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Characterization of the nuclear protein import mechanism using Ran mutants with altered nucleotide binding specificities

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The small nuclear GTP binding protein Ran is required for transport of nuclear proteins through the nuclear pore complex (NPC). Although it is known that GTP hydrolysis by Ran is essential for this reaction, it has been unclear whether additional energy-consuming steps are also required. To uncouple the energy requirements for Ran from other nucleoside triphosphatases, we constructed a mutant derivative of Ran that has an altered nucleotide specificity from GTP to xanthosine 5′ triphosphate. Using this Ran mutant, we demonstrate that nucleotide hydrolysis by Ran is sufficient to promote efficient nuclear protein import *in vitro*. Under these conditions, protein import could no longer be inhibited with non-hydrolysable nucleotide analogues, indicating that no Ran-independent energy-requiring steps are essential for the protein translocation reaction through the NPC. We further provide evidence that nuclear protein import requires Ran in the GDP form in the cytoplasm. This suggests that a coordinated exchange reaction from Ran-GDP to Ran-GTP at the pore is necessary for translocation into the nucleus.

Keywords: nuclear pore complex/nuclear protein import/Ran/translocation/XTP

Introduction

Transport of proteins into the nucleus occurs through a large, multiprotein structure called the nuclear pore complex (NPC). The NPC provides the framework that allows the translocation of karyophilic proteins through the nuclear membrane into the nucleoplasm (Forbes, 1992; Goldberg and Allen, 1995). Substrates for this transport pathway are proteins that contain in their primary sequence a nuclear localization sequence (NLS) which is usually characterized by one or two stretches of basic amino acid residues (Dingwall and Laskey, 1991). The transport reaction itself is a multistep process that requires energy (Newmeyer and Forbes, 1988; Richardson *et al.*, 1988; reviewed in Melchior and Gerace, 1995; Görlich and Mattaj, 1996).

The development of an *in vitro* transport system, based on digitonin-permeabilized mammalian cells (Adam *et al.*, 1990), has permitted the identification of soluble factors that mediate the protein import reaction *in vitro* (Adam and Gerace, 1991; Moore and Blobel, 1992; reviewed by Sweet and Gerace, 1995). The NLS motif is recognized in the cytoplasm by a heterodimeric receptor complex which targets the import substrate to the nuclear envelope (reviewed by Görlich and Mattaj, 1996). The smaller subunit of the NLS receptor is primarily responsible for NLS binding and has been identified from different species. It is a 50–60 kDa factor which belongs to a large protein family, all showing homology to the Srp1p protein from *Saccharomyces cerevisiae* (Yano *et al.*, 1992). The different homologues have been named the NLS receptor (Adam and Gerace, 1991), importin α (Görlich *et al.*, 1994), hSRP1α/Rch1 (Weis *et al.*, 1995; Cuomo *et al.*, 1994), hSRP1/NPI (Cortes *et al.*, 1994; O'Neil and Palese, 1995), karyopherin α (Moroianu *et al.*, 1995a), m-importin (Imamoto *et al.*, 1995b) or pendulin/OHO31 (Küssel and Frasch, 1995; Török *et al.*, 1995). The second subunit is 97 kDa in size, can bind directly to different repetitive motifs in nuclear pore proteins (Iovine *et al.*, 1995; Radu *et al.*, 1995; Rexach and Blobel, 1995) and is thus thought to mediate the interaction of the receptor complex with the NPC (Görlich and Mattaj, 1996). This factor has also been identified independently from different species and has been called either karyopherin β (Radu *et al.*, 1995), importin β (Görlich *et al.*, 1995a), p97 (Chi *et al.*, 1995; Weis *et al.*, 1996), PTAC 97 (Imamoto *et al.*, 1995a) or Kap95p (Enenkel *et al.*, 1995). The two subunits of the receptor complex seem to dissociate during the translocation through the NPC since the 60 kDa subunit is able to enter the nucleus together with the NLS substrate, whereas the p97 component remains bound at the nuclear envelope (Görlich *et al.*, 1995b; Moroianu *et al.*, 1995b). In the nucleus, the small subunit dissociates from its cargo and recycles back to the cytoplasm (Görlich *et al.*, 1996; Weis *et al.*, 1996). A short amino-terminal domain of the 60 kDa subunit is responsible for the interaction with p97, and this domain can also promote nuclear uptake of a cytoplasmic reporter protein by directly targeting it to p97 (Görlich *et al.*, 1996; Weis *et al.*, 1996).

Two additional factors have been identified which mediate the translocation of the substrate–NLS receptor complex through the NPC *in vitro*. One is the small GTP binding protein Ran (Melchior *et al.*, 1993a; Moore and Blobel, 1993), a predominantly nuclear protein (Bischoff and Ponstingl, 1991b) with homology to Ras and to other small GTPases (Rush *et al.*, 1996). Experiments both in the *in vitro* transport system (Moore and Blobel, 1993; Melchior *et al.*, 1993a) and in yeast (Schlenstedt *et al.*, 1995), have demonstrated that GTP hydrolysis by Ran is essential for nuclear protein import. Ran, like other GTPases, undergoes a constant cycle of GTP hydrolysis and nucleotide exchange, regulated by GTPase-activating proteins (GAPs) and by guanosine nucleotide exchange factors (GEFs), respectively (Rush *et al.*, 1996). A cyto-

