1. Nuclear transport/ The RanGTPase system

Text books:
1. Molecular biology of the Cell, Alberts, 4th edition
2. Molecular Cell Biology, Lodish, 4th edition
3. Cell Biology, Pollard and Earnshaw, 2002

Literature:

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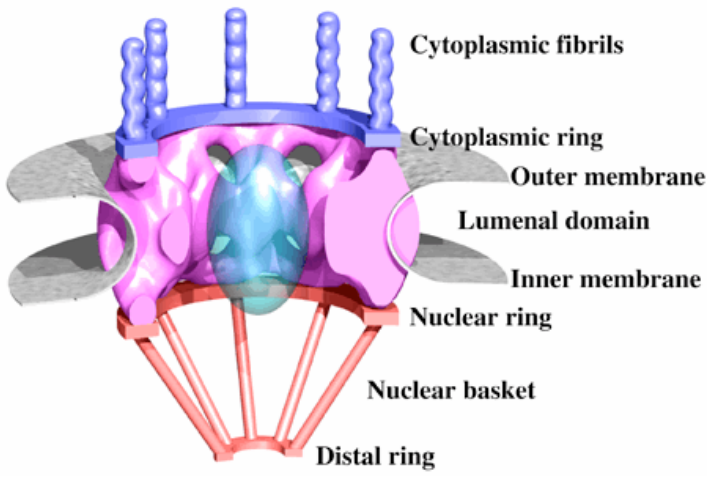
1.1. Introduction

In eukaryotic cells, the enclosure of the genetic information in the nucleus allows the spatial and temporal separation of DNA replication and transcription from cytoplasmic protein synthesis. This compartmentalization not only allows a high level of regulation of these processes but at the same time necessitates a system of selective macromolecular transport between the nucleus and the cytoplasm. Transfer of macromolecules between both compartments is mediated by soluble receptors that interact with components of nuclear pore complexes (NPC) to move their specific cargos. Transport occurs by way of a great variety of different pathways defined by individual receptors and accessory factors.

1.1.1. NPC

The exchange of material between the nucleus and cytoplasm occurs by way of nuclear pore complexes (NPC). The NPC is a massive structure ~15-30 times the size of a eukaryotic ribosome. Completely spanning the two membranes that separate the nucleus from the cytoplasm, the NPC houses a central ~65 nm-long aqueous channel. The NPC appears to remain open to free diffusion of low molecular weight solutes, bringing the nucleus and cytoplasm into continuous ionic contact.



The three-dimensional structure of the NPC has been obtained by analysis of electron microscopic images. Viewed along the axis of its central channel, the NPC exhibits an octagonal symmetry. The main mass of the pore complex forms a cylindrical structure, composed of spoke-ring complexes sandwiched between nuclear and cytoplasmic ring structures that are embedded in the nuclear envelope. The NPC is asymmetric with respect to its cytoplasmic and nuclear extensions. Attached to the cytoplasmic face are flexible filaments protruding into the cytoplasm, while fibrils emanating on the nuclear side converge at their ends to form a cage-like structure referred to as the nuclear basket. The vertebrate NPC is considerably larger than the yeast NPC, with an estimated molecular mass of 125 MDa for vertebrates compared to about 55 to 72 MDa for yeast.

Recent proteomic analysis of the composition of the NPC in both yeast and vertebrates has revealed that each NPC consists of about only 30 different proteins, the so-called nucleoporins, many of which are present at a copy number of eight or multiples of eight reflecting the eightfold symmetrical arrangement of the NPC. It can be estimated that a mammalian pore consists of a minimum number of 400 individual proteins.

