Nuclear protein import

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The defining feature of eukaryotic organisms is the cell nucleus. All nuclear proteins are synthesized in the cytoplasm and need to be imported through the nuclear pore complexes (NPCs) into the nucleus. Import can be directed by various signals, of which the classical nuclear localization signal (NLS) and the M9 import signal are the best characterized. The past year has provided insight into the functions of the key players in NLS- and M9-dependent import, the interactions of these key players and possible implications of these interactions for the import mechanism. Although an understanding of some of the steps in the import process is emerging, the molecular mechanism of the actual translocation through the NPC is still obscure.

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Abbreviations

GAP	GTPase-activating protein
GEF	guanine nucleotide exchange factor
hnRNP	heterogeneous nuclear RNP
IBB	importin-β-binding
NLS	'classical' nuclear localization signal
NPC	nuclear pore complex
NTF2	nuclear transport factor 2
Ran	ras-related nuclear protein
RanBP	Ran-binding protein
Ran-GDP	GDP-bound form of Ran
Ran-GTP	GTP-bound form of Ran
RCC1	regulator of chromosome condensation 1
RNP	ribonucleoprotein
Sc	*Saccharomyces cerevisiae*
snRNP	small nuclear RNP
SUMO-1	small ubiquitin-related modifier-1
XTP	xanthosine triphosphate

Introduction

The import of proteins into the nucleus occurs through the nuclear pore complexes (NPCs) which allow diffusion of small molecules and can accommodate the active transport of particles as large as several million dalton in weight or 25 nm in diameter [1]. The active import is energy-dependent and is mediated by saturable import receptors. Import into the nucleus can be conferred by several distinct import signals. The best characterized one is the 'classical' nuclear localization signal which consists of one or more clusters of basic amino acids [2]. It is referred to here as the NLS. The M9 domain is the import signal of the hnRNPA1 protein but it is unrelated in sequence to the 'classical' NLS [3]. NLS- and M9-containing proteins do not compete with each other for import and are recognized by distinct receptors ([4••] and see below). The import of U snRNPs (U-rich small nuclear ribonucleoproteins) defines another pathway into the nucleus [5,6] and even more are likely to exist.

An important contribution to the understanding of nuclear transport has been the concept of shuttling of import receptors [7], which appears to apply at least to NLS-dependent import. In this model, the import receptor initially binds its import substrate in the cytoplasm; it then carries the import substrate through the NPC into the nucleus, where the cargo is released from the transport receptor. To accomplish multiple rounds of transport, the import receptor finally has to return to the cytoplasm without the cargo. This model predicts asymmetric import/re-export cycles and implies that the binding of the transport receptor to its cargo is regulated by the different environments of the nucleus and cytoplasm.

A key step in the understanding of NLS-dependent protein import was the development of an *in vitro* system that faithfully reproduces the process [8]. In this system, a fluorescent import substrate is introduced into digitonin-permeabilized cells and its nuclear accumulation is monitored by fluorescence microscopy. A crucial consequence of plasma-membrane permeabilization is the depletion of the soluble contents of the cells. The observation that the active import depends on the readdition of cytosol or cytosolic fractions allowed the purification and the subsequent molecular characterization of four soluble factors presently known to be required for import, namely importins α and β which together constitute the NLS receptor, the GTPase Ran/TC4, and NTF2 (nuclear transport factor 2) [9–18]. Alternative names have been used for these components such as pp15 or p10 for NTF2, NLS receptor or karyopherin α for importin α, and p97 or karyopherin β for importin β.

In the following sections of this review, I will give an overview of NLS-dependent nuclear protein import, and describe some characteristics of the transport factors involved and the known interactions between them. The review is focused on the GTPase Ran, its interacting partners, and the possible roles of the Ran system in driving translocation and generating asymmetry in the transport cycles. Finally, the NLS-dependent import pathway will be compared with other import routes.