plasmic Ran GAP activity has been identified as the product of the RNA1 gene in yeast and its homologues from mouse and humans (Bischoff *et al.*, 1994, 1995a,b; Rush *et al.*, 1996). The only known GEF for Ran is the RCC1 protein (Bischoff and Ponstingl, 1991a; Dasso, 1993). Interestingly, RCC1 is a mainly nuclear protein (Bischoff and Ponstingl, 1991b; Dasso, 1993), whereas Rna1p is localized in the cytoplasm (Hopper *et al.*, 1990; Melchior *et al.*, 1993b; Bischoff *et al.*, 1995a), suggesting that Ran has to shuttle between the two compartments to interact with these factors (Izaurralde and Mattaj, 1995). However, a nuclear protein, Spa1, recently has been identified that exhibits GAP activity towards Ran (Hattori *et al.*, 1995). Mutations both in RCC1 and in Rna1p were shown to interfere with nuclear protein import, indicating a role for both of these proteins in this transport process (Tachibana *et al.*, 1994; Corbett *et al.*, 1995). It was shown that Ran, in its GTP-bound state, can bind directly to the p97 transport factor (Rexach and Blobel, 1995; Floer and Blobel, 1996; Lounsbury *et al.*, 1996) and this interaction leads to the dissociation of the two subunits of the NLS receptor complex in a solution binding assay (Rexach and Blobel, 1995). Also, binding of Ran to nuclear pore proteins has been demonstrated (Dingwall *et al.*, 1995; Wu *et al.*, 1995; Yokoyama *et al.*, 1995) and Ran was visualized at the NPC both by immunofluorescence studies and by electron microscopy (Melchior *et al.*, 1995; D.Görlich and R.Bischoff, personal communication). Apart from its role in nuclear transport, the GDP–GTP cycle of Ran has also been implicated in many other cellular processes including RNA processing, RNA export, DNA synthesis and the regulation of the cell cycle (Rush *et al.*, 1996).

The other transport factor that was shown to stimulate the translocation reaction *in vitro* is the small protein p10/NTF2 (Moore and Blobel, 1994; Paschal and Gerace, 1995). This factor can also interact with nuclear pore proteins (Paschal and Gerace, 1995; Nehrbass and Blobel, 1996), and it was demonstrated that yeast p10 is able to bind to Ran-GDP and to Kap95p, the homologue of p97 from yeast, and, thus, a pentameric complex including p10, Ran-GDP, the two NLS receptor subunits and the nuclear pore protein Nup36 could be formed *in vitro* (Nehrbass and Blobel, 1996).

Little is known about the actual translocation process by which the NLS substrate is transported over a distance of ~100 nm through the NPC (Forbes, 1992; Görlich and Mattaj, 1996). Although it was shown that GTP hydrolysis by Ran is necessary for this translocation process, it has been unclear whether other energy-consuming steps are required for nuclear protein import. In fact, experiments with non-hydrolysable GTP analogues indicated that GTP hydrolysis by Ran might be a very early event in protein import (Melchior *et al.*, 1995). It was also suggested that downstream of Ran further NTPases are required, e.g. for the channel gating step or for the opening of the pore structure to allow the passage of large NLS-containing substrates (Melchior *et al.*, 1995; Sweet and Gerace, 1996, for a recent discussion, see Powers and Forbes, 1994; Melchior and Gerace, 1995; Görlich and Mattaj, 1996). An alternative model was presented recently, in which nuclear protein import would occur as a stochastic process, in which repeated steps of docking and undocking reactions allow the translocation through the long NPC channel (Rexach and Blobel, 1995). However, from this model, it is less obvious how a directional movement of an NLS substrate into the nucleoplasm can be achieved.

Here, using a xanthosine 5′ triphosphate-dependent Ran mutant, we demonstrate that nucleotide hydrolysis by Ran alone provides a sufficient energy requirement for efficient nuclear protein import *in vitro*. We provide evidence that the transport reaction requires Ran in the GDP-bound state and free GTP in the cytoplasm and that cytoplasmic Ran-GTP is not able to promote nuclear protein import.

Results

The mutant Ran D125N protein has an altered nucleotide specificity from GTP to XTP

Since high levels of both endogenous GTPase and ATPase activities are still present in digitonin-permeabilized cells (K.Weis, unpublished), it is difficult to uncouple the energy requirements for Ran from other NTPases which might play a role in nuclear transport. Therefore, in order to characterize the energy requirements during nuclear protein import, a mutant derivative of the protein import factor Ran was constructed which altered the nucleotide specificity of Ran from GTP to xanthosine 5′ triphosphate (XTP).

The protein superfamily of GTPases is characterized by several conserved sequence motifs which are responsible for guanine nucleotide binding and which form direct contacts with either the nucleotide itself or with the cofactor Mg^{2+} (Wittinghofer and Pai, 1991). The crystal structure had revealed that Ran, like many other GTP binding proteins, binds to guanine by forming a double hydrogen bond between the aspartic acid residue in the highly conserved NKXD box and the endo- and exocyclic nitrogen of the guanine base (Scheffzek *et al.*, 1995). For the translation elongation factor EF-Tu, it was shown before that a single amino acid substitution from aspartic acid to asparagine in this guanine binding site was able to switch the nucleotide specifictiy of EF-Tu from GTP to XTP (Hwang and Miller, 1987; Weijland and Parmeggiani, 1993). We used this information to introduce, by site-directed mutagenesis, the equivalent aspartic acid to asparagine mutation into the Ran cDNA. The resulting mutant Ran protein, termed Ran D125N, was expressed in *Escherichia coli* and purified as described in Materials and methods (Figure 1A).

