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NTF2 mediates nuclear import of Ran

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Importin β family transport receptors shuttle between the nucleus and the cytoplasm and mediate transport of macromolecules through nuclear pore complexes (NPCs). The interactions between these receptors and their cargoes are regulated by binding RanGTP; all receptors probably exit the nucleus complexed with RanGTP, and so should deplete RanGTP continuously from the nucleus. We describe here the development of an *in vitro* system to study how nuclear Ran is replenished. Nuclear import of Ran does not rely on simple diffusion as Ran's small size would permit, but instead is stimulated by soluble transport factors. This facilitated import is specific for cytoplasmic RanGDP and employs nuclear transport factor 2 (NTF2) as the actual carrier. NTF2 binds RanGDP initially to NPCs and probably also mediates translocation of the NTF2–RanGDP complex to the nuclear side of the NPCs. A direct NTF2–RanGDP interaction is crucial for this process, since point mutations that disturb the RanGDP–NTF2 interaction also interfere with Ran import. The subsequent nuclear accumulation of Ran also requires GTP, but not GTP hydrolysis. The release of Ran from NTF2 into the nucleus, and thus the directionality of Ran import, probably involves nucleotide exchange to generate RanGTP, for which NTF2 has no detectable affinity, followed by binding of the RanGTP to an importin β family transport receptor.

Keywords: importin β/NTF2/nuclear pores/nuclear transport/Ran

Introduction

Eukaryotic cells are characterized by their separation into distinct nuclear and cytoplasmic compartments by the nuclear envelope. This spatial separation of transcription from translation provides great advantages in the coordination and regulation of cellular events, but it also necessitates constitutive and regulated import into and export out of the cell nucleus. The nucleocytoplasmic transport of macromolecules proceeds through nuclear pore complexes (NPCs) that perforate the double membrane of the nuclear envelope. NPCs allow active transport of particles as large as 25 nm in diameter (Feldherr *et al.*, 1984) and also provide a 9 nm diffusion channel for ions, metabolites and, in principle, also for macromolecules smaller than ~60 kDa (for a review see Bonner, 1978). However, the transport of small RNAs such as tRNA (Zasloff, 1983; Arts *et al.*, 1998; Kutay *et al.*, 1998) and small proteins such as histones (Breeuwer and Goldfarb, 1990) or ribosomal proteins (Rout *et al.*, 1997; Schlenstedt *et al.*, 1997; Jäkel and Görlich, 1998) is normally mediated by specific carriers.

The transport of macromolecules through NPCs is generally energy dependent and also requires a number of soluble nuclear transport factors that can be classified into three categories: transport receptors, adaptor molecules and components of the RanGTPase system (for recent reviews see Dahlberg and Lund, 1998; Görlich, 1998; Izaurralde and Adam, 1998; Mattaj and Englmeier, 1998). Transport receptors shuttle continuously between nucleus and cytoplasm, interact with NPCs, bind cargo molecules and facilitate their translocation through the NPCs. They can be grouped into nuclear import receptors (importins) and export receptors (exportins). In some cases, transport receptors do not interact with their substrates directly, but instead employ an adaptor molecule. For example, importin α binds classical nuclear localization signals (NLSs) and in turn interacts with the actual import receptor, importin β. Transport receptors form a superfamily of proteins (Fornerod *et al.*, 1997a; Görlich *et al.*, 1997) that are of similar size (90–130 kDa), share an importin β-like RanGTP-binding motif (Görlich *et al.*, 1997) and use this RanGTP binding to regulate the interactions with their cargoes or adaptor molecules.

Like all Ras superfamily GTPases, Ran switches between GDP- and GTP-bound states by nucleotide exchange and GTP hydrolysis. The intrinsic rates for these reactions are very low but are stimulated by approximately five orders of magnitude by specific factors (Klebe *et al.*, 1995). Ran's major nucleotide exchange factor is RCC1, which charges Ran with GTP (Bischoff and Ponstingl, 1991a). Its direct antagonist is the GTPase-activating protein RanGAP1, which converts RanGTP into RanGDP (Bischoff *et al.*, 1994, 1995a; Becker *et al.*, 1995). This GTPase activation is facilitated further by the RanGTP-binding protein RanBP1 (Coutavas *et al.*, 1993; Bischoff *et al.*, 1995b; Richards *et al.*, 1995; Schlenstedt *et al.*, 1995). The nuclear localization of RCC1 (Ohtsubo *et al.*, 1989) and the nuclear exclusion of RanGAP1 and RanBP1 (Hopper *et al.*, 1990; Melchior *et al.*, 1993b; Matunis *et al.*, 1996; Richards *et al.*, 1996; Mahajan *et al.*, 1997) would be anticipated to result in a high nuclear RanGTP concentration and very low RanGTP levels in the cytoplasm, although it has not been possible to establish this directly. This putative RanGTP gradient across the nuclear envelope has been proposed to be a key parameter that controls the directionality of nuclear transport (Görlich *et al.*, 1996a,b; Izaurralde *et al.*, 1997).

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Import receptors such as importin β or transportin bind their substrates only in the absence of RanGTP (i.e. in the cytoplasm) and release them upon direct interaction with RanGTP (Rexach and Blobel, 1995; Chi *et al.*, 1996; Görlich *et al.*, 1996b; Izaurralde *et al.*, 1997; Siomi *et al.*, 1997; Jäkel and Görlich, 1998). This substrate release should occur in the nucleus where the RanGTP concentration is predicted to be high. Importin β and transportin are probably exported to the cytoplasm as complexes with RanGTP (Izaurralde *et al.*, 1997), which should preclude their re-exporting the cargoes they previously carried in and thereby ensure productive transport cycles. Finally, cytoplasmic RanBP1 and RanGAP1 remove RanGTP from the import receptors and restore them to an import-competent form (Bischoff and Görlich, 1997; Floer *et al.*, 1997; Lounsbury and Macara, 1997). Alternatively, RanGTP complexes could be disassembled by the RanBP2–SUMO–RanGAP1 complex that is localized at the cytoplasmic filaments of the NPC (Matunis *et al.*, 1996; Mahajan *et al.*, 1997; Saitoh *et al.*, 1997).

Substrate binding to the exportins (CRM1, CAS, exportin-t) and subsequent release are regulated in the opposite way to those of the importins. Binding of export substrates is enhanced greatly by the simultaneous binding of RanGTP to the exportins (Fornerod *et al.*, 1997b; Kutay *et al.*, 1997b, 1998; Arts *et al.*, 1998). This property should favour substrate binding in the nucleus, where the RanGTP concentration is predicted to be high. The trimeric substrate–exportin–RanGTP complex is then transferred to the cytoplasm. There, GTP hydrolysis results in Ran's irreversible dissociation from the complex, which allows the exportin to release the substrate, re-enter the nucleus and bind and export the next cargo molecule.

A key element of current models for importin and exportin function is that these factors normally exit the nucleus as a complex with RanGTP and thus constantly deplete Ran from the nucleus. Ran's predominantly nuclear localization (Bischoff and Ponstingl, 1991b) can then only be maintained by efficient nuclear import of Ran. The assumption that each transport cycle of an importin β family transport receptor results in export of one Ran molecule would imply that Ran crosses the nuclear envelope as frequently as all these receptors together. For reasons of stoichiometry, it appears then impossible that Ran itself is imported in a conventional way by an importin β family transport receptor.

