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Biochemistry

Mitotic regulator protein RCC1 is complexed with a nuclear *ras*-related polypeptide

(Ran protein/amino acid sequence/GDP/GTP-binding protein/maturation-promoting factor)

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ABSTRACT **We previously reported the purification of a complex of two proteins from human chromatin, consisting of a 47-kDa component called RCC1, which is a negative regulator of mitosis, and a 25-kDa protein. Here we show that the 25-kDa protein has a *ras*-related sequence. It binds guanine nucleotides, and excess Mg^{2+} and GDP or GTP dissociate the complex. Immunofluorescence studies and biochemical properties indicate that this polypeptide, in contrast to most members of the Ras family, is present in the nucleoplasm as a soluble monomer, in 25-fold excess over the complexed form. We designate this polypeptide Ran, for *ras*-related nuclear protein.**

RCC1 has been identified in a baby hamster kidney cell line BHK-21 as a gene whose temperature-sensitive mutation tsBN2 induces mitotic events independent of completed DNA replication. In cells synchronized at the G_1/S boundary, shifting to the nonpermissive temperature results in loss of RCC1 protein. As a consequence, $p34^{cdc2}$ kinase is activated, causing a mitotic phenotype, with chromosome condensation, breakdown of the nuclear envelope, and formation of the mitotic spindle (1, 2). $p34^{cdc2}$ is the catalytic subunit of the maturation-promoting factor (MPF), a major regulator of mitosis. Its activity is regulated by phosphorylation and dephosphorylation and by subunit rearrangement (see refs. 3 and 4 for review). Induction of mitosis following RCC1 inactivation requires protein synthesis but occurs in the presence of inhibitors of transcription (5). Thus, the protein encoded by *RCC1* acts upstream of MPF/cdc2, presumably by controlling the expression of a mitotic inducer by an unknown regulatory mechanism.

A human genomic DNA fragment and a cDNA that complement the tsBN2 mutation in BHK21 cells (6, 7) encode a protein of 45 kDa that is located in the nucleus and is bound to chromatin (8). In a previous communication (9), we reported on the isolation of a protein from human chromatin that corresponds to RCC1 in all 205 positions of the amino acid sequence so far elucidated, in molecular mass, and in DNA binding properties. It was isolated as a complex with a polypeptide of 25 kDa (9). Here we present evidence that this 25-kDa component is a *ras*-related nuclear protein.

MATERIALS AND METHODS

Rabbit Antiserum to the Complex and Affinity Purification of Antibodies. A rabbit was immunized by injecting 75 μg of purified native 68-kDa complex subcutaneously, in Freund's complete adjuvant, and given a booster injection 4 weeks later with the same amount of antigen in Freund's incomplete adjuvant. The animal was bled 6 weeks after the first immunization. Preimmune serum was taken and tested with the complex. Antibodies affinity-purified on polypeptide bands immobilized on nitrocellulose were used as described (9).

Indirect Immunofluorescence. HeLa cells were grown in monolayer cultures, fixed with 3.7% paraformaldehyde in phosphate-buffered saline at 37°C, permeabilized in methanol/acetone, and stained as previously described (9) with fluorescein-conjugated second antibody to rabbit immunoglobulin (Amersham). DNA was stained by adding 50 ng of Hoechst 33342 to 1 ml of washing buffer. To show that the 25-kDa component was a soluble nucleoplasmic protein, cells were extracted with 0.5% Nonidet P-40 in 10 mM sodium phosphate (pH 7.2) at 37°C prior to fixation with 3.7% paraformaldehyde in the same buffer, followed by methanol at −20°C for 5 min.