The nucleotide binding specificity of the recombinant D125N protein was analysed and compared with the specificity of the wild-type protein in filter binding assays. Both the D125N mutant and the wild-type protein were loaded with either [α-^{32}P]XTP or [α-^{32}P]GTP, filtered onto nitrocellulose and the amount of bound nucleotide analysed (Figure 1B). As expected, the wild-type protein bound efficiently to the GTP nucleotide and could bind ~20 times more GTP than XTP. In contrast, the mutant D125N protein specifically interacted with the xanthine nucleotide. In this case, the amount of bound XTP was >100 times higher than the amount of bound GTP, and guanosine nucleotide binding could only be detected at background levels (Figure 1B). The total amount of XTP that was complexed with the D125N mutant was similar

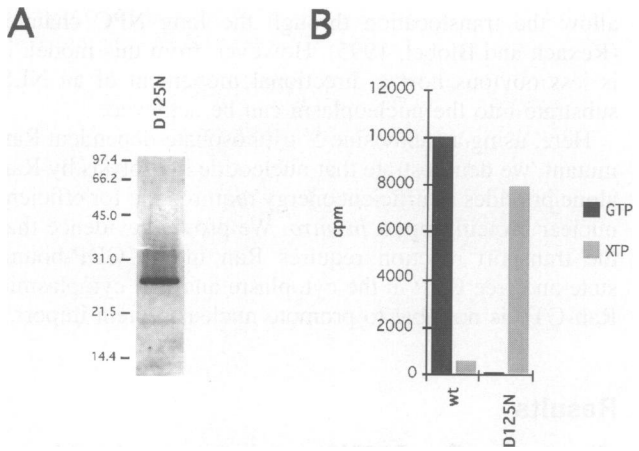

Fig. 1. (**A**) The recombinant Ran D125N protein has an altered nucleotide specificity. The Ran D125N mutant was expressed in *E.coli*, purified as described in Materials and methods and its purity was checked by Coomassie staining after separation on 15% SDS–PAGE. Molecular sizes are indicated in kDa. (**B**) Wild-type or D125N Ran were loaded with either [α-^{32}P]GTP (dark grey) or [α-^{32}P]XTP (light grey). Proteins were filtered onto nitrocellulose filters and the amount of bound nucleotide quantitated by liquid scintillation counting.

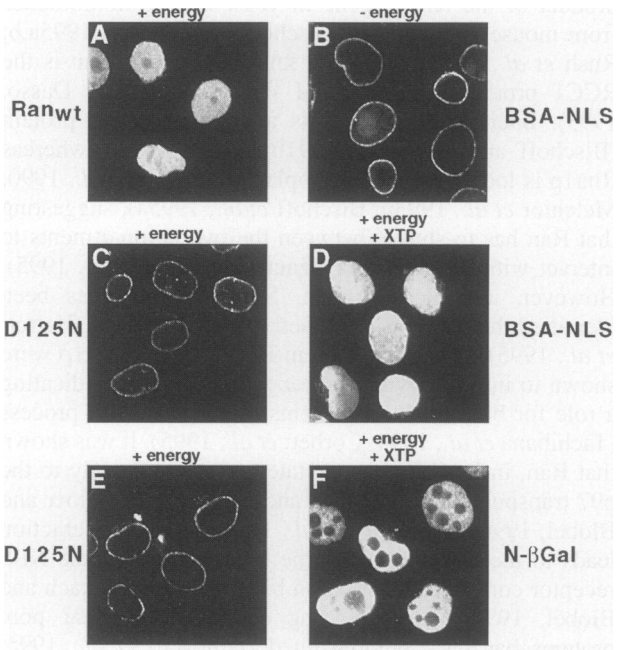

Fig. 2. Nuclear protein import is XTP dependent in the presence of the D125N mutant. Nuclear import of fluorescein-coupled BSA–NLS with hSRP1α (**A–D**) or of the N-βGal reporter (**E** and **F**) was assayed in the presence of the recombinant transport factors p97, p10 and either wild-type Ran-GDP (A and B) or D125N Ran-XDP (C–F). In the reactions shown in (A) and (C–F), energy, in the form of ATP, GTP and an energy-regenerating system, was present. In (D) and (F), 200 μM XTP was also included. In the transport assay shown in (B), no energy was added. Pictures were recorded with a confocal fluorescence microscope.

to the amount of GTP that was bound by the wild-type protein (Figure 1B).

Having established that the mutant D125N protein showed the predicted switch in nucleotide specificity, the effect of the D125N protein in *in vitro* protein import assays was tested. In agreement with previous data (Weis *et al.*, 1996), the four recombinant human proteins, hSRP1α, p97, p10 and Ran-GDP were sufficient to promote complete nuclear uptake of a fluorescently labelled bovine serum albumin (BSA)–NLS conjugate into the nuclei of digitonin-permeabilized HeLa cells (Figure 2A). This import reaction is dependent on the presence of energy which was added in the form of ATP, GTP, creatine phosphate and creatine kinase. In the absence of energy, only docking of the import substrate at the nuclear envelope was observed (Figure 2B). We next replaced wild-type Ran with the D125N mutant that was preloaded with XDP and performed an import reaction with recombinant proteins in the presence of ATP, GTP, creatine phosphate and creatine kinase (Figure 2C). Only docking of the BSA–NLS substrate at the nuclear envelope was observed, as would be expected from an import reaction lacking Ran (Moore and Blobel, 1993; Melchior *et al.*, 1993a). However, addition of XTP to this import reaction restored nuclear uptake of the import substrate to levels similar to those observed with the wild-type protein (Figure 2D). To demonstrate further the XTP dependence of the transport reaction in the presence of the mutant D125N protein, we also tested nuclear import of a β-galactosidase fusion protein that contains the first 65 amino acid residues of hSRP1α (N-βGal). We could show recently that this N-βGal fusion protein is transported efficiently into nuclei of digitonin-permeabilized cells in the presence of energy and the recombinant transport factors p97, Ran and p10 (Weis *et al.*, 1996). N-βGal binds directly to the transport factor p97, and is thus able to circumvent a normal NLS–receptor interaction. In the presence of the D125N mutant in its XDP form, p97, p10 and energy in the form of ATP, GTP, creatine phospate and creatine kinase, the N-βGal substrate also only docks at the nuclear envelope (Figure 2E). Complete nuclear import was obtained after the addition of XTP to this import reaction (Figure 2F). From these data, we conclude that the D125N mutant functions in nuclear protein import and renders the import reaction dependent on the presence of XTP.

