Ran/TC4: A Small Nuclear GTP-binding Protein That Regulates DNA Synthesis

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Abstract. Ran/TC4, first identified as a well-conserved gene distantly related to H-RAS, encodes a protein which has recently been shown in yeast and mammalian systems to interact with RCC1, a protein whose function is required for the normal coupling of the completion of DNA synthesis and the initiation of mitosis. Here, we present data indicating that the nuclear localization of Ran/TC4 requires the presence of RCC1. Transient expression of a Ran/TC4 protein with mutations expected to perturb GTP hydrolysis disrupts host cell DNA synthesis. These results suggest that Ran/TC4 and RCC1 are components of a GTPase switch that monitors the progress of DNA synthesis and couples the completion of DNA synthesis to the onset of mitosis.

Ran/TC4, was initially described as a RAS-related transcript of unknown function. It was identified in a human teratocarcinoma cell line but is abundant in a variety of cultured cell lines, and is of interest because it defines a new, evolutionarily well-conserved branch of the RAS gene superfamily (Drivas et al., 1990, 1991a). Recent genetic and biochemical analyses (Matsumoto and Beach, 1991; Bischoff and Ponstingl, 1991a) suggest that Ran/TC4 also plays a key role in the regulation of cell cycle progression in eukaryotes, and that this role depends on its nuclear localization and interaction with the product of a second gene, RCC1, defined by the tsBN2 mutation of BHK cells (Nishimoto et al., 1978; Uchida et al., 1990) and the piml mutation of the fission yeast Schizosaccharomyces pombe (Matsumoto and Beach, 1991). The compound name used here reflects this: a teratocarcinoma-derived cDNA clone that encodes a Ras-related nuclear (Ran) protein.

S. pombe pim1 (premature initiation of mitosis) mutants enter mitosis without completing chromosomal DNA replication. Overexpression of the wild-type allele of a second gene, spil (suppressor of pim1), suppresses the pim1 mutant phenotype. The predicted amino acid sequences of spil (yeast) and Ran/TC4 (human) are 80% identical. The fact that spil overexpression cannot rescue null mutants and the existence of a cold-sensitive mutation in spil suggest direct interaction between Pim1 and Spil proteins (Matsumoto and Beach, 1991).

The mammalian homolog of pim1 is RCC1 (regulator of chromosomal condensation), a gene originally defined by the tsBN2 mutation of BHK cells (Uchida et al., 1990; Nishitani et al., 1991). The wild-type activity of RCC1 is required both to initiate DNA synthesis (Dasso et al., 1992) and to prevent chromosome condensation until the completion of S phase (Uchida et al., 1990; Nishitani et al., 1991; Enoch and Nurse, 1991; Dasso and Newport, 1990). pim1 is predicted to encode a larger protein (539 aa) than RCC1 (421 aa), but over the region shared by the two proteins, their sequences are 30% identical and share a sequence motif repeated seven times in each protein (Matsumoto and Beach, 1991).

RCC1 protein can bind DNA and is associated with chromatin (Ohtsubo et al., 1989). It is present in Xenopus egg extracts in amounts sufficient to provide one molecule per nucleosome (Dasso et al., 1992), and can be purified from HeLa cell chromatin in the form of a complex with a 25-kD protein. Partial amino acid sequence data indicate that the latter is Ran/TC4 (Bischoff et al., 1990; Bischoff and Ponstingl, 1991a).

To further characterize the function of Ran/TC4 and its interaction with RCC1 in mammalian cells, we have now prepared Ran/TC4-specific antibodies and used them to demonstrate that nuclear localization of Ran/TC4 is dependent on expression of RCC1. We have also shown that expression of a mutant Ran/TC4 allele blocks cellular DNA replication.

Materials and Methods

DNA Sequencing and In Vitro Mutagenesis

DNA sequencing was performed as described previously (Drivas et al., 1990, 1991b) using double-stranded pMT2 or lambda phage templates, with Ran/TC4-specific oligonucleotides as primers.

In vitro mutagenesis was performed by oligonucleotide priming, using the 1.5-kb Ran/TC4 cDNA EcoRI fragment (Drivas et al., 1990) cloned in M13 as a template. Mutants were identified by differential hybridization with wild-type and variant oligonucleotide probes, and the mutated EcoRI fragments were isolated and cloned into the EcoRI site of pMT2. Mutant identifications were confirmed by DNA sequence analysis of both the initial M13 isolate and the pMT2 subclones.
Ran/TC4 and RCC1 Antibodies

The dodecamer peptide underlined in Fig. 1 was synthesized, coupled with glutaraldehyde to Keyhole Limpet hemocyanin, emulsified in complete Freund's adjuvant, and injected intradermally into two rabbits (Pocono Rabbit Farms). One yielded a high-titer polyclonal antisera used in all experiments described here. RCC1 antibody (Nishitani et al., 1991) was a generous gift of Dr. Takeharo Nishimoto.

