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Separate Domains of the Ran GTPase Interact with Different Factors To Regulate Nuclear Protein Import and RNA Processing

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The small Ras-related GTP binding and hydrolyzing protein Ran has been implicated in a variety of processes, including cell cycle progression, DNA synthesis, RNA processing, and nuclear-cytosolic trafficking of both RNA and proteins. Like other small GTPases, Ran appears to function as a switch: Ran-GTP and Ran-GDP levels are regulated both by guanine nucleotide exchange factors and GTPase activating proteins, and Ran-GTP and Ran-GDP interact differentially with one or more effectors. One such putative effector, Ran-binding protein 1 (RanBP1), interacts selectively with Ran-GTP. Ran proteins contain a diagnostic short, acidic, carboxyl-terminal domain, DEDDDL, which, at least in the case of human Ran, is required for its role in cell cycle regulation. We show here that this domain is required for the interaction between Ran and RanBP1 but not for the interaction between Ran and a Ran guanine nucleotide exchange factor or between Ran and a Ran GTPase activating protein. In addition, Ran lacking this carboxyl-terminal domain functions normally in an in vitro nuclear protein import assay. We also show that RanBP1 interacts with the mammalian homolog of yeast protein RNA1, a protein involved in RNA transport and processing. These results are consistent with the hypothesis that Ran functions directly in at least two pathways, one, dependent on RanBP1, that affects cell cycle progression and RNA export, and another, independent of RanBP1, that affects nuclear protein import.

Ran, a small GTPase located predominantly in the nucleus, was first discovered in a search for human cDNAs with homology to the nucleotide sequences encoding the guanine nucleotide binding site of the H-*RAS* proto-oncogene (19). This human protein is often referred to as Ran/TC4. Ran homologs have since been found in the budding and fission yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, plants, and vertebrate and invertebrate animals (reviewed in reference 12). Ran genes form an exceptionally well-conserved family: the human, fission yeast, and budding yeast genes encode proteins about 80% identical to each other, for example. Aside from the elements thought to encode the Ran guanine nucleotide binding site, family members show little amino acid sequence homology to other RAS-like or RAS-related proteins.

Like H-RAS and other members of the RAS family, Ran has the biochemical properties of a GTPase switch (9, 10). Ran binds guanine nucleotides, catalyzes the slow hydrolysis of bound GTP to GDP, and slowly exchanges bound for free nucleotide. Both guanine nucleotide exchange and GTP hydrolysis rates are increased sharply by accessory proteins (RCC1 [regulator of chromosome condensation 1] and RanGAP, respectively), and candidate effector proteins have been described (13, 14). Models for the function of a Ran GTPase switch (7, 42, 44) are based on biochemical analyses of these component proteins and on functional studies of yeasts, *Xenopus* egg extracts, and mammalian cells expressing various forms of Ran and RCC1.

RCC1, the Ran-specific guanine nucleotide exchange factor (GEF), was first identified as the product of a hamster gene whose wild-type (WT) function is required both to traverse the G_1-S boundary and to prevent chromosome condensation before the completion of DNA replication (15, 39, 41, 46). RCC1 proteins have since been identified in eukaryotes ranging from yeasts to humans (15). The human protein contains a single polypeptide chain of 421 amino acids. In the absence of guanine nucleotides and magnesium, it forms a stable heterodimeric complex with Ran, and under physiological conditions it can function as a Ran-specific GEF (6, 7). The presence of RCC1 is also required for DNA replication in *Xenopus* egg extracts in vitro (16, 17, 28).

A HeLa cell RanGAP activity (13) has been purified to homogeneity (5). It activates hydrolysis of GTP bound to WT Ran protein approximately 1,000-fold but has no effect on GTP hydrolysis by Ran proteins with missense mutations expected to inactivate Ran's GTPase activity without affecting its affinity for guanine nucleotides. RanGAP is a homodimer of 65-kDa subunits.

The search for downstream effectors of the Ran GTPase switch has centered on proteins that bind differentially to Ran-GTP and Ran-GDP. Binding assays performed with whole-cell extracts immobilized on nitrocellulose membranes have identified many such polypeptides (14, 29). One of these proteins, RanBP1, interacts particularly strongly and reproducibly with Ran-GTP. We have used this interaction to recover a full-length cDNA clone for it from a mouse expression cDNA library and to identify a fission yeast homolog of it (11a, 13). The mouse cDNA contains a 609-bp open reading frame encoding a highly charged (40% Glu plus Asp plus Lys plus Arg) and acidic protein with a predicted molecular weight of 23,600. It contains no clear diagnostic catalytic domains but does possess one region close to that for an RNA binding motif. Purified recombinant RanBP1 has no Ran-specific GAP activity

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and binds to GTP-charged, but not GDP-charged, Ran proteins.

A second potential effector, p10, smaller than any of the proteins detected in Ran-GTP binding assays, has been identified in studies of nuclear protein import. In digitonin-permeabilized buffalo rat liver cells supplemented with cytosolic fractions and purified proteins from *Xenopus* ovaries, import proceeds in two steps. First, protein destined for import binds to the nuclear envelope, a process that requires *Xenopus* cytosolic fraction A (35). The bound protein then translocates into the nucleus in a step which requires Ran protein, GTP hydrolysis (32, 36), and the presence of protein p10. In *Xenopus* ovaries, p10 occurs in a complex with Ran (38).

The first function associated with the Ran GTPase switch, regulation of cell cycle progression, was identified in studies with fission yeast and cultured mammalian cells. Specifically, Matsumoto and Beach (30) observed that fission yeast cells lacking RCC1 entered mitosis before completing DNA replication but were rescued by overexpression of WT Ran, suggesting that Ran and RCC1 interact to monitor the completion of DNA replication and onset of chromosome condensation. We observed that transient expression of relatively low levels of a GTPase-defective Ran mutant protein in COS and 293 cells blocked cell cycle progression, predominantly in G_2 (42, 43). Other studies of RCC1 and Ran in yeast, *Xenopus*, and mammalian systems have demonstrated roles for both proteins in additional aspects of cell cycle regulation (28, 31, 45), mRNA processing and transport (2–4, 22, 26), DNA synthesis (16, 17, 28), and as noted above, nuclear protein import (32, 36, 37, 48).

