Importin β, transportin, RanBP5 and RanBP7 mediate nuclear import of ribosomal proteins in mammalian cells

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The assembly of eukaryotic ribosomal subunits takes place in the nucleolus and requires nuclear import of ribosomal proteins. We have studied this import in a mammalian system and found that the classical nuclear import pathway using the importin α/β heterodimer apparently plays only a minor role. Instead, at least four importin β-like transport receptors, namely importin β itself, transportin, RanBP5 and RanBP7, directly bind and import ribosomal proteins. We found that the ribosomal proteins L23a, S7 and L5 can each be imported alternatively by any of the four receptors. We have studied rpL23a in detail and identified a very basic region to which each of the four import receptors bind avidly. This domain might be considered as an archetypal import signal that evolved before import receptors diverged in evolution. The presence of distinct binding sites for rpL23a and the M9 import signal in transportin, and for rpL23a and importin α in importin β might explain how a single receptor can recognize very different import signals.

Keywords: importin β/nuclear transport/RanBP7/ribosomes/transportin

Introduction

Transport between the cell nucleus and cytoplasm proceeds through the nuclear pore complexes (NPCs) and comprises a multitude of substrates (for recent reviews, see Corbett and Silver, 1997; Görlich, 1997; Nigg, 1997; Mattaj and Englmeier, 1998). Not only do all nuclear proteins need to be imported from the cytoplasm, but tRNA, rRNA and mRNA, which are synthesized in the nucleus, need to be exported to the cytoplasm where they function in translation. Indeed, the biogenesis of ribosomes even involves multiple crossings of the nuclear envelope; ribosomal proteins are first imported into the nucleus, assemble in the nucleolus with rRNA and finally are exported as ribosomal subunits to the cytoplasm (reviewed in Scheer and Weisenberger, 1994; Melese and Xue, 1995). In quantitative terms, this is a major activity. For a growing HeLa cell, one can estimate that each NPC has to import 100 ribosomal proteins and to export three ribosomal subunits per minute (see Görlich and Mattaj, 1996).

NPCs can accommodate active transport of particles as large as 25 nm in diameter or several million Daltons in molecular weight (Feldherr et al., 1984). This active transport is generally energy dependent and receptor mediated. In addition, NPCs provide a 9 nm diffusion channel for ions, metabolites and, in principle, also for macromolecules smaller than ≈60 kDa (Bonner, 1978). However, the transport of small RNAs such as tRNA and small proteins such as histones is normally active and carrier mediated (Zasloff, 1983; Breeuwer and Goldfarb, 1990).

Active transport across the NPC requires nuclear transport factors which fall into three categories: namely, transport receptors, adaptor molecules and the constituents of the RanGTPase system (reviewed in Görlich, 1997; Ullmann et al., 1997; Izaurralde and Adam, 1998; Mattaj and Englmeier, 1998). Transport receptors interact directly with NPCs, shuttle continuously between nucleus and cytoplasm, bind cargo molecules and carry them through the NPCs. In some cases, adaptors such as importin α have to bridge the interaction between a transport receptor and a substrate. Transport receptors form a protein superfamily whose members are of similar size (90–130 kDa) and have an importin β-like RanGTP-binding motif (Fornerod et al., 1997a; Görlich et al., 1997). Transport receptors use RanGTP binding as a means to regulate their interactions with cargoes or adaptor molecules. According to the direction in which they carry a cargo, they can be grouped into import receptors (importins) or export receptors (exportins).

Ran’s nucleotide-bound state is controlled by the nucleotide exchange factor RCC1, which can charge Ran with GTP (Bischoff and Ponnstingl, 1991), and the GTPase-activating protein RanGAP1, which converts RanGTP into RanGDP (Bischoff et al., 1994, 1995; Becker et al., 1995). The nuclear localization of RCC1 (Ohtsubo et al., 1989) and the nuclear exclusion of RanGAP1 (Hopper et al., 1996a,b; Melchior et al., 1993b; Matunis et al., 1996; Mahajan et al., 1997) should result in a very low cytoplasmic RanGTP concentration and high levels in the nucleus. This RanGTP gradient across the nuclear envelope has been proposed to control transport receptor–substrate interactions in a compartment-specific manner (Görlich et al., 1996b,c).