Purification of the Monomeric 25-kDa Component. Growth of HeLa cells, synchronization in mitosis, lysis in the presence of protease inhibitors, immunoblotting, and isolation of the complex were performed as described (9). For isolation of the monomeric form, lysate from 50 ml of synchronized mitotic (and in control experiments, unsynchronized) HeLa cells was centrifuged at 70.000 × *g* for 60 min, and the supernatant was made 35–55% saturated with ammonium sulfate. The precipitate was dissolved in 100 mM sodium phosphate (pH 7.0) containing 1 mM dithiothreitol and was incubated with 2 mM GDP for 30 min at 20°C to obtain a uniform nucleotide–protein complex. It was stabilized by 5 mM $MgCl_2$ and gel-filtered on a Sephacryl S-200 column (2.6 × 100 cm) at 4°C in the same buffer with a flow rate of 1 ml/min. The fraction containing the 25-kDa antigen was finally purified on a hydroxylapatite column (Merck; 10 × 150 mm, 10-μm particle size) with a linear gradient of 100–600 mM sodium phosphate (pH 7.0) containing 1 mM dithiothreitol and 1 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS; Boehringer Mannheim), at a flow rate of 2 ml/min.

Quantitation of Protein and of Immunostain. After electrophoresis, comparative gels were stained with Coomassie blue R250 and scanned in an LKB Ultroscan XL enhanced laser densitometer, with bovine serum albumin as a control. Immunoblots were stained with rabbit antiserum to the RCC1·Ran complex and the intensity of the stain was determined densitometrically.

Amino Acid Sequence Determination. The purified 25-kDa protein was reduced in 6 M guanidinium chloride/1 M Tris·HCl, pH 8.6/120 mM 2-mercaptoethanol/2.7 mM EDTA for 16 hr at room temperature. Iodoacetate (1.2 M in water) was added to a final concentration of 120 mM and alkylation was terminated after 15 min by addition of 2 μl of 2-mercaptoethanol. Protein was precipitated with methanol/chloroform (4:1, vol/vol) and cleaved with endoproteinase

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Abbreviation: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate.

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Asp-N or trypsin (Boehringer Mannheim), in 100 mM ammonium bicarbonate, pH 7.5/2 M deionized urea for 14 hr at 37°C and with CNBr in 70% formic acid in the dark for 24 hr at 20°C. The digest was lyophilized and fractionated on a reversed-phase column (ODP50, Asahipak, 4.6 × 150 mm) using a gradient of acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 1 ml/min. Peptides were sequenced on an Applied Biosystems 470A gas-phase sequencer and the resulting phenylthiohydantoins were identified and quantitated on-line.

Nucleotide Binding Assay. To determine the incorporation of nucleotides, free Ran protein or bovine serum albumin was incubated in 200 μl of 200 mM phosphate, pH 7.0/1 mM dithiothreitol/1 mM CHAPS/2.5 μM $MgCl_2$ with 350 pmol of [^{3}H]GDP (10 Ci/mmol; 1 Ci = 37 GBq) or [^{3}H]GTP (7 Ci/mmol) for 15 min at 20°C. RCC1–Ran complex was incubated with 350 pmol of [^{3}H]GDP in 100 μl of 20 mM Tris·HCl, pH 7.5/100 mM NaCl/5 mM $MgCl_2$/1 mM dithiothreitol/0.2 mM CHAPS. To determine the amount of ^{3}H-labeled nucleotide bound to protein, the samples were vacuum-filtered through nitrocellulose (Schleicher & Schuell; BA85, 0.45 μm). After washing with Tris buffer containing 5 mM $MgCl_2$, the protein-bound radioactivity retained by the filter was measured in a Packard 1500 Tri-Carb liquid scintillation analyzer.

After the final purification on hydroxylapatite the Ran protein was largely nucleotide-free. To obtain Ran·GDP complex, 5 ml of the hydroxylapatite fraction containing Ran was brought to 0.5 mM GDP and to 20 mM $MgCl_2$. To remove excess GDP and phosphate buffer, the fraction was gel-filtered on Sephacryl S-200 in 20 mM Tris·HCl, pH 7.5/100 mM NaCl/1 mM dithiothreitol/0.2 mM CHAPS. For quantitating the release of radioactive GDP, aliquots of 1 ml containing 24 pmol of Ran·GDP or 40 pmol of Ha-Ras p21·GDP were incubated with 200 pmol of [^{3}H]GDP (10 Ci/mmol) for 30 min at 30°C. To aliquots of 250 μl, 5 μl of $MgCl_2$ or EDTA was added to obtain final concentrations of 5 mM $MgCl_2$, 0.5 μM $MgCl_2$, or 1 mM EDTA. The exchange reaction was performed in the presence of 50 nmol of unlabeled GDP or of a mixture of 50 nmol each of ATP, CTP, and UTP. The protein-bound radioactivity was determined in 40-μl aliquots.

