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The Balance of RanBP1 and RCC1 Is Critical for Nuclear Assembly and Nuclear Transport

Robert T. Pu and Mary Dasso*

Laboratory of Molecular Embryology, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland 20892-5431

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Ran is a small GTPase that is essential for nuclear transport, mRNA processing, maintenance of structural integrity of nuclei, and cell cycle control. RanBP1 is a highly conserved Ran guanine nucleotide dissociation inhibitor. We sought to use *Xenopus* egg extracts for the development of an in vitro assay for RanBP1 activity in nuclear assembly, protein import, and DNA replication. Surprisingly, when we used anti-RanBP1 antibodies to immunodeplete RanBP1 from *Xenopus* egg extracts, we found that the extracts were also depleted of RCC1, Ran's guanine nucleotide exchange factor, suggesting that these proteins form a stable complex. In contrast to previous observations using extracts that had been depleted of RCC1 only, extracts lacking both RanBP1 and RCC1 (codepleted extracts) did not exhibit defects in assays of nuclear assembly, nuclear transport, or DNA replication. Addition of either recombinant RanBP1 or RCC1 to codepleted extracts to restore only one of the depleted proteins caused abnormal nuclear assembly and inhibited nuclear transport and DNA replication in a manner that could be rescued by further addition of RCC1 or RanBP1, respectively. Exogenous mutant Ran proteins could partially rescue nuclear function in extracts without RanBP1 or without RCC1, in a manner that was correlated with their nucleotide binding state. These results suggest that little RanBP1 or RCC1 is required for nuclear assembly, nuclear import, or DNA replication in the absence of the other protein. The results further suggest that the balance of GTP- and GDP-Ran is critical for proper nuclear assembly and function in vitro.

INTRODUCTION

Ran is a small GTPase that is essential for nuclear transport, mRNA processing, maintenance of structural integrity of nuclei, and cell cycle control (reviewed by Rush *et al.*, 1996; Sazer, 1996). The most characterized role of Ran is in nuclear protein import, and multiple lines of evidence suggest that Ran is required to sustain both active protein import (Melchior *et al.*, 1993; Moore and Blobel, 1993; Melchior *et al.*, 1995; Schlenstedt *et al.*, 1995a) and export (Moroianu and Blobel, 1995; Schlenstedt *et al.*, 1995a).

Like Ran, RanBP1 is ubiquitously expressed and highly conserved across species. RanBP1 is a guanine nucleotide dissociation inhibitor for GTP-Ran (Bischoff *et al.*, 1995b). RanBP1 acts a cofactor for RanGAP1, a Ran GTPase-activating protein, increasing Ran's in vitro rate of GAP-mediated hydrolysis by an order of magnitude (Bischoff *et al.*, 1995a). RanBP1 is encoded by an essential gene in yeast (Ouspenski *et al.*, 1995). Strains of *Saccharomyces cerevisiae* carrying temperature-sensitive alleles of the yeast RanBP1 homologue CST20/YRB1 show nuclear transport defects at the restrictive temperature (Schlenstedt *et al.*, 1995b). Yrb1p overproduction results in cell cycle defects: Overproducing strains undergo G_1 phase arrest and begin the inappropriate expression of mRNAs for proteins involved in mating (Hayashi *et al.*, 1995). This phenotype is strikingly similar to that of a mutant in the *S. cerevisiae* homologue of RCC1, srm1 (Clark and Sprague, 1989). RCC1 is the guanine nucleotide ex-

* Corresponding author: Laboratory of Molecular Embryology, National Institute of Child Health and Human Development, National Institutes of Health, Building 18, Room 106, Bethesda, MD 20892-5431.

change factor (GEF) for Ran (Bischoff and Ponstingl, 1991a). Yrb1p overproduction also results in increased sensitivity to the DNA replication inhibitor hydroxyurea and elevated mitotic recombination (Ouspenski *et al.*, 1995), consistent with overproduction affecting some aspect of DNA metabolism. Finally, Yrb1p overproduction causes increased sensitivity to the microtubule-depolymerizing drug benomyl and increased rates of mitotic chromosome nondisjunction, possibly indicating a requirement for RanBP1 in mitotic regulation (Ouspenski *et al.*, 1995).

Bischoff *et al.* (1995b) have analyzed the interactions of RanBP1, Ran, and RCC1 by using purified proteins. They found that RanBP1 has a high affinity for GTP-bound Ran and a low affinity for GDP-bound Ran. RanBP1 does not interact strongly with RCC1 in the absence of Ran. However, when Ran is in a nucleotide-free state RanBP1 forms a stable heterotrimeric complex with RCC1 and Ran. This complex rapidly dissociates with the addition of magnesium and GTP but not GDP. The association between GTP-Ran and RanBP1 stabilizes the bound nucleotide and inhibits further RCC1-induced exchange. It is still uncertain what role these interactions play in vivo, because Ran and RCC1 are predominantly nuclear proteins (Ohtsubo *et al.*, 1989; Bischoff and Ponstingl, 1991b) and RanBP1 is localized to the cytosol of yeast (Schlenstedt *et al.*, 1995b), mammalian cells (Richards *et al.*, 1996), and amphibian cells (our unpublished results). On the other hand, recent experiments have indicated that RanBP1 has both a nuclear export sequence and a cytosolic retention sequence, raising the question of whether RanBP1 may shuttle between the cytosol and the nucleus (Richards *et al.*, 1996).

A number of experiments have also examined the associations among RanBP1, Ran, RanGAP1, importin α, and importin β in vitro. Importin α and importin β form a heterodimeric complex, and they target proteins containing nuclear localization signals to the nuclear pore during protein import (reviewed by Gorlich and Mattaj, 1996). GTP-Ran binds avidly to importin β, causing it to dissociate from importin α (Rexach and Blobel, 1995; Nehrbass and Blobel, 1996). The association of GTP-Ran with importin β strongly inhibits RanGAP1-mediated GTP-Ran hydrolysis (Floer and Blobel 1996; Lounsbury and Macara, 1997). Lounsbury and Macara (1997) have suggested RanBP1 relieves the inhibition of RanGAP1 by importin β and thereby allows the release of importin β from its tight association with GTP-Ran. However, other studies did not find that RanBP1 significantly restored RanGAP1 activity in the presence of importin β (Gorlich *et al.*, 1996). GDP-Ran binds poorly to either RanBP1 or importin β individually, but Chi *et al.* (1996) have reported the efficient formation of complexes containing GDP-Ran, importin β, and RanBP1. The association of importin β, GDP-Ran, and RanBP1 does not appear to require the dissociation of the importin α/β heterodimer (Chi *et al.*, 1997). It has not yet been clearly established how these interactions promote nuclear transport.

Xenopus extracts offer an excellent system for the study of the Ran GTPase pathway (Smythe and Newport, 1991). Nuclei assembled in *Xenopus* egg extracts are both morphologically normal and functional for DNA replication and nuclear transport. The formation of functional nuclei in egg extracts has previously allowed the examination of the roles of RCC1 and Ran in interphase nuclei (Dasso *et al.*, 1992; Dasso *et al.*, 1994). After the immunodepletion of RCC1, nuclear assembly is highly abnormal, nuclear import is inhibited, and DNA replication does not occur. Similar results are observed when a dominant negative Ran mutant (RanT24N) that blocks RCC1's GEF activity is added to extracts. Egg extracts can also mimic cell cycle transitions of the early embryo, allowing an examination of the effects of mutant Ran proteins on the regulation of mitosis (Kornbluth *et al.*, 1994; Clarke *et al.*, 1995). In cycling extracts, mutant Ran proteins block the activation of cyclin B/$p34^{cdc2}$ as a mitotic kinase in the absence of DNA, indicating that Ran regulates mitosis in a manner that does not depend on nuclear transport.

To examine RanBP1's role in reconstituted nuclei, we cloned a *Xenopus* RanBP1 homologue and used it to generate recombinant RanBP1 protein and anti-RanBP1 antibodies. We removed RanBP1 from egg extracts by serial depletion with affinity-purified anti-RanBP1 antibodies. Surprisingly, immunodepletion of RanBP1 resulted in codepletion of RCC1, suggesting that RanBP1 and RCC1 can form a stable complex in extracts. Nuclei formed in extracts lacking both proteins (codepleted extracts) did not exhibit defects in assays of assembly, DNA replication, or nuclear transport. Nuclei from codepleted extracts also entered mitosis normally in response to the addition of recombinant cyclin B protein. Addition of either recombinant RanBP1 or RCC1 to codepleted interphase extracts blocked nuclear assembly, nuclear transport, and DNA replication in a manner that could be rescued by further addition of RCC1 or RanBP1, respectively. Although the abnormal nuclei formed in extracts lacking either RanBP1 or RCC1 appeared to be morphologically similar, their defects could be distinguished by their response to exogenous mutant Ran proteins. Our results demonstrate that little, if any, RanBP1 or RCC1 are required for interphase nuclear functions in the absence of the other protein. However, the results also suggest that the balance of RCC1 and RanBP1 is normally critical for proper nuclear assembly and function.