Another nuclear transport factor is NTF2. It was identified originally as an activity that stimulates import of NLS-containing proteins into nuclei of permeabilized mammalian cells (Moore and Blobel, 1994; Paschal and Gerace, 1995). It binds specifically the GDP-bound form of Ran (Clarkson *et al.*, 1996; Nehrbass and Blobel, 1996; Paschal *et al.*, 1996; Percipalle *et al.*, 1997; Wong *et al.*, 1997; Stewart *et al.*, 1998) and interacts with NPCs and isolated nuclear pore proteins containing xFxFG repeats (Paschal and Gerace, 1995; Clarkson *et al.*, 1996; Corbett and Silver, 1996). NTF2 is highly conserved in evolution, and the corresponding *Saccharomyces cerevisiae* gene is essential in most genetic backgrounds (Corbett and Silver, 1996; Paschal *et al.*, 1997). Gold-labelled NTF2 injected into *Xenopus* oocytes accumulates at the NPCs (Feldherr *et al.*, 1998), consistent with its binding to xFxFG-containing nucleoporins. Genetic and biochemical evidence suggests that NTF2 co-operates with the RanGTPase system, although its precise molecular function has remained obscure.

Here we show that NTF2 is a mediator of Ran import into the nucleus and that NTF2 is rate limiting for Ran import into nuclei of permeabilized cells. Moreover, a direct NTF2–RanGDP contact is crucial for Ran import because point mutations that disturb the interaction between Ran and NTF2 also interfere with Ran import. Nuclear accumulation of Ran requires the presence of GTP but not GTP hydrolysis, as judged from the effects of a non-hydrolysable GTP analogue. With exogenous NTF2 alone, Ran accumulates strongly at the nuclear envelope and in the nucleoli, together with a lower concentration in the nucleoplasm. Ran only becomes concentrated in the nucleoplasm of, for example, permeabilized HeLa cells, when abundant nuclear binding sites become available which apparently originate largely from importin β family transport receptors. This observation implies that a significant proportion of nuclear Ran is indeed already transport receptor-bound. We suggest a model for Ran import that includes the following steps: formation of a NTF2–RanGDP complex in the cytoplasm; binding of this complex to cytoplasmic portions of the NPC and translocation to the nucleoplasmic side; release of Ran from NTF2 into the nucleus by nucleotide exchange generating RanGTP for which NTF2 has no detectable affinity; and, finally, binding of RanGTP to importin β family transport receptors in the nucleus.

Results

Nuclear import of Ran from the cytoplasm requires soluble factors

Although they provide a coherent description of the manner in which macromolecules are transported across the nuclear envelope and receptors are recycled, current models of nucleocytoplasmic transport have not addressed the question of how Ran is re-imported into the nucleus after it has been carried to the cytoplasm. To study this process, we have developed an *in vitro* system for Ran import based on permeabilized mammalian cells (Adam *et al.*, 1990). This requires detection of Ran during its own import. Permeabilized cells, however, still contain variable amounts of Ran and it was therefore crucial to distinguish Ran that had been imported during the *in vitro* assay from any endogenous Ran that remained after permeabilization. We therefore modified wild-type RanGDP at a 1:1 molar ratio with fluorescein 5′ maleimide, which attaches specifically to protein SH groups at cysteine residues. The reaction was essentially quantitative (not shown). The X-ray crystal structure of RanGDP (Scheffzek *et al.*, 1995) shows that only one (Cys121) of its three cysteines is exposed to the solvent and thus available for modification, and so the reaction should result in a homogenous population of fluorescein-labelled RanGDP. Figure 1A shows that fluorescein–Ran promoted nuclear import of a Texas red-labelled IBB fusion (importin beta-binding domain fused to nucleoplasmin core) as efficiently as non-modified Ran. This crucial control verifies that the modification did not affect Ran's function in nuclear protein import.

We then tested how the fluorescent Ran would behave

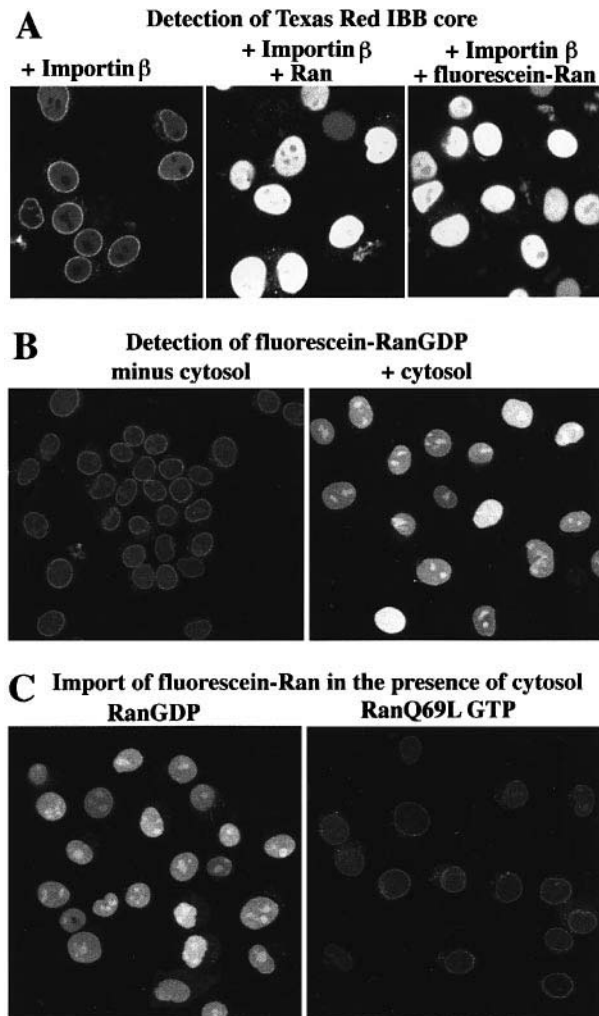


Fig. 1. (**A**) Fluorescein–Ran is functional. Nuclear import of a Texas red-labelled IBB–nucleoplasmin core fusion protein was performed in the presence of an energy-regenerating system and 0.6 μM importin β, 0.1 μM Rna1p, 0.3 μM RanBP1, 0.15 μM NTF2 (dimers) and, where indicated, 1.5 μM RanGDP or the same concentration of fluorescein–Ran. Import was for 5 min at 18°C, after which it was stopped by fixation with 4% paraformaldehyde on ice. Nuclei were spun onto coverslips and analysed by confocal fluorescence microscopy in the Texas red channel. Note, the IBB fusion substrate accumulated at the nuclear envelope in the absence of Ran, but efficiently entered the nucleus when non-modified Ran or fluorescein–Ran had been added. (**B**) Nuclear import of Ran requires soluble factors. Import of fluorescein–RanGDP (1.5 μM) into nuclei of permeabilized cells was performed in the presence of an energy-regenerating system and without or with addition of cytosol (reticulocyte lysate). Analysis was as in (A), except that fluorescein–Ran had been detected in the fluorescein channel. In the absence of cytosol, Ran gave only a faint nuclear pore staining, whereas in the presence of cytosol it accumulated efficiently inside the nuclei. (**C**) Import of Ran from the cytoplasm is specific for the GDP-bound form. Nuclear import of fluorescein–wild-type Ran pre-loaded with GDP was compared with that of the GTPase-deficient RanQ69L mutant pre-loaded with GTP. Import in the presence of cytosol was performed and analysed as in (B). Whereas wild-type RanGDP was imported efficiently, RanQ69LGTP was imported only very poorly.

during an import reaction into nuclei of permeabilized cells (Figure 1B). When the incubation was performed only in the presence of an energy-regenerating system and without any further addition, we observed some Ran binding to NPCs together with a very weak intranuclear accumulation. In the presence of reticulocyte lysate, however, Ran entered the nucleus efficiently, suggesting that the lysate contains at least one soluble factor that is limiting for Ran import. Other cytosol preparations, such as a cytoplasmic HeLa extract or *Xenopus* egg extract, also stimulated Ran import (data not shown, but see Figure 3).

