGAGAAACACCGGTGGCGAACCGGAAAGAAAAGCAACCGCGAAAGCGCGCGCCGCAGCCGC-9; 4) NLS of the maize Opaque-2 transcription factor (O2-NLS, monopartite type): MPTEERVRKRKESNRESAGCGAGCGAAACGAGAAACACCGGTGGCGAACCGGAAAGAAAAGCAACCGCGAAAGCGCGCGCCGCAGCCGC-9.

To generate GST-rice importin α1, a fragment of rice importin α1 cDNA with an artificial EcoRI site at the 5 ’-end was generated by PCR and inserted between the EcoRI and SmaI sites of pGEX-6p-1 to obtain an in-frame translation fusion with GST.

Expression and Purification of Fusion Proteins—The fusion proteins were expressed in Escherichia coli BL21 by growing in the presence of 0.5 mM isopropyl-β-D-thiogalactopyranoside for 4–6 h at 20 °C, and the proteins were purified essentially according to the manufacturer’s instructions (Amersham Pharmacia Biotech). All the procedures were carried out at 4 °C, and 1 mM EGTA and 2 mM dithiothreitol (DTT) were added in the solutions throughout the purification procedures. The GST portion of the GST-rice importin α1 fusion protein was cleaved by incubation of the fusion protein bound to glutathione-Sepharose 4B resin with 80 units/ml resin of Precllisis™ protease (Amersham Pharmacia Biotech) for 4 h at 5 °C. The Precllisis™ protease is a recombinant fusion protein with GST and hence can be easily removed by glutathione-Sepharose 4B. The purified proteins were concentrated by Millipore Ultrafree-MC (Millipore Corp., Bedford, MA) and finally suspended in 20 mM Hepes buffer (pH 7.3) containing 1 mM EGTA and 1 mM DTT.

Recombinant mouse importin α (PTAC58; Ref. 13), mouse importin β (PTAC97; Ref. 43), and Ran (44–46) were prepared as described previously.

In Vitro Binding Assay—In vitro protein binding was examined by native gel electrophoresis according to the method of Safer (47) with minor modifications. 20 pmol of each protein was mixed in 15 μl of transport buffer (TB) (20 mM Hepes (pH 7.3), 110 mM potassium acetate, 2 mM magnesium acetate, 5 mM sodium acetate, 0.5 mM EGTA, 2 mM DTT, 1 μg/ml each of aprotinin, leupeptin, and pepstatin A) supplemented with 250 mM sucrose and incubated for 1 h at room temperature. 7.5% polyacrylamide gels were run in the presence of 1 mM DTT and 1 mM EGTA in both the gels and the running buffer.

In some experiments, the protein band was excised from the native gel, placed on a 10% SDS-polyacrylamide gel with stacking gel, overlaid with SDS-PAGE sample buffer, and electrophoresis was carried out. 6 M urea was added in the concentration of which was adjusted with TB containing 2% BSA. For the nuclear-binding assay, the incubation was performed for 30 min at 37 °C, and 5% BSA was added in the concentration of which was adjusted with TB containing 2% BSA. After incubation, cells were fixed with 3.7% formaldehyde in TB. GST-NLS was detected by Axsight photo microscopy (Xarl Zeiss, Inc.).

RESULTS

Interaction between Rice Importin α1 and Plant NLSs—Protein import into the nucleus is initiated by recognition of and binding to the NLS of a nuclear protein by importin α in the cytoplasm. To address the function of rice importin α1, we investigated whether rice importin α1 could bind to an NLS in vitro. We chose three representative plant NLSs that were previously used for in vitro binding studies by Smith et al. (33): (i) T-NLS, the simian virus 40 large T-antigen NLS (a monopartite type NLS); (ii) O2-NLS, identified in the maize transcription factor Opaque-2 (a bipartite type NLS); (iii) R-NLS, identified in the maize transcription factor R (a Mata 2-type NLS). We also used Tm-NLS, a point mutant of the T-NLS, in which the sixth lysine residue of the T-NLS was replaced by a threonine, as a negative control. We inserted these NLS sequences between GST and GFP using recombinant DNA techniques to generate GST-NLS-GFP fusion proteins, rather than chemically conjugating NLSs to BSA protein. This would presumably result in fusion proteins that would function as more natural NLS substrates. For convenience in this paper, we have designated these NLS-GFP fusion proteins: T-, O2-, R-, and Tm-GFP, respectively.