In regard to the role of Ran in RNA transport, only a few other proteins have been identified as components of this process (20). Two of these, RCC1 (the Ran GEF) and protein RNA1, are special in that budding yeast cells expressing mutant forms of either or both have indistinguishable phenotypes (22). These phenotypes include defects in mRNA initiation, polyadenylation, and export. The normal role(s) of RCC1 and RNA1 in these processes are unknown, as is the nature of the functional link between them.

The multiple defects of yeast and mammalian cells expressing mutant *Ran* or *RCC1* genes, the findings that RCC1 and Ran proteins from one species can complement defective mutants of another species, and the fact that overexpression of Ran can suppress many, perhaps all, RCC1 mutant phenotypes (1, 4, 15, 17, 26, 30) suggest that Ran may normally play a role in all of these processes and thereby coordinate them. A range of models is possible to explain these effects of Ran. At one extreme, Ran could be imagined to have only one direct action (e.g., on RNA export), and the other perturbations observed in the presence of mutant Ran or RCC1 would be secondary consequences of this defect (e.g., other defects in RNA processing are due to the resulting abnormally high levels of nuclear RNAs, and defects in cell cycle progression are due to failure to export a critical mRNA species from the nucleus). At the other extreme, all of these actions could be direct, mediated by the interaction of Ran with multiple distinct effectors or by multiple Ran proteins (1, 4, 12), each with a distinct physiological effector specificity.

To distinguish among these models at a biochemical level, we have (i) compared the interactions of WT, carboxyl-terminal deletion (c-del) mutant, and missense mutant Ran proteins with RCC1, RanBP1, and RanGAP; (ii) shown that a Ran c-del mutant protein that does not affect cell cycle progression or interact with RanBP1 can still function normally in a nuclear protein import assay; and (iii) demonstrated that RanBP1 can interact with the mammalian homolog of the protein encoded by the budding yeast *RNA1* gene. The latter result is particularly striking in light of previous genetic studies that have suggested roles for RCC1, Ran, and RNA1 in a common RNA transport pathway (3, 4, 20, 22, 24, 33). Our results define the components of a plausible biochemical pathway linking the Ran GTPase switch to RNA processing and transport, namely, RCC1, Ran, RanBP1, and RNA1. By identifying a domain of the Ran protein, the carboxyl-terminal DEDDDL sequence, that is needed for this pathway but appears to be dispensable for nuclear protein import, our results provide biochemical evidence that the two pathways are separate.

MATERIALS AND METHODS

DNA sequencing. DNA sequencing was performed with Sequenase T7 DNA polymerase (U.S. Biochemicals). Alignments, translations, and comparisons of sequences were carried out with the Genetics Computer Group program package (version 7).

Generation of c-del mutations of Ran for bacterial expression. The PCR was used to generate a c-del (Δ211-216) in human Ran/TC4 WT and GTPase-deficient mutant (dm; G19V+Q69L [43]) cDNAs by mutating Asp^{-211} to a stop codon. The upstream primer, with an *Nde*I site for cloning, was 5′-GGCATAT GGCTGCGCAGGGAGAGCC-3′, and the downstream primer, with a *Bam*HI site, was 5′-GCGGATCCTCACGGGAGCAGTTGTC-3′. PCR conditions were 30 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 45 s. PCR products were subcloned into pET9c after digestion with *Nde*I and *Bam*HI.

Isolation of recombinant Ran proteins. WT, dm, c-del, and dm–c-del Ran/TC4 cDNAs, subcloned into bacterial expression vector pET9C under the control of a T7 promoter, were transformed into *Escherichia coli* BL21(DE3). In this strain, T7 polymerase is provided by a lysogen under control of an isopropylthio-β-D-galactoside-inducible β-galactosidase promoter, allowing high-level, inducible expression of the cloned cDNAs.

A 400-ml overnight culture grown from a single colony was diluted to 4 liters in fresh Luria broth, and isopropylthio-β-D-galactoside was added to 4 mM after the culture reached an optical density at 600 nm of 0.5 to 0.8 (measured in a 1-cm cuvette). Cells were collected 3 h after induction, washed once in phosphate-buffered saline; resuspended in 5 volumes of phosphate-buffered saline, and lysed in a French pressure cell. The lysate, typically 20 to 40 ml, was subjected to centrifugation at 100,000 × *g* for 90 min at 4°C. The high-speed supernatant was then subjected to ammonium sulfate fractionation. The precipitate from a 30 to 55% ammonium sulfate fraction was redissolved in 5 to 10 ml of S-300 buffer containing 10 mM sodium phosphate (pH 7.0), 150 mM NaCl, 1 mM dithiothreitol [DTT], 0.5 mM 3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate (CHAPS), and 1 mM GDP and incubated for 5 min on ice. Magnesium chloride was added to a final concentration of 10 mM, and the sample was subjected to gel filtration with a Sephacryl S-300 (Pharmacia) column (20 by 150 cm) with a flow rate of 2.5 ml/min at room temperature in S-300 buffer plus 1 mM magnesium acetate. Column fractions were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) to identify those containing Ran. These fractions were pooled and dialyzed into 10 mM sodium phosphate (pH 7.0)–1 mM DTT by using positive pressure in an Amicon concentrator cell with a YM-10 membrane (Amicon). This material was applied to a hydroxyapatite column (1 by 10 cm; Pentax) and eluted with a linear gradient of 10 to 500 mM sodium phosphate (pH 7.0) at 1 ml/min. Column fractions were assayed by SDS-PAGE. Ran protein usually eluted in two peaks, at approximate sodium phosphate concentrations of 125 and 180 mM. The peak fractions were pooled and concentrated with Amicon YM-10 Centricon units spun at 3,000 × *g* for 30 to 60 min at 4°C. The resulting material was filtered and applied to a Superdex HR75 gel filtration column (Pharmacia) in 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.5)–160 mM potassium acetate–1 mM DTT and eluted at 0.5 ml/min. Ran protein eluted from this column as a single peak with the mobility of a 25-kDa globular protein. The protein was stored in aliquots (2 to 3 mg/ml) at −80°C. One gram (wet weight) of *E. coli* typically yielded 5 mg of protein.