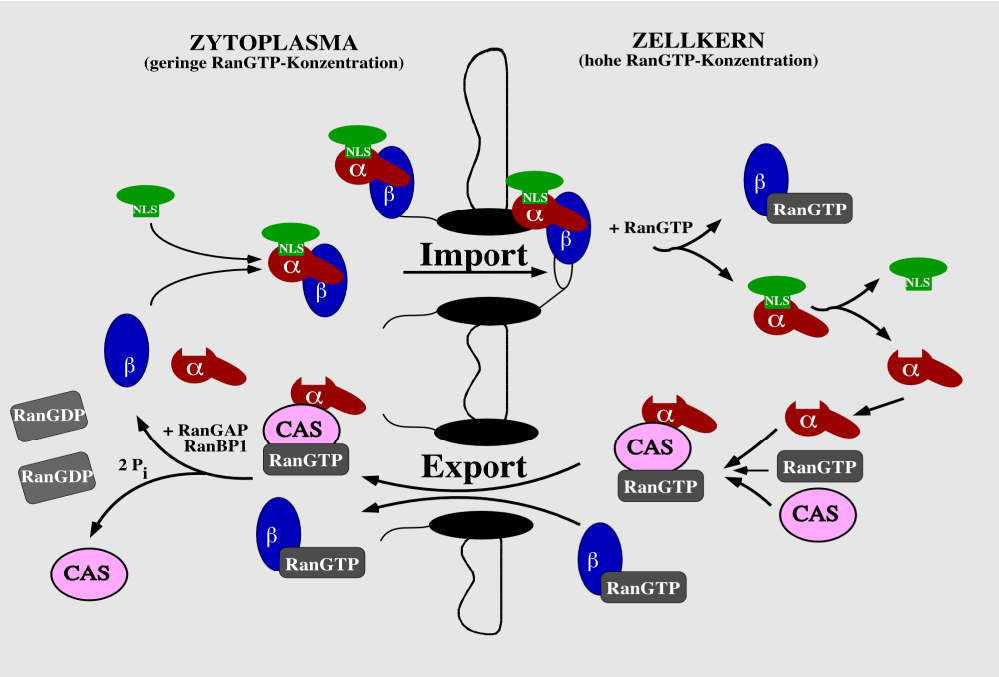
Based on their amino acid sequences, about two-thirds of the 30 different nucleoporins are reasonably conserved between yeast and mammals, although conservation is not as striking as for the soluble transport machinery. However, both types of NPCs are structurally and functionally similar. One important feature common to many nucleoporins from all species is that they contain numerous repeats of the amino acid sequences FG, FxFG or GLFG. These FG-repeats provide binding sites for nuclear transport receptors such as importins and exportins, NTF2 and others. It has been estimated that there are more than 1000 copies of such repeats within one NPC and that their local concentration in the interior of the central channel could approach 50 mM. This high concentration of FxFG repeats, of course, favors complex formation between individual repeats and nuclear transport receptors. To allow reasonable rates of transport, however, the interaction between transport receptors and FG repeats should be weak. Indeed, dissociation constants have been estimated to be in the micromolar range.

1.1.2. Transport receptors and directionality of transport

Proteins and RNA molecules generally do not diffuse across the NPC. Rather, like a parcel delivery service, macromolecules are carried through the central channel of the NPC by way of specific transport receptors (refer to Figure 1). These transport receptors identify their substrates by recognizing specific signals present within the cargo molecules. Transport requires input of energy, which is usually derived from GTP hydrolysis; however, the expenditure of high-energy phosphate is not used directly to move receptor-cargo complexes through the NPC. Rather, during transport, GTP is consumed by Ran, a member of the Ras-related GTPase superfamily. The interaction between RanGTP and receptors, and subsequent hydrolysis of GTP by Ran, dictates in which compartment a given receptor will bind its cargo and promotes cargo release in the opposite compartment. RanGTP-responsive transport accounts for the majority of nucleo-cytoplasmic exchange of macromolecules; however, Ran-independent transport receptors also exist.

A still useful paradigm to illustrate the general principles of nuclear transport and the way in which RanGTP and GTP hydrolysis confer directionality is the first transport pathway

that was delineated, involving the import receptor importin β and the import of proteins containing short, basic nuclear localization signals (NLS).