The import receptors such as importin β or transportin bind their substrates only in the absence of RanGTP, i.e. in the cytoplasm, and release them upon interaction with RanGTP which should happen in the nucleus where the RanGTP concentration is predicted to be high (Rexach and Blobel, 1995; Chi et al., 1996; Görlich et al., 1996b; Izaurralde et al., 1997; Siomi et al., 1997). Importin β and transportin are probably exported to the cytoplasm as RanGTP complexes (Izaurralde et al., 1997). This should preclude re-export of the cargoes they just carried in. Finally, cytoplasmic RanBP1 (Coutavas et al., 1993; Schlenstedt et al., 1995; Richards, et al., 1996) and RanGAP1 remove RanGTP from the import receptors and restore them to an import-competent form (Bischoff and...
Substrate binding to the exportins (CRM1/exportin 1, CAS, exportin-t) appears to be regulated in exactly the opposite way, in being greatly enhanced by simultaneous RanGTP binding (Fornerod et al., 1997b; Kutay et al., 1997b; Arts et al., 1998; Kutay et al., 1998). This should happen in the nucleus where the RanGTP concentration is predicted to be high. The trimeric substrate–exportin–RanGTP complex is then transferred to the cytoplasm. There, RanGTP needs to dissociate from the complex to allow the exportin to release the substrate, to re-enter the nucleus and to bind and export the next cargo molecule.

Import into the nucleus can proceed by several distinct pathways. The importin αβ heterodimer mediates import of proteins with a classical nuclear localization signal (NLS) (Adam and Adam, 1994; Görlich et al., 1994, 1995a; Chi et al., 1995; Imamoto et al., 1995a,b; Radu et al., 1995). The classical NLS contains either one cluster of basic amino acids (SV40-type NLS, Kalderon et al., 1984) or two basic clusters (bipartite NLS, Robbins et al., 1991; Makker et al., 1996). We can distinguish the following steps in this pathway. The initial cytoplasmic event is the binding of the import substrate to the importin αβ heterodimer. Importin α provides the NLS-binding site (Adam and Adam, 1994) and interacts via its importin beta-binding domain (IBB domain) with the β-subunit (Görlich et al., 1996a; Weis et al., 1996). Importin β in turn interacts with the NPC (Görlich et al., 1995b; Moroianu et al., 1995). The subsequent events include Ran-dependent steps (Melchior et al., 1993a, Moore and Blobel, 1993). The trimeric NLS–importin αβ complex is transferred into the nucleus where it meets an environment with a high RanGTP concentration. Direct binding of RanGTP to importin β displaces the α-subunit (Rexach and Blobel, 1995; Chi et al., 1996; Görlich et al., 1996b). The NLS protein dissociates from importin α, and importin α gets re-exported with the aid of its exportin CAS (Kutay et al., 1997b). The RanGTP–importin β complex is probably returned directly to the cytoplasm (Izaurralde et al., 1997).

Importin β can also bind to and import proteins independently of the importin α adaptor. This was first demonstrated for a fusion protein containing the IBB domain from importin α (Görlich et al., 1996a; Weis et al., 1996). Similarly, transportin, an importin β-related receptor, binds its import substrates (hnRNP proteins) directly (Pollard et al., 1996; Fridell et al., 1997). The hitherto best characterized import signal for transportin-dependent nuclear import is the glycine-rich M9 domain from hnRNP A1 (Siomi and Dreyfuss, 1995; Weighardt et al., 1995).

Further candidates for being import receptors are RanBP7 (Görlich et al., 1997) and RanBP5 (Deane et al., 1997; Yaseen and Blobel, 1997). RanBP5 is in sequence clearly related to importin β and to transportin. RanBP7 is more distantly related, and significant homology to importin β is restricted to the N-terminal Ran-binding domain. RanBP7 was identified originally as a protein from *Xenopus* that forms a stable heterodimer with importin β. The function of this heterodimer is still unknown. Both RanBP5 and RanBP7 bind NPCs; however, none of them has so far been shown to be a functional transport receptor.

