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MEDAL REVIEW

Transport into and out of the cell nucleus

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Introduction

A hallmark of eukaryotic cells is their separation into compartments. These compartments are surrounded by membranes that are impermeable to macromolecules. As most proteins are synthesised in the cytoplasm, specific transport systems have evolved to allow proteins to be imported from the cytoplasm into these compartments. I have been fascinated by this intracellular trafficking from the very beginning of my scientific life and had my first practical encounter with the problem in 1987 during a student project. This was in T.A.Rapoport's laboratory where, later, I also did my diploma and Ph.D. work on protein import into the rough endoplasmic reticulum (rER). During this time I identified and purified some of the key components of the machinery that translocates, for example, secretory proteins into the rER (Görlich *et al*., 1992a,b), and was finally able to reconstitute this 'translocon' from purified proteins and lipids (Görlich and Rapoport, 1993). After this, I began working on nucleocytoplasmic transport, from 1993 in R.A.Laskey's laboratory (Cambridge, UK) and from 1996 with my own laboratory at the ZMBH in Heidelberg. This review will focus on nuclear transport; however, to begin, a brief comparison of import into the various compartments might be useful.

Import, for example into mitochondria, chloroplasts or the rER, requires specific targeting sequences that are usually removed during import (for review see Rapoport *et al*., 1996; Schatz and Dobberstein, 1996). Once a protein has been imported, for example, into the rER, there is normally no return to the cytoplasm. In contrast, in the case of nuclear transport, import and export are equally major processes (for review see Görlich, 1997; Nigg, 1997; Mattaj and Englmeier, 1998). Many proteins need to be imported into the nucleus multiple times, as is the case for proteins which shuttle continuously between nucleus and cytoplasm. In addition, higher eukaryotes have an open mitosis and all not-chromatin-bound nuclear proteins need to be re-imported upon reformation of the nuclear envelope after mitosis. This may be a reason why nuclear import signals are not removed during import.

The nucleus is surrounded by the nuclear envelope (NE), a double membrane that is continuous with the rER. The NE is penetrated by nuclear pore complexes (NPCs) which are the sole sites of exchange between nucleus and cytoplasm (Feldherr *et al*., 1984). They have a mass of ~125 MDa in higher eukaryotes and are estimated to consist of 50–100 different proteins (for review see Doye and Hurt, 1997). NPCs provide a 9 nm diffusion channel for ions, metabolites and, in principle, also for macromolecules smaller than ~60 kDa. However, the transport of small RNAs such as tRNA, and small proteins such as histones, is normally active and carrier-mediated (Zasloff, 1983; Breeuwer and Goldfarb, 1990). NPCs can accommodate active transport of particles as large as 25 nm in diameter or several million Daltons in molecular weight. This transport of large protein assemblies or ribonucleoparticles (RNPs) through the NPCs is fundamentally different from protein import into mitochondria, chloroplasts or the rER, where proteins cross the membrane one by one and in a fully unfolded state.

Nucleocytoplasmic transport is a tremendous activity, comprising a multitude of substrates. Not only do all nuclear proteins need to be imported from the cytoplasm, but tRNA, rRNA and mRNA, which are synthesised in the nucleus, need to be exported to the cytoplasm where they function in translation. Indeed, the biogenesis of ribosomes even involves multiple crossings of the NE; ribosomal proteins are first imported into the nucleus, assemble in the nucleolus with rRNAs and finally are exported as ribosomal subunits to the cytoplasm.

An essential demand on the nuclear transport system is selectivity and specificity; the system has to ensure that only the 'correct' cargoes are imported or exported at the proper time. Specific transport signals contribute crucially to the fidelity of nuclear transport. The prototype of such a signal is the 'classical' nuclear localisation signal (NLS), which was first identified in nucleoplasmin and the SV40 large T antigen (reviewed in Dingwall and Laskey, 1991).

