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The importin β/importin 7 heterodimer is a functional nuclear import receptor for histone H1

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Import of proteins into the nucleus proceeds through nuclear pore complexes and is largely mediated by nuclear transport receptors of the importin β family that use direct RanGTP-binding to regulate the interaction with their cargoes. We investigated nuclear import of the linker histone H1 and found that two receptors, importin β (Impβ) and importin 7 (Imp7, RanBP7), play a critical role in this process. Individually, the two import receptors bind H1 weakly, but binding is strong for the Impβ/Imp7 heterodimer. Consistent with this, import of H1 into nuclei of permeabilized mammalian cells requires exogenous Impβ together with Imp7. Import by the Imp7/Impβ heterodimer is strictly Ran dependent, the Ran-requiring step most likely being the disassembly of the cargo–receptor complex following translocation into the nucleus. Disassembly is brought about by direct binding of RanGTP to Impβ and Imp7, whereby the two Ran-binding sites act synergistically. However, whereas an Impβ/RanGTP interaction appears essential for H1 import, Ran-binding to Imp7 is dispensable. Thus, Imp7 can function in two modes. Its Ran-binding site is essential when operating as an autonomous import receptor, i.e. independently of Impβ. Within the Impβ/Imp7 heterodimer, however, Imp7 plays a more passive role than Impβ and resembles an import adapter.

Keywords: histones/importin β/nuclear pore complex/nuclear transport/RanBP7

Introduction

Histones are the major structural proteins in eukaryotic chromosomes. This group of basic proteins comprises the core histones H2A, H2B, H3 and H4 which form the protein octamer of the nucleosomal core, and the H1 linker histones (Allan *et al*., 1986; Wolffe, 1995; Pruss *et al*., 1996). H1 histones interact with the DNA that links the core particles of the nucleosomal chromatin chain and are involved in the formation and maintenance of a higher order chromatin structure. The H1 class comprises seven different subtypes, termed H1.1–H1.5, $H1^0$ and H1t (for review see Doenecke *et al*., 1994). $H1^0$ is mostly confined to highly differentiated cells, it replaces main type H1 histones upon chromatin-remodelling and is therefore also referred to as a replacement histone.

Histones, as all nuclear proteins, are synthesized in the cytoplasm and need to be transported across the nuclear envelope (NE) into the nucleus in order to fulfil their function. Nuclear pore complexes (NPCs) penetrate the double membrane of the NE and constitute the sole sites of such nucleocytoplasmic exchange (Feldherr *et al*., 1984). NPCs allow diffusion of small molecules and can accommodate active transport of even large particles. Active transport requires nuclear transport factors which comprise a minimum of three categories, namely: transport receptors, the constituents of the RanGTPase system, and in some cases adapter molecules (for recent reviews see Dahlberg and Lund, 1998; Gorlich, 1998; Izaurralde and Adam, 1998; Mattaj and Englmeier, 1998).

Transport receptors bind cargo molecules on one side of the NE, translocate with them through the NPC, release them on the other side, and finally return to the original compartment, leaving the cargoes behind. According to the direction in which these receptors carry their cargo, they can be classified as importins or exportins. Transport receptors which are all, albeit often distantly, related to importin β (Impβ), form a superfamily (Fornerod *et al*., 1997b; Gorlich *et al*., 1997), with 13 family members in the yeast *Saccharomyces cerevisiae* and probably even more in higher eukaryotes. They are all characterized by an N-terminal sequence motif that accounts for binding of RanGTP (Gorlich *et al*., 1997) and strikingly, they use this RanGTP-binding to regulate interactions with their substrates or adapter molecules (Rexach and Blobel, 1995; Chi *et al*., 1996; Gorlich *et al*., 1996c; Fornerod *et al*., 1997a; Izaurralde *et al*., 1997; Kutay *et al*., 1997a, 1998; Schlenstedt *et al*., 1997; Siomi *et al*., 1997; Arts *et al*., 1998; Jakel and Gorlich, 1998).

The nucleotide-bound state of the GTPase Ran (Bischoff and Ponstingl, 1991b; Melchior *et al*., 1993; Moore and Blobel, 1993) is controlled by the GTPase activating protein RanGAP1 and the nucleotide exchange factor RCC1 (Bischoff and Ponstingl, 1991a; Bischoff *et al*., 1994). RCC1 is exclusively nuclear (Ohtsubo *et al*., 1989) and, thus, generates RanGTP only in the nucleus, while RanGAP1 is excluded from the nucleus (Matunis *et al*., 1996; Mahajan *et al*., 1997) and constantly depletes RanGTP from the cytoplasm. Therefore one would predict a steep RanGTP gradient across the NE with a high nuclear concentration and a very low level in the cytoplasm.

We have proposed previously that this gradient serves as a crucial determinant for the directionality of nuclear transport that regulates the binding of substrates to transport receptors in the correct compartment-specific manner (Gorlich *et al*., 1996b,c; Izaurralde *et al*., 1997). Indeed,

the RanGTP-bound and Ran-free forms of a given transport receptor have dramatically different affinities for their substrates. Importins bind cargoes in the Ran-free conformation which is favoured in the cytoplasm and release them in the nucleus upon encountering RanGTP (Rexach and Blobel, 1995; Chi *et al*., 1996; Gorlich *et al*., 1996c; Izaurralde *et al*., 1997; Siomi *et al*., 1997; Jakel and Gorlich, 1998). They exit the nucleus as RanGTP complexes without their substrate. RanGTP is removed from the transport receptor in the cytoplasm by RanGAP1 and RanBP1 (Bischoff and Gorlich, 1997; Floer *et al*., 1997; Lounsbury and Macara, 1997), which results in the hydrolysis of the Ran-bound GTP and restores the competence of the importins to bind another cargo molecule.

Binding of substrates to exportins is regulated in a converse manner to importins. Exportins bind their cargoes preferentially in the RanGTP-bound conformation (Fornerod *et al*., 1997a; Kutay *et al*., 1997a, 1998; Arts *et al*., 1998), i.e. in the nucleus, and release them in the cytoplasm when RanGTP is removed and Ran-bound GTP is hydrolysed. Both importins and exportins normally enter the nucleus in a Ran-free state and exit as complexes with RanGTP, thereby constantly depleting Ran from the nucleus. The maintenance of the RanGTP gradient should then require a very efficient re-import of Ran. Indeed, NTF2 (Moore and Blobel, 1994; Paschal and Gerace, 1995) has recently been demonstrated to be the carrier that replenishes the nucleus with Ran (Ribbeck *et al*., 1998).

A complete cycle of cargo-binding to and release from a β-family transport receptor should result in the hydrolysis of one molecule of GTP by Ran. Recent studies came to the conclusion that this is apparently the sole input of energy into the corresponding transport cycle and that the translocation through the NPC itself is not directly coupled to nucleotide hydrolysis, at least in the case of simple substrates (Schwoebel *et al.*, 1998; Englmeier *et al.*, 1999; Ribbeck *et al.*, 1999; see also Weis *et al.*, 1996b; Kose *et al.*, 1997; Nakielny and Dreyfuss, 1998; Ribbeck *et al.*, 1998).

Access to the nuclear transport machinery is highly regulated. For example, not all cytoplasmic proteins are imported into the nucleus, but only a subset that is distinguished by characteristic domains or import signals. These signals are, in simple cases, directly recognized by their cognate import receptors. Examples are the M9 domain of hnRNP A1 which confers binding to and import by transportin (Pollard *et al*., 1996; Fridell *et al*., 1997), or the BIB (beta-like import receptor binding) domain from rpL23a which can access at least four distinct import pathways in higher eukaryotes (Jakel and Gorlich, 1998), namely import by importin β, transportin, importin 5 (Imp5, formerly called RanBP5) and importin 7 (Imp7, formerly called RanBP7). Likewise, the import signal of yeast rpL25 can also be directly recognized by two receptors, Yrb4p or Pse1p (Rout *et al*., 1997; Schlenstedt *et al*., 1997).

Impβ (Chi *et al*., 1995; Gorlich *et al*., 1995a; Imamoto *et al*., 1995a; Radu *et al*., 1995) is exceptional among the transport receptors in that it can use adapters, such as importin α (Impα; Adam and Adam, 1994; Gorlich *et al*., 1994), in order to expand its substrate specificity. Impα binds the classical nuclear localization signal (NLS) and also Impβ. The trimeric complex consisting of the Impα/β heterodimer and the NLS protein is translocated into the nucleus, where the complex is dissociated by RanGTP and the NLS protein is released. The RanGTP/Impβ complex can directly exit the nucleus, whereas Impα employs a specialized exportin, CAS, for its re-export back to the cytoplasm (Kutay *et al*., 1997a).

We have reported previously the observation that Impβ can also form a stable complex with another β-family import receptor, Imp7 (Gorlich *et al*., 1997). Both transport receptors are equally highly abundant, each approaching a concentration of 3 μM in *Xenopus* eggs and in HeLa cells, which would suggest that the complex of the two is also very abundant. So far, no function has been assigned to this heterodimer. However, the observation that the Impβ/Imp7 dimer is dissociated by RanGTP (Gorlich *et al*., 1997), implies that it forms in the cytoplasm and decays in the nucleus, and thus suggests a role in import.

Here, we show that the Impβ/Imp7 heterodimer is a functional import receptor for the linker histone H1 and we suggest the following steps for the corresponding H1 import cycle. A trimeric complex consisting of Impβ, Imp7 and histone H1 assembles initially in the cytoplasm. The formation of the complex is highly co-operative with probably both importins contributing to substrate recognition. This complex is then translocated to the nuclear side of the NPC where RanGTP binding to Impβ releases the cargo–receptor complex from the NPC and Impβ from the remaining Imp7/H1 complex. The histone is then transferred from Imp7 onto DNA, whereby RanGTP facilitates the displacement of H1 from Imp7. The RanGTP complexes of Impβ and Imp7 have no detectable affinity for each other and are probably separately returned to the cytoplasm, where RanGTP is removed and thereby the competence of the two importins for heterodimer formation and/or substrate binding is restored.

Results

Previously we have characterized nuclear import of ribosomal proteins in higher eukaryotes and found that they can bind directly to and be imported by at least four different β-family import receptors, namely importin β (Impβ), transportin, importin 5 (Imp5, RanBP5) and importin 7 (Imp7, RanBP7) (Jakel and Gorlich, 1998). Ribosomal proteins are usually small and very basic, properties which are shared with histones. It was therefore interesting to know whether histones are imported in a way similar to ribosomal proteins.

