The EMBO Journal vol.15 no.20 pp.5584–5594, 1996

Identification of different roles for RanGDP and RanGTP in nuclear protein import

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The importin-α/β heterodimer and the GTPase Ran play key roles in nuclear protein import. Importin binds the nuclear localization signal (NLS). Translocation of the resulting import ligand complex through the nuclear pore complex (NPC) requires Ran and is terminated at the nucleoplasmic side by its disassembly. The principal GTP exchange factor for Ran is the nuclear protein RCC1, whereas the major RanGAP is cytoplasmic, predicting that nuclear Ran is mainly in the GTP form and cytoplasmic Ran is in the GDP-bound form. Here, we show that nuclear import depends on cytoplasmic RanGDP and free GTP, and that RanGDP binds to the NPC. Therefore, import might involve nucleotide exchange and GTP hydrolysis on NPC-bound Ran. RanGDP binding to the NPC is not mediated by the Ran binding sites of importin-β, suggesting that translocation is not driven from these sites. Consistently, a mutant importin-β deficient in Ran binding can deliver its cargo up to the nucleoplasmic side of the NPC. However, the mutant is unable to release the import substrate into the nucleoplasm. Thus, binding of nucleoplasmic RanGTP to importin-β probably triggers termination, i.e. the dissociation of importin-α from importin-β and the subsequent release of the import substrate into the nucleoplasm.

Keywords: GTPase/importin/nuclear import/Ran/RanGAP

Introduction

Nuclear protein import proceeds through the nuclear pore complexes (Feldherr *et al.*, 1984), is triggered by nuclear localization signals (NLSs) and is energy dependent (Dingwall *et al.*, 1982; Kalderon *et al.*, 1984; Newmeyer *et al.*, 1986; Newmeyer and Forbes, 1988; Richardson *et al.*, 1988; reviewed in Fabre and Hurt, 1994; Davis, 1995; Görlich and Mattaj, 1996; Panté and Aebi, 1996a). 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.*, 1995a) and pp15 (Moore and Blobel, 1994; Paschal and Gerace, 1995). Importin has also been called karyopherin and pp15 is also called NTF2 or p10.

For convenience, we distinguish four stages of nuclear protein import: (i) the NLS protein binds initially in the cytoplasm to the importin-α/β heterodimer, where importin-α harbours the NLS binding site (Adam and Gerace, 1991; Görlich *et al.*, 1995a; Weis *et al.*, 1995). (ii) This trimeric import ligand complex then docks to the cytoplasmic periphery of the NPC via importin-β (Görlich *et al.*, 1995b; Moroianu *et al.*, 1995) and (iii) subsequently is translocated through the NPC, probably as a single entity. These subsequent steps require Ran and GTP. Based on experiments using Ran with an altered nucleotide specificity, it was proposed that a second GTPase distinct from Ran is also involved in nuclear protein import (Sweet and Gerace, 1996). However, taking the same approach, another group concluded that only nucleotide hydrolysis by Ran is required (K.Weis, personal communication). It is thus still an open question as to whether Ran's GTP cycle is the sole energy source of the import reaction. The import ligand complex has to be moved over a distance of ~100 nm. Therefore, translocation is unlikely to be a single event, but rather a succession of smaller translocation steps. (iv) Finally, translocation is terminated at the nucleoplasmic side of the pore by the disassembly of the NLS–importin-α/β complex and the release of the NLS protein and importin-α into the nucleoplasm. Importin-β does not accumulate inside the nucleus and appears to be re-exported rapidly to the cytoplasm (Görlich *et al.*, 1995b; Moroianu *et al.*, 1995).

Importin-α binds to importin-β via an N-terminal motif, the importin-β binding domain (IBB domain) (Görlich *et al.*, 1996; Weis *et al.*, 1996). A fusion between this domain and a heterologous protein is transported into the nucleus in the same way as importin-α itself. Thus, binding to importin-β via the IBB domain is sufficient for nuclear entry. This implies that importin-β mediates the interactions with the NPC that drive translocation. Importin-β can bind to GLFG or FXFG repeat domains of several nucleoporins *in vitro* (Kraemer *et al.*, 1995; Radu *et al.*, 1995b; Rexach and Blobel, 1995). However, the *in vivo* and functional significance of these interactions for import still needs to be established.

Ran, the Ras-related nuclear protein (Drivas *et al.*, 1990; Bischoff and Ponstingl, 1991b) is a key component of the import reaction. It belongs to a class of small GTP binding proteins that cycle between a GTP- and a GDP-bound state. RCC1 is the principal mammalian guanine

nucleotide exchange factor (GEF) for Ran and stimulates the nucleotide exchange rate by five orders of magnitude (Bischoff and Ponstingl, 1991b; Klebe *et al.*, 1995). It is a nuclear, chromatin-bound protein (Ohtsubo *et al.*, 1989) and thus generates RanGTP inside the nucleus. The main antagonist of RCC1 is the GTPase-activating protein RanGAP1 (Rna1p in yeast). It converts RanGTP into RanGDP by a $>10^5$-fold activation of the low intrinsic Ran-GTPase activity (Bischoff *et al.*, 1994, 1995a; Becker *et al.*, 1995; Corbett *et al.*, 1995). Its localization is exclusively cytoplasmic and thus opposite to that of RCC1 (Hopper *et al.*, 1990; Melchior *et al.*, 1993b). This would predict that cytoplasmic Ran is predominantly in the GDP-bound form, and nuclear Ran mainly in its GTP-bound form.

Another interaction partner of Ran, the 23 kDa Ran binding protein RanBP1, was identified by its specific interaction with the GTP-bound form of Ran (Coutavas *et al.*, 1993). Its conserved Ran binding domain is found also in other proteins, such as the nuclear pore protein RanBP2 (Bischoff *et al.*, 1995b; Wu *et al.*, 1995; Yokoyama *et al.*, 1995). An isolated RanBP1 homology domain of RanBP2 behaves identically to the RanBP1 protein in biochemical assays (Beddow *et al.*, 1995; Bischoff *et al.*, 1995b; Schlenstedt *et al.*, 1995b).

The *Saccharomyces cerevisiae* homologues of RCC1, RanGAP1 and RanBP1 are encoded by the essential genes *PRP20*, *RNA1* and *YRB1*, respectively. Mutations in these genes and mutant forms of Ran have been shown to cause defects in nuclear protein import and in RNA export (Atkinson *et al.*, 1985; Forrester *et al.*, 1992; Kadowaki *et al.*, 1992; Amberg *et al.*, 1993; Tachibana *et al.*, 1994; Cheng *et al.*, 1995; Corbett *et al.*, 1995; Schlenstedt *et al.*, 1995a,b; Carey *et al.*, 1996; Palacios *et al.*, 1996).

Recently, importin-β was also found to bind RanGTP (Rexach and Blobel, 1995; Floer and Blobel, 1996; Lounsbury *et al.*, 1996). This binding was reported to cause the dissociation of yeast importin-β from the nuclear pore protein Nup1p and from SRP1p, the yeast homologue of importin-α (Rexach and Blobel, 1995). In addition, the binding of importin-β to RanGTP was found to inhibit the Ran-GTPase activation by the RanGAP1 homologue Rna1p (Floer and Blobel, 1996).