Ribosomal proteins (rps) are a very abundant class of import substrates. They are usually small and very basic proteins with apparently complex nuclear import signals (Schaap et al., 1991; Schmidt et al., 1995; Russo et al., 1997). Mechanistic aspects of their import so far have only been studied in yeast, where two importin β-like transport receptors have been implicated in rpl25 import, namely Yrb4p (also called Kap123p) and Pse1p, the homologue of RanBP5 (Rout et al., 1997; Schlenstedt et al., 1997).

Here we show that importin β, transportin, RanBP5 and RanBP7 mediate import of ribosomal proteins into nuclei of mammalian cells. This establishes first that RanBP5 and RanBP7 are functional importins, second that transportin’s range of substrates is not restricted to hnRNP proteins, and third that importin α-independent import is a major activity of importin β. Surprisingly, the three ribosomal proteins we tested, rpS7, rpL5 and rpL23a, can each be imported alternatively by any of the four import receptors. We have identified a 32 amino acid domain in rpL23a which conforms direct binding to and import by either importin β, transportin, RanBP5 or RanBP7. This domain might thus be considered as an archetypal import signal that evolved before these import receptors diverged in evolution. Furthermore, it points to a remarkable and unexpected functional conservation of these import receptors.

**Results**

We wanted to investigate by which import route(s) ribosomal proteins enter the nucleus in higher eukaryotes. For this purpose, we purified ribosomes from canine pancreas, modified the ribosomal proteins with fluorescein 5′ maleimide and extracted them from the rRNA (see Materials and methods). We then used this mixture of fluorescent ribosomal proteins as a substrate for import into nuclei of permeabilized HeLa cells (Figure 1). An energy-regenerating system was present in all incubations. Without addition of soluble transport factors, only a low level of import was evident. However, with reticulocyte lysate as a source of soluble transport factors, the ribosomal proteins efficiently entered the nuclei and accumulated as bright spots in the nucleoli, and the nucleoli even enlarged (see corresponding panels in Figure 1). Import was blocked completely by addition of the GTPase-deficient RanQ69L mutant (3 μM final). This was the expected result if import was mediated by importin β-like import receptors. RanGTP binding to importin β, for example, triggers substrate release. As detailed in the Introduction, RanGTP normally should only be available and stable in the nucleus, making this release normally a specific nuclear event that terminates import. RanQ69L, however, remains GTP-bound even in the presence of cytoplasmic RanGAP1 (Bischoff et al., 1994) and, therefore, acts as a dominant-negative mutant by promoting premature substrate release in the cytoplasm.

**Importin β, transportin, RanBP5 and RanBP7 are import receptors for ribosomal proteins**

We then tested if import of the ribosomal proteins can be reconstituted with recombinant transport factors (see corresponding panels in Figure 1). The addition of Ran alone already stimulated import weakly, probably because
Nuclear import of ribosomal proteins

**Fig. 1.** Nuclear import receptors for ribosomal proteins. Total, fluorescein-labelled ribosomal proteins were prepared from canine ribosomes as described in Materials and methods and used as the substrate for import into nuclei of permeabilized HeLa cells. Import was for 15 min at room temperature in the presence of an energy-regenerating system. Where indicated, the import reaction contained the following additions: reticulocyte lysate (Retic); 3 μM RanQ69L; a Ran-mix (Ran) containing 4 μM RanGDP, 0.4 μM RanBP1, 0.4 μM *Schizosaccharomyces pombe* Rna1p and 0.4 μM NTF2 (each final concentrations); 2 μM importin β; 2 μM importin β + 6 μM importin α; 3 μM transportin; 3 μM RanBP5; or 3 μM RanBP7. The reactions were stopped by fixation and import was analysed by confocal fluorescence microscopy. Note that efficient nucleolar accumulation of the ribosomal proteins occurred in the presence of either reticulocyte lysate, importin β, transportin, RanBP5 or RanBP7. The RanQ69L mutant had a strong dominant-negative effect.

Surprisingly, transportin also stimulated nuclear import of the ribosomal proteins (Figure 1), suggesting that transportin's range of substrates is not restricted to hnRNP proteins. To our knowledge, this is the first case of non-hnRNP proteins shown to be imported by transportin. Moreover, RanBP5 and RanBP7 also promoted import of ribosomal proteins into human cell nuclei, which is the first direct demonstration that RanBP5 and RanBP7 function as nuclear import receptors. It should be noted that import by importin β, transportin, RanBP5 or RanBP7 is Ran dependent and was much weaker if no exogenous Ran had been added.

