In vitro characterization of rice importin β1: molecular interaction with nuclear transport factors and mediation of nuclear protein import

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Abstract We recently isolated two cDNAs encoding importin β homologues (rice importin β1 and β2), the first such homologues identified in plants. To address the function of rice importin β1 in the process of nuclear import of proteins, we carried out in vitro binding and nuclear import assays. Recombinant protein of rice importin β1 assembled a complex (PTAC) with rice importin α1 and NLS protein, and also bound to the nuclear envelope of tobacco BY-2 cells. Ran-GTP, but not Ran-GDP, interacted with rice importin β1 and dissociated the heterodimer formed between rice importin α1 and rice importin β1. An in vitro nuclear import assay using digitonin-permeabilized HeLa cells revealed that rice importin β1 can mediate nuclear envelope docking of NLS proteins and their subsequent translocation into the nucleus. These data strongly suggest that rice importin β1 functions as a component of the NLS receptor in plant cells.

Key words: Importin β; Nuclear localization signal; Nuclear protein import; Rice (*Oryza sativa*)

1. Introduction

Nucleo-cytoplasmic transport of diverse proteins and RNA-protein complex takes place across the nuclear pore complex (NPC) [1–5]. The import of proteins containing a nuclear localization signal (NLS) into the nucleus is one of the best characterized transport pathways and is initiated by recognition of the NLS by the NLS receptor in cytoplasm. The NLS receptor exists as a heterodimer of importin α and importin β. Importin α recognizes and binds to the NLS of a nuclear protein, forming a stable pore-targeting complex (PTAC) in the cytoplasm [6–8], while importin β interacts directly with nucleoporins that contain FXFG or GLFG repeats, docking the PTAC to the cytoplasmic face of the NPC [9–11]. Translocation of the docked PTAC into the nucleus is an energy-dependent process, mediated by small GTPase Ran along with a homodimeric factor known as p10 or NTF2 [12–15]. Ran's nucleotide-bound state is regulated by two different proteins, the chromatin-bound exchange factor, RCC1, generating Ran-GTP in the nucleus [16,17], and the cytoplasmic GTPase activating protein, RanGAP1, which depletes Ran-GTP from the cytoplasm [12,18–22]. Ran-GTP binding to importin β has been shown to release importin α-substrate complex into the nucleus [23–25].

Utilizing both homology search and biochemical approaches, cDNAs for homologues of importin α [26–30], importin β [31], Ran [32–34] and Ran binding protein (RanBP1) [35] from diverse plant species have been identified and partially characterized. In addition, plant in vitro assays for the nuclear import of proteins have been developed using permeabilized, evacuolated protoplasts from tobacco BY-2 cell [26,36]. In these assays, however, in vitro import did not require exogenous cytosolic extract and ATP, suggesting that all necessary factors are retained within the permeabilized protoplasts in sufficient amount for efficient nuclear import. This makes such an assay unsuitable for elucidating the role of a specific factor in the process of nuclear import of proteins. In order to solve this problem, in our previous work we employed a vertebrate in vitro assay system using digitonin-permeabilized HeLa cells, and successfully demonstrated that the rice importin α1, in combination with vertebrate nuclear transport factors, can specifically bind to functional NLS-containing proteins and direct them into the nucleus [30].

Recently we isolated two cDNAs encoding the first known plant importin β homologues, rice importin β1 and β2 [31]. Recombinant rice importin β1 protein showed specific interaction with both the rice importin α1 and α2 [31]. In the present work, we show that the rice importin β1 interacts with importin α, the Ran GTPase and the nuclear envelope, and can mediate the nuclear import of NLS proteins in digitonin-permeabilized HeLa cells.

2. Materials and methods

2.1. DNA construction

GST-rice importin β1: a fragment of rice importin β1 cDNA [31] with an artificial *Eco*RI site at the 5′ end was generated by polymerase chain reaction (PCR) and inserted between the *Eco*RI and *Xho*I sites of pGEX-6p-3 (Pharmacia Biotech Inc.) to obtain an in-frame translation fusion with GST.