XTP is the only nucleotide required for nuclear protein import in vitro in the presence of the D125N protein

The XTP specificity of the D125N mutant protein was exploited to address whether other NTPases apart from Ran are required for nuclear protein import. First, the stability of XTP was analysed in the *in vitro* transport system. XTP was incubated at a final concentration of 1 mM in digitonin-permeabilized cells in the presence of small amounts of [α-^{32}P]XTP as a tracer. After 30 min, the reaction was stopped and the nucleotides were separated by thin layer chromatography (Figure 3). Quantitation of the amounts of XTP, XDP and XMP with a Phosphorimager showed that after a 30 min time course ~90% of the initial amount of XTP remained, indicating that there is no significant XTPase activity in the digitonin-permeabilized cells (Table I). In contrast, incubation of either [α-^{32}P]ATP or [α-^{32}P]GTP in the transport system showed that they were both hydrolysed rapidly in the absence of an energy regeneration system (data not shown). Since XTP was stable in our assay conditions, it was possible to perform

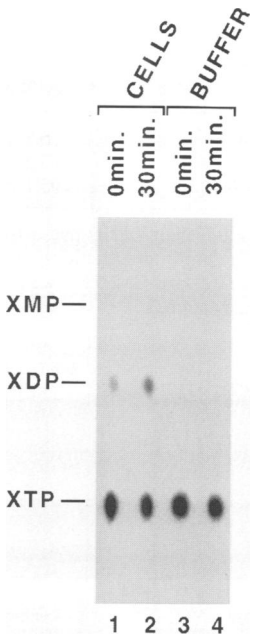

Fig. 3. XTP is stable in digitonin-permeabilized cells. Thin layer chromatography analysis of [α-^{32}P]XTP incubated under transport conditions either in the presence (lanes 1 and 2) or absence (lanes 3 and 4) of digitonin-permeabilized HeLa cells. Aliquots of the reactions containing 1 mM unlabelled XTP were separated on TLC plates immediately after the addition of the [α-^{32}P]XTP tracer (lanes 1 and 3) or after 30 min incubation at room temperature (lanes 2 and 4).

Table I. Quantitation of XTP hydrolysis in digitonin-permeabilized nuclei

Time (min)	XTP left	
	Buffer	Cells
0	100	98.9
5	100	99.5
15	100	95.8
30	100	93.7

The amount of [α-^{32}P]XTP that remained after incubation under transport conditions either in the absence (Buffer) or in the presence of digitonin-permeabilized HeLa cells (Cells) was quantified [in percentage of total radioactivity; XTP/(XTP + XDP + XMP)×100). Aliquots of the reactions containing 1 mM unlabelled XTP were taken at the indicated time points and separated on TLC plates.

import reactions in either the presence or absence of XTP as the only energy source (Figure 4). The import of a BSA–NLS conjugate was tested in the presence of the recombinant factors hSRP1α, p97, p10 and Ran D125N-XDP (Figure 4A). As expected, only docking at the nuclear envelope was observed without the addition of XTP. Addition of XTP promoted complete nuclear uptake of the import substrate after 30 min incubation at room temperature in the presence of Ran D125N (Figure 4B), whereas no import could be detected under these conditions with wild-type Ran protein (data not shown). Identical results were obtained with the N-βGal reporter system in the presence of recombinant p97, p10 and D125N proteins (Figure 4C and D). This suggested that the XTP hydrolysis by Ran D125N might be the only energy source needed to promote nuclear import. However, to exclude that XTP-induced phosphorylation of ADP or GDP, which might

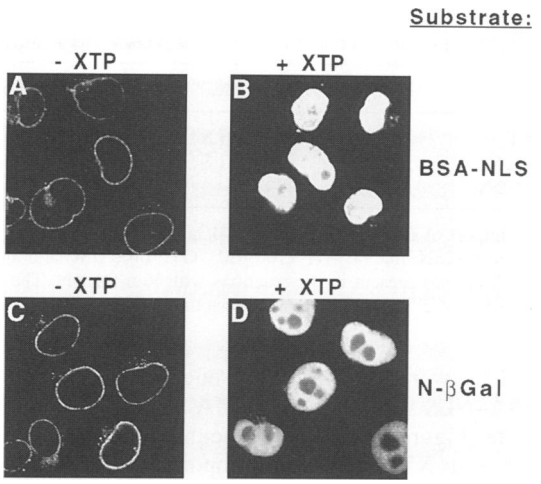

Fig. 4. XTP alone is sufficient to promote import in the presence of the D125N mutant. Nuclear protein import was assayed with digitonin-permeabilized cells in the presence of the recombinant human transport factors p97, p10 and the Ran D125N mutant loaded with XDP. In (**A**) and (**B**) the uptake of a fluorescein-labelled BSA–NLS conjugate was analysed in the presence of the NLS binding protein hSRP1α. In the reactions (**C**) and (**D**) the N-βGal reporter system was used to assay nuclear import. Import reactions were performed either without (A and C) or with the addition of 500 μM XTP (B and D). In all reactions neither ATP, GTP nor an energy regeneration system were added. Images were recorded and analysed with a confocal fluorescence microscope.