Here we show that nuclear protein import requires cytoplasmic RanGDP and free GTP. If cytoplasmic Ran is supplied in the GTP-bound form instead, then transport becomes fully dependent on RanGAP1 or Rna1p. RanGDP binds to sites at the NPCs which are distinct from NPC-bound importin-β, suggesting that translocation into the nucleus is not driven from the Ran binding site of importin-β. We have generated a mutant form of importin-β which lacks Ran binding. The mutant protein allows translocation from the cytoplasm up to the nucleoplasmic side of the NPC, but then fails to deliver its cargo to the nucleoplasm. This suggests the Ran binding site in importin-β to be essential for termination, i.e. the final disassembly of the import ligand complex following translocation. This is supported further by the fact that Ran binding to importin-β causes dissociation of the importin heterodimer. Given that the Ran binding site in importin-β is highly specific for the GTP-bound form, it is highly probable that the binding of nucleoplasmic RanGTP to importin-β releases both importin-α and the import substrate into the nucleoplasm. This requirement of nuclear protein import for cytoplasmic RanGDP and for nucleoplasmic RanGTP explains why the major RanGAP is cytoplasmic and the major RanGEF is nuclear.

Results

Protein import depends on cytoplasmic RanGDP and free GTP

Due to the asymmetric distribution of the major Ran GEF (chromatin-bound) and of the major Ran GAP (cytoplasmic), free RanGTP should only be stable within the nucleus and cytoplasmic Ran should be mainly in the GDP-bound form. In order to determine which form of cytoplasmic Ran (i.e. RanGDP, RanGTP or both) is utilized for the import of nuclear proteins, we prepared Ran with a defined nucleotide loading state (Bischoff and Ponstingl, 1995). By HPLC analysis, the bound nucleotide was found to be virtually 100% GDP for the RanGDP preparation and 96% GTP for the RanGTP preparation (Figure 1A). RanGDP and RanGTP were then tested for their ability to promote nuclear import of nucleoplasmin in permeabilized cells in the presence of importin-α/β, an energy-regenerating system and free GTP. The import reactions were stopped after 10 min by fixation, and the fluorescent nucleoplasmin was visualized by confocal microscopy. In the absence of Ran, docking to the nuclear envelope but no nuclear accumulation of nucleoplasmin was observed (upper panel Figure 1B). Strikingly, it made a crucial difference whether Ran was pre-loaded with GDP or GTP: only RanGDP supported import. No import was detected with RanGTP and, compared with the control without Ran, even docking to the nuclear envelope appeared to be reversed (see also below).

RanGAP1/Rna1p becomes essential for import when cytoplasmic Ran is supplied in the GTP-bound form

To rule out that an inhibitory contamination in the RanGTP preparation abolished import, we tested import of fluorescent nucleoplasmin in the presence of RanGTP together with Rna1p, the cytoplasmic, Ran-specific GAP from *Schizosaccharomyces pombe*, which allowed conversion to the GDP-bound form. This treatment restored import and proved that cytoplasmic RanGDP is essential for nuclear protein import (see Figure 1B, lower panel). This also confirms a previous report showing that Rna1p is essential for nuclear protein import in *S.cerevisiae* (Corbett *et al.*, 1995). Where Ran was already pre-loaded with GDP, the addition of Rna1p resulted only in a moderate further stimulation of import (compare corresponding panels in Figure 1).

The chromatin-bound GEF RCC1 generates RanGTP within the nucleus, and Ran would be small enough to diffuse into the cytoplasm. This could supply the cytoplasm with RanGTP even if only RanGDP is added. However, our data make it extremely unlikely that translocation requires cytoplasmic RanGTP. Not even the high concentration of 1 μM Rna1p caused detectable inhibition of import (not shown). The half-life of free, cytoplasmic RanGTP would be <1 s under these conditions. Therefore, nuclear protein import requires cytoplasmic Ran to be in the GDP form only.

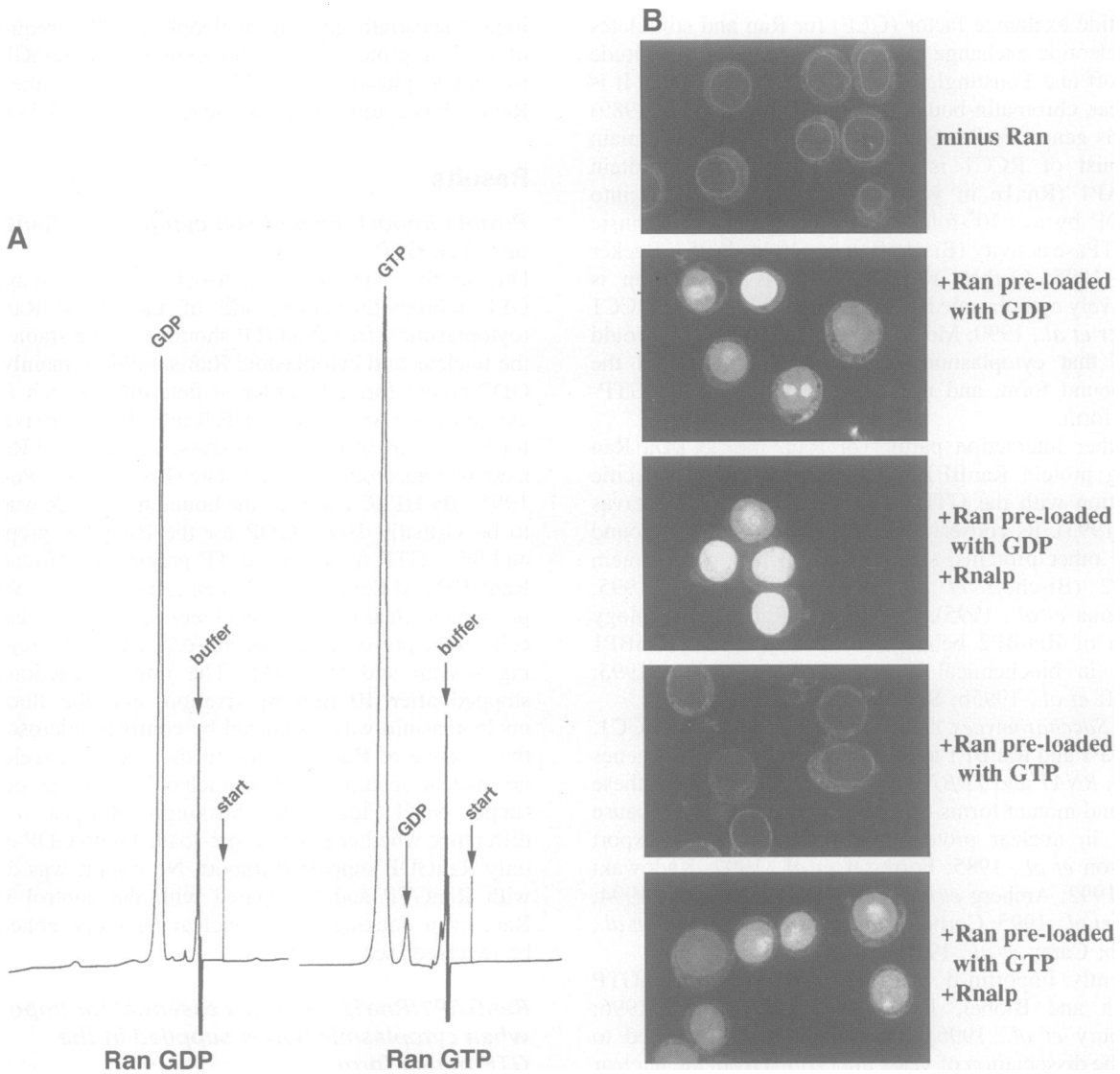

Fig. 1. Nuclear protein import requires cytoplasmic RanGDP. (**A**) To characterize the Ran preparations, the bound nucleotide was released by brief boiling and analysed by HPLC as described (Bischoff and Ponstingl, 1995). The positions of the GDP and GTP peaks are indicated. (**B**) Permeabilized cells were pre-incubated for 15 min on ice with a pre-formed complex of X-rhodamine-labelled nucleoplasmin, importin-α and -β. The indicated combinations of RanGDP, RanGTP and the RanGAP Rna1p were added and import was performed in the presence of energy for 10 min at 20°C. Nuclei were fixed, spun onto polylysine-coated coverslips and analysed by confocal microscopy. Final concentrations were: importin-β, 200 nM; importin-α, 500 nM; nucleoplasmin (pentamers), 500 nM; and, where indicated, 5 μM Ran and 120 nM Rna1p.