GST-NLS-GFP: oligonucleotides encoding either the NLS sequence of the SV40 large T-antigen (T-NLS: CTPPKKKRKV) or its point mutant, in which the sixth lysine residue of the T-NLS was replaced by a threonine (Tm-NLS: CTPPKTKRKV), were coupled to the 5′ end of the GFP gene by PCR using pS65T-C1 (Clontech Laboratories, Palo Alto, CA) as template DNA. The 5′ end primer used for the PCR incorporates an *Eco*RI site at the 5′ end followed in frame by the NLS-encoding nucleotide sequence. The 3′ end primer incorporates an *Xho*I site at its 5′ end. The PCR product was digested with *Eco*RI and *Xho*I restriction enzymes and cloned in frame at the 3′ end of GST in pGEX-4T-1 (Pharmacia Biotech Inc.). The 5′ end oligonucleotide primers used for the PCR were as follows: (1) T-NLS: 5′-ACG**GAATTC**TGCACCCCGCCGAAAAAAAAACGCAAAGTGATGAGTAAAGGAGAAGAACTTTTCACTGGA-3′; (2) Tm-NLS: 5′-ACG**GAATTC**TGCACCCCGCCGAAA**ACC**AAACGCAAAGTGATGAGTAAAGGAGAAGAACTTTTCACTGGA-3′. The 3′ end primer used was 5′-ACG**CTCGAG**TTATTTGTAGAGCT-

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CATCCATGCCATGTGT-3′. The nucleotide sequences encoding T-NLS and Tm-NLS are underlined and the endonuclease restriction sites are indicated by boldface.

2.2. Expression and purification of fusion proteins

The fusion proteins were expressed in *Escherichia coli* BL21 by growing in the presence of 0.5 mM IPTG for 4–6 h at 20°C and the proteins were purified essentially according to the manufacturer's instruction (Pharmacia Biotech). All the procedures were carried out at 4°C, and 1 mM EGTA and 2 mM dithiothreitol (DTT) were included in the solutions through out the purification procedures. The GST portion of the GST-rice importin α1 and rice importin β1 fusion protein was cleaved by incubation of the fusion protein bound to glutathione-Sepharose 4B resin with 80 U/ml resin of Precission protease (Pharmacia Biotech) for 4 h at 5°C. The Precission 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, Bedford, MA) and finally suspended in 20 mM HEPES buffer (pH 7.3) containing 1 mM EGTA and 1 mM DTT.

Recombinant rice importin α1 [30] and mouse Ran [20,37,38] were prepared as described previously.

2.3. In vitro binding assay

In vitro protein binding was examined by native gel electrophoresis as described previously [30]. 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, 1 mM EGTA and 2 mM magnesium acetate in both the gels and the running buffer. Protein bands on the gels were visualized by staining with Coomassie blue.

2.4. Fluorescence labeling of rice importin β1 and nuclear envelope binding assay

Rice importin β1 was labeled with Cy3 Dye (Amersham Life Science) essentially according to the manufacturer's instruction. The unconjugated dye was removed by Millipore Ultrafree-MC (Millipore, Bedford, MA) with five washes, and the labeled protein was suspended in 20 mM HEPES buffer (pH 7.3) containing 1 mM EGTA and 1 mM DTT.

Evacuolated protoplasts of BY-2 cells were isolated and permeabilized with Triton X-100 as described by Merkle et al. [36]. The Cy3-labeled rice importin β1, at final concentration of 10 μg/ml, either alone or with a four-fold excess of unlabeled rice importin β1, was mixed with the Triton-permeabilized, evacuolated BY-2 protoplasts and incubated for 20 min at room temperature. After fixation with 3.7% formaldehyde in TB for 30 min, the nuclei were stained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) at 0.5 μg/ml. Following twice washes with TB, an aliquot of the sample was transferred onto a slide glass and viewed under a microscope equipped with epifluorescence illumination (Olympus AX70). The microscopic images were taken with a MicroMAX digital CCD camera (Prinston Instruments, Trenton, NJ) and processed with the IP Lab image processing system (Scanalytics, Fairfax, VA).

