The small nuclear GTPase Ran: how much does it run?

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Summary

Ran is one of the most abundant and best conserved of the small GTP binding and hydrolyzing proteins of eukaryotes. It is located predominantly in cell nuclei. Ran is a member of the Ras family of GTPases, which includes the Ras and Ras-like proteins that regulate cell growth and division, the Rho and Rac proteins that regulate cytoskeletal organization and the Rab proteins that regulate vesicular sorting. Ran differs most obviously from other members of the Ras family in both its nuclear localization, and its lack of sites required for post-translational lipid modification. Ran is, however, similar to other Ras family members in requiring a specific guanine nucleotide exchange factor (GEF) and a specific GTPase activating protein (GAP) as stimulators of overall GTPase activity. In this review, the multiple cellular functions of Ran are evaluated with respect to its known biochemistry and molecular interactions.

Accepted
6 October 1996

Introduction

In the five years since its discovery, Ran, one of the most abundant and highly conserved small GTP binding proteins of eukaryotes, has been implicated in a wide variety of cellular processes including nuclear protein import, nuclear RNA export, cell cycle progression, mitotic regulation, DNA synthesis, RNA synthesis and processing, and maintenance of nuclear structure. The data suggesting these roles for Ran have come from diverse assay systems *in vivo* and *in vitro*, derived from mammals, amphibians and yeasts. This diversity of assays and phenomena has made it difficult to determine whether Ran is functioning directly in many different pathways or whether it is functioning indirectly in several. The goal of this review is to summarize and compare these data, in order to identify the key aspects of Ran function shared by these systems.

General concepts of regulatory GTPase function

The GTPase superfamily includes a diverse group of proteins such as the α-subunits of heterotrimeric G proteins, protein synthesis initiation, elongation and termination factors, the signal recognition particle and its receptor (proteins that modulate targeting to the endoplasmic reticulum), ADP ribosylation factors (proteins that modulate the coating of transport vesicles), and the Ras family (relatively small proteins that regulate such processes as cell growth and division, cytoskeletal organization and vesicular sorting). The subfamilies of Ras include the Ras and Ras-like proteins involved in cell growth and division, the Rho and Rac proteins involved in cytoskeletal organization, the Rab proteins involved in vesicular sorting, and the Ran proteins.

Like all regulatory GTPases, Ran binds GTP and catalyzes its hydrolysis:

$$\text{GTP} + \text{GTPase} \rightleftharpoons \text{GTPase}\cdot\text{GTP} \xrightarrow[\text{P}_\text{i}]{K_{\text{hyd}}} \text{GTPase}\cdot\text{GDP} \xrightarrow{K_{\text{diss}}} \text{GTPase} + \text{GDP},$$

where K_{hyd} is the specific reaction rate constant for GTP hydrolysis and K_{diss} is the specific reaction rate constant for GDP dissociation. The hydrolysis is effectively irreversible. The GDP dissociation step is not, but under physiological conditions, where the concentration of GTP is thirtyfold greater than that of GDP, binding of free GDP to the GTPase can have a negligible effect on the overall kinetics.

Regulatory GTPases are more than catalysts, however, since the enzyme-substrate (GTPase·GTP) and enzyme-product (GTPase·GDP) complexes are not solely intermediates in the catalytic pathway. These complexes also function as molecular switches that couple the binding and hydrolysis of GTP (the GTPase cycle) to one or more other cellular processes. Two distinct coupling mechanisms have been elucidated (Fig. 1). The first is based on a pathway in which the GTP-bound form of the GTPase interacts selectively with an effector molecule, usually an enzyme, causing the latter's activation and thus stimulating a particular bio-