RanGDP binds to nuclear pore complexes

Cytoplasmic RanGDP is needed for nuclear protein import and this might involve its binding to NPCs. To demonstrate this binding directly, import reactions containing the importin-α/β heterodimer and nucleoplasmin were carried out either in the presence or absence of RanGDP, and each assay was also performed either with an excess of GDP or in the presence of an energy-regenerating system and GTP. Each combination was then analysed in two ways as shown in Figure 2A: in the left-hand panels, the fluorescent import substrate was detected; in the right-hand panels, an unlabelled import substrate was used, and instead Ran was detected by immunofluorescence. As expected, in the absence of either Ran or an energy source, only docking of the import substrate occurred, whereas efficient nuclear accumulation was observed in the presence of both. Without addition of exogenous Ran, the anti-Ran antibody gave a faint intranuclear staining, probably representing RCC1-bound Ran that was not lost upon preparation of the permeabilized cells. Strikingly, whenever Ran was added to the reaction, Ran staining at the nuclear envelope was evident, as was also the case when only RanGDP and no free GTP was present. The signal reproducibly became somewhat brighter when the energy-regenerating system and free GTP were added, indicating that RanGTP can occupy additional sites at the NPC that are inaccessible to the GDP-bound form.

To show unequivocally that the staining pattern is due to RanGDP binding to NPCs, co-localization with a nuclear pore marker was performed. To ensure that all Ran was in the GDP-bound form, it was pre-incubated with Rna1p, the Ran-specific GAP from *S.pombe*, and the nuclei were pre-treated with apyrase. Binding of Ran to the nuclear envelope was performed in the presence of the import substrate nucleoplasmin and the importin-α/β heterodimer. After fixation, the sample was labelled with a

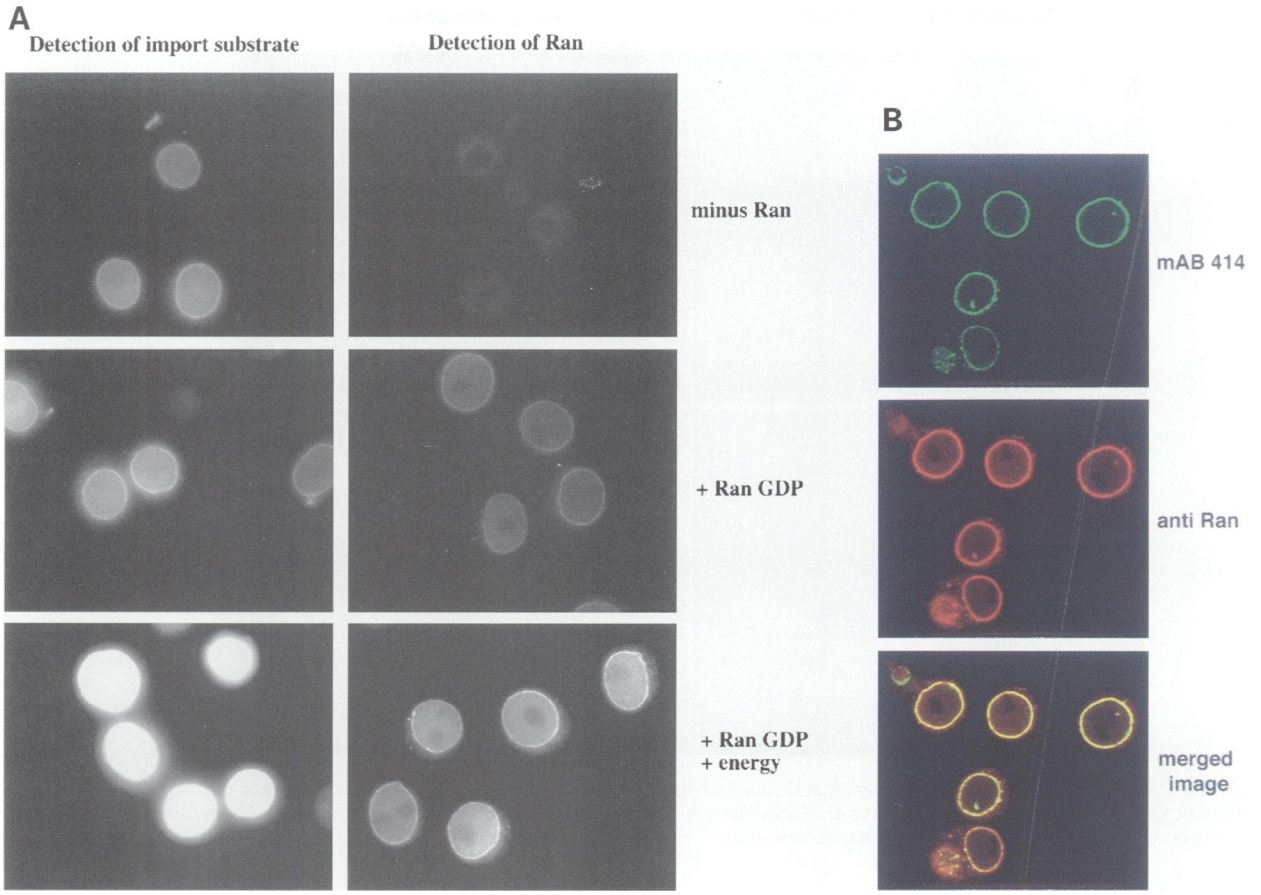

Fig. 2. RanGDP binds to nuclear pore complexes. (**A**) Nuclear import of nucleoplasmin was studied with or without 5 μM RanGDP, and with the addition of either 1 mM GDP or of an energy-regenerating system plus GTP. In the left panels, fluorescein-labelled nucleoplasmin was used and detected by conventional fluorescence microscopy. In the right panels, nucleoplasmin was unlabelled and Ran was detected by indirect immunofluorescence. All incubations contained 200 nM importin-β and 500 nM importin-α. (**B**) Import with importin, unlabelled nucleoplasmin and RanGDP, and in the absence of energy was as in (A), except for two modifications: the permeabilized cells were pre-treated with apyrase and 120 nM Rna1p was also present. Double immunofluorescence with the monoclonal antibody 414 as a nuclear pore marker and anti-Ran antibodies is shown.

mixture of the monoclonal antibody 414, which recognizes several repeat-containing nuclear pore proteins (Davis and Blobel, 1986), and an anti-Ran antibody, followed by fluorescent secondary antibodies. The nucleoporin marker and Ran were then detected by confocal laser scanning microscopy in the fluorescein and Texas red channels, respectively. Omission of each of the primary antibodies demonstrated that there was no spill-over between the channels (data not shown). As seen from Figure 2B, NPCs (upper panel) and RanGDP (middle panel) gave strikingly similar staining patterns, and merging the two fluorescence images revealed their nearly perfect co-localization.

Our data are in contrast to a previous report proposing that only RanGTP but not RanGDP would bind to the NPC (Melchior *et al.*, 1995). The antibody used for these studies was also reported to be specific for the GTP-bound form of Ran (Richards *et al.*, 1995), which would explain why RanGDP binding escaped detection.

The facts that nuclear protein import requires cytoplasmic RanGDP (Figure 1) and GTP hydrolysis by Ran (Melchior *et al.*, 1993a; Moore and Blobel, 1993, 1994; Sweet and Gerace, 1996), and that RanGDP actually binds to the NPC (Figure 2) make it probable that nucleotide exchange for GTP and GTP hydrolysis has to occur at NPC-bound Ran in order to achieve translocation.

