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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 Ponstingl, 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.*, 1990; 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

Görlich, 1997; Floer *et al.*, 1997; Lounsbury and Macara, 1997).

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 α/β 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; Makkerh *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 α/β 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 α/β 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 confers 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

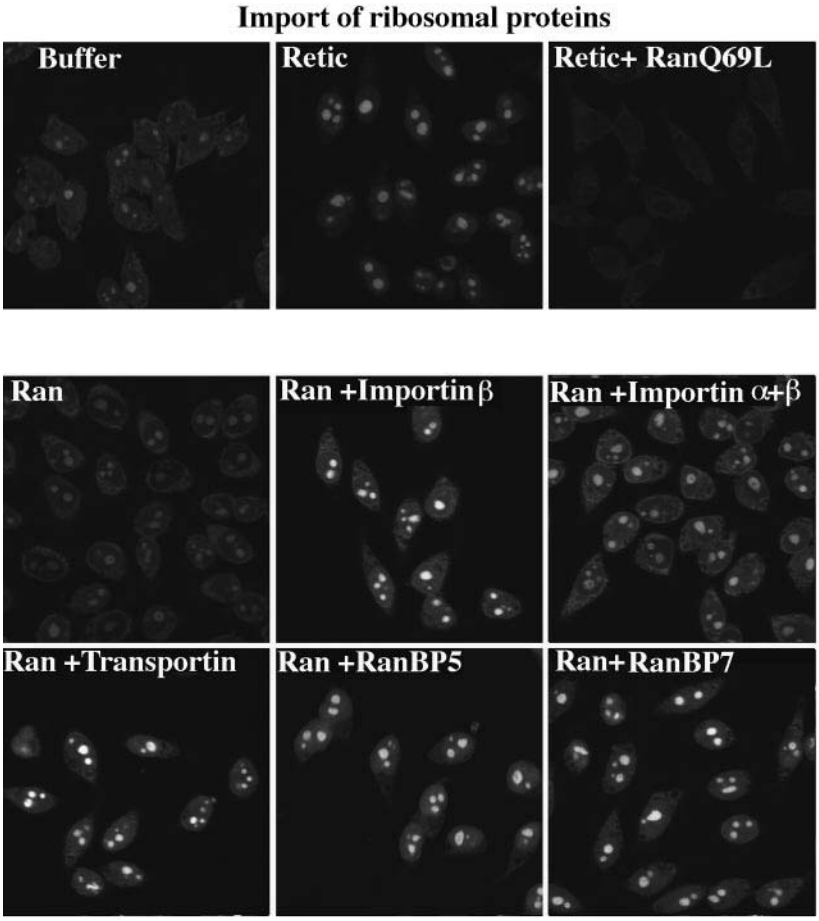

Fig. 1. Nuclear import receptors for ribosomal proteins. Total, fluorescein-labelled ribosomal proteins were prepared from canine ribosomes as described in Materials and methods and used as the substrate for import into nuclei of permeabilized HeLa cells. Import was for 15 min at room temperature in 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.

the permeabilized cells still contain a significant amount of transport receptors. If importin β was also added, then very efficient import was observed. The classical NLS import pathway requires importin α. However, in this case, exogenous importin α significantly reduced the capacity of importin β to promote import, suggesting that ribosomal proteins can bind importin β directly and without importin α.

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.

It might also be worth mentioning that import of ribosomal proteins is not an intrinsic activity of all importin β-like transport factors. The export receptors CAS and exportin-t, for example, do not import ribosomal proteins detectably (data not shown, but see Figure 2 below.).

