Ran and Nuclear Transport*

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The last several years have witnessed an explosion in our understanding of how proteins and RNAs traffic into and out of the nucleus. Although an increasing number of proteins have been implicated in different nuclear transport pathways, the small GTPase Ran appears to play a central role in coordinating and directing the transport of nuclear cargos. Recently there have been several excellent reviews describing the multiple pathways of nuclear transport (1–5); consequently this review will focus on what is currently known about Ran and its biochemical properties and what is presently understood of the role of the Ran GTPase cycle during nuclear transport.

Biochemical Properties of Ran

The 25-kDa protein Ran (Ras-related nuclear protein) was first cloned because of its homology to Ras (6). However, most of this homology is found within the conserved domains involved in guanine nucleotide binding, and Ran clearly constitutes its own family of small GTP-binding proteins (reviewed in Ref. 7). Ran is a typical G-protein in that it cycles between a GDP-bound and GTP-bound state; however, one feature that makes Ran unique is the location in the cell where these cycles are thought to occur. Ran, unlike the rest of the known G-proteins, appears to localize in two distinct cellular compartments in all known cell types and probably cycles back and forth between the two (see below). Ran is primarily nuclear during interphase with approximately 80% of it being measured inside the nuclear interior in a baby hamster kidney cell despite the fact it does not appear to contain a nuclear targeting signal in its sequence (Fig. 1) (8). At mitosis when the nuclear envelope breaks down, Ran appears dispersed throughout the cell (Fig. 1). Also unlike many G-proteins, Ran does not appear to undergo any post-translational lipid modifications and probably as a result does not bind membranes inside the cell or require lipids for its activity (reviewed in Ref. 7). Instead of a consensus prenylation domain, human Ran possesses an acidic -DEDDDL at its C terminus that is conserved across species (9).

Ran is a very abundant cellular protein and thus probably constitutes one of the most abundant G-proteins in the cell. HeLa cells, for example, contain an estimated 107 copies of Ran per cell (10). Additionally, Ran has been very conserved throughout evolution and has been found in every eukaryotic cell examined, although the amount decreases as one moves away from those larger than 50–60 kDa are too large to visibly diffuse in any reasonable time frame and must contain a targeting signal to be actively transported.

Active nuclear transport is by necessity highly selective if the distinct environments found in the cytoplasm and nucleus are to be maintained, and the cell shows great discrimination both in its choice of substrates to be transported and in the direction in which those substrates are moved. The types of transported molecules include proteins, mRNAs, tRNAs, ribosomal subunits, and some Usn ribonucleoproteins, and to handle such diverse cargo there appear to be a number of distinct but in many cases uncharacterized transport pathways (1–5). Much of the excitement in the nuclear transport field has resulted from the characterization of new transport pathways, each apparently using different but structurally related proteins as receptors and carriers for the different transport signals.

The first evidence for the involvement of Ran in nuclear transport came when Ran was shown to be essential for the nuclear import in permeabilized cells of a reporter construct containing the nuclear localization sequence (NLS) of the SV40 T antigen (PKKKRKV) (12, 13). This nuclear transport pathway remains the best characterized because for many years this type of NLS, consisting primarily of a single or bipartite stretch of basic amino acids, was the only nuclear transport signal that had been defined for some proteins at the sequence level (reviewed in Ref. 14). This type of basic NLS is recognized in the cytosol by its receptor karyopherin α (importin α, KAP60p in yeast) (15–18). In addition to binding the import substrate, karyopherin α forms a complex with a second protein called karyopherin β (importin β, KAP95p in yeast), and it is this interaction that targets the import complex to the cytoplasmic face of the NPC (19–23).

The karyopherin α subunit apparently enters the nuclear interior in association with the transport substrate prior to their dissociation, whereas the karyopherin β subunit appears to only travel as far as the nuclear tip of the NPC (24, 25). It may be a general phenomenon that the receptors for nuclear transport signals travel through the NPC in association with their substrates. In vitro, Ran is not required for the initial docking of the import substrate at the NPC but rather for the subsequent appearance of the import substrate in the nuclear interior (12). Ran’s mechanism of action during transport has been the subject of much investigation.