Ran binds to the NPC without importin-β

What is the RanGDP receptor at the NPC? Importin-β has been shown to bind Ran (Rexach and Blobel, 1995), and thus RanGDP binding via importin-β would be a possibility. In this case, the number of RanGDP binding sites should correlate with the importin-β concentration at the NPC. This was tested by the experiment shown in Figure 3. The addition of exogenous importin-β results in a >10-fold increase in nuclear pore-bound importin-β; however, it does not increase the level of Ran binding to the NPCs. It should also be noted that even in the presence of free GTP, addition of importin-β could not increase the number of Ran binding sites at the NPC (data not shown). Thus, most of the Ran at the nuclear pore has bound to sites that are distinct from importin-β. These sites might be unidentified nucleoporins or pp15 (alias NTF2/p10) which recently has been shown to bind RanGDP *in vitro* (Nehrbass and Blobel, 1996). The RanBP1 homology domains of the nucleoporin RanBP2 on their own should have only a negligible affinity for RanGDP. Thus, RanGDP could only bind via RanBP2 if a third activity stabilized this interaction.

Our data make it very unlikely that translocation is driven from the Ran binding sites of importin-β. Thus, the question of the cellular function of the Ran–importin-β

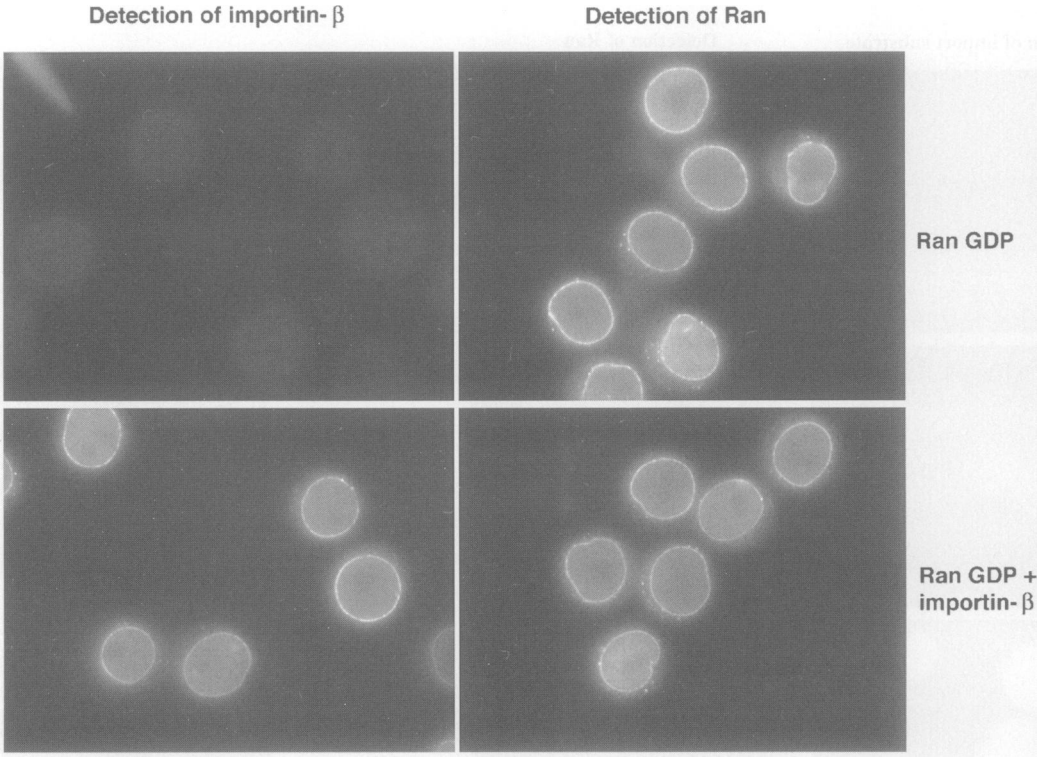

Fig. 3. Importin-β is not a major RanGDP receptor at the nuclear pore complex. Nuclei were incubated in the absence of energy with 5 μM RanGDP or with RanGDP plus 200 nM importin-β. Nucleoplasmin and importin-α were present in both incubations. Each sample was split, and either importin-β or Ran was detected by immunofluorescence.

interaction arises. We have addressed the issue in three steps. First, we have analysed the Ran–importin-β interaction biochemically. Then, we have characterized a mutant form of importin-β which cannot interact with Ran. Finally, we have determined the step of nuclear protein import that is affected by this mutation.

Importin-β binding inhibits exchange and hydrolysis of Ran-bound GTP

GDP or GTP are tightly bound to Ran if Mg^{2+} ions are present. Chelation of Mg^{2+} by EDTA as well as the physiological exchange factor RCC1 stimulate the nucleotide exchange by several orders of magnitude. Figure 4 shows that binding of importin-β to RanGTP strongly inhibits both the EDTA- and the RCC1-induced nucleotide exchange. From the dose dependence, we can estimate a dissociation constant for the importin-β–RanGTP complex of 0.3 nM, which is comparable with 0.13 nM for the RanBP1–RanGTP complex measured under identical conditions (for details, see Materials and methods). The affinity of importin-β for RanGDP can be estimated to be $>10^4$ times lower than for RanGTP (see Figure 4). RanGDP is, therefore, probably not a physiological ligand, adding to the evidence (Figure 3) that importin-β is not the NPC receptor for RanGDP.

Importin-β inhibits the intrinsic GTPase activity of Ran to a non-detectable level (Figure 5A). The RanGTP–importin-β complex is also fully resistant towards GTPase activation by human RanGAP1 or its *S.pombe* homologue Rna1p (Figure 5B, Floer and Blobel, 1996). The inhibition constant in this assay is again ~0.3 nM. The trimeric RanBP1–RanGTP–importin-β complex (Lounsbury *et al.*, 1996) behaves similarly and is also resistant to RanGAP-mediated GTPase activation (Figure 5B).

To date, we have not detected any direct binding of RCC1 or RanGAP1 to the importin-β–RanGTP complex, indicating that the importin-β binding site on Ran at least partially overlaps with the sites for RCC1 and RanGAP1 interaction. This would explain why importin-β inhibits the latter two activities.

The extreme N-terminus of importin-β is crucial for Ran binding

In the course of dissecting importin-β function, we noticed that a small deletion of the 44 N-terminal amino acid residues virtually prevents RanGTP binding. In Figure 6, the Ran binding of wild-type and mutant (ΔN44) importin-β is compared in an overlay assay (Lounsbury *et al.* 1994). The wild-type protein binds Ran·[γ-^{32}P]GTP strongly, whereas no signal is detectable for ΔN44 importin-β even with 10 times more protein loaded. Likewise, no interaction between RanGTP and ΔN44 importin-β is detectable by gel filtration. Using inhibition of GAP-stimulated RanGTPase as an assay, we estimate that Ran binding is ~400-fold lowered by the mutation (not shown). It should be noted that although the N-terminal 44 amino acids of importin-β are essential for Ran binding, they are not sufficient; the entire Ran binding domain is considerably larger (manuscript in preparation).