HeLa cell S-100 extract preparation. HeLa cells (5×10^6) suspended in 1 ml of 25 mM HEPES (pH 7.0)–150 mM NaCl–5 mM $MgCl_2$–3 mM DTT–100 U of aprotinin per ml–10 μg of leupeptin per ml were sonicated on ice and subjected to centrifugation (100,000 × *g*, 60 to 75 min, 4°C) to yield supernatant (S-100) and pellet fractions. Total protein concentrations of S-100 fractions were estimated with a Bio-Rad protein assay kit.

GTPase assay. Purified recombinant Ran proteins (approximately 1 μM) charged with [γ-^{32}P]GTP (0.5 μM; 1,000 Ci/mmol; NEN) by incubation for 20 min at 25°C in 50 mM Tris (pH 7.4–40 mM NaCl–0.5 mM EDTA–1 mM DTT–1 mg of bovine serum albumin (BSA) per ml were adjusted to 10 mM $MgCl_2$–5 mM NaCl and incubated at 25°C in the presence or absence of 1 μg of HeLa S-100 protein per 40 μl as a source of GAP activity (13). Reactions were terminated by addition of 1 ml of ice-cold 50 mM Tris (pH 7.4)–20 mM $MgCl_2$–1 mM DTT–2 mg of BSA per ml, washed through nitrocellulose filters, and sub-

jected to scintillation counting. Ran proteins charged with [α-^{32}P]GTP (instead of [γ-^{32}P]GTP) and assayed for GTPase activity in the presence of HeLa cell S-100 extract as described here exhibited no loss of bound radioactivity (data not shown).

Ligand blotting. Proteins fractionated by SDS-PAGE on 12% gels were electrophoretically transferred to nitrocellulose membranes in 25 mM Tris–192 mM glycine–15% methanol (pH 8.3) and renatured by 48 h of incubation at 4°C in 50 mM HEPES (pH 7.0)–100 mM potassium acetate–5 mM magnesium acetate–3 mM DTT–0.3% Tween 20–1% BSA (11). Blots were preincubated in renaturation buffer plus 0.1% Tween 20 and 0.25 mM GTP for 30 min at 25°C and then in the same buffer containing 0.5 μg of Ran–[γ-^{32}P]GTP (30 Ci/mmol) for 45 min at 4°C. Filters were washed five times at room temperature in preincubation buffer without GTP and autoradiographed.

Yeast interaction trap assays. Assays and cDNA selections utilizing the yeast interaction trap (two-hybrid) strategy were carried out by following the detailed protocol of Gyuris et al. (23). Briefly, PCR-generated fragments of Ran and RanBP1 were each cloned in frame either downstream of the *LexA* DNA binding domain in plasmid pEG202 or downstream of the B42 transcriptional activating domain in plasmid pJG4-5 (23) by using an upstream *Eco*RI site and a downstream *Xho*I site added to the respective PCR primers. WT Ran was generated either from a mouse Ran cDNA, Ran/M1, which encodes a protein with a sequence identical to that of human Ran/TC4, or from a mouse Ran cDNA, Ran/M2, which encodes a protein 95% identical to Ran/TC4 and Ran/M1 (12). c-del Ran and a dominant-negative mutant Ran (T24N, a generous gift from Mary Dasso) were generated from human Ran/TC4 cDNAs. A PCR-generated DNA fragment containing the complete open reading frame of a human RCC1 cDNA (41) with added upstream *Bam*HI and downstream *Xho*I sites was cloned in frame downstream of the DNA binding domain in plasmid pEG202. Pairs of plasmids encoding one each of the two types of hybrid proteins were then jointly transfected into *S. cerevisiae* EGY48 (*MATα his3 trp1 ura3 6LexAop-LEU2*:: pSH18-34), in which the chromosomal *LEU2* gene and a plasmid-borne β-galactosidase gene are each under control of multiple *LexA* operators (23). Expression of the transcriptional activating domain fusions is galactose inducible, so positive interactions between two hybrid proteins were detected by the presence of galactose-dependent growth on medium without leucine and galactose-dependent β-galactosidase activity as assayed by blue colony color on 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) plates (23). The numbers of pluses in Tables 1 and 3 refer to color intensity and are a rough measure of relative interaction affinity.

To screen for cDNAs encoding proteins that interact with RanBP1, a mouse MPC11 myeloma cDNA library was constructed (25) in plasmid pJG4-5 and cotransfected with the RanBP1-pEG202 plasmid into *S. cerevisiae* EGY48.

Nuclear import assay. Protein import measurements were performed as previously described (36, 38), with digitonin-permeabilized buffalo rat liver cells, *Xenopus* fraction A at 1.0 mg/ml, purified *Xenopus* p10 (38) at 2.25 μg/ml, various concentrations of purified WT or c-del Ran protein, and rhodamine-labeled human serum albumin coupled to a nuclear localization sequence peptide (5 μg/ml) as an import substrate.