The ability of the rice importin α1 to complex with NLS-GFP was assessed using native gel electrophoresis, in which complex formation between two proteins gives a new band with a mobility different from that of either protein alone. The rice importin α1 and each of the NLS-GFPs migrate as single bands on the nondenaturing gel as shown in Fig. 1, lanes 1, 2, 4, 6, and 8. A mixture of the rice importin α1 with either T-GFP or O2-GFP gives a major new band of retarded mobility with little of either unbound protein at the position of the control (Fig. 1, lanes 3 and 7). In contrast, a mixture of the rice importin α1 with either R-GFP or Tm-GFP gives no new visible bands, with migration of each proteins as in the control (Fig. 1, lanes 3 and 5). The complex of O2-GFP and rice importin α1 gives almost two bands on the gel. However, complex formation between the two proteins is apparent, as all of the rice importin α1 shifted upward, giving a much darker band. In addition, we also confirmed this using the GST-rice importin α1 fusion protein (GST-uncleaved), which gives a clear difference in mobility between the O2-GFP and its complex with rice importin α1 (data not shown). These data suggest that the rice importin α1 selectively binds to T-NLS and O2-NLS, but not to R-NLS. The binding was NLS-specific as the rice importin α1 did not bind to Tm-GFP (Fig. 1, lanes 8).
more than 50% of importin α1 binds to NLS- GFPs. Native gel electrophoresis showing rice importin α1, T-GFP, Tm-GFP, O2-GFP, and R-GFP run separately or as a mixture. T-GFP (lane 2) and O2-GFP (lane 6) migrate as single bands, whereas both of them in a mixture with rice importin α1 gives a major new band of retarded mobility (lanes 3 and 7, respectively). Tm-GFP (lane 4) and R-GFP (lane 8) migrate as single bands, but neither of the proteins resulted in any new band in a mixture with rice importin α1 (lanes 5 and 9, respectively). These data show that rice importin α1 selectively binds to T-GFP and O2-GFP, but not to Tm-GFP and R-GFP.

Complex Assembly of Importins α and β and NLS Substrate—Importin α simultaneously binds to NLS substrate at one site and importin β via its N-terminal, importin β-binding domain, forming the PTAC in the cytoplasm. We examined the interaction between rice importin α1 and mouse importin β and the effects of T-GFP on the interaction (Fig. 2A). As a 7.5% polyacrylamide gel did not give a clear separation for the mixture of rice importin α1 and mouse importin β (Fig. 2A, lane 5), a 9% polyacrylamide gel was run for this particular mixture (Fig. 2A, lane 10). A weak binding between the two proteins was observed as appearance of a minor intermediate band between the bands of the rice importin α1 and mouse importin β with migration of each protein as in the control (Fig. 2A, lane 10, arrowhead). There was some smearing of both of the two proteins toward the position of the complex, suggesting partial dissociation during electrophoresis. Addition of T-GFP to the mixture resulted in formation of a large complex with lower mobility on the gel (Fig. 2A, lane 6) relative to the complex consisting of rice importin α1 and T-GFP (Fig. 2A, lane 7). SDS-PAGE of the band corresponding to the large complex on the native gel revealed that the complex contained rice importin α1, mouse importin β, and T-GFP, demonstrating that a complex assembly (PTAC) occurred in the mixture (Fig. 2B). Moreover, addition of T-GFP to the mixture tended to increase the binding of importin β to importin α1, as the unbound importin β decreased correspondingly. These results suggest that NLS substrate may promote or stabilize the interaction between rice importin α1 and mouse importin β, and more importantly, that binding of rice importin α1 to mouse importin β is stable only when all the three proteins are present in the mixture.

It has been shown that mouse importin α associates directly with mouse importin β in a 1:1 ratio, even in the absence of NLS substrate (43). Consistent with this finding, much higher affinity between these two proteins, in contrast to that between rice importin α1 and mouse importin β, was observed on the native gel (Fig. 2C, lane 2). Roughly judged on darkness of unbound importin β band relative to that of control on the gels, more than 50% of importin β bound to mouse importin α1, in contrast to about 5–10% of that bound to rice importin α1. The low affinity between rice importin α1 and mouse importin β might be ascribed to the heterogeneity of their origin, in this case one from plant and the other from mouse. Interestingly, even with both importins from the same species, T-GFP was still able to enhance the binding of mouse importin β to mouse importin α1, as the unbound importin β decreased correspondingly (Fig. 2C, lane 4).