Competition studies have suggested that the histone H1 import pathway shares receptors with the classical, Impα/β-dependent pathway (Breeuwer and Goldfarb, 1990; Imamoto *et al*., 1995b; Schwamborn *et al*., 1998). On the other hand, it has also been shown that the H1 import signal is of a very complex nature (Schwamborn *et al*., 1998) and thus rather atypical for the classical pathway. To clarify this issue and to identify potential nuclear import receptors for the linker histone H1, we immobilized human histone $H1^0$ and calf thymus $H1^{TH}$, and tested which factors from a cytoplasmic HeLa extract they would bind (Figure 1). Immobilized rpL23a served as a positive control and bound Imp5, Imp7, Impβ and Impα, as

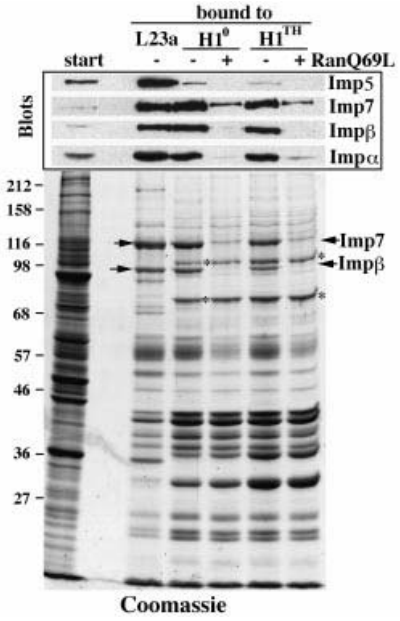

Fig. 1. Interactions of histone H1 with nuclear transport receptors. The replacement histone $H1^0$ and $H1^{TH}$ from calf thymus were biotinylated, immobilized on streptavidin agarose and tested for binding of transport receptors from a HeLa extract. Positive control for binding was the immobilized ribosomal protein L23a to which importin α (Impα), importin β (Impβ), importin 5 (Imp5, RanBP5) and importin 7 (Imp7, RanBP7) bind. Each 20 µl of the resins was incubated with 1 ml cytoplasmic HeLa cell extract. Where indicated, 15 µM RanQ69LGTP was also added. RanQ69L is a GTPase-deficient Ran mutant that remains GTP-bound even in the presence of cytoplasmic RanGAP1 (Bischoff *et al*., 1994). After extensive washing in 50 mM Tris–HCl pH 7.5, 200 mM NaCl, 5 mM $MgCl_2$, 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 Blue staining. Load in the bound fractions corresponds to 20× the starting material. Note, Impα and Impβ, as well as Imp7 were specifically recovered with the immobilized histones H1. RanQ69L displaced the import receptors. Asterisks indicate two forms of nucleolin that also specifically bound to the histones.

observed previously (Jakel and Gorlich, 1998). The histones bound only very low amounts of Imp5 (Figure 1) and essentially no transportin (not shown). However, binding of Imp7, Impβ and Impα was significant.

As detailed in the introduction, direct binding of RanGTP to β-family import receptors displaces import substrates from these receptors and is normally a specific nuclear event that follows translocation into the nucleus. As seen in Figure 1, the binding of both Impβ and Imp7 to histone H1 was sensitive to RanGTP and thus follows the paradigm of nuclear import receptor–cargo interactions. This sensitivity towards RanGTP can also be taken as a stringent control for the specificity of binding. The displacement of Impα, which itself cannot bind RanGTP, would suggest that Impα requires Impβ for efficient recovery in the bound fraction.

Impβ and Imp7 co-operate in nuclear import of histone H1

Import of fluorescent H1 into nuclei of permeabilized HeLa cells occurs efficiently in the presence of an energy-regenerating system and a source of soluble transport factors, e.g. a reticulocyte-lysate (Figure 2; Kurz *et al*., 1997; Schwamborn *et al*., 1998). The experiments in Figure 1 suggested Impα, Impβ and Imp7 as potential mediators of H1 import. We therefore tested if Ran, in any combination with these three factors, could reconstitute nuclear import of histone H1 (Figure 2). We had included a number of control substrates which behaved as reported before: import of nucleoplasmin was dependent on the Impα/β heterodimer (Gorlich *et al*., 1994, 1995a). Import of an artificial Impβ-specific substrate, containing the IBB domain (importin beta binding domain), was efficient with Impβ and competed by Impα (Gorlich *et al*., 1996a; Weis *et al*., 1996a). In both cases, the presence of Imp7 was slightly inhibitory. Import of a fusion protein containing the BIB domain from rpL23a was efficient with either Impβ or Imp7 (Jakel and Gorlich, 1998) and combining the two transport receptors did not further stimulate import. The requirements for import of the two histones H1 ($H1^0$ and H1 from thymus) were then strikingly different from that of any of the other substrates. Their import required the simultaneous presence of both exogenous Impβ and Imp7 and this combination was more efficient than the crude reticulocyte lysate system. The effect of co-operation between Impβ and Imp7 is probably still underestimated in this experiment because we used the heterodimer only at half the concentration of the individual receptors. We have reported previously that Impβ and Imp7 form a heterodimer (Gorlich *et al*., 1997). The import data from Figure 2 would now suggest that this heterodimer is the active species in histone H1 import.

Addition of Impα reduced H1 import by the Impβ/Imp7 heterodimer, which resembles the situation with rpL23a (Jakel and Gorlich, 1998) in that Impα can specifically be recovered on H1 or L23a columns, but apparently does not support import (Figures 1 and 2).

Co-operative binding of Impβ and Imp7 to histone H1

To characterize the H1–import receptor complex further, we expressed Impβ and Imp7 in *Escherichia coli* and used the corresponding lysates for binding assays with immobilized histone $H1^0$. Impβ alone bound to H1 only very weakly, while binding of Imp7 alone was moderate. However, when both importins were present simultaneously, their binding was very efficient (Figure 3), indicating the co-operative formation of a trimeric H1/Impβ/Imp7 complex.

There are two possibilities for how Imp7 and Impβ might co-operate in histone H1 binding. First, Impβ and Imp7 might each interact with distinct domains of histone H1. The heterodimer would then have a greater number of contacts with this substrate and therefore bind it more stably than can either Imp7 or Impβ alone. Secondly, Impβ could induce a conformational switch in Imp7 that increases the affinity for H1. One argument against such a general conformational switch is that co-operativity between Impβ and Imp7 is H1-specific and not detectable for BIB-binding or import (Figures 3 and 2). Such an argument is somewhat indirect; however, one can make a testable prediction to distinguish between the two models. If an Impβ-triggered conformational switch in Imp7 accounts for the co-operativity in H1 binding, then such a switch should also be induced by Impβ fragments as long as they bind Imp7 tightly. We decided to test this experimentally and determine the domains of Impβ

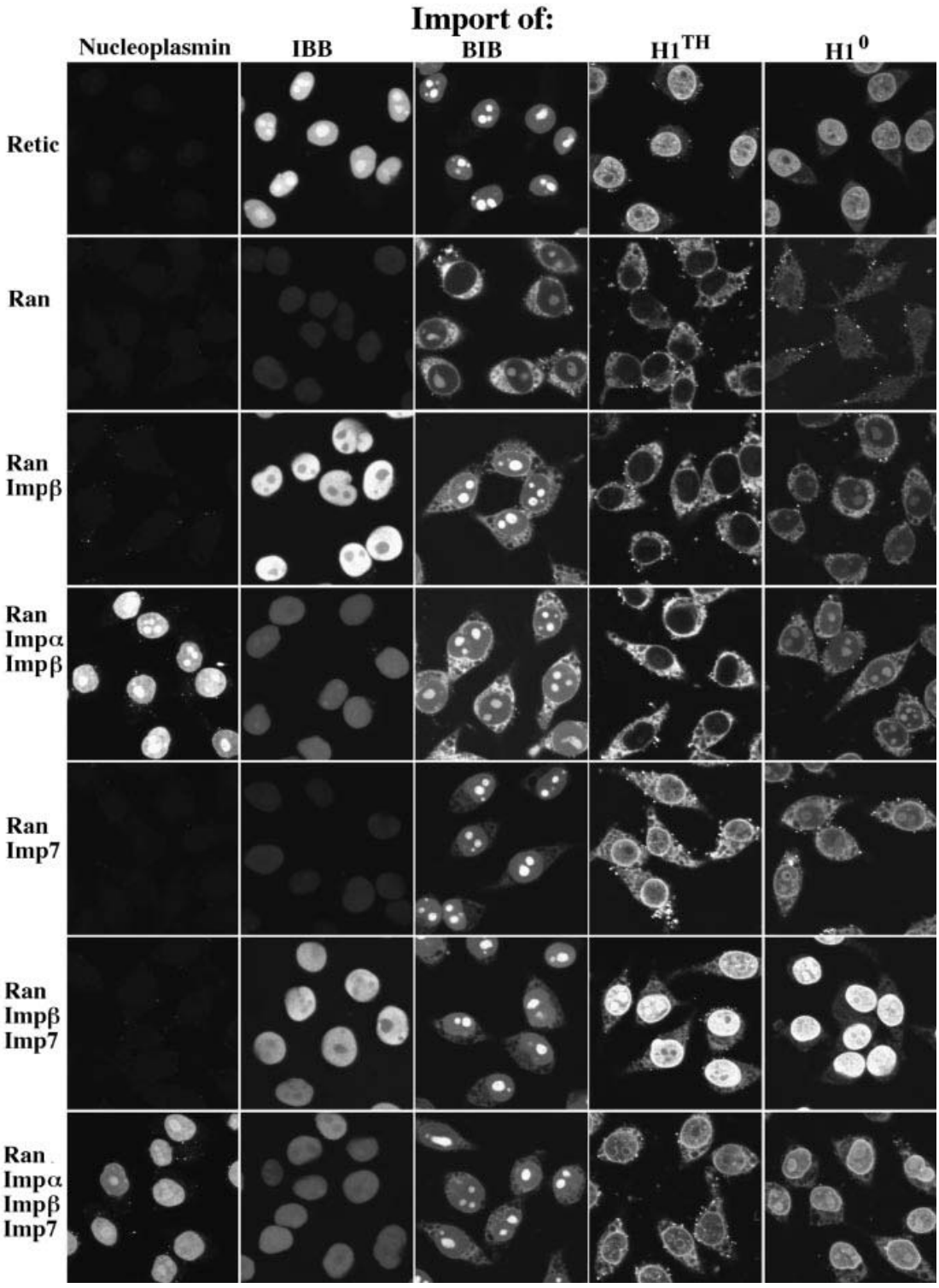

Fig. 2. The Impβ/Imp7 heterodimer is a functional import receptor for histone H1. Nuclear import of the indicated fluorescein-labelled import substrates was performed in the presence of an energy-regenerating system, and either reticulocyte lysate ('retic') or a Ran mix (see Materials and methods) plus the indicated combinations of import receptors at the following concentrations: Impα, 1.5 μM; Impβ, 0.5 μM; Imp7, 0.5 μM; Impβ/Imp7 heterodimer, 0.25 μM. The following concentrations of import substrates were used: nucleoplasmin, 2 μM; IBB core, 2 μM; 6z-BIB fusion, 1 μM; histones $H1^{TH}$ and $H1^{0}$ each 0.4 μM. Import was stopped after 20 min by fixation and analysed by confocal fluorescence microscopy.

required for Imp7 binding and for co-operative formation of the H1/Impβ/Imp7 complex.