One key observation was that karyopherin β binds GTP-Ran (but not GDP-Ran) with high affinity (26). Other pieces of the puzzle came from the cloning and sequence analysis of karyopherin β coupled with the identification and cloning of additional transport receptors. A homology search of proteins in the data base revealed a number of proteins that showed clustered regions of homology with karyopherin β, and at least some of this homology is now thought to relate to Ran binding ability (27, 28).

A great deal of excitement has resulted from the demonstration that a number of these homologous proteins function as receptors and carriers in other nuclear transport pathways; it is speculated that they may all do so. At the present time, various protein members of this group have been individually implicated in the nuclear transport of mRNA binding proteins (29–32), ribosomal proteins (33, 34), tRNA processing proteins (35), tRNAs (36), Usn RNAs (4), substrates containing the leucine-rich nuclear export sequence (37–40), and in the recycling of the karyopherin α subunit.
back to the cytoplasm (41). Efforts to define the transport substrates recognized by the remaining uncharacterized members of this group and in a number of cases to identify the specific signals recognized on the transport substrates by the characterized members continue at a rapid pace.

In most of the pathways that have been described to date, the receptor does double duty in that it seems to bind the targeting sequence (without an α subunit) and also interact with the NPC and Ran. All of the members of this family that have been directly examined for their ability to bind GTP-Ran appear to do so (with one exception) (28). It is speculated that the rest may, although the exact amino acid sequence conferring Ran binding ability on each has not yet been defined. This ability to bind Ran may be key to the regulation of these related proteins in nuclear transport, and this binding in turn is dependent on the Ran GTPase cycle.

The Ran GTPase Cycle

The three-dimensional structure of all G-proteins is dependent on which guanine nucleotide (GTP or GDP) they currently have bound, and it is this reversible change in structure that allows them to act as “switches” in so many diverse cellular pathways (reviewed in Ref. 42). Ran has been crystallized in the GDP-bound form (43), and although its structure in the GTP-bound form is not yet known, its binding affinity for different classes of proteins changes dramatically depending on whether it is in the GDP- or GTP-bound state. Like other members of the Ras superfamily, Ran both exchanges guanine nucleotides and hydrolyzes GTP very slowly at the cellular magnesium concentration of approximately 2 mM, but these rates can be speeded up dramatically by the presence of several additional proteins.

One protein that has been shown to function as a guanine nucleotide exchange factor (GEF) on Ran by increasing its rate of guanine nucleotide exchange approximately 500,000-fold is a nuclear protein called RCC1 (prp20p in yeast) (reviewed in Ref. 44). RCC1 (regulator of chromosome condensation) is a 45-kDa protein that contains a basic NLS and is found inside the nucleus bound to chromatin. Much of RCC1 consists of seven internal amino acid repeats, and its crystal structure has recently revealed that these repeats are organized in a propeller-like arrangement (45). In a HeLa cell, RCC1 is about 20 times less abundant than Ran, being present at about 500,000 copies/cell (44). RCC1 has been reported to bind to chromatin via protein-protein interactions, and whether this protein may play additional roles in the maintenance of chromosome structure (in addition to its role as the GEF of Ran) is the subject of an ongoing investigation (44).

RCC1 appears to play a pivotal role in the eukaryotic cell by catalyzing the conversion of nuclear GDP-Ran to GTP-Ran. There is a hamster cell line called tsBN2 that contains a point mutation in RCC1, which causes this protein to be rapidly degraded upon shifting the cells to the non-permissive temperature of 39.5 °C (44). After several hours at this temperature, Ran loses its primarily nuclear localization and spreads out equally into the cytoplasm, indicating that Ran may need to be in the GTP-bound form to remain concentrated in the nucleus (8). Although the exact reason(s) for the nuclear concentration of Ran at steady state remains undetermined, certainly the binding of GTP-Ran to other nuclear proteins would raise the effective size of Ran and slow its diffusion out of the nucleus. tsBN2 cells also exhibit profound changes in nuclear structure and in a variety of nuclear transport pathways at the non-permissive temperature, as do a number of yeast strains harboring mutations in the RCC1 homologue prp20p, in Ran itself, and in several other proteins that affect the Ran GTPase cycle (see below) (reviewed in Ref. 46). Ran has now been shown to interact with so many different proteins that it is perhaps not surprising in retrospect that altering its nucleotide state can result in such marked changes in the morphology and function of the nucleus.

