Import and export of the nuclear protein import receptor transportin by a mechanism independent of GTP hydrolysis

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Background: Nuclear protein import and export are mediated by receptor proteins that recognize nuclear localization sequences (NLSs) or nuclear export sequences (NESs) and target the NLS-bearing or NES-bearing protein to the nuclear pore complex (NPC). Temperature-dependent translocation of the receptor–cargo complex in both directions through the NPC requires the GTPase Ran, and it has been proposed that the Ran GTPase cycle mediates translocation. We have addressed the role of GTP hydrolysis in these processes by studying the import receptor transportin, which mediates the import of a group of abundant heterogeneous nuclear RNA-binding proteins bearing the M9 NLS.

Results: We investigated the transport properties of transportin and found that the carboxy-terminal region of transportin could, by itself, be imported into the nucleus. Transportin import and export were inhibited by low temperature *in vitro*, but were unaffected by the non-hydrolyzable GTP analogue GMP–PNP.

Conclusions: Temperature-dependent import and export through the NPC can be uncoupled from the Ran GTPase cycle and can occur without GTP hydrolysis.

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Received: **6 November 1997**
Revised: **20 November 1997**
Accepted: **24 November 1997**

Published: **16 December 1997**

Current Biology 1997, 8:89–95
http://biomednet.com/elecref/0960982200800089

Background

The division of the eukaryotic cell into nucleus and cytoplasm necessitates the nucleocytoplasmic transport of diverse proteins and RNA–protein complexes between their sites of synthesis and function [1–5]. The import of proteins into the nucleus is the best understood transport pathway across the nuclear envelope. Proteins larger than ~60 kDa are imported by a process that can be considered to comprise three steps. First, the protein bearing a nuclear localization sequence (NLS) is recognized in the cytoplasm by a specific receptor, and this cargo–receptor complex is then targeted to the nuclear pore complex (NPC). Second, the cargo–receptor complex is translocated through the NPC into the nucleoplasm by a temperature-dependent mechanism that requires the GTPase Ran [1–4,6–9]. Third, in the nucleus, the GTP-bound form of Ran binds to the receptor and triggers dissociation of the cargo–receptor complex, thereby terminating import by releasing the cargo into the nucleoplasm [10–12]. Ran is regulated by an exchange factor, regulator of chromosome condensation 1 (RCC1), which is a nuclear protein [13], and by a GTPase-activating protein (RanGAP1), which is largely cytoplasmic [14]; their distribution predicts a steep Ran–GTP gradient across the nuclear membrane, such that the concentration of Ran–GTP is high in the nucleus and low in the cytoplasm. Thus, the terminating effect of Ran–GTP on import is strictly a nucleoplasmic event.

Three nuclear import receptor systems have been described, each of which mediates the import of a distinct group of proteins. A dimeric receptor comprising importin α (that binds the NLS) and importin β (that mediates translocation) imports proteins bearing the classical NLS motif, which contains basic residues [1–4,15]. The importins are also known as karyopherins. By contrast, a monomeric receptor, transportin, which is distantly related to importin β, mediates the import of a group of abundant heterogeneous nuclear RNA-binding proteins (hnRNPs) that contain an NLS, termed M9, rich in glycine and aromatic amino acids [3,12,16–20]. The import of ribosomal proteins has also been reported to be mediated by monomeric receptors, termed Kap123 and Pse1, that are related to importin β [21].

Like nuclear protein import, protein export is also mediated by specific signals, termed nuclear export signals (NESs) [5]. Very recently, two NES receptors were identified, both of which are distantly related to importin β [22]. Exportin 1 (also known as CRM1) mediates the export of proteins, such as Rev, that contain leucine-rich NESs [23–26], and CAS1 exports importin α, whose NES remains to be identified [27]. In contrast to its dissociating effect on import cargo–receptor complexes, nuclear Ran–GTP stabilizes the interaction between export receptors and their cargo [22,24,27]. One critical role for Ran in nucleocytoplasmic transport is therefore to impose directionality on the traffic by dictating the

stability of cargo–receptor interactions in the nucleus and the cytoplasm.

The Ran GTPase cycle has also been proposed to mediate protein translocation through the NPC ([1,2,4] and references therein). Here, we have addressed the role of GTP hydrolysis in translocation by studying the transport properties of the import receptor transportin. We report that both import and export of transportin are temperature-dependent translocations that do not require GTP hydrolysis. In addition, we show that the carboxy-terminal region of transportin is a nuclear import module — it contains all that a protein needs to translocate into the nucleus.