RESULTS

The 25-kDa Ran Protein Is Present as Soluble Monomer in the Nucleoplasm in 25-fold Excess over the Complexed Form in Chromatin. We previously isolated a noncovalent complex of two proteins of 47 and 25 kDa from HeLa chromatin (9). The larger protein is recognized by autoimmune sera staining centromeres. It shares with RCC1 identical amino acid sequences in all 205 positions identified, molecular weight, and DNA-binding properties. In addition, it binds to an anti-RCC1 antibody kindly provided by T. Nishimoto. The 25-kDa component of the complex was not recognized by human autoimmune sera. To investigate the smaller polypeptide, an antiserum to the complex was raised in a rabbit. This serum strongly stained the RCC1 band on immunoblots but had a low titer for the 25-kDa protein (Fig. 1). Surprisingly, a large amount of the 25-kDa protein was detected in the soluble fraction of HeLa proteins. Whole rabbit antiserum reacted with three additional proteins of 43, 28, and 21 kDa, which were not further characterized, and with a faint band at 180 kDa, which by binding of a specific antibody (a gift from R. Knippers, University of Konstanz) was identified as DNA topoisomerase II. In contrast to the human autoimmune serum initially used, rabbit antiserum raised to the complex did not stain centromeres. Antibody that was affinity-purified on blots of the RCC1 protein crossreacted with all proteins identified with whole serum, except the 25-kDa protein.

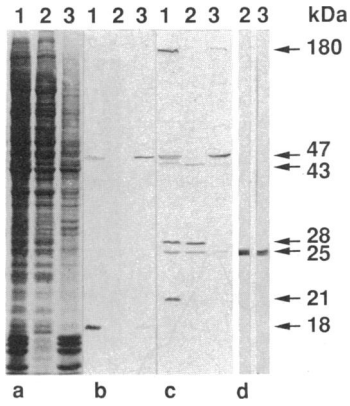

FIG. 1. Western blots of HeLa protein fractions. Blots of a 12% polyacrylamide gel were stained with Ponceau S (*a*), with human anti-centromere serum (*b*), with rabbit antiserum to the 68-kDa complex (*c*), or with affinity-purified anti-Ran rabbit antibody. Lanes: 1, total lysate; 2, fraction soluble in 10 mM Tris·HCl, pH 7.5/1 mM dithiothreitol/0.2 mM EDTA; 3, insoluble fraction.

Therefore, unambiguous localization of the 47-kDa component was not possible. Antibody eluted from the 25-kDa antigen was monospecific for that polypeptide. In interphase, immunofluorescence with this antibody was confined to the nuclei (Fig. 2), but when nuclei were prepared, a considerable amount of the 25-kDa protein appeared in immunoblots of the cytoplasmic fraction. Gel filtration of an aliquot of the soluble HeLa fraction on Superose 12 showed this protein to be almost exclusively in the monomeric state, which may enable it to readily penetrate the nuclear membrane. In mitosis, the staining was dispersed throughout the cell.

Initial attempts to fractionate the native 25-kDa component by ion-exchange and hydroxylapatite chromatography revealed three forms with widely differing elution positions, which, however, were coeluted in 8 M urea. This suggested the presence of ligands. Reversed-phase chromatography (data not shown) identified the presence of GDP and GTP. For purification, the protein was precipitated from the soluble fraction of HeLa lysate by ammonium sulfate, dissolved in 100 mM sodium phosphate (pH 7.0), incubated with GDP