Soluble transport factors

A great advance in studying nuclear protein import has been the development of an *in vitro* system based on permeabilised mammalian cells (Adam *et al*., 1990). The selective permeabilisation of the cholesterol-rich plasma membrane with digitonin has two major consequences. First, a fluorescent import substrate can be introduced into the cells and its uptake followed by fluorescence microscopy. Secondly, the cells are depleted of their soluble contents, allowing assay of essential, soluble transport factors. The use of this system has resulted in the purification, molecular cloning and functional characterisation of the key players in the classical nuclear import pathway, namely Ran, importin α, importin β and NTF2 (for references see below and Nigg, 1997).

Identification of importin

At the time that I began working on nuclear protein import, one essential nuclear transport factor, the GTPase Ran, had been identified (Melchior *et al*., 1993; Moore

and Blobel, 1993). In addition to Ran, the cytosol contained a then unknown number of additional essential import factors. To assay for the purification of one component, the system had to be simplified. I therefore tested various chromatographic resins for their capacity to deplete import activity from a cytosol, hoping to find a highly selective matrix to which only one essential import factor would bind. This strategy allowed me to purify another essential soluble import factor, importin α, on the basis of its unusual property of binding tightly to nickel-agarose (Görlich *et al*., 1994). The key observation was that a *Xenopus* egg extract lost its import activity when passed through the nickel column. Import activity was restored by re-addition of the nickel-bound fraction. As very few proteins bind under these conditions, the nickel-agarose step provided an almost single step purification of *Xenopus* importin α. Recombinant importin α could restore import activity of a nickel-depleted extract, proving that the importin α indeed accounts for this import activity. Sequencing of the corresponding gene revealed striking homology of importin α to a number of proteins of until then unknown function, such as SRP1p from *Saccharomyces cerevisiae* and mammalian Rch1p. These factors were subsequently shown to function in these organisms in the same way as importin α functions in *Xenopus* (Imamoto *et al*., 1995b; Loeb *et al*., 1995).

The β subunit of importin was cloned nearly simultaneously by a number of laboratories (Chi *et al*., 1995; Görlich *et al*., 1995a; Imamoto *et al*., 1995a; Radu *et al*., 1995). We identified importin β as stoichiometrically co-purifying with importin α on an anti-importin α immunoaffinity column.

Dissecting importin function

Having the key import factors in hand, we could begin to characterise them functionally and dissect the mechanism of import (Figure 1). The initial cytoplasmic event in NLS-dependent nuclear protein import turned out to be the binding of the import substrate to the importin α/β heterodimer (Görlich *et al*., 1995a; Imamoto *et al*., 1995c). Importin α provides the NLS-binding site (Adam and Adam, 1994) and interacts via its importin β-binding domain (IBB domain) with the β-subunit (Görlich *et al*., 1996a; Weis *et al*., 1996). Importin β in turn interacts with the NPC (Görlich *et al*., 1995b; Moroianu *et al*., 1995). The transfer of the trimeric NLS/importin α/β complex through the NPC is energy-dependent and appears to require GTP hydrolysis by Ran (Melchior *et al*., 1993; Moore and Blobel, 1993). However, the actual mechanism of the NPC passage is still far from clear. Translocation into the nucleus is terminated at the nuclear side of the NPC by disassembly of the trimeric NLS/importin α/β complex. The NLS/importin α complex behaves like an inert cargo during import and the actual movement through the NPC is generated by importin β. In fact, the requirement for importin α can even be bypassed in certain circumstances. For example, fusion proteins containing the IBB domain from importin α can be bound directly and imported by importin β (Görlich *et al*., 1996a; Weis *et al*., 1996).

After importin α and β have delivered their cargo into the nucleus, they need to return to the cytoplasm to mediate the next round of import. This raises the interesting problem of how importin α leaves the nucleus without the cargo it has just carried in. The question already implies that export of importin α cannot be simply the reversal of its entry and that importin α must return to the cytoplasm in a low-affinity form for NLS binding. It soon became clear that the importin transport cycle is indeed asymmetric in that the subunits of importin enter the nucleus together, but leave it via separate routes. They are separated from each other as a consequence of the import reaction and return to the cytoplasm at very different rates (Görlich *et al*., 1995b; Moroianu *et al*., 1995; Izaurralde *et al*., 1997). Furthermore, the IBB domain from importin α is sufficient for nuclear entry, but not for export, suggesting that importin α is exported from the nucleus independently of the β subunit (Görlich *et al*., 1996a; Weis *et al*., 1996).