Cell Lines
tsbN2 cells were a generous gift from Dr. Claudio Basilio. HeLa, COS, 3T3, and wild-type BHK21 hamster cells were from laboratory stock derived ultimately from American Type Culture Collection. Rockville, MD.

Immunoblotting

Total Cell Lysates. Cells at 50-75% confluence in 10-cm dishes were lysed in 0.5 ml of 2% SDS, 50 mM Tris, pH 7.5, boiled for 10 min, sonicated, and to an equal volume of 4% SDS, 0.05% Bromophenol blue, 40% glycerol, 10% B-mercaptoethanol, 200 mM Tris, pH 6.8 (2 x SDS-PAGE sample buffer). Aliquots corresponding to 50 µg total protein were then electrophoresed through 0.1% SDS, 15% polyacrylamide gels, and electroblotted onto nitrocellulose membranes. The membranes were incubated for 2 h at room temperature with 1:400 dialysis of rabbit anti-Ran/TC4 antisemur in PBS, pH 7.4, containing 5% (wt/vol) nonfat dry milk, and 0.3% Tween 20. Filters were washed with the same solution (minus antisemur), incubated with [125I]-protein A (ICN Biomedicals Inc., Costa Mesa, CA) for 2 h at room temperature, washed with 0.3% Tween 20 in PBS, and autoradiographed.

Digitonin-treated Cell Lysates. Cells at 50-75% confluence in 10-cm dishes were treated with digitonin in order to selectively permeabilize plasma membranes and release cytosolic components (Adam et al., 1990; Shi and Thomas, 1992; Walton et al., 1992). Specifically, cells were washed twice in 5 ml cold PBS and then incubated for 10 min in ice cold permeabilization buffer containing 25 µg/ml digitonin (Sigma Chem. Co., St. Louis, MO.), 160 mM KCl, 1 mM MgCl2, and 10 mM sodium phosphate, pH 7.0. Permeabilization buffer (containing cytosolic components) was removed, and cellular material remaining on the plate (including nuclei and other major membrane organelles) was solubilized in 1 ml 2% SDS, 50 mM Tris, pH 7.5. The proteins solubilized in the permeabilization buffer were precipitated in 10% TCA and redissolved in 1 ml 2% SDS, 50 mM Tris, pH 7.5. Solubilized proteins were then boiled for 2 min, sonicated, added to equal volumes of 2 x SDS-PAGE sample buffer, and analyzed for Ran/TC4 content by the immunoblotting procedures described above for total cell lysates.

Immunoprecipitation

Cells at 50-75% confluence in 3.5-cm dishes were washed with methionine-free DME, and incubated in 1 ml of the same supplemented with 125 µCi [35S]methionine (1,330 Ci/mmol) for 6 h at 37°C. Cells were washed twice in PBS, lysed in 0.1 ml 2% SDS, 50 mM Tris, pH 7.5, boiled 10 min, sonicated, and diluted 1:10 in 1.25% Triton X-100, 150 mM NaCl, 50 mM Tris, pH 7.5, 6 mMEDTA (solution A). Lysates were precleared with protein A-Sepharose beads, incubated for 1 h at room temperature with 1:200 rabbit anti-Ran/TC4 antisemur, and then for 1 h at room temperature with protein A-Sepharose. Precipitated beads were washed four times with solution A, and then with solution A containing 0.5 M NaCl, and once with PBS. Beads were then boiled in 50 µl SDS-PAGE sample buffer, and the eluate was electrophoresed through a 0.1% SDS, 15% polyacrylamide gel. The gel was fixed for 3 h in 25% methanol, 10% acetic acid, incubated 1 h in autoradiography enhancer (ENVANCE, DuPont Co., Wilmington, DE) and 1 h in water (all at room temperature), and then dried and autoradiographed (XAR film, Eastman Kodak Co., Rochester, NY).

Densitometry

Autoradiograms from Southern and Western blotting experiments were analyzed by a GS300 transmittance/reflectance scanning densitometer (Hoefer Sci. Inst., San Francisco, CA) coupled to a computer. Integrated absorbances for each band were recorded as bar graphs.