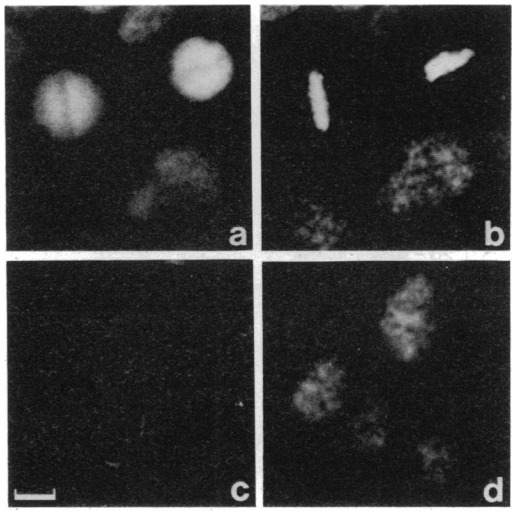

FIG. 2. DNA and anti-Ran immunofluorescence staining of HeLa cells fixed with paraformaldehyde (*Upper*) or fixed after extraction with 0.5% Nonidet P-40 in 10 mM phosphate (*Lower*). (*a* and *c*) Immunostaining with affinity-purified rabbit anti-Ran antibody and fluorescein-conjugated anti-rabbit immunoglobulin serum. (*b* and *d*) The same view fields, showing DNA stained with Hoechst 33342. (Bar = 10 μm.)

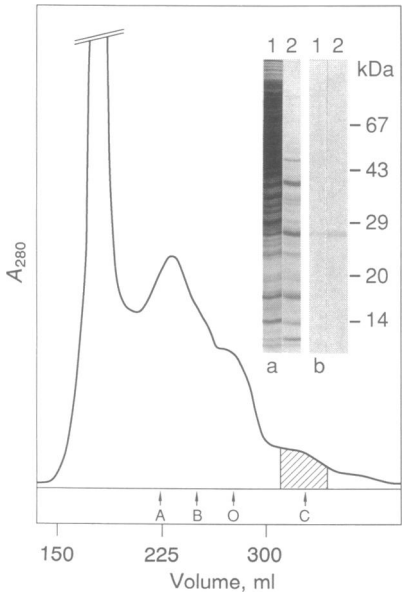

FIG. 3. Gel filtration of soluble 25-kDa Ran protein on Sephacryl S-200 in 100 mM phosphate at pH 7.0. (*Inset*) SDS/PAGE profile of proteins detected by Coomassie blue staining (*a*) or immunostaining with rabbit anti-complex antiserum (*b*). Lanes: 1, total sample applied to the column; 2, hatched antigen fraction. The markers indicated at the bottom were aldolase (A, 158 kDa), bovine serum albumin (B, 67 kDa), ovalbumin (O, 43 kDa), and chymotrypsinogen A, (C, 25 kDa).

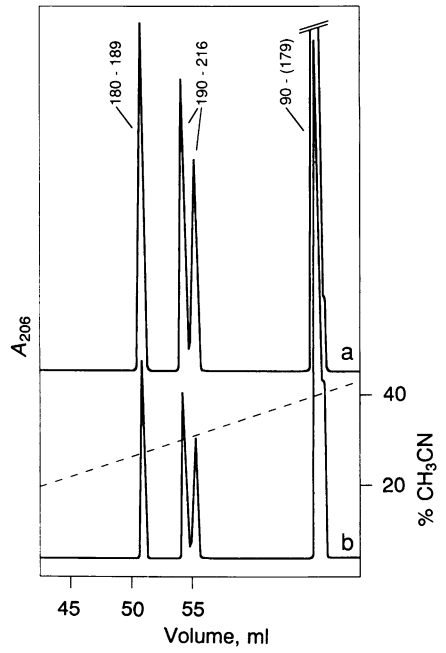

FIG. 5. CNBr peptide maps of the free (profile a) and complexed (profile b) form of Ran, shown by reversed-phase chromatography on a C_{18} column in 0.1% trifluoroacetic acid with a linear gradient of acetonitrile. Numbers next to the peaks indicate positions of the respective fragments in the sequence of the Ras-related protein.

to obtain uniform nucleotide binding, and gel-filtered on Sephacryl S-200 (Fig. 3). Purification to homogeneity was achieved on hydroxylapatite (Fig. 4).