In the cytoplasm, the activity of RanGAP should rapidly convert Ran to its GDP-bound form, and so we wondered if the Ran import system would be specific for the RanGDP. We therefore compared import of fluorescein-labelled wild-type RanGDP with that of the RanQ69L mutant pre-loaded with GTP. Previous studies have shown that this mutant has a low intrinsic GTPase activity that is not activated by RanGAP and so it should remain in the GTP-bound state, even in the presence of a high concentration of cytoplasmic RanGAP (Bischoff *et al.*, 1994; Klebe *et al.*, 1995). The import of Q69LRan in the presence of reticulocyte lysate and an energy-regenerating system was dramatically different from that observed for wild-type Ran (Figure 1C). Whereas RanGDP efficiently entered the nucleus, RanQ69L GTP did not and instead only gave some NPC staining that probably represented binding to the nuclear pore protein RanBP2.

Identification of NTF2 as a mediator of Ran import

From Figure 1C one can conclude that the Ran import system is apparently specific for cytoplasmic Ran in its GDP-bound form and that RanQ69LGTP is not an import substrate for this system. We then used this observation to identify the limiting, soluble import receptor for RanGDP. We immobilized wild-type RanGDP and RanQ69LGTP and passed reticulocyte lysate through each of these columns. Depletion of the cytosol with RanQ69LGTP had only a marginal effect on the Ran import activity (Figure 2A), and the eluate from this column showed the typical pattern of β-like transport receptors (Figure 2B). However, as we discuss in detail below, it is important to note that the system was not fully deprived of these receptors. Low affinity Ran binders such as Crm1p were hardly depleted from the cytosol, and also import of, for example, an IBB fusion substrate still occurred with reasonable efficiency, probably using importin β that was mobilized from the permeabilized cells (not shown).

Figure 2A shows that, in contrast to RanQ69L, depletion of the lysate with wild-type RanGDP essentially abolished Ran import activity, indicating that the RanGDP column had specifically depleted a limiting import receptor for Ran from the lysate. Analysis by SDS–PAGE and Coomassie staining revealed only one protein of apparently 10 kDa that had been retained by RanGDP but not by RanQ69L GTP (Figure 2B). This band was of similar size to NTF2 (Figure 2B, compare lanes 4 and 6), and Western blotting with anti-NTF2 antibodies confirmed its identity. Western blotting also confirmed that the immobilized RanGDP had depleted NTF2 from the lysate to undetectable levels, whereas the NTF2 concentrations in the RanQ69LGTP-depleted lysate was approximately the same as in the mock-treated one.

NTF2 forms homodimers and was identified originally as an activity that stimulates nuclear import of NLS-containing proteins (Moore and Blobel, 1994; Paschal and Gerace, 1995). It binds RanGDP and also interacts with

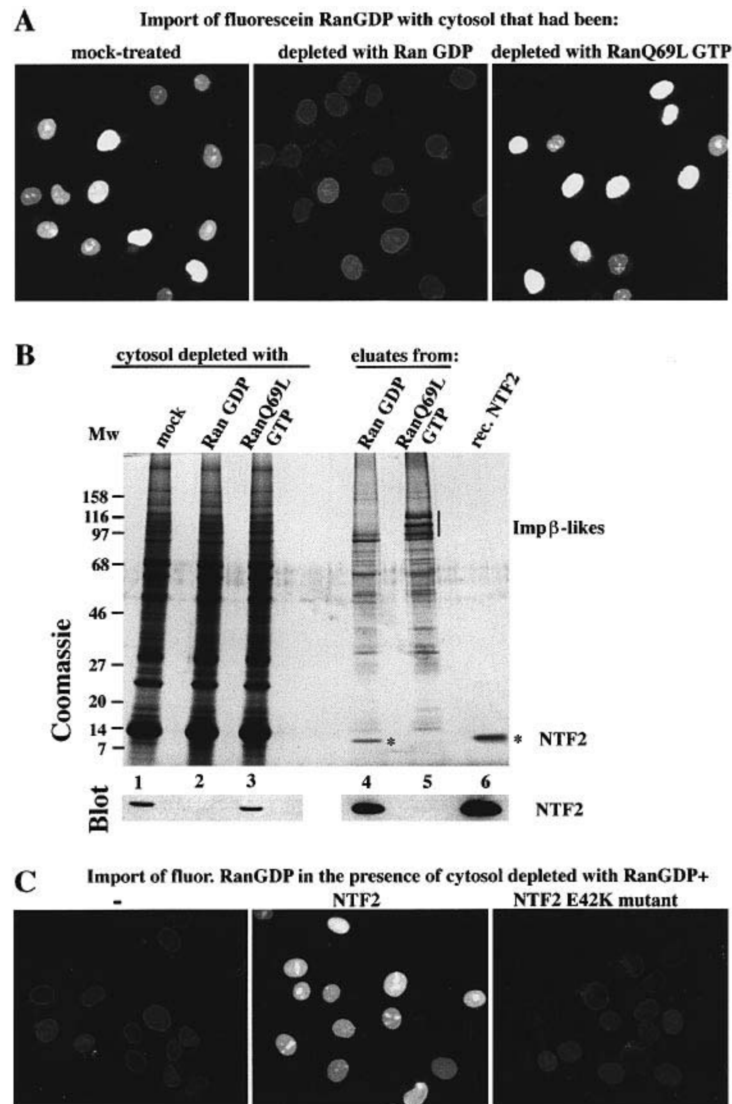


Fig. 2. (**A**) Cytosolic Ran import activity can be depleted with immobilized RanGDP. Import of fluorescein–RanGDP was performed in three different cytosols: reticulocyte lysate that had been mock treated (left), or passed through a wild-type RanGDP column (middle) or a RanQ69L GTP column (right). Analysis was as in Figure 1A. Immobilized RanGDP depleted the Ran import activity specifically. (**B**) Depletion of NTF2 from the cytosol correlates with loss of Ran import activity. The depleted cytosols from (A) and corresponding bound fractions were analysed by electrophoresis on a 7–15% polyacrylamide–SDS gel followed by Coomassie staining or Western blotting with anti-NTF2 antibodies. Load in the bound fractions corresponds to five times the starting material. Immobilized RanGDP, but not RanQ69L GTP, specifically depleted NTF2 from the lysate. (**C**) Recombinant NTF2 restores Ran import activity of the cytosol depleted by immobilized RanGDP. Import of fluorescent RanGDP was performed in the presence of the depleted reticulocyte lysate alone, or with addition of 0.75 μM NTF2 (dimers) or of the E42K NTF2, which is an engineered mutant deficient in RanGDP binding. Wild-type NTF2 stimulated Ran import, whereas the E42K mutant NTF2 did not.

isolated nuclear pore proteins containing xFxFG repeats (Paschal and Gerace, 1995; Clarkson *et al.*, 1996; Nehrbass and Blobel, 1996). However, its precise function in nuclear protein import has remained obscure. A central role in Ran import would be an attractive possibility for NTF2 function, and indeed Figure 2C demonstrates that Ran import activity of the lysate depleted with RanGDP could be restored by addition of recombinant NTF2.