It should be noted that the interaction of RCC1 and Ran in a test tube does not result in as specific a guanine nucleotide exchange reaction as is found for some other G-proteins. Ran actually has a 10-fold higher affinity for GDP than GTP, whereas RCC1 will bind either form of Ran with equal affinity (47). As a result, when presented with equal amounts of the two unbound nucleotides RCC1 will actually stimulate Ran to exchange any bound GTP for GDP, i.e. the reverse reaction of what is normally thought to happen inside the nucleus. What is thought to keep Ran continuously bound with GTP (rather than GDP) inside the cell is the estimated 20–30-fold higher concentration of GTP over GDP, which preferentially results in the loading of Ran with GTP because of the greater availability of this guanine nucleotide. This loading reaction may also be modulated (to an unknown extent) by other nuclear proteins inside the cell.

Although RCC1 catalyzes the conversion of GDP-Ran to GTP-Ran inside the nucleus, the Ran GTPase-activating protein (GAP) carries out the opposite reaction in the cytoplasm. RanGAP1 RNA1 in yeast is a 65-kDa protein whose presence has been shown to increase the rate of GTP hydrolysis by Ran approximately 100,000-fold (48). Significantly, in contrast to RCC1, the majority of RanGAP1 is localized in the cytoplasm by immunofluorescence microscopy although there has been a report that small amounts of this protein may also gain access to the nucleus (49). This arrangement of the majority of the RanGEF in the nucleus and the majority of the RanGAP in the cytoplasm is thought to play a role in determining the directionality of nuclear transport by setting up a gradient of GDP-Ran in the cytoplasm and GTP-Ran in the nucleus (see below).

There is another abundant cytosolic protein called RanBP1 (Ran binding protein 1) that binds GTP-Ran and when bound to Ran increases its affinity for RanGAP1 by about 10-fold (50). However, RanBP1 has also been reported to form a stable complex with GDP-Ran and karyopherin β, whereas GDP-Ran will bind neither protein alone (51). Formation of such a complex may well be important in the early stages of nuclear import when the predominant form of Ran present in the cytoplasm is thought to be GDP-Ran. RanBP1 has also been shown to modulate the binding of Ran to other members of the karyopherin β family, and it may well be these functions, in addition to its relatively modest ability to stimulate RanGAP1 activity, that give RanBP1 such observed profound influence over various nuclear transport pathways in vivo (51, 52).

The nucleoporin Nup358/RanBP2 located on the cytoplasmic filaments of the NPC contains four RanBP1 homologous domains (and thus binds Ran) and furthermore also binds RanGAP1 and karyopherin β, each by a different mechanism (see below) (4).

In vertebrate cells, a significant portion of the RanGAP1 undergoes post-translational modification in the form of a 121-amino acid...
ubiquitin-like addition (called SUMO-1) (53, 54). Interestingly, this modification does not appear to target this protein for degradation but instead targets it specifically to the filaments on the cytoplasmic face of the NPC itself. Somewhat confusingly, the yeast Ran-GAP (Ran1p) does not appear to undergo a similar modification, and the NPC protein to which the vertebrate GAP is targeted upon modification (Nup358/RanBP2) does not appear to have a yeast homologue. In general, NPC proteins show a surprisingly small degree of conservation across species, and the exact role of this RanGAP modification and targeting in vertebrate nuclear transport will be an interesting question. Certainly, this positioning of the RanGAP at the cytoplasmic entrance of the NPC puts it in an ideal spot to stimulate GTP hydrolysis on Ran molecules either entering and/or leaving the NPC.