Identity of the soluble monomeric form with the complexed 25-kDa protein from chromatin is indicated by immunological crossreaction, apparent molecular mass in gel electrophoresis, the elution pattern of CNBr fragments from a reversed-phase column (Fig. 5), the amino acid sequences (see below), and the isoelectric points (Fig. 6). Its amount per cell was calculated, from quantitative immunoblots and from gels stained with Coomassie blue, to be 0.36% of the total cellular protein or 10^7 copies per cell. This corresponds to a 25-fold excess over the complexed form (Table 1).

The 25-kDa Polypeptide Has a *ras*-Related Sequence. The structural basis for nucleotide binding became evident when we could identify 125 residues of the 25-kDa protein by sequencing peptides derived from cleavage with Asp-N protease, trypsin, or CNBr (Fig. 5). A search of data banks (EMBL, Genbank; November 27, 1990, and May 31, 1991) with these sequences revealed homology to the reading frame of a cDNA designated TC4 (Fig. 7; ref. 10). This *ras*-related sequence had been found by screening a human teratocarcinoma cDNA library with oligonucleotides coding for the Ras consensus sequence residues 57–62. There is identity with all residues identified in Ran up to Pro-180. Starting at the carboxyl end of that residue, however, all residues of the C-terminal region in Ran differ from the published reading frame. This difference can be accounted for by inserting a guanine in the first position of codon 181 in the published TC4

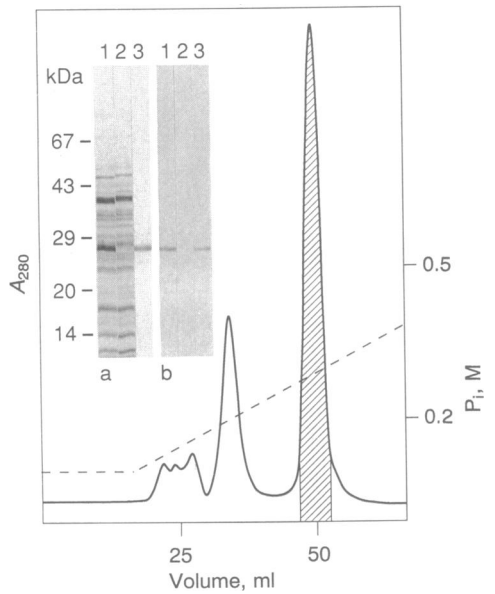

FIG. 4. Purification of Ran on hydroxylapatite. (*Inset*) SDS/PAGE profile of proteins detected by Coomassie staining (*a*) or immunostaining with rabbit anti-complex antiserum (*b*). Lanes: 1, total sample applied to the column; 2, flowthrough; 3, hatched antigen fraction.

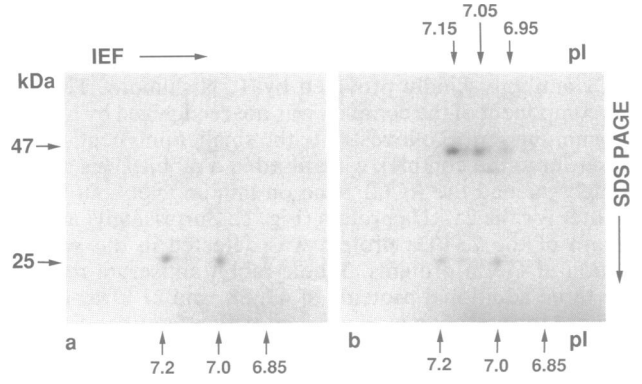

FIG. 6. Two-dimensional gel electrophoresis of the 25-kDa Ran protein nucleoplasmic monomer (*a*) or complexed with 47-kDa RCC1 (*b*). pI values were calibrated using Carbamylytes black (glyceraldehyde-3-phosphate dehydrogenase from Pharmacia). IEF, isoelectric focusing.