Mapping of functional domains in Impβ

Figure 4A shows that approximately amino acid residues 143–409 of Impβ are required for full Imp7 binding, whilst the minimum Imp7 binding domain comprises residues 203–362. Next we tested which Impβ fragments would assemble into a trimeric complex with H1 and Imp7. Co-operative complex formation was measured as binding of Imp7 together with the various Impβ fragments to immobilized H1. It was most efficient with full-length

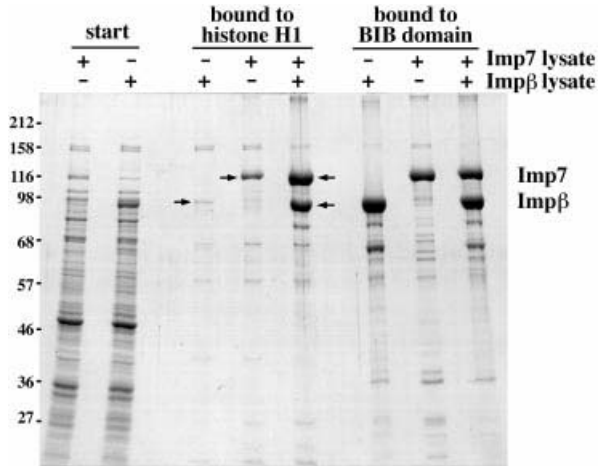

Fig. 3. The formation of the Impβ/Imp7/histone H1 complex is co-operative. Immobilized histone $H1^0$ and the BIB domain (import signal from rpL23a) were used to bind recombinant Impβ and Imp7 out of total *E.coli* lysates. Where indicated, 100 µl Impβ lysate and/or 300 µl Imp7 lysate (400 µl final volume) were incubated with 20 µl beads. After extensive washing in 50 mM Tris–HCl pH 7.5, 300 mM NaCl, 5 mM $MgCl_2$, bound proteins were eluted with 1.5 M $MgCl_2$ and analysed by SDS–PAGE followed by Coomassie staining. Note that Imp7 greatly enhanced Impβ binding to $H1^0$ and vice versa. In contrast, no co-operative effect was evident in the case of binding to the BIB domain.

Impβ (1–876) and somewhat reduced with a fragment lacking the 202 N-terminal residues. More extensive N-terminal deletions abolished trimeric complex formation (Figure 4B), probably because the Impβ/Imp7 interaction is then lost (Figure 4A). Deletions from Impβ's C-terminus suggest that residues 619–876 make a minor contribution to the trimeric complex formation, whereas amino acids 410–618 are essential. As residues 410–618 are dispensable for Imp7 binding (Figure 4A), we conclude they are directly involved in the interaction with the histone.

Taken together, we conclude that binding of Impβ to Imp7 is not sufficient to enhance the affinity of Imp7 for the histone and that a conformational switch in Imp7 is therefore an unlikely cause for the effect. Instead, the deletion analysis supports the assumption that the H1/Impβ/Imp7 complex formation is co-operative because each constituent of the complex directly contacts the other two.

Figure 4C depicts schematically Impβ's respective binding site for Imp7, H1 and the previously mapped interaction sites for RanGTP, Impα and NPCs (Chi and Adam, 1997; Kutay *et al.*, 1997b; Jakel and Gorlich, 1998).

The C-terminus of Impβ is dispensable for H1 import

We wanted to test next whether any of the Impβ fragments would support H1 import in conjunction with Imp7. As seen in Figure 5, N-terminal deletions of either 44, 202, 255 or 330 residues all abolished import activity. This is probably because the N-terminus of Impβ is required for RanGTP binding and the Ran-Impβ interaction is an essential event in H1 import (for detailed discussion see below). Surprisingly, deletion of the C-terminal 258 residues (Impβ 1–618) still allowed efficient H1 import to occur. This is, to our knowledge, the first case where a fragment of a β-family transport receptor has been shown to be functional. Further truncations from the C-terminus abolished import activity (Figure 5), just as they impaired formation of the H1/Imp7/Impβ complex (Figure 4B).

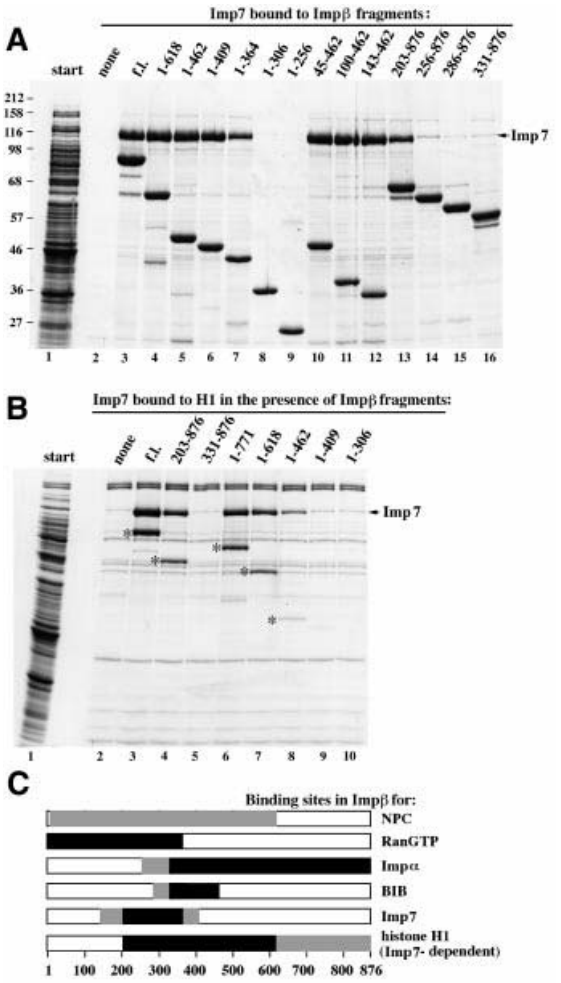

Fig. 4. (**A**) Mapping of the Imp7-binding domain in Impβ. zz-Imp7 was expressed in *E.coli* without a His-tag. A lysate was prepared in 50 mM Tris–HCl pH 7.5, 500 mM NaCl, 10 mM magnesium chloride, 20 mM imidazole–HCl pH 7.5 and passed through nickel agarose. Each 500 µl of the pre-cleared lysate (start) were supplemented as indicated with 1 µM wild-type Impβ or Impβ fragments. Complexes were allowed to form and were recovered with nickel agarose by virtue of the His-tag present in the Impβ derivatives. Elution was with 100 µl 0.5 M imidazole–HCl pH 7.5. Starting material and bound fractions were analysed by SDS–PAGE followed by Coomassie staining. Load in the bound fractions corresponds to 25 times the starting material. Note, Imp7 bound to Impβ fragments that contained residues 203–409 as efficiently as to wild-type Impβ. The minimum binding domain comprises residues 143–364. (**B**) Mapping of the histone H1 binding site in Impβ. An Imp7 lysate ('start') was supplemented with 1 µM of indicated Impβ fragments and subjected to binding to immobilized histone $H1^0$ as described in Figure 3. Note, trimeric Impβ/Imp7/H1 complex formation was most efficient with full-length Impβ, and slightly reduced for Impβ fragments comprising residues 203–618. Further truncations from either end essentially abolished binding. Asterisks indicate positions of bound Impβ fragments. (**C**) Schematic representation of the functional domains in Impβ. Residues shown in black are absolutely required for the indicated interaction, those in grey are required for binding at wild-type level [see (A) and (B) and Chi and Adam, 1997; Kutay *et al*., 1997b; Jakel and Gorlich, 1998]. Numbers indicate the positions within the 876 amino acids of the Impβ sequence.

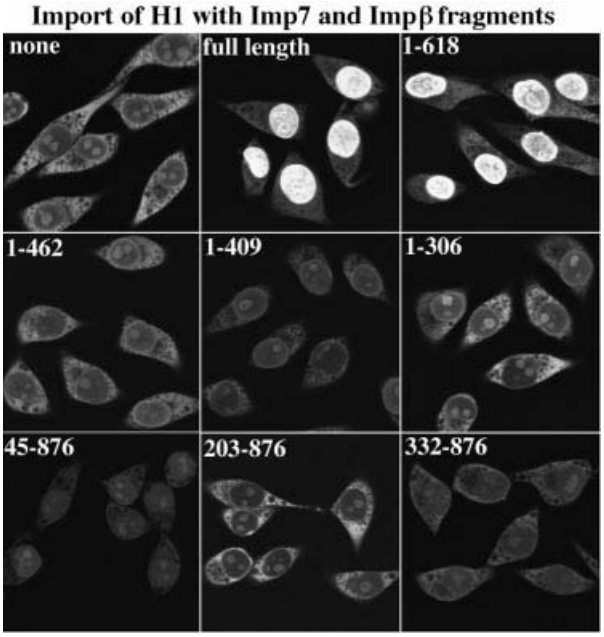

Fig. 5. Nuclear import of histone $H1^0$ by heterodimers formed by Imp7 and Impβ fragments. Import of 0.25 μM $H1^0$ into nuclei of permeabilized HeLa cells was performed in the presence of Ran, an energy-regenerating system, 0.25 μM Imp7 and 0.25 μM of indicated Impβ fragments. Note, import was efficient with wild-type Impβ and Impβ 1–618, further C-terminal deletions abolished import activity. None of the N-terminal deletion mutants supported import, probably because they are deficient in Ran binding.

The H1 and BIB binding sites in Impβ are distinct from that for Impα

The Impβ-dependent import pathway can be accessed by several different targeting signals, e.g. IBB (Impβ binding domain from Impα) and BIB (rpL23a import signal). Except for their basic nature, the two bear no resemblance to each other, raising the questions of how they can both bind to the same receptor. Figure 6A shows that BIB cannot compete the IBB–Impβ interaction. Conversely, the BIB–Impβ interaction is not competed by IBB (not shown). This suggests that Impβ uses distinct and largely non-overlapping recognition sites for BIB and IBB.

