The translocation of transportin–cargo complexes through nuclear pores is independent of both Ran and energy

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Active transport between nucleus and cytoplasm proceeds through nuclear pore complexes (NPCs) and is mediated largely by shuttling transport receptors that use direct RanGTP binding to coordinate loading and unloading of cargo [1–4]. Import receptors such as importin β or transportin bind their substrates at low RanGTP levels in the cytoplasm and release them upon encountering RanGTP in the nucleus, where a high RanGTP concentration is predicted. This substrate release is, in the case of import by the importin α/β heterodimer, coupled directly to importin β release from the NPCs. If the importin β–RanGTP interaction is prevented, import intermediates arrest at the nuclear side of the NPCs [5,6]. This arrest makes it difficult to probe directly the Ran and energy requirements of the actual translocation from the cytoplasmic to the nuclear side of the NPC, which immediately precedes substrate release. Here, we have shown that in the case of transportin, dissociation of transportin–substrate complexes is uncoupled from transportin release from NPCs. This allowed us to dissect the requirements of translocation through the NPC, substrate release and transportin recycling. Surprisingly, translocation of transportin–substrate complexes into the nucleus requires neither Ran nor nucleoside triphosphates (NTPs). It is only nuclear RanGTP, not GTP hydrolysis, that is needed for dissociation of transportin–substrate complexes and for re-export of transportin to the cytoplasm. GTP hydrolysis is apparently required only to restore the import competence of the re-exported transportin and, thus, for multiple rounds of transportin-dependent import. In addition, we provide evidence that at least one type of substrate can also complete NPC passage mediated by importin β independently of Ran and energy.

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Results and discussion

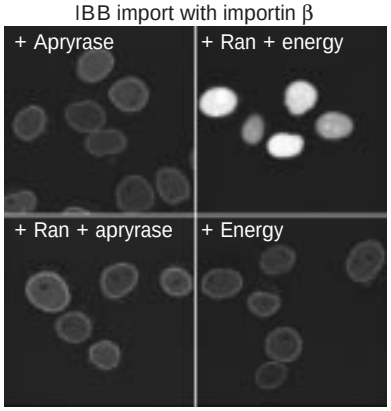
The mechanisms of carrier-mediated translocation of cargo through NPCs are one of the great mysteries in nucleocytoplasmic transport. These transport processes are generally considered to be active, but a direct input of metabolic energy could occur at many steps of a given transport cycle, including the actual nuclear pore passage. Whether and how NTP hydrolysis is coupled to translocation through NPCs is, clearly, absolutely crucial for any model of this process.

Importin β can function either as an autonomous import receptor or in conjunction with adapters, such as importin α in the case of the import of proteins with a classical nuclear localisation signal (NLS). Importin β binds cargo (or adapters) in the cytoplasm and mediates translocation through NPCs. The translocation is terminated at the nuclear side by direct binding of RanGTP to importin β, which leads to release of importin β from the nuclear pores and of importin α from importin β [6,7]. The importin β–RanGTP complex is then returned to the cytoplasm, where Ran is removed from importin β, Ran-bound GTP is hydrolysed, and the import competence of importin β is thereby restored [8–10]. GTP hydrolysis by Ran appears to be the main, if not sole, source of energy for importin β transport cycles [5,11,12].

Figure 1 shows importin-β-mediated nuclear import of a fluorescein-labelled IBB–core fusion protein (an engineered importin-β-specific substrate containing the importin-β-binding domain of importin α [13,14]). For this, and the following import experiments, we assessed import on unfixed samples by scanning directly through the import reactions using a confocal microscope. IBB import was very efficient in the presence of importin β, Ran and an energy-regenerating system. The omission of Ran and/or the energy-regenerating system prevented intranuclear accumulation and arrested the substrate at NPCs. This confirms the published data mentioned above and is an essential control here, because other transport-receptor–cargo combinations behave differently under identical conditions (see below).