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-

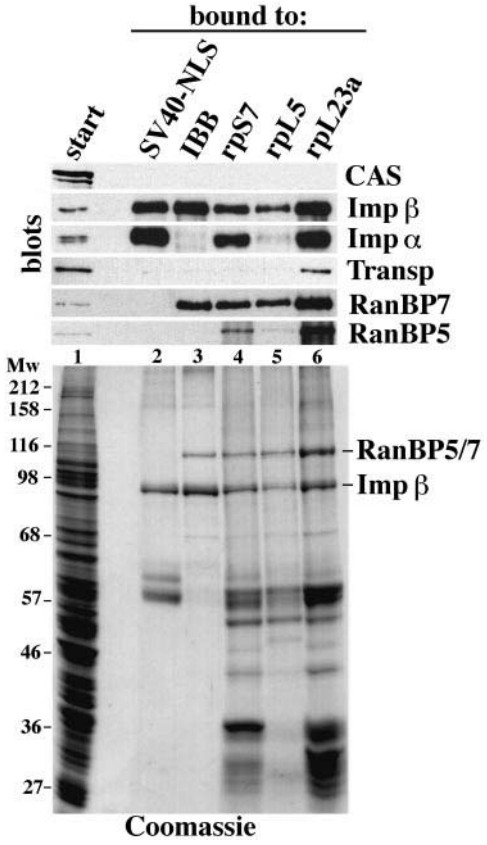

Fig. 2. Ribosomal proteins interact with multiple cytosolic transport receptors. The ribosomal proteins S7, L5 and L23a were immobilized and tested for binding of nuclear transport receptors from a *Xenopus* egg extract. Positive controls were binding to an immobilized SV40 NLS peptide–BSA conjugate (specific for the importin α/β heterodimer) or to the IBB domain from importin α (specific for importin β and the importin β–RanBP7 heterodimer). Each 20 µl of the resins was incubated with 500 µl of egg extract (HSS). After extensive washing, bound proteins were eluted with 1.5 M $MgCl_2$, precipitated and analysed by SDS–PAGE followed by immunoblotting with specific antibodies or by Coomassie staining. The load in the bound fractions corresponds to 25 times the starting material. The positions of RanBP5, RanBP7 and importin β in the Coomassie gel are indicated. Transportin would co-migrate with importin β.