2.5. HeLa cell culture and in vitro import assay

HeLa cells were cultured in 5% CO_2 at 37°C in Dulbecco's modified Eagle's essential medium supplemented with 5% fetal bovine serum (Life Technologies). Digitonin-permeabilized cells were prepared as described previously [39,40]. 10 μl sample solution contained 1 μl of GST-NLS-GFP (0.2 μg/μl) and appropriate transport factors were diluted with TB. For the nuclear binding assay, the incubation was performed on ice for 20 min in the presence of rice importin α1 (6 pmol) and rice importin β1 (6 pmol), the concentration of which was adjusted with TB containing 2% BSA. For the nuclear import assay, the incubation was performed at 25°C for 20 min in the presence of rice importin α1 (12 pmol), rice importin β1 (3 pmol), mouse Ran-GDP (42 pmol), 1 mM ATP, ATP regeneration system (20 units/ml creatine phosphokinase, 5 mM creatine phosphate), and 1 mM GTP, the concentration of which was adjusted with TB containing 2% BSA. After incubation, cells were fixed with 3.7% formaldehyde in TB. NLS-GFP was detected by Axiophot microscopy (Carl Zeiss).

2.6. Miscellaneous

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli [41]. Concentration of proteins were determined by the method of Bradford [42] using Bio-Rad dye reagent (Bio-Rad) and BSA as the standard.

3. Results and discussion

3.1. Complex assembly of importin α, β and NLS substrate

The NLS of a nuclear protein to be imported is recognized and complexed in cytoplasm by NLS receptor consisting importin α and β, thereby forming PTAC [6–8]. To address the ability of rice importin β1 to bind to importin α and assemble a PTAC in the presence of NLS substrate, an in vitro binding assay was carried out using native gel electrophoresis. On a native gel, complex formation between two proteins gives a new band with mobility different from either of the proteins alone. GST-T-NLS-GFP (T-GFP) and GST-Tm-NLS-GFP (Tm-GFP) fusion proteins were used as NLS substrates throughout the study, as positive and negative controls, respectively.

Rice importin β1, rice import α1, T-GFP and Tm-GFP migrate as single bands on a non-denaturing gel (Fig. 1, lanes 1, 2, 3 and 9). A mixture of rice importin β1 with rice importin α1 gave a new, major band of retarded mobility, with some unbound proteins migrating at the position of the controls (Fig. 1, lane 4), indicating a direct binding of the two proteins. Addition of T-GFP (Fig. 1, lane 5), but not Tm-GFP (Fig. 1, lane 7), to the mixture resulted in formation of a large complex with lower mobility on the gel relative to the complex formed either between rice importin β1 and α1 (Fig. 1, lane 4) or between rice importin α1 and T-GFP (Fig. 1, lane 6). This indicates that PTAC assembly occurred in the mixture. A mixture of rice importin β1 and T-GFP did not give any new visible band (Fig. 1, lane 10), suggesting that the PTAC assembly (lane 5) occurred through the rice importin α1 simultaneously binding to rice importin β1 at one site and T-GFP at another.

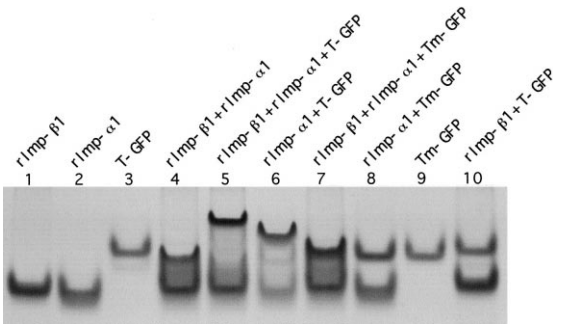

Fig. 1. Complex assembly of rice importin β1 with rice importin α1 and T-GFP. Native gel electrophoresis showing rice importin β1 (lane 1), rice importin α1 (lane 2), T-GFP (lane 3) and Tm-GFP (lane 9) run separately or as a mixture. The mixture of rice importin β1 and rice importin α1 (lane 4) gives a new band of retarded mobility, indicating binding between the two proteins. In the presence of T-GFP (lane 5), but not Tm-GFP (lane 7), it gives a major, new band of mobility lower than the complex composed of rice importin α1 and T-GFP (lane 6). These data show that rice importin β1 is capable of forming a complex with rice importin α1 and T-GFP. No direct interaction between rice importin β1 and T-GFP was observed (lane 10).

