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High Dosage of Rok1p, a Putative ATP-dependent RNA Helicase, Leads to a Cell Cycle Arrest at G1/S Stage in *Saccharomyces cerevisiae*

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The *ROK1* gene encodes a putative ATP-dependent RNA helicase which is essential for mitotic cell growth. *ROK1* has been thought to affect microtubule and spindle pole body (SPB) functions in *Saccharomyces cerevisiae*. To investigate the intracellular functions of *ROK1*, we varied the Rok1 protein dosage in a cell and analyzed its phenotypic effects. Overexpression of the *ROK1* gene by using a strong *GAL1* promoter was lethal, leading cells to arrest at the unbudded stage. This arrest phenotype is very similar to that of the *rok1* null mutation. Indirect immunofluorescence revealed that the majority of arrested cells contained a single SPB. Normal development of microtubules between the duplicated SPBs was rarely observed. Multinuclear cells with abnormal microtubule array were detected in small fraction. Taken together with the phenotype of the *rok1* null mutation, these results imply that *ROK1* is required for cell cycle progression at the G1/S stage.

Key words: *Saccharomyces cerevisiae*, RNA helicase, Rok1p, cell cycle arrest

In the yeast *Saccharomyces cerevisiae*, the spindle pole body (SPB) functions as a microtubule organizing center in a manner analogous to the centrosomes in higher eukaryotes (4, 5). Microtubules eminating from the SPB participate in mitosis, meiosis, and nuclear movements (11). The SPB is a discrete layered-structure embedded in the nuclear envelope and undergoes dramatic alterations in lateral size during the cell division cycle. During the G 1/S stage, the SPB makes a new copy of itself which is connected via a bridge. The duplicated SPBs remain attached in a side-by-side configuration until the completion of the S phase. The SPBs then migrate away from one another, locating themselves on opposite sides of the nucleus. Discontinuous microtubule spindles develop between the two SPBs.

In *S. cerevisiae*, progression through the cell cycle transitions requires CDC28 kinase in association with G1 or G2 cyclins, regulatory molecules synthesized transiently at the G1 or G2 stages of the cell cycle (10, 27, 35). The cell cycle regulatory machinery controls not only the periodicity of DNA replication and chromosome segregation but also SPB duplication (10, 30). The major class of *cdc28* mutations causes arrest at an early stage of SPB duplication (4, 5). SPB segregation has been reported to require dephosphorylation of the Cdc28 protein (23). A component protein of the SPB, Spc 110p, is phosphorylated in a cell cycle-dependent manner (7, 16). These data have suggested that the well defined cascade of cyclin activation of Cdk kinase activities may play an important role in the regulation of SPB duplication and spindle formation.

The *ROK1* gene was identified as a high-copy suppressor of the *kem1* null mutation (19). The *KEM1* gene has been reported to affect microtubule and spindle pole body functions during conjugation and mitotic cell growth (17, 18). This suggestion is based on a number of phenotypes of *kem1* mutations including hypersensitivity to microtubule-depolymerizing drug benomyl, chromosome loss, and defects in SPB duplication or nuclear fusion. We expect that the *ROK1* gene also affects microtubule and spindle pole body functions in a manner similar or related to those of *KEM1*.

The amino acid sequence of the Rok1 protein contains highly conserved domains found in the DEAD (Asp-Glu-Ala-Asp) protein family of ATP-dependent RNA helicases (24, 32, 34). Substitution of the first aspartic acid in the DEAD domain with glutamate (EEAD) was shown to be lethal to cells, suggesting that the RNA helicase domains contribute to the essential *ROK1* functions (14). The Rok1 protein expressed and purified in *Escherichia coli* exhibited both the ATP hydrolysis and RNA unwinding ac-

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tivities (to be published elsewhere). In our present study, the question to be addressed was how the Rok1 protein with RNA helicase activity is related to microtubule or spindle pole body mediated processes. The *ROK1* gene had already been shown to be essential for mitotic cell growth (34). We present here results which suggest the possible functions of *ROK1* in cell cycle progression.

Materials and Methods

Strains and media

The bacterial strain used for plasmid propagation was *E. coli* JM109 (*recA1 supE44 endA1 hsdR 17 gyrA96 relA1 thi*Δ*(lac-proAB) F'[traD36 proAB+ lacI*^{q} *lacZ*ΔM15]). *S. cerevisiae* strain F808 (*MATa ura3-52 leu2-3,112 his4-519 ade1-100 GAL*$^+$) was used for overexpression of *ROK1*.