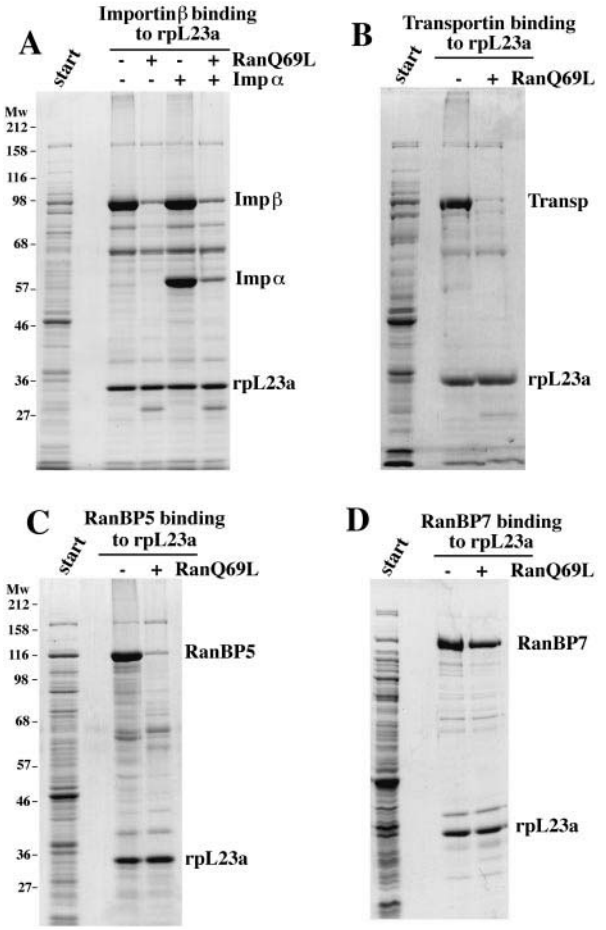

Fig. 3. Direct interaction of rpL23a with nuclear transport receptors. Immobilized rpL23a was used to bind either (**A**) recombinant importin β, (**B**) transportin, (**C**) RanBP5 or (**D**) RanBP7 out of total *E.coli* lysates. Where indicated, 10 µM RanQ69L GTP or 5 µM *Xenopus* importin α had also been added. Analysis of the starting material and bound fraction was by SDS–PAGE followed by Coomassie staining. The load in the bound fractions corresponds to 15 times the starting material. Note that importin β, the importin α/β heterodimer, transportin, RanBP5 and RanBP7 all bound specifically to rpL23a. RanQ69L strongly reduced binding of all these factors, except for RanBP7 where the effect was weaker. It should also be noted that the (minor) background bands in the bound fractions in (A) and (C) are mainly proteolysis products from importin β (A) or RanBP5 (C).

