A novel importin α from rice, a component involved in the process of nuclear protein transport

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Abstract In eukaryotes, nuclear proteins that are transported into nuclei have nuclear localization signals (NLSs), which are recognized by proteins called importin α. We isolated a rice cDNA, #61L, and the corresponding gene that encodes a protein, which shows significant homology to the importin α. Although the encoded protein had only 23–27% amino acid identity to the importin αs from various organisms including plants, the fusion protein with glutathione S-transferase showed a specific binding activity to the NLS of SV40 T-antigen. These results suggest that the rice #61L protein is a novel importin α in plants.

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

1. Introduction

In eukaryotic cells, the nuclear envelope defines the nuclear and cytoplasmic compartments. Nuclear proteins, which are synthesized in the cytoplasm, are selectively imported into nuclei through the nuclear pore complex (NPC). Most nuclear proteins contain a short stretch(es) of amino acid sequences, called a nuclear localization signal (NLS), which directs the NLS-containing proteins to nuclei [1]. By using digitonin-permeabilized mammalian cells as an in vitro system [2], the process of the nuclear protein import has been experimentally scrutinized, and the essential protein components required for nuclear transport have been identified [3]. In the cytoplasm, NLS-containing proteins bind to the nuclear protein import carrier, and then they are docked to the cytoplasmic side of the NPC. The import carrier is composed of two subunits, importin α and importin β, each of which is involved in the recognition of NLS sequences and the interaction with the NPC, respectively. Subsequently, NLS-containing proteins are transported along with importin α into the nucleoplasm with the aid of other import factors, GTPase Ran and NTF2 [4,5].

Several lines of evidence indicate that the mechanism of nuclear protein transport identified in animals and yeast is conserved in plants: (1) Some NLS sequences from animal and yeast proteins as well as the NLS of SV40 large T-antigen function in plant cells [6]. (2) In principle, the properties of in vitro nuclear transport systems in plant cells are similar to those in mammalian cells, in that they both are NLS-dependent and energy-requiring processes [7,8]. (3) The observation that the addition of cytoplasmic extract from plant cells conferred transport activity on an in vitro nuclear transport system in mammalian cells indicates that plant cells also contain essential import factors homologous to mammalian factors [9]. Indeed, plant cDNAs encoding proteins homologous to some components required for nuclear protein import have been identified as follows: importin α from *Arabidopsis thaliana* [7,10], Ran from *Arabidopsis* [11], tobacco [12], tomato [13] and *Vicia faba* [14], and Ran-binding proteins from *Arabidopsis* [11].

Importin α cDNAs have been identified in a wide variety of species, and the presence of multiple genes has been shown in animals [3,15]. To date, five importin α cDNAs have been identified both in mouse [16] and in humans [17], and differential tissue- and cell type-specific expression of the corresponding genes has been shown [16–18]. Moreover, it has been reported that multiple importin αs from humans differ in their recognition of some NLSs [19–22]. Here, we describe a rice cDNA and the corresponding gene encoding a novel importin α. The NLS-binding activity of the recombinant fusion protein was shown in an in vitro binding assay. These results indicate the presence of two importin α subfamilies in plants.

2. Materials and methods

2.1. Basic techniques

Rice plants (*Oryza sativa* L. cultivar 'Nipponbare') were grown as previously described [23]. Library construction, screening, sequence analysis, and DNA and RNA blot analysis were essentially carried out by the standard procedures [24]. DNA sequences were determined on both strands.

2.2. Recombinant protein production and NLS-binding assay

Recombinant fusion of glutathione S-transferase (GST) and the protein encoded by the rice cDNA was produced in *Escherichia coli* using the pGEX4T expression vector (Pharmacia Biotech, Uppsala). The fragment of rice #61L cDNA with an artificial 5′ *Bam*HI site was generated by polymerase chain reaction, and inserted between the *Bam*HI and *Sma*I sites of pGEX4T-1 to obtain in-frame translational fusion with GST. The plasmid was introduced into *E. coli* (JM109) and the production of the GST-importin α fusion protein was induced by the addition of isopropylthio-β-D-galactoside (1 mM). Extraction and affinity purification of the fusion protein with glutathione-Sepharose (Pharmacia Biotech) were carried out according to the manufac-

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Abbreviations: GST, glutathione S-transferase; IBB, importin β binding; NLS, nuclear localization signal; NPC, nuclear pore complex

DDBJ accession number: D78504 (#61L cDNA), AB006788 (#61L gene).