Importin-β cannot bind RanGTP and importin-α at the same time

We next tested the effect of RanGTP on the NLS–importin-α/β interaction. To give the system a choice and to ensure

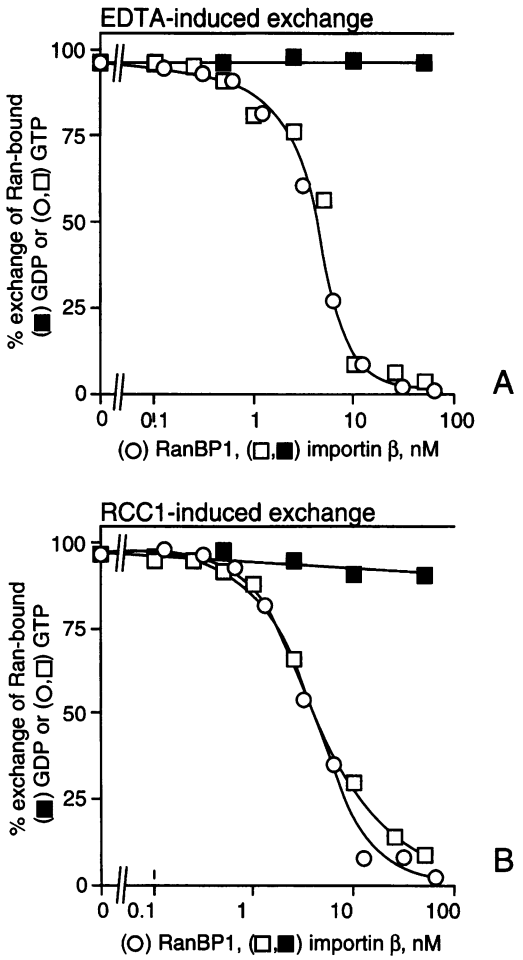

Fig. 4. Inhibition of GTP exchange on Ran by importin-β. Ran·[γ-^{32}P]GTP (open symbols) or Ran·[α-^{32}P]GDP (filled symbols) was pre-incubated with RanBP1 or importin-β. After 2 min, non-radioactive GDP was added to 200 μM and a 5 min exchange reaction was started by addition of either (**A**) 20 mM EDTA or (**B**) 2 nM RCC1. To determine nucleotide exchange on Ran, protein-bound radioactivity was measured. The final concentration of Ran was 10 nM. The concentrations of RanBP1 and importin-β are indicated in the diagram.

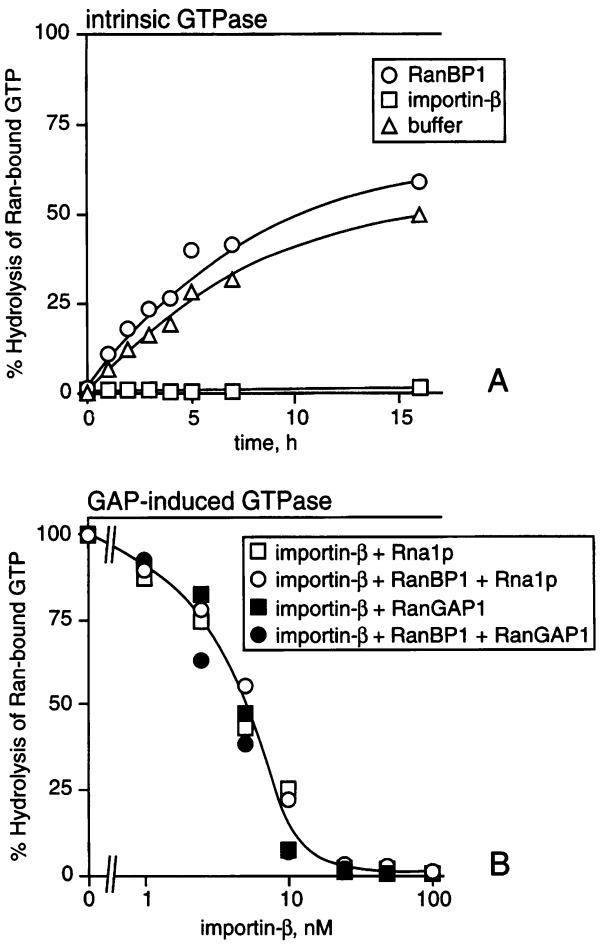

Fig. 5. Effects of importin-β on intrinsic and RanGAP-induced GTPase activity of Ran. (**A**) Effect on intrinsic GTPase. Ran·[γ-^{32}P]GTP (10 nM) was incubated with buffer, or in the presence of 40 nM importin-β or RanBP1 in a total volume of 500 μl. After the indicated times, released [^{32}P]phosphate was determined in 50 μl aliquots. (**B**) Effect on GAP-induced GTPase. Ran·[γ-^{32}P]GTP was pre-incubated for 2 min with buffer, or with a 4-fold molar excess of RanBP1. The concentrations of importin-β were adjusted as indicated. After another 2 min, 2 nM Rna1p or RanGAP1 was added and the reaction was allowed to proceed for 5 min. Hydrolysis of Ran-bound GTP was determined as released [^{32}P]phosphate. The final Ran concentration was 10 nM.

specificity, the experiments were performed in a complete cytosol, an egg extract. Since the high RanGAP1 activity in the extract would consume wild-type RanGTP, we used the GTPase-deficient RanQ69L mutant (Klebe *et al.*, 1995).

As can be seen from Figure 7, and as reported before (Görlich *et al.*, 1995a), complete cytosol allows efficient binding of the importin-α/β heterodimer to an immobilized bovine serum albumin (BSA)–NLS conjugate (lane 2). Likewise, importin-β is co-precipitated with antibodies against recombinant importin-α (lane 6). Addition of RanQ69L GTP to the extract had a very striking effect: importin-β became undetectable in the NLS-bound fraction (lane 3). Similarly, co-precipitation of importin-β with importin-α was abolished completely (lane 7). These effects could not be reversed by adding more wild-type importin-β (lanes 4 and 8). However, ΔN44 importin-β binds efficiently via importin-α to the NLS conjugate even with a 20-fold molar excess of RanQ69L GTP present (lanes 5 and 9). Fully consistent results were obtained using wild-type RanGTP with recombinant importin subunits (thus in the absence of interfering RanGAP1) and gel filtration as an assay (not shown). Taken together, these experiments confirm and establish several facts. First, they provide one explanation as to why the RanQ69L inhibits nuclear protein import in this dominant way, it is by dissociating the importin heterodimer in the cytoplasm. It also explains why RanGTP in the purified system prevents and reverses import substrate docking to the nuclear envelope (see Figure 1). Second, it is important to note that not even a complete cytosol contains any factor which would stabilize a RanGTP–importin-α/β complex; thus importin-β cannot bind RanGTP and importin-α at the same time (see also Rexach and Blobel, 1995). Third, the NLS affinity of importin-α is increased by importin-β binding (compare lanes 2 and 5 with lanes 3 and 4 in Figure 7). Finally, ΔN44 importin-β is functional in importin-α binding; however, the importin-α–ΔN44 importin-β heterodimer resists RanGTP-mediated dissociation.

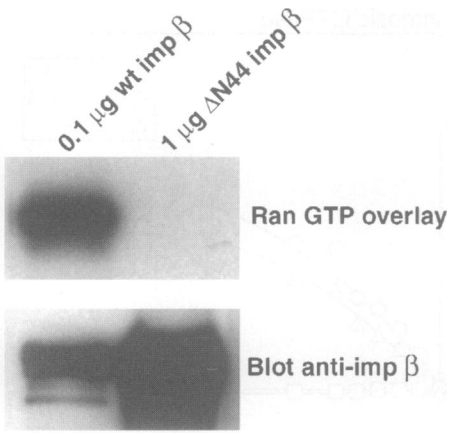

Fig. 6. A mutant importin-β deficient in Ran binding. Wild-type importin-β (0.1 μg) and ΔN44 importin-β (1 μg) were electrophoresed through an SDS gel, transferred onto nitrocellulose and subsequently probed with Ran·[γ-^{32}P]GTP. The upper panel shows the autoradiogram. The lower panel shows the same blot probed with an anti-importin-β antibody followed by ECL detection.