The importin α/β heterodimer is dissociated by direct binding of RanGTP to importin β (Rexach and Blobel, 1995; Chi *et al*., 1996; Görlich *et al*., 1996c). This reaction is a key step in nuclear protein import and explains at least part of its Ran-dependence. It had been suggested that multiple cycles of this dissociation reaction followed by re-association of the importin heterodimer would constitute the actual translocation of the import substrate through the NPC into the nucleus (Rexach and Blobel, 1995). For conceptual reasons we considered this model unlikely (Görlich and Mattaj, 1996). However, we wanted to test experimentally where at the NPC this dissociation takes place, and at which stage of import it is required. To address this question, we needed an importin β mutant deficient in Ran binding; we generated such a mutant by deleting the 44 N-terminal amino acids from importin β (Görlich *et al*., 1996c; Kutay *et al*., 1997a). Complexes of this ΔN44 importin β mutant and importin α were indeed resistant to dissociation by RanGTP. When tested in *in vitro* import assays, the mutant allowed docking of fluorescent import substrates to the NPC. However, it did not support complete import and transport intermediates arrested at the NE even in the presence of energy and Ran (Görlich *et al*., 1996c). Because fluorescence microscopy could not resolve the exact sites of the arrest, we repeated the experiment with gold-labelled substrates and visualised them by electron microscopy. We observed that the importin β mutant allowed translocation of the substrate through the NPC; however, it failed to release the substrate from the nuclear baskets of the NPC into the nucleoplasm.

Thus, the dissociation of the importin heterodimer by RanGTP is not driving the translocation through the NPC; instead, it terminates translocation at the nuclear side of the NPC and releases importin α together with the import substrate into the nucleus. To explain this order of events, we have suggested that the dissociation of the importin heterodimer occurs specifically in the nucleus because free RanGTP might be available only there (Görlich *et al*., 1996b,c); Ran itself is predominantly, though not exclusively, nuclear (Bischoff and Ponstingl, 1991b). Ran's major nucleotide exchange factor is the chromatin-bound RCC1, which generates RanGTP inside the nucleus (Ohtsubo *et al*., 1989; Bischoff and Ponstingl, 1991a). In contrast, Ran's principal GTPase activating protein, RanGAP1, is excluded from the nucleoplasm (Hopper *et al*., 1990; Matunis *et al*., 1996; Mahajan *et al*., 1997).