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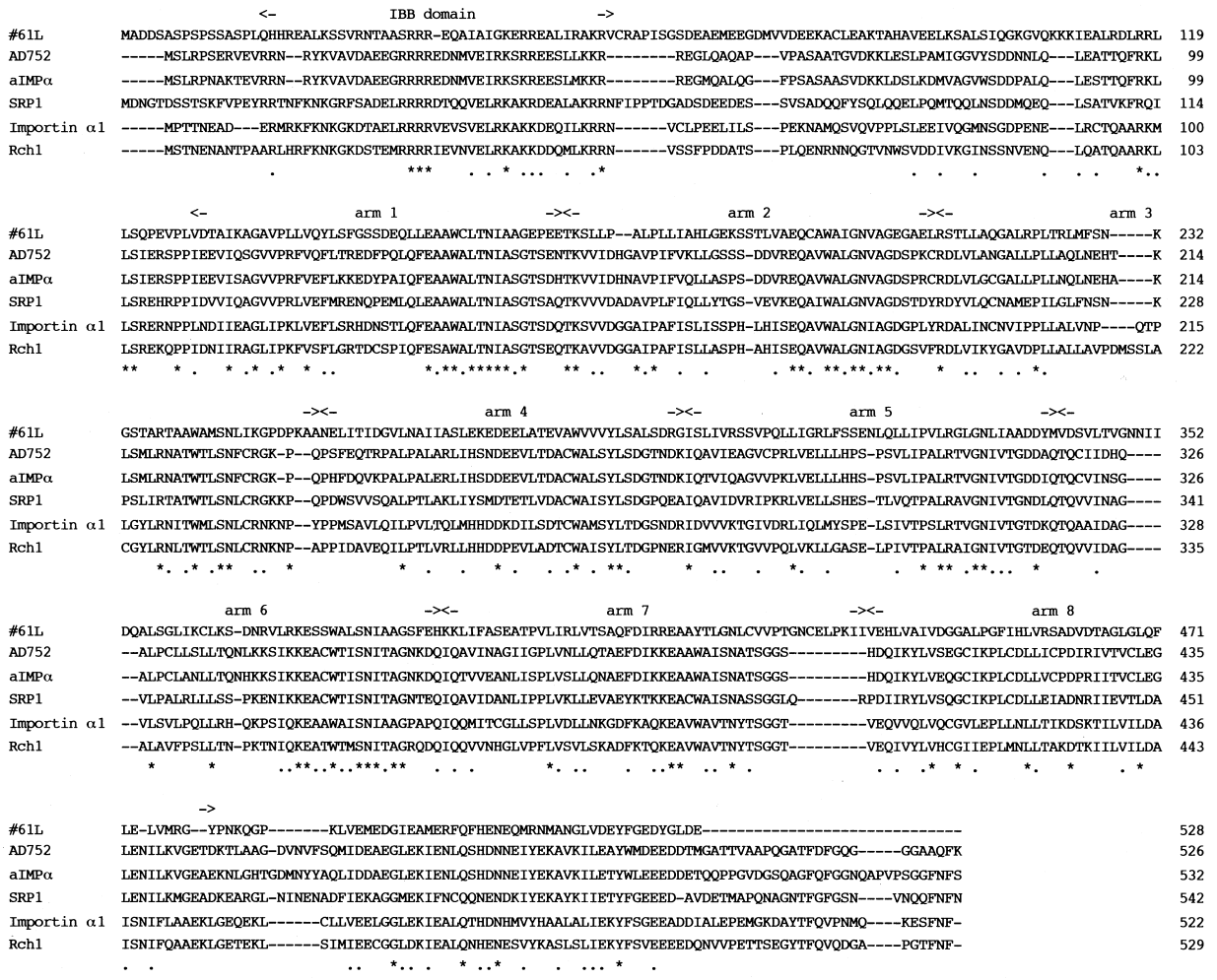

Fig. 1. Multiple alignment of importin α. The amino acid sequences of rice #61L, rice AD752 [29], *Arabidopsis* aIMPα [7], yeast SRP1 [26], *Xenopus* importin α1 [27] and human Rch1/hSRP1α [28] were aligned by the Clustal W (1.60) program with default parameters. Dashed lines indicate gaps inserted to achieve maximum homology. Identical residues are indicated with asterisks, similar residues with dots. The IBB domain and arm repeats are indicated above the alignment.

turer's instructions. The recombinant fusion protein is denoted as GST-rice importin α in the text.