We then tested BIB or IBB as competitors of H1-binding to the Impβ/Imp7 heterodimer. As seen from Figure 6B, BIB competed efficiently, but IBB did not, even at the high concentration of 20 μM used in this experiment. This suggested, first, that the BIB binding sites of Impβ and Imp7 also mediate interaction with H1, and secondly, that the IBB binding site of Impβ is not used for H1 binding.

If a quaternary IBB/Impβ/Imp7/H1 complex can form, the question arises as to whether it is also an active species in import. A transport receptor complex with several transport substrates bound would seem a very economical mode of transportation because more than just a single cargo molecule could be moved per transport cycle. Unfortunately, however, such a multi-cargo complex is apparently not productive as H1 import by the Impβ/7 heterodimer is easily blocked by 2.5 μM IBB (Figure 7). This inhibition of import was specific as verified by a crucial control: when full-length Impβ was replaced by the Impβ 1–618 fragment, which cannot bind IBB, then import also became resistant towards IBB (Figure 7).

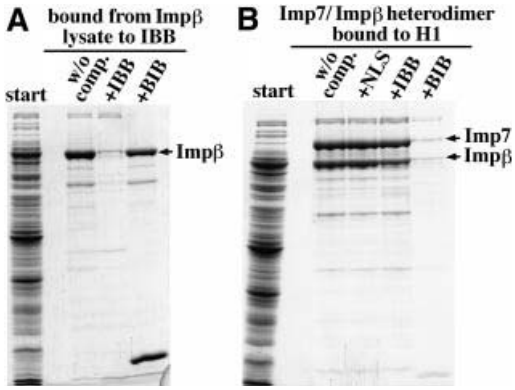

Fig. 6. (**A**) The BIB and IBB binding sites on Impβ are distinct and apparently non-overlapping. Impβ was expressed in *E.coli* and the resulting lysate used for binding to an immobilized IBB domain. Starting material and bound fractions were analysed by SDS–PAGE and Coomassie Blue staining. Note, binding of Impβ to IBB was competed by 20 μM of non-immobilized IBB, but not by 20 μM of the BIB domain, suggesting BIB and IBB do not compete for a common binding site on Impβ. (**B**) The sites for H1 and Impα binding on Impβ are distinct. The Impβ lysate was complemented with 0.5 μM Imp7 and used for binding to immobilized H1 in the presence of the indicated competitors (20 μM). NLS stands for BSA to which SV40-NLS peptides had been coupled. Note, binding of the Impβ/Imp7 heterodimer resisted competition by IBB, but was efficiently competed by excess of BIB. The BSA-NLS does not bind the Impβ/Imp7 heterodimer and was a control for non-specific effects of the competitors.

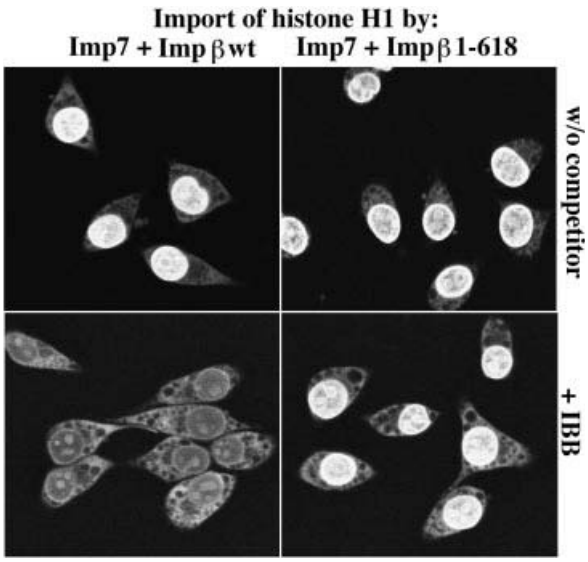

Fig. 7. Excess of IBB inhibits Impβ/Imp7-mediated H1-import in a non-competitive manner. $H1^0$ import was performed as described in Figures 2 and 5. Note, import by the wild-type Impβ/Imp7 heterodimer was strongly inhibited by the presence of 2.5 μM IBB domain. This inhibition of import is non-competitive because IBB does not displace the cargo from the transport receptors (see Figure 6B). Also note that import became resistant towards IBB inhibition when Impβ wild type was replaced by the Impβ 1–618 fragment that does not bind IBB.

The excess of IBB blocks H1 import by the wild-type Impβ/7 heterodimer not by displacing H1 from its import receptor (Figure 6B), but apparently because the cargo–receptor complex formed is non-productive. There is a precedent for such a situation: transportin can simultaneously bind an M9 domain (import signal of hnRNP A1) and a BIB domain, but apparently it cannot import the

two at the same time (Jakel and Gorlich, 1998). This could reflect inefficient translocation of such a complex through the NPC. Alternatively, the problem could be termination of import and unloading of the cargo; RanGTP might not bind the import receptor strongly enough to displace two substrates at a time.

The inhibition of Impβ/7-mediated H1 import by IBB (the Impβ binding domain of Impα) also gives an explanation for the inhibitory effect of Impα itself (see Figure 2). In this context, it is interesting to note that the Impα/β heterodimer can specifically bind histone H1 (not shown) but is not sufficient to mediate H1 import (Figure 2). The H1/Impα/β trimer is thus a further example of a non-productive cargo–transport receptor complex.

RanGTP binding to Impβ is an essential event in H1 import

Histone H1 import by the Impβ/Imp7 heterodimer is strictly GTP-dependent (not shown) and requires Ran (Figure 11). As the concentration of the two import receptors was not limiting in the assay, it can be concluded that Ran is required to complete the import reaction per se and not just for recycling of the receptors back to the cytoplasm after one round of import. Both Impβ and Imp7 are RanGTP-binding proteins and, therefore, we wondered which Ran-binding site(s) would actually be used during nuclear import of histone H1. Figure 5 showed that the ΔN44 Impβ mutant (Impβ 45–876) that is deficient in Ran-binding (Gorlich *et al*., 1996c) failed to promote H1 import. This strongly suggests that a RanGTP-importin β interaction is essential at some stage of import mediated by the Impβ/Imp7 heterodimer, probably to terminate translocation into the nucleus. However, the ΔN44 Impβ mutant also acts as a dominant-negative mutant in that it binds irreversibly to NPCs and blocks them for other transport pathways (Kutay *et al*., 1997b). One could therefore argue that the lack of H1 import in the presence of ΔN44 is due to this trans-dominant effect and does not necessarily reflect the Ran requirement. Figure 5 therefore shows the crucial control that the ΔN202 Impβ mutant, which has a much weaker inhibitory potential, was also unable to confer H1 import in conjunction with Imp7. Please note that ΔN202 deletion does not abolish Imp7 of H1 binding (see Figure 4A and B).

Generation of Imp7 point mutants that are deficient in RanGTP-binding

Next we wanted to test for a role of a Ran–Imp7 interaction in H1 import. The problem, however, was that no Imp7 mutants with Ran-binding defects have so far been described. Imp7 shares with Impβ the N-terminal Ran-binding motif and so deletions from Imp7's N-terminus would have been one approach to generate such mutants. Unfortunately, all tested deletions turned out to be insoluble when expressed in *E.coli*. However, two point mutations of a conserved residue in the Ran-binding motif had the desired effect: K61A and K61D lowered the affinity of Imp7 for Ran 15- and 70-fold, respectively (Figure 8). For reasons detailed below, it is important to note that these mutations do not impair the interaction of Imp7 with transport substrates or with Impβ (not shown, but see Figures 10 and 11).

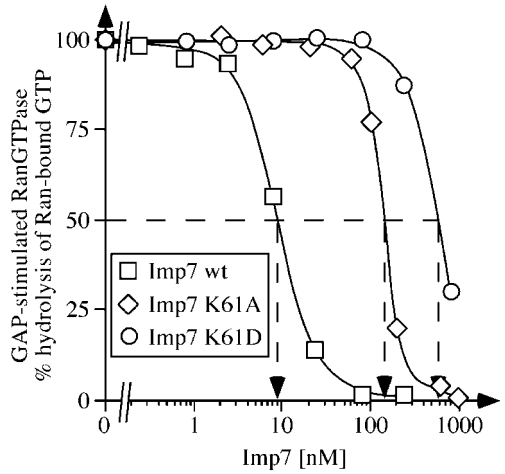

Fig. 8. Characterization of Imp7 mutants that are deficient in RanGTP-binding. The principle of the assay is that binding of Imp7 to RanGTP prevents GTPase activation by RanGAP. The figure shows the dose-dependence of the effect, from which apparent dissociation constants for the Imp7/RanGTP interactions can be estimated (interpolated half-maximum effect). Note, Imp7 wt binds RanGTP with a K_d of ~9 nM, Imp7 K61A with a K_d of ~150 nM and Imp7 K61D with a K_d of ~600 nM. For comparison, the apparent K_d for Impβ/RanGTP interaction is ~0.6 nM, when measured under identical conditions (not shown).

RanGTP binding is essential for Imp7 when operating as an autonomous import receptor

Imp7 is an import receptor for the ribosomal protein L23a. RanGTP can displace rpL23a from its import receptor (Jakel and Gorlich, 1998) and Figure 9 shows that L23a import by Imp7 is indeed Ran-dependent. The same figure also shows that the K61A and K61D mutations, which impair the Ran–Imp7 interaction, prevent L23a import. This is a crucial control for the experiments described below. It is unclear at present whether RanGTP-binding to Imp7 is already needed to complete NPC passage or just to release the cargo from Imp7 and allow deposition in the nucleoli.