Results

Transportin accumulates in the nucleus *in vitro*

We noticed that while transportin is required *in vitro* for the import of M9-bearing proteins such as hnRNP A1 [12,17,18], a fusion of transportin with glutathione *S*-transferase (GST–TRN) readily accumulated in the nucleus of digitonin-permeabilized cells, in the absence of exogenous factors (Figure 1b). GST itself does not accumulate in the nucleus ([17] and data not shown). The ability of GST–TRN to localize to the nucleus in this assay is consistent with the predominantly nuclear distribution of transportin *in vivo* [12]. By contrast, a fusion protein of GST and importin β accumulated largely at the rim of the nucleus *in vitro*, although some of it was also detected in the nucleoplasm (Figure 1c), reflecting the localization that is observed for importin β *in vivo* [12,28]. Production of the expected GST fusion protein was in this and all other cases verified by western blotting (Figure 1a and data not shown).

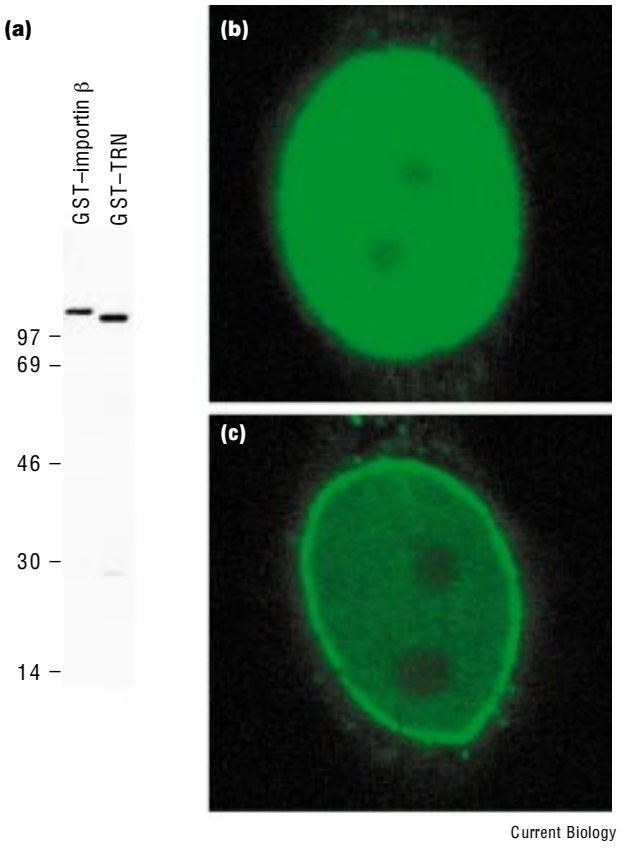
The carboxyl terminus of transportin contains an NLS

In order to identify the primary NLS of transportin, we fused segments of transportin to a normally cytoplasmic protein, pyruvate kinase (PK), and analyzed the localization of the fusion proteins. HeLa cells were transfected with plasmids encoding the fusion proteins, and the expressed proteins were detected by immunofluorescence microscopy. Whereas the amino-terminal region of transportin (amino acids 2–420) was not capable of localizing PK to the nucleus, a fusion of the carboxy-terminal portion (amino acids 421–890) with PK was localized to the nucleus (Figure 2). Large truncations into this carboxy-terminal region of transportin from either the amino or the carboxyl terminus reduced or abolished its capacity to localize PK to the nucleus (Figure 2). The carboxyl terminus of transportin (amino acids 421–890) therefore contains an NLS.

The carboxyl terminus of transportin is a temperature-dependent import module

To further understand the basic requirements for translocation of transportin into the nucleus, the behavior of a