The importin-dependent pathway

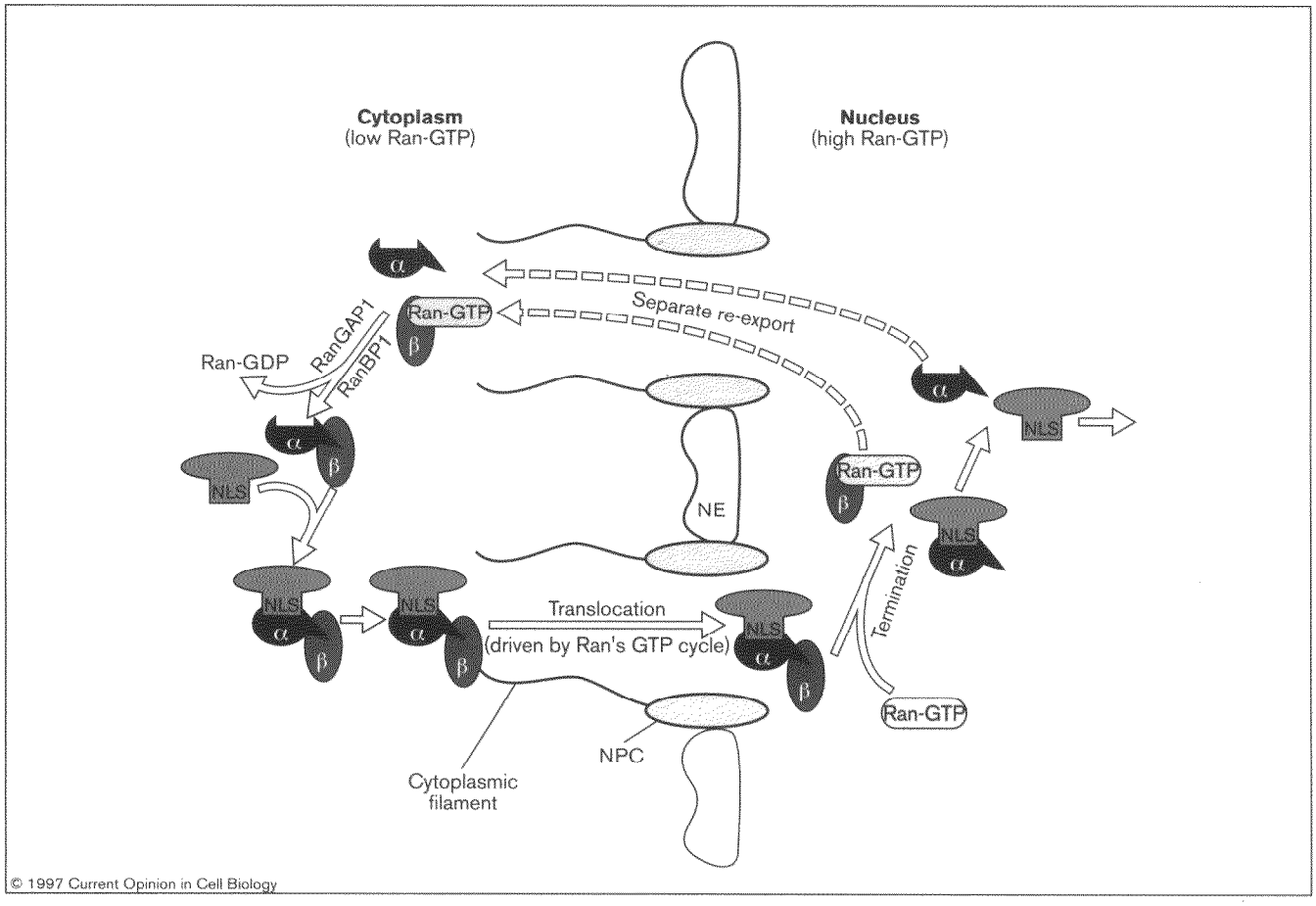
The following brief outline describes the stages of NLS-dependent import that have been distinguished experimentally (for details see below, Fig. 1, and [19] for a review). The initial, cytoplasmic event is the binding