The mixture of mouse importin β and T-GFP did not result in formation of any complex (Fig. 2A, lane 9), demonstrating that the PTAC was formed via rice importin α1 binding with both mouse importin β and T-GFP. No complex assembly was observed when Tm-GFP was substituted for T-GFP (Fig. 2A, lane 8).

Activity of Rice Importin α1 in the in Vitro Import Assay Using Digitonin-permeabilized HeLa Cells—The PTAC, formed in the cytoplasm by binding of importin α/b heterodimer to NLS protein, docks to the cytoplasmic surface of the NPC via importin β binding to nucleoporins and is then transported as a single entity into the nucleus through a process mediated by the GTPase, Ran. To assess the functional activity of rice importin α1 in the process of nuclear import of proteins, we performed an in vitro nuclear import assay. As has been shown, a sufficient amount of transport factors are retained in permeabilized plant protoplasts to allow efficient nuclear import of proteins to take place (34, 41), making it protoplasts unsuitable for characterization of putative transport factors. Therefore, we employed a vertebrate assay system using permeabilized HeLa cells, in combination with vertebrate transport factors, in which the rice importin α1 was substituted for vertebrate importin α. T-GFP and Tm-GFP were used as transport substrates, as positive and negative controls, respectively.
In the presence of rice importin α1 in the transport solution, T-GFP was translocated efficiently into the nucleus (Fig. 3B, panel a). The translocation of the substrate was rice importin α-dependent (Fig. 3B, panel e) and NLS-specific (Fig. 3B, panel e). Omission of Ran-GDP or depletion of ATP by hexokinase from the transport solution abolished translocation of the substrate into the nucleus (data not shown).

These data strongly suggest that the rice importin α1 can replace vertebrate importin α in the mediation of nuclear import of NLS substrates, implying that rice importin α functions as a NLS receptor in the process of nuclear import of proteins.

In agreement with the in vitro binding assay (Fig. 1), rice importin α1 mediated nuclear import of O2-GFP, but not R-GFP, into the nucleus in the import assay (data not shown).

**DISCUSSION**

Although recent efforts have led to the identification of a number of putative nuclear import factors from plants, no direct functional evidence has been presented. This is due mainly to the lack of an appropriate plant in vitro system in which a putative nuclear transport factor can be characterized. Recently, two groups have independently developed a plant in vitro system for the study of nuclear import of proteins utilizing evacuated tobacco protoplasts (34, 41) and with which some unique features of plant nuclear import of proteins have been successfully elucidated. However, it has been found that a sufficient amount of transport factors are retained in the permeabilized protoplasts to allow for efficient protein import into the nucleus without the addition of any exogenous factors. This makes such an assay unsuitable for elucidating the role of a specific factor in the process of nuclear import of proteins. For this reason, we employed a vertebrate in vitro nuclear transport assay system to elucidate the function of rice importin α1, which we had identified previously. We demonstrated that rice importin α1, in combination with vertebrate transport factors, can specifically bind to functional NLS-containing proteins and direct them into the nucleus. These data present direct evidence, strongly suggesting that rice importin α1 functions as an NLS receptor in the NLS-mediated nuclear import of proteins within living cells. To our knowledge, this is the first time that a plant importin α homologue has been directly demonstrated to be functional in the nuclear import of proteins.

Our in vitro binding assay revealed that rice importin α1 specifically binds to T-NLS and O2-NLS protein, but not to R-NLS protein. This appears to be contrary to previous binding studies by Smith et al. (33), in which an importin α homologue of Arabidopsis bound to all three types of plant NLSs conjugated to BSA. This discrepancy might be due to the different binding assays employed in the experiments. However, this is unlikely, because in both the assays it was clearly shown that the importin α homologues specifically bound to the functional NLS, but not to nonfunctional NLS mutants. Rather, this discrepancy seems likely to suggest the existence of diversity among different importin α homologues with respect to NLS recognition. To date a number of importin α homologues have been identified from a wide range of species, including vertebrates, yeast, and plants. In fact, in many species, multiple family members have been identified. Previous in vitro binding studies and nuclear import assays in vertebrate cells have suggested the existence of different specificities of NLS recognition even between importin α homologues of the same origin. For example, in human cells, three importin α family proteins have been identified, namely, Rch1, NPI-1, and Qip1. These human importin α homologues have been shown to be ex-

