The EMBO Journal Vol.16 No.6 pp.1153–1163, 1997

Dominant-negative mutants of importin-β block multiple pathways of import and export through the nuclear pore complex

Ulrike Kutay, Elisa Izaurralde[1,2], F.Ralf Bischoff[3], Iain W.Mattaj[1] and Dirk Görlich[4]

Zentrum für Molekulare Biologie der Universität Heidelberg, Im Neuenheimer Feld 282, 69120 Heidelberg, [1]European Molecular Biology Laboratory, Meyerhofstrasse 1, 69117 Heidelberg and [3]Abteilung Molekulare Biologie der Mitose, Deutsches Krebsforschungszentrum, Im Neuenheimer Feld 280, 69120 Heidelberg, Germany

[2]Present address: University of Geneva, Department of Molecular Biology, 30 quai Ernest-Ansermet, CH-1211 Geneva 4, Switzerland

[4]Corresponding author

Nuclear protein import proceeds through the nuclear pore complex (NPC). Importin-β mediates translocation via direct interaction with NPC components and carries importin-α with the NLS substrate from the cytoplasm into the nucleus. The import reaction is terminated by the direct binding of nuclear RanGTP to importin-β which dissociates the importin heterodimer. Here, we analyse the sites of interaction on importin-β for its multiple partners. Ran and importin-α respectively require residues 1–364 and 331–876 of importin-β for binding. Thus, RanGTP-mediated release of importin-α from importin-β is likely to be an active displacement rather than due to simple competition between Ran and importin-α for a common binding site. Importin-β has at least two non-overlapping sites of interaction with the NPC, which could potentially be used sequentially during translocation. Our data also suggest that termination of import involves a transient release of importin-β from the NPC. Importin-β fragments which bind to the NPC, but not to Ran, resist this release mechanism. As would be predicted from this, these importin-β mutants are very efficient inhibitors of NLS-dependent protein import. Surprisingly, however, they also inhibit M9 signal-mediated nuclear import as well as nuclear export of mRNA, U snRNA, and the NES-containing Rev protein. This suggests that mediators of these various transport events share binding sites on the NPC and/or that mechanisms exist to coordinate translocation through the NPC via different nucleocytoplasmic transport pathways.

Keywords: importin/nuclear pore complex/nuclear transport/RNA export

Introduction

Nuclear pore complexes (NPCs) are the sites of exchange of macromolecules between nucleus and cytoplasm (Feldherr *et al.*, 1984). NPCs allow diffusion of small molecules up to ~40–60 kDa and can accommodate active transport of particles as large as 25 nm in diameter or several million Daltons in molecular weight (reviewed in Bonner, 1978). Active transport across the NPC occurs in both directions, is generally saturable, and thus carrier-mediated. A number of kinetically distinct pathways have been characterized by competition experiments or by the signals that direct transport. Import of most nuclear proteins is triggered by nuclear localization signals (NLSs) consisting of one or more clusters of basic amino acids (for review, see Dingwall and Laskey, 1991). In the following, we will use the term 'NLS' in its strict sense, referring to this basic type of signal only. The M9 domain of hnRNP A1 is different in sequence and confers import into the nucleus as well as nuclear export (Michael *et al.*, 1995). Nuclear export of proteins like HIV Rev is by virtue of a short, leucine-rich nuclear export signal (NES) (Fischer *et al.*, 1995; Wen *et al.*, 1995). The term 'NES' is used here to describe this Rev-type signal only.

It is probable that the export of RNAs out of the nucleus is directed by associated proteins carrying NESs, M9 domains or perhaps still unidentified export signals (for reviews, see Gerace, 1995; Izaurralde and Mattaj, 1995; Görlich and Mattaj, 1996). For example, HIV Rev achieves export of viral RNAs containing Rev response elements. The best candidates to mediate export of cellular mRNAs are hnRNP A1 and its relatives (Piñol-Roma and Dreyfuss, 1993). The NES-containing TFIIIA contributes to nuclear export of 5S RNA (Guddat *et al.*, 1990), and the cap binding complex (CBC) is involved in export of capped U snRNAs (Izaurralde *et al.*, 1995). It is well established that the different classes of RNAs leave the nucleus via distinct routes. For example, export of tRNA is competed by excess tRNA but not by mRNAs, U snRNAs, 5S RNA or by rRNA (Zasloff, 1983; Jarmolowski *et al.*, 1994; Pokrywka and Goldfarb, 1995).

The import of NLS-containing proteins is the best-characterized nucleocytoplasmic transport pathway. Following the observation that import into the nuclei of digitonin-permeabilized cells depends on the re-addition of cytosol or cytosolic fractions (Adam *et al.*, 1990; Moore and Blobel, 1992; Adam and Adam, 1994), four soluble factors implicated in nuclear protein import were purified and the corresponding genes have been identified: the GTPase Ran/TC4 (Melchior *et al.*, 1993a; Moore and Blobel, 1993), importin-α (Görlich *et al.*, 1994; Imamoto *et al.*, 1995b), importin-β (Chi *et al.*, 1995; Görlich *et al.*, 1995a; Imamoto *et al.*, 1995a; Radu *et al.*, 1995) and NTF2 (Moore and Blobel, 1994; Paschal and Gerace, 1995). Importin has also been called karyopherin and NTF2 is also called pp15 or p10.

For import, the NLS-protein binds in the cytoplasm to the importin-α/β heterodimer (Görlich *et al.*, 1995a; Imamoto *et al.*, 1995c). Importin-α provides the NLS binding site (Adam and Gerace, 1991; Görlich *et al.*, 1995a; Weis *et al.*, 1995). Importin-α in turn interacts with importin-β via the N-terminal importin-β binding domain (IBB domain) (Görlich *et al.*, 1996a; Weis *et al.*, 1996). Binding to importin-β with this IBB domain is sufficient for efficient nuclear entry and the IBB domain can therefore be regarded as the nuclear targeting signal of importin-α. The trimeric NLS–importin-α/β complex docks via importin-β to the cytoplasmic periphery of the NPC (Görlich *et al.*, 1995b; Moroianu *et al.*, 1995) and is subsequently translocated through the NPC as a single entity. Translocation is finally terminated at the nucleoplasmic side of the nuclear pore by the disassembly of the NLS–importin-α/β complex and the release of the NLS-protein and importin-α into the nucleoplasm. The importin heterodimer is dissociated by direct binding of RanGTP to importin-β (Rexach and Blobel, 1995; Görlich *et al.*, 1996b). The inactivation of the Ran binding site in importin-β prevents termination, but not translocation, i.e. transport intermediates accumulate in the nuclear baskets of the NPC and are not released into the nucleoplasm (Görlich *et al.*, 1996b). This indicates that RanGTP normally binds to importin-β only in the nucleus. The two subunits of importin are probably returned separately from the nucleus back to the cytoplasm.

NLS-mediated nuclear protein import requires cytoplasmic RanGDP and, as discussed above, seems to depend on nuclear RanGTP (Görlich *et al.*, 1996b). This obligatory gradient in Ran's nucleotide binding state is generated by the nuclear GDP/GTP exchange factor for Ran, RCC1, and cytoplasmic RanGAP1 (Rna1p), the Ran-specific GTPase activating protein (Ohtsubo *et al.*, 1989; Hopper *et al.*, 1990; Bischoff and Ponstingl, 1991; Melchior *et al.*, 1993b; Bischoff *et al.*, 1994, 1995a; Corbett *et al.*, 1995). Low cytoplasmic RanGTP levels allow importin-α to bind importin-β, the high nuclear RanGTP concentration favours importin heterodimer dissociation in the nucleus. The RanGTP gradient thereby determines from which compartment the importin heterodimer can transport an NLS substrate.

In addition to the role of Ran in defining nuclear and cytoplasmic identity, Ran's GTP cycle probably also drives the translocation through the NPC. To achieve this, Ran would have to interact with components distinct from importin-β. Nuclear protein import requires cytoplasmic RanGDP (Görlich *et al.*, 1996b) and GTP hydrolysis by Ran (Melchior *et al.*, 1993a; Moore and Blobel, 1993). This, together with the observation that RanGDP binds to the NPC, suggests that translocation involves GDP/GTP exchange and GTP hydrolysis by Ran that is bound to the NPC. However, it is still unclear how the GTP cycle on NPC-bound Ran would translate into a directed movement.