MATERIALS AND METHODS

Buffers and Reagents

The 1× SDS sample buffer contains 80 mM Tris-HCl, pH 6.8, 350 mM 2-mercaptoethanol, 2% SDS, 0.1% bromophenol blue, and 10% glycerol. PBS contains 1.7 mM KH_2PO_4, 5 mM Na_2HPO_4, and 150 mM NaCl, pH 7.4. Wash buffer contains 50 mM Tris-HCl, pH 8.0, 80 mM NaCl, 10% glycerol, 2 mM $MgCl_2$, and 0.1% Triton X-100. XB contains 50 mM sucrose, 100 mM KCl, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, and 10 mM K-HEPES, pH 7.7. In all figures, proteins were analyzed on 4–20% gradient SDS-polyacrylamide gels (Novex, San Diego, CA). Protein concentrations were determined by using a protein assay kit from Bio-Rad Laboratories (Hercules, CA). Affinity-purified anti-*Xenopus* RCC1 antibodies were prepared as described in Dasso *et al.* (1992). Anti-B4 antibodies were prepared as described in Dimitrov *et al.* (1993). (B4 is a *Xenopus* early embryonic linker histone.) The anti-human RanBP1 antibodies were the kind gift of Dr. Mark Rush (NYU Medical Center, NY). The anti-Ran antibodies were as described in Kornbluth *et al.* (1994). The RanGAP1 antibodies were described in Saitoh *et al.* (1997). Anti-importin β is the kind gift of Dr. D. Gorlich (Universität Heidelberg, Germany). Other reagents were from Sigma (St. Louis, MO) unless otherwise stated.

cDNA Library Screening and Sequencing

Degenerate oligonucleotides corresponding to two well-conserved RanBP1 protein sequences (EWKERG and LKICAN) were designed. A reverse transcription-coupled PCR was performed with *Xenopus* oocyte RNA as a template under standard conditions with *Taq* polymerase (Perkin Elmer-Cetus, Foster City, CA). The amplified DNA fragment was subcloned into a pCR-I plasmid vector by using a TA cloning kit (Invitrogen, San Diego, CA). The fragment was radiolabeled by using a random primer labeling kit (Life Technologies-BRL, Gaithersburg, MD), and used to probe a Lambda-ZAP-based *Xenopus* oocyte cDNA library (the gift of D. Patterton and A. Wolffe, LME, NICHD, NIH). Primary and secondary screenings were performed by standard methods (Sambrook *et al.*, 1989), and 13 positive single plaques were purified. One clone (10–2-2) that contained the entire coding region of RanBP1 was sequenced on both strands using commercially available and custom synthesized primers. DNA sequence analysis and protein sequence alignment were performed by using the DNA Strider program and the GCG pileup program, respectively.

Construction of Plasmids for the Expression of RanBP1

The coding region of *Xenopus* RanBP1 in clone 10–2-2 was amplified by PCR using 5′-CCCAAGCTTCCATGGCCGATACCAAGGAT-3′ as the 5′ primer and a T7 promoter primer 5′-GTAATACGACTCACTATAGGGC-3′ as the 3′ primer. The PCR fragment was digested with *Nco*I/*Xho*I or *Hin*dIII/*Xho*I, and the resultant DNA fragments were subcloned into *Nco*I/*Xho*I- or *Hin*dIII/*Xho*I-cut pET28a plasmid vectors, respectively. The first plasmid allows the expression in *Escherichia coli* of RanBP1 from its own authentic initiation codon. The second plasmid construct gives a protein with a 43-amino acid N-terminal extension, including a 6-amino acid histidine tag. The plasmid constructs were sequenced to ensure that there was no change in the coding sequence due to mutations during PCR amplification. The plasmids were transformed into *E. coli* (strain BL21 DE3, pLysS), and RanBP1 expression was induced with 0.4 mM isopropyl β-D-thiogalactoside in cultures growing exponentially at 37°C (OD_{600} = 0.4–0.6). The incubations were continued for 4 h, and cells were harvested by centrifugation and kept at −80°C.

Production of Recombinant Proteins and Antibody Generation

For nontagged RanBP1 purification, frozen bacterial cells from 1 l of culture were thawed and resuspended in 20 ml of 50 mM Tris-HCl, pH 8.0, and 2 mM EDTA. Cells were then sonicated and centrifuged at 39,000 × *g* to separate soluble and insoluble fractions. Recombinant RanBP1 was found in the soluble fraction and such soluble bacterial extracts were used for the experiment shown in Figure 2B. Nontagged RanBP1 was further purified by 35–55% $(NH_4)_2SO_4$ precipitation, followed by separation on SDS-PAGE. The purified protein was excised from the gel and used as the immunogen for antibody production in two rabbits (Research Genetics, Huntsville, AL). His-tagged RanBP1 was purified on a Ni^{2+}-agarose column using a protein purification kit according to the manufacturer's instructions (Novagen, Madison, WI). After elution from the Ni^{2+}-agarose column, the protein was dialyzed into XB buffer. The protein was frozen and stored −80°C. Purified His-tagged RanBP1 were coupled to CNBr-activated Sepharose according to the manufacturer's suggestions (Pharmacia, New Brunswick, NJ), and used as a matrix for antibodies purification (Harlow and Lane, 1988). The glutathione *S*-transferase (GST)-Ran constructs used were as described (Dasso *et al.*, 1994). The GST moiety alone was expressed from a pGEX-KG vector without insert (Guan and Dixon, 1991). The GST-Ran and GST proteins were produced as described previously (Saitoh *et al.*, 1996). The bacterially expressed Ran proteins in Figure 7 were prepared as described in Dasso *et al.* (1994).

Ran and RanBP1 Interaction Assay

Approximately 100 μg of GST-Ran or GST and either 10 μl of soluble extracts of bacteria expressing recombinant RanBP1 protein or 35 μl of *Xenopus* egg extracts were incubated on ice for 1 h in 1 ml of wash buffer in the presence of 200 μM GTP. Glutathione-Sepharose beads (25–30 μl) were added, and the incubation was continued for 30 min with gentle rocking at 4°C. The beads were collected by centrifugation and washed three times with 1 ml of wash buffer. After the final wash, the beads were suspended in 30 μl of wash buffer and 50 μl of 2× SDS sample buffer were added. One-tenth of the sample was analyzed by SDS-PAGE followed by Western blotting and visualization using a LumiGLO chemiluminescence kit (Kirkegaard & Perry Laboratories, Gaithersburg, MD). For the experiment shown in Figure 2B, a rabbit anti-human RanBP1 antibody was used as the primary antibody at a dilution of 1:500 followed by a 1:30,000 dilution anti-rabbit secondary antibody conjugated to horseradish peroxidase.

Immunodepletion of RanBP1

Fractionated *Xenopus* egg extracts were prepared as described in Smythe and Newport (1991). The depletion of RanBP1 was carried out as follows: 200 μl of protein A-Sepharose beads (Pharmacia) were blocked by two 15-min incubations with 3.5 volumes of 10 mg/ml bovine serum albumin (BSA) in PBS. The beads were washed three times in PBS and incubated with 1.2 mg of affinity-purified anti-RanBP1 antibodies. The mixture was incubated for 1 h at room temperature with rotation and washed three times with XB. Beads for mock depletion were produced by coupling a similar amount of IgG from preimmune sera to beads instead of affinity-purified antibodies. To deplete cytosol, 4 volumes of cytosol and 1 volume of beads were incubated at 4°C for 1.5 h with rotation. The beads were pelleted by centrifugation, and the cytosol was removed and reincubated for an additional 1.5 h with 1 volume of fresh beads. After depletion, extracts were aliquoted and kept at −80°C until use.

Nuclear Assembly, DNA Replication, Histone H1 Kinase, and Nuclear Protein Import Assays

Assays for nuclear assembly, DNA replication, and histone H1 kinase activity were performed according to Smythe and Newport

(1991). In all cases, nuclear assembly reactions contained approximately 1000 sperm nuclei per μl of reaction mixture. Induction of mitosis was carried out by addition of nondegradable cyclin B (final concentration, 10 μg/ml) to assembled nuclei, and chromosome condensation and nuclear envelope breakdown (NEBD) were monitored. Nuclear protein import was assayed as described in Dasso *et al.* (1994).

GTP-Ran Overlay Assay

Ran overlay assays were performed according to the method of Lounsbury *et al.* (1994): Extracts were subjected to SDS-PAGE and proteins were transferred to a polyvinylidene difluoride (PVDF) filter in 20 mM phosphate buffer. The filter was then blocked by 4% BSA in PBST (PBS plus 0.2% Tween 20, 2.5 mM $MgCl_2$, and 1 mM dithiothreitol) for 1.5 h followed by three washes in PBST. The filter was incubated at room temperature for 20 min with 100 mg of GST-Ran preloaded with 50 μCi of [α-^{32}P]GTP. The filter was washed at least three times with PBST and autoradiographed.

RESULTS

Cloning and Expression of Xenopus laevis RanBP1 and Production of Anti-RanBP1 Antibodies

As a first step toward examining RanBP1's function in *Xenopus* egg extracts, we cloned the *Xenopus* homologue of RanBP1 (see MATERIALS AND METHODS). The clone encoded a peptide with a single open reading frame of 209 amino acids (Figure 1A). We believe that the ATG indicated in Figure 1A is the authentic initiation codon for this cDNA because of upstream termination codons in the same reading frame. Alignment of the encoded peptide with human, murine, and *S.cerevisiae* RanBP1 protein sequences reveals a high level of homology between species (80% identity to human and mouse; Figure 1B), and we will thus refer to the protein as *Xenopus* RanBP1. Two constructs were made by subcloning the full coding region of the cDNA into a pET28a plasmid vector, allowing the expression of RanBP1 protein with or without a histidine tag at its N terminus. Recombinant RanBP1 without the histidine tag was expressed in *E. coli*, purified, and used to produce affinity-purified polyclonal rabbit anti-RanBP1 antibodies. The anti-RanBP1 antibodies recognized a single band with an apparent molecular weight of 34 kDa on Western blots that was found almost exclusively in the soluble (cytoplasmic) fraction from *Xenopus* high-speed egg extracts (Figure 2A). RanBP1 was undetectable in sperm chromatin preparations by Western blotting, and it was present in the membrane fraction at less than one-tenth the level found in the cytoplasmic fraction. The ratio of the membrane to cytoplasmic fractions in nuclear assembly reactions is 1:10, and the membrane fraction, therefore, contributes less than 1% of the RanBP1 in assembly assays. Fortunately, this distribution allowed us to remove RanBP1 from nuclear assembly assays by immunodepletion from the soluble fraction without subjecting the membrane fraction to manipulations that might cause it to lose activity (see below).