We then tested whether a direct NTF2–RanGDP interaction is required for Ran import using engineered point mutations that interfere with this interaction (Clarkson *et al.*, 1997). The crystal structure of the NTF2–RanGDP complex has been solved and shows that there are two major components of the interaction interface (Stewart *et al.*, 1998). First, the aromatic side chain of Ran F72 inserts into a hydrophobic cavity in NTF2. Secondly, salt bridges are formed between K71 and R76 of Ran with D92, D94 and E42 of NTF2, respectively (residues listed as single letter codes). One of these salt bridges is abolished in the NTF2 E42K mutant, resulting in a drastically reduced affinity for Ran, and crucially X-ray crystallography has shown that this mutation does not alter the overall NTF2 structure (Clarkson *et al.*, 1997). Figure 2C shows that, in contrast to the wild-type NTF2 protein, the NTF2 E42K mutant failed to restore Ran import activity of the depleted cytosol. This was the expected result if the direct NTF2–RanGDP interaction was required for Ran import. The R76E Ran mutation also abolishes the salt bridge to E42 of NTF2. In Figure 3, we compared import of this mutant with that of wild-type Ran using *Xenopus* egg extract as a source of soluble transport factors. The experiment shows that this mutant

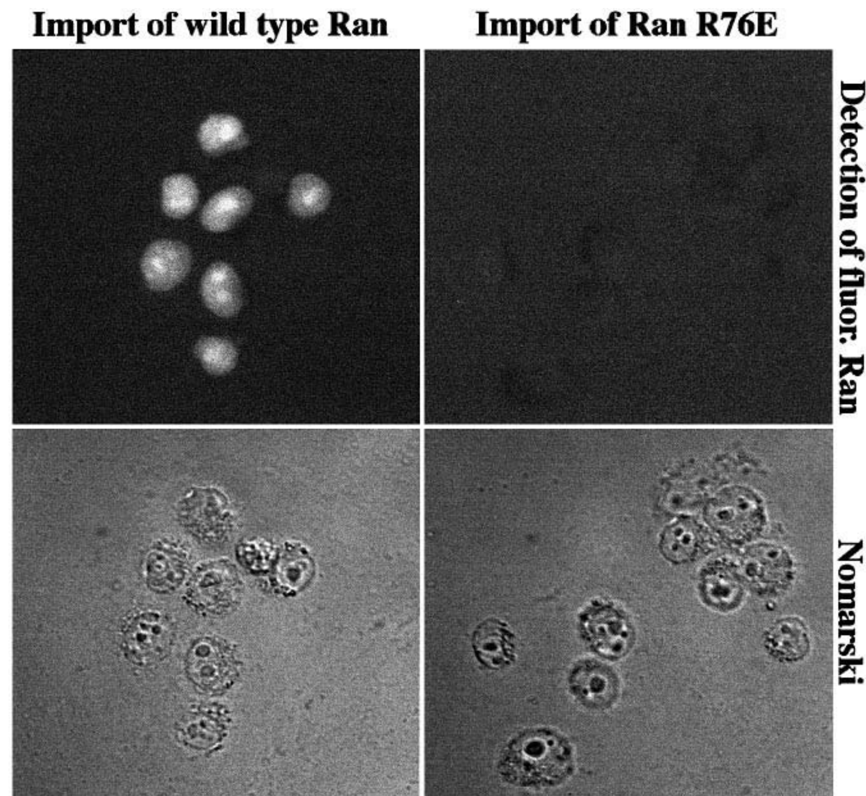


Fig. 3. The NTF2–RanGDP interaction is crucial for Ran import. Permeabilized cells were pre-incubated for 10 min at 18°C with a *Xenopus* egg extract and an energy-regenerating system. Fluorescein–Ran wild-type or fluorescein–RanR76E (1.5 µM) was added and their distribution was analysed 10 min later by confocal microscopy using a 40× dry objective. Upper panels show scans through the unfixed samples, thus allowing direct comparison between nuclear and cytoplasmic concentrations of the fluorescent probes. Positions of nuclei were recorded simultaneously by interference contrast (lower panels, 'Nomarski'). Only wild-type Ran accumulated in the nuclei. The R76E Ran point mutant, which is deficient in NTF2 binding, was not imported to a significant extent.

Ran failed to accumulate in the nuclei, confirming that the direct NTF2–RanGDP interaction is crucial for Ran import. In addition, we can conclude that NTF2 plays a key role in Ran import not only when using reticulocyte lysate as a source of transport factors, but also in the *Xenopus* system.

Nucleoplasmic accumulation of Ran also requires importin β family transport receptors

We next investigated whether the interaction with NTF2 would be sufficient for Ran import. Fluorescein–RanGDP was incubated with permeabilized cells and an energy-regenerating system (Figure 4). Without a further addition, Ran gave a weak staining at the NPCs and in the nucleoli. Both signals were increased ~3-fold upon NTF2 addition. There are probably several distinct populations of Ran at the NPC (which, for example, represent Ran on its way into the nucleus or Ran exiting the nucleus while bound to importin β family transport receptors etc.). Figures 4 and 2 together suggest that one of these Ran populations binds to NPCs via NTF2 and constitutes an intermediate of Ran import into the nucleus.

Addition of NTF2 alone also stimulated complete nuclear import of Ran as indicated by the increased signal in the nucleoli. However, it was not sufficient to reconstitute the *in vivo* situation where Ran is concentrated in the nucleoplasm. We therefore tested whether any of the established nuclear transport factors would stimulate nucleoplasmic accumulation of Ran. RanBP1 had no effect (not shown) but, when exogenous RanBP7 was added together with NTF2, strong nuclear accumulation of Ran was observed (Figure 4). In addition, the intranuclear Ran pattern changed from a nucleolar to a mainly nucleoplasmic staining, indicating that RanBP7 and Ran form a complex in the nucleus. This stimulation of nuclear Ran accumulation was not only observed with RanBP7 but also with transportin, importin β (Figure 4), an N-terminal importin β fragment that cannot bind importin α (see below, Figure 7B) and with other importin β family transport receptors such as exportin t and RanBP5 (not shown). These data suggest that stimulation of Ran import is an intrinsic activity of importin β family transport receptors. It is important to note that in the presence of transportin but absence of NTF2, no significant nuclear accumulation of Ran was observed (compare panels 'Transportin' and 'NTF2+Transportin'), which emphasizes the central role of NTF2 in Ran import.

The import reactions in Figure 4 were each initiated by the addition of fluorescein–Ran to the samples that already contained all other components. It is important to note that nuclear accumulation of Ran was transient and reached a maximum after only ~2 min incubation. The subsequent decrease in Ran nuclear staining probably reflects efficient nuclear export of RanGTP complexed to the importin β family transport receptors.