Importin-β consists of 876 amino acids and interacts with the NPC, importin-α and Ran. We have determined the domains required for these interactions: residues 1–364 are needed for high-affinity Ran binding, and residues 331–876 for importin-α binding. Release of importin-α upon RanGTP binding to importin-β (Rexach and Blobel, 1995; Görlich *et al.*, 1996b) is therefore probably explained by a conformational switch in importin-β. The NPC binding of importin-β survives deletion from either terminus and involves more than one interaction site. The 45–462 importin-β fragment binds to the NPC but does not interact with either importin-α or Ran. It has only a small effect on import substrate docking to the cytoplasmic side of the NPC, but blocks sites used at later import steps very efficiently. It therefore appears to bind irreversibly to that subset of sites at the NPC from which importin-β is normally released as an importin-β–RanGTP complex. This is consistent with a model for termination in which RanGTP binding to importin-β not only disassembles the importin heterodimer, but also releases importin-β from the NPC before re-export can occur. The 45–462 importin-β fragment efficiently blocks nucleocytoplasmic transport of several substrates, including hnRNP A1 M9-mediated import and the export of the NES-containing Rev protein, mRNA and U snRNA, but it only weakly blocks export of tRNA. This could indicate that importin-β binds to sites at the NPC which are shared with mediators of other transport pathways, i.e. that different nucleocytoplasmic transport pathways converge at the level of NPC binding. Alternatively, or in addition, it is also possible that a nuclear pore complex has to be cleared of one cargo before the next can pass the central transporter. The cross-competition of importin-β derivatives with other pathways could thus also be due to this 'traffic control'.

Results

Importin-β function requires intact N- and C-termini

Importin-β is a key component of nuclear protein import. In order to support the import reaction it has to interact with at least three components: importin-α, the nuclear pore complex and Ran. A more detailed molecular dissection of the import reaction requires knowledge of which part of importin-β accounts for which interaction. To approach the problem, we first tested bacterially expressed recombinant importin-β fragments for their ability to support complete import. Permeabilized HeLa cells were incubated with Ran, an energy regenerating system, fluorescent nucleoplasmin as the import substrate, importin-α, and full-length or truncated forms of importin-β. The reactions were stopped by fixation and import analysed by confocal fluorescence microscopy. The upper panel of Figure 1 shows that full-length importin-β supports import. No nuclear accumulation of nucleoplasmin occurs if full-length importin-β was replaced by truncated fragments lacking either the N- or the C-terminus.

The deletion of the first 44 residues of importin-β arrests import intermediates at the nuclear envelope and gives rise to the punctate, ring-like pattern typical for nuclear pore complexes. This confirms our previous report that 45–876 importin-β (ΔN44) is able to bind to importin-α and to the NPC (Görlich *et al.*, 1996b). It should be noted that deletion of only the N-terminal 32 or 10 amino acids results in a similar phenotype. However, the block is not as tight as for the deletion of the first 44 amino acids (not shown, but see below). An importin-β fragment lacking the N-terminal 330 residues still allowed importin-α binding and some NPC binding (Figure 1). However, efficient targeting of nucleoplasmin to the NPC requires a ~10-fold higher concentration of this fragment compared with wild-type protein. This lower targeting efficiency is mainly due to a reduced affinity for the NPC (not shown). Further N-terminal deletions could not be analysed as the

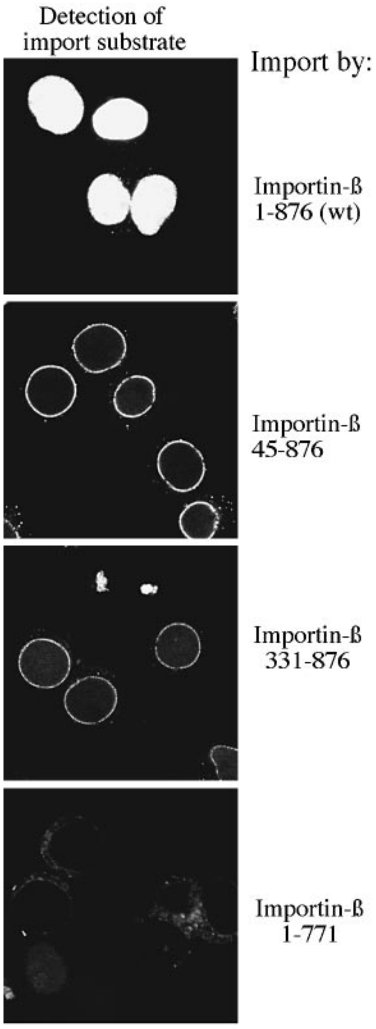

Fig. 1. Nuclear import function of importin-β requires intact N- and C-termini. The import activities of truncated forms of importin-β were compared with the wild-type protein (wt). Importin-β derivatives were added to 1 μM; importin-α to 3 μM; the substrate, fluorescein-labelled nucleoplasmin, was present at 3 μM (pentamers). Nucleoplasmin, importin-α and indicated importin-β derivatives were premixed. All reactions contained Ran, RanBP1, Rna1p, NTF2 and an energy regenerating system (see Materials and methods). Incubations with permeabilized HeLa cells were for 10 min at 23°C. Panels show confocal sections through the fixed nuclei.

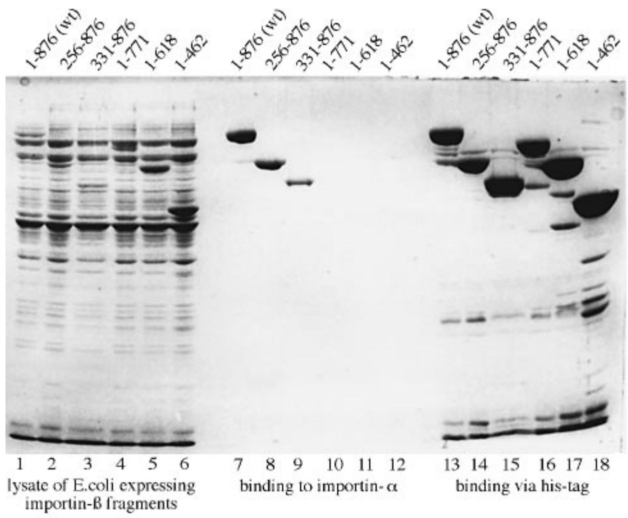

Fig. 2. The C-terminus of importin-β is essential for importin-α binding. Wild-type importin-β and indicated importin-β fragments were expressed with C-terminal his-tags in *E.coli*, and tested for their capacity to bind importin-α. The Coomassie-stained SDS gel shows the total lysates (lanes 1–6), the fractions that bind to importin-α (lanes 7–12) and as a positive control the fractions that bind to nickel agarose (lanes 13–18). Importin-α binding was monitored using an immobilized IBB domain, the importin-β binding domain in importin-α. The IBB domain (residues 1–55 of importin-α) was fused C-terminally to a z domain (IgG binding domain of protein A) and was pre-bound to IgG–Sepharose. Binding was in 50 mM Tris–HCl, pH 7.5, 350 mM NaCl, 1 mM mercaptoethanol. Elution was performed with 1 M $MgCl_2$, 50 mM Tris–HCl, pH 7.5. Load in the bound fractions corresponds to 20-fold that in the total lysates.

resulting fragments did not fold and were insoluble when expressed in *Escherichia coli*.

The phenotypes of C-terminal deletions were fundamentally different. If importin-β lacked the C-terminal 106 residues, the fluorescent signal in the confocal section showed only non-specific binding to the cytoplasmic remnants of the permeabilized cells (Figure 1). The fragment thus fails to target the import substrate to the NPC. This could be due to a failure to interact with either importin-α or the NPC, or both.

The importin-α binding site

To distinguish between the possibilities, we tested directly the importin-α binding of importin-β fragments (Figure 2). First, lysates were prepared from *E.coli* expressing the indicated importin-β derivatives (lanes 1–6). The fragments were then allowed to bind to an immobilized IBB domain (the importin-β binding domain of importin-α) or, as a control, to nickel agarose to which all the fragments bind by virtue of their his-tag.