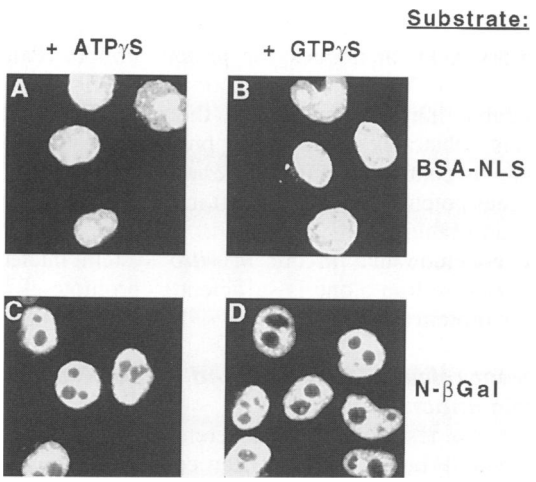

Fig. 5. Protein import is not inhibited by ATPγS and GTPγS in the presence of the Ran D125N mutant. Nuclear protein import was assayed with digitonin-permeabilized cells in the presence of the recombinant human transport factors p97, p10 and the Ran D125N mutant. In (**A**) and (**B**) the uptake of a fluorescein-labelled BSA–NLS conjugate was analysed in the presence of the NLS binding protein hSRP1α. In the reactions (**C**) and (**D**) the N-βGal reporter system was used to follow nuclear uptake. All reactions (A–D) were performed in the presence of 250 μM XTP. The non-hydrolysable analogues ATPγS (A and C) or GTPγS (B and D) were present at a final concentration of 2.5 mM. Images were recorded and analysed with a confocal fluorescence microscope.

be still present in low levels in the permeabilized cells, was sufficient to drive other energy-requiring events, we performed the import reaction also in the presence of an excess of non-hydrolysable ATP or GTP analogues. Analysis of the intranuclear fluorescence levels using confocal microscopy demonstrated that neither a large excess of ATPγS (Figure 5A and C) nor of GTPγS (Figure

Table II. Quantitation of protein import in the presence of non-hydrolysable GTP analogues

	Substrate	Control	GTPγS	GMPPNP
Ran WT	BSA–NLS	100 ± 11.3	8.7 ± 4.4	6.3 ± 5.2
Ran D125N	BSA–NLS	100 ± 10.4	99.2 ± 10.9	103.2 ± 11.8
Ran D125N	N-βGal	100 ± 13.1	106.3 ± 10.4	105.1 ± 10.0

Nuclear import of BSA–NLS and the N-βGal substrate was quantified in the presence of recombinant transport factors p97, p10 and either wild-type or the Ran D125N mutant. ATP (100 μM), GTP (100 μM) and an energy-regenerating system were present in the reactions with wild-type Ran, and 250 μM XTP was present in the reactions with Ran D125N. The non-hydrolysable GTP analogues GTPγS and GMPPNP were added at a final concentration of 2.5 mM. The table shows the average value of >100 cells. The errors are standard deviations.

5B and D) were able to inhibit nuclear accumulation of the BSA–NLS substrate (Figure 5A and B) or the N-βGal substrate (Figure 5C and D) in the presence of the D125N mutant and XTP. Even the import of the ~500 kDa tetrameric N-βGal fusion protein occurred efficiently in the minimal import system using XTP as the only energy source. In contrast, in the presence of wild-type Ran, protein import was strongly inhibited by non-hydrolysable NTP analogues, as was expected from previous studies (data not shown, but see Table II; Moore and Blobel, 1993; Melchior *et al.*, 1993a).

To test for a possible small inhibitory effect of the non-hydrolysable GTP analogues, import reactions with both the BSA–NLS and the N-βGal substrate were quantified (Table II) in the presence of 2.5 mM GTPγS or 2.5 mM GMPPNP (guanylyl imidodiphosphate). These two analogues were shown previously to be the two most potent inhibitory GTP analogues for protein import (Palacios *et al.*, 1996). Neither GTPγS nor GMPPNP showed a measurable inhibitory effect on the uptake of the two different substrates tested in the presence of the D125N mutant. In contrast, control experiments show that both analogues potently blocked import in the presence of wild-type Ran (Table II).

We conclude that in our *in vitro* system nucleotide hydrolysis by Ran alone is sufficient to promote efficient nuclear protein import.

Different roles for Ran-GDP and Ran-GTP during protein import in vitro

Next it was tested whether different roles for the GTP- and the GDP-bound forms of Ran could be distinguished during the *in vitro* transport reaction, since we previously had observed that Ran in its GDP state was a much more potent activator of nuclear transport than Ran that was pre-loaded with GTP (data not shown but see also Figure 6E and F). For this, two mutant Ran proteins were used that lock Ran either in the GTP- or in the GDP-bound state of the GTPase cycle. One is a point mutant that has the glutamine residue at position 69 changed to leucine (Ran Q69L). The Q69L mutant cannot be activated by the Ran GAP and persists in the GTP-bound state (Klebe *et al.*, 1995). A functionally equivalent mutant has been shown to act as a dominant-negative inhibitor for both protein import and RNA export in yeast (Schlenstedt *et al.*, 1995). The second mutant has a change of threonine at position 24 to asparagine (Ran T24N). This protein binds weakly to GTP, but has almost normal affinity towards GDP, and is thus predominantly in the GDP state (Klebe *et al.*, 1995). Both the Q69L and T24N mutants can act as dominant-negative inhibitors for both protein and snRNP