Phosphorimaging

Quantitation of Western blots to obtain the data shown in Fig. 7 was carried out using a Phosphorlmager unit (Molecular Dynamics, Sunnyvale, CA). Data are recorded as bar graphs, in arbitrary units.

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Results

Predicted Amino Acid Sequence of Human and Mouse Ran/TC4

We have resequenced our human Ran/TC4 clone (Drivas et al., 1990) on both strands using internal primers and have obtained a sequence that agrees well with those proposed for S. pombe Spil (Matsumoto and Beach, 1991) and human H-RAS (Barbacid, 1987). Dots indicate residues identical to those predicted for Ran/TC4; dashes indicate gaps introduced to maximize homology. The sequence used to generate an anti-Ran/TC4 peptide antibody is underlined. The Ran/TC4 residues altered in the “double-activated” mutant construct are indicated with asterisks. The corrected human Ran/TC4 cDNA sequence has been deposited with GenBank (accession number M31469).

A Ran/TC4-specific Antipeptide Antibody

Previously, antibodies specific for Ran/TC4 have been obtained only in limited amounts by adsorption from complex antisera (Bischoff and Ponstingl, 1991a). To generate monospecific antibodies directly, we took advantage of the divergence of the carboxy-terminal sequence of Ran/TC4 from those of other RAS-related proteins to generate polyclonal rabbit antibodies specific for Ran/TC4. A hydrophilic decapptide, indicated by underlining in Fig. 1, was synthesized, coupled to Keyhole Limpet hemocyanin, and used to immunize rabbits.

One of two rabbits tested yielded a high-titer, monospecific anti-Ran/TC4 antiserum. The antiserum reacted with a single 25-kD band in Western blots of extracts of bacteria and monkey cells harboring human Ran/TC4 cDNA in expression vectors. (The predicted molecular weight of Ran/TC4 is 24,423.) It also reacted with a single 25-kD band in Western blots of total cell lysates of untreated mouse (3T3), monkey (COS), and human (HeLa) cell lines (Fig. 2 A). The same band was visualized in immunoprecipitates of [35S]methionine-labeled COS cell extracts resolved by SDS-PAGE (Fig. 2 B).

When COS and HeLa cells were reacted with anti-Ran/TC4 antiserum plus fluorescein-labeled goat anti-rabbit Ig, staining was restricted to the nuclei of interphase cells. Mitotic cells were diffusely stained except that the metaphase chromosomes themselves were unstained (Fig. 3). Staining of 3T3 cells gave similar results (data not shown).

The absence of Ran/TC4 from metaphase chromosomes was confirmed using digitonin-permeabilized COS cells, in which most of the cytosol and background Ran/TC4 was removed (Fig. 4). Permeabilized mitotic cells were essentially unstained with anti-Ran/TC4, while metaphase chromosomes continued to be stained with anti-RCC1. Approximately 10 metaphases were examined with each antibody.

Dependence of the Nuclear Localization of Ran/TC4 on RCC1 Expression

Because of the genetic and in vitro biochemical evidence of interaction between Ran/TC4 and RCC1/Pim1 (Bischoff and Ponstingl, 1991a,b; Matsumoto and Beach, 1991), we asked whether the nuclear localization of Ran/TC4 is dependent on the presence of RCC1 in vivo. In the BHK cell line BHK21, Ran/TC4 and RCC1 proteins, as detected by immunofluorescence microscopy, were localized to cell nuclei at both 33.5 and 39.5°C (data not shown). The cell line tsBN2, a mutant derived from BHK21, synthesizes a temperature-sensitive RCC1 protein. Within 3 h of transfer from 33.5°C to the nonpermissive temperature of 39.5°C, immunologically detect-
Figure 3. Nuclear localization of Ran/TC4. COS and HeLa cells were fixed, permeabilized, stained with rabbit anti-Ran/TC4, or preimmune serum, plus FITC-conjugated goat anti-rabbit Ig, and photographed under phase contrast or epillumination. Arrows indicate mitotic cells.

Figure 4. Localization of Ran/TC4 and RCC1 in digitonin-permeabilized COS cells. Cells were permeabilized, fixed, and stained with anti-Ran/TC4 or anti-RCC1. Arrows indicate mitotic cells.
Figure 5. Subcellular localization of Ran/TC4 and RCC1 in tsBN2 cells—low magnification. Cells incubated for 0, 3, or 6 h at 39.5°C were fixed, permeabilized, and scored for Ran/TC4 and RCC1 localization as described in the legend to Fig. 3. DNA was localized by staining with HOECHST 33258 (Flow Labs., McLean, VA).
detectable and substantial Ran/TC4 staining of cytoplasm was detected (Figs. 5 and 6). Visual scoring of photographs such as those shown in Fig. 5 indicated that at least 90% of cells exhibited Ran/TC4 cytoplasmic staining after a 3-h incubation at 39.5°C. Total cellular levels of Ran/TC4 protein were essentially unchanged over the course of these experiments in both tsBN2 and BHK21 cells (Fig. 7).