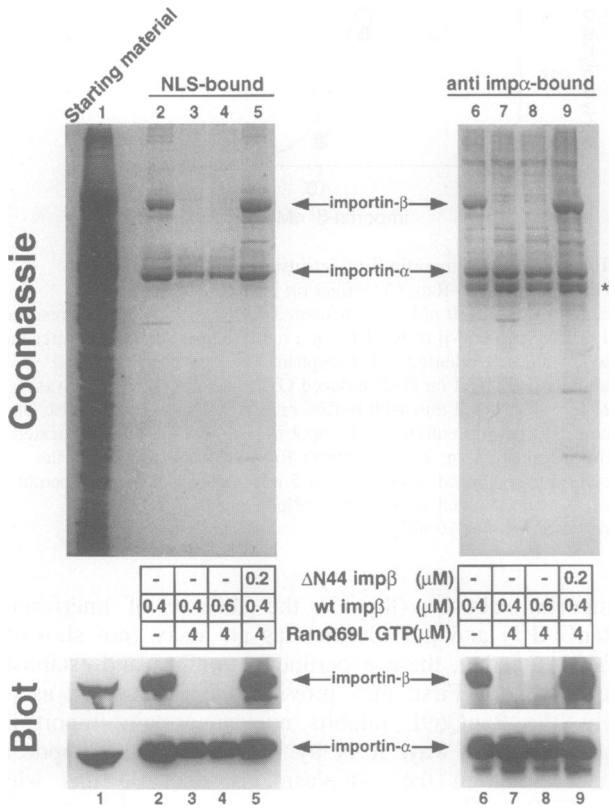

Fig. 7. Effects of RanQ69L GTP on importin-α/β interaction. A post-ribosomal supernatant (PRS) was prepared from *Xenopus* egg extract as described in Materials and methods. The endogenous importin-β concentration was ~0.4 μM, and each of the 1 ml incubations contained an energy-regenerating system. The following additions were made: lanes 2–5 and 6–9: 4 μM RanQ69L pre-loaded with GTP; lanes 4 and 8: 0.2 μM wild-type importin-β; lanes 5 and 9: 0.2 μM ΔN44 importin-β. Lane 1 shows the PRS as the starting material; lanes 2–5 the fractions bound to an NLS–BSA conjugate (Görlich *et al.*, 1995a); lanes 6–9 immunoprecipitations with antibodies raised against recombinant importin-α (Görlich *et al.*, 1995a). Analysis was by SDS–PAGE followed by Coomassie staining or Western blotting. The importin-α and -β bands are indicated. '*' is a background band.

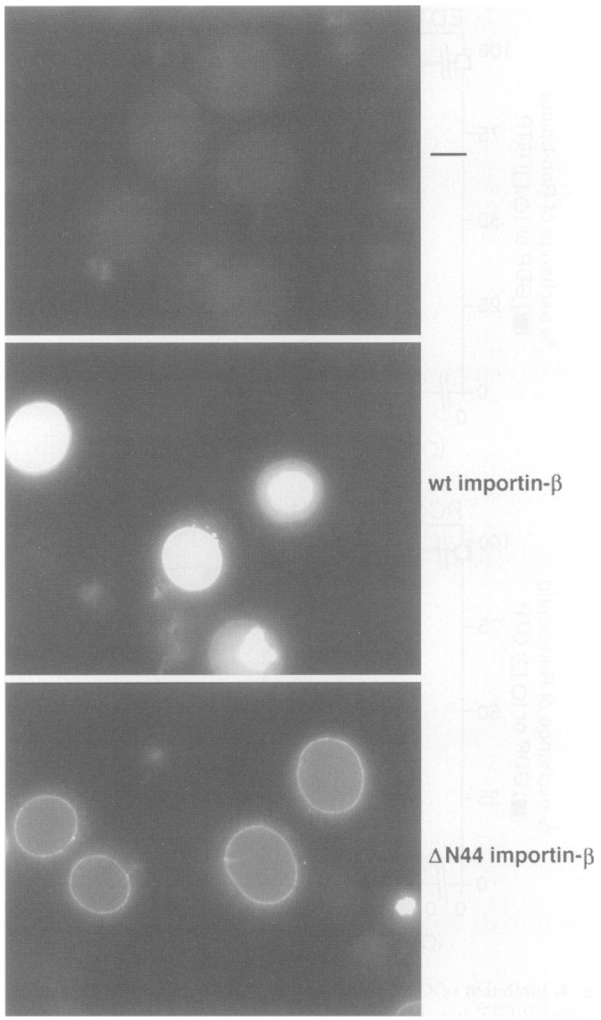

Fig. 8. The Ran binding site of importin-β is essential for nuclear protein import. Fluorescein-labelled nucleoplasmin and importin-α were pre-incubated alone, or with wild-type importin-β, or with a mutant form of importin-β lacking the first 44 amino acid residues. Import into nuclei of permeabilized cells was then allowed for 30 min at 23°C in the presence of an energy-regenerating system and RanGDP. Nuclei were then fixed, spun onto coverslips and analysed by fluorescence microscopy. Final concentrations were: Ran, 5 μM; importin-α, 500 nM; nucleoplasmin (pentamers), 500 nM; and, where indicated, 500 nM wild-type or mutant importin-β.

Ran binding to importin-β is required to deliver the import substrate to the nucleoplasm

To test how the ΔN44 importin-β deletion mutant behaves in nuclear protein import, complexes between wild-type or mutant importin-β, importin-α and fluorescent nucleoplasmin were pre-formed. Import into nuclei of permeabilized cells was then allowed to proceed in the presence of RanGDP and an energy-regenerating system plus GTP. Figure 8 demonstrates that only wild-type importin-β allowed nuclear accumulation of nucleoplasmin to occur. In the presence of ΔN44 importin-β, transport intermediates were trapped at the nuclear envelope, revealing the typical nuclear pore staining pattern.

Ran binding to importin-β is required at a late stage of the nuclear pore passage

In order to establish at which point at the NPC import intermediates get arrested if importin-β is deficient in Ran

binding, we carried out an *in vivo* experiment using *Xenopus* oocytes, which allow an excellent structural preservation of NPCs and are, therefore, the system of choice for this type of experiment. To have a strong but monovalent import substrate, we conjugated IBB domains, the nuclear targeting signal of importin-α (see Introduction), to 14 nm colloidal gold using conditions under which only a single ligand per gold particle was bound. As a control, we injected first the IBB–gold conjugate (IBB–gold) alone or after pre-incubation with wild-type importin-β into the cytoplasm of *Xenopus* oocytes and, as expected, efficient accumulation of IBB–gold inside the nucleus was observed (not shown), similarly to the fluorescence import assay (Figure 8, see also Görlich *et al.*, 1996).

IBB–gold was then pre-incubated at a 1:1 molar ratio with ΔN44 importin-β and injected into the cytoplasm of *Xenopus* oocytes. After 2 h incubation at room temperature (the conditions under which nuclear accumulation of IBB–gold was observed in the control experiment), the import reaction was stopped by fixation with cold glutaraldehyde (see Materials and methods) and the samples were processed for analysis by thin section electron microscopy. As documented in Figure 9A, there was no intranuclear accumulation of the IBB–gold, instead the IBB–gold became arrested at distinct positions at the nucleoplasmic side of the NPC, i.e. ~40 nm from its central plane, which might correspond to the terminal ring of the nuclear baskets (see arrowheads). Similar results were observed if the ΔN44 importin-β mutant itself was tagged with 14 nm colloidal gold and microinjected into the cytoplasm of *Xenopus* oocytes together with importin-α and nucleoplasmin (Figure 9B), or with isolated IBB domains (not shown). This distinct location for IBB–gold and ΔN44 importin-β–gold at the nucleoplasmic side of the NPC implies that the import ligand complex is translocated through the NPC even if importin-β is unable to bind Ran. This finding makes the Ran binding site of importin-β unlikely to be involved in the actual translocation of the import ligand complex through the NPC, and instead suggests that it mediates termination of import, i.e. the release of the import ligand from the NPC into the nucleus.