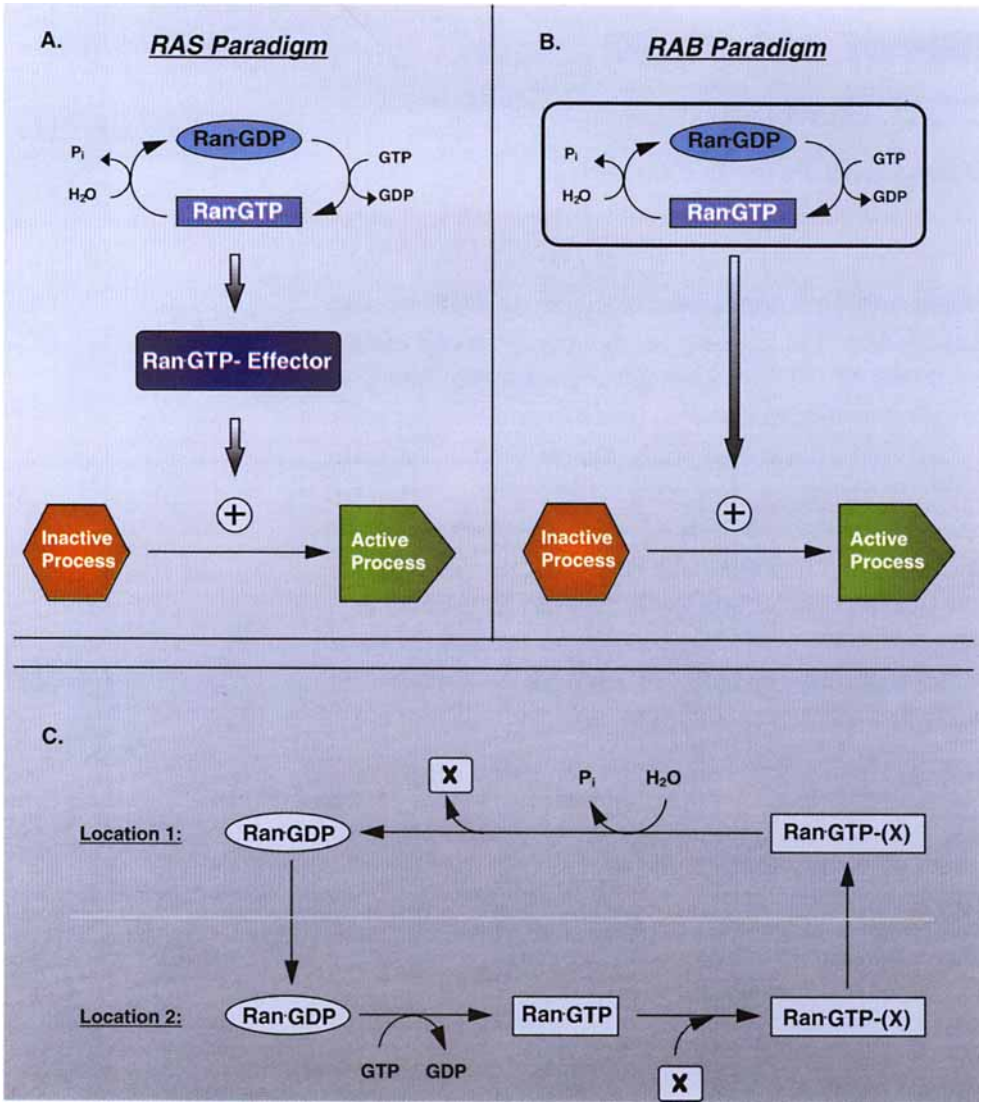

Fig. 1. Two models that could couple a Ran GTPase cycle to the regulation of a cellular process. (A) In the Ras paradigm, the absolute amount of Ran·GTP is the factor that determines the state of the cycle: high levels of Ran·GTP cause the binding and activation of large amounts of effector. (B) In the Rab paradigm, the continual binding and hydrolysis of GTP by Ran is needed for the cycle to function. Ran·GTP interacts with a specific effector, but generation and/or transmission of a signal to a downstream cellular process requires GTP hydrolysis. (C) When a factor stimulating guanine nucleotide exchange (a GEF) is present at one cellular location and a factor stimulating GTP hydrolysis (a GAP) is present at another, a Rab GTPase cycle can result in directional trafficking. In this trafficking schematic, molecule X, unable to move from location 2 to location 1 on its own, is transported by means of its interaction with Ran·GTP. (In principle, the locations of the GAP and the GEF could be reversed without affecting the direction of transport, but then X would be transported by means of its association with Ran·GDP.) A major operational distinction between the Ras and Rab paradigms is that coupling of a Ras type GTPase to activation of a process would be stimulated by blocking GTP hydrolysis and inhibited by blocking GTP exchange, while coupling of a Rab type GTPase cycle would be inhibited by blocking either hydrolysis or exchange.

logical process. The absolute amount of the GTPase·GTP complex is what matters, and this amount is regulated strictly by adjusting the rates of GTP hydrolysis (K_{hyd}) and GDP dissociation (K_{diss}). This regulation, in turn, is achieved by two accessory factors, GTPase activating proteins (GAPs) and guanine nucleotide exchange factors (GEFs), which interact with the GTPase. Stimulation of GTP hydrolysis by GAPs favors formation of GTPase·GDP, while stimulation of GDP release by GEFs favors formation of GTPase·GTP. Changes in the amounts and/or activities of GAPs and GEFs are the ultimate regulators in this mechanism of GTPase cycle coupling. This mechanism describes the role of true Ras proteins in intracellular signalling, so it is referred to here as the Ras paradigm (Fig. 1A).

The second mechanism for coupling a GTPase cycle to a cellular process requires the repeated and continual binding and hydrolysis of GTP. Both GTPase·GTP and GTPase·GDP complexes interact selectively with specific regulators and effectors, as in the Ras paradigm, but neither complex alone is sufficient to stimulate a cellular process. In essence, the entire cycle and not just one intermediate is involved in coupling. When intrinsic levels of GTP hydrolysis and GDP release are extremely low (as is the case for most GTPases and all known members of the Ras family), and when GAPs, GEFs and effectors are present in different cellular compartments, this mechanism is particularly suited for coupling a GTPase cycle to the unidirectional transport of cellular components. This mechanism describes the role of Rab proteins in vesicular sorting, so it is referred to here as the Rab paradigm (Fig. 1B).

The crucial difference between the two mechanisms is that in the Ras paradigm, the intensity of the signal varies with the amount of GTPase·GTP, while in the Rab para-

digm, it varies with the amount of turnover of GTPase·GTP. The Ras and Rab paradigms predict very different outcomes for a process regulated by a GTPase cycle should the GTPase itself, the GAP or the GEF, be functioning abnormally. For example, a GTPase obeying the Ras paradigm should be in a stable active state if trapped in its GTP-bound form (for example by inhibiting GAP activity), and in a stable inactive state if trapped in its GDP-bound form (by inhibiting GEF activity). In contrast, a GTPase obeying the Rab paradigm should be inactive if trapped in either form. Also, in the case of the Ras paradigm, addition of exogenous mutant GTPase trapped in its GTP-bound form to a system containing normal levels of the endogenous wild-type GTPase should stimulate the process regulated by the GTPase. In the case of the Rab paradigm, the added mutant GTPase should inhibit, either by binding to and sequestering GAP, or by binding to and sequestering an effector. These distinctive predictions will be useful in interpreting the data to be presented in the following sections.