**Interactions of ribosomal proteins with nuclear transport receptors**

The existence of four import carriers for ribosomal proteins raises two immediate questions. First, can a given ribosomal protein be imported by any of the four factors, or alternatively are there distinct populations each absolutely depending on one of the import receptors? Secondly, which import signals confer direct binding to and import by importin β, transportin, RanBP5 or RanBP7? In Figure 1, we analysed the import of a mixture of ribosomal proteins. To address the two questions, we decided to study a number of individual ribosomal proteins in more detail.

We expressed the ribosomal proteins S7, L5 and L23a in *Escherichia coli*, immobilized them and tested which factors from a *Xenopus* egg extract they would bind. The bound fractions were analysed by SDS–PAGE followed by Coomassie staining and by immunoblotting with various specific antibodies. We had included the following con-
controls. First, CAS did not bind significantly to any of the immobilized ribosomal proteins. Secondly, an immobilized bovine serum albumin (BSA)–SV40 NLS conjugate (a substrate of the classical import pathway) bound specifically the importin α/β heterodimer, but not transportin, RanBP5 or RanBP7. Thirdly, the IBB domain (from importin α) efficiently bound importin β and the importin β–RanBP7 heterodimer, as reported before (Görlich et al., 1997), but it did not bind importin α, transportin or RanBP5.

Each of the ribosomal proteins S7, L5 and L23a efficiently bound importin β and RanBP7 (Figure 2). Importin α was recovered with rpS7 and rpL23a, but not with rpL5. Significant transportin binding was detected only for rpL23a. It should be noted, however, that transportin binding is here probably underestimated because in a Xenopus egg extract most transportin appears inactive (see also below). Finally, RanBP5 bound very efficiently to rpL23a, more weakly to rpS7 and to a low, but still significant, extent to rpL5.

In conclusion, each of the ribosomal proteins apparently can interact with several of the transport receptors. The apparently extreme example was rpL23a to which importin α, importin β, transportin, RanBP5 and RanBP7 had bound efficiently. However, this binding activity from the egg extract did not yet prove direct interactions with rpL23a. First, because additional (mainly low molecular weight) proteins were also bound which could as well account for the recovery of the transport receptors. Secondly, importin β and RanBP7 can form a heterodimer and importin β could mediate the rpL23a binding of RanBP7, or vice versa. It was therefore crucial to test which of the transport receptors would bind individually to the ribosomal protein. For this purpose, we expressed importin β, transportin, RanBP5 and RanBP7 in E.coli and used the bacterial lysates as a starting material for the next binding experiments.

Importin β alone bound to rpL23a very efficiently...
Fig. 4. rPL23a can be imported into the nucleus via four distinct pathways. Nuclear import of the indicated fluorescein-labelled import substrates (~4 μM each) was performed in the presence of a Ran mix (see Figure 1), an energy-regenerating system and either buffer, 1.5 μM importin β, 1.5 μM importin β + 4.5 μM importin α, 4 μM transportin, 4 μM RanBP5 or 2.5 μM RanBP7. Reactions were stopped after 15 min by fixation, and import was analysed by confocal fluorescence microscopy. Note that efficient import of nucleoplasmin was only observed with importin α/β, that of the IBB fusion only with importin β, and that of the M9 fusion only with transportin. In contrast, import of the ribosomal protein L23a was efficient either with importin β, transportin, RanBP5 or with RanBP7.

(Figure 3A). The presence of 10 μM RanQ69L (GTP) essentially abolished binding. This was not only a stringent specificity control, it also established that RanGTP regulates the importin β–rPL23a interaction in the same way as that between importin α and β. When importin α was added to the importin β lysate, then importin α was recovered with the immobilized rPL23a stoichiometrically with the β subunit. It should be noted that importin α binding was strictly dependent on the presence of importin β (not shown), as if the importin α/β heterodimer would bind rPL23a only via the β subunit. Consistent with this, RanQ69L reduced the recovery of both importin β and α to the same low level (Figure 3A).