Bacterial media were made as described by Sambrook *et al.* (31). Yeast media were essentially as described by Sherman *et al.* (33). SC-Ura media consisted of yeast nitrogen base with all amino acids added except uracil. Galactose media contained 2% galactose instead of glucose. For growth prior to induction in galactose, a mixture of 2.5% ethanol and 2.5% glycerol was used. For galactose induction, galactose was added directly to this medium.

Transformation and DNA manipulation techniques

Yeast transformation was carried out by the lithium acetate method developed by Ito *et al.* (13) using 50 μg of sonicated calf thymus DNA (Sigma.) per transformation as carrier. *E. coli* transformation was performed by the calcium chloride procedure of Mandel and Higa (26). Rapid isolation of plasmid DNA was done by the modified alkaline lysis method (31). Restriction endonuclease analysis and agarose gel electrophoresis were carried out as described in Sambrook *et al.* (31).

Plasmid constructions

Plasmids pJI219 and pJI220 contained the *GAL 1::ROK1* fusion genes. Plasmid pJI219 was constructed by isolating the *Nco*I-*Sph*I fragment of *ROK1* from plasmid pJI216 and inserting it into the *Bam*HI and *Sph*I sites of plasmid CGS109. CGS109 carries the *Eco*RI-*Bam*HI fragment of the *GAL1* promoter sequence on a 2 μ-based high-copy plasmid. In plasmid pJI219, the *GAL1* promoter sequence resides right in front of the ATG initiation codon of *ROK1*. Plasmid pJI220 was constructed by isolating the *Eco*RI-*Sph*I fragment from pJI219 and inserting it into the *Eco*RI and *Sph*I sites of plasmid YCp50.

Protein preparation and western blot

Total yeast protein was obtained by trichloroacetic acid precipitation as described by Oashi *et al.* (28). Protein extracts were separated by SDS-PAGE and electrophoretically transferred to nitrocellulose sheets using Semi-dry transfer set (Hoeffer, TE70). Immunoblots were blocked for 2 h with a solution containing 5% instant nonfat milk and 0.2% Tween 20 in TBS (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 0.5% sodium azide). Rok1 antiserum was added (1:1000 dilution) and incubated for 4 h. Rinsed blots were incubated for 2 h with an anti-rabbit IgG conjugated to horseradish peroxidase (Bio-Rad, 1:2500 dilution) and subsequently incubated in a solution of 0.018% 4-chloro-1-naphtal and 30% H_2O_2 for 10 min (9).

Indirect immunofluorescence

Indirect immunofluorescence using formaldehyde fixation of intact cells was performed by the method of Adams and Pringle (1) with a few modifications (20). Rat anti-tubulin antibodies (YOL1/40) were used at a 1:50 dilution. Rhodamine-conjugated anti-rat IgG (KDL) as secondary antibody was used at a 1:500 or 1:1,000 dilution.

Results and Discussion

Cell lethality caused by the *ROK1* overexpression

To investigate the intracellular functions of the *ROK1* gene, we varied the Rok1 protein dosage in a cell and analyzed its phenotypic effects. As shown in Fig. 1A, the *GAL1::ROK1* fusion gene constructed and put on either a high-copy number vector (pJI219) or a single-copy number vector (pJI220). The *GAL1::ROK1* fusion gene expresses *ROK1* under the control of a strong *GAL1* promoter. Plasmid pSH70 is a high copy number *ROK1* plasmid (19). Growth patterns of Gal$^+$ yeast strains harboring each of these plasmids were compared on a galactose containing medium. As shown in Fig. 1B, the strain with pJI219 showed lethality on the galactose plate, indicating that the overexpression of *ROK1* is toxic to yeast cells. The strains carrying either plasmid pJI220 or plasmid pSH70 grew well on the galactose medium. The Rok1 polypeptides synthesized in these strains were analyzed by Western blot using anti-Rok1 antiserum (Fig. 2). Protein bands with an estimated molecular mass of 64 kDa were detected in whole cell lysate. After 12 h of induction in the