of the import substrate via its NLS to the importin α–β heterodimer [12,20], with importin α providing the NLS-binding site [21,22]. Importin β is responsible for the initial docking to the cytoplasmic filaments of the NPC [23,24] and the subsequent translocation through the nuclear pore complex [25•,26•]. The transfer through the NPC is energy-dependent, requires GTP hydrolysis by Ran [14,16], and appears to be facilitated by NTF2 [15,17]. The translocation ends approximately at the terminal ring of the nuclear baskets inside the nucleus where the termination reaction disassembles the importin heterodimer and releases the import substrate into the nucleoplasm [27•]. Finally, the importin subunits are separately returned to the cytoplasm [25•,26•], without the import substrate.

Transport factors and interactions between them

Importin α has two functional domains, an importin-β-binding domain (IBB domain) at the amino terminus and an NLS-binding site consisting of eight so-called arm repeats. A fusion between the IBB domain and a heterologous reporter is transported very efficiently into the nucleus, in a manner that is dependent on importin β but that bypasses the requirement for intact importin α [25•,26•]. This implies that importin β accounts for the

Figure 1



A model for the importin-dependent nuclear protein import cycle. The initial, cytoplasmic event in import is the binding of the import substrate to the importin α–β heterodimer; the α subunit provides the binding site for the NLS of the import substrate. The resulting trimeric complex then docks to the cytoplasmic periphery of the NPC, and is subsequently translocated to the nuclear side of the NPC. The translocation is mediated by importin β, requires energy which is probably provided by Ran's GDP/GTP cycles, and is finally terminated at the nuclear side of the NPC by direct binding of Ran-GTP to importin β, binding which disassembles the importin heterodimer. This disassembly appears to be a specific nuclear event because free Ran-GTP should be stable inside the nucleus only. The NLS-bearing imported protein is then released. The two importin subunits are returned to the cytoplasm by different routes; importin β is probably exported as a complex with Ran-GTP, thus precluding binding of importin α to importin β on the way out. The dotted arrow is used to differentiate importin export from import, and also to show that nothing definite is known about the export of importin to the cytoplasm. In the cytoplasm, the importin-β–Ran-GTP complex needs to be dissociated before the importin α–β heterodimer can re-form and accomplish the next round of import. The dissociation of importin β from Ran-GTP appears to involve the activities of RanGAP1 and RanBP1, which convert Ran-GTP into Ran-GDP. For details and references, see main text. α, importin α; β, importin β; NE, nuclear envelope.

interactions with the NPC that drive translocation. In fact, importin β binds directly to the NPC [23,24], and almost the entire molecule of importin β appears to be involved in the interaction with the NPC [28].

Importin β binds specifically to Ran-GTP [27•,29•,30–32]. It stabilizes Ran-GTP against nucleotide exchange, inhibits the intrinsic GTPase activity of Ran [27•] and prevents GTPase activation by RanGAP (GTPase-activating protein) 1 [30]. In turn, Ran-GTP binding to importin β dissociates the importin α–β heterodimer [27•,32]. Ran-GTP binds to the amino terminus of importin β, whereas importin α binds to the carboxy-terminal region. Release of importin α upon Ran-GTP binding to importin β is therefore probably explained by a conformational switch in importin β [28].