Wild-type importin-β binds efficiently to the IBB domain, as reported previously. In spite of the very basic nature of the IBB domain, this interaction is highly specific as only importin-β and virtually none of the *E.coli* proteins was recovered in the bound fractions. Importin-α binding was still retained if the N-terminal 255 or 330 amino acids of importin-β were deleted. Importin-β 331–876, however, binds less strongly than the wild-type protein. If the ionic strength was raised from 350 mM NaCl (as in Figure 2) to 650 mM, binding of the wild-type or 256–876 importin-β remained unaffected, but binding of 331–876 was severely reduced (not shown).

Even the importin-β derivative with the smallest C-terminal deletion tested, importin-β 1–771, fails to bind importin-α (Figure 2, compare lanes 4, 10 and 16). This explains the failure of this fragment to target import substrates to the NPC (Figure 1, bottom panel). Thus, it is approximately residues 331–876 of importin-β that are required for binding to importin-α.

Characterization of the NPC binding domain in importin-β

To set up an assay for NPC binding of importin-β derivatives, full-length and truncated forms of importin-β were expressed in *E.coli*, purified and modified with fluorescein 5′-maleimide such that, on average, a single fluorescein was attached per molecule. The fluorescent signal from these modified fragments is thus a direct measure of their local concentration. We could therefore use confocal fluorescence microscopy to compare the

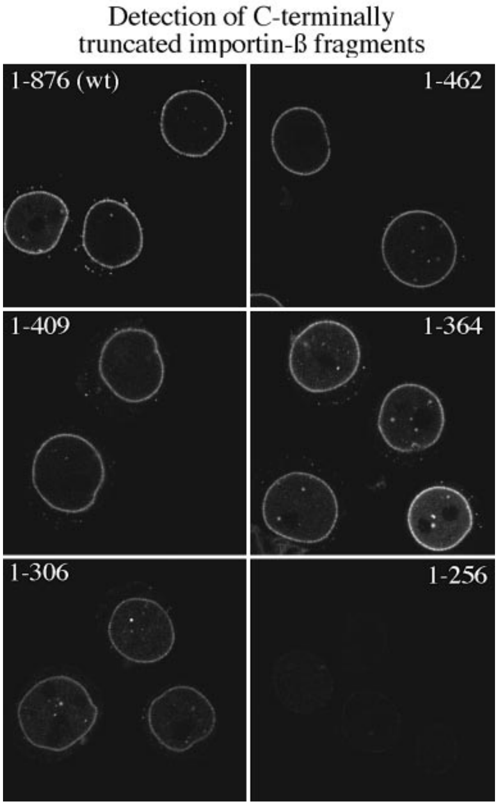

Fig. 3. Sequence requirements for importin-β binding to the NPC. 200 nM of each fluorescein-labelled wild-type importin-β or C-terminal truncations was incubated with permeabilized cells. Ran and an energy regenerating system was present, but no importin-α. Nuclear pore binding of the importin-β derivatives was detected after fixation by confocal microscopy.

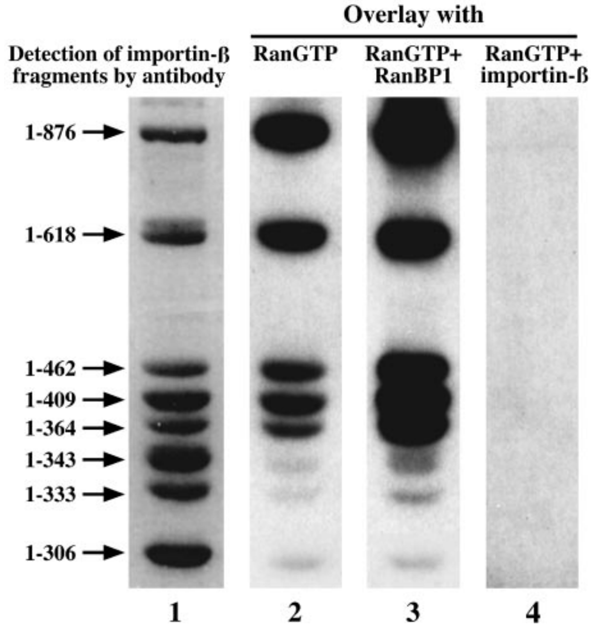

Fig. 4. The Ran binding domain of importin-β. The mixture of indicated importin-β fragments (numbers refer to amino acid residues) was separated by SDS–PAGE, transferred onto nitrocellulose and analysed in four ways. Lane 1, the amounts of transferred fragments was calibrated by Western blotting with an antibody against a peptide present in all fragments. Lane 2, Ran binding was monitored by probing the blot with Ran [γ-^{32}P]GTP followed by autoradiography. As controls, Ran [γ-^{32}P]GTP was pre-incubated with a 5000-fold excess of either RanBP1 (lane 3) or importin-β (lane 4). The specific signal is enhanced by RanBP1 but extinguished by importin-β.

efficiency with which different importin-β fragments bind to NPCs of permeabilized cells. We first noted that the capacity of the NPC to bind importin-β became considerably higher if Ran and an energy regenerating system were added (not shown). There are probably two contributions to this effect. First, translocation allows importin-β to occupy additional sites further inside the NPC. Second, translocation likely clears endogenous (unlabelled) importin-β from NPCs, making the sites available for the fluorescent proteins. In Figure 3, the NPC binding of some importin-β fragments under these conditions is shown. The shortest C-terminally truncated fragment with detectable NPC binding is importin-β 1–306. However, under more stringent conditions, i.e. a lower concentration of the fluorescent proteins, the gradual decrease in NPC binding from importin-β 1–618 to the 1–306 fragment was evident (not shown). In conclusion, it is approximately residues 1–618 of importin-β that are required for full NPC binding activity. Some binding was still observed for the N-terminal 306 residues (Figure 3), but also for amino acids 331–876 (Figure 1). This suggests that at least two separate sites of NPC interaction exist on importin-β.

Ran interacts with the N-terminus of importin-β

To determine which part of importin-β accounts for Ran binding, we separated a mixture of C-terminal importin-β truncations by SDS gel electrophoresis and transferred the fragments onto nitrocellulose. The amounts of the fragments were normalized by Western blotting with an antibody raised against a peptide present in all fragments (Figure 4). Ran interaction was then detected in an overlay blot with Ran [γ-^{32}P]GTP as a probe. As seen from Figure 4, all importin-β fragments down to 1–364 showed significant Ran interaction. However, the signal is essentially lost by the deletion of a further 21 amino acids. Thus, the C-terminal border of the Ran binding domain appears to be between residues 343 and 364.

Probing the blot with Ran [γ-^{32}P]GTP in the presence of a large excess of RanBP1 (Coutavas *et al.*, 1993; Bischoff *et al.*, 1995b; Schlenstedt *et al.*, 1995) resulted in an enhancement of the specific signal, as reported before (Lounsbury *et al.*, 1996). Thus, RanBP1 and importin-β do not compete for the same binding site on Ran. The amplification of the signal is probably due to the stabilization of RanGTP by RanBP1 and to cooperativity that favours the formation of the trimeric RanBP1–RanGTP–importin-β complex. This trimeric complex is also detectable by gel filtration and by affinity chromatography on immobilized RanBP1 (not shown). As expected, RanGTP binding to the importin-β fragments is competed by importin-β itself. It should be noted that the N-terminus of importin-β is absolutely crucial for interaction with Ran. No Ran binding is detectable in the overlay assay if as little as the 10 N-terminal amino acids are deleted (not shown, but see Görlich *et al.*, 1996b and below).