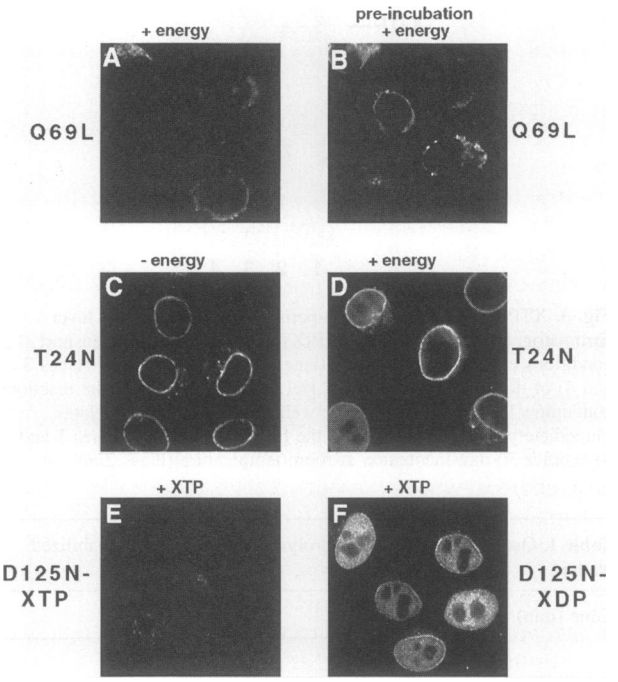

Fig. 6. Only cytoplasmic Ran-GDP is active for protein import *in vitro*. Nuclear import of the N-βGal reporter was analysed in the presence of recombinant transport factors p97, p10 and either the Ran Q69L mutant (**A** and **B**) or the Ran T24N mutant (**C** and **D**). Energy in the form of ATP, GTP and an energy regeneration system was present in the reactions shown in (A), (B) and (D). No energy was added in (C). In (B) N-βGal, p97 and p10 were pre-incubated for 10 min at room temperature to allow the docking of the substrate at the nuclear envelope, before the Q69L protein was added. *In vitro* nuclear import assays with N-βGal, p97, p10 and Ran D125N, pre-loaded either with XTP (**E**) or XDP (**F**), were performed for 10 min at room temperature. XTP was present at a concentration of 500 μM. Images were recorded with a confocal fluorescence microscope.

import when added to *in vitro* transport reactions with complete cytosolic extracts from *Xenopus* eggs (Palacios *et al.*, 1996). The effect of both mutants was assayed in the recombinant transport system with the N-βGal reporter in the absence of wild-type Ran protein (Figure 6A–D). When import assays were performed in the presence of N-βGal, p97, p10 and the Q69L protein, either without (data not shown) or with the addition of energy in the form of ATP, GTP and a regeneration system (Figure 6A), neither import nor docking at the nuclear envelope was observed, and only an increased cytoplasmic background staining could be detected under these conditions. This result was confirmed further when we pre-incubated the permeabilized cells with the import substrate for 10 min in the presence of p97 and p10 to allow the docking

reaction to occur and subsequently added the same amount of Q69L protein as before (Figure 6B). Again no substrate was detected at the envelope. We thus conclude that the Q69L mutant not only prevents docking of an import substrate at the nuclear envelope but is also able to cause the release of a pre-bound substrate from the docked state.

Transport experiments were next carried out with the T24N mutant (Figure 6C and D). When digitonin-permeabilized cells were incubated for 30 min with the N-βGal reporter, p97, p10 and the T24N protein, either in the absence (Figure 6C) or presence of energy (Figure 6D), only docking at the nuclear envelope was observed. In a small percentage of cells, weak nuclear fluorescence was detectable under the conditions where energy was present, presumably because of the residual affinity of the T24N protein towards GTP. However, the T24N protein clearly is not able to support efficient transport although it does not prevent docking of the import substrate at the nuclear envelope.

To characterize further this differential behaviour of the two mutant Ran proteins, import reactions using Ran proteins that were pre-loaded either with nucleoside triphosphates or nucleoside diphosphates were compared. First, the D125N protein was charged with XTP and purified by FPLC chromatography. D125N-XTP produced very poor import, as compared with reactions in which we added the D125N-XDP protein like those shown in Figures 2, 4 and 5 (data not shown). This difference was most obvious when transport reactions were performed under suboptimal conditions, i.e. for 10 min, since, under these conditions, basically no import of the N-βGal reporter could be detected with the D125N-XTP protein (Figure 6E). To exclude that this was due to an inactivation of the protein during the exchange reaction, the same batch of the D125N-XTP protein was reloaded with XDP, re-purified and used in import assays (Figure 6F). After 10 min, most cells had already imported the N-βGal substrate, although weak rim staining was still detectable at this timepoint (Figure 6F). Identical results were obtained with wild-type Ran protein pre-loaded with either GTP or GDP, respectively (data not shown).

Taken together, these results suggest that it is the Ran D125N-XDP or Ran-GDP complex which is required in the cytoplasm together with free XTP/GTP to allow the import reaction to proceed. The Ran-GTP/XTP complex on the other hand causes release of pre-bound substrate when added directly to the import reaction and is thus not able to promote transport.