Energy requirement of Ran import

The various nucleocytoplasmic transport events have different energy requirements. Nuclear export of tRNA, NES-

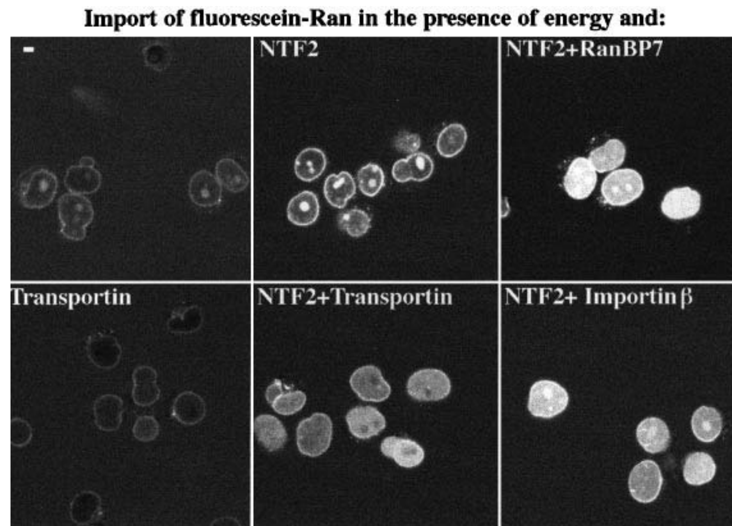


Fig. 4. Ran accumulates in the nucleoplasm when NTF2 and importin β family transport receptors are both present. Import of fluorescein–RanGDP was studied in the presence of an energy-regenerating system (0.5 mM GTP, 0.5 mM ATP, 10 mM creatine phosphate and 50 µg/ml creatine kinase), 0.7 µM Rna1p (RanGAP to keep cytoplasmic Ran in the GDP-bound form) and with the following additions as indicated: 2 µM NTF2 (dimers), 1.7 µM transportin, 2 µM importin β, 1.7 µM *Xenopus* RanBP7. Import was initiated at 18°C by the addition of 1.5 µM fluorescein–Ran. The panels show scans through the unfixed samples at a 90 s time point. A 63× objective with water immersion was used. Note, NTF2 alone increased the Ran accumulation at NPCs and in the nucleoli, but the nucleoplasmic Ran signal remained weak. Nucleoplasmic accumulation of Ran is observed in the presence of NTF2 plus either transportin, importin β or RanBP7.

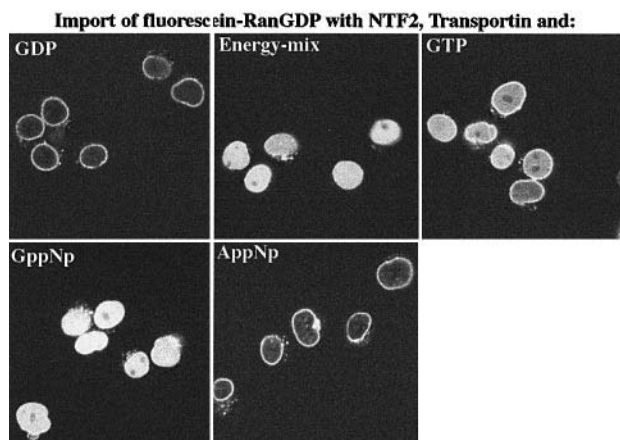


Fig. 5. Nucleotide requirement of Ran import. Import of fluorescein–RanGDP into nuclei of permeabilized cells was performed in the presence of 2 µM NTF2 (dimers), 1.7 µM transportin and 0.5 mM of the indicated nucleotides. 'Energy mix' stands for a mixture of 0.5 mM GTP, 0.5 mM ATP, 10 mM creatine phosphate and 50 µg/ml creatine kinase. Analysis was as in Figure 4. With GDP or AppNp, Ran is detected mainly at the nuclear envelope. In contrast, addition of either GTP, an energy-regenerating system or the non-hydrolysable GTP analogue GppNp allowed accumulation of Ran inside the nuclei.

containing proteins or importin α, for example, requires nuclear RanGTP, but not GTP hydrolysis by Ran (Izaurralde *et al.*, 1997; Richards *et al.*, 1997). NPC passage of 'empty' nuclear transport receptors such as importin β, transportin or exportin-t is apparently energy independent (Kose *et al.*, 1997; Kutay *et al.*, 1998; Nakielny and Dreyfuss, 1998). In contrast, import of NLS-containing proteins appears to require GTP hydrolysis by Ran (Melchior *et al.*, 1993a; Moore and Blobel, 1993; Palacios *et al.*, 1996; Weis *et al.*, 1996). We therefore investigated the energy requirements for Ran import. As illustrated in Figure 5, we examined Ran import in the presence of transportin, NTF2 and a range of different nucleotides. With GDP, no detectable import was observed and Ran remained at the nuclear envelope. In contrast, either an energy-regenerating system with ATP and GTP, or GTP alone, or the non-hydrolysable GTP analogue GppNp all supported import to similar levels, whereas the ATP analogue AppNp had no effect. This would be consistent with a model in which nucleotide exchange to generate RanGTP, but not GTP hydrolysis itself, is a prerequisite for nuclear accumulation of Ran.

Behaviour of NTF2 during nuclear import of Ran

Because the RanGDP–NTF2 interaction is crucial for Ran import, we investigated how NTF2 itself behaved during Ran import. For this purpose, we labelled NTF2 with fluorescein and added it to permeabilized cells (Figure 6). Without further addition, only a very weak binding to NPCs was evident and this weak signal was essentially lost upon transportin addition. However, when RanGDP was added, NTF2 accumulated brightly at the NPCs, consistent with recent observations using gold-labelled NTF2 microinjected into *Xenopus* oocytes (Feldherr *et al.*, 1998). The increased NTF2 accumulation we observed in the presence of Ran indicated that NTF2 binds RanGDP and a constituent(s) of the NPC in a co-operative manner. It further suggests that the NPC–NTF2 interaction is dynamic and that NTF2 may be released from the NPC after having delivered Ran into the nucleus. The NPC signal of NTF2 became slightly weaker when transportin was added together with RanGDP. It is important to note that there was no significant intranuclear NTF2 signal even in the presence of energy and transportin, when Ran would accumulate inside the nuclei (compare corresponding panels in Figures 4 and 6). This suggests that the separation of Ran from NTF2 occurs either at the NPC itself, or alternatively immediately after nuclear entry, and is then followed by a rapid return of NTF2 to the cytoplasm. A possible mechanism for Ran release from NTF2 during import might be nucleotide exchange to

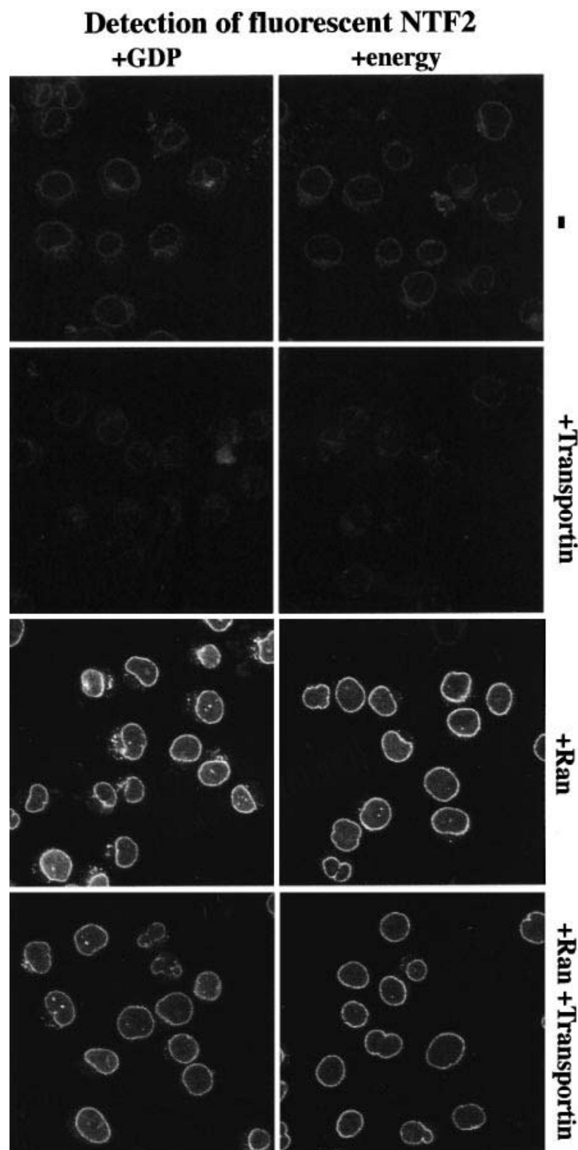


Fig. 6. Distribution of NTF2 during Ran import. Fluorescein-labelled NTF2 (2 μM; dimer) was incubated with permeabilized cells. Where indicated, the following additions had been made: 0.5 mM GDP, an energy-regenerating system (see Figure 4), 1.7 μM transportin, 2.3 μM RanGDP. Nuclei were fixed after 6 min with 4% paraformaldehyde, spun onto coverslips and analysed by confocal microscopy using a 63× objective and oil immersion. NPC binding of NTF2 was greatly enhanced by the addition of RanGDP, slightly reduced by transportin addition, and apparently unaffected by the presence of nucleoside triphosphates.