Mouse gene mapping. To localize the mouse *Rna1* gene in a chromosomal linkage group, the strategy of typing panels of recombinant inbred (RI) mouse strains (50) was used. Mice of the BXD (C57BL/6J × DBA/2J), BXH (C57BL/6J × C3H/HeJ), CXB (BALB/cBy × C57BL/6By), OXA (O20/A × AKR/A), AXB (A/J × C57BL/6J), BXA (C57BL/6J × A/J), and NXSM (NZB/B1NJ × SM/J) RI strain sets were obtained from J. Hilgers (The Netherlands Cancer Institute, Amsterdam; OXA strains) or The Jackson Laboratory (Bar Harbor, Maine; all other strains). Liver genomic DNA was digested with restriction endonuclease *Msp*I and analyzed by Southern blotting as described previously (8) to visualize a restriction fragment length variant associated with *Rna1*. To map the *Rna1* locus genetically, the distribution of this variant among RI strains was compared to those for 1,817 other loci in the New York University RI strain database (18a) by using the BAYLOC computer program (8).

Nucleotide sequence accession number. The mouse RNA1 sequence reported here (see Fig. 4) has been submitted to GenBank and assigned accession number U20857.

RESULTS

c-del Ran hydrolyzes GTP in response to GAP activity. GTPase-defective human Ran/TC4 protein loses its ability to inhibit entry of cultured human cells into mitosis when the carboxyl-terminal six residues of the protein, DEDDDL, are deleted (42). Two possible explanations for this change are that the deletion alters the Ran protein's ability to bind and hydrolyze GTP or that it alters the Ran protein's interaction with downstream effectors. To test the first possibility directly, we compared the GTPase activities of c-del variants of WT and dm Ran proteins (Fig. 1). GTP bound to c-del Ran protein was hydrolyzed briskly when the protein was incubated with an S-100 extract from HeLa cells known to contain Ran-specific

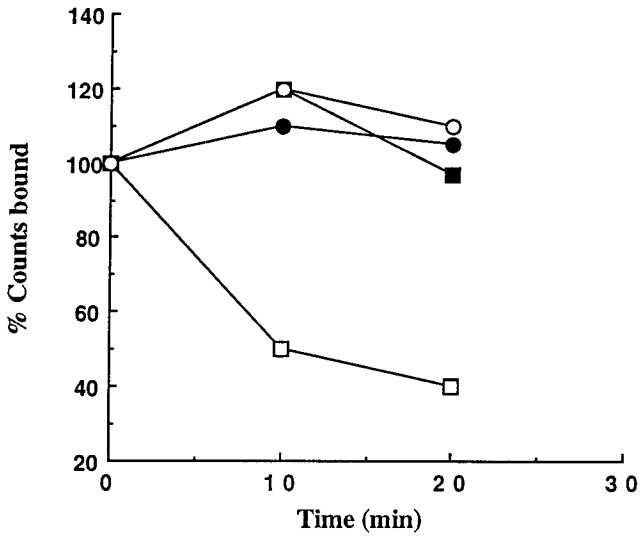

FIG. 1. GTP binding and hydrolysis by recombinant c-del Ran proteins. Recombinant c-del and c-del–dm Ran/TC4 proteins charged with [γ-^{32}P]GTP were incubated in the presence or absence of HeLa S-100 extract and assayed for GTPase activity (release of γ-phosphate) by filter binding as described in Materials and Methods. Symbols: ■, c-del; □, c-del–S100; ●, c-del–dm–S100; ○, c-del–dm.

GAP activity, but no GTP hydrolysis was observed in the absence of this extract. These are the properties previously described for intact WT Ran protein (13). When dm–c-del protein was tested, no hydrolysis of bound GTP was observed in either the presence or the absence of S-100 extract. These are the properties previously described for the full-length dm Ran protein. Together, these data suggest that the c-del does not significantly affect either the Ran protein's GTPase activity or its interaction with GAP.

c-del Ran does not bind to RanBP1 in vitro. Mammalian RanBP1 protein was first identified as a candidate Ran effector because it bound strongly to WT Ran-GTP but not to WT Ran-GDP (13). To test the possibility that deletion of the carboxyl-terminal six residues of Ran protein might perturb its interaction with RanBP1, purified recombinant WT and c-del Ran proteins charged with [γ-^{32}P]GTP were used to probe replicate samples of HeLa cell S-100 extract that had been fractionated by SDS-PAGE and transferred onto nitrocellulose membranes (Fig. 2). In this ligand blot assay, WT, but not c-del, Ran bound strongly to the HeLa cell S-100 band previously shown to be RanBP1 (13). This result indicates that the carboxyl terminus of the Ran protein is required for interaction with RanBP1.

Mutant and WT Ran proteins interact differentially with RanBP1 and RCC1 in vivo. To define better the interactions of WT and mutated Ran proteins with putative effector RanBP1 and GEF RCC1, the yeast interaction trap (two-hybrid) system was used as an assay (Table 1). The complete open reading frames of WT mouse Ran/M1 (identical in amino acid sequence to human Ran/TC4 [12]), mouse RanBP1, and human RCC1 cDNAs were cloned in frame downstream of the DNA binding domain in plasmid pEG202 and/or of the transcriptional activating domain in plasmid pJG4-5, as were the c-del and dominant-negative (T24N) mutant forms of Ran/TC4. Pairs of plasmids encoding one each of the two types of fusion proteins were transfected into *S. cerevisiae* and assayed for interaction, which was detected as leucine prototrophy and inducible β-galactosidase production. This strategy is based on

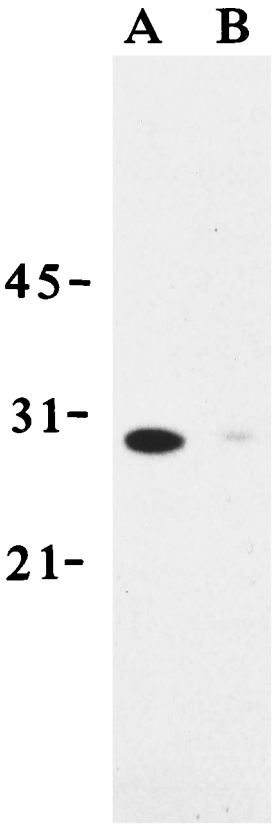

FIG. 2. The acidic carboxyl terminus of Ran is required for interaction with RanBP1. An autoradiograph of a blot of HeLa S-100 extract (20 μg per lane) probed with recombinant full-length Ran/TC4–[γ-^{32}P]GTP (lane A) or recombinant c-del Ran/TC4–[γ-^{32}P]GTP (lane B) is shown. Equal amounts of the probe were used in both experiments. The numbers on the left are molecular sizes in kilodaltons.