We confirmed that bacterially expressed *Xenopus* RanBP1 bound to GTP-Ran (Figure 2B). To assay for Ran binding, soluble extracts from bacteria expressing either wild-type or His-tagged RanBP1 were incubated with a GST-Ran fusion protein (GST-RanG19V, a mutant Ran that is constitutively in GTP form) or GST. Bacterially expressed RanBP1 and GST-RanG19V or GST were incubated for 1 h. Glutathione-Sepharose beads were added to the samples, and the incubation continued for 30 min. The beads were removed by centrifugation and washed. The bound proteins were analyzed on Western blots using anti-RanBP1 antibodies. Both wild-type RanBP1 (Figure 2B) and His-tagged RanBP1 (our unpublished results) bound to GST-RanG19V with high affinity but did not bind to GST. *Xenopus* RanBP1 from egg extracts specifically bound to GST-RanG19V in similarly prepared samples where the bacterial lysates were replaced by the cytoplasmic fraction from *Xenopus* egg high-speed extracts, and it showed the same electrophoretic mobility as the bacterially expressed protein (Figure 2B). These results confirmed that the recombinant RanBP1 behaved like the endogenous RanBP1 in egg extracts. Our results further indicated that the addition of a His-tag to the N terminus of the recombinant protein did not interfere with Ran binding, so we chose to use this protein for the studies described below because it could be easily purified in large quantities.

Immunofluorescent staining of interphase *Xenopus* A6 tissue culture cells with anti-RanBP1 antibodies showed a distribution similar to that observed in yeast (Schlenstedt *et al.*, 1995b) and mammalian cells (Richards *et al.*, 1996). Anti-RanBP1 antibodies give a diffuse pattern of cytosolic staining without obvious colocalization to cytoplasmic organelles or to distinct regions of the cytosol, as judged by either conventional or confocal microscopy (our unpublished results). In mitotic cells, RanBP1 was dispersed throughout the cell. These patterns suggest that RanBP1 may associate with nuclear proteins during mitosis but that the bulk of the RanBP1 does not colocalize with nuclear components during interphase. Thus, our data suggest that *Xenopus* RanBP1's biochemical behavior and subcellular localization are similar to those of RanBP1 homologues in other species.

Immunodepletion of RanBP1 Results in the Codepletion of RCC1

We were interested in determining what role RanBP1 has in nuclear assembly, nuclear transport, and other interphase functions. To address this question, we removed RanBP1 from cytosol by serial immunodepletions with affinity-purified anti-RanBP1 antibodies coupled to protein A-Sepharose beads (anti-

A

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  1 gaattcGGCACGAGCGACATTGTAGAAACAGAAGACCCGCCCGCGTAATCTGCCTCCATCCTGTGCCCG ATG GCC    75
  1                                                                        M   A     2

 76 GAT ACC AAG GAT AAA CCA GAA GAG CGT GAC ACC TCT GTT GAC AAT ACA GAA GAT TCA AAT  135
  3 D   T   K   D   K   P   E   E   R   D   T   S   V   D   N   T   E   D   S   N   22

136 CAT GAC CCT CAT TTT GAG CCC ATA GTT TCA CTG CCA GAG CAA GAG ATT AAG ACA CTT GAA  195
 23 H   D   P   H   F   E   P   I   V   S   L   P   E   Q   E   I   K   T   L   E   42

196 GAA GAT GAA GAA GAA CTA TTT AAA ATG CGT GCA AAG TTG TTT CGA TTT GCA TCA GAA AAT  255
 43 E   D   E   E   E   L   F   K   M   R   A   K   L   F   R   F   A   S   E   N   62

256 GAC CCA CCG GAA TGG AAA GAA CGG GGC ACA GGC GAT GTT AAA TTG CTG AAA CAC AAA GAG  315
 63 D   P   P   E   W   K   E   R   G   T   G   D   V   K   L   L   K   H   K   E   82

316 AAG GGA ACA ATT CGT CTC TTG ATG AGG AGA GAC AAG ACA CTG AAG ATA TGT GCA AAT CAT  375
 83 K   G   T   I   R   L   L   M   R   R   D   K   T   L   K   I   C   A   N   H  102

376 GCT ATT ACC CCT GTG ATG GAA CTG AAG CCT AAT GCG GGA AGT GAC CGG GCA TGG GTT TGG  435
103 A   I   T   P   V   M   E   L   K   P   N   A   G   S   D   R   A   W   V   W  122

436 AAC ACA TAT GCT GAT TAT GCA GAT GAA TTG CCA AAA CCT GAA CTA CTG GCT ATT CGA TTT  495
123 N   T   Y   A   D   Y   A   D   E   L   P   K   P   E   L   L   A   I   R   F  142

496 TTA AAT GCA GAA AAT GCA CAG AAG TTC AAG GCA AAA TTT GAA GAA TGC AGA AAT GAA GTG  555
143 L   N   A   E   N   A   Q   K   F   K   A   K   F   E   E   C   R   N   E   V  162

556 AAG AGT AAT AAA GAA AAA GAT ACA ACC AAA AAT GAT AGT GCC GAC AAA GTG GCA GAG AAA  615
163 K   S   N   K   E   K   D   T   T   K   N   D   S   A   D   K   V   A   E   K  182

616 CTA GAA GAG CTG TCT GTA AAG GAG GCA AAA GTG CAA GAC AAG CAA GAA GAC CCC AAG CAG  675
183 L   E   E   L   S   V   K   E   A   K   V   Q   D   K   Q   E   D   P   K   Q  202

676 GAT AAA GCA GAG GAA AAG CAA TAA AATCACCCAGAGCCctcgag                           719
203 D   K   A   E   E   K   Q   *                                                 210
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B

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HumBP1  ..........  ..........  ..........  .........M AAAKDTHEDH 11
MusBP1  ..........  ..........  ..........  .........M AAAKDSHEDH 11
XenBP1  ..........  ..........  ..........  .........M ADTKDKPEER 11
Yrb1p   MSSEDKKPVV DKKEEAAPKP PSSAVFSMFG GKKAEKPETK KDEEDTKEET 50
CONSEN                                                *  *D  E

HumBP1  DTSTENTDES NHDPQFEPIV SLPEQEIKTL EEDEEELFKM RAKLFRFASE 61
MusBP1  DTSTENADES NHDPQFEPIV SVPEQEIKTL EEDEEELFKM RAKLFRFASE 61
XenBP1  DTSVDNTEDS NHDPHFEPIV SLPEQEIKTL EEDEEELFKM RAKLFRFASE 61
Yrb1p   KKEGDDAPES .PDIHFEPVV HLEKVDVKTM EEDEEVLYKV RAKLFRF..D 97
CONSEN   ***  *  ~S **D* FEP*V *~*****KT* EEDEE*L*** RAKLFRF***

HumBP1  NDLPEWKERG TGDVKLLKHK EKGAIRLLMR RDKTLKICAN HYITPMMELK 111
MusBP1  NDLPEWKERG TGDVKLLKHK EKGTIRLLMR RDKTLKICAN HYITPMMELK 111
XenBP1  NDPPEWKERG TGDVKLLKHK EKGTIRLLMR RDKTLKICAN HAITPVMELK 111
Yrb1p   ADAKEWKERG TGDCKFLKNK KTNKVRILMR RDKTLKICAN HIIAPEYTLK 147
CONSEN  *D *EWKERG TGD*K*LK*K *** *R*LMR RDKTLKICAN H I*P **LK

HumBP1  PNAGSDRAWV WNTHADFADE CPKPELLAIR FLNAENAQKF KTKFEECRKE 161
MusBP1  PNAGSDRAWV WNTHADFADE CPKPELLAIR FLNAENAQKF KTKFEECRKE 161
XenBP1  PNAGSDRAWV WNTYADYADE LPKPELLAIR FLNAENAQKF KAKFEECRNE 161
Yrb1p   PNVGSDRSWV YACTADIAEG EAEAFTFAIR FGSKENADKF KEEFEKA... 194
CONSEN  PN*GSDR*WV *** AD A**  ******AIR F***ENA*KF K *FE*** *

HumBP1  IEEREKKAGS GKNDHAEKVA EKLEALSV.. .K......EE TKEDAEEKQ  201
MusBP1  IEEREKK.GP GKNDNAEKVA EKLEALSVRE AR......EE AEEKSEEKQ  203
XenBP1  VKS.NKEKDT TKNDSADKVA EKLEELSVKE AKVQDKQEDP KQDKAEEKQ  209
Yrb-1p  .QEINKKA.. .......... .......... .......... .........  201
CONSEN        K~     *** * *** **** ***                   ****
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Figure 1. DNA and protein sequences of *Xenopus* RanBP1. (A) DNA sequence of *Xenopus* RanBP1 cDNA. Lowercase type at both ends of the nucleotide sequence shows the restriction sites for *Eco*RI (5′) and *Xho*I (3′). The numbers correspond to nucleotide sequences (normal type) and amino acid sequences (boldface type). The underlined nucleotides indicate stop codons preceding the putative initiation codon. (B) Alignment of RanBP1 homologues. The protein encoded by the open reading frame for the *Xenopus* RanBP1 sequence (XenBP1, GenBank accession number AF015303) was aligned with RanBP1 proteins of human (HumBP1, GenBank accession number X83617), mouse (MusBP1, accession number L25255), and *S. cerevisiae* (Yrb1p, accession number X65925). Amino acids that are identical among the four homologues are indicated by type in the consensus sequence (CONSEN). The star indicates the amino acids that are identical among human, mouse, and *Xenopus* RanBP1 and the tilde indicates the other amino acids that are identical in any three homologues. Gaps inserted for optimal sequence alignment are indicated by periods.