The basic biochemistry of Ran and its GTPase cycle

Ran

The Ras-related nuclear protein Ran was first identified as an open reading frame in a human teratocarcinoma cDNA[1] and then as a purified protein isolated from HeLa cells[2]. Studies since then of Ran homologues in essentially all types of eukaryotes have established that Ran is one of the most highly conserved proteins of nucleated cells[3-17]. At >2×10^7 copies per cell in mammals, it is also one of the most abundant. Yeast and mammalian Rans are about 80% identical[4,11,12], while mouse and human Rans are either identical or 95% identical (two mouse Ran isoforms have been identified)[7]. Human Ran contains 216 amino acids and is about 30% identical to other members of the Ras family such as true Ras, Rho, Rac and Rab proteins. Its classification as a member of the Ras family is based primarily on its size, the presence and spacing of four conserved domains required for GTP binding and hydrolysis, and the overall three-dimensional structure of the protein as determined by X-ray crystallography[18]. The Ran protein's intrinsic rates of both GTP hydrolysis and GDP exchange are low, as is typical for GTPases of the Ras family[19].

Two characteristics of the Ran group distinguish them from other Ras family GTPases. First, while other members of the family are cytosolic or membrane-associated, Ran is located predominantly in the nucleus[2,20] (Fig. 2). Second, while Ras and Ras-like, Rho, Rac and Rab proteins contain a carboxyl terminal sequence that includes a signal and site for lipid modification (isoprenylation), Ran proteins contain an acidic carboxyl terminus (DEDDDL in human Ran). This sequence protrudes from the otherwise compact globular structure of Ran[18]. The DEDDDL sequence is not required for targeting to the nucleus: expression in human cells of a Ran protein lacking it had no apparent effect on nuclear localization of the protein[21]. Instead, this sequence may mediate interaction with a specific Ran effector[22,23], as discussed below.

Ran-specific GEFs and GAPs

The GEF and GAP factors that regulate the Ran GTPase cycle have been identified and isolated. The major, and perhaps the only, Ran GEF, known as RCC1 (Regulator of Chromosome Condensation-1)[24-26], is an abundant, nuclear, chromatin-associated, DNA binding protein[2,27]. Human RCC1 (421 amino acids) stimulates GDP release from Ran·GDP by a factor of 100,000[19], and the fact that yeast, *Drosophila*, *Xenopus* and mammalian homologues can complement one another *in vivo* (for a review and references, see ref. 28), strongly suggests that these proteins have been conserved functionally. There is also evidence that mammals may express a minor functional splice variant of RCC1, differing from the major form by an additional 13 (hamster) and 31 (human) amino acids[29]. Whether there is any relation between this observation and the fact that a variety of organisms including mammals[7], yeasts[4,11] and plants[3] express more than one Ran isoform, sometimes in a tissue-specific manner[7], remains to be examined.

Human RanGAP1, a homodimer of 65-kDa subunits[30], stimulates Ran·GTP hydrolysis by a factor of 100,000[19], and the fission yeast homologue is active with human Ran[31]. RanGAP1 and its yeast homologues are probably the major Ran-specific GAPs. These proteins are located predominantly in the cytosol, perhaps concentrated on the cytosolic face of the outer nuclear membrane[32,33]. A model that reconciles the cytosolic localization of RanGAP1 with a function in nuclear RNA metabolism, will be presented below in our discussion of biological functions of Ran. An additional nuclear RanGAP has recently been identified in mammals[34], but it is much less active, and possibly less specific, than RanGAP1.

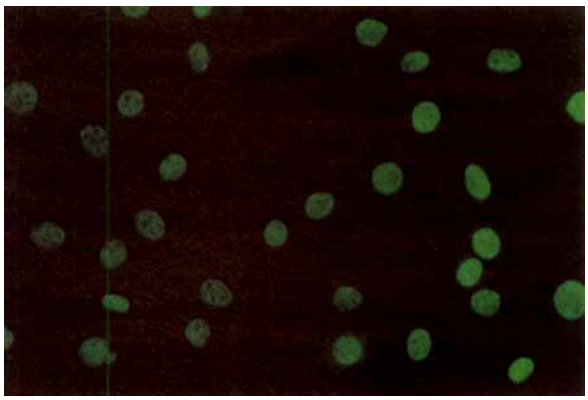

Fig. 2. Immunolocalization of Ran to cellular nuclei. Monkey kidney (COS) cells were incubated with a rabbit antibody specific for a Ran peptide, followed by fluorescently labeled goat anti-rabbit immunoglobulin. Only nuclear staining is visible in these photographs of nearly confluent cells.

Both RCC1 and RanGAP were characterized genetically before they were recognized as regulators of Ran. RCC1 (Ran GEF) was originally identified as the product of a hamster gene whose normal function is required to prevent the premature onset of mitosis during the DNA synthesis (S) phase of the cell cycle[24-26]. A similar function has been claimed for the fission yeast (*Schizosaccharomyces pombe*) RCC1 homologue[12]. RanGAP was identified originally in budding yeast (*Saccharomyces cerevisiae*) as the product of a gene, *RNA1*, whose normal function is required for multiple steps of RNA metabolism, including proper initiation and termination, processing and nuclear export[32,35,36]. The wild-type fission yeast *RNA1* gene can rescue a budding yeast *rna1* mutant[33]. RanGAP was also identified in the mouse as the product of a gene, '*Fug1*', whose absence leads to embryonic death before gastrulation[37].