The release of Ran/TC4 from the nuclei of tsBN2 cells at 39.5°C was analyzed more quantitatively by using immunoblotting to measure the fraction of Ran/TC4 present in cytosolic extracts of digitonin-permeabilized cells. As shown in Fig. 7, the total amount of Ran/TC4 remained constant over 6 h at 39.5°C, but the fraction of this protein extracted by digitonin treatment increased from 19 to 55%. This redistribution of Ran/TC4 was specific to tsBN2 cells, as the fraction of Ran/TC4 released from normal BHK21 ranged between 10 and 15% over 6 h at 39.5°C. Both immunostaining and cellular fractionation procedures thus demonstrate that a significant fraction of Ran/TC4 protein is released from the nuclei of tsBN2 cells at 39.5°C.

Neither cell death nor increased entry into mitosis at the nonpermissive temperature is sufficient to explain the redistribution of Ran/TC4 into the cytoplasm. tsBN2 cells exhibit normal RNA and protein synthesis for at least 12 h at 39.5°C (Nishimoto et al., 1978). Also, after 6 h at 39.5°C, no more than 5% of unsynchronized tsBN2 cells exhibit a mitotic appearance (Fig. 5), consistent with the previous observation that after 6 h at 39.5°C, tsBN2 cells are arrested in G1 (Nishimoto et al., 1978; Nishitani et al., 1991). Most of these cells would have been in G1 at the time of the temperature shift and would have arrested there; the minority in S or G2 would be expected to progress through M to arrest in G1.

**Effect of a Putative GTPase-deficient Mutant Ran/TC4 on Cellular DNA Synthesis**

Members of the GTPase superfamily, including translation factors, heterotrimeric G proteins, and RAS and RAS-related proteins, function as switches, changing state according to the nucleotide, GTP or GDP, bound to the GTPase. Ran/TC4 is known to bind and hydrolyze GTP, and exchange of bound GDP for GTP is accelerated by interaction with RCC1 (Bischoff and Ponstingl, 1991b). We have therefore examined the functional consequences in vivo of transient expression of wild-type and mutated forms of the gene in COS cells.

The human Ran/TC4 cDNA was mutated in vitro to specify Val at codon 19 (equivalent to H-RAS codon 12) and Leu at codon 69 (equivalent to H-RAS codon 61). In previously studied RAS and RAS-related proteins, these substitutions alone or in combination cause GTPase deficiency (Bourne et al., 1991; Kaziro et al., 1991). The double-mutant and wild-type cDNAs were cloned into the pMT2 expression vector, which contains the SV-40 origin of replication. These constructs were transfected into COS cells, and protein products in cell extracts prepared 48-70 h after transfection were analyzed by SDS-PAGE. This system should allow transient expression of large amounts of any protein en-
Figure 7. Quantitation of Ran/TC4 and RCC1 in tsBN2 and BHK21 Cells. (A) BHK21 and tsBN2 cells incubated for 0, 3, or 6 h at 39.5°C were analyzed for total Ran/TC4 and RCC1 proteins by immunoblotting and phosphorimaging. The bar graphs show the results of phosphorimaging in arbitrary units. (B) BHK21 and tsBN2 cells incubated for 0, 3, or 6 h at 39.5°C were treated with digitonin to generate cytosolic and residual fractions and the percentage of Ran/TC4 extracted in the cytosolic fraction was determined by immunoblotting and phosphorimaging.

Figure 8. Proteins made in COS cells transfected with Ran/TC4 gene constructs. (A) SDS-PAGE. COS cells were transfected with (1) nothing (mock); (2) pMT2-DHFR, which specifies an ~22 kD protein; (3) pMT2-wild type Ran/TC4, which specifies an ~25 kD protein; (4) pMT2-mutant Ran/TC4; (5) equal amounts of pMT2-DHFR and pMT2-wild-type Ran/TC4; and (6) equal amounts of pMT2-DHFR and pMT2-mutant Ran/TC4. Cultures were harvested 62 h after transfection and analyzed by SDS-PAGE and Coomassie blue staining. (B) Western blotting. Total cell lysates (50 μg protein) prepared from each of the transfected cell populations (lanes 1–6) were analyzed by Western blotting as described in the legend to Fig. 2A. The bar graph shows the results of densitometric analysis of the Western blot, in arbitrary units.