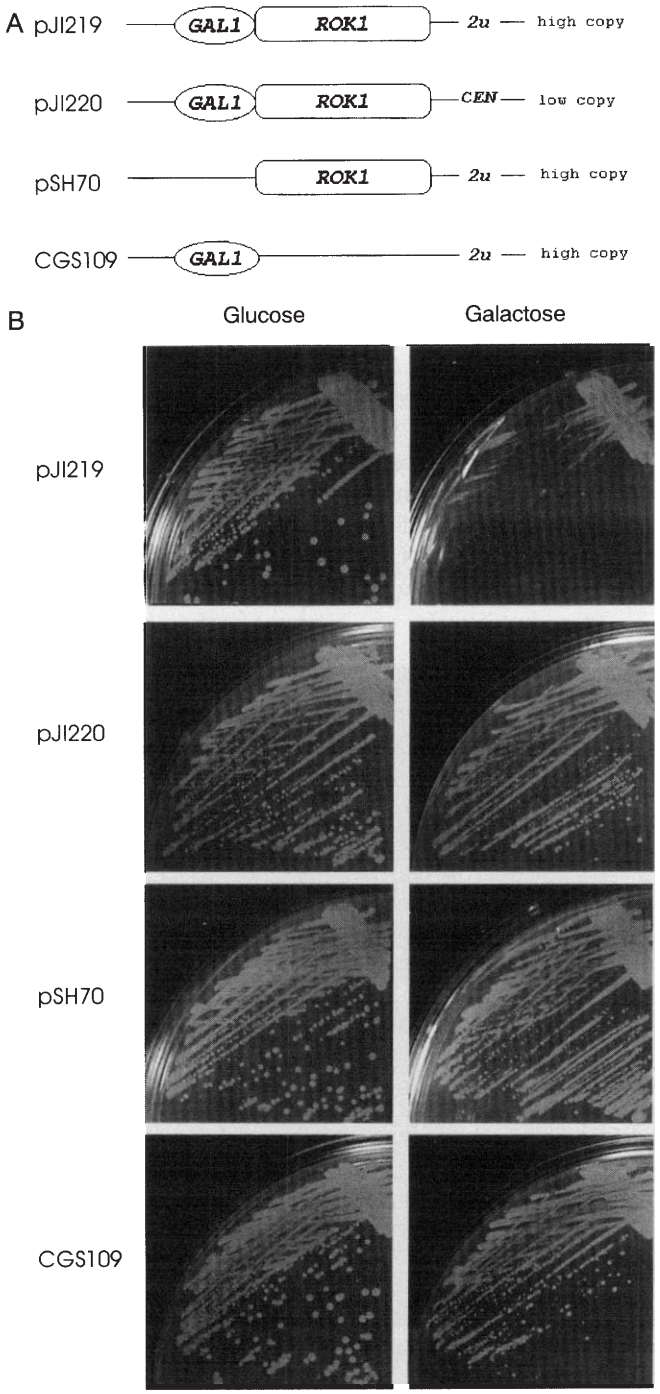

Fig. 1. Lethality caused by overexpression of *ROK1*. (A) Structures of the *ROK1* gene dosage plasmids. (B) Growth of the transformants carrying each plasmid on glucose or galactose plates.

galactose containing medium, the Rok1 protein level in the strain with pJI219 was highly increased. The amounts of Rok1 protein in the strains with pJI220 and pSH70 were similar to each other but much lower than that of pJI219. These results indicate that the amount of Rok1 protein in the cell

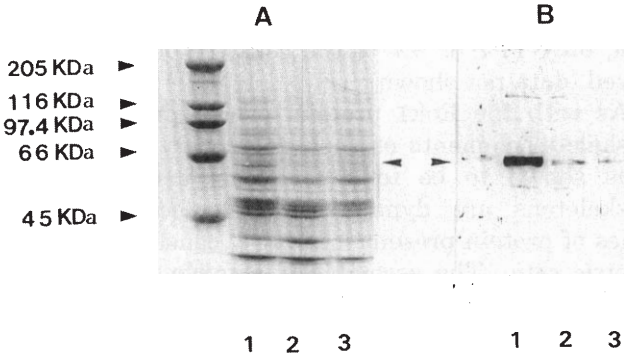

Fig. 2. Overproduction of Rok1p analyzed by SDS-PAGE (A) and Western blot (B). Lane 1, yeast total protein from a strain carrying pJI219; lane 2, pJI220; lane 3, pSH70.

is critical for cell viability.

The toxic effects of Rok1p overproduction were irreversible. When cultures of 6 h or 12 h induction