The NLS-binding activity of the GST-rice importin α was examined using artificial substrates as previously reported by Imamoto et al. [25]. Bovine serum albumin conjugated to synthetic peptides containing SV40 T-antigen NLS (BSA-SV40 T) was used as an artificial substrate. The BSA-SV40 T was labeled with ^{125}I by use of the Radioiodination System (DuPont/NEN, Wilmington, DE). The GST-rice importin α (480 pmol) was immobilized on glutathione-Sepharose beads (80 μl of packed volume) in 0.5 ml of a buffer (20 mM HEPES-KOH, pH 7.3, 110 mM potassium acetate, 5 mM sodium acetate, 2 mM magnesium acetate, 0.5 mM EGTA, 2 mM dithiothreitol, 2% BSA), and incubated on ice for 1 h. The beads were pelleted by centrifugation (500×*g*, 5 min), washed three times with 1 ml of the buffer, and suspended in the same buffer. GST-fusion protein trapped by beads (10 μl packed volume) was incubated with 320 fmol (~36 000 cpm) of ^{125}I-labeled BSA-SV40 T in 100 μl of the buffer on ice for 1 h. The beads were collected by centrifugation, washed three times with 0.5 ml of the buffer, and the radioactivity associated with the beads was counted by a γ-counter. To demonstrate the specificity of NLS-binding to importin α, 120 pmol of unlabeled wild-type, point-mutated or reverse sequence of SV40 T NLS conjugated to BSA were added as competitors to the in vitro binding assay system. The binding activity of GST-mouse importin α was examined as a positive control. The synthetic peptide sequences were as follows: wild-type, CYGGPKKKRKVEDP; point-mutated (mutant), CYGGPKTKRKVEDP; or reverse sequence, CYGGPDEVKRKKKP. BSA-$\overline{\text{S}}$V40 T conjugates and its derivatives, and the GST-mouse importin α were kindly provided by Dr. N. Imamoto, Osaka University, Osaka, Japan.

3. Results and discussion

3.1. Isolation of a rice cDNA encoding a protein homologous to importin α

In the course of characterizing genes expressed in rice seedling leaves, we isolated a novel cDNA (#61L) possibly encoding a homologue of importin α. The 1751 bp nucleotide sequence contains a 1587 bp open reading frame, which encodes a putative polypeptide of 528 amino acids with calculated molecular mass of 57 129 Da. Northern blot analysis using poly(A) RNA showed that a transcript of 1.7 kb in length, corresponding to that of the #61L cDNA, was detected in all tissues examined: cultured cells, roots, leaves of seedlings and mature plants, and flowers (data not shown). The level of the transcript was so low that it was not detected when total RNA was used (data not shown). A multiple alignment of amino acid sequences of importin α from rice, *Arabidopsis*

[7], yeast [26], *Xenopus laevis* [27] and human [28] is shown in Fig. 1. Recently, we have cloned another rice importin α cDNA (AD752; accession number AB004660) [29], and the deduced amino acid sequence was included in the alignment. The AD752 protein shares about 70% amino acid identity with importin α from *Arabidopsis*, and each of them has 40–50% amino acid identity with those from yeast and animals. In contrast, the #61L protein has only 23–27% amino acid identity with other importin αs including those from plants. In spite of the lower homology with other importin αs, the #61L protein exhibited overall similarity with importin α: (1) It contains eight degenerate repeats of 42 amino acid sequences, called arm motifs, which is a conserved characteristic of importin α [30]. (2) There is a stretch of basic amino acids in the N-terminal region, which is highly conserved among importin αs from various organisms, and has been identified as an importin β-binding (IBB) domain [31,32]. The overall sequence similarity suggests that the #61L protein is an importin α of rice.

3.2. Rice importin α binds SV40 T-antigen NLS

To determine if the protein encoded by the rice #61L cDNA has a function as an NLS receptor, the NLS-binding activity of the recombinant fusion protein with GST was examined in an in vitro assay system according to Imamoto et al. [25] (Fig. 2). The synthetic NLS of SV40 T-antigen conjugated with BSA was used as an artificial NLS protein, since SV40 T-antigen NLS has been well characterized and is also known to function as an NLS in plants [6]. Just as in the positive control, in which the binding activity of the GST-mouse importin α was confirmed (Fig. 2b), a significant amount of ^{125}I-labeled BSA-SV40 T was bound to the GST-fusion protein (Fig. 2a). The radioactivity bound to the fusion protein was reduced by the addition of an excess amount of non-labeled wild-type SV40 T conjugate as a competitor, but not by the addition of non-functional NLS substrates such as point-mutated or reverse sequence SV40 T conjugate. These results indicate that the rice protein has binding activity specific to the functional NLS. The NLS-binding activity observed with the rice protein is consistent with the understanding that #61L cDNA encodes an importin α in rice plants.