GTPase-ones) all produced high levels of the expected proteins (Zeng, J., and M. Ren, unpublished observations).

This reduction was due to failure of the transfected plasmids to replicate. Specifically, to demonstrate that transcription and translation of plasmid genes was normal, pMT2-Ran/TC4 mutant was cotransfected into COS cells with plasmid constructs whose product could be detected in the absence of plasmid replication. pRSV constructs encoding TH, β-galactosidase, or chloramphenicol acetyltransferase programmed the synthesis of proteins that could be detected even at low levels by immunofluorescence, β-galactosidase enzymatic activity, or chloramphenicol acetyltransferase assay, respectively. Equal levels of the products, as determined by densitometric analysis, were detected in COS cells transfected with the pRSV construct alone or with the pRSV construct plus pMT2-Ran/TC4 mutant (data not shown).

To show directly that the pMT2-Ran/TC4 mutant product blocked plasmid DNA replication, parallel cultures of COS cells were transfected with bacterially methylated pMT2-Ran/TC4 wild-type and mutant constructs. Supernatants prepared 60 h after transfection (Hirt, 1967) were digested with EcoRI (to linearize all plasmid molecules) plus DpnI (to digest methylated, hence unreplicated, DNA into multi-
Figure 9. Correlation of transfection with mutant Ran/TC4 and cessation of DNA replication: cell populations. Supernatants (Hirt, 1967) from COS cells transfected with pMT2 constructs as described in Fig. 8 were analyzed by Southern blotting. Cells were transfected with nothing (mock), pMT2-DHFR, pMT2-wild-type Ran/TC4, pMT2-mutant Ran/TC4, equal amounts of pMT2-DHFR and pMT2-wild-type Ran/TC4, and equal amounts of pMT2-DHFR and pMT2-mutant Ran/TC4. The two leftmost tracks on the gel are loaded with 300- and 10-ng quantities of pure methylated pMT2 plasmid processed in parallel with the Hirt supernatants but digested only with EcoRI. The bar graph shows the results of densitometric analysis of the blot. Amounts of fragment in each track were calculated relative to the intensity of the 300-ng control fragment.

Discussion

Ran/TC4 is a remarkably well-conserved member of the RAS supergene family, specifying proteins of identical sequence in humans and mice and 80% identical between these species and S. pombe (Fig. 1). Using a dodecapeptide from its carboxy-terminal region, high-titer, monospecific polyclonal antibodies to Ran/TC4 were readily obtained (Figs. 2 and 3). Immunostaining experiments showed that Ran/TC4 is primarily restricted to the nuclei of interphase cells, and is diffusely distributed in mitotic cells (but excluded from the chromosomes). These staining patterns are consistent with the ones reported by Bischoff and Ponstingl (1991a) for antibodies extracted from a complex antiserum by adsorption to purified Ran/TC4 protein.

Ran/TC4 protein has been suggested to interact with RCC1 protein. As a structural correlate of that interaction, we have shown that nuclear localization of Ran/TC4 requires the presence of functional RCC1 protein (Figs. 5 and 6). The nuclear localization of Ran/TC4 and RCC1 is intriguing in two respects. First, although the amino-terminal DNA binding domain of RCC1 functions as a nuclear localization signal (Seino et al., 1992), neither protein includes clearly identifiable nuclear localization signals such as the stretches of arginine and lysine residues found in many nuclear proteins (Fig. 1; Bischoff et al., 1990; Garcia-Bustos et al., 1991). Second, while RCC1 protein is required to maintain the nuclear localization of Ran/TC4, the cellular molar ratio of Ran/TC4 to RCC1 is at least 10:1 and Ran/TC4·RCC1 complexes in vitro contain a 1:1 protein ratio (Bischoff and Ponstingl, 1990, 1991a). RCC1 must therefore be modulating the nuclear localization of Ran/TC4 by mechanisms in addition to simple binding.