The small GTPase Ran appears to be involved in multiple reactions following the initial docking of the import substrate to the cytoplasmic periphery of the NPC [14,16,27•,33•]. Like all G proteins, Ran switches between a GDP-bound and a GTP-bound state by nucleotide exchange and GTP hydrolysis. The intrinsic rates for these reactions are very low and have to be facilitated by specific factors (see Table 1). RCC1 (regulator of chromosome condensation 1) is Ran's major guanine nucleotide exchange factor (GEF) and generates Ran-GTP [34]. RanGAP1 is, so far, the only known GTPase-activating protein and causes conversion of Ran-GTP into Ran-GDP [35]. Another constituent of the Ran system is the Ran-binding protein RanBP1, which preferentially binds to Ran-GTP and facilitates the GTPase activation by RanGAP1 [36–38]. The *Saccharomyces cerevisiae* (Sc) homologues of Ran, RCC1, RanGAP1 and RanBP1 are encoded by the genes *GSP1*, *PRP20*, *RNA1* and *YRB1*, respectively, each of which is essential for viability. A variety of studies has demonstrated that the functional products of these genes are required for transport into and out of the nucleus (see Table 1 and [39–41,42•,43,44,45•,46–48]).

A characteristic feature of the Ran system is the asymmetric cellular distribution of its constituents. Ran itself is concentrated in the nucleus [49], although it is also detectable at the NPC [23,24,50] and in the cytoplasm. The RanGEF RCC1 is chromatin-bound [51] and generates Ran-GTP in the nucleus. In contrast, RanGAP1 and RanBP1 are excluded from the nucleus [52,53•] and deplete Ran-GTP from the cytoplasm. This predicts a steep Ran-GTP gradient across the nuclear envelope with a low Ran-GTP concentration in the cytoplasm and a high concentration in the nucleus. This probably explains why the Ran-GTP-mediated dissociation of the importin heterodimer is a specifically nuclear event that follows cargo translocation into the nucleus.

Interestingly, there is not only a soluble cytoplasmic pool of RanGAP1 but also a distinct proportion of RanGAP1 that is bound to RanBP2/Nup358 at the cytoplasmic filaments of the NPC [54•,55•]. RanBP2 [56,57] contains four RanBP1-homology domains that behave in biochemical assays identically to RanBP1 [36,58]. The close proximity of RanGAP1 with its coactivator RanBP2 in this complex should be particularly efficient in converting Ran-GTP to the GDP-bound form and in preventing nuclear Ran-GTP from reaching the cytoplasm. The NPC localization is conferred to RanGAP1 by a covalent modification

Table 1

The Ran system*.

Name (species)	Cellular localization	Effects on the RanGTPase	Effect on import
Ran (vertebrates) Gsp1p (Sc)	Mainly nuclear, but also found at the NPC and in the cytoplasm	–	Essential *in vivo* and *in vitro*. Provides energy for translocation; regulates interactions between importin α and importin β
RCC1 (vertebrates) Prp20p (Sc)	Nuclear, chromatin-bound	GDP/GTP-exchange factor. Stimulates nucleotide exchange 10^5-fold	Essential *in vivo*; no need for exogenous RCC1 *in vitro* because not depleted in permeabilized cells. Generates Ran-GTP gradient across the NE
RanGAP1 (vertebrates) Rna1p (Sc)	Soluble in the cytoplasm and found at the cytoplasmic filaments of the NPC	Activates the RanGTPase 10^5-fold, that is, it converts Ran-GTP into Ran-GDP	Essential *in vivo*; stimulatory *in vitro* (not completely depleted in permeabilized cells). Generates Ran-GTP gradient across the NE by depleting Ran-GTP from the cytoplasm
RanBP1 (vertebrates) Yrb1p (Sc)	Cytoplasmic. Excluded from the nuclei by a nuclear export signal and by cytoplasmic retention	Binds specifically to Ran-GTP (K_D≈0.3 nM); blocks nucleotide exchange; 10-fold stimulates GTPase activation by RanGAP1	Essential *in vivo*; stimulatory *in vitro*. Contributes to the Ran-GTP gradient across the NE
RanBP2 (vertebrates only)	At the tip of the cytoplasmic filaments of the NPC	Contains four RanBP1-homology domains that behave identically to RanBP1	Might constitute an initial cytoplasmic docking site for the NLS–receptor complex. Probably functional overlap with RanBP1. No equivalent in *S. cerevisiae*
Importin β (vertebrates, Sc)	NPC; shuttles between nucleus and cytoplasm	Binds specifically to Ran-GTP (K_D≈1 nM); blocks nucleotide exchange; inhibits intrinsic GTPase; prevents GTPase activation by RanGAP1. Can form a trimeric importin-β–Ran-GTP–RanBP1 complex	Key mediator of NLS-dependent protein import; essential *in vivo* and *in vitro*. Interactions with Ran-GTP required for termination of import
NTF2 (vertebrates, Sc)	NPC and soluble	No obvious effects on the RanGTPase	Normally essential *in vivo*. Requirement for NTF2 can be bypassed by Gsp1p overexpression. Stimulatory *in vitro*