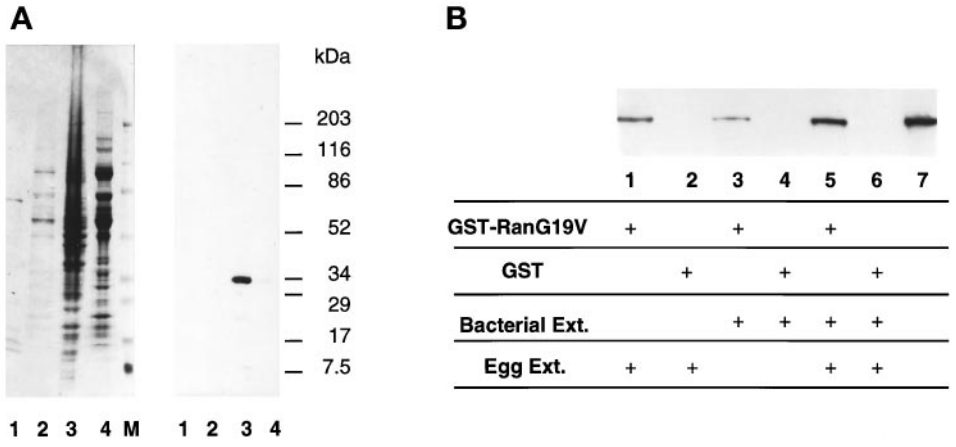


Figure 2. *Xenopus* RanBP1 is a soluble Ran-binding protein. (A) RanBP1 is found in the cytosolic fraction of reconstituted extracts. Amounts of sperm chromatin, membranes, and cytosol equivalent to those in 1 μl of reconstituted extract were separated on SDS-PAGE followed by blotting to a PVDF membrane filter (lane 1, 1000 sperm nuclei; lane 2, 0.1 μl of membranes; lane 3, 0.9 μl of cytosol). Lane 4 contains 1.0 μl of the membrane fraction. The filter was stained with India ink (left lanes) followed by Western blotting with anti-*Xenopus* RanBP1 antibodies (right lanes). M represents protein molecular size standards in kilodaltons. (B) *Xenopus* RanBP1 binds GST-RanG19V. Egg cytosol (lanes 1 and 2), extracts of bacteria expressing *Xenopus* RanBP1 (lanes 3 and 4), or both (lane 5 and 6) were incubated with GST-RanG19V (lanes 1, 3, and 5) or GST (lanes 2, 4, and 6). Glutathione-Sepharose was added to the incubations to remove the GST-RanG19V- and GST-associated proteins. Proteins bound to the beads were eluted with sample buffer and separated by SDS-PAGE. Proteins from the gel were transferred to a PVDF membrane, and Western blotting analysis was performed with anti-human RanBP1 antibodies. (Identical results were obtained when the experiment was performed with anti-*Xenopus* RanBP1 antibodies; our unpublished results). Lane 7 contained 1 μl of egg cytosol. GST-RanG19V was preloaded with GTP prior to this experiment. Both wild-type and his-tagged RanBP1 proteins also bound to wild-type GST-Ran but with a lower affinity that probably reflects the fact that wild-type Ran would exist as a mixed population of GTP- and GDP-bound forms (our unpublished results).

RanBP1 beads; see MATERIALS AND METHODS). We confirmed that more than 98% of the RanBP1 had been removed in two ways: First, we analyzed the immunoprecipitates and depleted cytosol on Western blots. We found that RanBP1 was specifically and quantitatively immunoprecipitated by affinity-purified anti-RanBP1 antibody but not by preimmune serum (Figure 3). Second, we performed a Ran overlay blot (Figure 3C). [α-^{32}P]GTP-Ran binds to renatured Ran-binding proteins from mammalian cells that have been separated by SDS-PAGE and blotted to PVDF membrane (Lounsbury *et al.*, 1994; Beddow *et al.*, 1995). Two prominent bands corresponding to RanBP1 and RanBP2 are found on blots when similar experiments are performed with *Xenopus* egg extracts (Saitoh *et al.*, 1996). [α-^{32}P]GTP-Ran overlay blots revealed equal amounts of RanBP2 in mock- and RanBP1-depleted extracts (Figure 3C), and we confirmed that the concentration of RanBP2 was the same in both extracts by Western blotting (our unpublished results). By contrast, the RanBP1 band was absent in depleted extracts (Figure 3C). This experiment independently demonstrated that all RanBP1 was depleted from the cytosol and that there were no RanBP1-like proteins remaining that failed to cross-react with our antibodies.

As controls for the specificity of RanBP1 depletion, we examined whether the concentrations of importin β, RanGAP1, RCC1, histone B4, and Ran were altered when RanBP1 was immunodepleted. The levels of importin β, RanGAP1, histone B4, and Ran were indistinguishable in RanBP1- and mock-depleted extracts, indicating that the concentrations of these proteins were not significantly affected by the removal of RanBP1 (Figure 3B). RanGAP1 has two forms in egg extracts: an unmodified form with an apparent molecular weight of 65 kDa and a more abundant modified form that migrates with an apparent molecular weight of 88 kDa (Matinus *et al.*, 1996; Mahajan *et al.*, 1997; Saitoh *et al.*, 1997). Depletion of RanBP1 did not influence the distribution of RanGAP1 between the higher and lower molecular weight forms. In contrast, RCC1 was removed during the immunodepletion process (Figure 3). Biochemical analysis has shown that purified RanBP1 can form heterotrimeric complexes with Ran and RCC1 under conditions where Ran is nucleotide free but has also suggested that these complexes dissociate after the addition of magnesium and GTP (Bischoff *et al.*, 1995b). Because egg extracts contain magnesium and GTP, we had not anticipated that RCC1 would be removed in association with RanBP1. On the other hand, RanBP1 was found in a two-hybrid screen using RCC1 (Hayashi *et al.*, 1995), suggesting that complexes containing RCC1 and RanBP1 may be considerably more stable in vivo than would be anticipated from in vitro observations.

The unexpected codepletion of RCC1 indicates either that it can form stable complexes with RanBP1 in egg extracts or that the antibodies used to immunodeplete RanBP1 may have promoted formation of an artificially stable complex containing RCC1 and RanBP1. It is unlikely that the anti-RanBP1 antibodies bound to RCC1 directly, because they neither recognized RCC1 on Western blots (Figure 2A) nor were

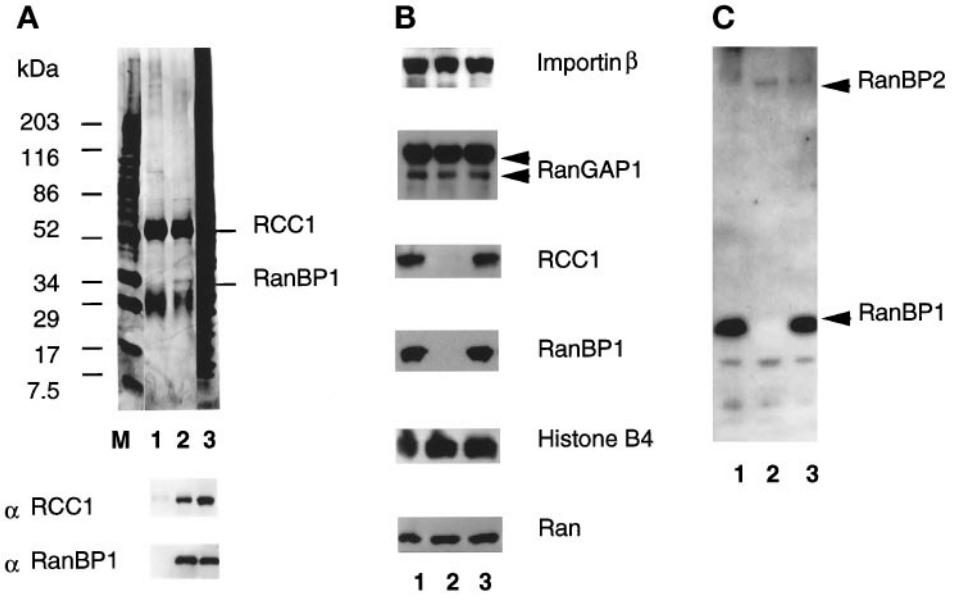


Figure 3. Depletion of RanBP1 results in the codepletion of RCC1. (A) Anti-RanBP1 antibodies immunoprecipitate both RanBP1 and RCC1. Immunoprecipitates of anti-RanBP1 (lane 2) or preimmune sera (lane 1) were analyzed by SDS-PAGE together with 1 μl egg extract (lane 3). M indicates molecular weight marker. Top, a silver stained gel of the samples, with the position of RCC1 and RanBP1 indicated. Bottom, duplicate samples analyzed by Western blotting with anti-RCC1 and anti-RanBP1 antibodies. The faint band in lane 1 of the anti-RCC1 Western blot is the immunoglobulin heavy chain. (B) RCC1 is specifically and quantitatively codepleted with RanBP1. One μl of control (lane 1), immunodepleted (lane 2), and mock depleted (lane 3) cytosol were subjected to SDS-PAGE and Western blotting analysis with antibodies against importin β, RanGAP1, RCC1, RanBP1, histone B4, and Ran as indicated. (C) No RanBP1-like proteins remain in *Xenopus* egg cytosol after RanBP1 immunodepletion. One-microliter samples of control (lane 1), immunodepleted (lane 2), and mock-depleted extracts were subjected to SDS-PAGE and transferred to a PVDF membrane. The filter was incubated with [α-^{32}P]GTP-bound GST-Ran to allow RanBP1 detection (see MATERIALS AND METHODS). RanBP1-depleted extracts show no low molecular weight Ran-binding proteins in this assay.

able to immunoprecipitate purified *Xenopus* RCC1 protein in the absence of other extract components (our unpublished results). We believe that RCC1 was depleted as part of a complex containing Ran, because Ran could also be detected in association with the anti-RanBP1 beads (our unpublished results). However, the amount of Ran in the anti-RanBP1 immunoprecipitates was small compared with the total Ran in the cytosol, consistent with the fact that Ran is in a large molar excess over the other two proteins in egg extracts.

RanBP1 was not removed to a detectable extent when we depleted RCC1 by using previously published protocols (our unpublished results; Dasso *et al.*, 1992) nor could we detect RanBP1 in anti-RCC1 immunoprecipitates (our unpublished results). We previously found that RanBP1 can associate at least transiently with GST-RCC1 in egg extracts (Saitoh and Dasso, 1995), so we suspect that RanBP1 was not precipitated by our anti-RCC1 antibodies because those antibodies disrupted complexes between RCC1 and other proteins. We also note that RCC1-depleted extracts might have retained significant concentrations of RanBP1 because the RanBP1 pools are considerably larger than RCC1 pools in *Xenopus* extracts. (We estimate that RanBP1 is in a five- to sevenfold molar excess over RCC1 [our unpublished results].) Given the larger RanBP1 pools, only a small fraction of the total RanBP1 may be associated with RCC1 and thus subject to codepletion.