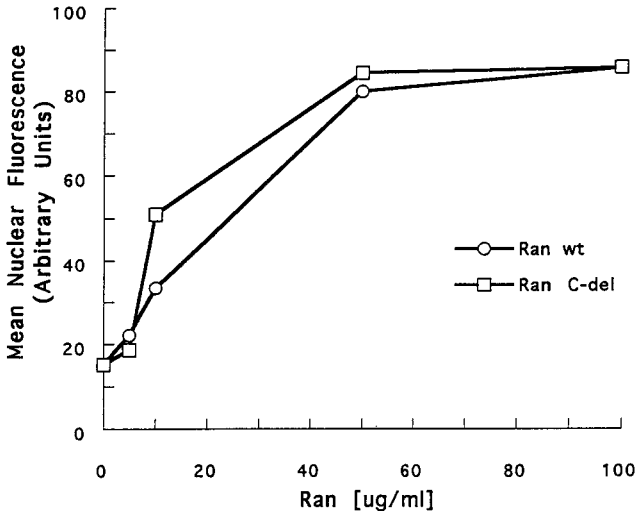

FIG. 3. Nuclear import stimulated by both WT and c-del Ran proteins. Import was assayed as described in Materials and Methods, in the presence of increasing amounts of either the WT or c-del Ran/TC4 protein.

the observation that transcription factors can contain physically distinct DNA binding and transcriptional activation domains which retain their individual functions when expressed as parts of fusion proteins. A protein such as Ran can be expressed in yeast cells as a fusion with the DNA binding domain of a transcription factor (the bait). A test protein, cloned as a cDNA fused to the transcriptional activating domain of a transcription factor, can be coexpressed in the same yeast. Whenever the bait interacts with the test protein, the DNA binding and transcriptional activating domains are brought together, reconstituting a functional transcription factor and turning on a yeast reporter gene under the control of that factor. Interactions between WT and mutant forms of pairs of proteins can thereby be screened in the physiological environment of a eukaryotic cell (21, 23).

TABLE 1. Yeast interaction trap assays[a]

pEG202 LexA construct	Affinity for pJG4-5 B42 construct:			
	WT Ran	c-del Ran	T24N Ran	RanBP1
RCC1	+	++	+++	−
WT Ran	−	ND	ND	+++
c-del Ran	ND	−	−	−
T24N Ran	ND	−	−	−
RanBP1	+++	−	−	−

[a] Assays were performed as described in Materials and Methods. Minuses indicate no detectable interaction, and pluses indicate a clearly detectable interaction. Numbers of pluses indicate color intensity and are a rough measure of relative interaction affinity. Each of the pairwise tests shown in this grid was performed at least in duplicate. ND, not done.

WT Ran interacts weakly with RCC1 and strongly with RanBP1, c-del Ran interacts moderately strongly with RCC1 but not detectably with RanBP1, and dominant-negative mutant Ran interacts strongly with RCC1 but not detectably with RanBP1. Dominant-negative mutant Ran, like its Ras dominant-negative mutant homolog, binds preferentially to GDP (17), so the failure of dominant-negative Ran to interact with RanBP1 supports our previous finding that RanBP1 binds to Ran-GTP but not to Ran-GDP (13). The weak interaction between WT Ran and RCC1 is consistent with previous reports that WT Ran and RCC1 form stable complexes only in the absence of guanine nucleotides (6). The strong interaction between dominant-negative Ran and RCC1 is consistent with the observation that many mutant or modified forms of GTPases, including Ran (17), can form stable complexes with their exchange factors. The failure of c-del Ran to interact with RanBP1 in vivo confirms the results of ligand-blotting assays in vitro (Fig. 2) and suggests that the interaction of RanBP1 with Ran-GTP is mediated by the carboxyl-terminal region of the latter.

c-del Ran functions as well as WT Ran in a Ran-dependent in vitro nuclear import assay. Recent studies have shown that Ran protein is required for nuclear protein import and suggest that importation is coupled to GTP binding and hydrolysis by Ran (32, 36). To test whether RanBP1 is involved in this process, we compared the efficiency of nuclear protein import in the presence of WT or c-del Ran protein, reasoning that the mutant Ran protein would be unable to carry out any RanBP1-dependent processes. As shown in Fig. 3, the WT and c-del proteins functioned equally well in this assay, suggesting that Ran's effect on nuclear protein import occurs independently of RanBP1.