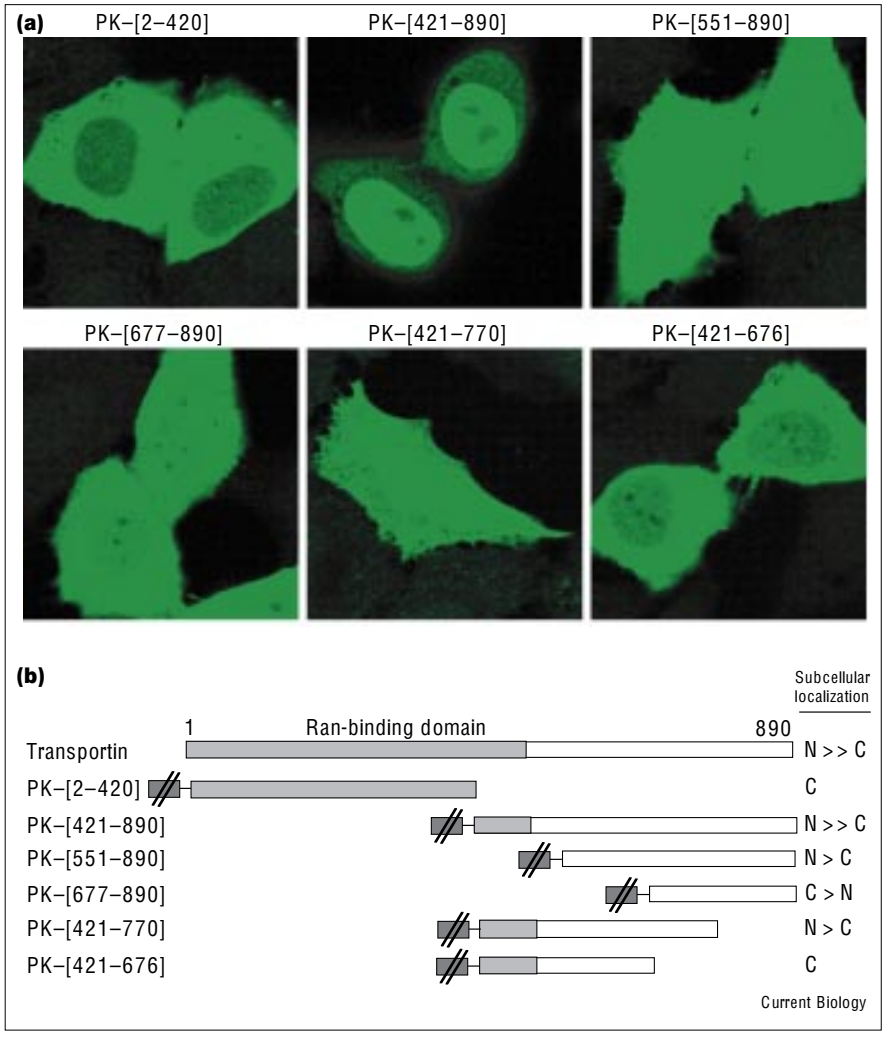
Figure 1



GST–TRN accumulates in the nucleus *in vitro*. **(a)** Western blot analysis of GST fusion proteins. GST–importin β (~1 μg) and GST–TRN (~1.5 μg) were detected with an anti-GST monoclonal antibody (Santa Cruz Biotechnology). The migration of molecular mass markers is indicated (in kDa). **(b,c)** *In vitro* import assays. Permeabilized HeLa cells were incubated with (b) 0.4 μM GST–TRN or (c) 0.4 μM GST–importin β. Both incubations were performed in the presence of an energy-regenerating system. The GST fusion proteins were detected by immunofluorescence using an anti-GST monoclonal antibody.

fusion protein of GST and the transportin carboxyl terminus (GST–C-TRN) was tested in an *in vitro* import assay. Like GST–TRN, GST–C-TRN (76 kDa) accumulated in the nucleus *in vitro*, in the absence of any exogenous factors (Figure 3g). Therefore, the import of PK–C-TRN *in vivo* cannot be explained by a 'piggy-back' mechanism in which it is carried into the nucleus by binding to endogenous transportin–A1 complexes. The import of GST–TRN and GST–C-TRN *in vitro* was abolished both by the lectin wheat germ agglutinin (WGA, an inhibitor of most facilitated and active nucleocytoplasmic transport [29]), and by a fragment of importin β comprising amino acids 45–462 (an inhibitor of all tested nucleocytoplasmic transport pathways, hereafter referred to as ΔN44 [30]), and was severely inhibited when the import assays were conducted on ice (Figure 3). Neither WGA nor ΔN44 blocks movement through the NPC by passive diffusion

Figure 2



A carboxy-terminal portion of transportin is sufficient for nuclear localization *in vivo*. **(a)** HeLa cells were transfected with plasmids expressing Myc-tagged fusion proteins of PK and the indicated amino acids of transportin. Approximately 20 h post-transfection, the intracellular distributions of the expressed proteins were analyzed by immunofluorescence microscopy using an anti-Myc tag monoclonal antibody, 9E10. **(b)** Summary of the subcellular localization analyses. The light gray portion of transportin represents the Ran-binding domain (amino acids 1–517), and the dark slashed box represents PK. N, nucleus; C, cytoplasm.