A Ran/TC4 mutant homologous to GTPase-defective H-RAS (Fig. 1) was constructed. When plasmids containing the mutant construct were transfected into COS cells, replication of the plasmid was blocked, as was replication of any cotransfected plasmid and replication of the host cell chromosomes (Figs. 8–10). The blockade appears to be replication specific, as transcription and translation of plasmid genes were not detectably affected. A crucial problem that remains unsolved is to identify the point(s) in the cell cycle at which cells arrest in response to mutant Ran/TC4 protein. Data from the experiments reported here are consistent with arrest at the G1/S boundary or in S caused by a direct effect of the Ran/TC4 mu-
tient on DNA synthesis, but arrest elsewhere in the cell cycle cannot be excluded. Extension of the FACS® analysis of COS cells to measure DNA content was uninformative because the cells are heterogeneous and polyploid. Resolution of this issue will probably require studies of uniformly diploid cell populations.

A Hypothetical GTPase Switch Using Ran/TC4 and RCC1 to Monitor the Progress of DNA Replication

Guanine nucleotide binding proteins, whether they are true RAS proteins, members of the YPT/Rab subfamily involved in vesicle sorting, heterotrimeric G proteins, or protein synthesis initiation and elongation factors, function as switches whose GTP- and GDP-bound forms interact differentially with effectors and regulators (Bourne et al., 1990; Kaziro et al., 1991). Most guanine nucleotide binding proteins have low intrinsic rates of GTP hydrolysis and nucleotide exchange, so that the functional state of the switch is regulated by associated proteins that increase GTPase activity (GTPase activating proteins, or GAPs), and that inhibit or promote turnover of bound nucleotide (guanine nucleotide dissociation inhibitors, or GDIs, and guanine nucleotide release proteins, or GNRP, respectively). For example, in the case of the bacterial protein synthesis elongation factor EF-TU, EF-TU-GTP binds to aminoacyl-tRNA, GAP and GDI activities are associated with the ribosome, and GNRP activity is provided by EF-TS. In the case of eukaryotic vesicle sorting, (G-GDP)-GDI complexes appear to be soluble, while GNRP and GAP activities may be associated with donor or acceptor membranes (for review see Pfeffer, 1992).

By analogy to these systems, models can be constructed in which Ran/TC4-RCC1 monitors the progress of DNA synthesis and couples the completion of DNA synthesis to the activation of mitosis/maturation promoting factor (MPF). MPF is a complex of two proteins, a cyclin and a serine-threonine protein kinase p34cdc2 (for review see Freeman and Donoghue, 1991). MPF plays a key role in cell cycle control in all eukaryotes. Its regulation involves complex interactions among tyrosine and serine-threonine protein kinases, protein phosphatases, a cyclin protease, and other activators and inhibitors. MPF activation, required in prophase, involves at least cyclin-p34cdc2 complex formation, cyclin phosphorylation, and activation of p34cdc2 kinase via removal of esterified phosphate from a tyrosine residue. MPF inactivation, in anaphase, requires destruction of cyclin.

Two alternative models that both use RCC1 as a monitor of the completion of DNA synthesis, and that couple the cell cycle and a GTPase cycle involving Ran/TC4 and RCC1 are shown in Fig. 11. The models differ in the timing of GNRP and GAP activities and in the role of Ran/TC4-GTP as either an inhibitor or activator of MPF. Thus, in the upper model, START (the beginning of DNA synthesis or a signal that commits the cell to DNA synthesis) activates a GNRP activity, possibly RCC1, that stimulates the conversion of Ran/TC4 to its GTP-bound state, which in turn inhibits MPF activity. FINISH (a signal such as the elimination of single-stranded DNA or the disassembly of replication complexes that indicates the completion of DNA synthesis) attenuates GNRP activity, activates GAP, and thus sharply reduces the concentration of Ran/TC4-GTP. This in turn releases the inhibition of MPF. In the lower model, START activates GAP, FINISH activates GNRP, and Ran/TC4-GTP both stimulates MPF activation and directly inhibits DNA replication. A key feature of the lower model is the autoregulation of Ran/TC4-GTP function through the activation of a GDI that binds to Ran/TC4-GTP and prevents the further activation of MPF.

Our demonstration that expression of putative GTPase-defective Ran/TC4 protein inhibits DNA synthesis is consistent with either of these models.