RanBP1, a putative Ran effector, selects both Ran- and RNA1-expressing clones in a yeast interaction trap screen. To extend our analysis of the Ran GTPase-dependent pathway that functions through RanBP1, we screened for proteins that interact with RanBP1. The entire 203-amino-acid open reading

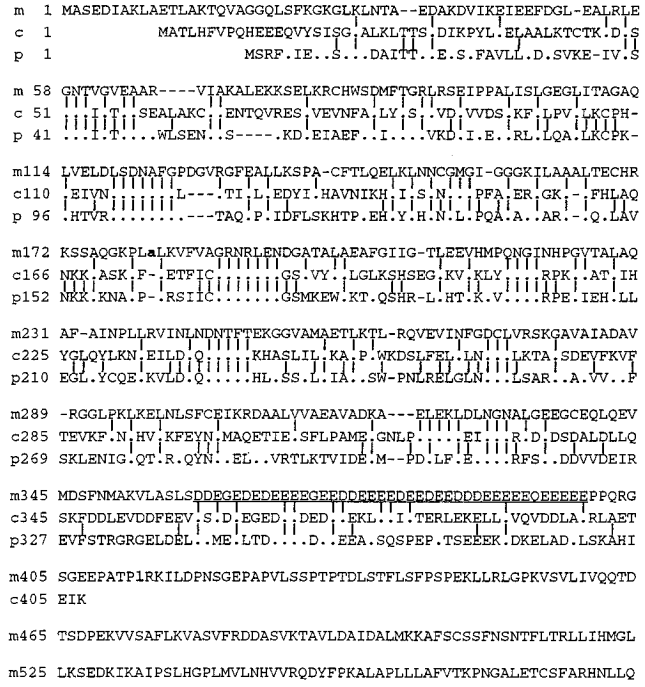

FIG. 4. Alignment of predicted mouse and yeast RNA1 amino acid sequences. The sequence for mouse RNA1 (m) (GenBank no. U20857) is aligned with those of the WT forms of *S. cerevisiae* (51) (c) and *S. pombe* (33) (p) RNA1 proteins (GenBank no. M27142 and X69882, respectively). In the mouse sequence, lowercase letters indicate the two differences from the sequence predicted for FUG1 (18) and underlining indicates the acidic domain. In the alignment, dots indicate residues identical to the mouse sequence, vertical bars indicate identities between adjacent sequences (m and c; c and p), and dashes indicate gaps introduced to maximize homology.

frame of mouse RanBP1 cDNA (13) was subcloned in frame downstream of a LexA DNA binding domain open reading frame to produce a fusion protein for use as bait in a yeast interaction trap screen (23) of a mouse myeloma cDNA expression library. Of 20 positive clones identified in this way (of 500,000 screened) and characterized by partial DNA sequencing, 18 coded for Ran/M1, a mouse Ran with an amino acid sequence identical to that of human Ran/TC4 (12) and two coded for the entire open reading frame of a protein homologous to the *S. cerevisiae RNA1* gene product (51) (Fig. 4).

To locate the mouse *Rna1* gene on a chromosome and thus determine whether it could be the WT allele of any known, visible mouse mutation, the cDNA clone was used to probe Southern blots of *Msp*I-digested genomic DNAs from inbred strains of mice to identify a restriction fragment length variant. The variant segregated as a single genetic locus in RI strains of mice, and comparison of the RI strain *Rna1* typing patterns to those previously determined for 1,817 other loci distributed over the 19 mouse autosomes and the X chromosome revealed tight linkage of *Rna1* to several markers of distal chromosome 15, including *Pdgfb* (platelet-derived growth factor b, the Sis proto-oncogene) (Table 2). These results do not indicate tight linkage of *Rna1* and any of the visible mutations mapped to chromosome 15 (34) but do suggest possible locations for the human homolog of *Rna1*, on the long arms of either chromosome 8 or 22.

The nucleotide sequence of one of the mouse *RNA1*-like clones was determined by using seven unique primers. A database search revealed near identity of our mouse clone to a newly listed mouse gene, *Fug1*, which was also identified as an *RNA1* homolog (18). Disruption of *Fug1* is associated with the death of mouse embryos before gastrulation (18). The sequence spanned the entire open reading frame plus 232 residues of the 5′-untranslated sequence and 174 residues of the 3′-untranslated sequence. It was identical to that of *Fug1* (GenBank accession no. U08110) except for the substitutions of GC for CG at positions 815 and 816, causing the substitution R181A, and T for C at position 1512, causing the substitution S413L (*Fug1* numbering). Genomic Southern blotting experiments suggest that the sequence differences between *Rna1* and *Fug1* reflect polymorphism between the BALB/c and 129 mouse strains from which *Rna1* and *Fug1* were cloned. Consistent with this suggestion, the fragment pattern observed when Southern blots of *Eco*RI-digested mouse genomic DNA were probed with *Rna1* was the same as that reported for *Fug1* (18) (data not shown) and the genetic localization determined for *Fug1* through analysis of interspecific backcross progeny (18) is indistinguishable from that determined for *Rna1* through analysis of RI strains (Table 2).

TABLE 2. Localization of *Rna1* on distal mouse chromosome 15[a]

Marker	R/N	Probability	Distance, cM (95% CI)	Human homolog
Pmv17	14/79	<0.00001	6.0 (3.0–12.0)	
D15Mit1	2/22	0.00941	2.6 (0.3–13.0)	
D15Ncvs20	2/25	0.00174	2.3 (0.3–10.7)	
Pmv50	0/15	0.00365	0.0 (<8.1)	
Amh-rs7	0/15	0.00365	0.0 (<8.1)	
Gpt1	0/15	0.00365	0.0 (<8.1)	8q24-qter
Atf4	0/23	0.00002	0.0 (<4.8)	
D15Mit2	0/22	0.00004	0.0 (<5.0)	
Cyp2d	0/22	0.00004	0.0 (<5.0)	22q11
Pdgfb	2/69	<0.00001	0.8 (0.0–3.0)	22q12.3-q13.1
Ins3	1/20	0.00300	1.4 (0.0–9.9)	

[a] RNA was typed in mice from the BXD, BXH, CXB, OXA, AXB, BXA, and NXSM RI strain sets (a total of 107 strains). Typing results were compared to those for 1,817 other loci in the New York University RI strain database (18a). For each matching locus, its recombination fraction with *Rna1* (number of recombinant strains found/number of strains compared [R/N]) is shown, together with the probability that that fraction or a smaller one would be found by chance (8) and the estimated map distance (in centimorgans [cM]) (50) between the locus and *Rna1* together with the 95% confidence interval (CI) of the estimate (47). Loci are ordered on the basis of all available linkage data (34), but estimated map distances are derived from RI strain typing data only. Chromosomal assignments of human homologs of these loci are shown where these have been defined (34).