*For details and references, see main text. NE, nuclear envelope.

with a ubiquitin-related protein called SUMO-1 (small ubiquitin-related modifier-1) or GMP1 (GAP-modifying protein 1) [54•,55•].

A number of studies confirm that the function of the Ran system indeed crucially depends on the proper intracellular localization of its constituents. For example, RanBP1 is normally cytoplasmic by virtue of its carboxy-terminal domain containing a nuclear export signal and a cytoplasmic retention signal [53•]. If this carboxy-terminal domain is deleted, RanBP1 accumulates inside the nucleus and then has a strong dominant-negative effect on nuclear transport. Likewise, the breakdown of the Ran-GTP gradient across the nuclear envelope inhibits transport into and out of the nucleus. If the cytoplasmic Ran-GTP concentration is raised with a GAP-resistant Ran mutant, then nuclear import becomes blocked [42•,45•,46], probably primarily because Ran-GTP dissociates the importin heterodimer in the cytoplasm. The opposite experiment of lowering the nuclear Ran-GTP concentration also inhibits multiple nuclear transport pathways. This has been shown, for example, in the tsBN2 cell line which is temperature-sensitive for the RanGEF RCC1 [41,59] or by injecting RanGAP into nuclei of *Xenopus* oocytes (E Izaurralde, personal communication).

NTF2 was originally identified as an activity that stimulates nuclear import *in vitro* [15,17]. It was subsequently shown to interact with NPCs [60•,61•], isolated nucleoporins, Ran-GDP, and importin β *in vitro* [31,60•,62]. Yeast NTF2 is essential both for viability and for nuclear transport [60•,61•]. However, the NTF2 null allele can be completely suppressed by overexpression of the yeast Ran Gsp1p (PA Silver, personal communication). The crystal structure of NTF2 has recently been solved [62].

What are the translocation intermediates?

The early steps of nuclear import are reasonably well understood. First, the import substrate binds to the importin α–β heterodimer which is stable in the cytoplasm because RanGAP1 and RanBP1 (and RanBP2) keep levels of cytoplasmic Ran-GTP very low, which otherwise would dissociate the complex. The trimeric NLS–importin-α–importin-β complex then docks via the importin β subunit to the cytoplasmic filaments of the NPC and is translocated through the NPC, probably as a single entity.

The actual nuclear passage can be visualized by using gold-labelled import substrates and electron microscopy [63]. The substrate is transported over a rather long distance of more than 100 nm. The translocation is, therefore, probably not a single event, but instead may involve a number of import intermediates. An 'elementary' translocation step would be the transition from one intermediate to the next. Which and how many import intermediates are observed seems to depend on the experimental conditions. If only a small number of gold particles cross the NPC, intermediates are preferentially seen at three distinct positions: firstly, at the tip of the cytoplasmic filaments; secondly, near the central channel; and thirdly, occasionally also at the nuclear baskets [64•]. This might suggest that as few as three intermediates exist and that the single translocation steps are rather long. However, it is also possible that these distinct positions mark the rate-limiting steps only and that other intermediates are too transient to be detected frequently. This second possibility is supported by earlier studies that show multiple 'lined-up' gold grains at single NPCs, with the gold lines beginning at the cytoplasmic filaments and spanning the entire NPC up to its nuclear side [63,65].