The Ran-binding sites in Impβ and Imp7 synergize in the RanGTP-mediated dissociation of the H1/Impβ/Imp7 complex

RanGTP apparently triggers several events in H1 import that follow the actual translocation through the NPC: namely the release of the cargo–receptor complex from the NPC into the nucleoplasm (termination of import), the dissociation of Impβ from Imp7, and the displacement of the importins from the histone. In Figure 10 we compared the contributions of the RanGTP binding sites in Impβ and Imp7 to the release of H1 from the importins. Impβ wild type and ΔN44, Imp7 wild type, K61A, and K61D were used to assemble various combinations of H1/Impβ/Imp7 complexes, in which none, one or both Ran-binding sites were inactivated. Dissociation by RanGTP was measured as the release of the importins from the immobilized histone. The Ran-resistant fraction was subsequently eluted under denaturing conditions (Figure 10). As expected, dissociation by RanGTP was very efficient for the wild-type heterodimer, and not detectable if the Ran-binding sites in both Impβ and Imp7 were inactivated. Dissociation was weak for the combination of Impβ ΔN44/Imp7 wild type. The most interesting combination,

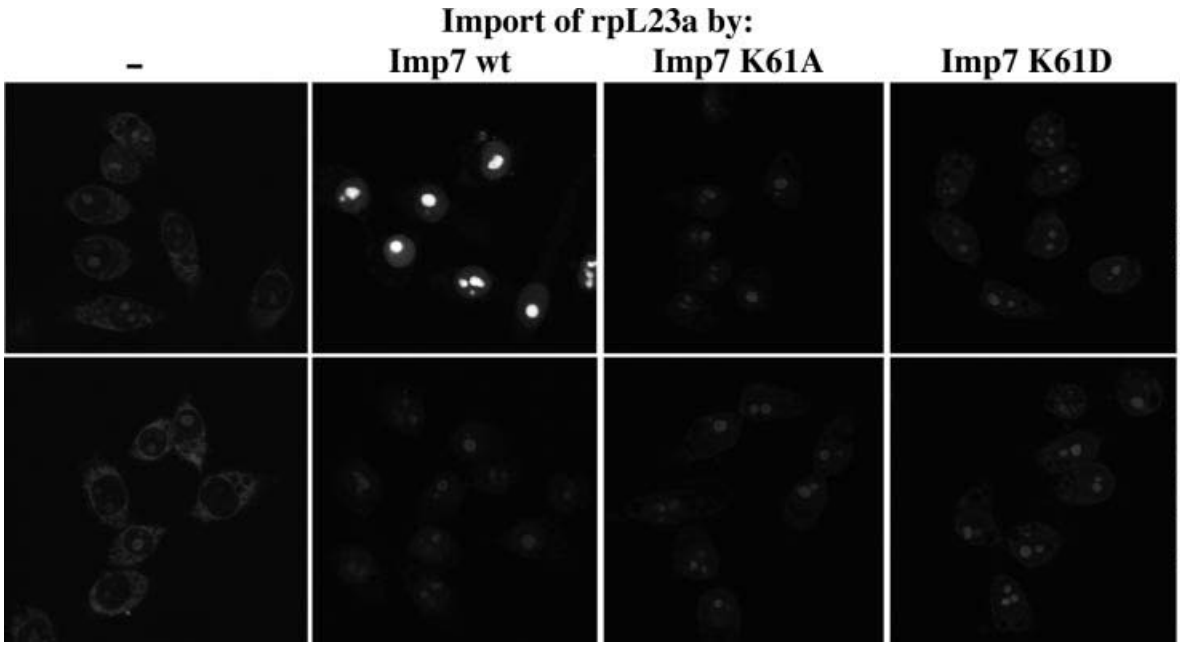

Fig. 9. RanGTP-binding is essential for Imp7 to mediate nuclear import of rpL23a. Import of fluorescent rpL23a (0.8 μM) was performed in the presence of the indicated combinations of 1.5 μM Ran with 1 μM Imp7 wild type, Imp7 K61A, or Imp7 K61D. An energy-regenerating system, NTF2, RanBP1 and Rna1p was present in all incubations. Note, import of rpL23a was Ran-dependent. Import was supported by wild-type Imp7 but not by the K61A or K61D mutants that are deficient in Ran-binding.

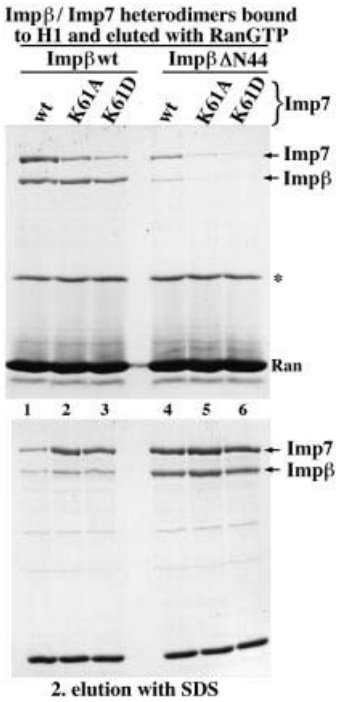

Fig. 10. Disassembly of the Impβ/Imp7/histone H1 complex by RanGTP. Wild-type or mutant Impβ/Imp7 heterodimers were formed by addition of 80 pmol of purified Imp7 wt, K61A, or K61D to 80 μl of Impβ wild type or ΔN44 lysates ('start'). The mixtures were diluted to 300 μl in binding buffer, the heterodimers were bound to immobilized histone H1 (Figure 3) and eluted in two steps: the initial elution was with 1.8 nmol RanQ69L GTP in 50 μl binding buffer. The RanGTP-resistant fraction was subsequently released with SDS-sample buffer. Analysis was by SDS–PAGE followed by Coomassie staining. Load in the eluted fractions corresponds to 8× the starting material. A Ran-dimer is indicated by an asterisk.

however, was that of wild-type Impβ with the Imp7 mutants. In this case, RanGTP dissociated Impβ efficiently from the complex, whereas the Imp7 mutants remained largely H1-bound. It should be noted, however, that the H1/Imp7 complex is already considerably destabilized compared with the trimeric H1/Impβ/Imp7 complex (e.g. Figure 3).

RanGTP-binding appears required for Imp7 when operating as an autonomous import receptor, but not for H1 import in conjunction with Impβ

We next tested import of H1 by the Imp7 K61A and K61D mutants in the presence of wild-type Impβ. Surprisingly, they showed wild-type activity (Figure 11), suggesting that the Ran–Imp7 interaction is dispensable for H1 import. It is important to note that H1 import in the presence of Imp7 K61A or K61D was still strictly Ran-dependent (Figure 11) and was thus solely driven from Impβ's Ran-binding site (see Figure 5). Imp7 would then behave in this situation like an import adapter. However, one could argue that the Ran-binding of the Imp7 mutants was not completely abolished (Figure 8) and that the low, residual Ran-binding activity accounts for the effect. This appears unlikely for several reasons. First, the mutants had wild-type import activity even when the Ran concentration was reduced to a limiting level (0.4 μM, Figure 11). Secondly, the same mutations prevented rpL23a import by Imp7 (Figure 9). In addition, one should consider that the affinity of wild-type Imp7 for RanGTP (K_d ~9 nM) is already ~15 times lower than that of Impβ (K_d ~0.6 nM), the differences relative to the K61A and K61D Imp7 mutants are then factors of 250 and 1000, respectively.

RanGTP binding to Impβ probably releases an H1–Imp7 complex into the nucleoplasm (see Figure 11), which

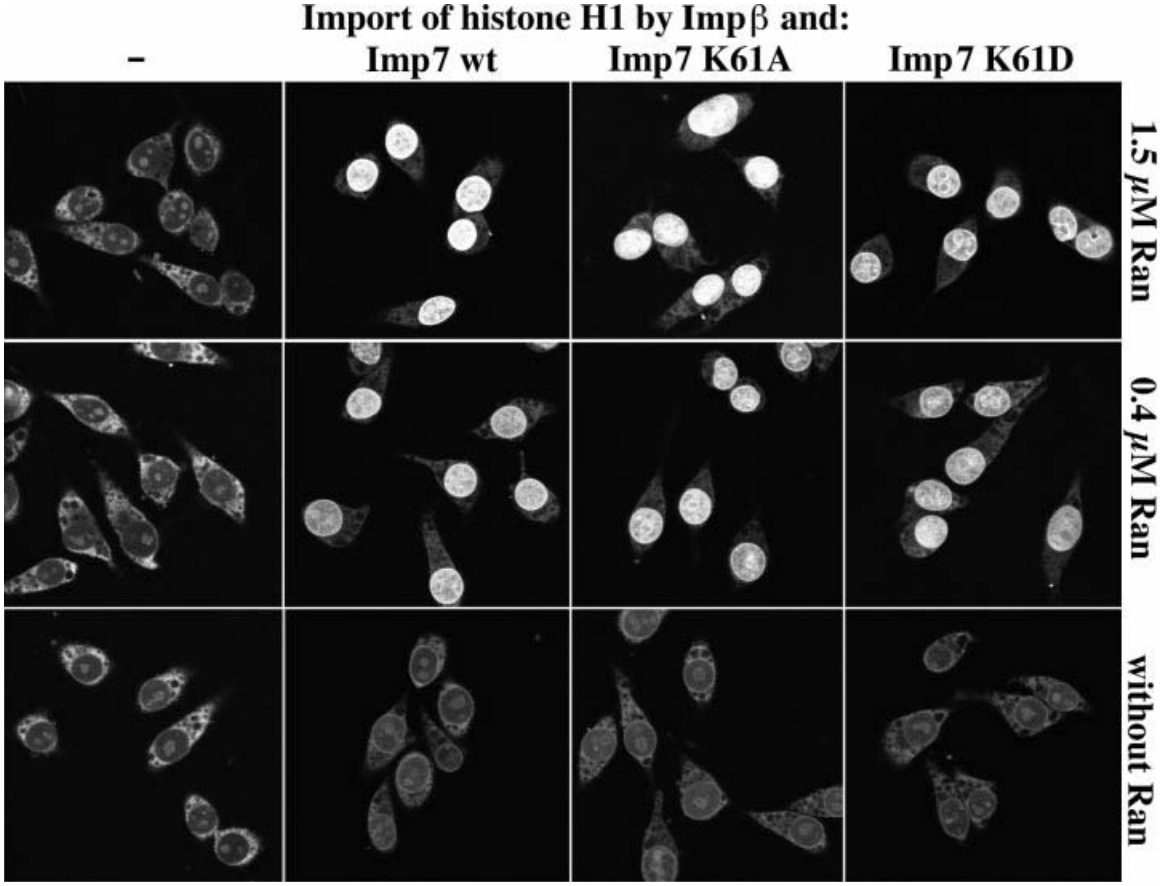

Fig. 11. The Imp7 mutants defective in Ran-binding are functional in H1 import. Import of fluorescent $H1^0$ (0.25 μM) was performed as described in Figures 2 and 5 with the indicated combinations of transport factors. NTF2, RNA1, RanBP1, an energy-regenerating system and 0.3 μM Impβ were present in all incubations. Imp7 derivatives were used at 0.3 μM. Note, H1 import by the Impβ/Imp7 heterodimer is strictly Ran-dependent. Although the K61A and K61D mutants of Imp7 are deficient in Ran-binding, they promoted H1 import nearly as efficiently as wild-type Imp7, suggesting that the Ran-dependence of this import is largely accounted for by Impβ (see also Figure 5).

leaves us with the questions of how this 'residual complex' dissociates. A simple solution to the problem might be that binding of DNA and Imp7 to histone H1 appear mutually exclusive (our unpublished observation). Incorporation of H1 into chromatin would thus also release the import receptor from its cargo. With wild-type proteins, however, this would be aided by Ran. Import of H1 by the Impβ/Imp7 heterodimer is summarized schematically in Figure 12.