Table 1. Enrichment of Ran protein

Fraction	Total protein, mg	Ran, mg	Yield, %	Enrichment factor
Free Ran				
Complete lysate	2520	9.0	100	
Supernatant	1018	6.5	72	1.8
$(NH_4)_2SO_4$ precipitate	513	4.8	53	2.6
Sephacryl S-200	9.4	1.81	20	54
Hydroxylapatite		1.04	11.6	280
Ran complexed with RCC1				
Complete lysate	2520	0.37	100	
Purified RCC1·Ran	0.175	0.06	16.5	2377

Values refer to 50 ml of packed HeLa cells ($\approx 2 \times 10^{10}$ cells) as starting material. Ran was quantitated by staining of gels with Coomassie blue and of immunoblots with rabbit antiserum to the RCC1·Ran complex. The results for free Ran refer to the free protein as reported in this communication. For comparison, RCC1·Ran complex was isolated as described in ref. 9.

nucleotide sequence. As a consequence, codon 201 codes for leucine instead of termination and the sequence extends beyond the published reading frame to residue 216.

We did not find any of the common signal sequences for posttranslational modification at the C terminus that is required for attachment to membranes. This agrees with the intranuclear location, but we have no proof that we have indeed established the C-terminal residue of the protein. The intact protein was blocked at the N terminus; thus a different posttranslational modification is to be expected at this site. In addition, we found two main spots, with isoelectric points of 7.2 and 7.0, upon two-dimensional gel electrophoresis under denaturating conditions. The position of these spots was the same for the complexed and the monomeric form and did not change upon treatment with alkaline phosphatase. Two different peaks on reversed-phase chromatography (Fig. 5) for the C-terminal CNBr fragment correspond to the two isoelectric points; therefore the difference is likely to reside in residues 190–216.

Guanine Nucleotide Binding Properties of Free Ran and RCC1·Ran Complex. Free Ran was used as a nucleotide-free

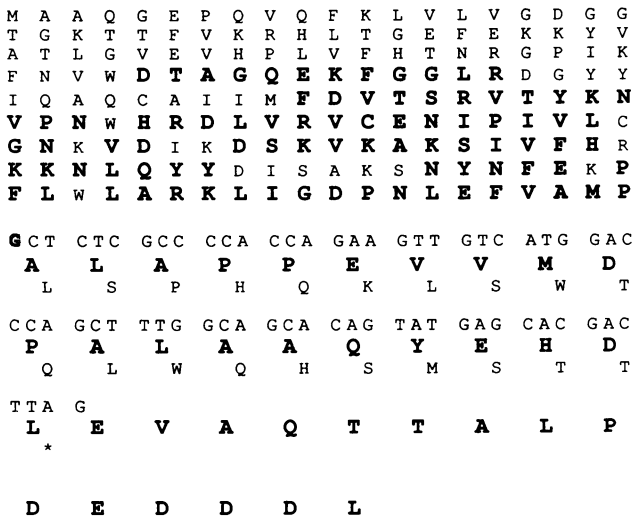

FIG. 7. (*Upper*) Amino acid sequence of the translated reading frame of TC4 (ref. 10, lightface letters) and sequences derived from peptides of Ran (boldface). Single lightface letters within boldface sequences indicate positions not identified by protein sequencing. (*Lower*) Insertion of a single G in the first position of codon 181 may account for the altered sequence in the C-terminal region of the protein.

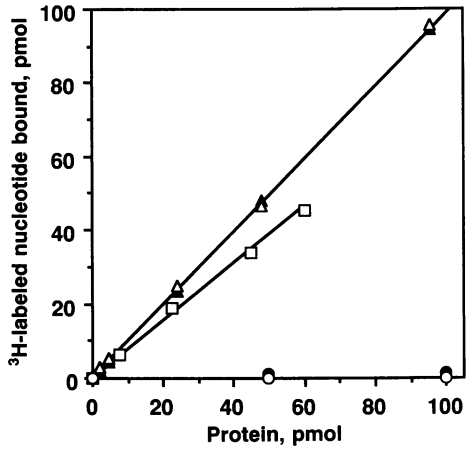

FIG. 8. Nucleotide binding to free and complexed Ran. Free Ran and bovine serum albumin (control) were incubated with 350 pmol of [^{3}H]GDP or [^{3}H]GTP in the presence of 2.5 μM $MgCl_2$. RCC1·Ran complex was incubated in buffer containing 5 mM $MgCl_2$. △, GDP bound to free Ran; ○, GDP bound to albumin; ▲, GTP bound to free Ran; ●, GTP bound to albumin; □, GDP bound to Ran complexed to RCC1.