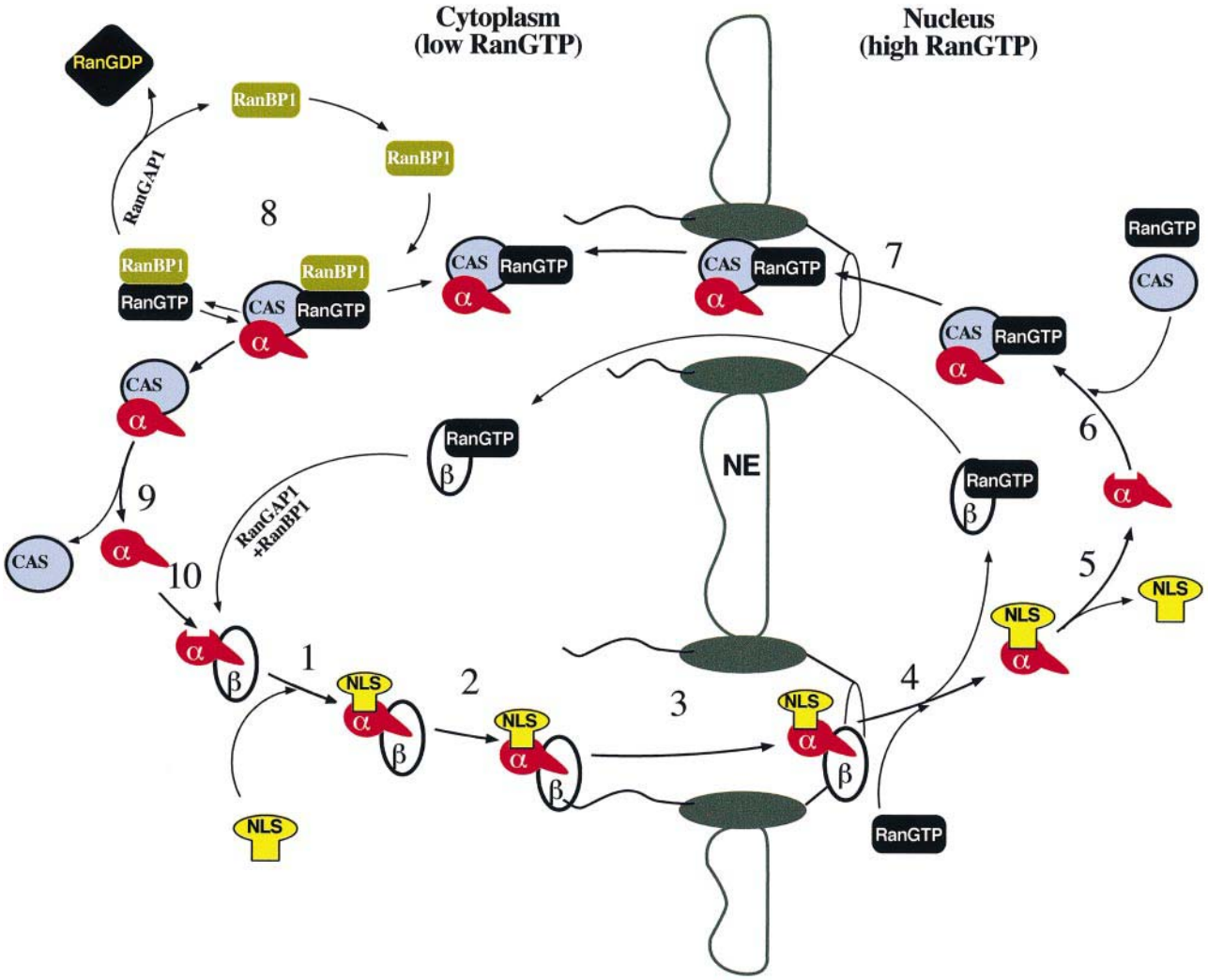

Fig. 1. Scheme of the importin α transport cycle. (1) The initial, cytoplasmic event in NLS-dependent import is the binding of the import substrate to the importin-α/β heterodimer, where the importin-α subunit provides the NLS binding site. (2) The resulting trimeric complex then docks via importin β to the cytoplasmic periphery of the NPC and (3) is translocated to the nuclear side where it meets an environment with a high RanGTP concentration. The mechanism of translocation is not yet understood. (4) The direct binding of nuclear RanGTP to importin-β terminates the translocation and disassembles the importin heterodimer. (5) The NLS protein is released from importin α. (6) Importin α is incorporated into a trimeric RanGTP/CAS/importin α complex and (7) CAS promotes export to the cytoplasm. (8) In the cytoplasm, RanBP1 causes the dissociation of RanGTP from the RanGTP/CAS/importin α complex which is subsequently made irreversible by RanGAP1-triggered GTP hydrolysis. (9) The Ran-free CAS is now in the low-affinity form for importin α binding, importin α is released and (10) can combine with importin β to re-form the importin heterodimer and participate in another round of import. Importin β probably accounts for its own export as a RanGTP complex. The efficient cytoplasmic release of RanGTP from importin β requires RanBP1, RanGAP1, and also the presence of importin α. Cargo-free CAS can apparently cross the NPC on its own and independently of NTP hydrolysis. Two molecules of Ran appear to be exported per transport cycle. It is still not known how Ran is replenished in the nucleus.