Discussion

The cell nucleus requires import of a great many proteins. In proliferating cells, histones are some of the most abundant import substrates and during S-phase in HeLa cells one can estimate that each nuclear pore complex has to accomplish import of, on average, one histone molecule per second. Here, we have studied nuclear import of the linker histone H1 and found that this substrate follows a novel import pathway that requires the heterodimerization of two distinct β-family transport receptors, namely Imp7 (RanBP7) and Impβ (see Figure 12 for a scheme). The Impβ/Imp7 heterodimer is stable only in the..absence of RanGTP, i.e. in a cytoplasmic environment (Gorlich *et al*., 1997; also see Introduction). The heterodimer is the active species in H1 import and binds H1 more efficiently than either Impβ or Imp7 alone. The probable explanation for the greater stability of the trimeric receptor/H1 complex is that Impβ and Imp7 both contribute to substrate recognition and each contact distinct domains of H1. The binding energy for the interaction of H1 with the Impβ/Imp7 heterodimer would then be approximately the sum of binding energies of the individual H1/Impβ and H1/Imp7 interactions.

Once the substrate–receptor complex has assembled, it can dock to the cytoplasmic periphery of the NPC, which might involve the NPC binding sites of both Impβ and Imp7, and becomes translocated to the nuclear side of the nuclear pore complex. There, it meets an environment of high RanGTP concentration which disassembles the trimeric complex into its constituents and allows histone H1 to be deposited onto DNA. This series of events is strictly Ran dependent (Figure 11) and requires GTP (not shown), most probably to generate the RanGTP in the nucleus.

There can be subtle differences between different import receptors with respect to exactly how and when Ran is utilized. In the case of M9 import by transportin, the only function of RanGTP is to displace the substrates from the receptor and this may occur far inside the nucleus (Izaurralde *et al.*, 1997; Siomi *et al.*, 1997; Englmeier *et al.*, 1999; Ribbeck *et al.*, 1999). In the absence of Ran, M9/transportin complexes accumulate inside the nucleus, i.e. Ran is not immediately required for nuclear

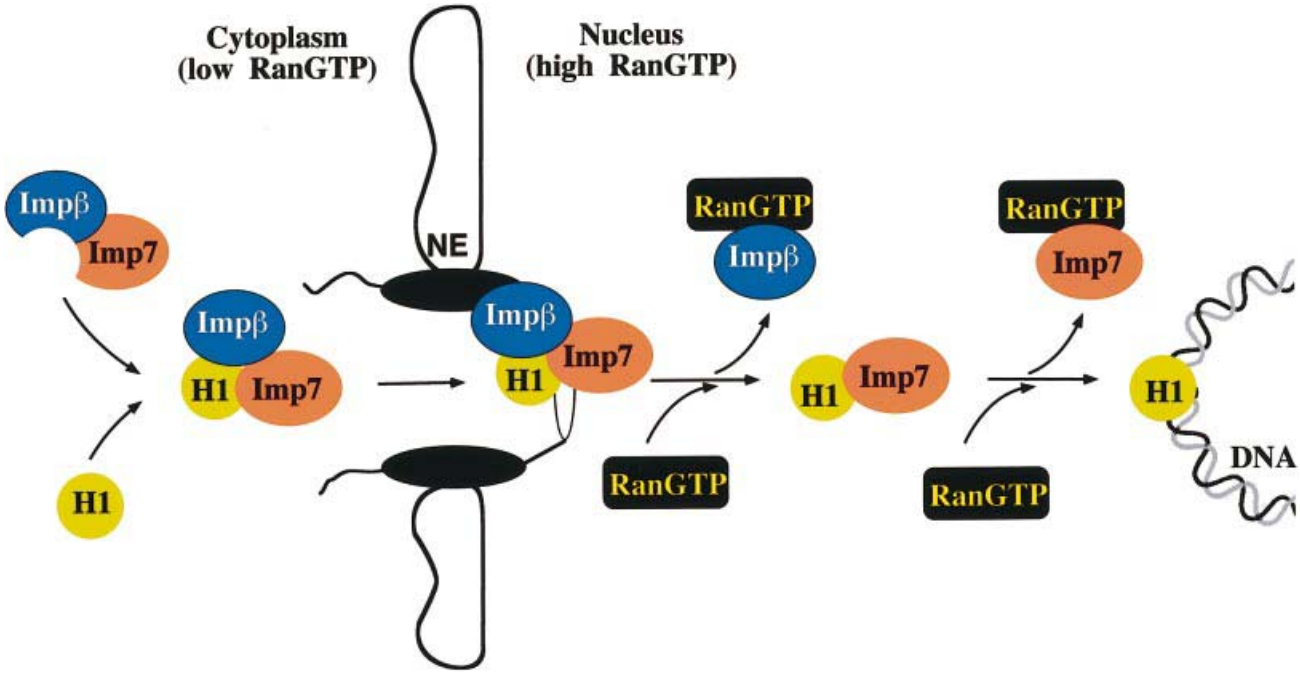

Fig. 12. Schematic presentation of histone H1 import into the nucleus. A trimeric H1/Impβ/Imp7 complex is assembled in the cytoplasm and translocates to the nuclear side of the NPC. Direct binding of RanGTP to Impβ is required to complete NPC passage. This results in the detachment of the import complex from the NPC and the dissociation of Impβ from the remaining H1/Imp7 complex. The transfer of the histone from Imp7 onto DNA is facilitated by RanGTP-binding to Imp7.

accumulation of the import substrate. The second category is exemplified by Impβ-mediated IBB import, where cargo release from Impβ is directly coupled to Impβ-release from the NPC, i.e. the RanGTP–Impβ interaction is already required to complete NPC passage (Moore and Blobel, 1993; Gorlich *et al.*, 1996c). The data from Figure 12 suggest that H1 import by the Impβ/Imp7 heterodimer is apparently another example for this second category.

The trimeric H1/Impβ/Imp7 complex is disassembled by direct binding of RanGTP to Impβ and Imp7 (Figures 1 and 10). Both Ran-binding sites promote a disassembly of the complex; however, their contribution to the overall import reaction is not the same. The Ran–Impβ interaction is absolutely required, whilst binding of Ran to Imp7 is not. How can this be explained? We would suggest that RanGTP-binding to Impβ and to Imp7 are successive events and that RanGTP-binding to Impβ is essential because it triggers release of the cargo–receptor complex from nuclear pores. An Imp7/H1 sub-complex would be released, from which the histone can subsequently be transferred onto DNA. This transfer is normally aided by RanGTP, but such a 'support' is obviously not rate-limiting. A delayed dissociation of H1 from Imp7 after NPC passage might actually be an advantage. Imp7 could then act in a chaperonin-like fashion also far inside the nucleus and accompany the histone until assembly into chromatin.

The RanGTP complexes of Impβ and Imp7 have no detectable affinity for each other (Gorlich *et al.*, 1997) and therefore are probably returned to the cytoplasm separately, where RanGTP is removed to restore import competence of Impβ and Imp7. The heterodimer might reform and accomplish import of another H1 molecule. Alternatively, Impβ and Imp7 might independently participate in other nuclear import pathways.

The flexible use of the two import receptors is indeed quite remarkable. Imp7 is also an autonomous import receptor for ribosomal proteins and possibly for further substrates as well. However, Impβ is clearly the most versatile of all nuclear import receptors in higher eukaryotes. On its own it can import ribosomal proteins; furthermore, it can combine with at least six alternative adapter molecules, namely with (at least) five distinct Impα subunits to mediate import by the classical pathway (see for example Gorlich *et al*., 1994; Kohler *et al*., 1997; Tsuji *et al*., 1997; Nachury *et al*., 1998), or with snurportin 1 to accomplish nuclear import of m_3G capped snRNPs (Palacios *et al*., 1997; Huber *et al*., 1998). In addition, Impβ can form heterodimeric complexes with two other β-family receptors: as shown here, Impβ binds Imp7 to mediate nuclear import of histone H1 and perhaps also import of other substrates. Within this heterodimer, Imp7 plays a more passive role than Impβ, its RanGTP-binding is 15-fold weaker compared with Impβ and is not essential for H1 import. Imp7 could therefore also be considered an adapter molecule. Impα would then be an example for the consequent further evolution, where the Ran-binding has been completely lost. The fact that Impα can directly interact with some nucleoporins, such as Nup2p (Belanger *et al.*, 1994; Gorlich *et al.*, 1996b), might be a relict of its past as an autonomous import receptor.

Impβ can also form a complex with RanBP8 that is 61% identical to either *Xenopus* or human Imp7 (Gorlich *et al*., 1997). Despite this similarity, the Impβ/RanBP8 complex neither binds nor imports histone H1 (not shown). It will thus be interesting to see which cargo this heterodimer might carry. The combinatorial flexibility of Impβ in higher eukaryotes is in apparently sharp contrast to the situation in *S.cerevisiae*. This yeast employs only a single Impα subunit (Yano *et al*., 1992; Loeb *et al*.,

1995) and lacks snurportin. In addition, yeast apparently does not use an equivalent of the Impβ/Imp7 heterodimer, since such a complex cannot be purified from yeast cytosol and yeast Impβ has no detectable affinity for Imp7 from *Xenopus* (not shown). This would suggest that the heterodimerization is not conserved between higher eukaryotes and yeast. In this context it is interesting to note that *S.cerevisiae* has no linker histone H1. Furthermore, human $H1^0$, expressed in *S.cerevisiae*, does not accumulate in the yeast nuclei, but aggregates in the cytoplasm (Albig *et al*., 1998 and our unpublished observation). Lack of nuclear accumulation could indeed reflect the absence of an appropriate import receptor such as the Impβ/Imp7 heterodimer.

In principle, nuclear pore complexes allow the passive diffusion of macromolecules up to 40–60 kDa (for a review see Bonner, 1978). Nevertheless, it has become clear that proteins or RNAs that need to cross the nuclear envelope normally use specific carrier systems even if they are small enough for passive diffusion. This has been shown e.g. for histone H1 (Breeuwer and Goldfarb, 1990; Kurz *et al*., 1997; this study), for tRNA (Zasloff, 1983; Arts *et al*., 1998; Kutay *et al*., 1998), ribosomal proteins (Rout *et al*., 1997; Schlenstedt *et al*., 1997; Jakel and Gorlich, 1998) and for Ran (Ribbeck *et al*., 1998). The carriers clearly facilitate the crossing of the nuclear envelope. However, one should also consider a second effect, namely that transport receptors could cover 'sticky' domains of a cargo and thereby prevent undesired interactions before the cargo reaches its final destination. This might be particularly crucial for very basic proteins such as histones or ribosomal proteins, which have a high tendency to precipitate and aggregate at physiological salt concentration. The receptors should then cover as much as possible of such basic domains. This would explain why the 'import signals' of e.g. ribosomal proteins are that large. In the case of histone H1, a single import receptor might not suffice to completely wrap the extended and extremely basic domain of this cargo, so that the employment of the Impβ/Imp7 heterodimer for H1 import may be considered a good solution to such a problem.