Unfortunately, none of the translocation intermediates have been characterized at molecular resolution, for example, by a nearest-neighbour analysis of NPC-bound importin β. That is, we can only guess which nucleoporins actually constitute the intermediate binding sites in intact and functional NPCs. However, this knowledge would be a prerequisite to understanding the translocation steps *per se*, that is, the transitions between the intermediates.

What drives translocation?

GTP hydrolysis by Ran is essential for passage through the nuclear pore [14–16]; it might even constitute the sole source of energy for translocation [33•]. The latter conclusion has been derived from a number of observations. Import with wild-type transport factors can be blocked with nonhydrolyzable GTP analogues; ATP analogues have no effect, suggesting that only GTP and not ATP is hydrolyzed to provide energy for import. To test if Ran was the only GTP-consuming component, a mutant Ran that utilizes XTP instead of GTP was used [33•]. Import now became XTP-dependent and resistant to inhibition by GTP analogues, suggesting that the rate-limiting steps of import are driven solely by nucleotide hydrolysis by Ran and not by other NTPases.

As Ran appears to be needed for translocation through the cytoplasmic and the nuclear parts of the NPC, it is probably required on both sides of the NPC. Import requires cytoplasmic Ran to be in its GDP-bound form [27•,33•]. Consistent with the requirement that Ran be in its GDP-bound form for import, Ran-GDP has indeed been shown to bind to NPCs [27•]. As import requires Ran to go through its entire GTP/GDP-bound cycle, one has to postulate a local nucleotide exchange and GTP hydrolysis at the nuclear pore complex. However, the identity of the Ran receptor(s) at the NPC that would couple Ran's GTP/GDP cycle to the actual translocation process is still obscure. Although three candidates have been suggested for this function, namely, NTF2, importin β, and RanBP2, their known properties and interactions can hardly explain the Ran dependence of translocation. Yeast are viable without NTF2, provided that Gsp1p is overexpressed (PA Silver, personal communication). This

makes it unlikely that NTF2 is the central component that recruits Ran for translocation.

Importin β in turn is also unlikely, for several reasons, to translate Ran's GTP cycle into a directed movement. First, the Ran that is bound to importin β is unable to hydrolyze GTP [27•,30]. Second, the binding of Ran-GDP to NPCs does not depend on importin β [27•]. Third, importin β has only a very low affinity for Ran-GDP; binding of the GTP-bound form to importin β, however, dissociates importin α from importin β [27•,29•,32], terminating translocation. This should not happen before the nuclear side of the NPC is reached. Finally, an importin β mutant that is deficient in Ran-GTP binding can cross the nuclear envelope and only gets arrested at a very late stage of import [27•].

S. cerevisiae has no equivalent to RanBP2, only two distantly related proteins, Nup2p and Yrb2p, whose affinities for Ran (Gsp1p) are negligibly low (e.g. the affinity of Nup2p for Gsp1p is 2500 times lower than is the affinity of yeast RanBP1 for Gsp1p; FR Bischoff, personal communication). Neither Nup2p nor Yrb2p is essential and even the double-deletion strain is viable and without obvious import defects (G Schlenstedt, personal communication). Assuming that translocation is essential and conserved between yeast and mammals, Ran binding to RanBP2 appears unlikely to explain translocation into the nucleus. Also, a Ran-binding domain of RanBP2 alone has a high affinity for only Ran-GTP and not for Ran-GDP [36,58], which predominates in the cytoplasm.

Importin β can participate in a tetrameric importin-α–importin-β–Ran-GDP–RanBP1 complex [29•]. However, this does not solve the problem of coupling the GTP cycle to translocation, because if the nucleotide were exchanged to GTP then importin α would be released and translocation aborted. Furthermore, it remains to be shown how a model in which the importin-α–importin-β–Ran-GDP–RanBP1 complex moves through the NPC can be reconciled with RanBP1 containing a cytoplasmic retention signal [53•] which should prevent it from entering the nucleus.