The overlay blot measures two effects. First, renaturation of the blotted proteins and second, the presence of the Ran binding domain. To quantify Ran binding more precisely and independently of refolding, we employed an

Table I. Properties of the importin-β fragments

Importin-β fragment	Supports import?	Affinity for RanGTP (wt = 100)	Importin-α binding	NPC binding	Remarks
1–876	yes	100	yes	+++	full-length
11–876	partially	10	yes	+++	
33–876	hardly	6	yes	+++	
45–876	no	0.3	yes	++++[a]	
256–876	no	0	yes	++	
331–876	no	0	weaker than wt	+	
347–876	–	–	–	–	no folding in *E.coli*
1–771	no	40	no	+++	
1–618	no	40	no	+++	
1–462	no	95	no	++	
45–462	no	0	no	++++[a]	
1–409	no	65	no	++	
1–364	no	33	no	++	
1–343	no	0.04	no	–/+	
1–331	no	0.06	no	+	
1–306	no	0.01	no	+	
1–256	no	0	no	–	

[a]Bind irreversibly to the Ran-dependent sites at the NPC.

enzymatic assay: binding of importin-β protects RanGTP against GTPase activation by RanGAP1(Rna1p) (Floer and Blobel, 1996). The proportion of protected RanGTP can be measured and we have calculated the binding constants from the dose dependence of protection. Table I shows relative affinities for the importin-β fragments. The affinity of the wild-type protein was set to 100. The numbers confirm residues 1–364 as the Ran binding domain. There is a sharp transition at its C-terminal border. The Ran affinity of importin-β 1–343 is three orders of magnitude lower than that of 1–364. That the 1–771 and 1–618 fragments have a slightly lower affinity for Ran than importin-β 1–462 is probably explained by a poorly folded C-terminus of the longer fragments influencing the folding of the Ran binding domain.

Deletion of as few as 10 amino acids from the importin-β N-terminus results in a 10-fold drop in affinity for Ran, consistent with our observation that the import activity of this mutant is severely affected. Inhibition of importin-β transport activity is complete if the first 44 amino acids are deleted (Figure 1).

Importin-β is released from a subset of NPC binding sites as a complex with RanGTP

The Ran binding site and the nuclear pore binding site overlap in importin-β. This might indicate that RanGTP binding and binding to the NPC influence one another. However, at least three different classes of importin-β binding sites at the NPC might exist, that show different effects of RanGTP. First, as importin-β does not have to bind Ran on its way into the nucleus (Görlich *et al.*, 1996b), there have to be sites at which normally no interaction between Ran and importin-β must occur. Second, the termination sites are the sites where importin-β interaction with RanGTP releases importin-α. At termination, importin-β could either remain NPC-bound and return directly from the termination site to the cytoplasm, or alternatively, importin-β could be detached from the NPC before re-export. These two alternatives are tested below. Third, if we assume that cytoplasmic RanGAP1 is one factor required for the disassembly of the importin-β–RanGTP complex, then the importin-β–RanGTP complex has to be returned to the cytoplasm and has to bind to the NPC on its way out of the nucleus.

Different effects of the Ran–importin-β interaction on importin-β complexes with isolated nucleoporins have indeed been observed: RanGTP releases importin-β from the yeast nucleoporin Nup1p (Rexach and Blobel, 1995). Likewise, a similar effect has been seen for the p62–importin-β interaction in gel filtration experiments (F.R. Bischoff and V.Cordes, unpublished results). On the other hand, importin-β can simultaneously bind RanGTP and certain nuclear pore proteins, because a number of nucleoporins are detectable on overlay blots using a complex between Ran [γ-^{32}P]GTP and importin-β as a probe (Lounsbury *et al.*, 1996; our unpublished data). However, in all these studies it is unclear if binding sites found in isolated or partially denatured nucleoporins are accessible and functional in intact nuclear pore complexes.

Looking at intact nuclear pore complexes, we wanted to know if the termination reaction would transiently release importin-β from the NPC. Mutant forms of importin-β which do not bind Ran, should, in this case, resist the normal release mechanism and bind to the termination sites irreversibly. We have tested this in two sets of experiments, the first of which is shown in Figure 5. Permeabilized cells were incubated in *Xenopus* egg extract together with an energy regenerating system, and NPC binding of three fluorescent importin-β derivatives was tested. The fluorescent proteins were added to a concentration of 1 μM and had to compete with ~3 μM unlabelled, endogenous wild-type importin-β present in the extract.

Without further addition, the binding of the importin-β 1–462 fragment was reduced compared with that of the wild-type protein, probably because the fragment does not contain the entire NPC binding site. The binding of importin-β 45–462 (which does not bind Ran) was somewhat heterogeneous; on average, however, it was ~2-fold stronger than that of the full-length protein.

These differences became striking in the presence of 10 μM RanQ69L, a Ran mutant which is GTPase-deficient

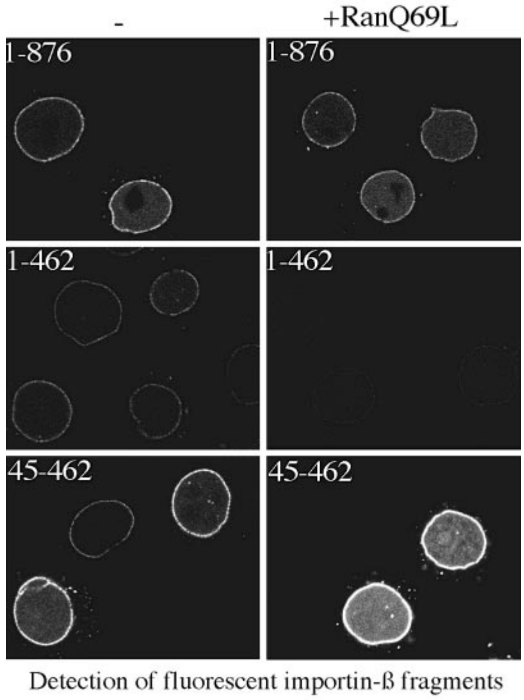

Fig. 5. Effects of RanQ69L GTP on the importin-β interaction with the NPC. Permeabilized cells were incubated in *Xenopus* egg extract with an energy regenerating system. 10 μM RanQ69L GTP was present in the right-hand panels. After 2 min, 1 μM of each indicated fluorescent importin-β fragment was added. After another 15 min, the reaction was stopped by fixation and NPC binding was monitored by confocal fluorescence microscopy. Note that the NPC binding of importin-β 45–462 in the presence of RanQ69L is underestimated because the signal was off scale at the settings required to detect the other signals.

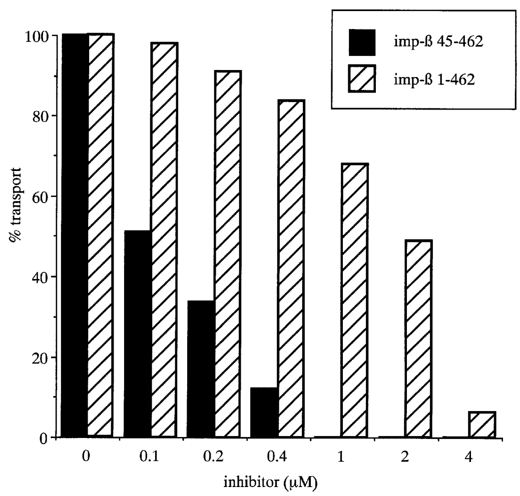

Fig. 6. Dose dependence of import inhibition by importin-β 45–462. Quantitation of import of a BSA–NLS conjugate. Import without inhibitor was set to 100. Numbers refer to import in the presence of the indicated concentrations of the importin-β fragments. Permeabilized cells were incubated with *Xenopus* egg extract at 19°C in the presence of an energy regenerating system; 10 min later, buffer or importin-β fragments were added and after another 10 min the import reaction was started by the addition of rhodamine-labelled BSA–NLS conjugate. The reaction was stopped after 15 min by fixation and nuclei were spun onto coverslips. Fluorescence intensities were recorded with a fluorescence microscope equipped with a CCD camera and mean nuclear accumulation of the import substrate was calculated using IPlab software (version 3.0).