As documented in the gallery of selected examples in Figure 9B, gold particles were also observed at the cytoplasmic entry of the central gated channel and at the cytoplasmic filaments of the NPC. It appears that these incoming import ligand complexes have to wait at the cytoplasmic parts of the NPC until the nuclear baskets are cleared.

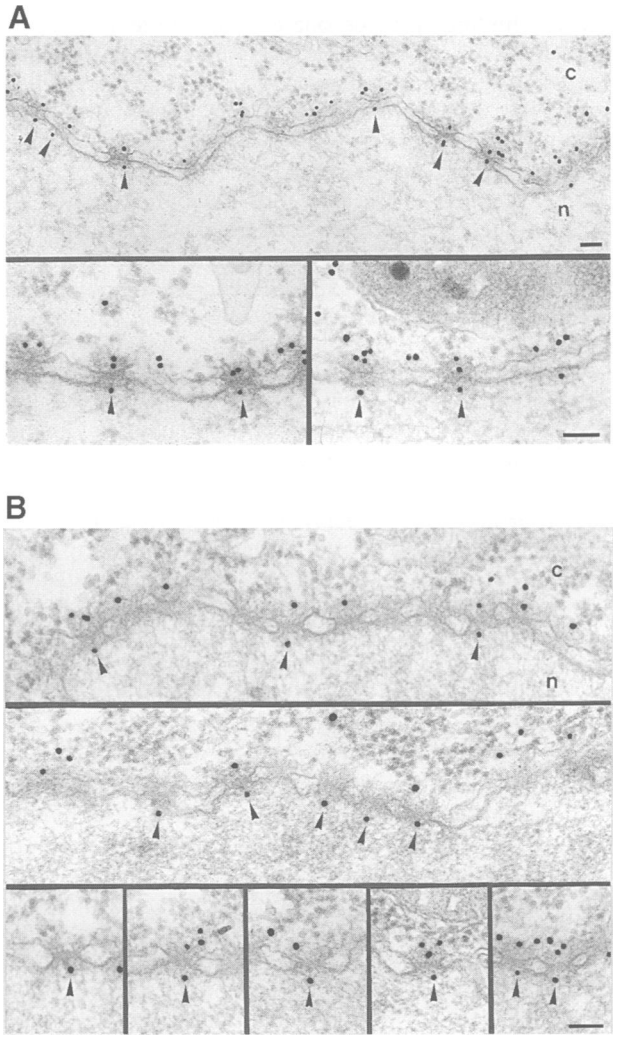

Fig. 9. Importin-β which is deficient in Ran binding can translocate import ligand complex through the NPC, but fails to release it into the nucleus. (**A**) The IBB domain, which targets importin-α into the nucleus, was conjugated to 14 nm colloidal gold, incubated with ΔN44 importin-β (molar ratio 1:1) and injected into the cytoplasm of *X.laevis* oocytes. After incubation at room temperature for 2 h, the injected oocytes were fixed and processed for thin section electron microscopy as described in Materials and methods. Panels show cross-sections through the nuclear envelope. (**B**) The ΔN44 importin-β mutant protein was coated to 14 nm colloidal gold and injected into the cytoplasm of *X.laevis* oocytes together with a 1:1:1 molar ratio of importin-α and nucleoplasmin. After 1 h (upper panel), 2 h (middle panel) or 4 h (lower gallery), samples were fixed and analysed as in (A). Arrowheads indicate gold particles at the nuclear side of the NPC. 'c' stands for cytoplasm, 'n' for nucleoplasm. Scale bars = 100 nm.

Discussion

We have shown that nuclear protein import is at least 2-fold Ran dependent: first, import requires cytoplasmic RanGDP, second, inactivation of the RanGTP binding site in importin-β arrests the import substrate in the nuclear baskets of the NPC. This directly implies that nuclear RanGTP is required to complete the import reaction. RanGTP binding to importin-β causes the release of importin-α and very probably constitutes the termination reaction, i.e. the final disassembly of the NLS–importin complex following its translocation through the NPC.

Cytoplasmic RanGTP disassembles the importin-α/β heterodimer already in the cytoplasm, and thus causes termination of import on the 'wrong side' of the NPC. This is normally prevented by the cytoplasmic RanGAP1, the major Ran-specific GTPase-activating protein. Genetic and biochemical studies have demonstrated that Rna1p, the *S.cerevisiae* RanGAP1 homologue, is essential for nuclear protein import (Corbett *et al.*, 1995). Our findings support these data and show one clear function for cytoplasmic RanGAP1/Rna1p: it is the consumption of cytoplasmic RanGTP, that would otherwise prematurely terminate translocation, and the generation of RanGDP, that is required on the cytoplasmic side of the NPC for nuclear import.

These findings provide one explanation as to why the major Ran GEF, RCC1, is nuclear, while RanGAP1/Rna1p is cytoplasmic: it is to generate a steep RanGTP gradient across the nuclear envelope which allows the NLS–importin-α/β complex to form in the cytoplasm but forces its dissociation in the nucleoplasm.

However, this is certainly not the only function for nuclear RanGTP. For example, the re-export of importin-α from the nucleus appears to require an even higher concentration of nuclear RanGTP than the dissociation of importin-α from importin-β (Koepp *et al.*, 1996; our unpublished data).

Nuclear protein import requires free GTP which cannot be replaced by non-hydrolysable GTP analogues (Melchior *et al.*, 1993a; Moore and Blobel, 1993, 1994). Likewise, the GTPase-deficient RanQ69L mutant pre-loaded with GDP cannot substitute for wild-type RanGDP in an import assay (our unpublished data). This strongly suggests that nuclear import requires the entire GTP cycle of Ran. Because cytoplasmic RanGDP is not only essential for import but also binds to NPCs, it appears likely that nuclear import involves nucleotide exchange and GTP hydrolysis on NPC-bound Ran. However, this still needs to be demonstrated directly. In this case, the Ran binding sites involved in import should induce nucleotide exchange and need to have an affinity for both forms of Ran.

RanGDP binds to NPCs but not via importin-β. This implies that the RanGTP produced by a subsequent exchange reaction is not generated at importin-β. The RanGTP produced is also unlikely to be handed over to importin-β, as this would result in premature termination. The RanGDP receptor at the NPC, the mechanism by which Ran exchanges its nucleotide and the mechanism of the subsequent translocation-coupled GTP hydrolysis still remain to be identified. The same applies to the central question of how this RanGTP cycle at the NPC translates into the generation of a directed movement.

Our data strongly suggest that once the NLS–importin-α/β complex has docked to the NPC, it is transferred into the nucleus as a single entity, without intermediate disassembly, until the nucleoplasmic side of the pore is reached. Figure 9 shows that at least three translocation intermediates exist: at the terminal end of the cytoplasmic filaments, at the cytoplasmic entry of the central gated channel and at the nuclear baskets. The first two intermediate states can also be visualized when nuclear import is inhibited by chilling or by the lectin wheat germ agglutinin (Panté and Aebi, 1996b). The release of the import ligand complex from the nuclear baskets occurs by direct binding of RanGTP to importin-β which disassembles the NLS–receptor complex and allows importin-α to diffuse into the nucleoplasm. Because the isolated α subunit has a lower NLS affinity than the importin-α/β heterodimer (see Figure 7), the termination reaction should also facilitate the release of the NLS from its receptor. One could speculate that importin-β is returned to the cytoplasm with RanGTP bound, which would ensure that importin-α is not re-exported the same way as it entered the nucleus.

The termination of import generates the RanGAP1-resistant and RCC1-resistant importin-β–RanGTP complex which would constitute an irreversible sink for Ran and importin-β unless some mechanism for its disassembly exists. Indeed, the GTP in the importin-β–RanGTP complex is hydrolysed rapidly when added to a complete cytosol. We are presently characterizing this activity which allows the recovery of Ran and importin-β from the termination complex.