Thus, the question of which components couple translocation through the NPC to the GTP cycle of Ran still remains to be answered. This not only applies to the problem of nucleotide exchange at the NPC, which is certainly not catalyzed by chromatin-bound RCC1; it is also by no means clear if all Ran-dependent GTP-hydrolysis steps required for translocation are actually triggered by RanGAP1.

Termination of import and recycling

The translocation into the nucleus is terminated when the NLS–receptor complex has reached the nuclear side of the NPC. This involves the dissociation of the importin α–β heterodimer by direct binding of nuclear Ran-GTP to importin β. As a result, importin α is released into the nucleoplasm. As the isolated importin α subunit has a lower affinity for NLS than does the heterodimer [27•,32], termination should also facilitate the release of the NLS from its receptor.

If termination is prevented, as in the case of an importin β mutant that is deficient in Ran binding, then import intermediates become arrested at the nuclear baskets [27•], blocking the nuclear pore complex for subsequent transport events [28]. Interestingly, not only are NLS–importin complexes then prevented from crossing the nuclear envelope, but the NPC is also blocked for M9-dependent import and export of NES (nuclear export signal)-containing proteins, mRNA and U snRNA [28]. Only tRNA can still pass through the NPC, and diffusion remains unaffected.

Once termination has occurred, the importin subunits need to be recycled back to the cytoplasm. A number of indications suggest that importin α and β are re-exported separately. First, importin β is re-exported more quickly than is the α subunit [23,24]. Second, the IBB domain of importin α confers import into but not export out of the nucleus [25•,26•]. The simplest explanation would be if importin β were exported as part of a Ran-GTP complex which would preclude importin α binding to importin β on the way out. The importin-β–Ran-GTP complex would then have to be disassembled in the cytoplasm. The importin-β–Ran-GTP complex itself is GAP-resistant [30]. However, it appears that RanGAP1 can cooperate with RanBP1 and a third factor to allow GTP hydrolysis and the release of free importin β [27•,66], and hence allow a new import cycle to begin.

M9-dependent import

The 38-residue M9 domain of hnRNPA1 is sufficient to confer import into the nucleus, but it bears no sequence similarity to classical NLSs [3]. Recently, transportin, the import receptor of the M9 pathway, was identified [4••]. Human transportin is 24% identical to importin β and it binds directly to the M9 signal with no equivalent of importin α being involved. A parallel study [67•] identified the *S. cerevisiae* homologue of transportin, also called Kap104, as a mediator of nuclear import of Nab2p and Hrp1p, which are two mRNA-binding proteins that are related to human hnRNP proteins. It seems likely that, in addition to NLS- and M9-dependent import, even more pathways into the nucleus exist.

Why would cells need several distinct nuclear import pathways? One possible answer is that it might be advantageous to regulate import of distinct classes of substrates separately. For example, transportin carries M9-containing proteins like hnRNPA1 into the nucleus where they assemble with hnRNA (heterogeneous nuclear RNA) and probably contribute to mRNA export. hnRNPA1 shuttles between nucleus and cytoplasm, whereas

its import depends on ongoing transcription by RNA polymerase II [3,68]. It obviously makes perfect sense to adjust the capacity of hnRNP import to the needs for hnRNP proteins in the nucleus, without at the same time affecting import in general.

Conclusions

The past year has seen great progress in the characterization of the factors involved in NLS- and M9-dependent protein transport into the nucleus. We are about to understand some of the steps of nuclear protein import. However, the mechanism of the actual translocation will continue to challenge the field in the foreseeable future. Import requires not only the presence of the essential transport factors, but also their proper distribution between nucleus and cytoplasm. This implies that the simplest system to reproduce the import reaction will probably be as complex as intact nuclei. After having identified and characterized the key import factors, we now have to study their interactions with functional nuclear pore complexes at molecular resolution.

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