trols. 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

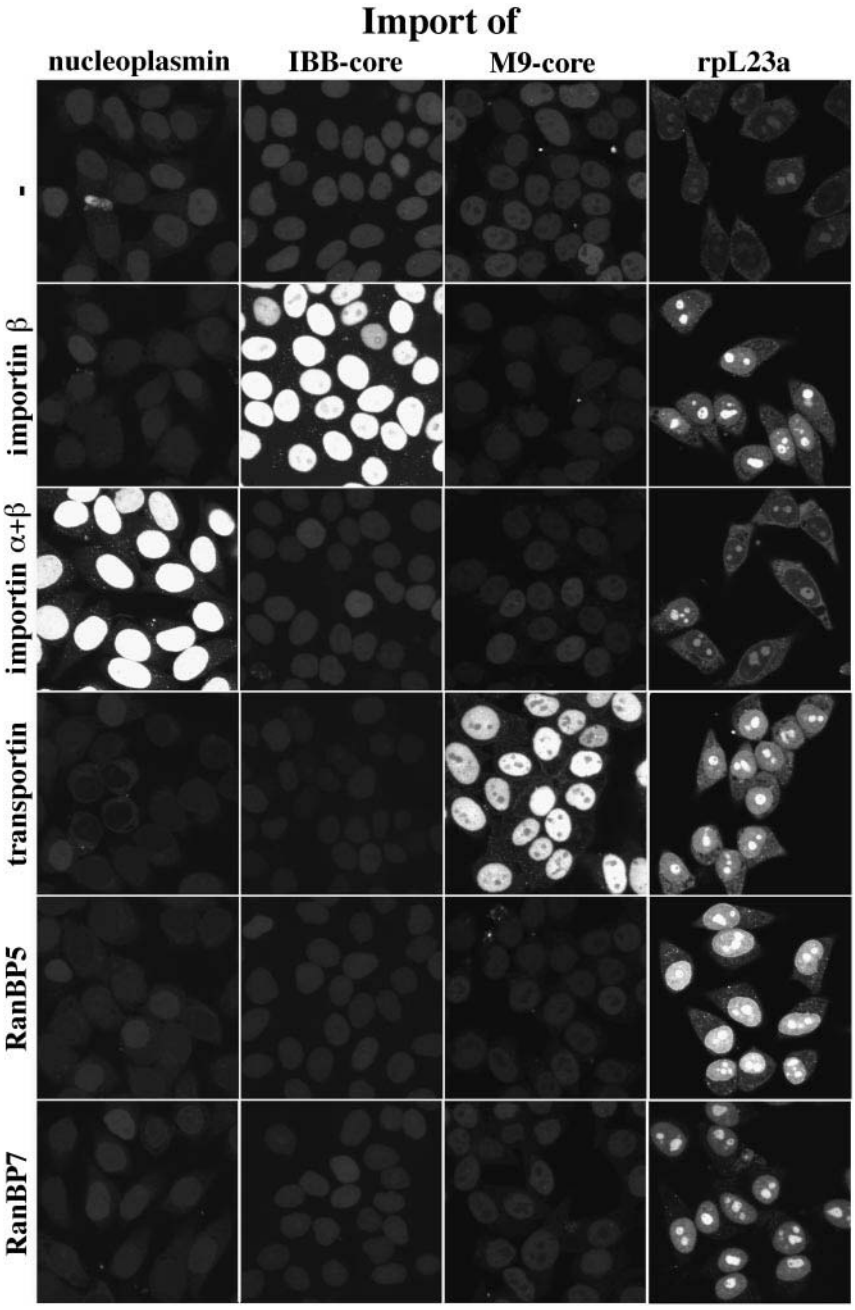

Fig. 4. rpL23a can be imported into the nucleus via four distinct pathways. Nuclear import of the indicated fluorescein-labelled import substrates (≈4 μM each) was performed in the presence of a Ran mix (see Figure 1), an energy-regenerating system and either buffer, 1.5 μM importin β, 1.5 μM importin 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

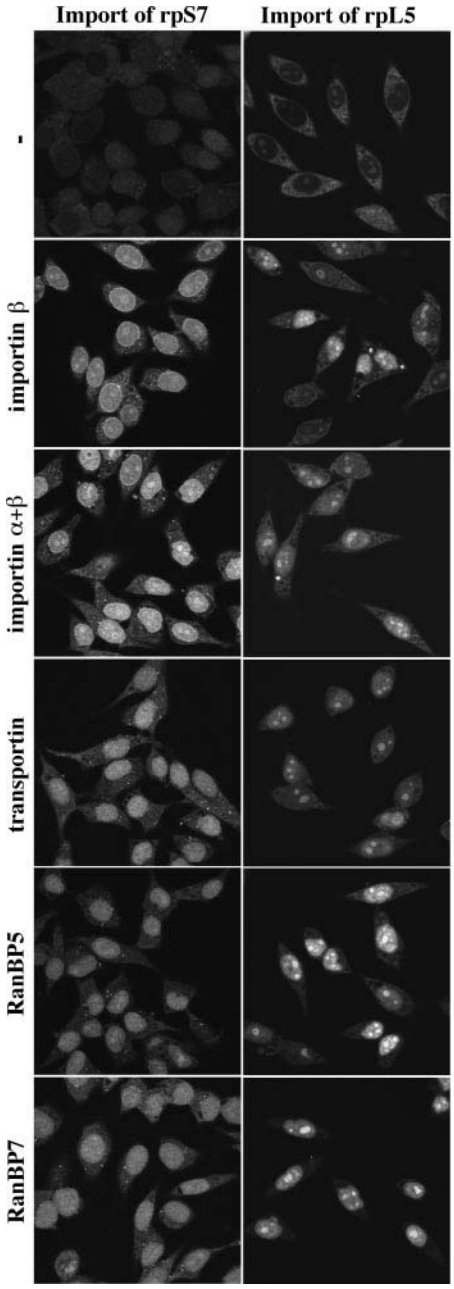

Fig. 5. Nuclear import of ribosomal proteins S7 and L5. Import in the presence of energy and Ran was performed and analysed as in Figure 4, using fluorescent fusions with rpS7 and rpL5 as substrates.

for RanGTP (K_d = 25 nM) is ~30-fold lower than that of importin β (K_d = 0.8 nM) (Görlich *et al.*, 1997). Thus RanGTP might not bind RanBP7 strongly enough to counteract the interaction with the avidly binding substrate (for discussion see below).

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.

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

rpL23a fragment	Binding to			
	Importin β	Transportin	RanBP5	RanBP7
1–156 (full-length)	+++++	+++++	+++++	+++++
32–156	+++++	+++++	+++++	+++++
43–156	++	++	+++	++++
75–156	–	–	–	–
1–31	–	–	–	–
1–42	–	(+)	++	+
1–74	+++++	+++++	+++++	+++++
32–74	+++++	+++++	+++++	+++++
43–74	+++	+++	+++	+++
52–74	+	+	++	–
62–74	–	–	–	–
52–64	–	–	–	–
42–52	–	–	–	–

The rpL23a N-terminal domain is IPPKAKKEAP[10] APPKAEAKAK[20] ALKAKKAVLK[30] GVHSHKKKKI[40] RTSPTFRRPK[50] TLRLRRQPKY[60] PRKSAPRRNK[70] 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).