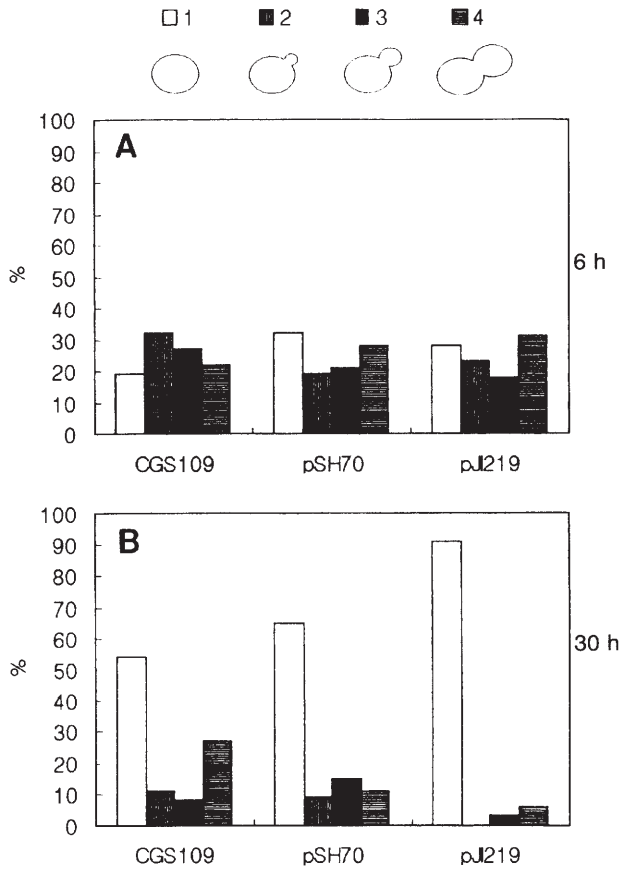

Fig. 3. Cell cycle arrest phenotype caused by overexpression of *ROK1*. (A) The distribution of cells in strains carrying plasmid CGS109, pSH70, or pJI219 was calculated after 6 h induction in galactose. (B) 30 h induction in galactose. more than 300 cells were counted for each strain, and the percentage of each type of cell is shown. The pictures above the graph represent the following: 1, unbudded cells; 2, tiny-budded cells; 3, small-budded cells; 4, large-budded cells.

were plated on repressing glucose containing medium, only 11% or 9% of the cells, respectively, survived (data not shown).

As with the Rok1 protein, overproduction of cytoskeletal elements or their regulatory components was shown to be toxic for yeast cells (25). Cytoskeletons are dynamic macromolecular assemblies of protein present in a fairly constant stoichiometric ratio. The assembly is a fairly ordered process and is determined by the affinity and concentration of the subunits present. Therefore, cells might be particularly sensitive to the overexpression of genes encoding these structural components. In agreement with this concept, the screening of a *GAL1*-regulated yeast cDNA expression library for genes whose overexpression causes lethality in yeast detected 15 clones including *ACT1* (actins), *TUB1* (tubulin), and *ABP1* (actin-binding protein) (25). In subsequent reports, the overexpressions of the most of the SPB components (Kar 1p, Spc42p, Spc97p, Spc98p, Dsk2p, etc.) were shown to be toxic (3, 6, 8, 21, 29, 30). All these reports support our expectation that the *ROK1* gene also affects microtubule and spindle pole body functions.

ROK1 is required for cell cycle progression at G1/S stage

To carefully assess the growth defects associated with *ROK1* overexpression, the cell cycle arrest phenotype was examined after induction with galactose. Cells from strains harboring *ROK1*-dosage plasmids were grown to early logarithmic phase in ethanol/glycerol media. Galactose was added to half of the culture. After 30 h, the culture consisted of a homogeneous collection of cells with a unique cell division cycle morphology. Greater than 90% of the cells were arrested at the unbudded stage (Fig. 3). This