In budding yeast, RCC1 and RNA1 mutants have indistinguishable defects in all aspects of RNA synthesis, transport and processing[35], an observation that can now be rationalized by the fact that both proteins are functional components of the Ran GTPase switch. That is, all or most of the phenotypes associated with both RCC1 and RanGAP/RNA1 defects may be due to their roles in regulating the Ran GTPase cycle. Problems associated with interpreting these multiple phenotypes are transferred to Ran.

Putative Ran effector proteins

Effectors of regulatory GTPases are usually defined as proteins other than GEFs and GAPs that interact selectively with either the GTP- or GDP-bound form of the GTPases, as parts of the mechanism for coupling the GTPase cycle to a cellular process (see Fig. 1). The definition can be too restrictive, as some effectors also have GEF or GAP activity, but the operational definition of effectors as proteins that exhibit selective binding to either GTPase·GTP or GTPase·GDP complexes has proved to be a valuable tool for isolating them and for elucidating many coupled pathways. This is also proving to be true in the case of Ran.

The first putative Ran effector to be identified, Ran Binding Protein-1 (RanBP1), was recovered from a mouse expression cDNA library by screening for clones whose products could bind to Ran charged with $\alpha[^{32}P]GTP$[22]. RanBP1 is a small (203 amino acids), highly polar, highly acidic protein, that binds to Ran·GTP but not to Ran·GDP[22,38]. It had been identified previously as the product of a highly expressed gene of unknown function[39]. RanBP1 is both nuclear and cytosolic[23]. The budding yeast homologue of RanBP1 (50% identical to mouse)[40,41] is essential for cell viability, and was identified in a screen for proteins whose over-expression in yeast results in chromosome instability[41]. (The other protein selected in this screen was one of the two budding yeast homologues of Ran itself.)

RanBP1 interacts not only with Ran·GTP but also with RanGAP1. Specifically, when mouse RanBP1 was used as bait in a yeast double hybrid screen of a mouse cDNA library, both Ran and RanGAP1 were cloned[42]. Although RanBP1 is not itself a GAP, purified RanBP1 can stimulate RanGAP1 activity[38]. RanBP1 could serve as an adapter to increase the affinity between Ran and its GAP or to direct Ran to its GAP, but more traditional effector roles both for it and for RanGAP1 itself, seem likely.

In addition to RanBP1, larger and predominantly nuclear mammalian proteins that interact selectively with Ran·GTP have been detected by probing renatured Western blots of cell extracts[23], and several Ran·GTP-binding polypeptides have been cloned by probing cDNA expression libraries[38,43]. Alignment of their predicted amino acid sequences with those of mammalian and yeast RanBP1 proteins revealed several regions of conserved sequence. One of these, in binding studies with mammalian RanBP1 fragments, was shown to be a Ran binding domain[43]. Searches of computer data bases, in turn, revealed significant homology between this domain and sequences within a nucleoporin (nuclear pore protein) gene from budding yeast, and within a nucleoporin-like open reading frame of unknown function from the nematode *Caenorhabditis elegans*[44]. In addition, a human nucleoporin was cloned through the use of Ran as bait in a yeast double hybrid screen of a cDNA library[45], and through use of Ran·GTP as a probe of a cDNA expression library[46]. This large nucleoporin, referred to as RanBP2 or Nup358, contains four RanBP1 homologous domains as well as zinc-finger, XFXFG and leucine-rich repeat motifs similar to those found in other nucleoporins. It is located on the cytosolic face of the nuclear pore, and antibodies directed against it inhibit import of a protein containing a polybasic nuclear localization signal.

The implication of these studies of Ran-binding proteins is that Ran may be targeted to multiple sites in the cell with multiple distinct functions. This will be a key point in our discussion of the biological functions of Ran, below.

Complexes containing Ran

In addition to its interactions with these defined partners, Ran can form various other relatively stable complexes. The constituents of these complexes vary with the cell type being studied and the conditions of the analysis. When Ran is purified from HeLa cells under conditions that prevent nucleotide binding, it occurs in the form of a heterodimeric complex with RCC1[2], and can also be shown *in vitro* in the absence of bound nucleotide to form a heterotrimeric complex with RCC1 and RanBP1[38]. RCC1 added to *Xenopus* egg extracts under conditions in which Ran should be nucleotide-free, binds not only Ran and RanBP1 but also the *Xenopus* homologue of the human nucleoporin identified as a possible Ran effector[47]. Even more complex aggregates have been identified in the chromatin of budding yeast[48]. The physiological signifi-

cance of these complexes remains unclear, but they clearly demonstrate that the list of Ran binding partners remains incomplete.

Biological functions attributed to Ran

Most of the biological functions so far associated with Ran, and the organisms in which these functions have been studied, are listed in Table 1. An association between a change in the state of Ran and a change in a cellular process does not of course demonstrate that Ran directly regulates the process. Indeed, only in the case of nuclear protein import has a direct biochemical coupling between Ran and a cellular process been shown. It seems unlikely that a similar direct link exists between Ran and all of the other processes shown in Table 1. We will approach this key issue of the physiological target(s) of the Ran GTPase switch by summarizing some of the experimental observations that support the five functions shown in Table 1.

Table 1. *Representative functions attributed to the Ran GTPase cycle, and the organisms in which those functions have been demonstrated* in vivo *or* in vitro

Function	Organism
Nuclear protein import	Budding yeast, *Xenopus*, mammals
Nuclear RNA export, RNA synthesis and processing	Budding yeast, fission yeast, mammals
Cell cycle progression	Fission yeast, *Xenopus*, mammals
Mitotic regulation, nuclear structure	Budding yeast, fission yeast, *Xenopus*, mammals
DNA synthesis	*Xenopus*

Inferred functions based on studies of defective Ran proteins are not distinguished from inferred functions based on studies of defects in other cycle components.