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, RanBP5, RanBP7 or transportin, despite the observation that transportin binding was weak when tested in egg extract (Figure 2). In addition, rpS7 apparently can also use the importin α/β heterodimer for nuclear entry.

rpL5 behaved somewhat differently. RanBP7 allowed efficient import, such that rpL5 accumulated evenly in all nuclei and the cytoplasmic signal was completely lost. Import was also efficient with RanBP5. With importin β, however, nuclear accumulation was heterogeneous in that some nuclei stained very brightly but others only faintly. The reason for this heterogeneity is unclear, but different cell cycle positions of the individual nuclei might have caused the effect. rpL5 import with transportin was weak, but still above background.

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

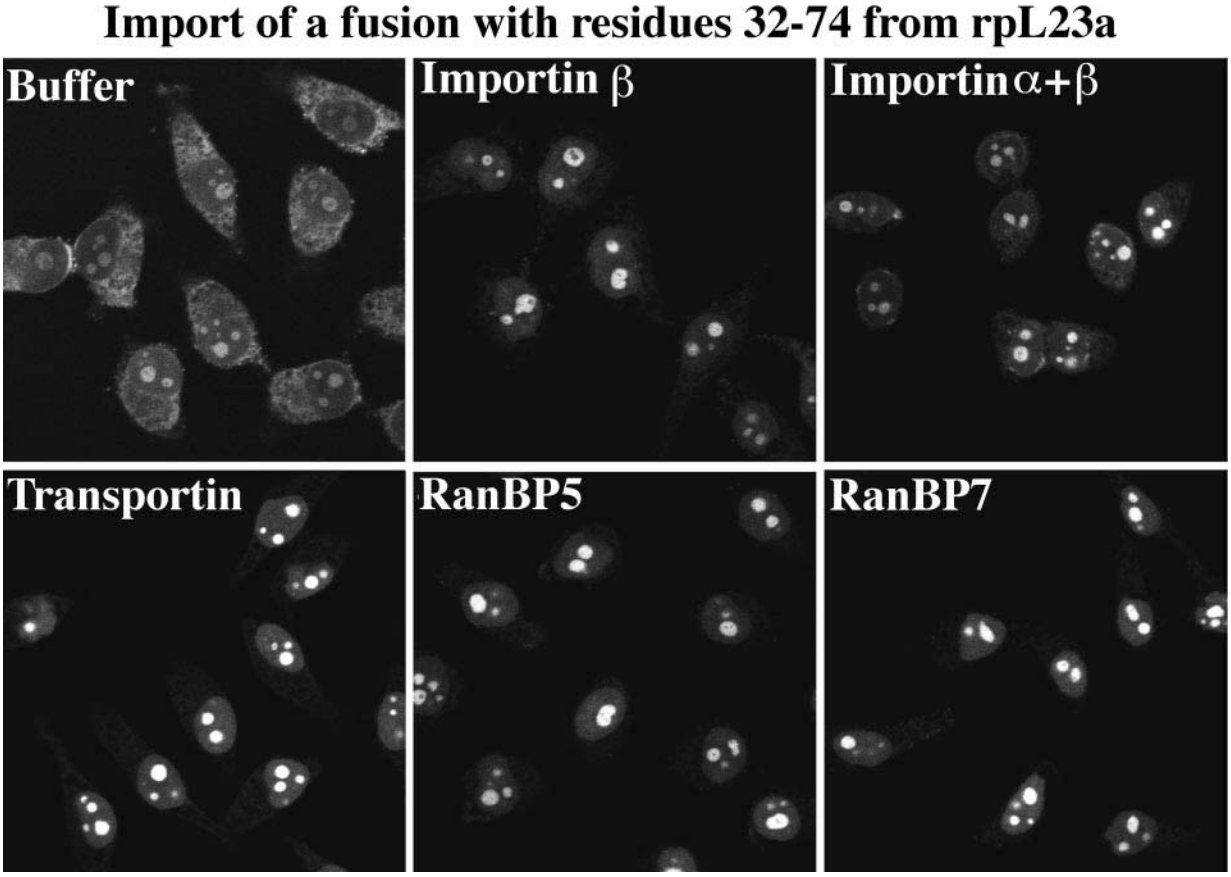

Fig. 6. Residues 32–74 of rpL23a constitute a universal NLS that accesses multiple import pathways. Import was performed and analysed essentially as in Figure 4, using a fluorescent fusion with residues 32–74 from rpL23a (i.e. the BIB domain) as a substrate.