RanGAP1 stimulates conversion of RanGTP to the GDP-bound form (Bischoff *et al*., 1994) and thereby depletes RanGTP from the cytoplasm. This reaction is further facilitated by another cytoplasmic factor, the Ran-binding protein RanBP1 (Coutavas *et al*., 1993; Bischoff *et al*., 1995; Richards *et al*., 1996). This asymmetric distribution of Ran, RCC1, RanGAP1 and RanBP1 should result in a steep RanGTP gradient across the nuclear envelope with a high nuclear concentration and a very low level in the cytoplasm, which allows the importin heterodimer to form in the cytoplasm and forces its dissociation in the nucleus. In our model, the local RanGTP concentration is a marker for a nuclear or a cytoplasmic environment which the Ran-binding site in importin β can 'sense'. Furthermore, the RanGTP gradient apparently confers directionality not only to import, but also to exportin-mediated transport out of the nucleus (see below).

Importin β is probably exported to the cytoplasm as a complex with RanGTP, which would prevent importin β from exporting the α subunit (Izaurralde *et al*., 1997). RanGTP is released from importin β in the cytoplasm with the aid of RanBP1, RanGAP1 and importin α, restoring importin β in an import-competent form (Bischoff and Görlich, 1997; Floer *et al*., 1997; Lounsbury and Macara, 1997).

Recycling of importin α

The export of importin α to the cytoplasm turned out to be a very interesting process. Not only is it an essential

part of the importin-transport cycle, but it also provided novel insights into nuclear export in general. We found that importin α export requires a specific nuclear export receptor, namely CAS (Kutay *et al*., 1997b). This export activity first became apparent when we studied importin α export with various sources of soluble transport factors. In the presence of *Xenopus* egg extract, the import substrate accumulated in the nuclei of permeabilised cells, and importin α was efficiently re-exported. In contrast, when import was performed with recombinant transport factors (e.g. importin, Ran), then import of the NLS substrate was fully reconstituted, but importin α export was not. This suggested that nuclear export of importin α is mediated by a soluble export factor which is abundant in the egg extract, but limiting in permeabilised cells. The fact that depletion of nuclear RanGTP blocked importin α export from *Xenopus* oocyte nuclei (Görlich *et al*., 1997; Izaurralde *et al*., 1997) further suggested that importin α's export receptor might have to interact with RanGTP in order to bind and export importin α. With the aid of immobilised RanGTP we could then isolate such a trimeric 'export complex' consisting of RanGTP, CAS and importin α, and thereby identify CAS as the importin α-specific exportin. Exogenous, recombinant CAS greatly stimulated importin α export *in vitro*, confirming its identity with the limiting soluble export factor. Furthermore, recent genetic evidence suggests that Cse1p, the *S.cerevisiae* homologue of CAS, mediates nuclear export of importin α in yeast (G.Schlenstedt, personal communication).

Both CAS and importin β have separate binding sites for importin α and RanGTP. However, RanGTP regulates importin α binding to CAS and to importin β in exactly the opposite manner; RanGTP dissociates importin α from β. RanGTP increases CAS' affinity for importin α by ~300-fold (Kutay *et al*., 1997b). The RanGTP gradient across the NE with a high nuclear concentration would thus ensure that importin α binds tightly to β in the cytoplasm, but preferentially to CAS inside the nucleus. Once the trimeric RanGTP/CAS/importin α complex has formed, it is probably transferred directly to the cytoplasm. It is likely that CAS makes a direct contact with the NPC and drives the translocation out of the nucleus. CAS' preference for NLS-free importin α probably allows importin α to leave the nucleus without the cargo it just carried in. In the cytoplasm, importin α needs to be released from its export mediator. This cytoplasmic release is accomplished by RanBP1 which initially binds Ran in the trimeric RanGTP/CAS/importin α complex (Figure 1; Bischoff and Görlich, 1997). A RanBP1/RanGTP complex is transiently released and attacked by RanGAP1, which triggers GTP hydrolysis and makes the dissociation irreversible. The Ran-free CAS is now in the low-affinity form for importin α binding, and can release its cargo and re-enter the nucleus to mediate another round of export. Interestingly, for nuclear entry of CAS, no other soluble factor and/or energy in the form of nucleoside triphosphates appears to be required (Kutay *et al*., 1997b). Indeed it seems to be a general phenomenon that 'empty' transport receptors can pass the NPC in such an energy-independent way (Kose *et al*., 1997; Kutay *et al*., 1998; Nakielny and Dreyfuss, 1998).