Nuclear protein import

The import of proteins that contain a polybasic nuclear localization signal is an energy-requiring, saturable process that occurs in two steps. (For a review and references, see refs 49 and 50.) The first is energy-independent and involves binding (docking) of the karyophilic protein to the nuclear pore complex. The second is energy-dependent and involves transport of the protein through the pore. The process has been extensively studied using a digitonin-permeabilized cultured cell model. When cells are treated with digitonin, their plasma membranes become permeable to macromolecules and many endogenous soluble cytoplasmic and nuclear components are lost, while nuclei and most cytoskeletal structures remain morphologically intact. Such permeabilized cells fail to import certain karyophilic proteins into their nuclei, but import can be restored by addition of exogenous cytosolic extract. Such an *in vitro* system has allowed the assay and purification of the factors required for the two steps of nuclear protein import. Specifically, addition of Ran[51,52], a small Ran-interacting protein known as pp15[49,53], GTP and ATP (which probably functions to regenerate GTP[54]), are necessary and sufficient for the nuclear import of proteins already docked at the nuclear pore. (Docking itself requires a different set of factors, which includes the importins, a class of nuclear localization signal receptors.) Addition of a non-hydrolyzable GTP analogue inhibits import[51,52], suggesting that both binding and hydrolysis of GTP by Ran are required. This suggestion is supported by two *in vivo* studies, one using mammalian cells in culture and the other, budding yeast. The first supports a requirement for GTP binding and the second for GTP hydrolysis.

The hamster kidney cell line tsBN2 contains a temperature-sensitive RCC1 (the Ran GEF). At the non-permissive temperature (39°C) the mutant protein is degraded, and within 2-3 hours can no longer be detected immunologically or enzymatically. Microinjection of a karyophilic protein into the cytosol of these and wild-type cells at either permissive (33°C) or non-permissive temperatures, followed by monitoring nuclear uptake of this protein, has shown a dramatic loss of protein import in tsBN2 cells under non-permissive conditions, that follows the loss of RCC1[55]. Ran itself, which is at least 85% nuclear in tsBN2 and wild-type cells at 33°C, also accumulates selectively in the cytosol of tsBN2 cells at 39°C[20,55].

A budding yeast strain containing an inducible plasmid-borne Ran gene coding for a GTPase defective mutant protein (defective in GTP hydrolysis in both the presence and absence of GAP) has been constructed. In the absence of inducer, Ran is provided by the constitutively expressed chromosomal genes, and nuclear protein import is normal. In the presence of inducer, Ran is provided by both wild-type chromosomal genes and the mutant plasmid gene, and nuclear protein import is strongly and rapidly inhibited[56].

Taken together, the data presented in this section, along with the observation that a variety of nucleoporins (including one from mammals and one from budding yeast) contain Ran-binding domains, support a direct role for Ran in nuclear protein import. Also, the requirement for both binding and hydrolysis of GTP supports a Rab paradigm, although it is not known whether Ran itself escorts proteins through the pore. One of many other possibilities would be the requirement for GTP exchange at the pore to release the docked import substrate for transport, while GTP hydrolysis would be required to release Ran from an effector. The latter model is analogous to that proposed for the signal recognition particle and its receptor in targeting proteins to the endoplasmic reticulum.

Nuclear RNA export, and RNA synthesis and processing

Nuclear RNA export, like protein import, is an energy-requiring saturable process, but very few factors involved in RNA transport have been identified and characterized. (For a

review and references, see ref. 57.) Significantly, three of these factors are components of the Ran GTPase cycle: Ran itself, RCC1 and RanGAP1. Budding and fission yeast expressing temperature-sensitive mutant RCC1 proteins, accumulate large amounts of nuclear polyadenylated RNA within minutes of a shift to the non-permissive temperature[4,11,35,58,59]. The same result has been obtained with the mammalian RCC1 temperature-sensitive mutant cell line tsBN2[11,59], which is also defective in export of small nuclear RNA (snRNA), but not of tRNA[60]. Budding yeast expressing an inducible wild-type Ran, but grown in the absence of inducer (an effective Ran null mutant), also accumulates large amounts of nuclear polyadenylated RNA[11]. This last finding supports the view that the RCC1 mutant phenotype reflects solely its role as a Ran GEF. This view is further supported by the finding that over-expression of wild-type Ran in budding yeast can suppress the RCC1 mutant phenotype[4,11].

These experiments indicate a role for Ran at least in export of polyadenylated RNA (mRNA) and snRNA, and demonstrate a requirement for GTP exchange in the process. Two additional studies support this role for Ran and demonstrate a requirement for GTP hydrolysis as well. First, when a budding yeast strain containing an inducible plasmid-borne Ran gene coding for a GTPase-deficient mutant protein (the same strain in which nuclear protein import is inhibited upon induction) is grown in the absence of inducer, nuclear mRNA export is normal. In the presence of inducer, nuclear mRNA export is inhibited strongly and rapidly[56]. Second, when a budding yeast strain expressing a temperature-sensitive RanGAP1 (RNA1) protein is grown at the non-permissive temperature, nuclei accumulate large amounts of polyadenylated RNA within minutes of the shift[35,36]. As noted previously in our discussion of RanGAP1, this and other phenotypes of RanGAP1 mutants are essentially indistinguishable from those of RCC1 mutants.

Taken together, the data presented here support a role for Ran in nuclear RNA export, and the requirement for both binding and hydrolysis of GTP is consistent with a Rab paradigm. As in the case of protein import, it is not known whether Ran itself must escort RNAs through the pore.