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. Import signals have been mapped for a number of ribosomal proteins, such as for human rpS6 (Schmidt *et al.* 1995) or human rpL7a (Russo *et al.*, 1997). A common feature is their very basic nature and a greater complexity as compared with the classical NLS. Considering this and extrapolating from our results with rpL5, rpL23a, rpS7 and total ribosomal proteins, we would suspect that rpS6, rpL7a and the bulk of ribosomal proteins also use non-classical pathways for nuclear entry.

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

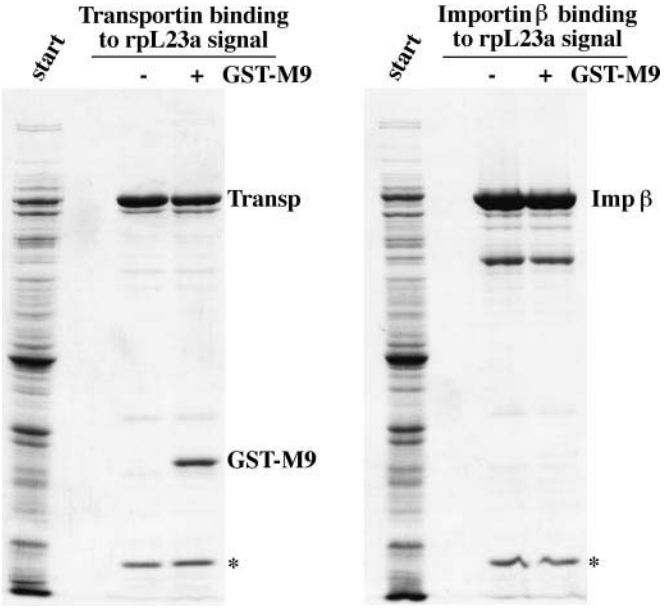

Fig. 7. Transportin has distinct binding sites for the M9 domain and the L23a interaction domain. Binding of transportin or importin β to residues 32–74 of rpL23a (BIB domain) was performed as described in Figure 4. Note that the addition of 5 μM of the GST–M9 fusion did not compete the rpL23a–transportin interaction. Instead, GST–M9 was recovered in the bound fraction, indicating the formation of a trimeric L23a–transportin–M9 complex. As a control, GST–M9 was not bound if transportin was replaced by importin β (right panel). The asterisk denotes 2z–BIB fusion protein that had leaked from the column.

trimeric complex (not shown). Thus one can conclude that transportin simultaneously can bind an M9 domain and the BIB domain from rpL23a. The binding sites for the two signals in transportin are, therefore, distinct and non-overlapping.

Even though transportin can bind an M9 signal and the BIB domain simultaneously, it appears unlikely that transportin normally would import the two substrates at the same time: import of the trimeric M9–transportin–BIB complex is apparently much less efficient than import of, for example, an M9–transportin complex (not shown).