Discussion

Here we show that nucleotide hydrolysis by Ran is sufficient for nuclear protein import *in vitro*. The data indicate that no additional NTPase other than Ran is required for protein import in the *in vitro* system used in this study. This conclusion stems largely from the analysis of a mutant Ran protein, termed Ran D125N, which has a single amino acid substitution of asparagine for aspartate at position 125. The introduction of the D125N mutation in Ran drastically weakens its binding to GTP while enhancing its binding to XTP. This is consistent with the previously observed switch from GTP to XTP binding seen when equivalent mutations were made in the translocation elongation factor EF-Tu (Hwang and Miller, 1987; Weijland and Parmeggiani, 1993), in the adenylosuccinate synthetase (Kang *et al.*, 1994) or in the FtsY protein from *E.coli* (Powers and Walter, 1995). The Ran D125N mutation thus allowed the uncoupling of the nucleotide hydrolysis by Ran from other putative NTPases involved in nuclear protein import. This was not possible previously, since in the digitonin-permeabilized cells, which are used for the nuclear transport assay, both GTPase and ATPase activities are still present, and interconversion reactions between these two nucleotides by nucleoside diphosphokinases can occur rapidly (K.Weis, unpublished; for a recent discussion, see Melchior *et al.*, 1995). In a reconstituted import assay with recombinant transport factors in the presence of the D125N mutant, protein transport becomes dependent on the addition of XTP and, furthermore, addition of XTP alone allows complete and efficient nuclear import. Under these conditions, even a large excess of non-hydrolysable GTP or ATP analogues no longer inhibits import, strongly arguing that under these *in vitro* conditions no further ATP- or GTP-dependent reaction is required for the uptake of the import substrates used in this study.

While this work was in progress, a separate study by Sweet and Gerace (1996) was published in which the authors, using the same Ran D125N mutant, reached the opposite conclusion to us, i.e. that one or more as yet uncharacterized GTPases, other than Ran, are involved in nuclear protein import. In their *in vitro* system, protein import was partially inhibited by non-hydrolysable GTP analogues in the presence of the D125N mutant. At present, the reason for the apparent discrepancy between the results we report here and the data of Sweet and Gerace is unclear. However, it should be pointed out that there are clear differences between the *in vitro* transport systems used in these two studies. For example, we have used exclusively a transport assay in which only recombinant transport factors are added, namely hSRP1α or N-βGal, p97, Ran and p10. This supports protein import in the presence of XTP without any other NTPs or an energy-regenerating system. In contrast, Sweet and Gerace use, in most of their experiments, cytosolic extracts depleted of Ran as their source of transport factors, and all experiments are performed in the presence of ATP and an energy-regenerating system. Nonetheless, Sweet and Gerace also observe a partial inhibitory effect of GTP analogues when they use a recombinant import system with hSRP1α, Ran D125N and p10/NTF2. Here there is still a clear difference with our transport assay since Sweet and Gerace do not add the transport factor p97, which does not stimulate protein import under their conditions (Sweet and Gerace, 1996). However, in our transport system, protein import is stimulated greatly upon addition of p97 (Weis *et al.*, 1996, but see also Görlich *et al.*, 1995a; Radu *et al.*, 1995). Based on the data we report here, we conclude that there is a nuclear protein import pathway in which nucleotide hydrolysis by Ran alone is sufficient to translocate proteins through the NPC. However, we cannot exclude that parallel or alternative protein transport pathways might also exist which differ in their sensitivity towards non-hydrolysable GTP analogues or in the involvement of GTPases which are downstream of the Ran-GTPase.

The data presented here indicate that Ran has to be in the GDP-bound state in the cytoplasm to be active for nuclear protein import and that Ran-GTP, when added exogenously, causes a non-productive release of the import substrate from the nuclear envelope. This conclusion was based on two types of experiments. First, two different mutant Ran proteins were used that lock Ran either in the GTP state (Q69L) or in the GDP state (T24N) (Klebe *et al.*, 1995). Although both mutants act as dominant-negative inhibitors when added to import assays with complete cytosolic extracts or recombinant proteins (Palacios *et al.*, 1996 and K.Weis, unpublished), they show a differential effect on the distribution of the import substrate. Whereas the Q69L protein causes a release of the substrate from the docked state at the nuclear envelope, the T24N protein does not interfere with the docking but also cannot support efficient nuclear transport. From this, it can be concluded that the complete GDP–GTP cycle of Ran is required for protein import and neither Ran-GTP nor Ran-GDP alone support complete nuclear import of an NLS-containing substrate. Since the Q69L mutant is unable to hydrolyse GTP, the observed release effect could be explained by an uncoupling of Ran-GTP binding and GTP hydrolysis. However, this is unlikely since, in a second set of experiments with Ran proteins that were either pre-loaded with the tri- or the diphosphate nucleoside, we again observed a strong difference in the effect of these two forms on nuclear import. Whereas the XDP/GDP state allowed rapid and efficient nuclear import in the presence of free XTP/GTP, the XTP/GTP form did not support efficient import. If cytoplasmic Ran-GTP was the active form for nuclear import exactly the opposite behaviour would be expected. The effect, however, was not as strong as with the Q69L mutant, and was most obvious when import experiments were compared which were carried out under suboptimal conditions, e.g. after 10 min at room temperature. There are several possible explanations for this. One likely explanation is that both the wild-type and the D125N protein are not locked in their nucleotide state which makes them accessible to exchange factor(s) or GAP activities. Any residual GAP activity present in the permeabilized cell system and the intrinsic GTPase activity of Ran itself will allow, therefore, the gradual reformation of the 'active' GDP state which then allows the import reaction to proceed over time. On the other hand, Ran is able to diffuse between the cytoplasmic and the nuclear compartment and, therefore, one can expect that Ran-GDP/XDP gets access to the nuclear protein RCC1 which would be able to reconvert it into the nucleoside triphosphate state and thereby change the ratio of the two different Ran states.