Indeed, in the absence of an *in vitro* RNA export assay, conclusions regarding a direct role for Ran in RNA transport may be premature. This need for caution is reinforced by the fact that, in some systems, defects in the Ran GTPase cycle have been associated with pleiotropic defects in RNA synthesis and processing. The mammalian cell line tsBN2, which contains a mutant RCC1 and is defective in RNA export, also exhibits defects in intra-nuclear snRNA transport and ribosomal RNA processing[60]. The budding yeast strains with mutations in either RCC1 or RNA1 (RanGAP1) exhibit defects not only in mRNA export, but also in mRNA initiation, mRNA termination, polyadenylation, general RNA splicing and ribosomal RNA processing[35]. The multiple phenotypes attributed to the RNA1 mutant are particularly surprising at first glance since, as noted above, this protein is predominantly cytosolic. However, the identification of RNA1 as RanGAP1, and the possibility that the many RNA metabolism phenotypes associated with its loss are secondary to its role in nuclear RNA export or protein import, may help in resolving this apparent anomaly.

Cell cycle progression

Except at the earliest stages of embryonic development, eukaryotic cells express a checkpoint control that prevents the onset of mitosis before the completion of DNA synthesis. This is achieved in many organisms by maintaining the mitosis promoting factor (MPF) (a dimer consisting of a mitotic cyclin and the cyclin-dependent kinase CDK1) in an inactive form. Normal cells arrested in S phase by hydroxyurea treatment do not enter mitosis, and maintain MPF in an inactivated state. However, when the mammalian cell line tsBN2 is arrested in S phase and then shifted to 39°, thereby inactivating its temperature-sensitive RCC1 (Ran GEF) protein, the cells undergo a rapid premature mitosis complete with MPF activation and chromosome condensation[24-26]. Fission yeast strains with temperature-sensitive RCC1 mutations likewise proceed abnormally through mitosis at the non-permissive temperature[12,61]. These results, the association of RCC1 with chromatin, and the observation that over-expression of wild-type Ran can suppress the fission yeast RCC1-defective phenotype, are consistent with the hypothesis that RCC1 and Ran are intrinsic components of a mitotic checkpoint control. A plausible specific form of the hypothesis is that activation of RCC1 during S phase generates Ran·GTP, which in turn acts through an effector to inhibit activation of MPF; inactivation of RCC1 at the end of S phase would terminate the inhibitory signal. This model of Ran function, based on a Ras paradigm, is supported by the finding that over-expression of an exogenous GTPase-deficient Ran in mammalian cells inhibits cell division and arrests cells predominantly in the G_2 phase of the cell cycle[21].

Additional studies of the effects of a so-called dominant negative mutant Ran protein in *Xenopus* cell-free systems, however, do not support such a model. Dominant-negative Ran mutant protein has an activity that inhibits an effect expected from the action of endogenous wild-type Ran. Specifically, the mutant protein does not bind GTP, and binds GDP relatively poorly. Under physiological conditions it probably exists to a significant extent in an uncharged form, allowing it to trap RCC1 in relatively stable, inactive complexes[19,62]. DNA-free extracts prepared from activated *Xenopus* eggs can reform apparently normal nuclei upon the addition of chromatin. These extracts can also undergo repeated rounds of MPF activation and inactivation, in either the presence or the absence of reconstituted nuclei. Addition of dominant negative Ran protein to *Xenopus* egg extracts, either in the presence or absence of

reconstituted nuclei, inhibits MPF activation[63,64]. This result is consistent with a model in which RCC1 and Ran are intrinsic components of a mitotic checkpoint control where inactivation of RCC1 during S phase generates Ran·GDP, which in turn acts through an effector to inhibit the activation of MPF. Alternatively, inactivation of RCC1 during S phase could deplete Ran·GTP, which in turn would no longer act through an effector to stimulate the activation of MPF. This model is the exact opposite of that proposed above for mammalian cells. A possible resolution of this discrepancy is that Ran is functioning *via* a Rab rather than a Ras paradigm to regulate MPF at more than one point in the cell cycle. It may bind and hydrolyze GTP during S phase to prevent the premature initiation of mitosis, and may bind and hydrolyze GTP during G_2 phase to allow mitosis.

Whatever the case, the demonstration of a role for Ran in MPF activation, even in the absence of nuclei, supports the view that this function is not secondary to nuclear RNA or protein trafficking. The *Xenopus* cell-free system should provide a tractable *in vitro* assay for elucidating the biochemical linkage between the Ran GTPase and the state of MPF.

Mitotic regulation and nuclear structure

The major biological role of mitosis is to ensure a proper distribution of chromosomes and other nuclear components to daughter cells. Cells of fission yeast with a temperature-sensitive mutation in RCC1 (Ran GEF) are defective in decondensing their chromatin at mitotic exit and arrest early in G_1 phase, with small nuclei that possess abnormal, disrupted membranes[61,65]. Budding yeast expressing such RCC1 mutants appear to enter mitosis normally and also appear to arrest with nuclei that possess a variety of structural abnormalities[4,58]. (Chromatin condensation and decondensation are difficult to visualize in budding yeast and were not examined in the studies reviewed here.) Finally, nuclei reconstituted in *Xenopus* egg extracts that are either depleted of RCC1, or that contain added dominant negative Ran, are small and structurally abnormal[62,64], as are the nuclei that form in the mammalian cell line tsBN2 following the premature initiation of mitosis. These results, in conjunction with the finding that over-expression of wild-type Ran in budding and fission yeasts can suppress their RCC1 mutant phenotypes, while the addition of wild-type Ran to the RCC1-depleted *Xenopus* egg extracts can suppress its phenotype, suggest a role for the Ran GTPase cycle in regulating nuclear structure and organization.