Unlike many other transport signals, the short, basic NLS do not bind importin β directly. Instead, these NLS are recognized by a transport adaptor, importin α, that associates with importin β by virtue of its N-terminal importin β binding domain (IBB). The trimeric complex consisting of the NLS-protein/importin a/importin β is targeted to the NPC by importin β. This receptor-cargo complex then translocates to the nuclear side of the NPC by an as yet unresolved mechanism involving interaction with proteins form the NPC central channel. In the nucleus, importin β binds RanGTP, which causes release of the importin α/cargo protein complex. The cargo protein itself is released from importin α, since, in the absence of importin β binding, the affinity of importin α for NLS cargo drops due to auto-inhibition by the IBB domain, which folds back into the NLS substrate binding pocket of importin α. Importin β, still bound to RanGTP, is recycled back to the cytoplasm, again by translocation through the NPC central channel. Importin α cannot translocate through the NPC on its own; thus, importin α, binds to a specific export receptor CAS. The affinity of CAS for importin α is dramatically enhanced by cooperative binding with RanGTP. The CAS/RanGTP/importin a complex then similarly travels through the central channel to the cytoplasm. In the cytoplasm, importin β- and CAS-bound Ran is stimulated to hydrolyze its GTP, causing dissociation of Ran from the export complexes. Dissociation of Ran from importin β permits importin β to form a new trimeric import complex with cargo and importin a, while dissociation of Ran from CAS releases importin a permitting CAS to return on its own to the nucleus.

Importin β and CAS are members of the *importin ß/karyopherin* superfamily of nuclear transport receptors. The importin β/karyopherin family mediates the majority of nuclear transport processes in metazoans and yeast. All members of this superfamily perform three functions: (1) they bind cargo by way of their non-conserved C-terminal domains, (2) they use RanGTP binding through their weakly conserved N-terminal domains to control cargo association, and (3) they bind a specific group of proteins, the FG-repeat nucleoporins, contained within the NPC. While RanGTP-responsive transport accounts for the majority of nucleocytoplasmic exchange of macromolecules, other Ran-independent transport receptors exist and their properties will be examined as well.

In summary, for the most part, factors that transfer cargo from the cytoplasm to the nucleus, which are referred to as importins, require an environment free of RanGTP to bind their cargo, a condition satisfied in the cytoplasm, and they release their cargo in the nucleus in which RanGTP is plentiful; conversely, factors that transfer cargo from the nucleus to the cytoplasm, exportins, require RanGTP to bind their cargo and they release their cargo in an environment in which RanGTP is converted to RanGDP. Thus, while importins and exportins likely navigate the central channel by the same mechanism, it is the asymmetrical distribution of RanGTP and the opposite responses of transport receptors to RanGTP in cargo binding that regulates the direction of cargo movement and allows for receptor recycling.