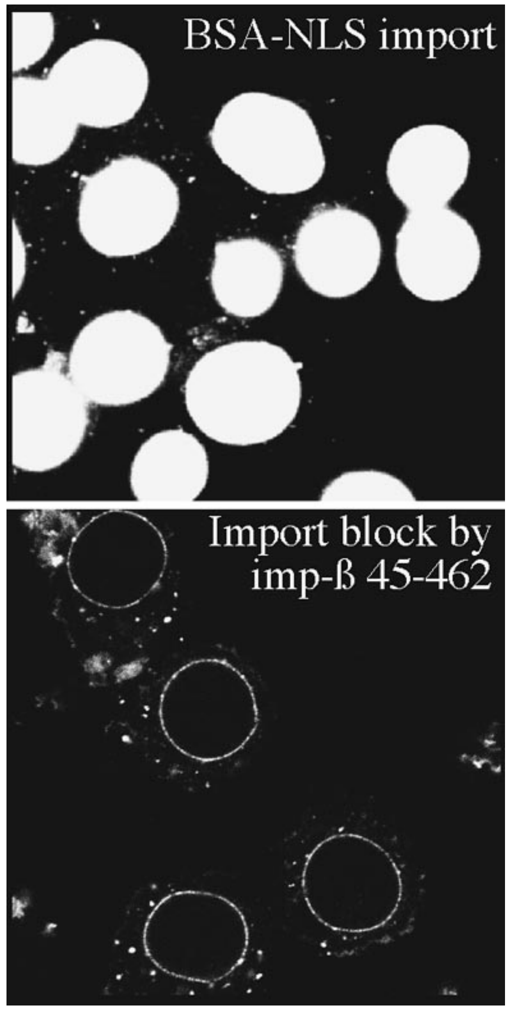

Fig. 7. Importin-β 45–462 inhibits protein import in a dominant-negative way. Permeabilized cells were pre-incubated with a *Xenopus* egg extract and an energy regenerating system; 10 min later either buffer (upper panel) or 1 μM 45–462 importin-β fragment (lower panel) was added. After another 10 min, fluorescent BSA–NLS conjugate was added and import allowed for 20 min at 19°C. The reaction was stopped by fixation, the nuclei were spun onto coverslips and viewed by confocal microscopy. Note that, in the presence of importin-β 45–462, the import substrate did not accumulate inside the nuclei, but at the nuclear envelope.

and therefore fixed in its GTP-bound form (Klebe *et al.*, 1995). This concentration of RanQ69L dissociates the importin heterodimer in the extract completely (not shown, but see Görlich *et al.*, 1996b), i.e. most importin-β should be complexed with RanGTP. RanQ69L addition caused only a slight decrease in NPC binding of wild-type importin-β, consistent with the assumption that the importin-β–RanGTP complex can bind to at least some sites at the NPC (perhaps those on the export route). Binding of 1–462 was severely reduced by addition of the Ran mutant, demonstrating that RanGTP can release importin-β from at least some sites at the NPC. In contrast, binding of 45–462 was enhanced, probably because the fragment could now occupy sites from which RanQ69L GTP has cleared endogenous importin-β and related transport factors.

Taken together, two possible conclusions are suggested from this experiment. First, importin-β is released as an importin-β–RanGTP complex from a subset of sites at

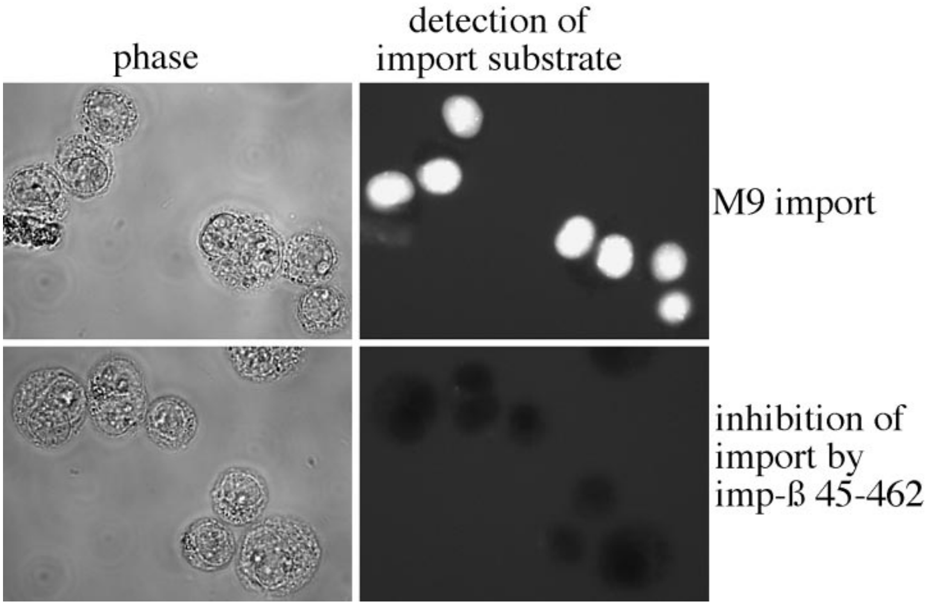

Fig. 8. Block of M9 import by 45–462 importin-β. The upper panels show nuclear import of a fluorescent nucleoplasmin core–M9 fusion protein in the presence of an energy regenerating system and HeLa cell extract. In the lower panels, 45–462 importin-β was added to 3 μM. Analysis was by phase contrast (phase, left panels) and by conventional fluorescence microscopy detecting the M9 import substrate (right panels).

the NPC, probably the termination sites. Second, the importin-β 45–462 fragment, as a consequence of its lack of response to Ran, binds to these sites much more stably.

Inhibition of NLS import by importin-β dominant-negatives

Both the 1–462 and 45–462 importin-β fragments bind to the NPC but not to importin-α. They should therefore inhibit protein import in a dominant-negative way. Figure 6 shows the dose dependence of the inhibition. Import of fluorescent BSA–NLS conjugate into the nuclei of permeabilized cells was measured in the presence of an energy regenerating system and a *Xenopus* egg extract which contains ~3 μM endogenous importin-β. A 50% inhibition was observed with a 2 μM concentration of the 1–462 fragment. Importin-β 45–462 is a far more potent inhibitor, a concentration of only 0.1 μM being required for 50% inhibition. The import block was complete at 1 μM (see Figures 6 and 7), a finding consistent with the assumption that importin-β 45–462 binds to sites at the NPC from which it cannot be cleared. Confocal microscopy revealed that the initial docking of the import substrate to nuclear pores was not yet affected at this concentration of the 45–462 fragment (Figure 7). This is consistent with the assumption that the 45–462 fragment binds irreversibly only to sites far inside the NPC where termination normally occurs and where RanGTP interaction is required to detach importin-β from the NPC (see also Görlich *et al.*, 1996b).

Importin-β dominant-negatives block major nucleocytoplasmic transport pathways

Next we wanted to know if the importin-β dominant-negatives inhibit only the importin-dependent pathway or if there is cross-competition between different pathways. Figure 8 shows nuclear import of a fusion protein composed of the nucleoplasmin core and an M9 domain, the nuclear targeting signal of hnRNP A1 (Michael *et al.*, 1995; see Introduction). The panels are views on unfixed import mixtures, i.e. nuclear and cytoplasmic concentrations of the fluorescent fusion protein are directly shown. Without an inhibitor, a clear nuclear accumulation is observed. If, however, the dominant-negative importin-β 45–462 was added, import was completely abolished and the probe remained excluded from the nuclei. It should be noted that wild-type importin-β at higher concentrations also competitively inhibits M9-mediated import (not shown).

To test whether export out of the nucleus would also be affected, we performed micro-injection experiments into *Xenopus* oocytes. Initial experiment established that the importin-β dominant-negatives block nuclear export of the Rev protein with the same efficiency as they inhibit NLS- and M9-dependent protein import (not shown). In Figure 9A we tested the effects on nuclear export of DHFR and histone mRNA, U1 and U5 snRNA, and a tRNA. As a control for nuclear integrity, the mixture also contained U6 snRNA, an RNA which is not exported from the nucleus. The RNAs were co-injected into the nucleus together with buffer, wild-type importin-β, the 1–462 fragment or the 45–462 fragment. Full-length importin-β left tRNA export largely unaffected, but significantly inhibited the export of mRNA and U snRNA. When importin-β is injected into the nucleus, it is rapidly exported (not shown) and importin-β re-export to the cytoplasm probably competes with mediators of mRNA and U snRNA export for binding sites at the NPC. The injection of importin-β 1–462 at this concentration had less effect on RNA export than full-length importin-β, probably because the affinity of this fragment for the NPC is lower than that of wild-type importin-β. Co-injection of 45–462, however, caused a complete inhibition of mRNA and U snRNA export. The concentration needed to block export is similar to that required to prevent NLS-dependent protein import into oocyte nuclei (not shown). At this concentration (5.8 μM in the injected sample)