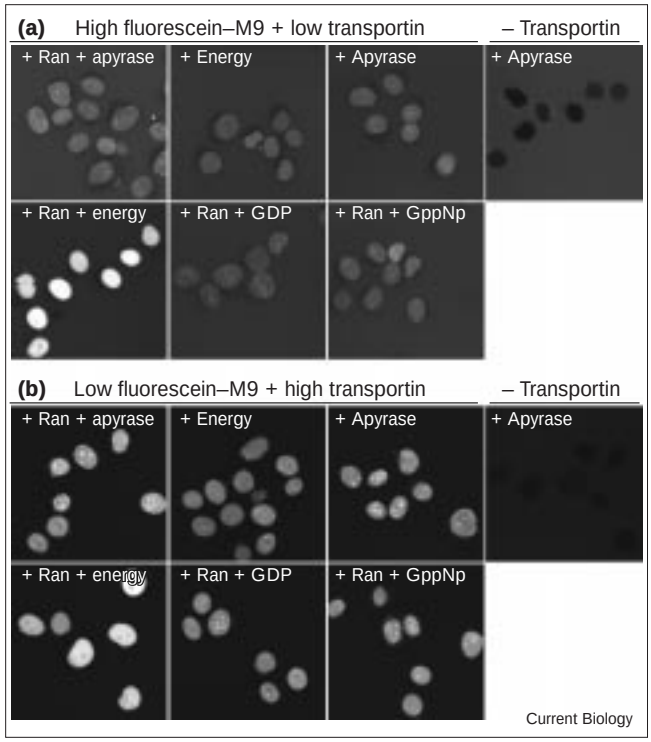
Transportin is related to importin β [15] and also releases its import substrate upon encountering RanGTP [16,17]. Figure 2a shows the import of a transportin-specific substrate — a core–M9 fusion protein. In the presence of transportin, Ran and energy, nuclear accumulation of M9 was very efficient. We then omitted Ran, or energy, or replaced the energy mix with GDP, the non-hydrolysable GTP analogue GppNp or apyrase (which depletes both

Figure 1



Nuclear pore passage of an IBB–core–importin β complex into the nucleus is Ran-dependent. Import of 0.7 μM fluorescein-labelled IBB–core pentamers into nuclei of permeabilised cells was performed in the presence of 0.8 μM importin β and with the indicated additions: 'Ran', a Ran mix containing 3 μM RanGDP, 0.3 μM NTF2, 0.3 μM RanBP1 and 0.2 μM Rna1p; 'energy', an energy mix of 1 mM ATP, 1 mM GTP, 20 mM creatine phosphate and 50 μg/ml creatine kinase; apyrase, used at 0.1 units/μl to deplete residual ATP and GTP. Import was for 5 min at 19°C. Panels show confocal scans directly through the import mixtures, detecting the IBB–core fusion protein in the fluorescein channel. (For further details on the *in vitro* import assay, see [23].)

Figure 2



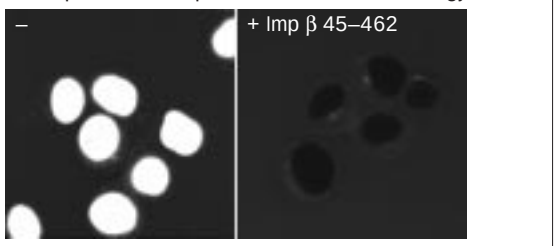
Translocation of a transportin–M9 complex into the nucleus is independent of Ran and energy. Import was performed as in Figure 1, using a fluorescein-labelled core–M9 fusion protein as import substrate and transportin as an import receptor. GDP and the GTP analogue GppNp were used at 0.5 mM. In (a), the import substrate (4.8 μM core–M9 pentamers) was in excess to transportin (1 μM), making efficient nuclear accumulation of M9 dependent on receptor recycling. In (b), transportin (2 μM) was in slight excess to the substrate (1.6 μM core–M9 pentamers), so import was independent of recycling. Note that transportin-mediated M9 import in the absence of Ran and energy is significant (compare corresponding panels with the control without transportin). At a high transportin-to-M9 ratio, transport was nearly as efficient as with Ran and energy.

ATP and GTP). All these treatments reduced M9 import to the same lower level, but crucial differences to IBB–core import by importin β became evident. In the absence of Ran or energy, no intermediates accumulated visibly at NPCs and, most importantly, nuclear accumulation was still significant when compared to the control case without transportin, in which M9 remained excluded from the nuclei. This suggests that a complete translocation of the transportin–M9 complex might occur in the absence of Ran and nucleotide hydrolysis. The fraction of M9 that accumulated was low, however, compared to the control case with Ran and energy. The import mix contained core–M9 pentamers in fivefold molar excess over transportin and a complete nuclear accumulation of the M9 substrate could have been accomplished only by multiple rounds of import. This, however, would involve the dissociation of receptor–substrate complexes in the nucleus by RanGTP after each round of import, followed by export of the transportin–RanGTP complex and the removal of RanGTP from transportin in the cytoplasm, which includes GTP hydrolysis. That Ran and hydrolysable GTP are essential for multi-round import is clearly evident (Figure 2).