We performed Western blot analysis to test whether anti-RanBP1 beads precipitated either RanGAP1 or importin β, because both of these proteins have been reported to form complexes with RanBP1 (Lounsbury *et al.*, 1995; Lounsbury and Macara, 1997). We could detect some RanGAP1 and importin β in the anti-RanBP1 immunoprecipitates but at levels that were very low compared with those found in egg extracts (our unpublished results). The relative amounts of modified and unmodified RanGAP1 associated with anti-RanBP1 beads were roughly proportional to the distribution between these forms in egg extracts, arguing against a preferential association of either form with RanBP1. However, we do not wish to draw any broad conclusions regarding the association of RanBP1 with RanGAP1 or importin β, because it is difficult to estimate the stoichiometry of components in any putative RanBP1-containing complexes, given the different titers of the antisera used and difficulty assuring that the complexes remain stable when the beads are removed from the extract and washed.

Thus, these data indicate that RanBP1 was quantitatively immunodepleted from egg extracts by anti-RanBP1 beads and that RCC1 was also quantitatively removed in association with RanBP1. This gave us a convenient way to examine defects created by the absence of both proteins (see below). By contrast, the concentrations of importin β, RanGAP1, histone B4, and Ran in the cytosol were essentially unaffected by depletion (Figure 3B). It is therefore not possible to

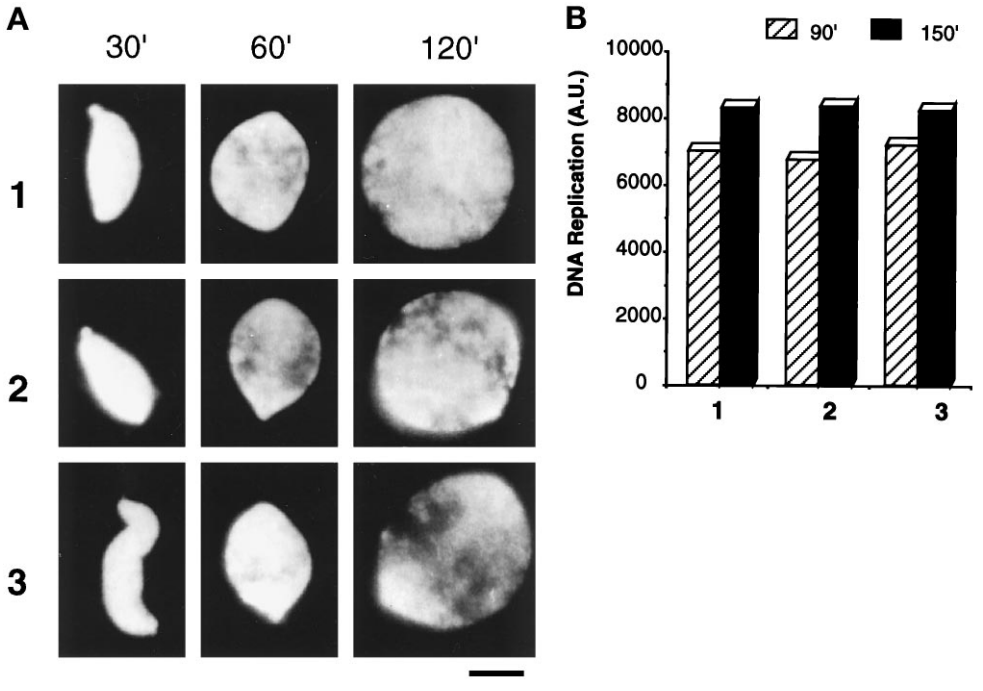


Figure 4. Normal nuclear assembly and function in the absence of RanBP1 and RCC1. (A) Nuclear assembly occurs normally in the absence of both RanBP1 and RCC1. Control (top), codepleted (middle), and mock-depleted (bottom) cytosol were used for a standard nuclear assembly assays. After 30, 60, and 120 min of incubation, nuclei were stained with Hoechst 33258 DNA dye. Bar, 2.6×10^{-6} m. (B) DNA replication occurs normally in the absence of both RanBP1 and RCC1. Nuclei assembled as in A were allowed to carry out DNA replication in the presence of [α-^{32}P]dCTP. At 90 min and 150 min, DNA samples from control (row 1), codepleted (row 2), and mock-depleted (row 3) reactions were taken for analysis of ^{32}P incorporation on agarose gels. The samples were treated as previously described (Smythe and Newport, 1991), and replication was quantified using a Molecular Dynamics Phosphorimager.

ascribe any defects of depleted extracts to a lack of any of these proteins. On the basis of results described below, we do not believe that any other proteins required for nuclear assembly or function are significantly removed in association with anti-RanBP1 beads.

Extracts Depleted of RanBP1 and RCC1 Retain Their Capacity for Interphase Nuclear Assembly, Mitotic NEBD, and Chromosome Condensation

The co- and mock-depleted cytosol were used in a standard nuclear assembly assay (Smythe and Newport, 1991). Sperm chromatin decondenses when added to reconstituted egg extracts and forms nuclei that are competent for nuclear growth, nuclear transport, and DNA replication. Surprisingly, there were no detectable defects in any aspects of the nuclear assembly assay when depleted extracts were compared with the control and mock-treated extracts: The rate of nuclear assembly and the final nuclear morphology were indistinguishable among these extracts (Figure 4A and see Figure 6A). DNA replication was not affected by the loss of both proteins, as measured by incorporation of [α-^{32}P]dCTP into high molecular weight DNA (Figure 4B), nor was nuclear protein import diminished by their depletion (see Figure 6, A, row 1, and B). Although a very small amount of RanBP1 or RCC1 may remain in our depleted extracts (we estimate $<2\%$), these observation clearly demonstrate that little of either protein is required for interphase nuclear assembly and function in egg extracts. These data also argue that no other protein that is essential for nuclear assembly or function is quantitatively removed by immunodepletion with anti-RanBP1 beads. The formation of normal nuclei after the removal of both proteins was striking because extracts lacking RCC1 have essentially no Ran nucleotide exchange activity in Ran GEF assays (Dasso *et al.*, 1994) and because nuclear formation in RCC1-depleted extracts is highly aberrant (Dasso *et al.*, 1992).

To determine whether RanBP1 or RCC1 are required for entry into mitosis or chromosome condensation, we assayed histone H1 kinase activation, NEBD, and chromosome condensation in codepleted extracts in response to an added nondegradable cyclin B protein (Figure 5). We found that histone H1 kinase activity reached mitotic levels within 30 min after cyclin B addition in untreated, codepleted, and mock-depleted extracts, suggesting that activation of the cyclin B/p34^{cdc2} kinase was indistinguishable in the three reactions (Figure 5A). Nuclei that had been assembled in each of the three reactions underwent NEBD with similar kinetics in response to cyclin B. The level of chromosome condensation and morphology of chromosomes after NEBD were also indistinguishable among the three reactions (Figure 5B). These observations clearly argue against a role for RanBP1 or RCC1 in NEBD or chromosome condensation.