Figure 1 summarises the transport cycle that results in nuclear accumulation of proteins with a classical NLS. An obvious, open question is how Ran, which appears to be constantly exported, is replenished in the nucleus. Because an importin β-like transport receptor would also export one RanGTP per transport cycle, we would assume that Ran does not employ this type of receptor for its own import.

The importin-dependent transport cycle involving an actual import receptor, namely importin β, and an additional adaptor molecule, namely importin α, appears rather complicated. However, importin β can also directly bind and import proteins independently of importin α. This was first demonstrated with an artificial fusion protein containing the IBB domain from importin α (Görlich *et al*., 1996a; Weis *et al*., 1996). More recent data suggest that importin β alone also accounts for nuclear import of the HIV Rev protein (Henderson and Percipalle, 1997) and a number of ribosomal proteins (S.Jäkel and D.Görlich, unpublished). Similarly, transportin, an importin β-related factor, binds its import substrates (hnRNP proteins) directly (Pollard *et al*., 1996). This mechanism of import is probably evolutionarily ancient and raises the question of why importin β uses the importin α adaptor for import of most of its substrates. The answer might be that import signals, such as those found in ribosomal proteins, are rather large and complex. The use of several distinct species of importin α apparently allows recognition of a wider range of simpler signals, such as the classical NLS.

Export of tRNA from the nucleus

A nuclear transport pathway that has been studied for a long time is nuclear export of tRNA. It was actually the first pathway shown to be saturable and thus to be mediated by a specific transport carrier (Zasloff, 1983). Furthermore, the observation that excess tRNA saturates only its own export, but not that of, for example, mRNA, U snRNA or ribosomal subunits, led to the conclusion that the putative tRNA-export receptor can discriminate tRNA from other export substrates and is thus class-specific (Jarmolowski *et al*., 1994).

We were interested in tRNA export because it became blocked upon depletion of nuclear RanGTP by microinjection of RanGAP into the nucleus (Izaurralde *et al*., 1997). Export could be rescued by co-injection of the GTPase-deficient and RanGAP-resistant RanQ69L mutant. This suggested that tRNA is exported by a CAS-like exportin. Because tRNA export represents a major activity, we expected the corresponding exportin to be an abundant RanGTP-binding protein. At that time we undertook a brute-force approach to identify RanGTP-binding proteins from HeLa cell extracts by systematically sequencing proteins that were retained on a RanGTP column. One of the novel importin β-like proteins turned out to be particularly interesting: exportin-t (exportin specific for tRNA) (Arts *et al*., 1998; Kutay *et al*., 1998). This protein bound efficiently out of the HeLa extract to the RanGTP column. However, when we tested purified, recombinant exportin-t for binding to RanGTP, we observed only weak binding. The obvious explanation was that HeLa extract contains a co-operative binding partner which increases the affinity of exportin-t for RanGTP. This would be analogous to Crm1p and CAS whose RanGTP binding is