([29] and U. Kutay and D. Görlich, personal communication). These observations show that GST–TRN and GST–C-TRN are imported through NPCs in a facilitated or active temperature-dependent manner. The carboxyl terminus of transportin is therefore a nuclear import module: that is, it contains all that a protein requires to interact with NPC components, and to be translocated through the NPC. As the overall structure of transportin is similar to that of other members of the importin β receptor family [17,31,32], it is likely that the corresponding carboxy-terminal domains of all the proteins in this family function as import modules.

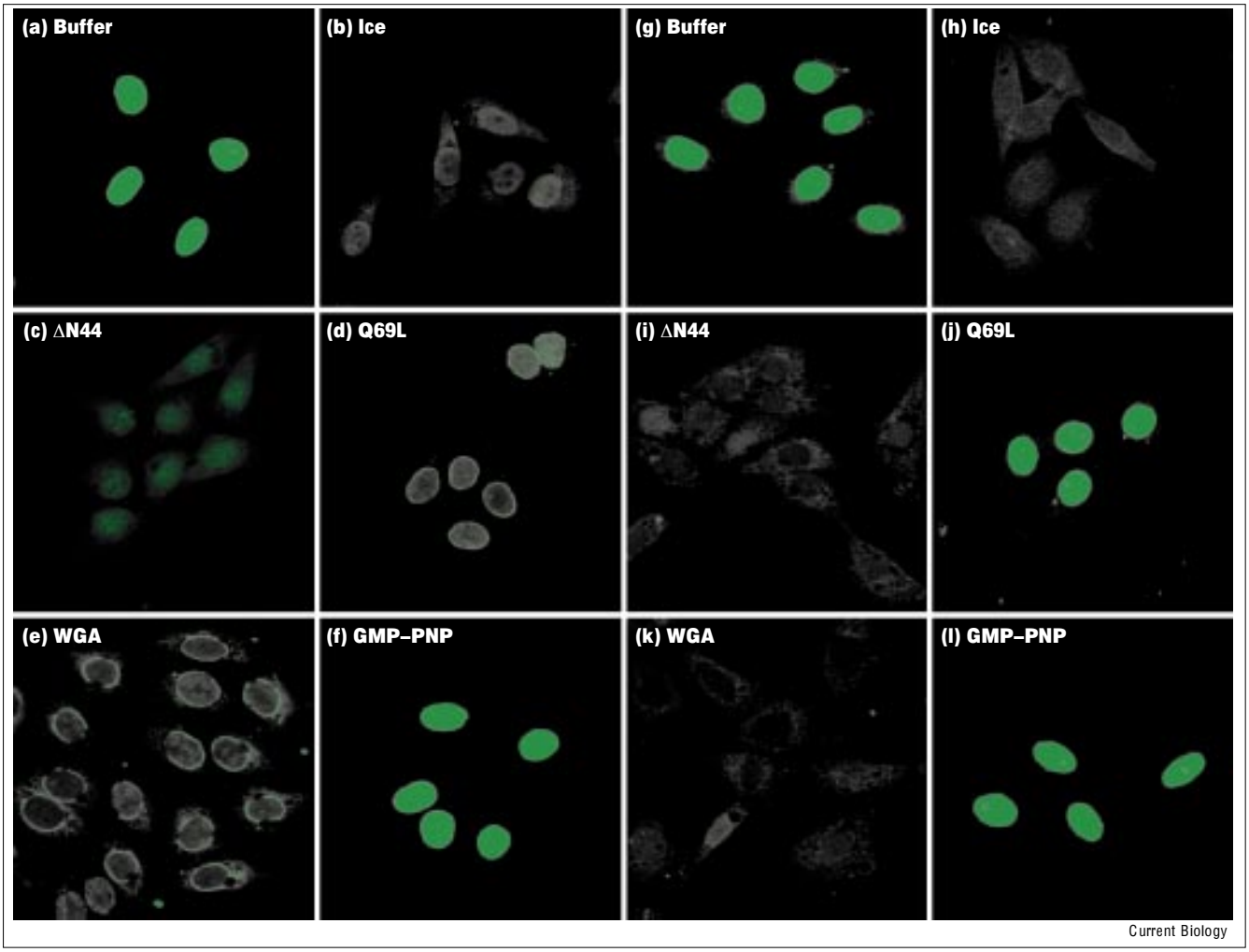
Transportin import does not require GTP hydrolysis