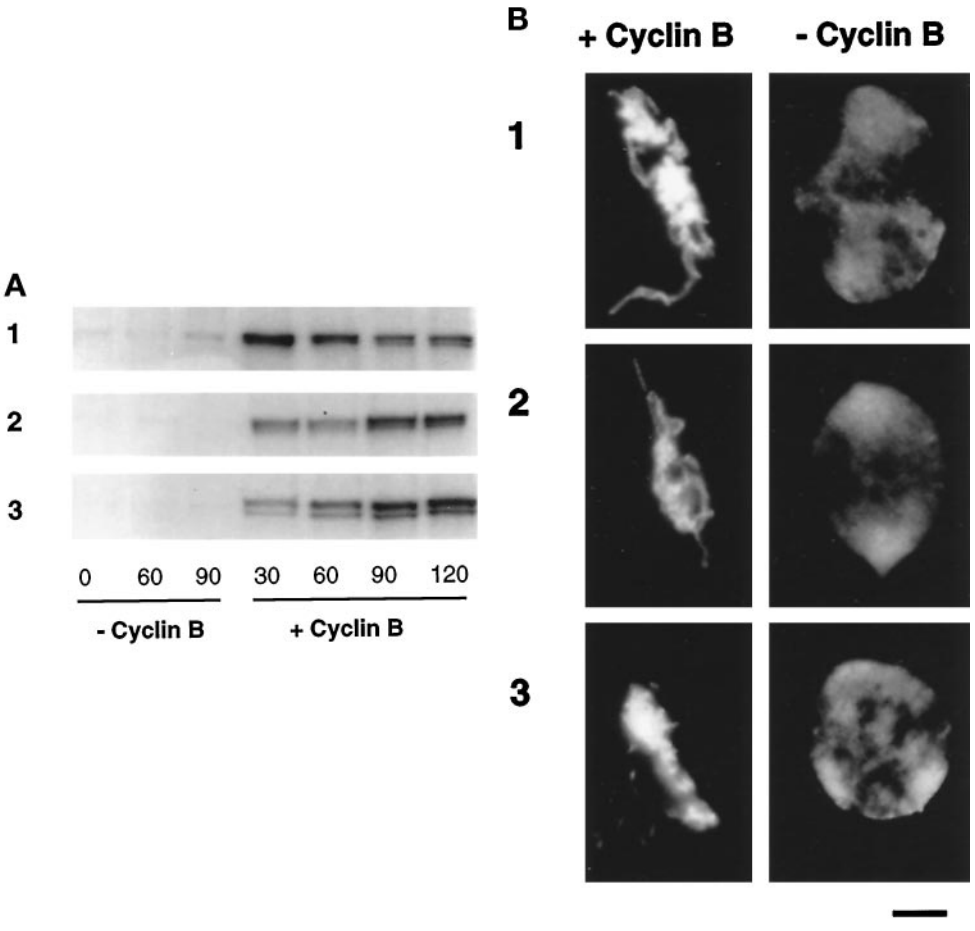
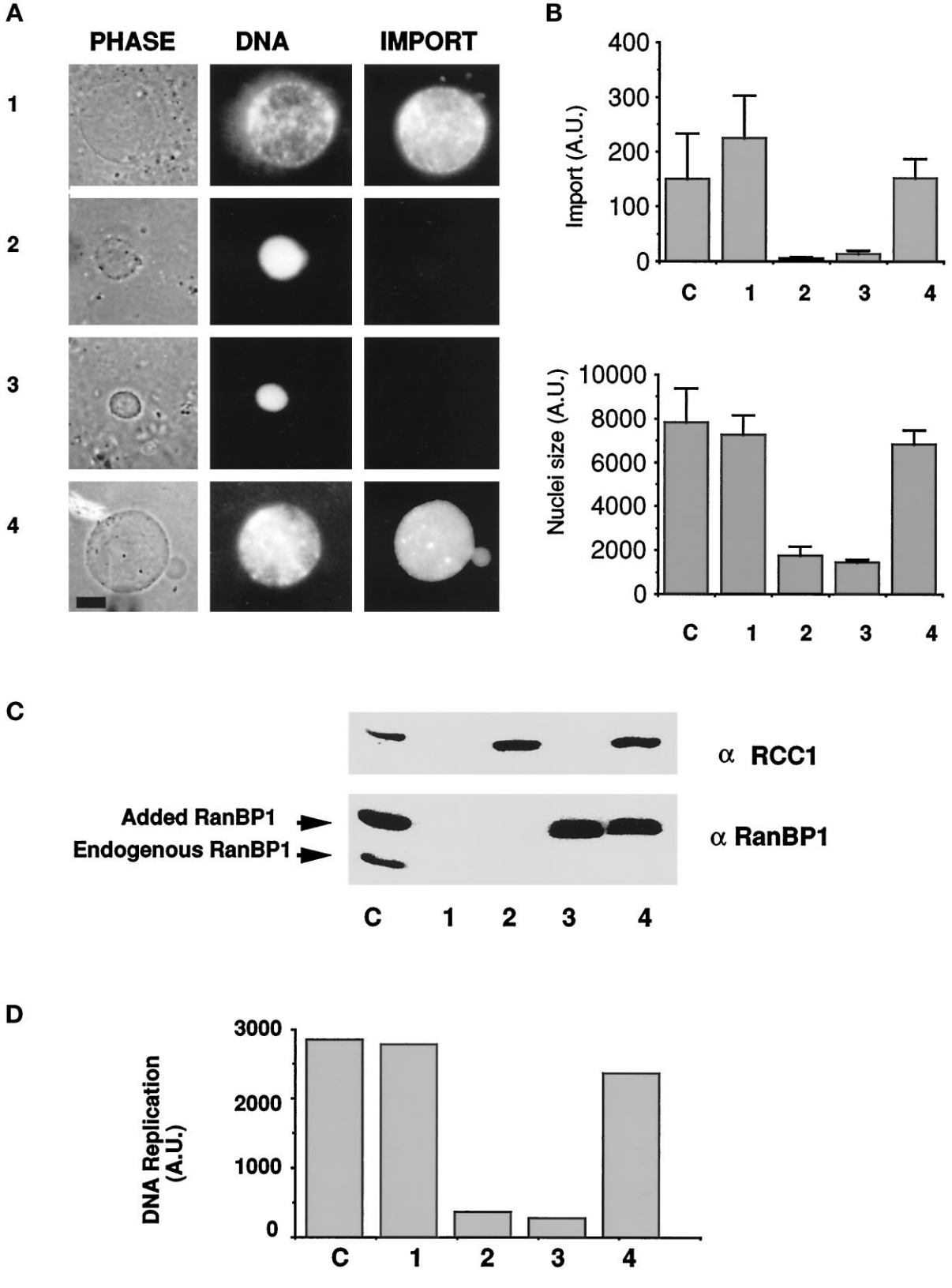


Figure 5. Cyclin B-induced mitotic events occur normally in the absence of RanBP1 and RCC1. (A) The activation of histone H1 kinase is not affected by the depletion of RanBP1 and RCC1. Nuclear assembly reactions were reconstituted with untreated (row 1), codepleted (row 2), or mock-depleted (row 3) cytosol. Nuclear formation was allowed to proceed for 60 min before addition of nondegradable cyclin B to initiate mitosis in half of the reaction mix (+ Cyclin B). The remainder of the sample was allowed to continue in the absence of cyclin B (− Cyclin B). At the times after cyclin B addition indicated (minutes), samples from each reaction were assayed for the H1 kinase activity. We observed that histone H1 kinase activity was induced synchronously in all of the reactions containing cyclin B. (B) The induction of NEBD and chromosome condensation are not affected by the depletion of RanBP1 and RCC1. Nuclear assembly was allowed to proceed for 60 min in extracts reconstituted with untreated (top), codepleted (middle), or mock-depleted (bottom) cytosol. Nondegradable cyclin B was added to half (left) of each nuclear assembly reaction, and buffer was added to the other half of the reaction (right). Nuclear breakdown occurred synchronously in the samples with cyclin B, and the nuclei remained intact in the samples without cyclin B. Each sample was stained with Hoechst 33258 DNA dye 90 min after cyclin B addition and photographed by fluorescence microscopy. Bar, 2.6×10^{-6} m.

The Depletion of RCC1 Compensates for the Depletion of RanBP1

Given the surprising observation that codepleted extracts assembled functional nuclei, we wondered whether the depletion of each protein compensated for the depletion of the other, thereby allowing normal nuclear assembly and transport. To test this idea, purified bacterially expressed RanBP1 and RCC1 were added back separately or together to depleted extracts lacking both proteins (Figure 6). After the addition of either RCC1 or RanBP1 at concentrations roughly equivalent to the original endogenous concentrations (Figure 6C), nuclei did not form properly (Figure 6A). In codepleted extracts with RCC1 added, sperm chromatin decondensed similarly to control extracts and became enclosed within a nuclear envelope. However, these nuclei did not grow significantly during the course of the experiment (Figure 6, A, row 2, and B). They were inhibited in their capacity to perform nuclear protein import, as measured by the uptake of a fluorescently labeled transport substrate (Figure 6B). Their level of DNA replication was typically more than 70% inhibited in comparison to that observed in extracts lacking both RCC1 and RanBP1, as measured by the incorporation of radiolabeled dCTP (Figure 6D). The same level of RCC1 addition did not inhibit nuclear formation, protein import, or DNA replication in untreated or mock-depleted extracts (Dasso *et al.*, 1992). When RanBP1 was added to the codepleted extract, we found that the nuclei resembled those previously observed in extracts where RCC1 alone was depleted (Dasso *et al.*, 1992). The sperm chromatin decondensed and formed an enclosed nuclear envelope, but the nuclei did not grow during the course of the experiment and they were inhibited in their capacity for nuclear protein import (Figure 6, A, row 3, and B). The replication of these nuclei was more than 80% inhibited (Figure 6D), and a twofold higher concentration of exogenous RanBP1 completely abolished DNA replication. There were no obvious defects in nuclear


A
PHASE
DNA
IMPORT
1
2
3
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B
Import (A.U.)
400
300
200
100
0
C 1 2 3 4
Nuclei size (A.U.)
10000
8000
6000
4000
2000
0
C 1 2 3 4
C
α RCC1
Added RanBP1
Endogenous RanBP1
α RanBP1
C 1 2 3 4
D
DNA Replication (A.U.)
3000
2000
1000
0
C 1 2 3 4

Figure 6.

formation, transport, or DNA replication when the same concentrations of RanBP1 were added to mock-depleted or untreated extracts (our unpublished results).

When both proteins were simultaneously added to codepleted extracts, the nuclei that formed were morphologically and functionally indistinguishable from those in control or mock-depleted extracts. Nuclei grew at the same rate as those in codepleted extracts and imported nuclear protein substrate similarly (Figure 6, A, row 4, and B), and they also replicated their DNA to the same extent (Figure 6D). The capacity of exogenous RanBP1 and RCC1 together to restore nuclear assembly and function argues against the possibility that the loss of assembly observed with addition of either protein alone was due to nonspecific contamination of the protein preparations used. It also argues against the possibility that the nuclear assembly defects arise from the loss of an unknown protein during the depletion with anti-RanBP1 beads. It appears that the relative level of these two proteins is critical for the formation and function of nuclei: When intermediate concentrations of RCC1 or RanBP1 were separately added to codepleted extracts, we observed that nuclear assembly, nuclear transport, and DNA replication were increasingly disrupted with increasing concentrations of the exogenous proteins (our unpublished results). Consistent with this notion, we also found that inhibition of nuclear assembly, nuclear transport, and DNA replication occurred when very high concentrations of either RanBP1 or RCC1 were added to untreated extracts (our unpublished results).

Thus, these results demonstrate that RanBP1 and RCC1 are each required for nuclear formation, nuclear transport, and DNA replication in the presence of the other protein and that the activity of these two proteins must be correctly balanced for nuclei to assemble and function properly. The concentrations of RCC1 and RanBP1 can vary at least 50-fold without disrupting nuclear processes, as long as a balance between the two proteins is maintained; indeed, it is possible that neither protein would be required for nuclear processes in the total absence of the other protein, although it is functionally impossible to test this speculation in egg extracts because a minute amount of RanBP1 or RCC1 may always remain after even the most rigorous immunodepletion protocols.