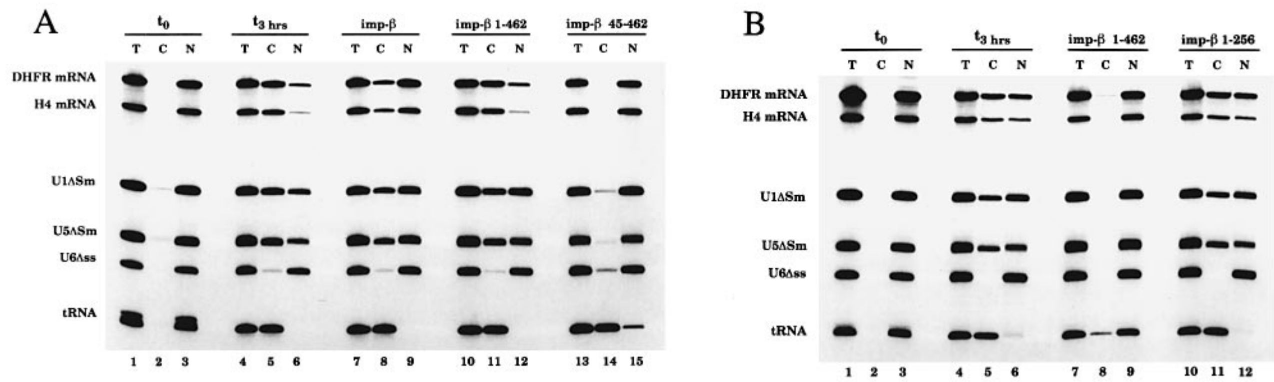

Fig. 9. Inhibition of RNA nuclear export by importin-β mutants. (**A**) The dominant-negative importin-β mutant 45–462 inhibits RNA nuclear export. Buffer or the indicated importin-β fragments were injected into oocyte nuclei together with a mixture of the following radioactively labelled RNAs: DHFR mRNA, histone H4 mRNA, U1ΔSm, U5ΔSm, U6Δss and human initiator methionyl tRNA. U6Δss does not leave the nucleus and is an internal control for nuclear integrity. Synthesis of DHFR, histone H4, U1ΔSm and U5ΔSm RNAs was primed with the m^7GpppG cap dinucleotide, whereas synthesis of U6Δss RNA was primed with γ-mGTP. The concentration of the importin-β fragments in the injected samples was 5.8 μM. Approximately 15 nl were injected, which compares with ~125 nl nuclear volume and 1000 nl total volume of an oocyte. In lanes 4–15, RNA was extracted 180 min after injection, in lanes 1–3, RNA was extracted immediately after injection. 'T', 'C' and 'N' labels above the lanes indicate RNA extracted from total oocytes, or, after dissection, from cytoplasmic or nuclear fractions, respectively. (**B**) Inhibition of RNA nuclear export by high concentrations of importin-β 1–462. *Xenopus laevis* oocytes were pre-injected with the importin-β fragments indicated above the lanes, followed 1 h later by nuclear injection of the mixture of radioactively labelled RNAs described in (A). The concentration of the importin-β fragments in the injected samples was 50 μM. The injected volume was ~30 nl, corresponding to about 1/30 of the volume of an oocyte. In lanes 4–12, RNA was extracted 180 min after injection; in lanes 1–3, RNA was extracted immediately after injection.

importin-β 45–462 also reduced tRNA export to some extent.

To determine whether a higher concentration of the importin-β mutants would have a greater effect on tRNA export, we injected importin-β 1–462 and, as a control 1–256, at 50 μM. Importin-β 1–256 does not interact with Ran, importin-α or the NPC, and is therefore a good negative control. The 45–462 fragment at this concentration was toxic to the oocytes, and could therefore not be used. While 1–256 had no effect on RNA export, even at this high concentration (Figure 9B, compare lanes 4–6 with lanes 10–12) the 1–462 fragment blocked mRNA, U snRNA and tRNA export completely (lanes 7–9). Thus, the dominant-negative importin-β mutants have a general impact on many distinct nucleocytoplasmic transport pathways. For the block of tRNA export by high concentrations of importin-β 1–462 it is not yet clear if the inhibition is due to a competition for NPC binding or because importin-β 1–462 titrates free RanGTP, for which it has a high affinity. We are currently testing these possibilities.

Discussion

We have mapped domains in importin-β which interact with Ran, importin-α and with the nuclear pore complex. A region of the protein encompassing approximately amino acids 331–876 accounts for interaction with importin-α and residues 1–364 account for RanGTP binding, whereas NPC binding appears complex and can be mediated by at least two non-overlapping sites on importin-β.

RanGTP dissociates importin-α from importin-β. This could be explained in two ways. First, Ran and importin-α could compete for the same binding site at importin-β. In this model, dissociation of the importin heterodimer would have to occur spontaneously and RanGTP would prevent re-binding of importin-α. Second, RanGTP binding to importin-β could cause a conformational change in importin-β that releases the alpha subunit. In this model, RanGTP could force the importin heterodimer to dissociate. Our observation of largely non-overlapping sequence requirements for Ran and importin-α binding suggests that Ran binding to importin-β causes a conformational change that inactivates the importin-α binding site located elsewhere on the molecule. Such an active displacement can of course be much more efficient than simple competition for re-binding after spontaneous dissociation. A previous study of the binding sites on importin-β for RanGTP and importin-α, based on the use of a single mutant β-protein and on competition assays with short peptides derived from β, reached the opposite conclusion as to the relative orientation of the binding sites on β for the two proteins (Moroianu *et al.*, 1996). Although we have no good explanation for this discrepancy, we feel the methods used in our study, and the larger numbers of different mutants and binding assays employed, allow us to define the regions required for the specific protein–protein interactions with some confidence.

Importin-β mediates translocation into the nucleus and makes contact with the nuclear pore complex. This translocation involves binding to a number of intermediate sites on the NPC (Görlich *et al.*, 1996b; Panté and Aebi, 1996). As discussed previously (Görlich and Mattaj, 1996) a directed transfer from one site to the next, without intermediate detachment from the nuclear pore complex, would require more than one NPC binding site within importin-β. Our observation that the N-terminal 306 residues are sufficient for NPC binding and that importin-β still binds the NPC without the first 330 amino acids indicates that distinct NPC binding sites on importin-β exist. These sites could potentially be used independently and possibly alternately to move from one NPC site to the next. We are currently using the different importin-β fragments to determine which parts of the NPC binding sites are required for the actual translocation reaction.

Ran has probably two distinct functions in nuclear protein import. First, Ran's GTP cycle probably drives translocation of the NLS–importin-α/β complex into the nucleus. This appears to involve binding of RanGDP to the NPC, followed by nucleotide exchange and GTP hydrolysis, but not binding of RanGTP to importin-β (Görlich *et al.*, 1996b). Second, Ran regulates the interaction between importin-α and importin-β. It is the binding of nuclear RanGTP to importin-β that finally terminates the import reaction and disassembles the importin heterodimer. There are indications to suggest that importin-β might return to the cytoplasm as a complex with RanGTP. First, importin-β is probably recycled to the cytoplasm without the alpha subunit. Re-export of importin-β complexed with RanGTP would explain why. Second, although the importin-β–RanGTP complex forms in the nucleus, its disassembly is likely to be a cytoplasmic event as it requires two cytoplasmic proteins, RanGAP1 and RanBP1, plus an auxiliary factor (F.R.Bischoff, unpublished results). In addition, our observation that RanGTP causes the release of importin-β from only a subset of sites at the NPC implies that the importin-β–RanGTP complex could be a species that moves through the NPC.

Here, we provide evidence that termination, i.e. dissociation of the importin heterodimer on the nuclear side of the NPC, also involves at least a transient release of importin-β from the nuclear pore complex. All importin-β fragments which have retained nuclear pore binding but do not interact with either Ran or importin-α, inhibit protein import in a dominant-negative way. Those fragments, however, which have lost Ran binding (e.g. importin-β 45–876 and 45–462) are by far the most potent inhibitors. Since mutants that do not bind RanGTP can be visualized on the nuclear face of the NPC (Görlich *et al.*, 1996b) they probably bind to the sites of termination such that release by the normal mechanism via interaction with nuclear RanGTP is not possible.