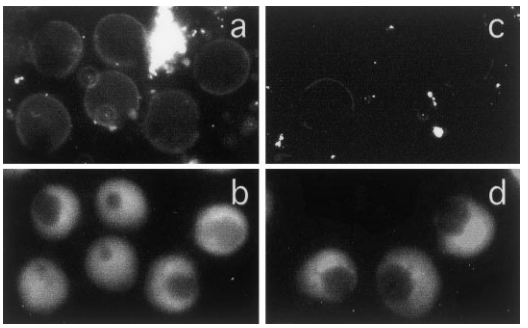

Fig. 2. Association of rice importin β1 with the nuclear envelope of tobacco BY-2 cells. Rice importin β1 was labeled with Cy3 dye and applied, either alone (a,b) or with a four-fold excess of unlabeled rice importin β1 (c,d), to Triton-permeabilized, evacuolated protoplasts of BY-2 cells. The Cy3-labeled rice importin β1 accumulated predominantly at the nuclear rim (a). Such accumulation was drastically reduced by the addition of unlabeled rice importin β1 (c), indicating specific binding of rice importin β1 to the nuclear envelope. Panels b and d show nuclei stained with DAPI.

3.2. Association of rice importin β1 to plant nuclear envelope

Importin β interacts directly with nucleoporins at the nuclear envelope, docking the PTAC to the cytoplasmic face of the NPC [9–11]. To assess if rice importin β1 can bind to the nuclear envelope, we labeled the rice importin β1 with the fluorescent dye, Cy3, and applied to Triton-permeabilized, evacuolated BY-2 protoplasts. As shown in Fig. 2, the rice importin β1 accumulated predominantly at the nuclear rim (Fig. 2a). Such accumulation was rice importin β1-specific, as addition of a four-fold excess unlabeled rice importin β1 drastically reduced the accumulation of Cy3 Dye at the nuclear rim (Fig. 2c). This observation suggests a specific binding of rice importin β1 to the nuclear envelope, probably via interaction with some yet unidentified nucleoporins.

3.3. Interaction between rice importin β1 and Ran

The GTPase Ran has been shown to be involved in multiple reactions in the translocation of the PTAC into the nucleus. In its GTP-bound state, Ran has been shown to bind directly to importin β1 and dissociate the importin α/β heterodimer

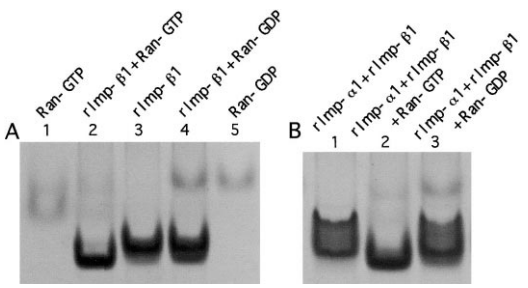

Fig. 3. Interaction between rice importin β1 and Ran. A: Native gel electrophoresis showing rice importin β1, Ran-GTP and Ran-GDP run separately or as a mixture. Ran-GDP gives a single band (lane 5), while the Ran-GTP sample appears as doublet bands (lane 1) due to the presence of some GDP-bound form in the sample. The lower band represents Ran-GTP, which is more negatively charged, and the upper band represents Ran-GDP. A mixture of rice importin β1 and Ran-GTP gives a new, major band with low mobility (lane 2), indicating interaction between the two proteins. No such interaction was observed with Ran-GDP (lane 4). B: Native gel electrophoresis showing Ran-GTP (lane 2), but not Ran-GDP (lane 3), dissociates the heterodimer of rice importin β1 and rice importin α1.