Figure 6 (facing page). Balance of RCC1 and RanBP1 is critical for nuclear assembly and function. (A) Imbalance of RanBP1 and RCC1 causes defects in nuclear assembly and protein import. Equal volumes of XB Buffer (row 1), recombinant RCC1 (final concentration = 10 μg/ml; row 2), recombinant RanBP1 (final concentration = 50–70 μg/ml; row 3), or both RCC1 and RanBP1 (row 4) were added to codepleted cytosol and incubated for 15 min on ice. The cytosol was then used in nuclear assembly reactions. A rhodamine-labeled import substrate was added 60 min after nuclear assembly began, and 120 min after the reaction began, each sample was assayed for nuclear morphology and protein import. Phase-contrast images of typical nuclei are shown on the left; corresponding photographs of nuclear DNA (stained with Hoechst 33258) and protein import assays are shown in the middle and right, respectively. Bar, 2.6×10^{-6} m. [As a control, BSA was added to codepleted extracts. BSA neither inhibited nuclear assembly in codepleted extracts nor restored nuclear assembly in codepleted extracts to which either RanBP1 or RCC1 had been added (our unpublished results).] (B) Quantitation of protein import and nuclear size among control and experimental samples. A set of samples similar to those in A (bars 1–4) plus a control (bar C) was allowed to form nuclei under the conditions described above. After 90 min, images of at least 25 nuclei from each sample were randomly selected and captured by using an IP Labs Spectrum Imaging System with a Photometrics cooled charge-coupled device camera. Capture was performed under identical conditions for each sample and the intensity of the signal was not saturating the imaging system. Nuclear size was measured as the number of pixels occupied by the nucleus at its maximal cross-sectional area. Import was measured as pixel intensity within the nucleus, corrected for background fluorescence. The mean and standard deviations were calculated to obtain the relative levels of import (top) and nuclear size (bottom). (C) RanBP1 and RCC1 protein levels. A 1-μl volume from each of the nuclear assembly reactions shown in A was subjected to SDS-PAGE and Western blot analysis with anti-RCC1 or anti-*Xenopus* RanBP1 antibodies, as indicated. Lane C shows the amount and positions of endogenous RCC1, RanBP1, and added recombinant RanBP1 (bigger than endogenous RanBP1 because of the tag). (D) DNA replication is inhibited by the addition of either RCC1 or RanBP1 but restored by the addition of both. Reactions of a control extract and those shown in A were allowed to undergo DNA replication in the presence of [α-^{32}P]dCTP. At 180 min, samples from the control (bar C) and reactions of codepleted extract containing XB buffer (bar 1), recombinant RCC1 (bar 2), recombinant RanBP1 (bar 3), or both RCC1 and RanBP1 (bar 4) were taken for analysis of ^{32}P incorporation. The samples were treated as previously described (Smythe and Newport, 1991) and the amount of ^{32}P incorporated into high molecular weight DNA was quantified using a Phosphorimager.

Mutant Ran Proteins Partially Rescue Extract without RCC1 or RanBP1

Although nuclei assembled in RanBP1-depleted extracts look similar to those assembled in RCC1-depleted extracts, the molecular defects of the nuclei in these two cases should be distinct: Extracts without RCC1 should accumulate GDP-Ran, but extracts without RanBP1 should be deficient in GAP-mediated GTP-Ran hydrolysis and should have high levels of GTP-Ran. We hypothesized that the distribution of Ran between GTP- and GDP-bound pools may be more important in egg extracts than the net flux between the two forms of Ran and that nuclear transport may be defective if the ratio of GTP- to GDP-Ran is imbalanced. In this case, removing both RanBP1 and RCC1 might bring the GTP- and GDP-Ran pools into balance and thereby allow normal nuclear transport. To test this idea, we added bacterially expressed RanG19V, a mutant Ran protein that is deficient in GAP-mediated GTP hydrolysis (Schlenstedt *et al.*, 1995a), and RanT24N, a mutant Ran protein that behaves as a nucleotide free form of Ran and that, there-

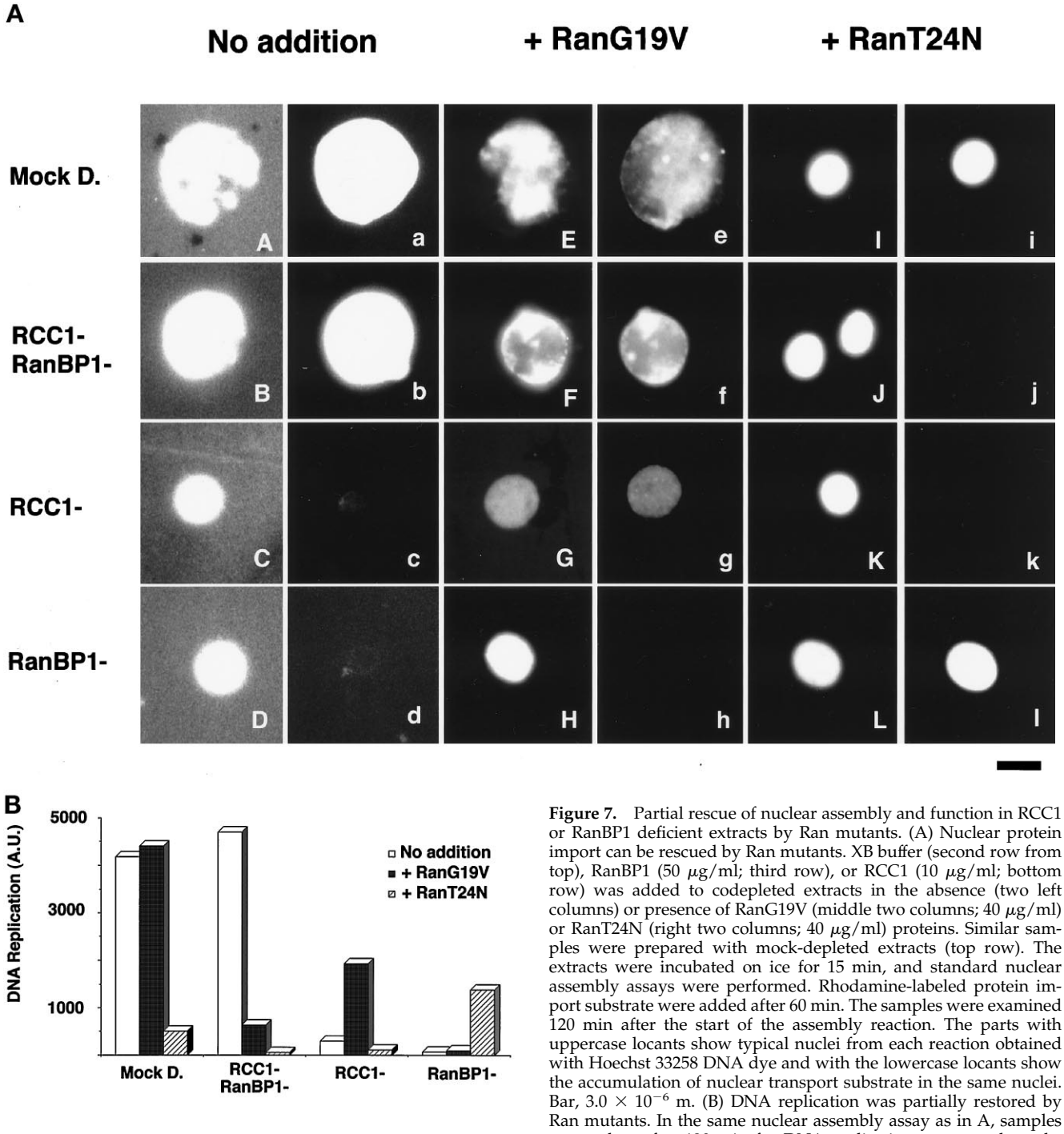

Figure 7. Partial rescue of nuclear assembly and function in RCC1 or RanBP1 deficient extracts by Ran mutants. (A) Nuclear protein import can be rescued by Ran mutants. XB buffer (second row from top), RanBP1 (50 μg/ml; third row), or RCC1 (10 μg/ml; bottom row) was added to codepleted extracts in the absence (two left columns) or presence of RanG19V (middle two columns; 40 μg/ml) or RanT24N (right two columns; 40 μg/ml) proteins. Similar samples were prepared with mock-depleted extracts (top row). The extracts were incubated on ice for 15 min, and standard nuclear assembly assays were performed. Rhodamine-labeled protein import substrate were added after 60 min. The samples were examined 120 min after the start of the assembly reaction. The parts with uppercase locants show typical nuclei from each reaction obtained with Hoechst 33258 DNA dye and with the lowercase locants show the accumulation of nuclear transport substrate in the same nuclei. Bar, 3.0×10^{-6} m. (B) DNA replication was partially restored by Ran mutants. In the same nuclear assembly assay as in A, samples were taken after 120 min for DNA replication assays and results were quantified on a Phosphorimager.

fore, binds exchange factors tightly and inhibits their activity (Klebe *et al.*, 1995).

RanG19V showed some inhibition of nuclear assembly and DNA replication in codepleted extracts (Figure 7, A, part F, and B), although it did not have any detectable effect on mock depleted extracts (Figure 7, part E; Kornbluth *et al.*, 1994). These data suggest that some GTP-Ran hydrolysis is required for nuclear assembly and function and that codepleted extracts showed enhanced sensitivity to inhibition by

RanG19V, perhaps because they possessed a lowered overall rate of GTP-Ran hydrolysis. Consistent with this idea, RanG19V did not restore function in nuclei lacking RanBP1 (Figure 7A, part H). By contrast, we found that the addition of RanG19V to extracts lacking RCC1 partially rescued both transport and DNA replication (Figure 7, A, part G, and B). This result was striking because RanG19V could not compensate for the lack of hydrolysis by wild-type GTP-bound Ran. We consider two mechanisms to be the most likely explanation of RanG19V's positive effect on extracts lacking RCC1: RanG19V could partially restore the ratio of GTP- to GDP-bound Ran pools by inhibiting GAP-mediated nucleotide hydrolysis or it could restore nuclear assembly and function by associating with and activating some essential GTP-Ran-binding protein.

RanT24N inhibited nuclear assembly and DNA replication in codepleted extracts in a manner similar to its inhibition of mock-depleted extracts. Furthermore, RanT24N inhibited nuclear transport more effectively in codepleted extracts than in mock-depleted extracts (Figure 7A, part J). These observations imply that some GEF activity in codepleted extracts was required for nuclear assembly and function and that RanT24N blocked these processes by inhibiting it. The enhanced sensitivity of codepleted extracts to RanT24N inhibition of transport may result from a lowered overall level of RanGEF activity after the depletion of RCC1. Consistent with this idea, RanT24N did not restore transport, nuclear assembly, or DNA replication in nuclei lacking RCC1 (Figure 7, A, part K, and B). On the other hand, the addition of RanT24N to extracts lacking RanBP1 partially rescued both transport and DNA replication (Figure 7, A, part L, and B). This result was interesting because RanT24N is very inhibitory for nuclear assembly and DNA replication in undepleted extracts. The most likely explanation for RanT24N's capacity to restore nuclear function in extracts without RanBP1 is that it inhibits RCC1 or other RanGEFs, restoring the balance between Ran nucleotide exchange and hydrolysis. This result strongly supports the conclusion that the levels of GTP- and GDP-bound Ran must be carefully balanced for the Ran GTPase pathway to support nuclear assembly, transport, and other nuclear functions.