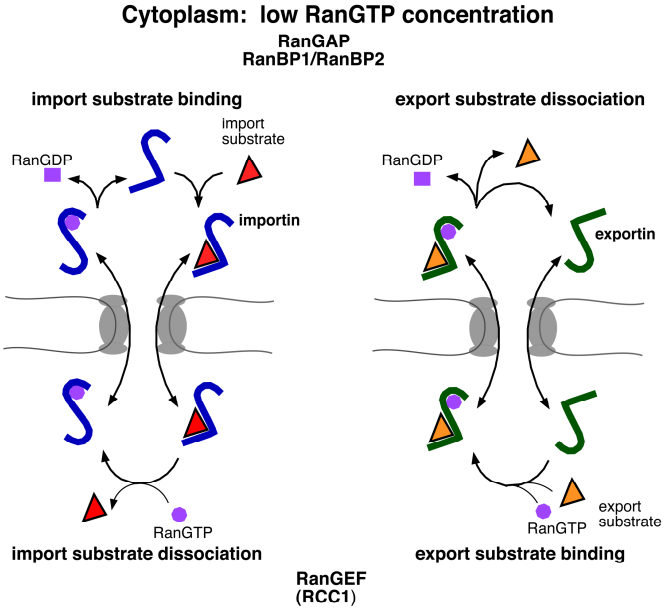


Figure 1: Control of cargo binding to importins and exportins by Ran. Transport receptors mediate nuclear pore passage of substrates by a diffusional mechanism involving contacts with FxFG containing nucleoporins. The directionality of the transport reaction is dictated by the differential localization of RanGTP. In the nucleus, the RanGTP concentration is kept high by chromatin associated RanGEF. In the cytoplasm, RanGAP, aided by RanBP1 and/or RanBP2, converts RanGTP to RanGDP. In general, importins (dark blue) have a high affinity for GTP-bound Ran. RanGTP binding to importins causes dissociation of the import substrate. Importins bind a substrate (triangle) in the cytoplasm where the RanGTP concentration is low. Upon arrival in the nucleus, they associate with RanGTP and most likely a conformational change in the substrate-binding domain leads to cargo release. The importin/RanGTP complex translocates back to the cytoplasm where RanGAP//RanBP1/RanBP2 dissociate RanGTP from the importin. Most exportins (light blue) have a low affinity for RanGTP and substrate. Both bind co-operatively to the exportin in the nucleus. Conformational changes in the transport receptor lead to the formation of a stable export complex. Export complex formation in the nucleus is favored by the high nuclear RanGTP concentration. After export to the cytoplasm, the export complex is disassembled by RanGAP assisted by RanBP1 and/or RanBP2. The exportin re-enters the nucleus on its own.

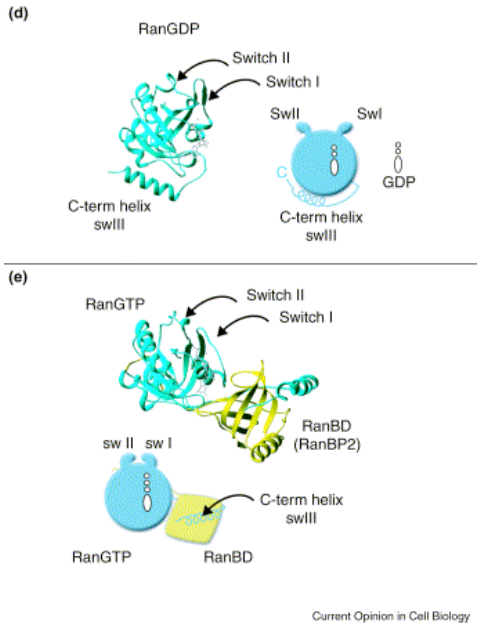
2. The RanGTPase system

As indicated, crucial for the directionality of nuclear transport is the asymmetrical distribution of RanGTP between the nucleus and cytoplasm. The maintenance of this asymmetry involves the spatial separation of regulators that control Ran's nucleotide state and its subcellular location.

Ran (Ras-related nuclear protein)

Ran (25kDa) was identified in a cDNA library screen searching for small GTPases similar to Ras. The encoded protein was mainly found in the nucleus and was therefore designated Ran (Ras-related nuclear protein). Ran does not have a signal sequence for conveying membrane anchors, in contrast, an acidic region is found at the C terminus, which is essential for binding a specific class of effectors (the RanBP1 family members).

Ran binds guanine nucleotides with high affinity and specificity (pico- to nanomolar range). Crystal structures have been solved in the GDP and GTP bound form. Five parallel β–strands and one antiparallel strand contribute to a central b-sheet, which is surrounded by five α-helices forming the G-domain, which is conserved in all known GTP-binding proteins. The two switch regions adopt different conformations in response to its nucleotide state. In addition, Ran has a carboxy-terminal tail of about 40 residues that functions as an extra switch (switch III), responding to the nucleotide state of the GTPase. When GDP is bound, the switch I and switch II regions move away from the nucleotide, adopting an open conformation, whereas the carboxy-terminal tail packs as an α-helix against the G domain. When GTP is bound, the switch regions I and II move closer to the nucleotide to interact with its γ-phosphate, whereas the carboxy-terminal tail is flipped away from the G domain.

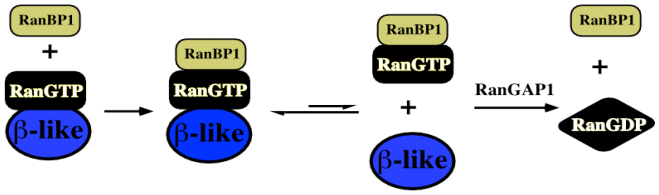


RanBP1 and RanBP2

Binding of importins and exportins to RanGTP inhibits RanGAP-induced GTP hydrolysis by Ran. This inhibition appears to be due to mutually exclusive binding of importins or exportins and RanGAP to the same site in Ran. Furthermore, RanGTP–importin complexes are stable, with half-lives of several hours. The binding of importins and exportins to RanGTP and exclusion of RanGAP would ordinarily make it impossible for transport receptors and Ran to disengage from each other and carry out repeated rounds of transport.