Transportin, RanBP5 and RanBP7 could each bind efficiently to rPL23a (Figure 3B and C). RanQ69L completely abolished the binding of transportin and RanBP5. However, in the case of RanBP7, RanQ69L caused only a slight reduction in the binding. The affinity of RanBP7
**Fig. 5.** Nuclear import of ribosomal proteins S7 and L5. Import in the presence of energy and Ran was performed and analysed as in Figure 4, using fluorescent fusions with rpS7 and rpL5 as substrates.

importin β and that of the M9 fusion only with transportin. RanBP5 and RanBP7 had no effect on import of any of these three control substrates. In contrast, import of rpL23a was efficient either with importin β, transportin, RanBP5 or RanBP7 (Figure 4). Importin α did not stimulate, but instead reduced the effect of importin β. Thus, although the importin α/β heterodimer can bind rpL23a (Figures 2 and 3A), it is in this case apparently not an active species in import.

It is indeed remarkable that rpL23a can ‘choose’ between at least four nuclear import receptors. Figure 5 shows that this phenomenon is not limited to rpL23a. Import of rpS7 was evident with either importin β, transportin, RanBP5 or RanBP7 (Figure 4). Importin α did not stimulate, but instead reduced the effect of importin β. Thus, although the importin α/β heterodimer can bind rpL23a (Figures 2 and 3A), it is in this case apparently not an active species in import.

**RanBP7, RanBP5, transportin and importin β can each mediate nuclear import of rpL23a, rpS7 or rpL5**

Having established that rpL23a can bind several importin β-like factors directly, we wanted to compare its import with that of well-characterized import substrates (Figure 4). The controls behaved as expected, and efficient import of nucleoplasmin was observed only with the importin α/β heterodimer, that of the IBB fusion only with importin β and that of the M9 fusion only with transportin. RanBP5 and RanBP7 had no effect on import of any of these three control substrates. In contrast, import of rpL23a was efficient either with importin β, transportin, RanBP5 or RanBP7 (Figure 4). Importin α did not stimulate, but instead reduced the effect of importin β. Thus, although the importin α/β heterodimer can bind rpL23a (Figures 2 and 3A), it is in this case apparently not an active species in import.

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**A ‘universal’ NLS in rpL23a that accesses multiple import pathways**

There are two extreme possible explanations for how the interaction between rpL23a and four different import receptors can be explained. First, there could be several distinct binding sites in rpL23a, each specific for one of the receptors. Secondly, there could be one ‘universal’ binding site for all the receptors. To distinguish between the possibilities, we expressed fragments of rpL23a in

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**Table I.** Mapping the binding site in rpL23a for nuclear import receptors

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<th>rpL23a fragment</th>
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<td>Importin β</td>
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The rpL23a N-terminal domain is IPPAKKEAP10 APPKAEEKAK20 ALKAKAVLKL30 GVHSHKKK40 RTSPTFRRPK50 TLRLRQPKY60 PRKSPKNK70 LDHY......

The indicated rpL23a fragments were expressed as 2z-tagged fusions in E.coli, immobilized and used to bind recombinant importin β, transportin, RanBP5 or RanBP7 out of bacterial lysates as in Figure 4. Binding was scored from ‘+ + + + + +’ (binding equally efficient as to full-length rpL23a) to ‘-’ (no detectable binding).

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Import of a fusion with residues 32–74 from rpL23a

E. coli, immobilized them and tested their capacity to bind importin β, transportin, RanBP5 or RanBP7. The results are summarized in Table I. rpL23a is 156 amino acids long and consists of two domains. The C-terminal domain (residues 75–156), which is conserved between eukaryotes, eu- and archaebacteria, did not bind any of the nuclear import receptors detectably. The N-terminal half (residues 1–74), which is absent in bacteria, but conserved between rpL23a in higher eukaryotes and yeast rpL25, accounts for all the interactions with the four import receptors. The binding region could be narrowed down to further residues 32–74. Further truncations resulted in a partial or complete loss of binding. However, at this resolution, the effects were slightly different for the different import receptors. For example, residues 52–74 still bound RanBP5 to some extent, but RanBP7 binding was already lost.