protein obtained from the final hydroxylapatite purification. Independent of the concentration of Mg^{2+}, this form was saturated with nucleotide in <1 min and bound equimolar amounts of either GDP or GTP (Fig. 8). In an experiment with [γ-^{32}P]GTP (data not shown), nucleotide hydrolysis upon binding was found to be very low. Very similar nucleotide binding was obtained with the RCC1·Ran complex, which, however, requires the presence of >1 mM Mg^{2+}.

The exchange of bound nucleotide on Ran depended on the concentration of Mg^{2+} in a manner similar to that observed for the *ras* gene product p21 (Fig. 9). In the presence of 5 mM Mg^{2+}, nucleotides were very slowly exchanged on both proteins, with a half-life of about 3 hr. Reduction of the Mg^{2+} concentration to 0.5 μM promoted exchange, but even in the absence of Mg^{2+} nucleotides were exchanged on Ran with a rate similar to that on Ras p21 in the presence of 0.5 μM Mg^{2+}, indicating a higher affinity for Ran. The RCC1·Ran complex was stable in 3 M guanidinium chloride in the absence of nucleotide (9) and in the presence of 2 mM Mg^{2+}

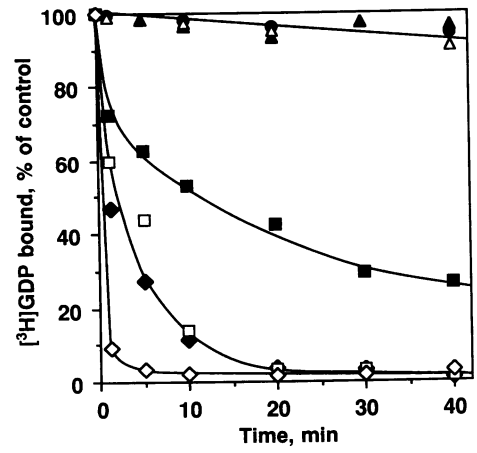

FIG. 9. Effect of Mg^{2+} on nucleotide exchange on free Ran p25 and Ras p21 (11). For measuring the release of radioactive GDP, Ran·[^{3}H]GDP (filled symbols) or Ha-Ras·[^{3}H]GDP (open symbols) were diluted to 5 mM (▲, △) or 0.5 μM (■, ●, □) $MgCl_2$, or to 1 mM EDTA (◆, ◇). The exchange reaction was performed in the presence of 50 nmol of unlabeled GDP (■, ▲, ◆, □, △, ◇) or with a mixture of 50 nmol each of ATP, CTP, and UTP (●). Protein-bound radioactivity was determined in 40-μl aliquots.

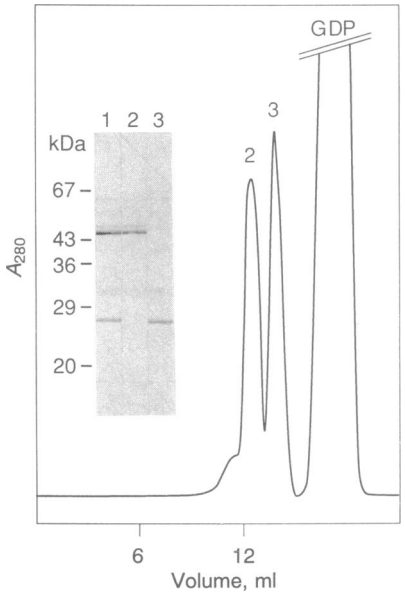

FIG. 10. Dissociation of the RCC1·Ran complex by GDP. Sixty-six picomoles of the complex in 100 μl of 10 mM Tris·HCl, pH 7.5/300 mM NaCl/1 mM dithiothreitol/1 mM CHAPS/2 mM $MgCl_2$ with 10 μg of aprotinin as carrier was incubated with 1 mM GDP for 30 min at 20°C and chromatographed in the same buffer containing 100 μM GDP on Superose 12 (Pharmacia, 10 × 300 mm, flow rate of 0.2 ml/min). (*Inset*) Gel stained with Coomassie blue R250. Lanes: 1, sample applied; 2, RCC1; 3, Ran. Peak numbers in the chromatogram correspond to the lane numbers.