DISCUSSION

We sought to use RanBP1-immunodepleted *Xenopus* egg extracts to develop an in vitro assay for RanBP1's activity in nuclei. Surprisingly, depletion of RanBP1 from *Xenopus* egg extracts resulted in codepletion of RCC1, suggesting that these proteins could exist as a stable complex in egg extracts. The capacity to remove both proteins simultaneously may be a fortuitous consequence of the way in which egg extracts are prepared—RCC1 and RanBP1 are normally localized to different cellular compartments during interphase (Ohtsubo *et al.*, 1989; Richards *et al.*, 1996), but both are contained in the soluble fraction of egg extract, allowing them to associate freely. We therefore do not wish to interpret our findings as evidence supporting or disproving a tight association between RanBP1 and RCC1 during interphase in vivo. Rather, we took advantage of the codepletion of RanBP1 and RCC1 as a convenient way to examine the functional consequences of removing both proteins simultaneously from egg extracts. Codepleted extracts, which contained $<2\%$ of the normal levels of RanBP1 and RCC1, were able to sustain nuclear assembly, DNA replication, and nuclear transport. When either RanBP1 or RCC1 were added back to codepleted extracts, generating extracts lacking solely either RCC1 or RanBP1, nuclei did not assemble properly, undergo DNA replication, or carry out nuclear protein import. These observations showed that RanBP1 and RCC1 are necessary for nuclear assembly and transport and that the requirement for either protein is dependent upon the concentration of the other protein. Codepleted extracts also provide an in vitro assay for the analysis of how RanBP1 and RCC1 function in nuclear assembly and protein import. We have used this assay to examine the effect of recombinant mutant Ran proteins on nuclei lacking RanBP1 or RCC1.

Our experiments show that the relative levels of RCC1 and RanBP1 must be carefully balanced. Genetic data from both mammalian tissue culture cells and yeast have also suggested that the activities of RanBP1 and RCC1 must be balanced. Hayashi *et al.* (1995) demonstrated that the overexpression of RanBP1 in *ts*BN2 cells, a mutant BHK cell line that has a temperature-sensitive allele of RCC1, results in an inhibition of cell growth even at the permissive temperature. A similar level of RanBP1 overexpression in wild-type BHK cells does not inhibit growth, indicating that defects caused by RanBP1 expression are enhanced when the level of RCC1 activity is lowered. In yeast, overexpression of RanBP1 gives a phenotype that is remarkably similar to that of a temperature-sensitive RCC1 mutant, *srm*1: The cells undergo G_1 phase arrest and induce the expression of FUS1, a mating-specific transcript (Hayashi *et al.*, 1995). These results were interpreted as showing that RanBP1 inhibits RCC1 through a heterotrimeric complex containing Ran. However, our finding that nuclear assembly and protein import occur normally when both proteins are depleted is not consistent with a simple model wherein RanBP1 works predominantly by associating with and inhibiting RCC1. If RanBP1 worked simply by inhibition of RCC1, we would have anticipated that codepleted extracts would be unable to assemble functional nuclei.

Consistent with the fact that both the homologues of RanBP1 and RCC1 are essential in yeast (Clark *et al.*, 1991; Ouspenski *et al.*, 1995), our experiments show that a lack of either protein causes abnormal nuclear assembly, inhibits transport, and blocks DNA replication. However, we had not expected the finding that nuclear assembly, transport, and DNA replication were normal when both proteins were depleted from the extract. Because we estimate that both proteins were >98% depleted, this result implies that a 50-fold reduction in the activity of the Ran GTPase pathway has little effect on the assembly and function of nuclei. It also poses the following paradox: If extracts lacking either RanBP1 or RCC1 individually cannot assemble nuclei or promote transport, how can extracts lacking both proteins be fully functional? One hypothesis that might explain our findings is that the net flux through the Ran GTPase pathway may be less important than the balance of the GTP- and GDP-bound forms of Ran. Consistent with this idea, we find that addition of RanG19V (a constitutively GTP-bound mutant Ran protein; Schlenstedt *et al.*, 1995a) partially rescued nuclear transport and DNA replication in extracts lacking RCC1 (Figure 7). Further, addition of RanT24N (a mutant Ran protein with a reduced affinity for nucleotides; Klebe *et al.*, 1995) partially restored transport and replication in extracts lacking RanBP1 (Figure 7). It is likely that RanG19V and RanT24N restored nuclear function by inhibiting RanGAP1 and RCC1, respectively, thereby restoring the balance of Ran nucleotide hydrolysis and exchange, rather than by substituting for the endogenous Ran protein.

Small GTPases of the Ras superfamily act as molecular switches to couple the binding and hydrolysis of GTP to multiple cellular processes. Two mechanisms for this coupling have been shown for members of this superfamily (reviewed in Rush *et al.*, 1996): Some Ras-like GTPases involved in signal transduction (i.e., Ras) associate with and activate effector molecules when they are in the GTP-bound form. The GAP-stimulated hydrolysis of GTP releases these GTPases from the effector and thereby leads to its inactivation. In this case, although the GEF and GAP activities regulate the activity of the GTPase, the strength of the signal is ultimately determined by the absolute level of the GTP-bound form. For these proteins, mutations that block GTP hydrolysis cause the signal transduction pathway to be perpetually active. Other members of the Ras superfamily directly couple the hydrolysis of GTP to processes such as the unidirectional transport of cellular proteins (i.e., Rab). In this case, blocking the GTPase pathway at any point will prevent the GTPase from accomplishing its function, such that GTPase deficient mutants are inactive. GAP and GEF mutants for this class of Ras-like GTPases would be predicted to show very similar phenotypes. A number of previous reports on Ran suggest that it functions according to the latter mechanism of action (Rush *et al.*, 1996): Mutants in RCC1 and RanGAP1 block nuclear transport and show very similar phenotypes (Matynia *et al.*, 1996). GTPase-deficient Ran mutants inhibit both import and export (Ren *et al.*, 1994; Schlenstedt *et al.*, 1995a), and nonhydrolyzable GTP analogs inhibit protein import (Melchior *et al.*, 1993). Our findings that extracts lacking RCC1 or RanBP1 individually showed very similar nuclear assembly defects was also consistent with a Rab-like mechanism for Ran's action.

However, the normal assembly of functional nuclei in extracts lacking both proteins is inconsistent with a Rab-like mechanism, as was the capacity of RanG19V to restore nuclear function to extracts lacking RCC1 only and the capacity of RanT24N to restore nuclear function to extracts lacking RanBP1 only. We do not have an explanation to easily reconcile these observations with previously proposed models for the Ran GTPase pathway. Proposing a model for Ran's role in protein import is also difficult because reports in the literature show conflicting requirements for GTP- and GDP-bound Ran and because the sites of Ran nucleotide exchange and hydrolysis remain controversial. For instance, Melchior *et al.* (1995) have presented data indicating that GTP-Ran hydrolysis occurs on the cytosolic face of the nuclear pore and that it is required for the translocation of transport substrates into the nucleus. On the other hand, Gorlich *et al.* (1996) have found that GTP-Ran causes the abortive release of transport substrates docked at the nuclear pore and that GDP-Ran promotes translocation of nuclear import substrates. They have also observed that a mutant form of importin β that is unable to bind Ran can translocate to the nuclear face of the pore, suggesting that it is unlikely that an importin β-Ran interaction is required for the actual translocation of import substrates into the nucleus (Gorlich *et al.*, 1996). We suspect that the conflicting conclusions reached in different experiments reflect divergent experimental conditions and Ran concentrations, and we feel that experiments in the *Xenopus* egg extract system are valuable because they are undertaken with roughly physiological levels of all of the components of the nucleus and cytosolic transport factors, as well as any accessory factors that may modulate the activity of the Ran pathway.

Although it is currently difficult to present a definitive model for the Ran GTPase pathway, it is possible to speculate why the GTP-Ran to GDP-Ran ratio might be critical for nuclear assembly and transport. One possibility that has been suggested is that the high concentration of nuclear GTP-Ran may serve to define compartmental identity between the nucleus and the cytosol (Gorlich *et al.*, 1996). Loss of RCC1 activity in temperature-sensitive mammalian cell lines or overexpression of RanGAP1 in *Schizosaccharomyces pombe* results in redistribution of Ran protein from the nucleus

to the cytosol (Ren *et al.*, 1993; Matynia *et al.*, 1996). These observations imply that GTP-Ran generated by RCC1 is largely restricted to the nucleus and that decay of GTP-Ran to GDP-Ran resultant from RCC1 inactivation or RanGAP1 overexpression allows the diffusion or transport of Ran to the cytosol. Rexach and Blobel (1995) recently reported that GTP-Ran disrupts complexes between importin α and β by binding tightly to importin β. They proposed that the cytosolic concentration of GTP-Ran would, therefore, normally be low, because high levels of GTP-Ran would cause the dissociation of the importin heterodimer and thereby abort nuclear transport prematurely. Conversely, the high levels of GTP-Ran within the nucleus may serve to keep the importin subunits dissociated from each other and facilitate their recycling back to the cytosol after each round of protein import. Such a role for GTP-Ran would be supported by recent observations in *S. cerevisiae*, where mutants in the RCC1 homologue prp20 specifically sequester importin α within their nuclei (Koepp *et al.*, 1996). The accumulation of importin α does not simply result from a lack of transport but from a lack of RCC1 per se, because other transport deficient mutants do not show this phenotype. Our data may suggest that both GTP-Ran and GDP-Ran play essential roles in defining compartmental identity and that the absence of either form of Ran results in deficient nuclear transport.

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