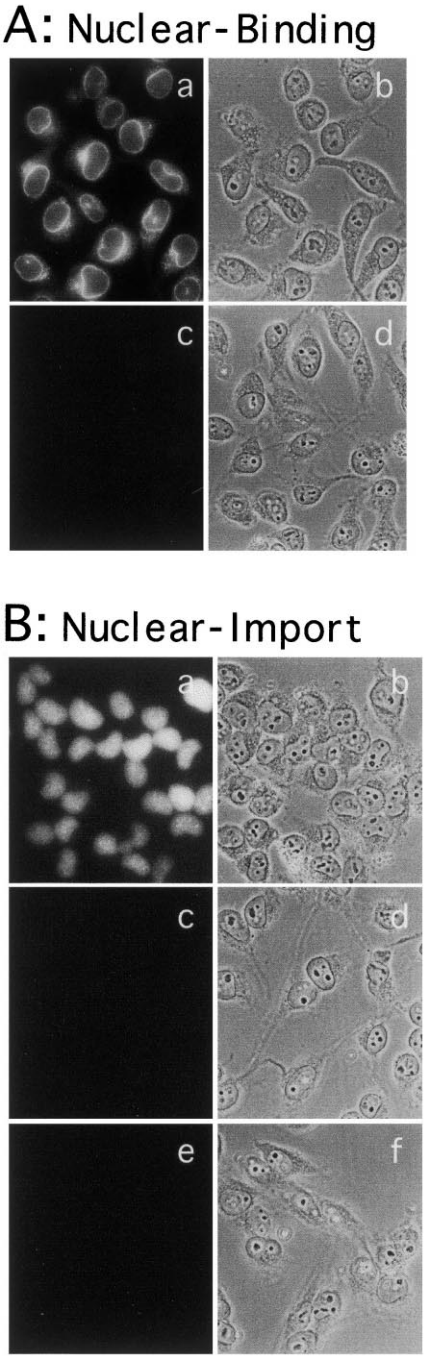

Fig. 4. Rice importin β1 supports the nuclear import of NLS-GFP in a vertebrate in vitro assay system. A: Rice importin α1 alone does not dock (c,d), whereas in combination with rice importin β1, it can dock the T-GFP to the nuclear envelope (a,b). B: Rice importin β1, in conjunction with rice importin α1 and mouse Ran-GDP, mediates translocation of T-GFP (a,b), but not Tm-GFP (e,f), into the nucleus. The translocation did not occur in the absence of rice importin β1 (c,d). Panels a, c and e: fluorescence images; panels b, d and f: phase contrast images.

[23–25]. We therefore examined the interaction between rice importin β1 and Ran, as well as the effect of Ran on the interaction between rice importin β1 and rice importin α1.

Ran-GDP migrates as single band on the gel (Fig. 3A, lane 5), while Ran-GTP sample appears as doublet bands (Fig. 3A, lane 1). The lower band represents Ran-GTP, which is more

negatively charged, while the upper band represents Ran-GDP. In our Ran-GTP preparation, about 60–70% is in the GTP-bound form, while the other 30–40% is in the GDP-bound form. A mixture of rice importin β1 with Ran-GTP gave a major band of high mobility (Fig. 3A, lane 2). In contrast, no such new major band was observed in a mixture of rice importin β1 with Ran-GDP (Fig. 3A, lane 4). This result indicates that rice importin β1 specifically interacts with Ran-GTP but not with Ran-GDP. There was a new minor band with the same mobility of rice importin β1/Ran-GTP complex in lane 4. However, this was most likely due to the presence of trace amounts of Ran-GTP in the Ran-GDP sample.

The specific interaction of Ran-GTP with the rice importin β1 completely dissociated the heterodimer of rice importin α1 and rice importin β1 (Fig. 3B, lane 2). In contrast, no apparent effect was observed with the Ran-GDP (Fig. 3B, lane 3).

3.4. Activity of the rice importin β1 in the in vitro import assay using digitonin-permeabilized HeLa cells

The PTAC, formed in the cytoplasm by the binding of importin α/β heterodimer to NLS protein, docks at the cytoplasmic surface of NPC via binding of importin β to nucleoporins. The PTAC is then transported as a single entity into the nucleus in a process mediated by the GTPase Ran. To assess the functional activity of the rice importin β1 in the process of nuclear import of proteins, we performed an in vitro nuclear import assay using digitonin-permeabilized HeLa cells.

In the nuclear binding assay as shown in the Fig. 4A, the rice importin α1 alone was not sufficient to direct the substrate to the nuclear rim (Fig. 4Ac). However, addition of the rice importin β1 to the mixture resulted in efficient accumulation of T-GFP at the nuclear rim (Fig. 4Aa).

In the presence of the rice importin β1 in the transport solution, T-GFP was translocated efficiently into the nucleus (Fig. 4Ba). The translocation of the substrate was rice importin β1-dependent (Fig. 4Bc) and NLS-specific (Fig. 4Be).

In summary, we have shown here that rice importin β1 shares most of the molecular interactions common to all known importin βs from vertebrates and yeast, specifically, interaction with importin α, the nuclear envelope and Ran-GTP. In addition, the in vitro transport assay demonstrated that rice importin β1, in conjunction with importin α and Ran factor, can mediate the import of NLS protein into the nucleus. These data strongly suggest that rice importin β1 functions as a component of the NLS receptor in plant cells.

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