**Fig. 3.** Rice importin α1 supported nuclear import of NLS-GFP in vertebrate in vitro assay system. A, mouse importin β alone does not dock (panels c and d), whereas in combination with rice importin α1, it does dock the T-GFP to the nuclear envelope (panels a and b). Such docking did not occur when Tm-GFP was used as substrate (panels e and f). B, rice importin α1 mediates translocation of T-GFP (panels a and b), but not Tm-GFP (panels e and f), into the nucleus in conjunction with mouse importin β and Ran-GDP. The translocation did not occur in the absence of rice importin α1 (panels c and d). Panels a, c, and e, fluorescence images; panels b, d, and f, phase contrast images.

In the nuclear binding assay as shown in the Fig. 3A, the mouse importin β alone was not sufficient to direct the substrate to the nuclear rim (Fig. 3A, panel c). However, addition of the rice importin α1 in the mixture resulted in efficient accumulation of T-GFP at the nuclear rim (Fig. 3A, panel a). In contrast, such accumulation did not occur when Tm-GFP was used as substrate (Fig. 3A, panel e).
pressed differentially in different tissues and cell lines (52, 53) and also have differential affinities for distinct types of NLSs (46, 49). We have shown in a previous paper (36) that the transcription level of rice importin α is light-regulated. All these previous and present data together suggest that the nuclear import of proteins is regulated at multiple levels, including the tissue-specific expression of importin α, as well as NLS-selective recognition by different importin α homologues.

We have shown previously that nuclear protein forms a stable PTAC with importin α and importin β heterodimer in the cytoplasm prior to nuclear pore binding (14). To determine whether rice importin α, like vertebrate and yeast importin α, assembles to form a complex with NLS substrate and importin β, we performed an in vitro binding assay. As shown in the Fig. 2, rice importin α bound directly to mouse importin β. However, the affinity was fairly low, compared with that between importin α and importin β of mouse (Fig. 2C and Ref. 43). We would ascribe the low affinity between rice importin α and mouse importin β to the heterogeneity of their origins. Although rice importin α shares about 48% identity in amino acid sequence with mouse importin α over the entire length, this may not be sufficient for rice importin α to form a tight complex with mouse importin β.

In the presence of NLS substrate, a large complex composed of rice importin α, mouse importin β, and the T-GFP formed in the mixture (Fig. 2A, lane 6, and Fig. 2B). Mouse importin β alone did not bind to NLS substrate (Fig. 2A, lane 9), indicating that the complex formed via rice importin α binding simultaneously to the NLS at one site and to importin β at another site. Moreover, the presence of the NLS-substrate in the mixture appeared to enhance the binding affinity between importin α and importin β (Fig. 2A, lane 6, and Fig. 2C, lane 4), suggesting that the NLS substrate may promote or stabilize the complex formation. It has been reported that the yeast importin β homologue, Kap 95, enhances binding of the yeast importin α homologue, Kap 60, to NLS-substrate, forming a more stable protein complex (29). Previously, we also found that mouse importin αβ complex binds at the nuclear rim only in the presence of the nuclear import substrate (43). Taken together, it appears that the protein complex is most stable when all the PTAC components, importin α, importin β, and the NLS substrate, are present in the complex. These findings suggest a cooperative interaction between NLSs and NLS receptor (importin αβ), in which the importin β enhances binding of importin α to NLSs (29), and the NLSs, in turn, enhance binding affinity between importin α and β (present study), consequently leading to formation of stable PTAC in the cytoplasm and docking the PTAC to the NPC (43).

Employing the vertebrate system in the present work allowed us to demonstrate that rice importin α is capable of mediating nuclear import of NLS-substrate into the nucleus, a strong implication of rice importin α as a plant NLS receptor in the process of nuclear import of proteins. We found that, in comparison with mouse importin α, a much higher concentration of rice importin α (about four times that of mouse importin α) is necessary to obtain equivalent transport. This could be accounted for by the low affinity between rice importin α and mouse importin β. However, the compatibility of rice importin α with vertebrate transport factors in our assay suggests that the nuclear import pathway for NLS proteins is well conserved between vertebrate and plant.

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