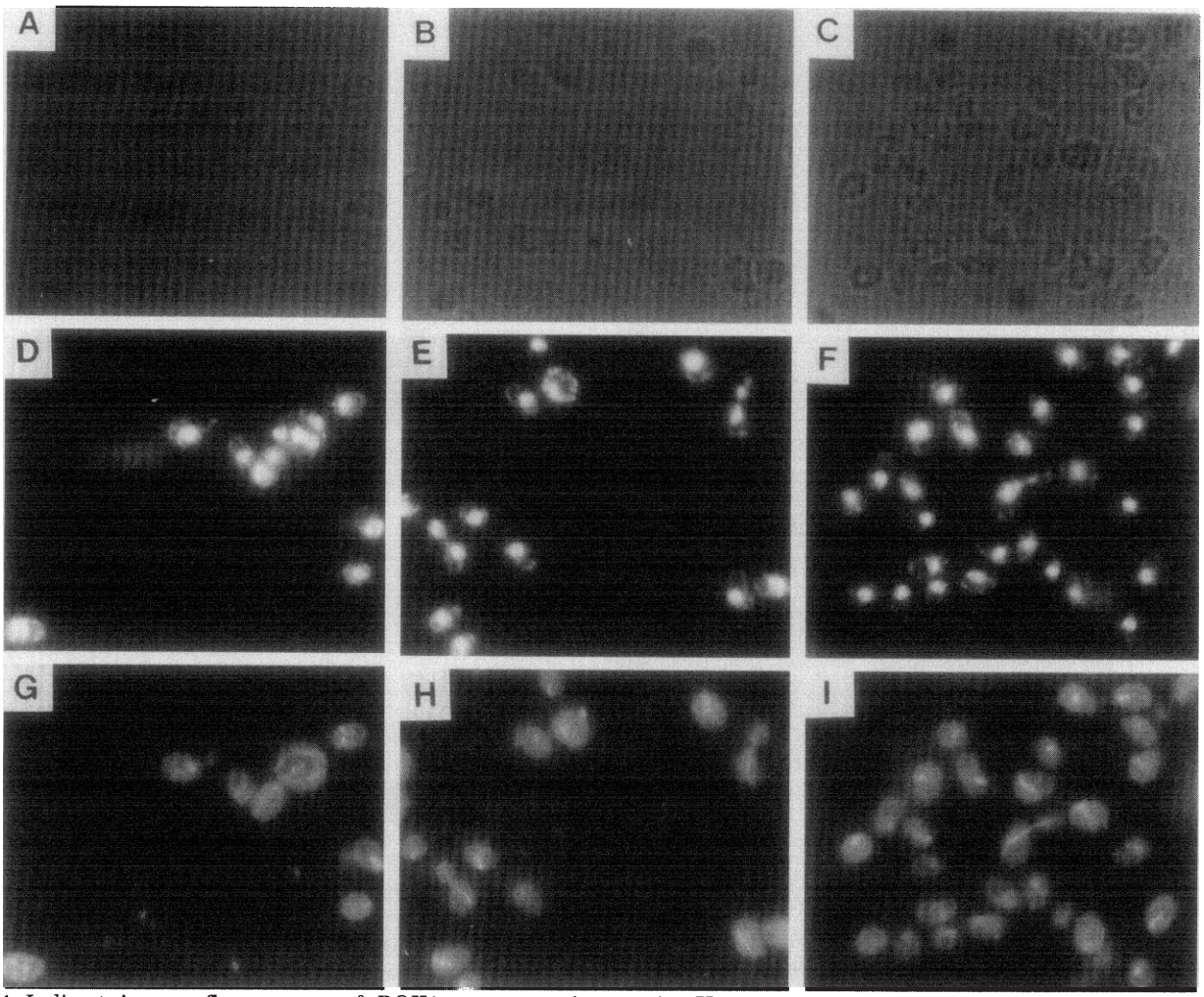

Fig. 4. Indirect immunofluorescence of *ROK1* overexpression strain. Yeast strain F808 harboring *ROK1* overexpression plasmid, pJI219, (A-B, D-E, G-H) or control plasmid CGS109 (C, F, I) was grown in galactose for 12 h and fixed for indirect immunofluorescence. Fixed cells were stained with DAPI (D, E, F) and anti-tubulin antibody (G, H, I).

arrest phenotype is very similar to that of the *rok1* null mutation (14, 34).

To determine if the arrest phenotype of *ROK1* overexpression was due to a defect in SPB duplication, cells grown in galactose were processed for indirect immunofluorescence. Staining with anti-tubulin antibodies revealed that the majority of arrested cells had a single SPB or duplicated but unseparated SPBs (Fig. 4, G and H). Normal development of microtubules between the duplicated SPBs was rarely observed. Multinuclear cells with abnormal microtubule array were detected in small fraction (Fig. 4, G). Taken together with the phenotype of the *rok1* null mutation, these results imply that *ROK1* is required for cell cycle progression at the G1/S stage. Whether the defect is in SPB duplication or separation of duplicated SPBs could be answered by serial section EM.

The *KEM1* gene has also been known to affect SPB duplication/separation (12, 17). *kem1* null mutants have shown increased populations of unbudded cells with a single SPB or duplicated/but unseparated SPBs. We report here that *ROK1*, a high-copy suppressor gene of the *kem1* null mutation, affects the processes related to *KEM1* in the yeast cell division cycle. The Kem1 protein has been reported to be a microtubule-associated protein (12). There are also a number of results suggesting that the Kem1 protein exerts a cytoplasmic exoribonuclease with possible roles in mRNA turnover (2, 15, 22). We suspect that the functions of *KEM1* could be rather specific to SPB components under certain conditions instead of just serving as a general exoribonuclease. Since the Rok1 protein is considered to be an ATP-dependent RNA helicase which unwinds double strand RNA, the Rok1 protein could also participate in *KEM1*-mediated processes. Taken together, we can postulate that both *ROK1* and *KEM1* affect SPB duplication at the G_1/S stage of the cell cycle by regulating SPB specific gene expression.

Acknowledgments

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