Over the region spanned by the *S. pombe* RNA1 protein, the predicted sequences of the mouse and *S. pombe* proteins are 32% identical, the mouse and *S. cerevisiae* proteins are 28% identical, and the two yeast proteins are 37% identical, a degree of homology similar to that between mammalian and yeast RCC1 proteins (15) and about half that of RAN proteins (12, 43). The three RNA1 proteins contain leucine-rich repeats (27) and a large acidic domain (18, 33, 51) (Fig. 4).

The use of the yeast interaction trap system with RanBP1 as bait to screen a mouse myeloma cDNA library thus confirmed the previously defined interaction between the RanBP1 and Ran proteins and identified a new interaction, between RanBP1 and the mouse homolog of the yeast *RNA1* gene product. Our failure to recover Ran/M2, a mouse Ran isoform that exhibits 95% amino acid sequence identity to human Ran/TC4 and mouse Ran/M1, is consistent with the restriction of Ran/M2 expression to the testis (12).

RNA1 interacts with RanBP1 but not Ran or RCC1. To test

TABLE 3. Interactions between mouse Ran, RanBP1, and RNA1 proteins in the yeast interaction trap assay system[a]

pEG202 LexA construct	Affinity for pJG4-5 B42 construct:			
	RanBP1	Ran/M1	Ran/M2	RNA1
RanBP1	−	+++	+++	++
Ran/M1	+++	−	−	−
Ran/M2	+++	−	−	−
RCC1	−	+	++	−

[a] Interactions were assayed as described in Materials and Methods. The number of pluses refers to color intensity and is a rough measure of relative interaction affinity.

the interactions of the mouse RNA1 protein with the known components of the Ran GTPase switch, the interaction trap system was used as an assay (Table 3). The complete open reading frames of mouse Ran/M1 (identical to human Ran/TC4), mouse Ran/M2 (95% identical to human Ran/TC4), mouse RanBP1, and human RCC1 cDNAs were cloned in frame downstream of a DNA binding domain (plasmid pEG202) and/or a transcriptional activating domain (plasmid pJG4-5). Pairs of plasmids encoding one each of the two types of fusion proteins were transfected into *S. cerevisiae* and assayed for interaction. RCC1 interacts with Ran but not with RanBP1 or RNA1, RanBP1 interacts with both Ran and RNA1, and RNA1 interacts only with RanBP1. The strongest interaction was reproducibly that between Ran and RanBP1. These results are consistent with the role of Ran as a GTPase switch: the exchange factor, RCC1, interacts with the GTPase, Ran, but not with the latter's putative effector, RanBP1, while RanBP1 interacts with both Ran and an additional component (RNA1) of a pathway that may regulate or be regulated by Ran.

DISCUSSION

The data presented here are consistent with the existence of separate domains in the Ran protein that mediate the functions of (i) nucleotide binding and hydrolysis and (ii) interaction with downstream effectors. They are also consistent with the hypothesis that the regulatory effects of the Ran GTPase switch on other cellular processes are mediated by at least two pathways, one that proceeds via the interaction of Ran-GTP with RanBP1 to affect RNA processing and cell cycle progression and one that proceeds independently of RanBP1 to affect nuclear protein import. Specifically, Ran protein lacking six carboxyl-terminal amino acids (DEDDDL) functions as well as the full-length protein in a Ran-dependent nuclear import assay (Fig. 3) but does not function in regulating cell cycle progression (42). At a molecular level, the deletion mutant protein interacts with regulatory factors RCC1 and RanGAP but fails to interact with RanBP1.

Lounsbury et al. (29) have also characterized a c-del Ran protein (ΔPDEDDDL), observing that GTP-charged deletion mutant Ran failed to interact with any of the array of hamster proteins that bound WT Ran-GTP under their assay conditions and that GDP-charged mutant Ran could be stimulated to release the bound nucleotide in the presence of a hamster cell extract. Their observations appear to be consistent with our analysis of the interactions of WT and mutant Ran proteins with RanBP1 and RCC1 (Fig. 2 and Table 1). The interaction data, as a whole, are consistent with the hypothesis that the acidic carboxyl-terminal residues of Ran have no role in determining the ratio of Ran-GTP to Ran-GDP through GEF and GAP activities but are required for transducing a signal from Ran to effectors such as RanBP1. The biological activity data suggest that the carboxyl-terminal residues of Ran are not required for stimulating nuclear import (Fig. 3) but may be required for regulating cell cycle progression.

The dichotomy of biological functions suggested by our mutagenesis studies supports the hypothesis that the Ran GTPase switch acts in at least two separate pathways, and biochemical studies may provide useful clues to the identity of the pathway-specific downstream effectors. A key result in support of this hypothesis is our discovery of the mouse homolog of yeast protein RNA1 in the course of a search for proteins other than Ran that interact with RanBP1 (Fig. 4 and Table 3). *S. cerevisiae RNA1* mutants are defective in aspects of RNA metabolism including mRNA initiation, termination, and polyadenylation; tRNA splicing; rRNA processing; and mRNA nuclear-cytosolic transport (22), and the *S. pombe RNA1* homolog can restore function to *RNA1*-deficient *S. cerevisiae* (33). Also, as previously noted, the same array of RNA processing and transport defects observed in *S. cerevisiae RNA1* mutants are observed in yeast cells expressing mutant RCC1 or both mutant proteins (22). This epistatic relationship between RNA1 and RCC1 strongly suggests that both are part of the same biochemical pathway. Our studies of protein-protein interactions in vivo (Table 3) suggest that the functional link between RCC1 and RNA1 is their interaction with Ran or RanBP1. Indeed, other components of the pathway may remain to be identified. These possibilities need to be explored through in vitro binding and reconstitution studies, but it is already clear that RNA1, RanBP1, Ran, and RCC1 are linked in a biochemical pathway.