In conclusion, rpL23a does not have separate binding sites for each of the four import receptors. Instead, importin β, transportin, RanBP5 and RanBP7 bind to the same region in rpL23a. We will refer to this region as the BIB domain (beta-like import receptor binding domain). Figure 6 shows that the BIB domain is not only sufficient for binding to the four receptors, but also confers import by any of these factors. For example, residues 32–74 still bound RanBP5 to some extent, but RanBP7 binding was already lost.

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**Transportin has two distinct substrate-binding sites**

Transportin can bind the M9 domain of hnRNP A1 and the BIB domain of rpL23a. The BIB domain is exceptionally basic (IP 12.2), whereas the M9 domain is not basic but instead is rich in glycine. This raises the question as to how transportin can recognize such very different signals. Does it have one very ‘flexible’ binding site for both types of signal, or distinct binding sites each specific for one of the signals? To distinguish between the two possibilities, we tested whether the M9 and BIB domain compete for each others binding to transportin. The BIB domain from rpL23a was immobilized and used to bind transportin out of an E. coli lysate. As can be seen from Figure 7 (left panel), an excess of a GST–M9 fusion did not compete transportin binding. Instead, the M9 fusion appeared in the bound fraction, indicating the formation of a trimeric M9–transportin–rpL23a complex. The recovery of the GST–M9 fusion on the rpL23a beads was dependent on the specific M9–transportin interaction, as verified by two controls: First, the GST–M9 fusion was not bound if transportin was replaced by importin β (Figure 7, right panel). Secondly, the G274A M9 mutant (Michael et al., 1995), which is deficient in transportin binding (Pollard et al., 1996), did not engage into the
Importin α and rpl23a bind to different parts of importin β

Importin β also binds two types of import signals, the BIB domain from rpl23a and the IBB domain from importin α. Both are very basic, but otherwise unrelated in sequence, and so we also wondered in this case if the binding sites on importin β are distinct or the same. We therefore tested various importin β fragments for their capacity to bind rpl23a. As seen from Figure 8, residues 1–462 of importin β bound equally efficiently as the full-length protein (residues 1–876). A further C-terminal deletion of 53 amino acids (1–409) essentially abolished binding and thus defined a C-terminal border of the binding domain. A deletion of the N-terminal 285 residues (286–876) had no effect on the importin β–rpl23a interaction; binding to the 331–876 fragment of importin β was, however, significantly less efficient. In conclusion, approximately residues 286–462 of importin β are essential for high affinity binding to rpl23a. This is in clear contrast to the IBB–importin β interaction which requires importin β’s intact C-terminus (residues 286–876, Chi and Adam, 1997; Kutay et al., 1997a). Thus the IBB and BIB domains do not bind to identical sites on importin β.

Discussion

Import into the cell nucleus can occur through several, distinct pathways. The classical import pathway is mediated by the importin αβ heterodimer, where importin α recognizes NLSs such as those found in the SV40 large T antigen with one cluster of basic amino acids, or the bipartite NLS from nucleoplasmin with two stretches of basic residues. A distinct pathway is mediated by transportin which imports hnRNP proteins such as A1. The import signal of hnRNP A1 is the glycine-rich M9 domain. Here, we have investigated the import of ribosomal proteins into mammalian cell nuclei. Ribosomal proteins are evolutionary ancient, normally very basic (average IP for human ribosomal proteins is 10.1) and most of them would be small enough to diffuse through the NPC into the nucleus. However, nuclear import of ribosomal proteins is active and receptor mediated (see, for example, Figure 1). This is similar to histone H1 which is also small and where active import into the nucleus dominates over diffusion (Breeuwer and Goldfarb, 1990). Binding to an import receptor might not only make nuclear entry more rapid, it could also help to prevent undesired interactions in the cytoplasm.