Such a role in budding yeast has been reinforced with the demonstration that over-expression of Ran or of RanBP1 (the putative Ran effector that binds selectively to Ran·GTP) in an otherwise wild-type background, results in chromosomal instability, as reflected in increased rates of chromosomal loss and nondisjunction[41]. Also, two other proteins associated with nuclear organization and/or mitotic function have been implicated as possible targets or regulators of the Ran GTPase cycle[66-68]. Ran need not function directly in any of the processes noted in this section. They are all almost certainly dependent on nuclear protein import, for example, so it is tempting to speculate that the role of Ran in them is secondary to its role in nuclear transport. However, since dominant negative Ran does not appear to block nuclear protein import in *Xenopus* extracts[64], but does cause nuclear formation abnormalities, the latter are probably not due simply to a lack of transport *per se*.

DNA synthesis

DNA synthesis in eukaryotes is an extremely complex process, requiring multiple polymerases and polymerase-associated factors that are almost certainly regulated by components of the basic cell cycle machinery such as the cyclins and cyclin dependent kinases. Thus, although Ran does not appear to be one of the purified components of *in vitro* reconstituted DNA synthesis systems, it might play a role *in vivo*. Such a role for Ran has been demonstrated to date only in *Xenopus*. Addition of dominant negative Ran proteins to cycling *Xenopus* egg extracts in the presence of reconstituted nuclei inhibits DNA synthesis[64], and this inhibition can be relieved by the addition of excess RCC1[62]. Conversely, nuclei reconstituted in RCC1-depleted extracts are defective in DNA synthesis[69], and this defect can be relieved by the addition of excess wild-type Ran[62]. It is not known whether the role of Ran in this system is direct or indirect, but it does appear to depend on the presence of reconstituted nuclei.

Does Ran function directly in one, or more than one, pathway?

It is highly unlikely that Ran functions directly in all of the processes with which it has been associated (Fig. 3). At the same time, several experimental approaches have yielded compelling evidence that Ran is in fact functioning directly in more than one of these processes. These approaches are: (1) *in vitro* assays using purified components; (2) time course studies; (3) identification of effectors or putative effectors; and (4) analysis of mutants defective in only one function. In this section, we will use data from these approaches to argue that Ran is functioning directly in nuclear protein import, nuclear RNA export and possibly MPF regulation.

Experiments of the first three types relate to the role of Ran in nuclear transport and have already been presented. In brief, Ran is an essential component of permeabilized cell protein import systems; defects in nuclear RNA export and protein import are very early, if not the earliest, phenotypes associated with loss of Ran GEF (RCC1) activity, and nucleoporins have been identified as putative Ran effectors.

Experiments of the fourth type, parts of which have already been presented, relate specifically to separating the role of Ran in protein import and in cell cycle regulation. First, when dominant negative Ran is added to *Xenopus*

egg extracts in the presence of reconstituted nuclei, MPF activation is inhibited while nuclear protein import appears to be normal[64]. Also, MPF inhibition occurs even in the absence of nuclei[63,64]. Second, the cell cycle arrest induced in human cells in culture by expressing GTPase defective Ran is silenced by deleting the six carboxyl terminal amino acids of the GTPase-defective mutant. Nevertheless, Ran lacking these six carboxyl terminal amino acids binds GTP and responds to RCC1 and RanGAP1 relatively normally, and also functions apparently normally in an *in vitro* permeabilized cell nuclear protein import assay[42]. However, the deleted Ran, charged with GTP, binds very poorly to RanBP1, the putative effector that itself interacts with RanGAP1[23,42]. Taken together, these results are consistent with the view that the role of Ran in cell cycle regulation is direct and not secondary to its role in nuclear transport, particularly protein import.

Before leaving this discussion, we must emphasize that our view is a working model. It has the virtue of simplicity, but is far from proved. Comparisons of function based on different organisms and different assays are subject to multiple interpretations, as we shall see in the following section. The issue is likely to be resolved only by a combination of studies of purified components in fully reconstituted systems and of intact cells expressing single mutant proteins at physiological levels under otherwise wild-type conditions.

Does Ran act like Ras or Rab?

The other key issue of Ran function is that of mechanism: does Ran act through a Ras paradigm or a Rab paradigm (Fig. 1)? In discussing individual processes affected by Ran, above, we have indicated in each case that the available data are most consistent with a Rab paradigm. Again, however, while this working model has the virtues of simplicity and consistency, it is not proved. When processes are analyzed in detail, problems arise that are even more taxing than those encountered when evaluating data in relation to the direct roles of Ran. These problems are once again based on the use of different organisms and different assays. There is almost always one piece of data that doesn't quite fit, but as is often the case, focusing on the inconsistent result helps define the problem. One example is particularly instructive.