Dissociation of RanGTP from these transport receptors is promoted by another family of Ran-binding proteins, represented by RanBP1 and RanBP2/Nup358. RanBP1 is 23 kDa and contains a single Ran-binding site; RanBP2 is 356 kDa and contains four Ran-binding domains homologous to the RanBP1 Ran-binding domain. RanBP1 or isolated Ran-binding domains of RanBP2 bind RanGTP but, rather than inhibiting the GTPase like importins, they stimulate GTPase activity by about an order of magnitude. Furthermore, RanBP1 can form a heterotrimeric complexes with RanGTP and importins. Binding of RanBP1 destabilizes the RanGTP/ transport receptor complexes and thereby renders RanGTP in the complex accessible to RanGAP. Thus, in effect, RanBP1 removes RanGTP from importin β-like transport receptors allowing RanGAP to activate Ran's GTPase. While RanBP1 is a soluble protein, RanBP2/Nup358 is a major component of the of the NPC in higher eukaryotes. Since *S. cerevisiae* lacks RanBP2, the RanBP1 homologue Yrb1p handles all of the RanGTP-dissociating duty; indeed, deletion of *YRB1* is lethal.

Cytoplasmic disassembly of RanGTP/ transport receptor complexes



Unexpectedly, the RanBD1 domain has the β-barrel topology of a pleckstrin-homology (PH) domain, although the sequence shows no detectable homology to these protein domains. PH domains are found in several proteins involved in the regulation of signal transduction by small GTP-binding proteins, such as the Ras p120GAP, the rasGEF Sos, and all of the rhoGEFs (GEF, guanine-nucleotide-exchange factor). PH domains bind phosphatidylinositol lipids. In contrast, the PH-like domain of RanBD interacts with the effector loop of Ran.

RanGAP

As required for the asymmetrical distribution of RanGTP, RanGAP is confined to the cytoplasm. Yeast RanGAP, Rna1p, contains a nuclear export signal that binds to the export factor Xpo1p, which rapidly exports any Rna1p that might enter the nucleus. Mammalian RanGAP's localization is restricted to the cytoplasmic periphery of the NPC; the C-terminal domain of mammalian RanGAP is modified by covalent attachment of the ubiquitin-like protein SUMO-1 and this modification targets RanGAP to the cytoplasmic face of the NPC.

There are no sequence homologies to RasGAP, nor extensive similarities in the three-dimensional structure. In the interaction between Ras and its GAP, the functionally most important residue Q61 (Q69 in Ran) is stabilized to position a water molecule that represents the attacking nucleophile. Most importantly, RasGAP contributes an arginine residue essential for catalysis at the tip of a "finger," and only RasGAP together with Ras forms an efficient GTPase. Arginine introduces a positive charge into the g-phosphate-binding site of the GTPase. This stabilizes a negative charge developing in the transition state of the phospho transfer reaction (GTPase reaction) and stimulates GTP hydrolysis. GTPase-activating proteins (GAPs) generally increase the rate of GTP hydrolysis on guanine nucleotide-binding proteins by many orders of magnitude.

Ran hydrolyzes GTP very slowly; the half-life of the RanGTP complex is several hours. This feature of Ran is but one means to ensure that RanGTP concentrated in the nucleus remains as RanGTP. In the cytoplasm, where GTP hydrolysis must occur, the Ran-specific GTPase activating protein, RanGAP1, increases Ran's rate of GTP hydrolysis by five orders of magnitude (from 1.8×10^{-5} s^{-1} to 2.1 s^{-1} at 25 °C).

RanGAP consists of three domains. The N-terminal domain, which comprises 385 residues and contains eleven leucine-rich repeats, is responsible for the GAP activity. The three-dimensional structure of this domain has been analyzed for the yeast orthologue Rna1p. The repeats form a crescent, the individual repeats consisting of an α-helix and a β-strand each.

RanGTP is hydrolysed by the combined action of Ran-binding proteins (RanBPs, see above) and RanGAP. **RanGAP does not act through an arginine finger.** The basic machinery for fast GTP hydrolysis is provided exclusively by Ran and correct positioning of the catalytic glutamine (Q69) is essential for catalysis.
Instead of an arginine, Tyr 39 of Ran forms hydrogen bonds with both the g-phosphate oxygen and the Gln 69 side chain of Ran.