Materials and methods

Recombinant protein expression and protein purification

The following proteins were expressed in *E.coli* and purified as described: C-his *Xenopus* Impα (Gorlich *et al*., 1994), Imp5 (RanBP5), N-His Imp7 (RanBP7) (Jakel and Gorlich, 1998), Ran, NTF2, RanBP1, Rna1p and human Impβ (Kutay *et al*., 1997b). Histone $H1^0$ containing an extra cysteine was expressed in *S.cerevisiae* and purified as described (Albig *et al*., 1998). The cysteine was introduced by site-directed mutagenesis, replacing glycine in position 190.

The following expression constructs are newly described in this study: the imp7 K61A and K61D mutants are derived from Imp7-pQE9. The lysine codon AAG at position 61 was changed to GCA (coding for alanine) or to GAC (coding for aspartic acid), using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's instructions. The Impβ fragments 100–462, 143–462 and 203–876 were cloned into the *Nco*I–*Bam*HI sites of pQE60 (Qiagen). All other Impβ fragments have been described previously (Kutay *et al*., 1997b). 2z-Imp7 w/o his was generated by cloning the Imp7 coding sequence into the *Bam*HI–*Hin*dIII sites of p2z60 (Gorlich *et al*., 1996a). The construct allows expression of an Imp7 that has two N-terminal z-tags but lacks a His-tag.

Preparation of labelled import substrates

Preparation of fluorescent nucleoplasmin, IBB core fusion (Gorlich *et al*., 1996a) and 6z BIB (Jakel and Gorlich, 1998) has been described previously. Histone H1 from calf thymus (Boehringer #223549), dissolved in 50 mM HEPES–KOH pH 7.5, 500 mM NaCl) was modified at a 1:1 molar ratio with either Fluos [carboxy fluorescein *N*-hydroxysuccinimide ester, dissolved in dimethylsulfoxide (DMSO)] or biotinamidocaproic acid *N*-hydroxy succinimido ester (dissolved in DMSO). Protein was separated from free label on a Sephadex G25 column. Histone $H1^0$ was modified through its engineered cysteine using fluorescein 5′ maleimide or biotin maleimide, respectively.

Import assays

Permeabilized cells were prepared using the protocol of Adam *et al*. (1990) with a number of modifications (Jakel and Gorlich, 1998). The energy regenerating system consists of the following components: 0.5 mM ATP, 0.5 mM GTP, 10 mM creatine phosphate and 50 µg/ml creatine kinase. The Ran-mix consists of the following constituents: 3 µM RanGDP, 0.3 µM RanBP1, 0.2 µM *Schizosaccharomyces pombe* Rna1p, 0.4 µM NTF2 (each final concentrations). Import buffers contained 20 mM potassium phosphate pH 7.2, 200 mM potassium acetate, 5 mM magnesium acetate, 0.5 mM EGTA and 250 mM sucrose. Import of rpL23a was at 250 mM potassium acetate. Import of nucleoplasmin and the IBB core fusion was performed at 140 mM potassium acetate in the presence of nucleoplasmin core as a non-specific competitor.

Binding assays

The following matrices were used: biotinylated $H1^0$ or biotinylated histone H1 from calf thymus pre-bound to Streptavidin agarose, or zz-tagged L23a or zz-BIB pre-bound to IgG Sepharose (all at ~2mg/ml). IBB immobilized to sulfoLink was described previously (Gorlich *et al*., 1996a). For each binding, 15–20 µl of affinity matrix were rotated for 3 h with the starting material. The beads were recovered by gentle centrifugation and washed four times with 1 ml of binding buffer and eluted as described in the figure legends. For further details see main text and figure legends.

GTPase assays were carried out as described previously (Kutay *et al*., 1997b) with the modification that incubations were performed in 20 mM HEPES–KOH pH 7.4, 50 mM potassium acetate, 1 mM magnesium acetate, 1 mM sodium azide, 0.05% hydrolysed gelatine and at a temperature of 15°C to minimize protein denaturation. GTPase reactions were shortened to 30 s.

Antibodies

The following antibodies have been described previously: anti-Imp5 (RanBP5) (Jakel and Gorlich, 1998), anti-Imp7 (RanBP7) (Gorlich *et al*., 1997) anti-Impβ (Gorlich *et al*., 1995b). Antibodies against human Impα (Rch1p) were raised in rabbits against the recombinant protein. All antibodies were used after affinity purification on the respective immobilized antigens.

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References

Adam,E.J.H. and Adam,S.A. (1994) Identification of cytosolic factors required for nuclear location sequence-mediated binding to the nuclear envelope. *J. Cell Biol*., **125**, 547–555.

Adam,S.A., Sterne Marr,R. and Gerace,L. (1990) Nuclear protein import in permeabilized mammalian cells requires soluble cytoplasmic factors. *J. Cell Biol*., **111**, 807–816.

Albig,W., Runge,D.M., Kratzmeier,M. and Doenecke,D. (1998) Heterologous expression of human H1 histones in yeast. *FEBS Lett*., **435**, 245–250.

Allan,J., Mitchell,T., Harborne,N., Bohm,L. and Crane-Robinson,C. (1986) Roles of H1 domains in determining higher order chromatin structure and H1 location. *J. Mol. Biol*., **187**, 591–601.

Arts,G.-J., Fornerod,M. and Mattaj,I.W. (1998) Identification of a nuclear export receptor for tRNA. *Curr. Biol*., **8**, 305–314.

Belanger,K.D., Kenna,M.A., Wei,S. and Davis,L.I. (1994) Genetic and physical interactions between srp1p and nuclear pore complex proteins nup1p and nup2p. *J. Cell Biol.*, **126**, 619–630.

Bischoff,F.R. and Ponstingl,H. (1991a) Catalysis of guanine nucleotide exchange on Ran by the mitotic regulator RCC1. *Nature*, **354**, 80–82.

Bischoff,F.R. and Ponstingl,H. (1991b) Mitotic regulator protein RCC1 is complexed with a nuclear ras-related polypeptide. *Proc. Natl Acad. Sci. USA*, **88**, 10830–10834.

Bischoff,F.R. and Gorlich,D. (1997) RanBP1 is crucial for the release of RanGTP from importin β-related nuclear transport factors. *FEBS Lett.*, **419**, 249–254.

Bischoff,F.R., Klebe,C., Kretschmer,J., Wittinghofer,A. and Ponstingl,H. (1994) RanGAP1 induces GTPase activity of nuclear ras-related Ran. *Proc. Natl Acad. Sci. USA*, **91**, 2587–2591.

Bonner,W.M. (1978) Protein migration and accumulation in nuclei. In Busch,H. (ed.), *The Cell Nucleus*. Vol. 6, part C. Academic Press, New York, NY, pp. 97–148.

Breeuwer,M. and Goldfarb,D. (1990) Facilitated nuclear transport of histone H1 and other small nucleophilic proteins. *Cell*, **60**, 999-1008.

Chi,N.C. and Adam,S.A. (1997). Functional domains in nuclear import factor p97 for binding the nuclear localization sequence receptor and the nuclear pore. *Mol. Biol. Cell*, **8**, 945–956.

Chi,N.C., Adam,E.J.H. and Adam,S.A. (1995) Sequence and characterization of cytoplasmic nuclear import factor p97. *J. Cell Biol.*, **130**, 265–274.

Chi,N.C., Adam,E.J.H., Visser,G.D. and Adam,S.A. (1996) RanBP1 stabilises the interaction of Ran with p97 in nuclear protein import. *J. Cell Biol.*, **135**, 559–569.

Dahlberg,J.E. and Lund,E. (1998) Functions of the GTPase Ran in RNA export from the nucleus. *Curr. Opin. Cell Biol.*, **10**, 400–408.

Doenecke,D., Albig,W., Bouterfa,H. and Drabent,B. (1994) Organization and expression of H1 histone and H1 replacement histone genes. *J. Cell. Biochem.*, **54**, 423–431.

Englmeier,L., Olivo,J.C. and Mattaj,I.W. (1999) Receptor-mediated substrate translocation through the nuclear pore complex without nucleotide triphosphate hydrolysis. *Curr. Biol.*, **9**, 30–41.

Feldherr,C.M., Kallenbach,E. and Schultz,N. (1984) Movement of karyophilic protein through the nuclear pores of oocytes. *J. Cell Biol.*, **99**, 2216–2222.

Floer,M., Blobel,G. and Rexach,M. (1997) Disassembly of RanGTP-karyopherin β complex, an intermediate in nuclear protein import. *J. Biol. Chem.*, **272**, 19538–19546.

Fornerod,M., Ohno,M., Yoshida,M. and Mattaj,I.W. (1997a) Crm1 is an export receptor for leucine rich nuclear export signals. *Cell*, **90**, 1051–1060.

Fornerod,M., Vandeursen,J., Vanbaal,S., Reynolds,A., Davis,D., Murti,K.G., Fransen,J. and Grosveld,G. (1997b) The human homologue of yeast Crm1 is in a dynamic subcomplex with Can/Nup214 and a novel nuclear pore component Nup88. *EMBO J.*, **16**, 807–816.

Fridell,R.A., Truant,R., Thorne,L., Benson,R.E. and Cullen,B.R. (1997) Nuclear import of hnRNP A1 is mediated by a novel cellular cofactor related to karyopherin-β. *J. Cell Sci.*, **110**, 1325–1331.

Gorlich,D. (1998) Transport into and out of the cell nucleus. *EMBO J.*, **17**, 2721–2727.

Gorlich,D., Prehn,S., Laskey,R.A. and Hartmann,E. (1994) Isolation of a protein that is essential for the first step of nuclear protein import. *Cell*, **79**, 767–778.

Gorlich,D., Kostka,S., Kraft,R., Dingwall,C., Laskey,R.A., Hartmann,E. and Prehn,S. (1995a) Two different subunits of importin cooperate to recognize nuclear localization signals and bind them to the nuclear envelope. *Curr. Biol.*, **5**, 383–392.

Gorlich,D., Vogel,F., Mills,A.D., Hartmann,E. and Laskey,R.A. (1995b) Distinct functions for the two importin subunits in nuclear protein import. *Nature*, **377**, 246–248.