Further analysis of the nuclear import of GST–TRN and GST–C-TRN, and comparison with the import of a classical NLS-bearing protein, revealed striking differences in their mechanisms of translocation. First, RanQ69L, a mutant that is locked in the GTP-bound form [33], abolished classical NLS and M9 protein import ([18,34] and data not shown) and severely inhibited GST–TRN import, but had no effect on the import of GST–C-TRN (Figure 3). Second, 5′-guanylyl-imidodiphosphate (GMP–PNP), a non-hydrolyzable analogue of GTP, abolished classical NLS import ([7] and data not shown), but, under the conditions employed here (that is, assays performed in the absence of exogenous factors), GMP–PNP had no effect on the import of GST–TRN or GST–C-TRN (Figure 3). Thus, these proteins are imported in a temperature-dependent but GTP hydrolysis independent manner. The import of calmodulin *in vivo* has been reported to be temperature sensitive, yet insensitive to ATP-depleting metabolic poisons [35].

Transportin export is temperature dependent, but does not require the hydrolysis of GTP or ATP

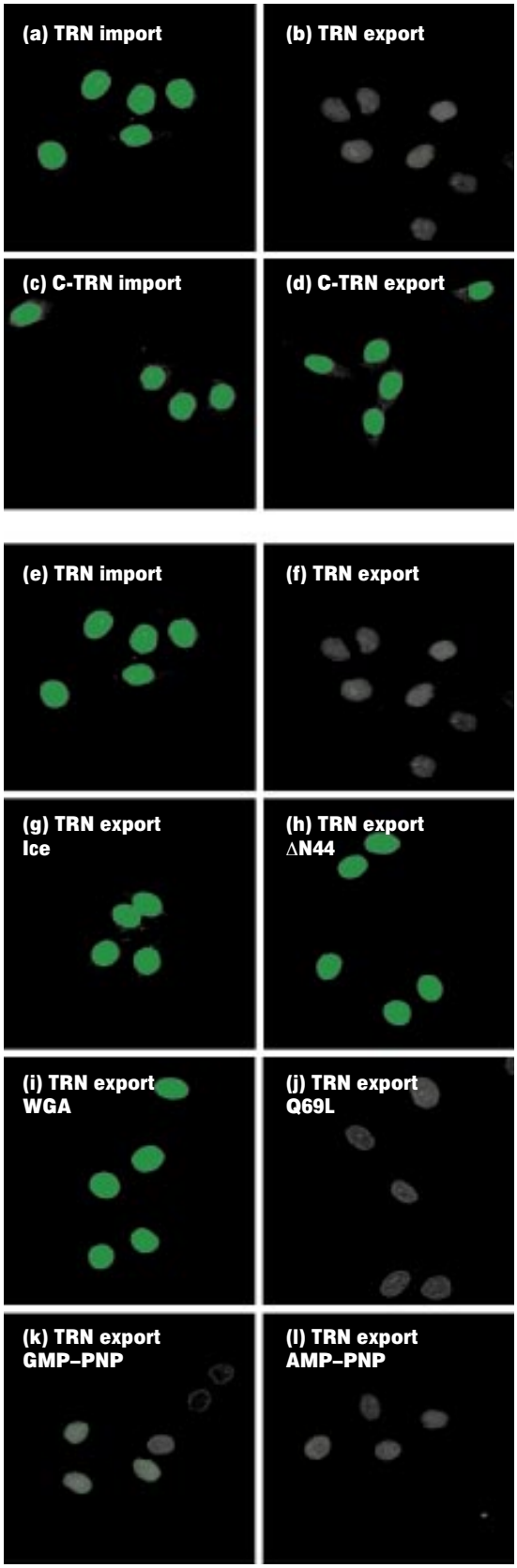
After transporting cargo into the nucleoplasm, import receptors must be exported to allow them to perform the next round of protein import. To study transportin export,