alone, but it completely dissociated in the presence of excess GDP or GTP plus Mg^{2+} (Fig. 10). Using radioactive nucleotide for dissociation and gel filtration of the mixture in the absence of nucleotides in the buffer, we found reassociation of RCC1·Ran, and radioactivity was found only in the remaining 10% of free Ran.

The RCC1·Ran complex binds to DNA-cellulose (8, 9), whereas under identical conditions monomeric Ran is not bound (data not shown). Thus, DNA binding appears to be mediated by RCC1.

DISCUSSION

Ras and Ras-related proteins act through a cycle of GDP/GTP exchange and GTP hydrolysis that is coupled to a signal-transduction pathway by interaction with other proteins (see ref. 12 for review). In their inactive form, they are complexed with GDP. Activation results from the exchange of GDP for GTP, which is induced by nucleotide-exchange factors and causes a conformational change. Interaction of active Ras p21 with an as yet unidentified effector is believed to generate a mitogenic signal. GTP hydrolysis then returns the protein to the inactive state. This inactivation is accelerated by the interaction of Ras p21 with a GTPase-activating protein, GAP. In oncogenic Ras mutants GTP hydrolysis is impaired and the protein remains in a permanently active state.

Our data demonstrate that the 25-kDa component tightly complexed to RCC1 is a Ras-related protein. The sequence is closely homologous to the translated reading frame of TC4, a cDNA identified in teratocarcinoma cells (10). Characteristically it binds guanine nucleotides and thereby the complex with RCC1 dissociates. The Mg^{2+} dependence of nucleotide binding also resembles that of Ras p21. When dissociated RCC1·Ran complex is gel-filtered in the absence of nucleotide, most of the protein reassociates and nucleotide is bound only to free Ran. The RCC1·Ran complex is devoid of nucleotide. In addition to the complexed form, free Ran exists in considerable excess as a soluble protein. Immunofluorescence results imply that it is confined to the nucleus, whereas immunoblots of cell fractions indicate Ran in the cytoplasmic fraction as well.

Both the complexed and the free form of Ran display the same two isoelectric points of 7.2 and 7.0. Two CNBr-derived fragments, separated by reversed-phase chromatography and comprising the C-terminal 27 residues, suggest that a difference, presumably a posttranslational modification, resides in that region. All other Ras-related proteins so far identified have a sequence motif for posttranslational modification involving a cysteine close to their C terminus. We have not found such a motif in Ran. This may explain why it is a readily soluble nuclear polypeptide.

In tsBN2 cells arrested in S phase by inhibitors of DNA synthesis, loss of the mutated RCC1 gene product results in premature activation of the $p34^{cdc2}$ kinase and consequently induction of mitotic events depending on protein synthesis (1). This indicates that active RCC1 is required to prevent induction of mitosis before DNA replication is complete. We have found that RCC1 catalyzes the exchange of guanine nucleotides on Ran (13). By analogy to the Ras system, binding of GTP should activate the Ran protein. Thus, in S phase, RCC1 might be responsible for maintaining a high intracellular concentration of Ran·GTP, which is necessary to prevent the synthesis of a mitotic inducer. Upon completion of DNA replication the pool of activated Ran protein could be depleted by the inactivation of RCC1 and/or by modulating the activity of a Ran-specific GTPase-activating protein.

Note Added in Proof. In a recent publication (14) genes corresponding to mammalian RCC1 and TC4 have been characterized in *Schizosaccharomyces pombe* as being involved in the initiation of mitosis. They are designated *pim*1 and *spi*1, respectively. The authors also provided a corrected amino acid sequence for TC4, which differs only in positions 206 and 207 (Asn-Pro) from that given for Ran in Fig. 7 (Thr-Thr).

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