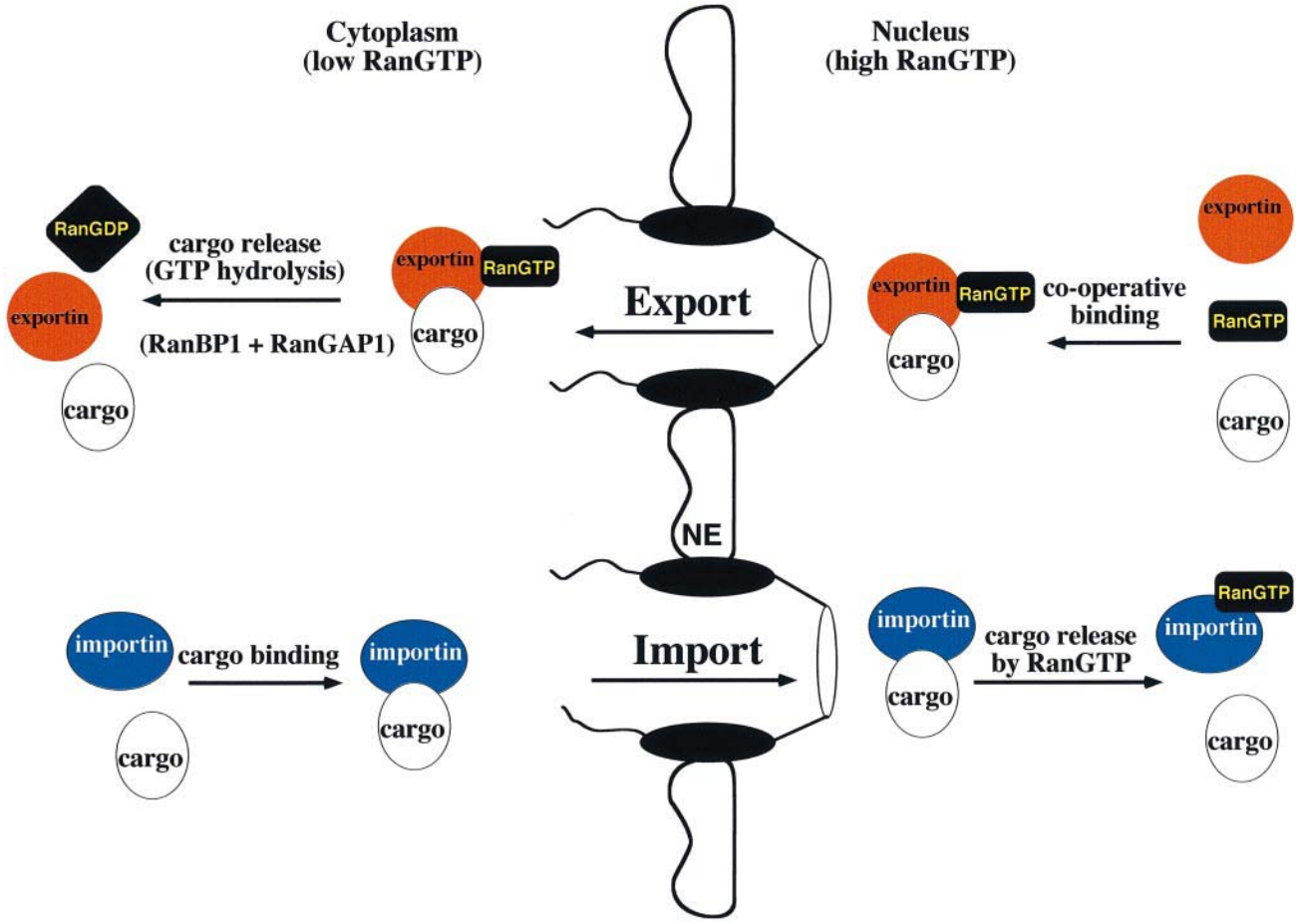

Fig. 2. Generalised model of function for import factors (importins) and export factors (exportins). For details see main text.

weak in the absence of their export substrates and where the stable RanGTP complex includes RanGTP, the exportin and the substrate (Fornerod *et al*., 1997a; Kutay *et al*., 1997b). It turned out that tRNA was the missing co-operative binding partner and the export substrate for exportin-t. Microinjection experiments in *Xenopus* oocytes confirmed exportin-t to be the rate-limiting factor for export of all tRNAs tested (Arts *et al*., 1998; Kutay *et al*., 1998). RanGTP regulates the substrate–exportin-t interaction such that tRNA can be bound preferentially in the nucleus and released in the cytoplasm. So far, exportin-t is the only exportin found which binds an RNA directly. Furthermore, it can discriminate clearly between tRNA and other highly structured RNA species and is thus the class-specific, saturable export carrier whose existence had been predicted by Zasloff (1983) and Jarmolowski *et al*. (1994).