That cytoplasmic Ran functions in the GDP state is consistent with the cytoplasmic localization of the Ran GAP protein Rna1p. Thus, a compartmentalized Ran GAP activity would keep the cytoplasmic levels of Ran-GTP low and would thereby prevent the unproductive release reaction induced by free Ran-GTP. This result has two consequences. First, it should be postulated that a coordinated nucleotide exchange reaction occurs at the NPC itself and second, that Ran also interacts, either directly or indirectly, with the NPC in its GDP-bound state. It was reported that only Ran-GTP binds to the NPC (Melchior *et al.*, 1995). However, recently it was shown that Ran-GDP could form an *in vitro* complex with the nuclear pore protein Nup36, via its interaction with p10 and p97 (Nehrbass and Blobel, 1996). Also, by immunofluorescence, Ran-GDP could be detected at the nuclear envelope (D.Görlich and R.Bischoff, personal communication). Interestingly, it was also reported recently that the GEF for Ran, i.e. RCC1, can bind to the the nuclear pore protein RanBP2/Nup358 from *Xenopus* (Saitoh and Dasso, 1995). It is possible, therefore, that a nucleotide exchange reaction could occur directly at the NPC.

The present data have important consequences for our understanding of how nuclear proteins are translocated through the NPC. Since the energy requirement for the full *in vitro* import mechanism can be supplied through GTP hydrolysis by Ran alone, no additional energy is required for molecules like motor proteins or for the opening of the NPC to allow the passage of large molecules into the nucleus. In fact it is shown here that XTP alone is sufficient to promote efficient transport of a 500 kDa protein complex in our *in vitro* import system. However, it is possible that the Ran cycle could have differential effects during different stages of the translocation, e.g. Ran could play a different role on the cytoplasmic and the nucleoplasmic side of the NPC. Further studies are required to understand fully the complex mechanism underlying the translocation reaction of nuclear proteins through the NPC.

Materials and methods

Mutagenesis and recombinant protein expression

To introduce the D125N mutation, the full-length cDNA of Ran (Klebe *et al.*, 1993) was subcloned into the pBluescript phagemid (Stratagene) and single-stranded DNA was prepared according to the method described by Kunkel (1985). The oligo 5′-GTCCTTAATGTTCACTTTGTTGCC-3′ was used for the site-directed mutagenesis (mutagenic bases are underlined) and isolated clones were analysed by DNA sequencing. The clone pKW299 was selected and the complete cDNA subcloned into the pET3d expression vector using the *NcoI–PstI* and *PstI–PstI* fragments of pKW299. The resulting clone pKW307 was again sequenced and used for the expression of the recombinant D125N mutant in *E.coli* BL21 [LysS]. Transformed bacteria were grown to an OD_{600} of 0.4 and induced for 6 h with 100 μM IPTG. Cells were harvested, resuspended in 30 mM potassium phosphate pH 6.8, 30 mM KCl, 2 mM β-mercaptoethanol, 0.5 mM $MgCl_2$, 5 μM XDP, 8.7% glycerol in the presence of protease inhibitors and lysed with a French press. After removal of bacterial debris, soluble Ran D125N was enriched by S100 FPLC gel filtration using the same buffer. Peak fractions were loaded onto a MonoS column (PC1.6/5) using the Smart system (Pharmacia) and eluted with a gradient from 30 to 500 mM KCl in 10 column volumes.

The Ran Q69L and T24N proteins were prepared as described by Palacios *et al.* (1996). All other recombinant proteins were purified as described previously (Weis *et al.*, 1995, 1996).

Nucleotide binding assays

To load wild-type Ran or the D125N mutant with [α-^{32}P]GTP or [α-^{32}P]XTP (both Amersham), 2 μM protein was incubated for 30 min at room temperature in 30 μl of exchange buffer (30 mM potassium phosphate pH 6.8, 50 mM KCl, 2 mM β-mercaptoethanol, 2 mM EDTA) in the presence of 100 nM α-labelled nucleotide (100 Ci/mmol). After addition of 30 mM $MgCl_2$, the reaction was diluted to 1 ml with ice-cold filtration buffer (30 mM potassium phosphate pH 6.8, 50 mM KCl, 2 mM $MgCl_2$), filtered through nitrocellulose filters (Schleicher and Schuell NC45), washed with 10 ml filtration buffer and dried. Bound radioactivity was measured by liquid scintillation counting.

To change the nucleotide state of the Ran D125N protein, the protein was incubated in exchange buffer with 5 mM EDTA and a 10-fold molar excess of XTP or XDP for 1 h at 4°C. The exchange reaction was stopped by addition of 20 mM $MgCl_2$. The protein was concentrated

and the excess of nucleotide removed by MonoS chromatography using the Smart System (Pharmacia).

Transport assays

Transport assays were performed as described previously (Weis *et al.*, 1996). The following amounts of proteins were used for the import reactions: 50 μg/ml fluorescently labelled BSA–NLS conjugate, 30 μg/ml hSRP1, 30 μg/ml N-βGal, 50 μg/ml p97, 5 μg/ml p10, 100 μg/ml wild-type Ran or 100 μg/ml mutant Ran proteins. Unless otherwise stated, wild-type and D125N Ran were always used in the GDP- or XDP-bound state, respectively. The import reactions were performed in import buffer containing 110 mM potassium acetate, 5 mM magnesium acetate, 2 mM dithiothreitol for 30 min at room temperature. If not indicated differently, XTP (Sigma) was added at a concentration of 500 μM. One hundred μM ATP, 100 μM GTP, 10 mM creatine phosphate, 5 μg/ml creatine kinase were used under full energy conditions. The non-hydrolysable analogues ATPγS and GTPγS were added at 2.5 mM final concentrations.

Quantitations of the import reactions were performed as described (Weis *et al.*, 1996).

Thin layer chromatography

Aliquots of the reactions (1/10 of each sample) were stopped by the addition of 0.5% SDS, heated for 5 min at 75°C and separated by thin layer chromatography on PEI-cellulose F (Merck), developed using 600 mM sodium phosphate pH 3.4. Plates were dried and radioactivity quantitated with a PhosphorImager (Molecular Dynamics). Standards of unlabelled XTP, XDP and XMP (all Sigma) were located, after drying, with a 254 nm UV lamp.

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