A third class of proteins that have been shown to interact with small G-proteins are the so-called guanine nucleotide dissociation inhibitors (42). Although members of this group have been shown to play an important role in other protein trafficking pathways, no Ran guanine nucleotide dissociation inhibitors have been described as such to date. Only one protein in the cell has been identified so far that has a significantly higher affinity for GDP-Ran rather than GTP-Ran, and this protein is called p10/NTF2. p10/NTF2 is a 14-kDa dimeric protein that has been implicated in NLS-mediated nuclear import both biochemically and genetically (55–57). Like Ran but unlike any of the karyopherins or other Ran-binding proteins, this protein does not appear to contain any type of targeting domain small enough to freely diffuse through the NPC. Strikingly, however, p10/NTF2 coupled to gold can be observed by electron microscopy to concentrate to a large extent all through the NPC when microinjected into either the cytoplasm or nucleus of Xenopus oocytes (58). This may be due in part to the reported ability of p10/NTF2 to interact with a particular class of NPC proteins (56, 59) (see below), although its exact functions in this import pathway and potential involvement in additional nuclear transport pathways remain for the most part unknown.

The Role of Ran during Nuclear Transport

Import substrates moving from the cytoplasm to the nuclear interior pass several morphologically (and biochemically) distinct regions of the NPC during their journey including the cytoplasmic filaments, the central transporter region, and the large basket-shaped structure on the nucleoplasmic side. To cover the not insignificant distance through the NPC of 100–200 nm, export substrates on the NPC in the opposite direction and all of the available evidence suggests that the same NPC carries out active transport in both directions (1, 4). How does this happen and what is the role of Ran in this process?

As mentioned above, the NPC is a very complicated structure, and an unknown number of NPC proteins remains uncharacterized. The exact identity and sequence of NPC proteins that a transport substrate? or its carrier? or Ran? interacts with during transport is unknown, but some individual NPC proteins appear to be critical in certain pathways but not in others (46). One family of NPC proteins whose members have been implicated in multiple nuclear transport pathways are the so-called repeat-containing nucleoporins. These NPC proteins contain many copies of a repeat motif, which appears in various forms but always ends in the amino acid sequence FG. A number of these proteins have been cloned from both vertebrate cells and yeast (Nup358/RanBP2 is one), and significantly both biochemical and genetic data indicate that these nucleoporins can specifically bind certain members of the karyopherin β family (4, 5, 19, 26). p10/NTF2 has also been reported to interact with members of this family (56, 59). As these repeat-containing nucleoporins are localized throughout the length of the NPC, this group of proteins may form different “tracks” through the NPC that different transport substrates follow as they move through the NPC. How exactly a transport substrate moves from any NPC site to the next and how many biochemical reactions are required to move a transport substrate from one end of the NPC to the other remain unknown.

One key function of Ran in nuclear transport must lie in its ability to trigger the assembly and disassembly of transport complexes. In nuclear import for example, the addition of GTP-Ran (but not GDP-Ran) has been shown to disassemble the karyopherin αβ complex and to release the β subunit from a repeat-containing nucleoporin (4, 26, 60). Consistent with this, the addition of Ran in the GTP-bound form to the cytoplasmic side of the nuclear envelope triggers dissociation of docked import substrates and inhibits nuclear import (58). Conversely, several export substrates have been shown to only bind their substrates in the presence of GTP-Ran, as would occur in the nucleus, and release them in the presence of GDP-Ran, as would occur upon reaching GDP-Ran in the cytoplasm (52). A requirement for low GTP-Ran in the cytoplasm versus a requirement for high GTP-Ran inside the nucleus during nuclear transport has been further demonstrated by lowering the GTP-Ran level inside the nucleus by microinjecting RanGAP, RanBP1, or mutant Rans with defective GTPase cycles and showing that this blocks the subsequent nuclear export of a number of classes of RNAs (62). These results indicate that the apparent asymmetry in the cellular Ran distribution may be an important way that the cell regulates the directionality of nuclear transport.