Materials and methods

Protein expression and purification

Preparation of the following proteins was as described: C-terminally his-tagged *Xenopus* importin-α (Görlich *et al.*, 1994); recombinant human Ran (Bischoff and Ponstingl, 1995), recombinant *S.pombe* Rna1p (Bischoff *et al.*, 1995a), recombinant murine RanBP1 (Bischoff *et al.*, 1995b); native RanGAP1 from HeLa cells (Bischoff *et al.*, 1994); and recombinant human RCC1 (Klebe *et al.*, 1993). Expression of wild-type human importin-β was as described (Görlich *et al.*, 1996) but its purification from the bacterial lysate was modified as follows. Lysis by sonication was performed in 50 mM Tris–HCl pH 7.5, 200 mM NaCl and 2 mM dithiothreitol (DTT), and a post-ribosomal supernatant was prepared and applied to Q-Sepharose equilibrated in lysis buffer. A gradient ending at 1 M NaCl was applied and the importin-β-containing fractions eluting at ~360 mM NaCl were pooled and loaded onto a 2 ml sulfo-Link column (Pierce) to which a synthetic peptide corresponding to the IBB domain of importin-α had been coupled. The column was washed in lysis buffer and elution was performed with 1 M $MgCl_2$, 50 mM Tris–HCl pH 7.5. The importin-β-containing pool was then dialysed against 50 mM Tris–HCl pH 7.5, 250 mM sucrose, 2 mM β-mercaptoethanol and was frozen in small aliquots.

ΔN44 importin-β was expressed from pQE60 with a C-terminal his-tag at 23°C. Purification utilized nickel–agarose, buffer exchange for lysis buffer on Sephadex G25 and affinity chromatography on IBB–sulfo-Link as described for the wild-type protein.

Enzymatic assays

Ran-bound guanine nucleotide was determined by reversed phase chromatography as described (Bischoff and Ponstingl, 1995). Charging of Ran with [γ-^{32}P]GTP and [α-^{32}P]GDP was carried as described (Bischoff *et al.*, 1994, 1995b). The concentration of Ran·[γ-^{32}P] was adjusted by addition of unlabelled RanGTP. Nucleotide exchange assays and GTPase assays were performed essentially as described (Bischoff and Ponstingl, 1991a; Bischoff *et al.*, 1994). Unless otherwise indicated, enzyme assays were performed in a total volume of 50 µl. Thirty µl aliquots of 17 nM Ran·[γ-^{32}P]GTP or Ran·[α-^{32}P]GDP were pre-incubated at 25°C with 10 µl aliquots of importin-β, the ΔN44 importin-β mutant or RanBP1, respectively, in incubation buffer (20 mM HEPES–NaOH, pH 7.4, 100 mM NaCl, 1 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 0.02% sodium azide, 0.05% hydrolysed gelatine). Exchange reactions were started by addition of 10 µl of 1 mM GDP containing either 100 mM EDTA pH 7.4 or 10 nM RCC1–Ran. For GTPase assays, 10 µl aliquots of 10 nM Rna1p or RanGAP1 were added. At various time points, samples were filtered through BA85 nitrocellulose and washed with rinsing buffer (incubation buffer without gelatine). Alternatively, released [^{32}P]phosphate was recovered using the charcoal assay as described (Bischoff *et al.*, 1995b).

The dissociation constants of the RanGTP–importin-β and the RanGTP–RanBP1 complexes were determined by measuring the importin-β- or RanBP1-mediated inhibition of EDTA-induced GTP exchange on Ran. For RanGTP–importin-β, the k_d was also assayed by inhibition of RCC1-induced GTP exchange and Rna1p-induced GTP hydrolysis. The experiments were performed in 50 µl volumes as described above, with the following modifications: 60 pM Ran·[γ-^{32}P]GTP was used and the pre-incubation of Ran·[γ-^{32}P]GTP with importin-β or RanBP1 was extended to 30 min.

Import assays

Import reactions into nuclei of permeabilized cells were performed as described (Görlich *et al.*, 1995b, 1996). The standard assay contained 20 mM HEPES–KOH pH 7.5, 80 mM potassium acetate, 4 mM magnesium acetate, 1 mM DTT, 250 mM sucrose, and 2 mg/ml nucleoplasmin core to block non-specifc binding of proteins. The energy-regenerating system plus GTP contains 10 mM creatine phosphate, 0.5 mM ATP, 0.5 mM GTP and 50 µg/ml creatine kinase. Concentrations of import factors, their order of addition, duration and temperature of incubation are specified in the figure legends. Immunofluorescence was done essentially as described (Görlich *et al.*, 1995b) with two

modifications for the anti-Ran antibody; it was used after affinity purification on immobilized Ran and the nuclear envelope was permeabilized with 0.01% digitonin immediately before addition of fixative, because otherwise nucleoplasmic Ran occasionally obscures the nuclear envelope staining.

Direct conjugation of the IBB domain and ΔN44 importin-β with colloidal gold.

Colloidal gold particles, ~14 nm in diameter, were prepared by reduction of tetrachloroauric acid with sodium citrate in the presence of tannic acid (Slot and Geuze, 1985). IBB and ΔN44 importin-β were conjugated to colloidal gold particles as described (Baschong and Wrigley, 1990). After gold conjugation, the complexes were centrifuged at 45 000 *g* for 15 min, the soft pellet was resuspended in low-salt buffer (LSB) containing 1 mM KCl, 0.5 mM $MgCl_2$, 10 mM HEPES, pH 7.5. For microinjection experiments, gold-tagged IBB was pre-incubated at 4°C for 1 h with a 1:1 molar ratio of wild-type importin-β or ΔN44 importin-β. Similarly, gold-tagged ΔN44 importin-β was pre-incubated with a 1:1:1 molar ratio of nucleoplasmin and importin-α.

Oocyte microinjection and preparation of samples for electron microscopy

Mature (stage six) oocytes were surgically removed from female *Xenopus laevis* as described previously (Jarnik and Aebi, 1991), and stored in modified Barth's saline (MBS) containing 88 mM NaCl, 1 mM KCl, 0.82 mM $MgSO_4$, 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 10 mM HEPES, pH 7.5. Oocytes were defolliculated by treatment with 5 mg/ml collagenase (Sigma, St Louis, MO) in calcium-free MBS for 3 h. Oocytes were then washed with MBS and used for microinjection within the following 2 days. Fifty nl of gold-tagged IBB (IBB–gold) or a mixture (see above) containing IBB–gold and wild type importin-β or ΔN44 importin-β was microinjected into the cytoplasm of each oocyte. For visualization of the mutant importin-β, a mixture containing gold-tagged ΔN44 importin-β + nucleoplasmin + importin-α was used for microinjections. The injected oocytes were incubated in MBS buffer at room temperature for the indicated times and then fixed overnight at 4°C in MBS containing 2% glutaraldehyde. The oocytes were then washed three times with MBS, and the nuclei with their surrounding cytoplasm were then dissected and fixed again with 2% glutaraldehyde in LSB for 1 h at 4°C. Samples were washed three times with LSB, post-fixed for 1 h with 1% OsO_4 in LSB, and processed for thin section electron microscopy by conventional procedures as described (Panté *et al.*, 1994).

Acknowledgements

We would like to thank Herwig Ponstingl and Ron Laskey, in whose laboratories part of the work was performed, for generous support. We are grateful to Marylena Dabrowski, Róisín Deane, Ron Laskey, Liz Müller and Herwig Ponstingl for critical reading of the manuscript. We thank Yves Cully for help with the figures. This work was supported by the Kanton Basel-Stadt, the M.E.Müller Foundation of Switzerland, and by research grants from the Swiss National Science Foundation (SNF), the Human Frontier Science Programme Organisation (HFSPO) and the Deutsche Forschungsgemeinschaft (DFG).

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Received on May 15, 1996; revised on July 4, 1996