Figure 3



(a–f) GST–TRN import *in vitro* is temperature dependent and independent of GTP hydrolysis. Permeabilized HeLa cells were incubated with GST–TRN either alone (a,b), or with 1 μM of an importin β fragment comprising amino acids 45–462 (c), 2 μM of a Ran mutant containing a Gln69→Leu substitution (d), 0.2 mg/ml wheat germ agglutinin (WGA; e), or 2 mM of a non-hydrolyzable GTP analogue (f). An energy-regenerating system was included in all assays, and GST–TRN was detected by immunofluorescence microscopy. **(g–l)** GST–C-TRN is imported *in vitro* in a temperature-dependent and GTP hydrolysis independent manner. Permeabilized HeLa cells were incubated with GST–C-TRN either alone (g,h), or together with (i–l) the reagents described in (c–f).

nuclei were loaded *in vitro* with GST–TRN or GST–C-TRN (Figure 4a,c). The loaded nuclei were washed and then incubated for a further 20 minutes in transport buffer containing an ATP-regenerating system, then fixed and examined by immunofluorescence microscopy. Only ~30% or less of the GST–TRN remained in the nucleus following this incubation. In contrast, ~90% of the GST–C-TRN was nucleoplasmic (Figure 4b,d). In order to investigate the mechanism of the dramatic decrease of nuclear GST–TRN following incubation, we analyzed the effects of several nucleocytoplasmic transport effectors on the process. The loss of GST–TRN from the nucleus was completely prevented by the addition of either importin β ΔN44 or WGA, or by conducting the incubation on ice. These observations demonstrate that the decrease in nuclear GST–TRN is not due to passive diffusion, damage to the nuclear membrane, or to degradation of the protein. Rather, GST-TRN is exported through NPCs, in a temperature-dependent manner. Since GST–C-TRN does not appear to be exported, the carboxy-terminal region of transportin is presumably not sufficient for export. Although we cannot yet entirely exclude the possibility that GST–C-TRN is exported and rapidly re-imported in this assay, we believe that this is unlikely, as conducting the export incubation in a much larger buffer volume does not decrease the nuclear signal of GST–C-TRN (data not shown). The export of GST–TRN *in vitro* was not inhibited by



RanQ69L or by the non-hydrolyzable nucleotide analogues GMP–PNP and AMP–PNP (Figure 4j–l), suggesting that the transportin export pathway does not use either GTP or ATP as an energy source. While this manuscript was in preparation, protein export mediated by a leucine-rich NES *in vivo*, and the export of transportin, importins α and β, Rev and tRNA in *Xenopus* oocytes were reported to be independent of Ran–GTP hydrolysis [36,37]. Our observations further suggest that GTP hydrolysis is not required at all for either transportin export or import.

Discussion

Although transportin does not have a discernible consensus Ran-binding motif [31,32], like the other importin β-related receptors, it binds Ran–GTP through its amino-terminal domain (amino acids 1–517), with a similar affinity to that with which importin β binds Ran–GTP (F.R. Bischoff, S.N. and G.D., unpublished observations). Here we show that the carboxy-terminal region of transportin, comprising amino acids 421–890, is a nuclear transport module that can function independently and does not require GTP hydrolysis for its translocation. RanQ69L, but not GMP–PNP, severely inhibits the import of full length transportin, whereas the import of the transportin carboxyl terminus is insensitive to both RanQ69L and GMP–PNP. In addition, the nuclear export of transportin is unaffected by these reagents.

Two distinct functions for Ran in nucleocytoplasmic transport have been proposed. First, the Ran GTPase cycle is believed to drive the translocation of cargo–receptor complexes through the NPC, although the molecular mechanism is unknown ([1,2,4] and references therein). Second, a steep gradient of Ran–GTP across the nuclear membrane imposes directionality on NPC traffic (see Background). We find that transportin import and export do not require GTP hydrolysis and therefore propose that the Ran GTPase cycle does not drive translocation through the NPC. The function of Ran appears therefore to be

Figure 4

Transportin is exported from the nucleus *in vitro* in a temperature-dependent and GTP hydrolysis independent manner. Nuclei of permeabilized HeLa cells were loaded under import assay conditions with either **(a)** GST–TRN or **(c)** GST–C-TRN, and the fusion proteins were detected by immunofluorescence. An identical pair of assays was performed with nuclei loaded in the same manner with either **(b)** GST–TRN or **(d)** GST–C TRN, then the cells were washed and incubated for a further 20 min before immunofluorescence analysis. Both import and export incubations included an energy-regenerating system. **(e)** Nuclei of permeabilized HeLa cells were loaded with GST–TRN as in (a). Identical assays were washed, and then incubated as in (b), either **(f)** in the presence of buffer, **(g)** on ice, or in the presence of either **(h)** 1 μM ΔN44, **(i)** 0.25 mg/ml WGA, **(j)** 10 μM RanQ69L, **(k)** 4 mM GMP–PNP, or **(l)** 4 mM AMP–PNP. GST–TRN was detected by immunofluorescence microscopy.