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–L23a 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 α/β 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-

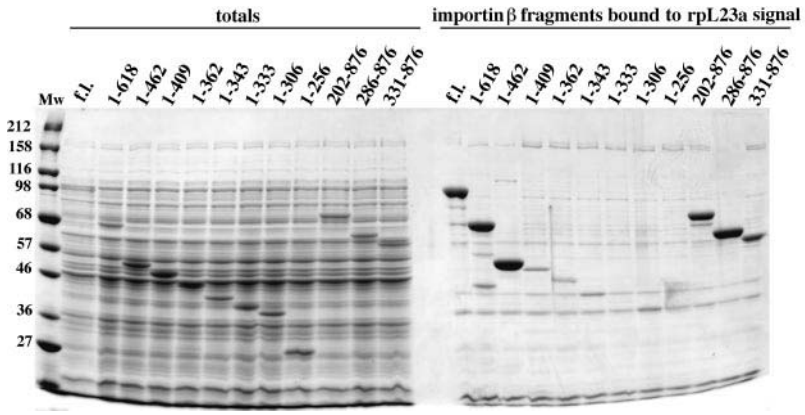

Fig. 8. Definition of a binding domain in importin β for rpL23a. The importin β fragments, as indicated above the lanes, were tested for binding to the immobilized BIB domain from rpL23a (residues 32–74). f.l. stands for full-length importin β, i.e. residues 1–876. Starting materials and bound fractions were analysed by SDS–PAGE and Coomassie staining. The load in the bound fractions corresponds to 10 times the starting material. Note that residues 286–462 of importin β are essential for high-affinity interaction with the rpL23a import signal.

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 diverged 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. Thus, the number of import signals recognized by a given receptor potentially might be great.

Nuclear import of *S.cerevisiae* rpL25, the yeast homologue of rpL23a, previously had been studied in yeast. Efficient import of a reporter protein containing the rpL25 NLS (Schaap *et al.*, 1991) was found to require the Yrb4 protein which binds the rpL25 signal directly (Rout *et al.*, 1997; Schlenstedt *et al.*, 1997). No immediate human homologue of Yrb4p has been reported so far; however, the protein shows clear overall similarity to importin β, transportin and RanBP5. An obvious parallel between the yeast and the mammalian system is that yeast rpL25 can also be imported via at least two parallel pathways using either Yrb4p or Pse1p, the yeast homologue of RanBP5: Yrb4p is not essential for viability, and the import defect in a Yrb4 deletion strain can be largely suppressed by overexpression of Pse1p (Rout *et al.*, 1997; Schlenstedt *et al.*, 1997).

To mediate multiple rounds of import, import receptors need to shuttle continuously between nucleus and cytoplasm. They bind their cargoes initially in the cytoplasm and take them into the nucleus. Nuclear RanGTP then causes cargo release by direct binding to the import receptors. The receptors then finally return to the cytoplasm without the cargo they just carried in. RanGTP released rpL23a very efficiently from importin β, transportin or RanBP5 (Figure 3). However, release from importin 7 (RanBP7) was not very efficient, probably because of the low affinity of importin 7 (RanBP7) for RanGTP. In this case, it is possible that substrate release needs to be facilitated by a co-operating, nuclear factor. For example, the nucleoporin at which import is terminated might stabilize the RanGTP-bound conformation of importin 7 (RanBP7), facilitating substrate release. Alternatively, it is quite possible that ribosomal proteins are not 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: p2z60 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 p2z60, but provides four N-terminal z tags, a C-terminal cysteine for covalent modification, followed by a His tag. p6zCys 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′ *Nco*I and a 3′ *Bam*HI site. The amplified fragments were cloned into the *Nco*I–*Bam*HI 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 AGCGGTACCGCAGCAGCAGCAGCAGAGCAGCAACAGTTC (upstream) and GCGCTCGAGTGGAGTTAGTTTTCTGGTGGGTGA-CATTAAGG (downstream). The coding sequence was cloned as a *Kpn*I–*Xho*I fragment into the *Kpn*I–*Sal*I 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 *Bam*HI–*Hin*dIII 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 pQE9 and pQE32, respectively. Purification was with nickel-NTA agarose (Qiagen), followed by precipitation with ammonium sulfate (35% saturation) and chromatography on Superdex 200 (Pharmacia).

Ribosomal proteins and fragments of ribosomal proteins were expressed in *E.coli* with a C-terminal His tag, and with N-terminal z tags (for details, see Plasmids and constructs). Disruption of cells and purification on nickel agarose was in the presence of 1 M lithium chloride.