A single round of import should be most efficient when transportin is present stoichiometrically to the import substrate. Indeed, when transportin was used in slight molar excess over core–M9, then nuclear accumulation in the absence of Ran and energy was nearly as efficient as in their presence (Figure 2b). It is important to note that this apparently energy-independent translocation of the transportin–M9 complex requires functional NPCs (Figure 3), as judged by its sensitivity to a dominant-negative importin β mutant [18]. It is thus fundamentally different from the passive diffusion of dextrans or small proteins, which is not affected by this mutant (U.K. and D.G., unpublished observation).

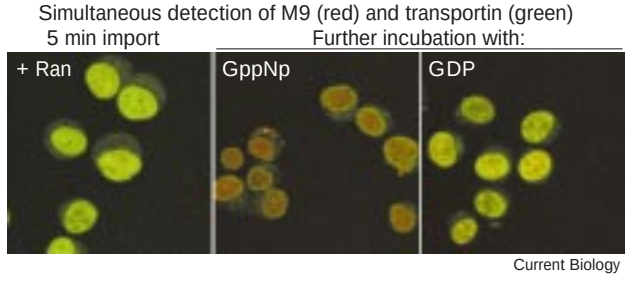
In Figure 4, we show the import of Texas-red-labelled core–M9 fusion protein with fluorescein-labelled transportin. The two labelled proteins were simultaneously detected in separate channels of a confocal microscope. Import was initially performed for 5 minutes in the presence of Ran but without exogenous NTPs. Both labelled proteins accumulated and co-localised in the nuclei. The sample was then split and either GppNp or GDP was

Figure 3



Energy-independent translocation of transportin–M9 complexes requires functional NPCs. Import of 1.6 μM fluorescein-labelled core–M9 pentamers with 2 μM transportin was performed as in Figure 2. Ran was not added and residual NTPs had been depleted with apyrase. Note that pre-incubation of the nuclei with 2 μM of the dominant-negative importin β mutant – comprising residues 45–462 (Imp β 45–462) – completely abolished import.

Figure 4



Dissociation of the transportin–M9 complex in the nucleus requires GTP, but not GTP hydrolysis. Import of 1.6 μM Texas-red-labelled core–M9 pentamers into nuclei of permeabilised cells was performed in the presence of 0.5 μM fluorescein-labelled transportin and the Ran mix but without energy. Both labels were detected in separate channels of the confocal microscope. Note that both transportin (green) and M9 (red) efficiently entered the nuclei under these conditions. Subsequent addition of the non-hydrolysable GTP analogue GppNp caused dissociation of the complex and re-export of transportin, whereas M9 remained nuclear. Addition of GDP had no effect.

added. GDP did not change the pattern; with GppNp, however, transportin was largely depleted from the nucleoplasm and gave NPC staining, whereas M9 remained nucleoplasmic. This effect was strictly dependent on prior addition of Ran (data not shown) and probably reflects the generation of RanGppNp in the nucleus. RanGppNp dissociates M9 from transportin and allows the transportin–Ran complex to exit the nucleus without M9. As seen from Figure 2, however, a multi-round import requires GTP hydrolysis and does not occur with GppNp. To enable cytoplasmic transportin to bind another M9 substrate, RanGTP must first be removed, a process that involves hydrolysis of the Ran-bound GTP. In the case of GppNp, 'dead-end' transportin–RanGppNp complexes accumulate and only a single round of import is possible.

Our data suggest that transportin-mediated translocation of M9 proteins into the nucleus is apparently independent of Ran and NTP hydrolysis. It could be argued, however, that this translocation is energy-dependent but that there is no requirement for exogenous NTPs because a sufficient amount was left in the permeabilised cells. We therefore measured, using a luciferase-based assay, how much ATP (as a representative for other NTPs) remained in the cells. We found that permeabilised cells contribute about 0.5–1 nM of available ATP to the reaction. This level dropped to below 0.1 nM after treatment with apyrase, which depletes ATP and GTP. Considering that micromolar concentrations of the substrates are transported, this does not permit a direct involvement of NTP hydrolysis, even if one assumes that all the remaining NTP molecules were consumed in the translocation.