restricted to dictating the direction of transport through the NPC, and effecting net accumulation of cargo on either side of the NPC. While this manuscript was under review, importin β import was reported to show some similar properties to those described here for transportin, leading to the conclusion that importin β can translocate into the nucleus without Ran–GTP hydrolysis [38].

RanGAP1 is localized to the cytoplasm [14], and a proportion of it is also targeted to the nucleoporin RanBP2, which is positioned at the cytoplasmic face of the NPC [39,40]. Ran–GTP hydrolysis at RanBP2 has been proposed to function early in protein import, to commit cargo–receptor complexes to translocation through the NPC [41]. It is also possible that GAP-catalyzed Ran–GTP hydrolysis at RanBP2 serves as a localized purging mechanism, to remove any Ran–GTP, and thus prevent dissociation of incoming cargo–receptor import complexes. RanQ69L probably inhibits the import of full length transportin by preventing its release from Ran–GTP-binding nucleoporins such as RanBP2, producing irreversibly bound receptor–nucleoporin complexes. The presence of irreversibly bound receptors at the NPC is expected to inhibit all active transport, as importin β mutants that cannot be released from the NPC block all active traffic [30]. Consistent with this observation, we found that high levels of RanQ69L or GMP–PNP caused slight inhibition of GST–C-TRN import, and when the assays were performed in the presence of reticulocyte lysate (which contains transportin and other import receptors), high levels of RanQ69L abolished GST–C-TRN import (data not shown).

Conclusions

We report that translocation through the NPC in both directions can be uncoupled from the Ran GTPase cycle. By studying the transport of the import receptor transportin, we found that the temperature dependence of receptor transport does not reflect a requirement for GTP hydrolysis. There is no indication of a requirement for any NTP hydrolysis, leaving the possibility that either a less common high energy bond is used, or that temperature-sensitive conformational changes in NPC components drive the translocation. The transport system that we describe here is the simplest that can be envisioned at present. It defines the core of the nuclear transport machinery and will allow detailed studies of a fundamental unresolved issue in nucleocytoplasmic transport, that of the molecular mechanism of translocation through the NPC.

Materials and methods

Preparation of GST and PK fusion constructs

The plasmid encoding GST–TRN was made by isolating a *Bam*HI–*Sal*I fragment from the His–TRN plasmid ([17], this is the TRN1 sequence in [12]), and inserting it into *Bam*HI–*Sal*I-digested pGEX-5X-3. GST–C-TRN was made by pfu PCR amplification of the transportin cDNA to generate at the 5′ end a *Bam*HI site immediately upstream of amino acid 421 and at the 3′ end an *Xho*I site downstream of a termination codon. This fragment was inserted into pGEX-5X-3 digested with *Bam*HI and *Xho*I. To produce GST–importin β, full length importin β cDNA [17] was amplified by pfu PCR to generate a 5′ *Bam*HI site and a 3′ *Not*I site immediately downstream of the termination codon. This fragment was inserted into *Bam*HI–*Not*I-digested pGEX-5X-3. To generate pcDNA3-Myc-PK-TRN fusions, fragments of transportin with an *Xho*I site at the 5′ end and an *Xba*I site downstream of a termination codon at the 3′ end were generated by pfu PCR amplification of transportin. These fragments were cloned into pcDNA3-Myc-PK [42].