RanGTP for which NTF2 has no detectable affinity (see Paschal *et al.*, 1996; Figure 2B).

The role of importin β family transport receptors in nuclear accumulation of Ran

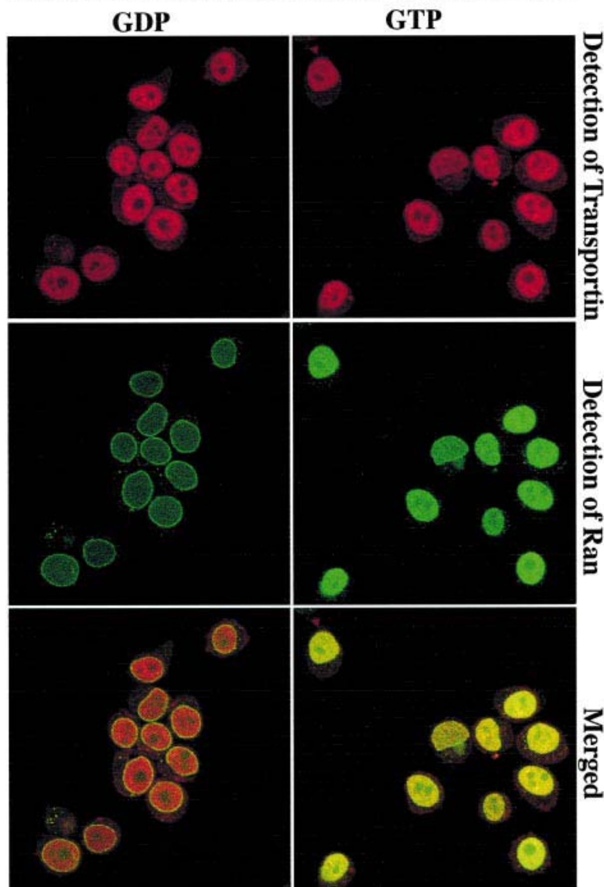
There are three possible models that could account for how importin β family receptors co-operate with NTF2 in Ran import and one can, in principle, discriminate between them experimentally. First, a trimeric complex consisting of an importin β family receptor, NTF2 and RanGDP might be the species that is moved through the NPC. In this case, import of Ran would be expected to be tightly coupled to the nuclear entry of the importin β family transport receptor. However, when we pre-incubated Texas red-labelled transportin with NTF2 and fluorescein–RanGDP and added the mixture together with either GDP or GTP to permeabilized cells (Figure 7A), in both cases transportin accumulated to similar levels in the nuclei. In contrast, nuclear accumulation of Ran was weak in the presence of GDP, but strong with GTP. This observation, therefore, was not consistent with the hypothesis that Ran is moved into the nucleus as a transportin–NTF2–RanGDP complex. Further arguments against this model are that NTF2 does not accumulate with transportin in the nuclei (see Figure 6), that no stable transportin–NTF2–RanGDP complex can be formed in solution (not shown) and that such complexes probably also do not form at NPCs because transportin promotes binding of neither NTF2 (see Figure 6) nor RanGDP to NPCs [not shown, but see Görlich *et al.* (1996b)].

A second model would be that the importin β family transport factors need to bind NPCs and thereby trigger a 'gating' that allows NPC passage of the NTF2–Ran complex. In this case, no direct contact between NTF2 and the importin β family receptor would be required during NPC passage. However, one would anticipate that importin β mutants or fragments would still stimulate Ran import provided they bound NPCs tightly. Figure 7B shows that an importin β fragment comprising the 462 N-terminal residues promoted nuclear accumulation of Ran, but importin β 45–462 did not. The difference between these two constructs lies in the fact that importin β 45–462 does not bind RanGTP and therefore binds irreversibly to NPCs (Kutay *et al.*, 1997a). Therefore, these data do not provide direct support for a gating model and suggest that NPC binding of the importin β family transport factor is not sufficient to promote Ran import. In addition, these data indicate that the capacity of the importin β family receptor to bind RanGTP is necessary to promote Ran import.

If the importin β family transport receptors are not directly involved in the actual translocation of the NTF2–RanGDP complex through the NPC, then there remains a third possibility. The importin β family transport receptors might come into play after NPC passage and stimulate nuclear accumulation of RanGTP simply by providing binding sites inside the nucleus. In fact, this model is consistent with all observations described above.

Discussion

Importin β family transport receptors, such as importin β, transportin, exportin-t, exportin-1 (CRM1) and RanBP7, play a key role in transport of proteins and RNAs between nucleus and cytoplasm. All of these receptors bind RanGTP in a common way and use this RanGTP binding to regulate interactions with cargo molecules such that importins confer import into and exportins export out of the nucleus (reviewed by Dahlberg and Lund, 1998; Görlich, 1998; Izaurralde and Adam, 1998; Mattaj and Englmeier, 1998). These receptors enter the nucleus without RanGTP and leave the nucleus as RanGTP complexes, and thereby continuously deplete nuclear RanGTP. Nevertheless, Ran is a predominantly nuclear protein (Bischoff and Ponstingl, 1991b), which implies that the replenishment of the nuclei with Ran must be a very efficient process. We have addressed here the question of how Ran



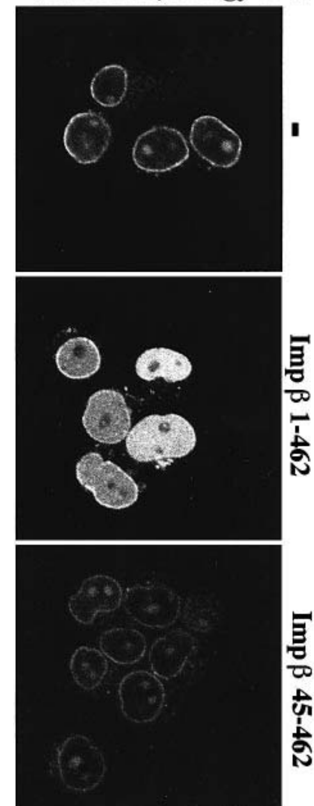


Fig. 7. (**A**) Import of Ran is not coupled directly to nuclear entry of an importin β family transport receptor. Fluorescein–Ran (1.5 μM), Texas red–transportin (1.7 μM) and unlabelled NTF2 (2 μM; dimers) were pre-incubated and added to permeabilized cells. As indicated, either 1 mM GDP or GTP was also added. After 6 min incubation, the samples were fixed, nuclei were spun onto coverslips, and the distributions of Ran and transportin detected by confocal microscopy in the fluorescein and Texas red channels, respectively. Transportin efficiently entered the nuclei with either GDP or GTP present. In contrast, Ran accumulated inside the nuclei only when GTP had been added. (**B**) Importin β requires a functional RanGTP-binding site to promote nuclear accumulation of Ran. Import of fluorescein–Ran into nuclei of permeabilized cells was assayed in the presence of an energy-regenerating system, 2 μM NTF2 and, where indicated, 2 μM of the importin β 1–462 or 45–462 fragment. The figure shows scans through the unfixed samples. Note that the 1–462 importin β construct is functional in RanGTP binding and promoted nuclear accumulation of Ran. In contrast, the importin β 45–462 construct is deficient in RanGTP binding and failed to stimulate Ran import.