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 (Calbiochem) 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 M 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 rER preparation from canine pancreas. A total of 50 000 equivalents of rER membranes (for definition, see Walter and Blobel, 1983) were adjusted to 500 mM potassium acetate, 5 mM magnesium acetate, 1 mM puromycin, 1 mM GTP, layered on top of a 10 ml cushion containing 1.2 M sucrose, 50 mM HEPES–KOH pH 7.5, 500 mM potassium acetate, 5 mM magnesium acetate and spun in a Ti50.2 rotor for 6 h at 50 000 r.p.m., 15°C. The clear ribosome pellet was resuspended in 50 mM HEPES–KOH pH 7.5 and spun for 3 h at 100 000 r.p.m., 15°C in a TLA100.3 rotor through a cushion containing 1.6 M sucrose, 50 mM HEPES–KOH pH 7.5, 5 mM magnesium chloride. The ribosome pellet was resuspended as before and the concentration of ribosomes was determined from the UV absorption at 260 nm, assuming a molar extinction coefficient of 10^8. Fluorescein 5′ maleimide was added in a 200-fold molar excess over ribosomes and the mixture was incubated overnight on ice. Ribosomes were recovered by sedimenting them through 0.5 M sucrose, 50 mM HEPES–KOH pH 7.5. The pellet was resuspended and adjusted to 1 M lithium chloride, which dissociates the bulk of ribosomal proteins from the rRNA. The rRNA was pelleted by ultracentrifugation and the supernatant containing the labelled proteins was dialysed against 50 mM HEPES–KOH pH 7.5, 300 mM potassium acetate, 5 mM magnesium acetate.

Import assays

Permeabilized cells were prepared similarly to the method described by Adam *et al.* (1990). Briefly, HeLa cells (# 85060701, European Collection of Cell Cultures) were grown on 12 mm coverslips to 30–80% confluence, washed once in ice-cold phosphate-buffrered saline (PBS) and permeabilized for 8 min in ice-cold 20 mM HEPES–KOH pH 7.5, 110 mM potassium acetate, 5 mM magnesium acetate, 250 mM sucrose, 40 μg/ml digitonin (Sigma #D5628). The coverslips were washed three times in permeabilization buffer minus digitonin. Each coverslip was then incubated with 20 μl of import mixture for the indicated time periods at room temperature. Reactions were stopped by fixation with 2% paraformaldehyde (w/v) in PBS, washed in PBS and water, and mounted with 2 μl of vectorshield mounting medium (Vector).

The energy-regenerating system consists of the following components: 0.5 mM ATP, 0.5 mM GTP, 10 mM creatine phosphate, 50 μg/ml creatine kinase.

It turned out that the optimum import buffer was different for different substrates. All buffers contained 250 mM sucrose and 5 mM magnesium 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.

Binding assays

The following affinity matrices were used: a biotinylated BSA–NLS conjugate bound to streptavidin–agarose (Görlich *et al.*, 1995a), a synthetic peptide corresponding to the IBB domain from *Xenopus* importin α (Görlich *et al.*, 1996a) and 2z-tagged ribosomal proteins or fragments of rpL23a pre-bound to IgG–Sepharose 4B. The z domain is the IgG-binding domain from *Staphylococcus aureus* protein A. The ligands were immobilized at a concentration of ~2 mg/ ml of resin. Binding to the BSA–NLS conjugate was in 50 mM Tris–HCl pH 7.5, 80 mM NaCl, 5 mM magnesium acetate. All other bindings were performed in 50 mM Tris–HCl pH 7.5, 300 mM NaCl, 5 mM magnesium acetate. Each 20 μl of affinity matrix was rotated with 500–1000 μl of starting material for 3 h at 4°C. The beads were recovered by gentle centrifugation and washed four times with 1 ml of binding buffer. Elution was with 150 μl of 1.5M magnesium chloride, 50 mM Tris–HCl pH 7.5. Proteins were precipitated with 90% isopropanol (final), dissolved in SDS sample buffer and analysed as described in the figure legends.

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