Gorlich,D., Henklein,P., Laskey,R.A. and Hartmann,E. (1996a) A 41 amino acid motif in importin α confers binding to importin β and hence transit into the nucleus. *EMBO J.*, **15**, 1810–1817.

Gorlich,D., Kraft,R., Kostka,S., Vogel,F., Hartmann,E., Laskey,R.A., Mattaj,I.W. and Izaurralde,E. (1996b) Importin provides a link between nuclear protein import and U snRNA export. *Cell*, **87**, 21–32.

Gorlich,D., Pante,N., Kutay,U., Aebi,U. and Bischoff,F.R. (1996c) Identification of different roles for RanGDP and RanGTP in nuclear protein import. *EMBO J.*, **15**, 5584–5594.

Gorlich,D., Dabrowski,M., Bischoff,F.R., Kutay,U., Bork,P., Hartmann,E., Prehn,S. and Izaurralde,E. (1997) A novel class of RanGTP binding proteins. *J. Cell Biol.*, **138**, 65–80.

Huber,J., Cronshagen,U., Kadokura,M., Marshallsay,C., Wada,T., Sekine,M. and Luhrmann,R. (1998) Snurportin1, an m3G-cap-specific nuclear import receptor with a novel domain structure. *EMBO J.*, **17**, 4114–4126.

Imamoto,N., Shimamoto,T., Kose,S., Takao,T., Tachibana,T., Matsubae,M., Sekimoto,T., Shimonishi,Y. and Yoneda,Y. (1995a) The nuclear pore targeting complex binds to nuclear pores after association with a karyophile. *FEBS Lett.*, **368**, 415–419.

Imamoto,N., Tachibana,T., Matsubae,M. and Yoneda,Y. (1995b) A karyophilic protein forms a stable complex with cytoplasmic components prior to nuclear pore binding. *J. Biol. Chem.*, **270**, 8559–8565.

Izaurralde,E. and Adam,S.A. (1998) Transport of macromolecules between the nucleus and the cytoplasm. *RNA J.*, **4**, 351–364.

Izaurralde,E., Kutay,U., von Kobbe,C., Mattaj,I.W. and Gorlich,D. (1997) The asymmetric distribution of the constituents of the Ran system is essential for transport into and out of the nucleus. *EMBO J.*, **16**, 6535–6547.

Jakel,S. and Gorlich,D. (1998) Importin β, transportin, RanBP5 and RanBP7 mediate nuclear import of ribosomal proteins in mammalian cells. *EMBO J.*, **17**, 4491–4502.

Kohler,M., Ansieau,S., Prehn,S., Leutz,A., Haller,H. and Hartmann,E. (1997) Cloning of two novel human importin α subunits and analysis of the expression pattern of the importin α protein family. *FEBS Lett.*, **417**, 104–108.

Kose,S., Imamoto,N., Tachibana,T., Shimamoto,T. and Yoneda,Y. (1997) Ran-unassisted nuclear migration of a 97-kD component of nuclear pore-targeting complex. *J. Cell Biol.*, **139**, 841–849.

Kurz,M., Doenecke,D. and Albig,W. (1997) Nuclear transport of H1 histones meets the criteria of a nuclear localization signal-mediated process. *J. Cell. Biochem.*, **64**, 573–578.

Kutay,U., Bischoff,F.R., Kostka,S., Kraft,R. and Gorlich,D. (1997a) Export of importin α from the nucleus is mediated by a specific nuclear transport factor. *Cell*, **90**, 1061–1071.

Kutay,U., Izaurralde,E., Bischoff,F.R., Mattaj,I.W. and Gorlich,D. (1997b) Dominant-negative mutants of importin-β block multiple pathways of import and export through the nuclear pore complex. *EMBO J.*, **16**, 1153–1163.

Kutay,U., Lipowsky,G., Izaurralde,E., Bischoff,F.R., Schwarzmaier,P., Hartmann,E. and Gorlich,D. (1998) Identification of a tRNA-specific nuclear export receptor. *Mol. Cell*, **1**, 359–369.

Loeb,J.D.J., Schlenstedt,G., Pellman,D., Kornitzer,D., Silver,P.A. and Fink,G.R. (1995) The yeast nuclear import receptor is required for mitosis. *Proc. Natl Acad. Sci. USA*, **92**, 7647–7651.

Lounsbury,K.M. and Macara,I.G. (1997) Ran-binding protein 1 (RanBP1) forms a ternary complex with Ran and Karyopherin β and reduces GTPase-activating protein (RanGAP) inhibition by karyopherin β. *J. Biol. Chem.*, **272**, 551–555.

Mahajan,R., Delphin,C., Guan,T., Gerace,L. and Melchior,F. (1997) A small ubiquitin-related polypeptide involved in targeting RanGAP1 to nuclear pore complex protein RanBP2. *Cell*, **88**, 97–107.

Mattaj,I.W. and Englmeier,L. (1998) Nucleocytoplasmic transport: the soluble phase. *Annu. Rev. Biochem.*, **67**, 265–306.

Matunis,M.J., Coutavas,E. and Blobel,G. (1996) A novel ubiquitin-like modification modulates the partitioning of the Ran-GTPase-activating protein between cytosol and nuclear pore complex. *J. Cell Biol.*, **135**, 1457–1470.

Melchior,F., Paschal,B., Evans,E. and Gerace,L. (1993) Inhibition of nuclear protein import by nonhydrolyzable analogs of GTP and identification of the small GTPase Ran/TC4 as an essential transport factor. *J. Cell Biol.*, **123**, 1649–1659.

Moore,M.S. and Blobel,G. (1993) The GTP-binding protein Ran/TC4 is required for protein import into the nucleus. *Nature*, **365**, 661–663.

Moore,M.S. and Blobel,G. (1994) Purification of a Ran-interacting protein that is required for protein import into the nucleus. *Proc. Natl Acad. Sci. USA*, **91**, 10212–10216.

Nachury,M.V., Ryder,U.W., Lamond,A.I. and Weis,K. (1998) Cloning and characterization of hSRP1 gamma, a tissue-specific nuclear transport factor. *Proc. Natl Acad. Sci. USA*, **95**, 582–587.

Nakielny,S. and Dreyfuss,G. (1998) Import and export of the nuclear protein import receptor transportin by a mechanism independent of GTP hydrolysis. *Curr. Biol.*, **8**, 89–95.

Ohtsubo,M., Okazaki,H. and Nishimoto,T. (1989) The RCC1 protein, a regulator for the onset of chromosome condensation locates in the nucleus and binds to DNA. *J. Cell Biol.*, **109**, 1389–1397.

Palacios,I., Hetzer,M., Adam,S.A. and Mattaj,I.W. (1997) Nuclear import of U snRNPs requires importin beta. *EMBO J.*, **16**, 6783–6792.

Paschal,B.M. and Gerace,L. (1995) Identification of NTF2, a cytosolic factor for nuclear import that interacts with nuclear pore protein p62. *J. Cell Biol*., **129**, 925–937.

Pollard,V.W., Michael,W.M., Nakielny,S., Siomi,M.C., Wang,F. and Dreyfuss,G. (1996) A novel receptor mediated nuclear protein import pathway. *Cell*, **88**, 985–994.

Pruss,D., Bartholomew,B., Persinger,J., Hayes,J., Arents,G., Moudrianakis,E.N. and Wolffe,A.P. (1996) An asymmetric model for the nucleosome: a binding site for linker histones inside the DNA gyres. *Science*, **274**, 614–617.

Radu,A., Blobel,G. and Moore,M.S. (1995) Identification of a protein complex that is required for nuclear protein import and mediates docking of import substrate to distinct nucleoporins. *Proc. Natl Acad. Sci. USA*, **92**, 1769–1773.

Rexach,M. and Blobel,G. (1995) Protein import into nuclei: association and dissociation reactions involving transport substrate, transport factors and nucleoporins. *Cell*, **83**, 683–692.

Ribbeck,K., Lipowsky,G., Kent,H.M., Stewart,M. and Gorlich,D. (1998) NTF2 mediates nuclear import of Ran. *EMBO J*., **17**, 6587–6598.

Ribbeck,K., Kutay,U., Paraskeva,E. and Gorlich,D. (1999) The translocation of transportin–cargo complexes through nuclear pores is independent of both Ran and energy. *Curr. Biol.*, **9**, 47–50.

Rout,M.P., Blobel,G. and Aitchison,J.D. (1997) A distinct nuclear import pathway used by ribosomal proteins. *Cell*, **89**, 715–725.

Schlenstedt,G., Smirnova,E., Deane,R., Solsbacher,J., Kutay,U., Gorlich,D., Ponstingl,H. and Bischoff,F.R. (1997) Yrb4p, a Yeast Ran GTP binding protein involved in import of ribosomal protein L25 into the nucleus. *EMBO J*., **16**, 6237–6249.

Schwamborn,K., Albig,W. and Doenecke,D. (1998) The histone H1 degrees contains multiple sequence elements for nuclear targeting. *Exp. Cell Res*., **244**, 206–217.

Schwoebel,E.D., Talcott,B., Cushman,I. and Moore,M.S. (1998) Ran-dependent signal-mediated nuclear import does not require GTP hydrolysis by Ran. *J. Biol. Chem.*, **273**, 35170–35175.

Siomi,M.C., Eder,P.S., Kataoka,N., Wan,L., Liu,Q. and Dreyfuss,G. (1997) Transportin-mediated nuclear import of heterogenous RNP proteins. *J. Cell. Biol*., **138**, 1181–1192.

Tsuji,L., Takumi,T., Imamoto,N. and Yoneda,Y. (1997) Identification of novel homologues of mouse importin α, the α subunit of the nuclear pore-targeting complex and their tissue-specific expression. *FEBS Lett*., **416**, 30–34.

Weis,K., Ryder,U. and Lamond,A.I. (1996a) The conserved amino terminal domain of hSRP1a is essential for nuclear protein import. *EMBO J*., **15**, 1818–1825.

Weis,K., Dingwall,C. and Lamond,A.I. (1996b) Characterization of the nuclear protein import mechanism using Ran mutants with altered binding specificities. *EMBO J*., **15**, 7120–7128.

Wolffe,A. (1995) *Chromatin: Structure and Function*. Academic Press, London, UK.

Yano,R., Oakes,M., Yamaghishi,M., Dodd,J.A. and Nomura,M. (1992) Cloning and characterization of SRP1, a suppressor of temperature-sensitive RNA polymerase I mutations, in *Saccharomyces cerevisiae*. *Mol. Cell. Biol*., **12**, 5640–5651.

Zasloff,M. (1983) tRNA transport from the nucleus in a eukaryotic cell: carrier-mediated translocation process. *Proc. Natl Acad. Sci. USA*, **80**, 6436–6440.

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