is re-imported into the nucleus. Ran's size of 25 kDa is below the diffusion limit of NPCs of ~40–60 kDa (reviewed by Bonner, 1978). However, we show here that Ran does not rely on simple diffusion for nuclear entry; instead, the import of Ran is carrier mediated and so is similar to the import of other small proteins such as histones or ribosomal proteins. For reasons of stoichiometry, it appears to be very unlikely that Ran could possibly use an importin β family receptor for its own import, and we show here that instead NTF2 is a principal mediator of Ran import. We found the NTF2 concentration to be rate limiting for Ran import into nuclei of permeabilized cells. In addition, point mutants in either NTF2 or Ran that interfere with the RanGDP–NTF2 interaction also interfered with Ran import. Recent studies (Feldherr *et al.*, 1998) have shown that gold-labelled NTF2 microinjected into *Xenopus* oocytes accumulates strongly at NPCs, which would also be consistent with its facilitating Ran's binding to and translocation through the NPC into the nucleus.

NTF2 was identified originally as an activity that stimulates import of NLS-containing proteins (Moore and Blobel, 1994; Paschal and Gerace, 1995). Nuclear import of Ran is clearly a pre-requisite for protein import along the importin-dependent pathway. Therefore, the stimulatory effect of NTF2 on NLS import can probably be explained, at least in part, by its raising nuclear RanGTP levels. Although exogenous NTF2 is not essential for *in vitro* nuclear import of NLS-containing proteins, it does appear to be required for efficient transport in the absence of high levels of added Ran (see, for example, Clarkson *et al.*, 1997). In this context, it is also interesting to note that the R76E Ran mutant that fails to bind NTF2 (see Figure 3) does support nuclear import of NLS proteins (not shown). However, the optimum concentration of R76E in this assay is shifted to a 3–5 times higher concentration as compared with wild-type Ran. This emphasizes that passive diffusion of Ran into the nucleus remains a low efficiency alternative when NTF2-mediated nuclear import of Ran fails.

NTF2 is conserved in evolution, and the *S.cerevisiae NTF2* gene is essential for viability and nuclear import

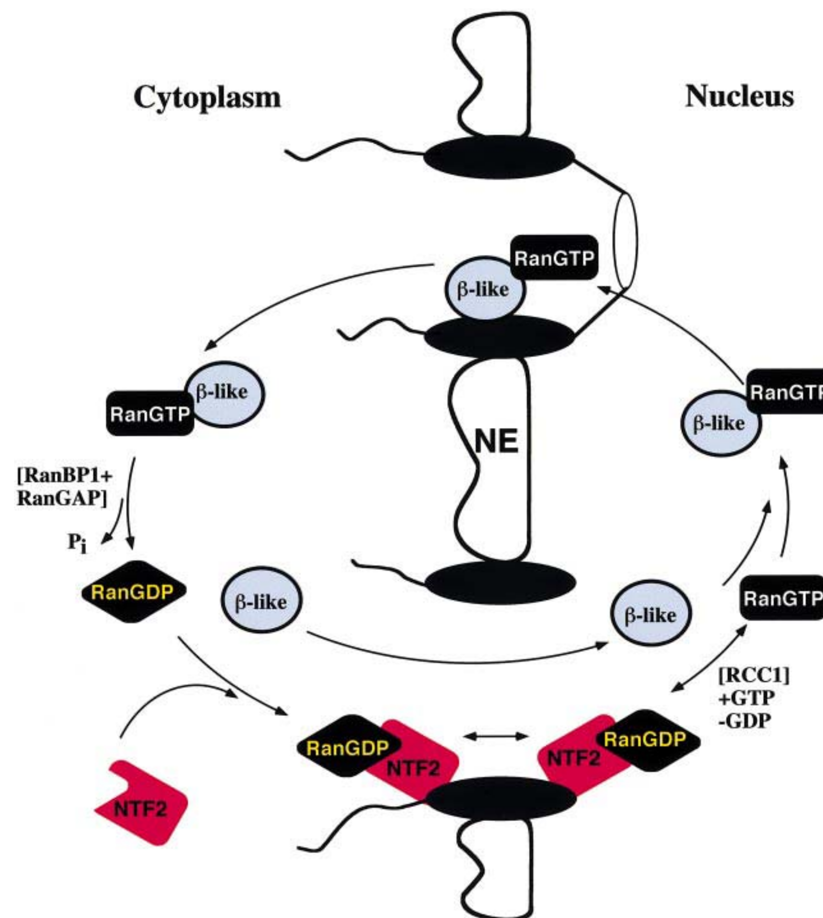


Fig. 8. Schematic illustration of Ran's nucleocytoplasmic transport cycles. RanGTP exits from the nucleus bound to import/export factors such as importin β, transportin, RanBP7 and CAS. Cytoplasmic RanGAP and RanBP1 together remove RanGTP from the import/export receptor and trigger GTP hydrolysis to form RanGDP. RanGDP then binds to NTF2 and is re-imported through NPCs into the nucleus where the complex dissociates and RanGTP is regenerated by RCC1. This re-importation step probably involves an initial targeting of Ran to the NPC (through the ability of NTF2 to bind RanGDP and FxFxG nucleoporins simultaneously) followed by a translocation step for which, like the translocation of other substrates, precise molecular details remain obscure.

(Corbett and Silver, 1996). However, the null allele can be suppressed by overexpression of Gsp1p, the yeast homologue of Ran (Paschal *et al.*, 1997). In the absence of NTF2, one would anticipate that nuclear Gsp1p would be reduced, which could be compensated for by an increased total cellular Gsp1p concentration. This points again to a low efficiency alternative to NTF2-mediated nuclear import of Ran, which probably is simple diffusion, since Ran's size of 25 kDa is below the exclusion limit observed for NPCs. However, there might also be a still uncharacterized active mechanism of nuclear Ran accumulation that is independent of NTF2. Interestingly, the genetic interaction between NTF2 and Gsp1p resembles that between yeast importin α (SRP1) and its nuclear export receptor CAS/Cse1 (Kutay *et al.*, 1997b). The *cse1-1* cold-sensitive allele could be suppressed by overexpression of Srp1p (Xiao *et al.*, 1993) which then probably compensates for the shortage of Srp1p in the cytoplasm caused by the Srp1p export defect.

Our data suggest the model illustrated in Figure 8 to account for Ran's nucleocytoplasmic transport cycles. Initially, NTF2 binds cytoplasmic RanGDP which results in an increased affinity of NTF2 for components of the NPC. The NTF2–RanGDP complex then docks on the cytoplasmic side of the NPC and is transferred to the nuclear side. The precise molecular mechanism of this translocation currently is obscure, as indeed it is for the translocation through NPCs of any other substrate (reviewed by Nakielny *et al.*, 1997; Dahlberg and Lund, 1998; Görlich, 1998; Izaurralde and Adam, 1998; Mattaj and Englmeier, 1998). The translocation of the NTF2–RanGDP complex does not appear to be coupled directly to nucleoside triphosphate hydrolysis although the Second Law of Thermodynamics would be violated if energy was not consumed at some stage of the cycle. Therefore, RanGDP is not actively 'pumped' into the nucleus, but instead its nuclear entry probably proceeds through a series of reversible equilibrium states, resulting in a kind of facilitated diffusion. An interesting parallel to this is the NPC passage of cargo-free importin β family transport receptors which also constitutes a highly selective but apparently energy-independent translocation (Kose *et al.*, 1997; Kutay *et al.*, 1998; Nakielny and Dreyfuss, 1998).