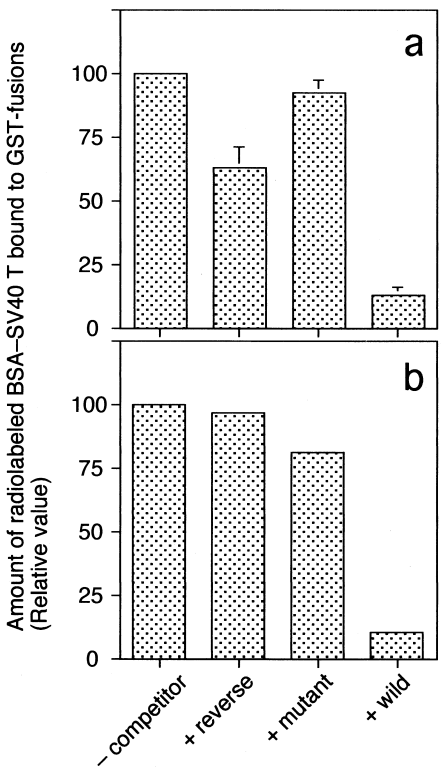

Fig. 2. NLS-binding activities of GST-rice importin α (a) and GST-mouse importin α (b). Amounts of radiolabeled BSA-SV40 T bound to each GST-fusion protein that had been immobilized to glutathione-Sepharose beads, in the absence of competitor, or in the presence of 375-fold molar excess of non-labeled BSA-SV40 T of reverse, mutant, or wild-type sequence, are shown as relative values. One hundred percent corresponds to 77 fmol and 130 fmol of the radiolabeled BSA-SV40 T in panels (a) and (b), respectively. The vertical lines in (a) represent standard deviations ($n = 3$).

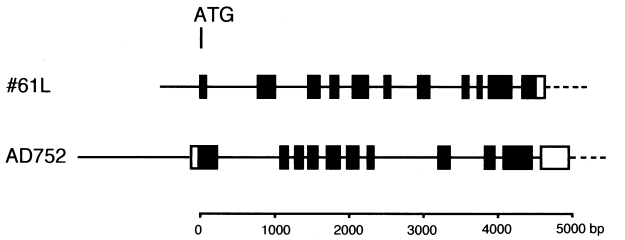

Fig. 3. Schematic structure of two importin α genes of rice. Solid lines indicate introns and franking DNA. Open and solid boxes represent untranslated and translated exons, respectively. Dashed lines indicate unsequenced DNA.

3.3. Multiple genes encoding importin α in rice

A genomic clone encoding #61L importin α was isolated and sequenced. In order to estimate the copy number of the #61L gene, we carried out Southern blot analysis using the #61L cDNA probe that was not hybridized to AD752 cDNA (data not shown). Sizes of the hybridized bands in the genomic Southern blot analysis (data not shown) were consistent with the prediction from the nucleotide sequence of the cloned #61L gene, indicating that the #61L gene is a single copy gene in rice. Structure of the gene encoding #61L importin α was compared to that of the gene encoding AD752 importin α ([29]; accession number AB004814). The relative organization of exons and introns is not conserved between #61L and AD752 genes; the number of the protein-coding exons are 11 and 10, respectively (Fig. 3). In addition, the positions of intron insertion relative to the domain structure of the encoded proteins was not conserved in two rice importin α genes (data not shown). These results suggest that these two genes have evolved independently.

The phylogenetic tree of importin α shows that the rice #61L protein is the most distant member among known importin αs from various organisms (Fig. 4). Although two importin αs have been reported in *Arabidopsis* [10,33], we could find another EST from *Arabidopsis* (accession number F15465), which has about 60% identity with #61L at the level of both nucleotide and amino acid sequences. It might be interpreted that importin α homologous to rice #61L is also present in *Arabidopsis*, and that the #61L protein and the homologue are functionally differentiated from the known importin α. Clarification of the functions of this novel importin α will facilitate more detailed understanding of the nuclear import in plants.

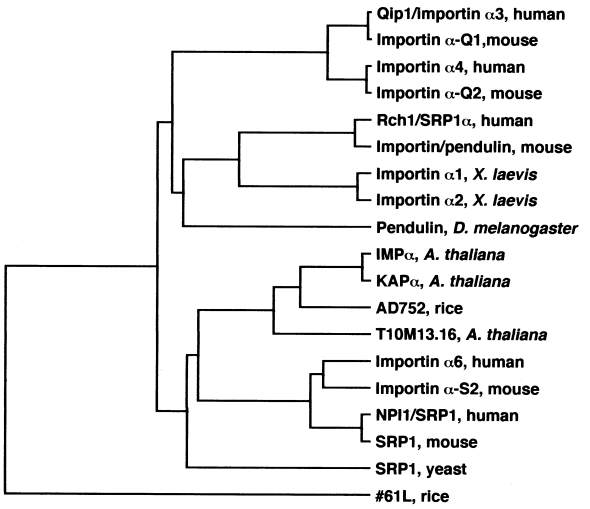

Fig. 4. Phylogenetic tree of importin α. The tree was constructed by the UPGMA method using the GENETYX-MAC 7.3 software (Software Development Co., Tokyo) with default parameters. The accession number of the putative open reading frame T10M13.16 which is predicted from the genomic sequence is AF001308.

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