The importin-β dominant-negatives not only block NLS-dependent import, but with the same efficiency they also block other pathways, namely M9 import, NES-dependent nuclear export, and export of mRNAs, U snRNAs and, though less efficiently, tRNAs. Aside from the export of U snRNAs (Görlich *et al.*, 1996c; Izaurralde *et al.*, 1997) none of these pathways is affected by inhibition of NLS-mediated protein import with saturating amounts of NLS conjugates or IBB domain (Görlich *et al.*, 1996c; Pollard *et al.*, 1996; Izaurralde *et al.*, 1997). Thus, each is mediated by a saturable receptor distinct from importin α/β. The fact that the importin-β mutants block these transport events in spite of this may be explained in two ways. First, although different transporter molecules bind different substrates they might take a similar path through the NPC and function according to similar principles. This is supported by the recent identification of the M9 import receptor, transportin, which is distantly related to importin-β (Pollard *et al.*, 1996). The cross-competition could indicate that at least some of the intermediate binding sites on the NPC are shared by the distinct transport mediators. Second, the cross-inhibition might be a reflection of how the NPC coordinates trafficking between nucleus and cytoplasm. For example, when a large RNP is being exported, then incoming particles may have to be excluded until the central transporter of the NPC has been cleared. The information that a cargo is not yet released from a NPC might be transmitted to more distant parts of the NPC, preventing further transport substrates from entering the same NPC.

Importin-β is at the heart of the NLS-mediated import pathway. As such, importin-β has to interact with multiple factors involved in transport and to help coordinate these factors to orchestrate directed movement of NLS-containing proteins to the nucleus from the cytoplasm. Our results cast light on how importin-β achieves these multiple interactions, and the effects of the dominant mutants suggest that other transport mediators will share at least some of these properties.

Materials and methods

Import assays with permeabilized HeLa cells

The basic method was described previously (Görlich *et al.*, 1994, 1996a,b). Unless indicated otherwise, reactions were performed at 23°C and contained an energy regenerating system, i.e. 10 mM creatine phosphate, 0.5 mM ATP, 0.5 mM GTP, 50 μg/ml creatine kinase. When using *Xenopus* egg extracts (LSS) salts were not adjusted. When using hypotonic HeLa cell extract the ionic conditions were: 20 mM HEPES–KOH, pH 7.5, 140 mM potassium acetate, 5 mM magnesium acetate, 1 mM dithiothreitol (DTT), 250 mM sucrose. The standard assay based on recombinant import factors contained: 20 mM HEPES–KOH, pH 7.5, 80 mM potassium acetate, 4 mM magnesium acetate, 1 mM DTT, 250 mM sucrose, 1.5 μM Ran (GDP form), 150 nM RanBP1, 150 nM Rna1p, 150 nM pp15 and 2 mg/ml nucleoplasmin core to block non-specific binding of proteins. For the concentrations of the other components see the figure legends.

Oocyte injections

Oocyte injections and analysis of microinjected RNA by denaturing gel electrophoresis, autoradiography or phosphoimager analysis were performed as described (Jarmolowski *et al.*, 1994). The histone H4 mRNA was generated by transcription of the mRNA coding portion of the S3 clone (Meier *et al.*, 1989), which was amplified by PCR and inserted into the *Bam*HI site of the pBS-vector. The concentrations of the recombinant proteins in the injected samples are indicated in the figure legends.

Recombinant expression and protein purification

Preparation of the following proteins was as described: C-terminally his-tagged *Xenopus* importin-α, nucleoplasmin, nucleoplasmin core (Görlich *et al.*, 1994), untagged recombinant, human Ran, *Schizosaccharomyces pombe* Rna1p and murine RanBP1 (Bischoff *et al.*, 1995b). For import assays, Ran was expressed with an N-terminal his-tag, and purified on nickel agarose as described (Görlich *et al.*, 1994). The bound nucleotide was exchanged for GDP using the EDTA method and final purification achieved on Superdex 75 equilibrated in 50 mM potassium phosphate, pH 7.2, 0.5 mM magnesium acetate. *S.pombe* Rna1p was expressed as a fusion with a z domain on the N-terminus and a his-tag on the C-terminus. Purification was with nickel agarose and activity verified with an enzymatic GAP assay. NTF2 was expressed untagged. It was bound from the *E.coli* lysate (post-ribosomal supernatant) to Q Sepharose FF equilibrated in 50 mM Tris–HCl, pH 8.0, and eluted in the NaCl gradient at 230 mM NaCl. Final purification was on Superdex 75 where it eluted at a position expected for the homodimer. The M9 fusion was prepared as follows: a *Bam*HI–*Hin*dIII DNA fragment that codes for residues 255–320 of human hnRNP A1 and includes the M9 domain was generated by PCR. The fragment was then fused to the C-terminus of the nucleoplasmin core domain. Expression was from the pQE9 vector (Qiagen) with an N-terminal his tag. Fluorescein labelling of the purified fusion protein was with fluorescein 5′-maleimide.

Expression of importin-β fragments

Fragments were generated by PCR using Pwo proof-reading DNA polymerase and were cloned into the *Nco*I–*Bam*HI sites of pQE60. The constructs were verified by DNA sequencing. Expression was in the BLR/Rep4 *E.coli* strain at 17°C for 4 h. 2 mM PMSF was added immediately before the culture was chilled on ice. After centrifugation, the bacterial pellet was resuspended in 50 mM Tris–HCl, pH 7.5, 20 mM imidazole, 200 mM NaCl, 5 mM mercaptoethanol, and lysis was performed by sonication. The lysate was cleared by ultracentrifugation in a Ti50.2 rotor at 50 000 r.p.m. for 3 h and loaded overnight onto nickel agarose equilibrated in sonication buffer. The column was washed in the same buffer and eluted with a gradient ending at 400 mM imidazole. Peak fractions were pooled and buffer was exchanged for 50 mM Tris–HCl, 250 mM sucrose. The concentration of the fragments was determined by photometry at 280 nm using the molar extinction coefficient calculated from the amino acid composition (Edelhoch, 1967). For fluorescence labelling, fluorescein 5′-maleimide dissolved in dimethylformamide was added at an equimolar ratio to the protein and the mixture was incubated for 1 h on ice. The reaction was quenched with 50 mM mercaptoethanol and free label separated on a PD10 column equilibrated in 50 mM Tris–HCl, pH 7.5, 250 mM sucrose. Efficiency of labelling was close to 100% for all of the fragments.

Enzymatic assays

Labelling of Ran with [γ-^{32}P]GTP and GTPase assays were as described (Bischoff *et al.*, 1994, 1995b; Görlich *et al.*, 1996b). The reaction was performed in 20 mM HEPES–KOH, pH 7.4, 100 mM NaCl, 1 mM $MgCl_2$, 1 mM sodium azide, 0.05% hydrolysed gelatin. Ran was used at 60 pM, far below the K_d of the importin-β–RanGTP complex. Ran was pre-incubated for 30 min with the importin-β fragments before 2 nM Rna1p was added. After 5 min, GTP hydrolysis was measured as released γ-^{32}P-labelled phosphate. The dissociation constants of various importin-β derivatives from RanGTP was interpolated from dilution series as the concentration that protects 50% RanGTP against GTPase activation by Rna1p. The affinity for RanGTP is then inversely proportional to K_d. For convenience, the affinity of the wild-type protein was set to 100 in the listing shown in Table I. The values were found to be highly reproducible in three independent series of measurements and the averages are shown.

Overlay blots

The basic method followed a published protocol (Lounsbury *et al.*, 1996) with some modifications. Briefly, proteins were separated by SDS–PAGE and transferred onto nitrocellulose. The blot was then incubated for 1 h at 4°C in renaturation buffer containing 20 mM MOPS, pH 7.1, 100 mM sodium acetate, 5 mM magnesium acetate, 5 mM DTT, 0.5% bovine serum albumin, 0.05% Tween 20, and subsequently for 30 min at 25°C in renaturation buffer plus 100 μM unlabelled GTP (20 ml total volume). 10 min later, 100 μl 1 nM Ran [γ-^{32}P]GTP was added alone, or with 5 μM RanBP1 or with 5 μM importin-β. After another 10 min the blot was washed five times in renaturation buffer and subjected to autoradiography at –70°C.