Essentially all of the available data indicate that the role of Ran in nuclear transport requires both the binding and hydrolysis of GTP, and thus follows a Rab paradigm. GEF mutants, GAP mutants and GTPase-defective Ran mutants all inhibit RNA export, and GEF mutants, GTPase defective Ran mutants and non-hydrolyzable analogues of GTP inhibit protein import. Also, the major GEF and GAP activities are present in different cellular locations. The piece of data that doesn't quite fit is that addition of dominant negative Ran protein to *Xenopus* egg extracts containing recon-

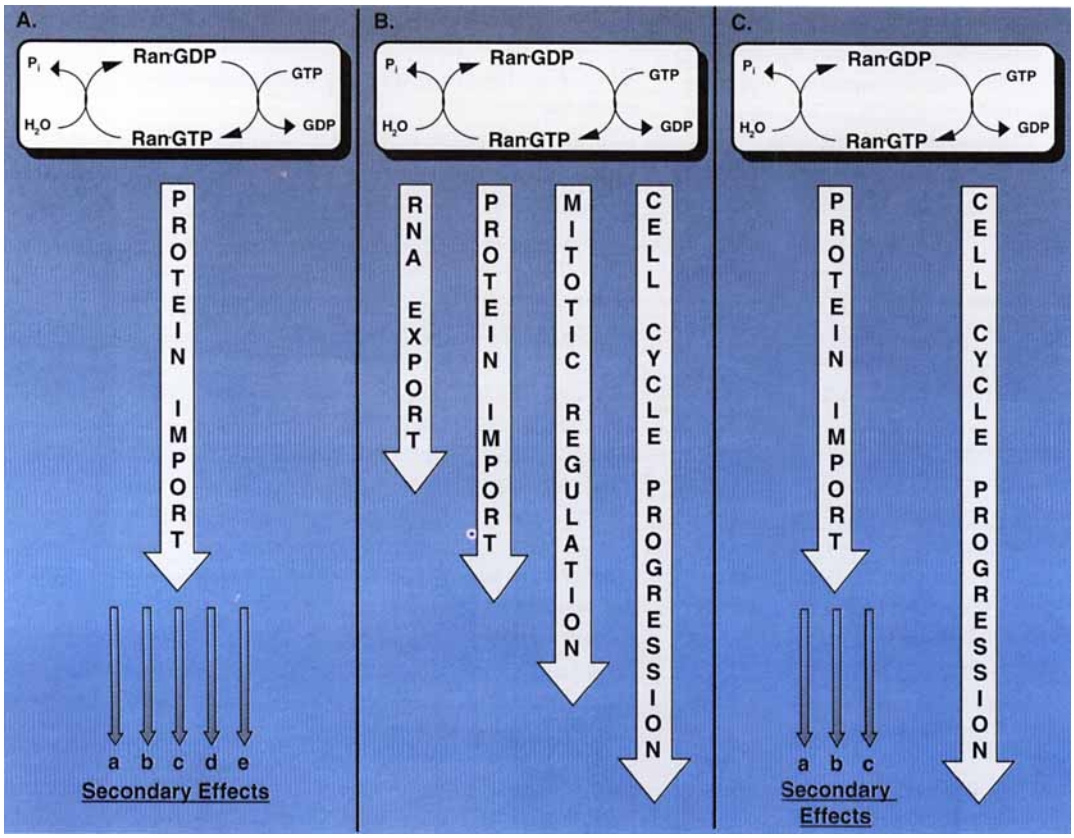

Fig. 3. Three possible explanations for the pleiotropic effects of alterations in the Ran GTPase cycle. (A) Ran functions directly in only one pathway (for example, nuclear protein import), and all other effects (a, b, c, d, e) are secondary (due, for example to depletion of nuclear proteins directly required for these other processes). (B) Ran functions directly in many pathways, and most of its roles are also direct. (C) Ran functions directly in more than one pathway, but many of its roles are secondary to its requirement in at least one of these direct pathways.

stituted nuclei does not appear to inhibit protein import[64]. As previously noted, this mutant inhibits GEF (RCC1) activity in the extracts, and affects nuclear structure, DNA synthesis and MPF activation. It should have the same effect as RCC1 immunodepletion, which inhibits both DNA synthesis and protein import[62]. One likely explanation for the unexpected result is that protein import, as measured in this assay, is less sensitive to Ran GEF levels than are the other processes.

The physiological functions of Ran clearly must be explained at a quantitative level, but available assays for these functions yield essentially qualitative results, and reconciliation of results from different assay systems can call for considerable subjective judgement. On the basis of studies using only *Xenopus* egg extracts in the presence of dominant negative Ran, one might conclude that Ran does not function in nuclear protein import. The Rab paradigm for Ran function is consistent with the available data, and helps reconcile some apparently conflicting observations especially, as noted previously, the action of Ran in cell cycle progression in mammalian cell culture and *Xenopus* egg extracts. At the same time, further work with purified proteins *in vitro* and rigorously characterized mutants *in vivo* will be needed to establish these conclusions definitively.

Perspectives

This review has attempted to present the rapidly accumulating information regarding Ran in the framework of a classic GTPase cycle. The experimental data have been organized to address two major issues: the number of biological processes regulated directly by Ran, and the overall mechanism by which Ran regulates these processes. A plausible working model is that Ran probably functions directly in more than one process, and that it probably operates through a continual binding and hydrolysis of GTP (a Rab paradigm). The provisional nature of these conclusions almost certainly reflects the complexities of available assay systems, rather than intrinsic complexities of the Ran GTPase cycle.

The abundance and complexity of the mechanistic data make it easy to lose perspective. It is easy to forget that Ran was discovered only five years ago, although it is probably the most abundant and most highly conserved of all the regulatory GTPases. It is also the only characterized GTPase with a predominantly nuclear localization. Perhaps it should not have been surprising that so many investigators studying nuclear function have run into Ran, either directly or through its regulators and putative effectors. Indeed, due to the abundance of Ran and other components of the Ran GTPase cycle, it remains possible and even likely that some phenotypes associated with their mutation and/or loss are due to purely structural nuclear alterations[61,65,70]. As with all developing fields, the number of Ran-related questions greatly exceeds the number of answers. For the first nuclear regulatory GTPase, this is to be expected.

Acknowledgements

We thank Dr Mindong Ren for many helpful discussions. Our research was supported by the American Cancer Society.

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