Preparation of recombinant proteins

GST fusion proteins were expressed in BL21(DE3) cells and purified according to the manufacturers' instructions with the following modifications. GST–TRN was induced at 37°C overnight, and GST–C-TRN was induced at 17°C for 4 h. Purification was performed in the presence of 10 μg/ml each of leupeptin and pepstatin, 1% aprotinin, 1 mM DTT and 0.1 mM EDTA. BSA was added to the purified proteins to a final concentration of 15 mg/ml, and they were stored on ice at 4°C. To prepare lysate from bacteria expressing GST–TRN, a cell pellet from 30 ml induced bacteria was washed once with 20 ml transport buffer (TB; 20 mM HEPES pH 7.3, 110 mM potassium acetate, 5 mM sodium acetate, 2 mM magnesium acetate, 0.5 mM EGTA) containing 1 mM DTT, 10 μg/ml each leupeptin and pepstatin and 1% aprotinin, and then resuspended in 1 ml of the same buffer. Lysozyme was added to 120 000 U/ml, the solution was incubated on ice for 1 h, and then centrifuged for 20 min at 14 000 *g*. The supernatant was quick frozen in liquid nitrogen and stored at –70°C. His–RanQ69L was expressed in M15[pREP4] cells at 37°C. The cell pellet was resuspended in 50 mM HEPES pH 7.4, 100 mM NaCl, 10 mM magnesium acetate, 5 mM β-mercaptoethanol, and 0.1 mM GTP (buffer A), then sonicated and centrifuged at 40 000 *g* for 30 min. The supernatant was loaded onto His bind resin charged and equilibrated according to the Novagen instructions, and the resin was washed with buffer A plus 400 mM NaCl. RanQ69L was eluted with buffer A plus 400 mM NaCl and 60 mM imidazole and then dialyzed against TB containing 0.1 mM GTP and 5 mM β-mercaptoethanol.

Cell culture and transfection

HeLa cells were cultured and transfected as described previously [42].

In vitro *import assay*

HeLa cells were grown on glass coverslips (Figure 1) or 10-well slides (all other figures) and permeabilized with digitonin as described [43]. The permeabilized cells were washed in TB containing 2 mM DTT, 1 μg/ml each leupeptin and pepstatin and 1% aprotinin and incubated for 15 min at 30°C with the same buffer plus an energy regenerating system (5 mM creatine phosphate, 20 U/ml creatine phosphokinase, 1 mM ATP) and 10 mg/ml BSA, along with 0.4 to 0.8 μM of the indicated GST fusion protein. For GST–TRN, the figures document experiments performed with bacterial lysate expressing this protein. Purified GST–TRN gave the same results (data not shown). Where indicated, importin β ΔN44 (amino acids 45–462) was included at 1 μM, RanQ69L at 2 μM, GMP–PNP (Calbiochem) at 2 mM and WGA (Sigma) at 0.2 mg/ml. Import assays were terminated by washing the cells in cold PBS followed by 30 min fixation in 2% formaldehyde/PBS.

In vitro *export assay*

Nuclei of digitonin-permeabilized cells were loaded for 15 min at 30°C with either GST–TRN or GST–C-TRN as described above. After washing in cold PBS, the cells were incubated for a further 20 min at 30°C in TB containing 2 mM DTT, 1 μg/ml each leupeptin and pepstatin and 1% aprotinin plus an energy regenerating system (see import assay) and 10 mg/ml BSA. The energy regenerating system can be omitted without effect on the observed export. Where indicated, importin β ΔN44 (amino acids 45–462) was included at 1 μM, WGA at 0.25 mg/ml, RanQ69L at 10 μM, and AMP–PNP (Calbiochem) or GMP–PNP at 4 mM. Export assays were terminated in the same way as import assays.

Immunofluorescence staining, confocal microscopy and western blotting

Fixed cells were permeabilized for 3 min in –20°C acetone, incubated for 30 min with primary antibody diluted in 3% BSA/PBS (1:100 anti-GST monoclonal antibody, Santa Cruz Biotechnology, 1:3000 anti-Myc monoclonal antibody 9E10), washed in PBS and incubated for 30 min in 1:50 FITC-conjugated goat anti-mouse secondary antibody (Cappel Laboratories). Laser scanning confocal microscopy was performed with a Leica TCS NT confocal microscope. Western blotting was performed as described previously [16].

Acknowledgements

We thank Dirk Görlich for the recombinant importin β ΔN44 protein and for the bacterial expression plasmid encoding His–RanQ69L, Steve Adam for GST–importin β, all members of the laboratory for enjoyable discussions, and Paul Eder, Mikiko Siomi, Naoyuki Kataoka and Haruhiko Siomi in particular for critical reading of the manuscript, and Sue Kelchner for secretarial assistance. S.N. thanks Naoyuki Kataoka for encouragement during this project. This work was funded by the Howard Hughes Medical Institute, and by a grant from the National Institutes of Health.

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