One could further argue that translocation is NTP-dependent but that M9 import occurs in the absence of exogenous NTPs because NPCs can store 'conformational energy' for this purpose. An average HeLa cell nucleus has a volume of 10^{-12} litres and accumulated, in the absence of energy, the core–M9 pentamers to achieve a nuclear concentration of about 8 μM (data from Figure 2). This corresponds to 4.8×10^6 molecules per nucleus, translocated through approximately 5000 NPCs. Thus, on average, each NPC accomplished import of ~1000 substrate molecules. That a single NPC could possibly store conformational energy for this number of translocation events appears to be highly unlikely.

The second law of thermodynamics would be violated if, in the absence of an energy input, the M9 substrate accumulated against a gradient of chemical activity. Thus, under these conditions, NPCs can allow only a reversible equilibration of transportin–M9 complexes between nucleus and cytoplasm. The end point would be a steady state at which import and export rates are the same. That the end point of the equilibrium is a 1.5–4 times higher nuclear than cytoplasmic concentration (depending on the transportin-to-M9 ratio) clearly indicates the presence of intranuclear binding sites for the transportin–M9 complexes.

We do not yet understand the mechanisms of translocation through NPCs, but the observation that, at least in the case of transportin, there is no direct coupling to NTP hydrolysis and the RanGTPase cycle, greatly simplifies possible models. Perhaps the most appropriate description of the process is that of a facilitated diffusion that involves reversible binding of the transportin–M9 complex to at least one intermediate binding site at the NPC. Binding to this site (or sites) is sensitive to competition by importin β fragments (see Figure 3). The release from this site can occur

either back into the cytoplasm or into the nucleus and, as a result, the transportin–M9 complex equilibrates between the two compartments. Directionality of import, however, requires cooperation with the RanGTPase system.

We would not envisage import mediated by importin β to be fundamentally different from that mediated by transportin. The difference might simply be due to the fact that the importin β–IBB–core complex or the importin α/β–NLS complex binds to one of the intermediate sites so tightly that spontaneous release occurs only very slowly, and RanGTP-mediated release from nuclear pores becomes rate-limiting even for a single round of import. However, cargo-free importin β, at least, does not get trapped at these sites and can traverse the NPC in a Ran-independent manner [19]. This raises the issue of whether some substrate could also be carried into the nucleus by importin β in this mode. Indeed, as shown in the Supplementary material (published with this article on the internet), we could identify such a substrate, namely snurportin, the import adapter for U small nuclear ribonucleoproteins [20]. Single-round import of snurportin was dependent on importin β, but did not require the addition of Ran or energy. Taken together, one can conclude that a given transport receptor can use different modes of nuclear passage. Whether the completion of NPC passage of an import-receptor–cargo complex is Ran-dependent depends not only on the receptor, but also on the cargo it carries.

Precedents for translocations that are independent of NTP hydrolysis are the NPC passage of cargo-free β-family transport receptors such as importin β, transportin, or exportin-t [19,21,22], and the NTF2-mediated nuclear import of Ran [23]. What is new in this study is that a larger receptor–cargo complex can also use such a mode of translocation. The core–M9–transportin complex has a molecular weight of 220 kDa. Assuming a globular shape, this would correspond to a particle of 10.4 nm. Its NPC passage should already require some conformational change of the nuclear pore. NPCs can, however, accommodate transport of much larger particles, up to 25 nm in diameter [24]. It will be interesting to see whether the translocation of such large cargoes is qualitatively different.

Supplementary material

A supplementary figure showing that the translocation of snurportin through NPCs is independent of Ran and NTPs is available as Supplementary material published with this paper on the internet.

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Supplementary material

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Figure S1

Translocation of a snurportin–importin β complex through NPCs is independent of Ran and NTPs. Single-round import was performed as described in Figure 2b, except that 0.35 μM fluorescent snurportin was the import substrate and 0.5 μM importin β was used as the import receptor. Note that, under these conditions, nuclear accumulation of snuportin is dependent on importin β, but is independent of Ran and NTPs.