We show here that at least four importin β-like import receptors mediate nuclear import of ribosomal proteins, namely importin β, transportin, RanBP5 and RanBP7 (Figures 1, 4 and 5). All of them, even importin β, bind ribosomal proteins directly without an adaptor. The ability of importin β to function without an α-subunit was first observed for an artificial substrate, namely a fusion containing the IBB domain from importin α (Görlich et al., 1996a; Weis et al., 1996). Likewise, it has been shown that human importin β can bind and import yeast Nab2p directly (Truant et al., 1998). A more physiological substrate for direct, importin β-dependent import might be the HIV Rev protein (Henderson and Percipalle, 1997). Our data now suggest that importin α-independent import is a major activity of importin β. This more simple mode of import appears evolutionarily ancient, and it seems very likely that importin β had already played a role in nuclear import of ribosomal proteins before importin α evolved. That transportin also imports ribosomal proteins came as quite a surprise. To our knowledge, rpL23a and rPS7 are the first non-hnRNP proteins shown to be imported by transportin. RanBP7 has been known to interact specifically with NPCs and with RanGTP, and on these grounds was proposed to be a nuclear transport receptor (Görlich et al., 1997). Here we show that RanBP7 is indeed a functional importin. We therefore suggest to refer to RanBP7 as importin 7. RanBP5 previously has been shown to interact with NPCs, RanGTP and denatured ribosomal proteins (Deane et al., 1997; Yaseen and Blobel, 1997). We provide here the functional import data that demonstrate that RanBP5 is an import receptor for ribosomal proteins.

RanBP5 and transportin show significant overall sequence homology to importin β (Pollard et al., 1996; Deane et al., 1997; Yaseen and Blobel, 1997). Importin 7 (RanBP7) is evolutionarily more distant, and the recogniz-
able homology is restricted to the N-terminal RanGTP-binding motif (Görlich et al., 1997). The functional conservation, however, is quite remarkable in that any of the four import receptors can accomplish import of rpL23a, rpS7 and rpL5. Moreover, at least in the case of rpL23a, the receptors recognize essentially the same import signal, which we will refer to as the BIB domain. The BIB domain is more complex than a classical NLS, is extremely basic and might be considered as an archetypal import signal that evolved before these import receptors have diversified in evolution. Ribosomal proteins most likely were already import substrates of the progenitor of present importin β-like import receptors. While these receptors diversified in evolution, they acquired specialized binding sites such as that for the M9 domain in transportin, or that for importin α in the case of importin β; but obviously they also maintained their capacity to bind and import ribosomal proteins.

The binding sites in transportin for the M9 domain and for rpL23a are distinct. Transportin is also the import receptor for the hnRNP F protein, and possibly also for hnRNP D and E (Siomi et al., 1997). These proteins have no obvious sequence similarity to either the M9 domain or to rpL23a. This raises the question as to what range of substrates a single import receptor potentially can import. In principle, one could imagine that any substrate that binds specifically the Ran-free conformation of the receptor can be imported, provided it does not interfere with the NPC passage or disturb the interaction with RanGTP. Alternatively, it is quite possible that ribosomal proteins are released immediately after NPC passage. Indeed, a delayed release from the receptor might be an advantage. Ribosomal proteins are very sticky, and importin 7 (RanBP7) could also shield them from undesired interactions on the way from the NPC to the nucleolus. The ribosomal protein could then be passed from the import receptor to an assembly factor (chaperonin), or be transferred directly to the rRNA. We currently are investigating these possibilities.

Materials and methods

Plasmids and constructs

For recombinant expression of ribosomal proteins, we have used the following vectors: p2x60 is a pQE60 (Qiagen) derivative for the expression of fusions with two consecutive z tags (IgG-binding domain from protein A) at the N-terminus and a C-terminal His tag (Görlich et al., 1997). p4zCys is analogous to p2x60, but provides four N-terminal z tags, a C-terminal cysteine for covalent modification, followed by a His tag. p6Cys is identical to p4zCys, except that six z tags are fused to the N-terminus. It is important to note that the z domains alone neither bind to any of the transport receptors nor get imported by them (not shown).

The expression constructs for ribosomal proteins were generated as follows. The coding sequences for rpS7, rpL5 and rpL23a, or fragments from rpL23a were amplified from HeLa cDNA using the Boehringer
high fidelity PCR system and specific primer pairs that each introduced a 5′ Ncol and a 3′ BamHI site. The amplified fragments were cloned into the Ncol–BamHI sites of the expression vectors. Expression of ribosomal proteins was from p2z60 for all binding experiments (Figures 2, 3, 7, and 8) and to prepare the rpL5 import substrate, p4zCys was used to express rpL23a for import assays and p6zCys was used to express rpS7 and the 32–74 rpL23a fragment (BIB domain) for import assays.