In regard to the mechanism of MPF activation, our models predict that the unreplicated DNA present between START and FINISH alters levels of Ran/TC4-GTP. We speculate that this change is an essential step of a pathway that leads...
Figure 11. Two alternative models for coupling the cell cycle and a GTPase cycle through the nuclear proteins Ran/TC4 and RCC1. GAP and GDI are as-yet-identified proteins hypothesized to interact specifically with Ran/TC4 to promote GTP hydrolysis, and to inhibit effector interaction and nucleotide exchange, respectively. START and FINISH mark the beginning and end of DNA replication. GNRP activity appears to be provided by RCC1. In the lower model, (-) indicates the direct inhibitory effect of Ran/TC4-GTP on DNA synthesis and (+) indicates the release of this inhibition. Ran/TC4 is abbreviated simply as TC4 here.

to MPF inactivation, although there may be multiple intermediate steps. For example, considering the upper model of Fig. 11, Ran/TC4-GTP could stimulate, either directly or through regulatory kinases and phosphatases, the activation of the kinase that phosphorylates p34^Cdc2 on tyrosine, thus inactivating MPF (Smythe and Newport, 1992). Ran/TC4-GTP could also inhibit, either directly or indirectly, the tyrosine phosphatase that dephosphorylates p34^Cdc2 and is required for MPF activation (Millar and Russell, 1992). Finally, since the induction of premature chromosome condensation in tsBN2 cells at 39.5°C requires new protein synthesis (Uchida et al., 1990), the possibility that Ran/TC4 might regulate the synthesis of cell cycle control proteins cannot be excluded.

These models draw on results from both yeast and mammalian systems. There are important differences between these systems, however. pim1 is mutants of yeast under nonpermissive conditions undergo chromosome condensation from any phase of the cell cycle (G1, G2, or S) and cells arrest with condensed chromosomes (Matsumoto and Beach, 1991). In hamster cell RCC1 is mutants under nonpermissive conditions, only cells in S and G2 proceed to chromosome condensation and mitosis, and cell arrest occurs at a point in G1 possibly analogous to the "start" point of the yeast cell cycle (Nishitani et al., 1991). The reasons for the yeast-hamster differences are unknown and may be specific to the mutant alleles studied in each species. The differences, however, do not alter the conclusions that RCC1 and Ran/TC4 play key roles in the orderly progression of the phases of the cell cycle.

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References

Adam, S. A., R. S. Marr, and L. Gerace. 1990. Nuclear protein import in permeabilized cells requires soluble cytoplasmic factors. J. Cell Biol. 111:807-816.

Aubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, I. Smith, and K. Struhl. 1989. Introduction of DNA into mammalian cells. In Current Protocols in Molecular Biology. Wiley-Liss, Inc., New York. 9.0.1-9.14.3.

Barbacid, M. 1987. ras genes. Annu. Rev. Biochem. 56:779-827.

Bischoff, F. R., and H. Ponstingl. 1991a. Mitotic regulator protein RCC1 is complexed with a nuclear ras-related polypeptide. Proc. Natl. Acad. Sci. USA. 88:10830-10834.

Bischoff, F. R., and H. Ponstingl. 1991b. Catalysis of guanosine nucleotide exchange on Ran by the mitotic regulator RCC1. Nature (Lond.). 354:80-82.

Bischoff, F. R., G. Maier, G. Tilz, and H. Ponstingl. 1990. A 47-kDa human nuclear protein recognized by antikinetochore autoimmune sera is homologous with the protein encoded by RCC1, a gene implicated in the onset of chromosomal condensation. Proc. Natl. Acad. Sci. USA. 87:8617-8621.

Bourne, H. R., D. A. Sanders, and F. McCormick. 1990. The GTPase superfamily: a conserved switch for diverse cell functions. Nature (Lond.). 348:125-132.

Bourne, H. R., D. A. Sanders, and F. McCormick. 1991. The GTPase superfAMILY: conserved structure and molecular mechanism. Nature (Lond.). 349:117-127.

Dasso, M., and J. W. Newport. 1990. Completion of DNA replication is monitored by a feedback system that controls the initiation of mitosis in vitro: studies in Xenopus. Cell. 61:811-823.

Dasso, M., H. Nishitani, S. Kornbluth, T. Nishimoto, and J. W. Newport. 1992. RCC1, a regulator of mitosis, is essential for DNA replication. Mol. Cell. Biol. 12:3337-3345.

Der, C. J., and A. Cox. 1991. Isoprenoid modification and plasma membrane association: critical factors for ras oncogenicity. Cancer Cells. 3:331-340.

Drivas, G. T., A. Shill, E. E. Coutavas, M. G. Rush, and P. D'Eustachio. 1990. Characterization of four novel RAS-related genes expressed in a human teratocarcinoma cell line. Mol. Cell. Biol. 10:1793-1798.

Drivas, G. T., S. Palmieri, P. D'Eustachio, and M. G. Rush. 1991a. Evolutionary grouping of the RAS-protein family. Biochim. Biophys. Res. Commun. 176:1130-1135.