Following translocation through the NPC, Ran needs to be released from NTF2 into the nucleus. A plausible mechanism would be nucleotide exchange to generate RanGTP for which NTF2 has no detectable affinity (Paschal *et al.*, 1996; Figure 2B). This hypothesis is supported by the observations that free GTP is required for nuclear Ran accumulation (Figures 5 and 7A) and that

loss of RCC1 function abolishes the predominantly nuclear localization of Ran (Ren *et al.*, 1993). RanGDP could transiently dissociate from NTF2 into the nucleus and its conversion to RanGTP by RCC1 then prevent re-binding. Alternatively, RCC1-mediated nucleotide exchange could actively displace Ran from its import carrier. Further experimental work will be required to distinguish between these possibilities

The NTF2 carrier system coupled to nucleotide exchange is apparently sufficient to supply importin β family transport receptors with RanGTP in the nucleus and, in principle, should be able to maintain free nuclear RanGTP at a low micromolar concentration which should suffice to saturate importin β family receptors which normally bind RanGTP with a lower nanomolar dissociation constant (see for example Görlich *et al.*, 1997; Kutay *et al.*, 1997b). However, the phenomenon of Ran being concentrated in the nucleoplasm of, for example, intact HeLa cells, seems to require abundant nuclear binding sites which apparently largely originate from importin β family transport receptors. This would imply that a significant proportion of nuclear Ran is indeed already transport receptor-bound. In terms of abundance, this seems to be a reasonable hypothesis. For *Xenopus* eggs, we would estimate that the various importin β family transport receptors together account for a total concentration of 10–20 μM (alone importin β, RanBP7 and CAS account each for ~3 μM). This would be the same order of magnitude as for Ran in HeLa cells of ~7 μM (Bischoff and Ponstingl, 1991b). In this context, it might also be worth noting that several of the very abundant importin β family transport receptors, such as CAS, exportin-t or transportin, are clearly concentrated in the nucleoplasm (Fridell *et al.*, 1997; Kutay *et al.*, 1998; our unpublished data).

The next step in Ran's transport cycle would be to exit from the nucleus as RanGTP–importin or RanGTP–exportin–export substrate complexes. Cytoplasmic RanBP1 (RanBP2) together with RanGAP1 then disassemble the export complexes, trigger GTP hydrolysis and allow the released RanGDP to enter another transport cycle. Whether this one molecule of GTP that is hydrolysed per transport cycle is sufficient to drive the cycle remains to be determined.

Materials and methods

Recombinant protein expression and purification

Preparation of recombinant importin β, transportin, RanBP1 and RNA1p have been described previously (Görlich *et al.*, 1996b, 1997). N-terminally his-tagged Ran (wild-type and Q69L) was expressed in *Escherichia coli*, purified on Ni-NTA–agarose and then applied to Mono S. Ran was eluted from Mono S with a linear gradient from A: 20 mM potassium phosphate pH 7.0, 0.5 mM $MgCl_2$, 5% glycerol to B: 0.5 M potassium phosphate, 0.5 mM $MgCl_2$. RanGDP elutes earlier in this procedure than RanGTP (F.R.Bischoff and D.Görlich, unpublished). R76E Ran cDNA was produced by PCR-based site-specific mutagenesis as described (Clarkson *et al.*, 1997) and sequenced to confirm that this was the only mutation introduced. This cDNA was introduced into the pET-based vector pMW172 and transformed into BL21(DE3) cells as described (Clarkson *et al.*, 1997). Untagged Ran (wild-type and R76E) were purified as follows: the bacterial lysate was prepared in 20 mM HEPES–KOH pH 7.0, 5% glycerol, 2 mM dithiothreitol (DTT) and applied to SP Sepharose FF equilibrated in the same buffer. Ran was eluted from the column with a linear gradient from A: 20 mM potassium phosphate pH 7.0, 0.5 mM $MgCl_2$, 50 μM GDP, 5% glycerol to B: 0.5 M potassium phosphate, 0.5 mM $MgCl_2$. Peak fractions were pooled and purified further on a 16/60 Superdex 75 column equilibrated in 50 mM potassium phosphate pH 7.0, 5% glycerol, 0.5 mM $MgCl_2$. The characterization and binding properties of R76E Ran will be described in detail elsewhere. NTF2 was expressed untagged from a pET expression vector (Kent *et al.*, 1996). NTF2 was precipitated from the bacterial lysate with ammonium sulfate (50% saturation), the pellet was dissolved in 50 mM Tris pH 8.0, NTF2 was then bound 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. Alternatively, wild-type and E42K NTF2 were prepared as described by Clarkson *et al.* (1997).

Fluorescein labelling

Ran or Ran mutants loaded with GDP or GTP were labelled in a potassium phosphate buffer pH 7.0 containing 2 mM magnesium acetate. An equimolar amount of fluorescein-5′-maleimide (dissolved in dimethyl formamide) was added and the reaction was allowed to proceed for 2 h on ice. Non-incorporated label was removed by gel filtration on a Nap5 column (Pharmacia) equilibrated with 50 mM potassium phosphate pH 7.0, 5 mM magnesium acetate, 250 mM sucrose and 200 μM of the corresponding nucleotide. Modification was quantitative for the GDP-bound forms of Ran and ~60% for GTP-bound forms. Labelling of NTF2 was also with fluorescein-5′-maleimide, aiming at one fluorescein molecule per NTF2 homodimer. Fluorescent IBB core fusion has been described previously (Görlich *et al.*, 1996b).

Antibodies

Anti-NTF2 antibodies were raised in rabbits against the recombinant protein and used after affinity purification on the immobilized antigen.

Depletion of cytosol

Starting material was a post-ribosomal supernatant prepared from a reticulocyte lysate (#L4151 Promega). For each sample, 1.5 ml were passed through IgG–Sepharose to which either zz-RanGDP or zzRanQ69L had been pre-bound (Kutay *et al.*, 1997b). The columns had been equilibrated in 20 mM HEPES–KOH pH 7.5, 6 mM magnesium acetate, 120 mM potassium acetate, 250 mM sucrose, 0.5 M EGTA. Ran wild-type had been brought to the GDP-bound state using the RanGTPase-activating protein RNA1 (from *Schizosaccharomyces pombe*). The nucleotide of RanQ69L had been exchanged to GTP using the nucleotide exchange factor RCC1 and GTP. The flow-through fractions were pooled and adjusted to identical protein concentrations as estimated from their UV absorbance. Bound fractions were eluted after extensive washing by 1.5 M $MgCl_2$. They were precipitated with 90% isopropanol (final concentration) and analysed together with the flow-through fractions as described in the legend to Figure 2.

Import assays

Import into nuclei of permeabilized cells was performed in suspension at 18°C essentially as described previously (Görlich *et al.*, 1996b, 1997; Kutay *et al.*, 1997b). The import buffer had the following composition: 2 mg/ml nucleoplasmin core (to block non-specific binding), 20 mM HEPES–KOH pH 7.5, 120 mM potassium acetate, 5 mM magnesium acetate, 250 mM sucrose, 0.5 mM EGTA. As GppNp frequently is contaminated with GDP, it was used after purification on Mono Q (operated in potassium phosphate buffer pH 7.0).

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