RanBP5 was amplified by PCR from HeLa cDNA using the primers AGCGGTACCGCAACGAAGCAGCAGCAGCAACAGTTC (upstream) and GCGCTCGAGGATGATTGTTTCTGTTGTGA-TATTAGG (downstream). The coding sequence was cloned as a KpnI–Xhol fragment into the KpnI–SalI sites of pQE32 (Qiagen), allowing expression with an N-terminal His tag. Note that the upstream primer changed the codon usage of the five N-terminal alanines. Xenopus RanBP7 was cloned into the BamHI–HindIII sites of pQE9 (Qiagen) allowing expression with an N-terminal His tag.

**Recombinant protein expression and protein purification**

The following proteins were expressed in E.coli BLR/Rep4 and purified as previously described: Xenopus importin α (Görlich et al., 1994), human importin β (Görlich et al., 1996b), importin β fragments (Kutay et al., 1997a) and transportin (Izaurralde et al., 1997). RanBP7 and RanBP5 were expressed with an N-terminal His tag from pQE32 (Qiagen), allowing expression with an N-terminal His tag. Note that the upstream primer changed the codon usage of the five N-terminal alanines. Xenopus RanBP7 was cloned into the BamHI–HindIII sites of pQE9 (Qiagen) allowing expression with an N-terminal His tag.

**Preparation of labelled recombinant import substrates**

The preparation of fluorescent nucleoplasmin (Görlich et al., 1994), IBB-nucleoplasmin core fusion (Görlich et al., 1996a) and core M9 fusion (Kutay et al., 1997a) has been described previously. Labelling with fluorescein 5′ maleimide (Calbiotech) of 4z-rpL23a, 6z-S7, 6z-32–74 rpL23a (6z-BIB) was performed in 50 mM HEPES–KOH pH 7.5, 300 mM potassium acetate, 5 mM magnesium acetate. Free label was removed by gel filtration on an NAP5 column equilibrated in 20 mM potassium phosphate pH 7.2, 300 mM potassium acetate, 5 mM magnesium acetate.

Labelling with FLUOS (Boehringer) of 2z-rpL5 was performed in 50 mM HEPES–KOH pH 7.5, 1 mM lithium chloride, 5 mM magnesium acetate. Free label was removed with an NAP5 column equilibrated in 50 mM HEPES–KOH pH 7.0, 300 mM potassium acetate, 5 mM magnesium acetate.

**Preparation of fluorescein-labelled total ribosomal proteins**

Ribosomes were purified by stripping them from an E. coli preparation from canine pancreas. A total of 50 000 equivalents of E. coli membranes (for definition, see Walter and Blobel, 1983) were adjusted to 500 mM potassium phosphate pH 7.2, 150 mM potassium acetate. The import buffers for the various substrates contained, in addition, the following salts: for total ribosomal proteins (Figure 1), 20 mM potassium phosphate pH 7.2, 200 mM potassium acetate; for 4z-rpL23a and 6z-rpS7 (Figures 4 and 5), 20 mM HEPES–KOH pH 7.5, potassium acetate 250 mM; for 2z-rpL5 (Figure 5), 50 mM potassium phosphate pH 7.2, 250 mM potassium acetate; for nucleoplasmin, IBB core and M9 core, 20 mM HEPES–KOH pH 7.5, 150 mM potassium acetate (Figure 4); for the 6z-32–74 rpL23a fragment (6z-BIB, Figure 6), 20 mM potassium phosphate pH 7.2, 150 mM potassium acetate.

Because nucleoplasmin core binds avidly to ribosomal proteins and inhibits their import, it was omitted in the import reactions for ribosomal proteins.

**Antibodies**

Antibodies against the following antigens have been described previously: Xenopus importin α, human importin β (Görlich et al., 1995b) and Xenopus RanBP7 (Görlich et al., 1997). Antibodies against recombinant human CAS, human transportin and human RanBP5 were raised in rabbits. Affinity purification was on sulfoLink (Pierce) to which the antigens had been coupled.

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**References**


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