It might be pointed out, however, that because of a technical inability to inhibit RanGAP activity during the time it takes to isolate cellular Ran for analysis, the exact percentage of Ran in the GTP-bound form inside the cell at any given time (and its distribution) remain unknown (63). Additionally, because of its small size Ran is capable of fast diffusion through the NPC. This is demonstrated by the rapid loss of nuclear Ran at 4 °C upon digitonin permeabilization of the plasma membrane, a treatment that is known to maintain an intact nuclear envelope (1, 12). To what extent the movement of Ran through the NPC in vivo is driven by RanGAP in complex with other proteins during GTPase cycles and what percent represents diffusion remains undetermined. In either case a considerable amount of Ran localizes throughout the length of the NPC in living cells based on electron microscopy.² It is the nucleotide-bound state of the Ran within the NPC associated with a particular transport complex that likely influences the movement of that complex through the NPC. Whether that stays the same during movement through the NPC or changes is not known.

One indication of the enormous complexity found in nuclear transport is that even the energy requirements for the most well understood pathway of basic NLS-mediated nuclear import remain unresolved. For example, the addition of either a non-hydrolyzable GTP analog or mutant Ran unable to hydrolyze GTP has been reported to block basic NLS-mediated import (12, 13). In contrast, GTP hydrolysis has been reported not to be required in another nuclear import pathway (64). Also, certain forms of nuclear export appear to require Ran in the GTP-bound form (based on microinjection) but do not appear to require GTP hydrolysis by Ran (65). Whether this means that Ran has to hydrolyze bound GTP in some nuclear transport pathways, but not in others, is not clear. It is also not clear whether the nucleotide state of Ran has to change only once or more than once, in adenine nucleotide transport from the cytoplasm to the nuclear interior (or vice versa). For nuclear import, GDP-Ran on the cytoplasmic side of the nuclear envelope plus a source of GTP appear to be required for import whereas for nuclear export, Ran in the GTP-bound form inside the nucleus appears to be essential. Although GTP-Ran may only be required during import for the final release of the import substrate into the nuclear interior and/or recycling of the import factors (61), the exact mechanism of Ran action during nuclear transport remains unknown. It remains possible that Ran may have additional functions inside the NPC, in addition to its demonstrated ability to interact with transport complexes.

In addition to Ran, whether there are any other GTP (or ATP)-requiring proteins required for nuclear transport remains unresolved. An old observation that still remains unexplained is that under conditions of energy depletion, import substrates have been reported to accumulate at the cytoplasmic face of the NPC. This implies an energy requirement for translocation of an import substrate across the midplane of the NPC (66, 67). An ATP requirement in nuclear transport remains to be specifically demonstrated. Basic NLS-mediated nuclear import does not require ATP generated by non-hydrolyzable ATP analogs (as long as GTP is present) (68), but the export of some very large ribonucleoproteins is accompa-

² M. S. Moore, unpublished data.
nied by significant shape changes of the NPC itself, which may indicate additional energy requirements (69). Different nuclear transport pathways, with different size substrates of different compositions, may have different energy requirements.

Two groups have addressed the question of additional GTPases (besides Ran) being required for nuclear import by introducing a mutation into Ran (D125N) that alters its nucleotide specificity from GTP to non-hydrolyzable GTP analogs no longer inhibit basic NLS-mediated nuclear import (68). Conversely, another group has reported that nuclear import achieved with the same targeting signal under similar but not identical conditions is still inhibited by non-hydrolyzable GTP analogs (70). To date the question of multiple GTPases (in this and other nuclear transport pathways) remains unresolved.

These and other conflicting reports about nuclear transport probably reflect the fact that any experimental system that maintains an intact nuclear envelope and a functioning NPC (which is necessary to maintain and measure nuclear transport in vitro) is by necessity a complex system containing many proteins whose identity in a number of cases is unknown. In vivo studies possess an even greater degree of complexity, and a further complication is the realization that many (if not all) nuclear transport pathways are probably interdependent. For example, the continued transport of a substrate in one direction may be dependent on the continued recycling of a necessary component of this pathway in the other direction (41). One of the continuing challenges therefore in defining the mechanisms of nuclear transport is to correctly distinguish between what is a primary and necessary component of a particular transport pathway and what may be affecting a pathway by modulatory or secondary effects. In summary, Ran appears to play a crucial role in many nuclear transport pathways, but its exact functions remain to be defined.

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