RanGEF

Ran binds GTP specifically and with high affinity (> $10^9 M^{-1}$). In addition, a low dissociation rate leads to an almost irreversible binding of the guanine nucleotide, the half-life of RanGDP and RanGTP complexes being in the range of several hours. Interestingly, the higher dissociation rate of GTP (1.1 x 10-4 s^{-1}) as compared to that of GDP (1.5 x 10^{-5} s^{-1} at 25°C) indicates a 7-fold higher affinity of Ran for GDP.

The RanGEF, RCC1 (metazoans) and Prp20p (yeast), stimulates dissociation of Ran-bound nucleotide (either GDP or GTP) and the subsequent exchange reaction by stabilizing the nucleotide-free form of Ran. *In vivo*, Ran binds GTP, which is present in high concentration in the cell. As required for maintenance of the asymmetrical distribution of RanGTP, RanGEF is restricted to the nucleus and bound to chromatin. Recent data show that RCC1 is actually a mobile enzyme that can cycle on and off chromatin [41]. Although RCC1 is highly active as isolated protein *in vitro*, chromatin association appears to be coupled to nucleotide exchange on Ran in living cells, which might provide an additional mechanism to ensure generation of RanGTP in the vicinity of chromosomes. Actually, RanGTP can be considered as a marker for the identity of the nuclear compartment or, more precisely, chromatin localization. The RanGTP halo around chromatin is used to provide a spatial cue in processes like mitotic spindle formation and nuclear envelope assembly following mitosis in higher eukaryotes.

RanGEF specifically binds Ran and stimulates dissociation of the bound nucleotide. The exchange factor in turn is replaced from the intermediary nucleotide-free GTPase-exchange factor complex by the guanine nucleotide, which is present in the cell in high concentration.

RanGEF has been crystallized and its three-dimensional structure has been determined by X-ray crystallography. β-strands are forming a propeller of seven blades, similar to that observed in the β-subunit of heterotrimeric G-proteins. All blades of the RCC1 β propeller participate in the Ran interaction. One key element of the interface, the protruding β wedge, buries 423 $Å^2$ of accessible surface upon complex formation. It interacts with P loop, switch II, and helix α3. Ran shows drastic changes on complex formation, RCC1 is rather used as a rigid scaffold that barely changes its structure.
Mechanistically, the β-wedge on RCC1 plays the decisive in catalysis by pushing aside the P-loop.

NTF2

The continuous outflow of exportin-cargo complexes and cargo-free importins from the nucleus would lead to depletion of nuclear RanGTP and eventual shut down of all nuclear transport, thus necessitating that Ran be restored to the nucleus. The return of Ran is mediated by another transport factor, NTF2. NTF2 is unrelated to the importin β family and it binds only RanGDP. Upon entering the nucleus, NTF2 and RanGDP must dissociate from each other and Ran's GDP must be replaced with GTP. Both steps are fostered by a Ran-specific guanine nucleotide exchange factor (RanGEF) present in the nucleus.

Mog1p

Mog1p is a Ran-binding protein isolated as a multicopy suppressor of temperature sensitive mutations in *Saccharomyces cerevisiae* Ran, *GSP1* [45]. Mog1p is an evolutionarily conserved nuclear protein; it binds to both RanGTP and RanGDP, and competes with NTF2 for binding to RanGDP, even though Mog1p and NTF2 bind different sites in Ran. Overexpression of *NTF2* or *GSP1* suppresses the effects of *MOG1* deletion in *S. cerevisiae* while overexpression of Ran suppresses *S. pombe MOG1* mutant phenotypes. Mog1p stimulates GTP release from Ran and forms a stable complex with nucleotide-free Ran. Consistent with this activity, mutations in *MOG1* are synthetically lethal with *PRP20* (RanGEF) [42]. These observations coupled with the genetic interaction between *NTF2* and *MOG1* suggest that Mog1p plays a role in release of RanGDP from NTF2 and in subsequent exchange of GDP for GTP promoted by RanGEF.

2. Ran in mitosis

The small GTPase Ran: interpreting the signs

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