Acknowledgements

We thank Petra Schwarzmaier for technical help, and Drs M.Dabrowski, R.A.Laskey and T.Sommer for critical reading of the manuscript. We are grateful to Drs E.Darzynkiewicz and J.Stepinski for the gift of the 7-methyl GpppG and gamma methyl GTP cap analogues, and D.Schümperli for the Histone H4 clone. This work was supported by the Deutsche Forschungsgemeinschaft (DFG).

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. and Gerace,L. (1991) Cytosolic proteins that specifically bind nuclear location signals are receptors for nuclear import. *Cell*, **66**, 837–847.

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.

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

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.

Bischoff,F.R., Krebber,H., Kempf,T., Hermes,I. and Ponstingl,H. (1995a) Human RanGTPase activating protein RanGAP1 is a homolog of yeast RNA1p involved in messenger RNA processing and transport. *Proc. Natl Acad. Sci. USA*, **92**, 1749–1753.

Bischoff,F.R., Krebber,H., Smirnova,E., Dong,W.H. and Ponstingl,H. (1995b) Coactivation of RanGTPase and inhibition of GTP dissociation by Ran GTP binding protein RanBP1. *EMBO J.*, **14**, 705–715.

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

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.

Corbett,A.H., Koepp,D.M., Schlenstedt,G., Lee,M.S., Hopper,A.K. and Silver,P.A. (1995) Rna1p, a Ran/TC4 GTPase activating protein, is required for nuclear import. *J. Cell Biol.*, **130**, 1017–1026.

Coutavas,E., Ren,M., Oppenheim,J.D., D'Eustachio,P. and Rush,M.G. (1993) Characterization of proteins that interact with the cell-cycle regulatory protein Ran/TC4. *Nature*, **366**, 585–587.

Dingwall,C. and Laskey,R.A. (1991) Nuclear targeting sequences – a consensus? *Trends Biochem. Sci.*, **16**, 478–481.

Edelhoch,H. (1967) Spectroscopic determination of tryptophan and tyrosine in proteins. *Biochemistry*, **6**, 1948–1954.

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.

Fischer,U., Huber,J., Boelens,W.C., Mattaj,I.W. and Lührmann,R. (1995) The HIV-1 Rev activation domain is a nuclear export signal that accesses an export pathway used by specific cellular RNAs. *Cell*, **82**, 475–483.

Floer,M. and Blobel,G. (1996) The nuclear transport factor karyopherin β binds stoichiometrically to Ran-GTP and inhibits the Ran GTPase activating protein. *J. Biol. Chem.*, **271**, 5313–5316.

Gerace,L. (1995) Nuclear export signals and the fast track to the cytoplasm. *Cell*, **82**, 341–344.

Görlich,D. and Mattaj,I.W. (1996) Nucleocytoplasmic transport. *Science*, **271**, 1513–1518.

Görlich,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.

Görlich,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.

Görlich,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.

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

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

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

Guddat,U., Bakken,A.H. and Pieler,T. (1990) Protein-mediated nuclear export of RNA: 5S rRNA containing small RNPs in *Xenopus* oocytes. *Cell*, **60**, 619–628.

Hopper,A.K., Traglia,H.M. and Dunst,R.W. (1990) The yeast RNA1 gene product necessary for RNA processing is located in the cytosol and apparently excluded from the nucleus. *J. Cell Biol.*, **111**, 309–321.

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., Shimamoto,T., Takao,T., Tachibana,T., Kose,S., Matsubae,M., Sekimoto,T., Shimonishi,Y. and Yoneda,Y. (1995b) *In vivo* evidence for involvement of a 58 kDa component of nuclear pore targeting complex in nuclear protein import. *EMBO J.*, **14**, 3617–3626.

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

Izaurralde,E. and Mattaj,I.W. (1995) RNA export. *Cell*, **81**, 153–159.

Izaurralde,E., Lewis,J., Gamberi,C., Jarmolowski,A., McGuigan,C. and Mattaj,I.W. (1995) A cap-binding protein complex mediating U snRNA export. *Nature*, **376**, 709–712.

Izaurralde,E., Jarmolowski,A., Beisel,C., Mattaj,I.W., Dreyfuss,G. and Fischer,U. (1997) A role for the M9 transport signal of hnRNP A1 in mRNA nuclear export. *J. Cell Biol.*, in press.

Jarmolowski,A., Boelens,W.C., Izaurralde,E. and Mattaj,I.W. (1994) Nuclear export of different classes of RNA is mediated by specific factors. *J. Cell Biol*., **124**, 627–635.

Klebe,C., Bischoff,F.R., Ponstingl,H. and Wittinghofer,A. (1995) Interaction of the nuclear GTP-binding protein Ran with its regulatory proteins RCC1 and RanGAP1. *Biochemistry*, **34**, 639–647.

Lounsbury,K.M., Richards,S.A., Perlungher,R.R. and Macara,I.G. (1996) Ran binding domains promote the interaction of ran with p97/beta-karyopherin, linking the docking and translocation steps of nuclear import. *J. Biol. Chem*., **271**, 2357–2360.

Meier,V.S., Bohni,R. and Schümperli,D. (1989) Nucleotide sequence of two mouse histone H4 genes. *Nucleic Acids Res*., **17**, 795.

Melchior,F., Paschal,B., Evans,E. and Gerace,L. (1993a) 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.

Melchior,F., Weber,K. and Gerke,V. (1993b) A functional homologue of the RNA1 gene product in *Schizosaccharomyces pombe*: purification, biochemical characterization, and identification of a leucine-rich repeat motif. *Mol. Biol. Cell*, **4**, 569–581.

Michael,W.M., Choi,M. and Dreyfuss,G. (1995) A nuclear export signal in hnRNP A1: a signal-mediated, temperature-dependent nuclear protein export pathway. *Cell*, **83**, 415–422.

Moore,M.S. and Blobel,G. (1992) The two steps of nuclear import, targeting to the nuclear envelope and translocation through the nuclear pore, require different cytosolic factors. *Cell*, **69**, 939–950.

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.

Moroianu,J., Hijikata,M., Blobel,G. and Radu,A. (1995) Mammalian karyopherin a1b and a2b heterodimers: a1 or a2 subunit binds nuclear localization sequence and b subunit interacts with peptide repeat containing nucleoporins. *Proc. Natl Acad. Sci. USA*, **92**, 6532–6536.

Moroianu,J., Blobel,G. and Radu,A. (1996) Nuclear protein import: Ran-GTP dissociates the karyopherin αβ heterodimer by displacing α from an overlapping binding site on β. *Proc. Natl Acad. Sci. USA*, **93**, 7059–7062.

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.

Panté,N. and Aebi,U. (1996) Sequential binding of import ligands to distinct nucleopore regions during their nuclear import. *Science*, **273**, 1729–1732.

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.

Piñol-Roma,S. and Dreyfuss,G. (1993) hnRNP proteins: localization and transport between the nucleus and the cytoplasm. *Trends Cell Biol*., **3**, 151–155.

Pokrywka,N.J. and Goldfarb,D.S. (1995) Nuclear export pathways of tRNA and 40 S ribosomes include both common and specific intermediates. *J. Biol. Chem*., **270**, 3619–3624.

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.

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.

Schlenstedt,G., Wong,D.H., Koepp,D.M. and Silver,P.A. (1995) Mutants in a yeast Ran binding protein are defective in nuclear transport. *EMBO J*., **14**, 5367–5378.

Weis,K., Mattaj,I.W. and Lamond,A.I. (1995) Identification of hSRP1α as a functional receptor for nuclear localization sequences. *Science*, **268**, 1049–1053.

Weis,K., Ryder,U. and Lamond,A.I. (1996) The conserved amino terminal domain of hSRP1α is essential for nuclear protein import. *EMBO J*., **15**, 1818–1825.

Wen,W., Meinkoth,J.L., Tsien,R.Y. and Taylor,S.S. (1995) Identification of a signal for rapid export of proteins from the nucleus. *Cell*, **82**, 463–473.

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

Received on September 24, 1996; revised on November 26, 1996