We suggest that RanBP1 and RNA1 may be joint effectors and/or regulators of Ran that control nuclear RNA export and that the involvement of Ran in the checkpoint that prevents the onset of mitosis until the completion of DNA synthesis may be secondary to RNA transport. That is, a defect in RNA transport and the resulting buildup of nuclear RNAs would interfere with normal RNA synthesis and processing. The consequent defect in the transport of mRNAs required for cell cycle regulation would result in faulty mitotic control.

As schematized in Fig. 5, the presence of Ran-GTP during DNA replication would ensure the export of an mRNA whose continued expression is required to prevent the onset of mitosis. Consistent with this possibility is the fact that, in both yeast and mammalian cells, temperature-sensitive RCC1 mutants accumulate nuclear poly(A)$^+$ RNA and undergo premature initiation of mitosis at the nonpermissive temperature, when Ran-GTP levels should be low (3, 22, 26). Under these conditions, nuclear RNA accumulation clearly precedes premature initiation of mitosis. Two apparently different Ran functions (RNA transport and mitotic checkpoint control) would thus be linked by a single Ran biochemical pathway.

In regard to nuclear protein import, Ran GTPase is clearly essential (32, 36) but three lines of evidence suggest that except for the involvement of Ran, the nuclear protein import process is separate from that of RNA processing and export. First, c-del Ran (which cannot interact with RanBP1 [Fig. 2 and Table 1]) is as effective as WT Ran in a protein import assay (Fig. 3). Second, addition of sufficient amounts of dominant-negative (T24N) Ran/TC4 protein to a *Xenopus* egg extract to inhibit cell cycle progression does not appear to affect nuclear protein import (28). Third, nuclear protein import in digitonin-permeabilized buffalo rat liver cells does not require addition of RanBP1 (38a) but does require a novel Ran-interacting protein, p10 (38).

The model presented here, in which the carboxyl terminus of

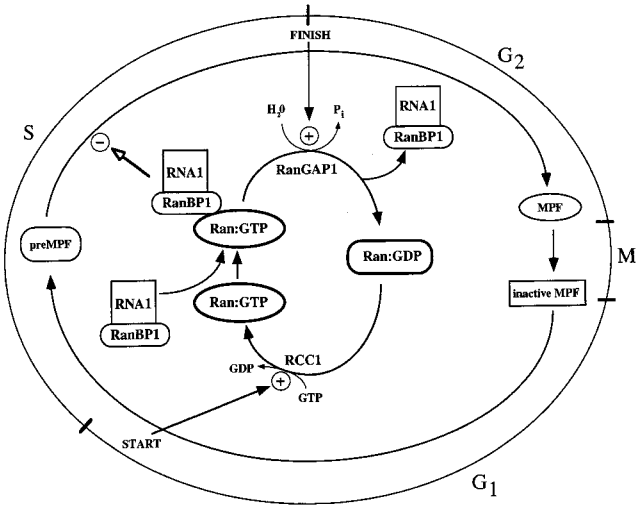

FIG. 5. Schematic representation of a Ran GTPase cycle coupled to the mitosis-promoting factor (MPF) and cell cycles. The inner circle represents the Ran GTPase cycle and includes a GEF, RCC1; a Ran effector complex, RanBP1-RNA1; and a Ran GTPase-activating protein, RanGAP. The middle cycle represents the MPF cycle (40), in which MPF is maintained in an inactive form, preMPF, until completion of the S phase and in which its cyclin component is degraded during exit from metaphase (inactive MPF). The outer circle represents the cell cycle, in which START and FINISH indicate the commitment to and termination of DNA synthesis, respectively. In this model the effector complex RanBP1-RNA1, perhaps through a mechanism regulating mRNA transport, would prevent conversion of preMPF to MPF until completion of DNA synthesis. See the text for details.

Ran plays a direct role in RNA export from the nucleus via its interactions with RanBP1 and RNA1 but has no direct role in protein import into the nucleus, makes the assumption that the Ran carboxyl-terminal deletion has no effect on Ran's GTPase cycle. Changes in the rate of nucleotide exchange, the rate of nucleotide hydrolysis, or overall nucleotide binding affinities could alter the in vivo ratio of [c-del Ran–GTP]/[c-del Ran–GDP] such that any process dependent on Ran could be affected. Such alterations, if in fact they occur, could have no detectable effects on our in vitro nuclear protein import assay while significantly affecting import in vivo. In this regard, however, it should be noted that expression in human cells of c-del Ran at a level well above that of endogenous WT Ran had no apparent effect on either cell division or the intracellular distribution of the mutant and WT proteins (42).

A quite different reason to search for a possible role for the carboxyl terminus of Ran in regulating its GTPase cycle is the recent observation that RNA1 can function as a RanGAP (49). RanBP1 might thus serve as an adaptor directing Ran to at least this one of its GAPs. RanBP1, RNA1, or both might still be specific downstream effectors of Ran, but it is also possible that an additional effector(s) mediating the role of Ran in RNA transport and cell cycle control could be among the other proteins whose interaction with Ran depends on the presence of Ran's carboxyl terminus (14, 29).

Together, Ran, RCC1, and RanBP1 constitute between 0.5 and 1% of total cellular protein, many species express more than one Ran isoform, and although Ran is predominantly nuclear it is by no means restricted to the nucleus. The possibility that Ran participates in more than one pathway is not surprising, and the identification and characterization of the other proteins with which it interacts is proving to be a fruitful approach to defining its multiple roles in the cell.

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