Drivas, G. T., A. Shih, E. Coutavas, P. D'Eustachio, and M. G. Rush. 1991b. Identification and characterization of a human homolog of the Schizosaccharomyces pombe ras-like gene YPT-3. Oncogene. 6:3-9.

Enoch, T., and P. Nurse. 1991. Coupling M phase and S phase: controls maintaining the dependence of mitosis on chromosome replication. Cell. 65: 921-923.

Fischer, R. S., and D. J. Donoghue. 1991. Protein kinases and protooncogenes: biochemical regulators of the eukaryotic cell cycle. Biochemistry. 30:2293-2302.

Garcia-Bustos, J., J. Heitman, and M. N. Hall. 1991. Nuclear protein localization. Biochem. Biophys. Acta. 1071:83-101.

Gorman, C. M., G. T. Merlino, M. C. Willingham, I. Pastan, and B. H. Howard. 1982. The Rous sarcoma virus long terminal repeat is a strong promoter when introduced into a variety of eukaryotic cells by DNA-mediated transfection. Proc. Natl. Acad. Sci. USA. 79:6777-6781.

Hirt, B. 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. J. Mol. Biol. 10:791-798.

Kaufman, R. J. 1990. Vectors used for expression in mammalian cells. Methods Enzymol. 185:487-511.

Kaziro, Y., H. Itoh, T. Kozasa, M. Kakafuku, and T. Satoh. 1991. Structure and function of signal-transducing GTP-binding proteins. Annu. Rev. Biochem. 60:349-400.

Matsumoto, T., and D. Beach. 1991. Premature initiation of mitosis in yeast lacking RCC1 or an interacting GTPase. Cell. 66:347-360.
Mellon, P., V. Parker, Y. Gluzman, and T. Maniatis. 1981. Identification of DNA sequences required for transcription of the human α-globin gene in a new SV40 host-vector system. *Cell.* 27:279–288.

Millar, J. B. A., and P. Russell. 1992. The cdk25 M-phase inducer: an unconventional protein phosphatase. *Cell.* 68:407–410.

Nishimoto, T., E. Eilen, and C. Basilico. 1978. Premature chromosome condensation in a ts DNA mutant of BHK cells. *Cell.* 15:475–483.

Nishitani, H., M. Ohtsubo, K. Yamashita, H. Iida, J. Pines, H. Yasudo, Y. Shibata, T. Hunter, and T. Nishimoto. 1991. Loss of RCC1, a nuclear DNA binding protein, uncouples the completion of DNA replication from the activation of cdc2 protein kinase and mitosis. *EMBO (Eur. Mol. Biol. Organ.)* J. 10:1555–1564.

Ohtsubo, M., H. Okazaki, and T. Nishimoto. 1989. The RCC1 protein, a regulator for the onset of chromosome condensation locates in the nucleus and binds to DNA. *J. Cell Biol.* 109:1389–1397.

Pfeffer, S. R. 1992. GTP-binding proteins in intracellular transport. *Trends Cell Biol.* 2:41–45.

Rindler, M. J., S. S. Naik, N. Li, T. C. Hoops, and M.-N. Peraldi. 1990. Uromodulin (Tamm-Horsfall glycoprotein/uromucoid) is a phosphatidylinositol-linked membrane protein. *J. Biol. Chem.* 265:20784–20789.

Seino, H., N. Hisamoto, S. Uzawa, T. Sekiguchi, and T. Nishimoto. 1992. DNA-binding domain of RCC1 protein is not essential for coupling mitosis with DNA replication. *J. Cell Sci.* 102:393–400.

Shi, Y., and J. O. Thomas. 1992. The transport of proteins into the nucleus requires the 70-kilodalton heat shock protein or its cytosolic cognate. *Mol. Cell. Biol.* 12:2186–2192.

Smythe, C., and J. W. Newport. 1992. Coupling of mitosis to the completion of S phase in Xenopus occurs via modulation of the tyrosine kinase that phosphorylates p34cdc2. *Cell.* 68:787–797.

Uchida, S., T. Sekiguchi, H. Nishitani, K. Miyachi, M. Ohtsubo, and T. Nishimoto. 1990. Premature chromosome condensation is induced by a point mutation in the hamster RCC1 gene. *Mol. Cell. Biol.* 10:577–584.

Walton, P. A., S. J. Gould, J. R. Feramisco, and S. Subramani. 1992. Transport of microinjected proteins into peroxisomes of mammalian cells: inability of Zellweger cell lines to import proteins with the SKL tripeptide peroxisomal targeting signal. *Mol. Cell. Biol.* 12:531–541.