Exportin-t is distantly related to the *S.cerevisiae* Los1 protein with which it shares 19% sequence identity. Los1p was originally identified as the los1-1 mutation which causes inefficient tRNA-mediated suppression of nonsense mutations (Hopper *et al*., 1980). A role for Los1p in tRNA biogenesis has since been suggested, although its precise function remained poorly defined. It now appears likely that yeast Los1p functions similarly to its mammalian counterpart as a tRNA export receptor. This view is further supported by Los1p interacting with NPCs (Simos *et al*., 1996) and having an importin β-like RanGTP-binding motif (Görlich *et al*., 1997).

tRNAs are initially synthesised by RNA polymerase III. Whilst still in the nucleus they undergo a series of maturation steps which involves trimming of the 5′ and 3′ ends, base and ribose modifications, addition of the 3′CCA end, and, in some cases, the removal of a small intron. Only mature tRNAs are finally exported to the cytoplasm (see for example Melton *et al*., 1980). Interestingly, exportin-t appears to discriminate between mature and immature tRNA molecules (Kutay *et al*., 1998). The higher affinity for the mature form is probably part of the ‘quality control’ that ensures that only fully processed tRNA species reach the cytoplasm. Exportin-t is thus a primary example of an export factor which directly helps to co-ordinate RNA processing with export.

The superfamily of importin β-like nuclear transport receptors

Importin β, CAS and exportin-t belong to a superfamily of putative or already proven transport factors (Fornerod *et al*., 1997b; Görlich *et al*., 1997). There are 13 such factors in yeast and probably even more in higher eukaryotes. They are of similar size (90–130 kDa), appear to have a common evolutionary origin, are characterised by an N-terminal RanGTP-binding motif, and, in all cases

tested, interact with NPCs and use RanGTP-binding as a means to regulate interaction with their cargoes. According to the direction in which they carry a substrate, the transport factors can be grouped into exportins or importins, which in turn depends on whether RanGTP promotes cargo binding or release (Figure 2). Two importin β-like factors, CAS and importin β itself, are involved in the import of NLS proteins. Transportin accomplishes import of hnRNP proteins (Pollard *et al*., 1996) and Crm1p, the export of proteins with leucine-rich NES (Fornerod *et al*., 1997a; Fukuda *et al*., 1997; Stade *et al*., 1997). Exportin-t exports tRNA (Arts *et al*., 1998; Kutay *et al*., 1998) and RanBP7 promotes nuclear import of ribosomal proteins (S.Jäkel and D.Görlich, unpublished). The function of at least seven mammalian factors remains to be defined. Conversely, no exportin for ribosomes or mRNA in higher eukaryotes has yet been reported.

Outlook

The last few years have provided considerable insights into how at least some cargoes are selected for nuclear import or export; however, there is probably still a long way to go before we really understand what governs nuclear export of complex substrates such as mRNAs and ribosomes. We have learned much about the soluble factors involved in nuclear transport and we understand the basic framework of a nuclear transport cycle. However, the mechanisms of the actual nuclear pore passage are still fully mysterious and remain the principal challenge in the field. Major obstacles are the enormous complexity of the NPC and the fact that intact nuclei are so far the simplest system in which NPCs can be shown to function. An understanding of translocation through the NPCs will require far more knowledge of the structure of NPC and that of the interacting soluble factors. In addition, we will need to follow the actual NPC passage of cargo molecules at a molecular or even atomic resolution. A combined approach using transport factor mutants, *in vitro* transport systems, electron microscopy and analysis of transport intermediates by chemical cross-linking might help.

Note

This review describes the field from a personal point of view and is focused on nuclear transport in higher eukaryotes. For more balanced reviews see, for example, Nigg (1997), Görlich *et al*. (1997) or Mattaj and Englmeier (1998).

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The EMBO Medal for 1997 has been awarded to Dirk Görlich of the Zentrum für Molekulare Biologie der Universität Heidelberg in Heidelberg, Germany for his work on nucleocytoplasmic transport, about which he writes in the following review.