Fission Yeast Slp1: An Effector of the Mad2-Dependent Spindle Checkpoint

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Mad2 is a component of the spindle checkpoint, which delays the onset of anaphase until all chromosomes are attached to the spindle. Mad2 formed a complex with Slp1, a WD (tryptophan–aspartic acid)–repeat protein essential for the onset of anaphase. When the physical interaction between the two proteins was disrupted, the spindle checkpoint was no longer functional. Post-anaphase events such as chromosome decondensation and the next round of DNA replication were not delayed even when the spindle assembly was incomplete. This relief of dependence appears to be a result of deregulation of ubiquitin-dependent proteolysis mediated by the anaphase-promoting complex.

Duplicated chromosomes segregate equally in anaphase. The spindle checkpoint delays the onset of anaphase until all chromosomes are attached to the spindle (*1*) and prevents production of cells with missing or extra chromosomes. Mad2 is a component of the spindle checkpoint conserved in eukaryotes (*2*). It associates with unattached kinetochores and disappears as mitosis proceeds normally. Fission yeast Mad2 causes a metaphase arrest when it is overexpressed (*3*). Thus, Mad2 has a key function in a signaling pathway that delays the onset of anaphase in response to a spindle defect. However, the nature of this function has not yet been described.

The fission yeast *slp1*$^+$ gene encodes a protein containing repeating units that often end with the sequence Trp-Asp (WD repeat). The *slp1*$^+$ gene is essential for cell growth, and a temperature-sensitive mutant (*slp1-362*) was arrested before anaphase (*4*). Slp1 is a member of a family of proteins that includes Fzy, Cdc20, p55CDC (*5*), Hct1/Cdh1, and Fzr (*6*). Members of this family may function as regulators of the ubiquitin-dependent proteolysis during mitosis (*6*). We isolated the fission yeast Mad2 in the yeast two-hybrid system (*7–9*) with *slp1*$^+$ as bait. Slp1 and Mad2 interacted strongly and gave rise to histidine-prototrophic growth (Fig. 1A) and increased β-galactosidase (LacZ) enzymatic activity (Fig. 1B) in a specific manner. The physical interaction between the two proteins was confirmed with a fusion protein of glutathione-S-transferase with Slp1 (GST-Slp1), and Mad2 protein translated in vitro in the presence of ^{35}S-methionine (*10*). ^{35}S-labeled Mad2 bound to GST-Slp1, but not to GST (Fig. 1C). We generated a series of truncated Slp1 mutants and tested them for binding activity in the yeast two-hybrid system. F6, a 66–amino acid polypeptide, interacted with Mad2 (Fig. 1D). Deletion of 29 amino acids (Δ118–146) resulted in a loss of the binding activity (Fig. 1D), suggesting that these amino acids are important for binding to Mad2.

Deletion of *mad2*$^+$ (*11*) (Fig. 2A) did not cause noticeable defects under normal growth conditions. The *cut7*$^+$ gene encodes a kinesin, and the *cut7* mutant fails in interdigitating the mitotic spindles (*12*). The *cut7* mutants arrest as septated cells with overcondensed chromosomes, implying that loss of *cut7*$^+$ is normally recognized by the spindle checkpoint and results in a delay of chromosome decondensation. Therefore, Δ*mad2* was expected to abolish the metaphase arrest seen in the *cut7* mutant at the restrictive temperature. Indeed, there was no indication of chromosome overcondensation in the double mutants, *cut7* Δ*mad2*, over a period of 4.5 hours after the shift to the restrictive temperature (Fig. 2, C and D). Their nuclei appeared noncondensed and hemispheric. In the *cut7* Δ*mad2* mutants, the dependence of chromosome decondensation on formation of a mitotic spindle was relieved. In contrast, 3 hours after the shift, 60% of the *cut7* single-mutant cells exhibited overcondensed chromosomes that appeared dense and fibrous. Most of the double-mutant cells contained nearly 4N DNA content 2 hours after the shift (Fig. 3), indicating that the double mutant can initiate the next round of DNA replication even when the spindle assembly is incomplete. Thus, the fission yeast *mad2*$^+$ functions as a component of the spindle checkpoint. These results are consistent with the previous report (*3*).

Overexpression of *mad2*$^+$ causes a metaphase arrest (*3*), perhaps because Mad2 binds to Slp1 and inhibits its activity to promote the transition from metaphase to anaphase. We therefore generated and characterized an *slp1.* mutant that is

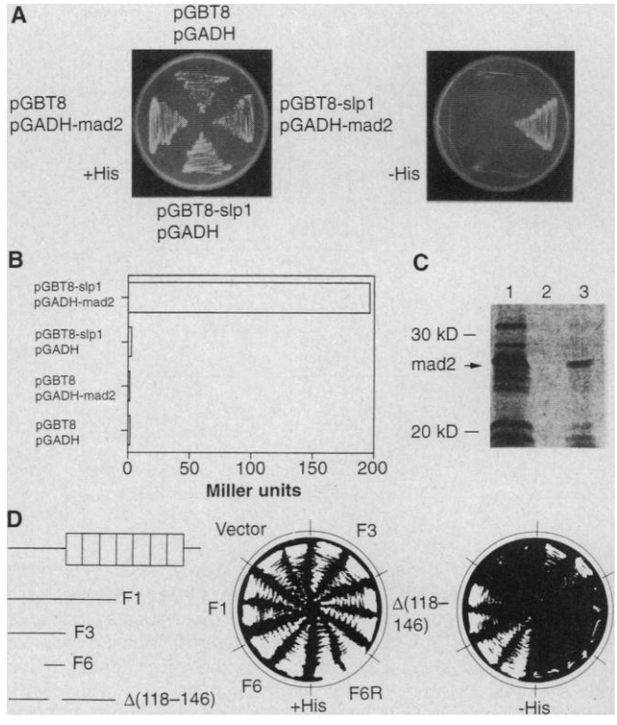

Fig. 1. Binding of Mad2 to Slp1. (**A** and **B**) Yeast two-hybrid system. A tester strain was transformed by the combination of plasmids as indicated. In the transformants with pGBT-slp1 and pGAD-mad2, two reporter genes, *HIS3* and *LacZ*, were highly expressed. The specific activity of β-galactosidase (indicated as Miller units) was determined as described (*22*). Similar results were obtained in three independent experiments. (**C**) Binding of in vitro–translated *mad2* gene product labeled by ^{35}S (lane 1) to GST protein or a fusion protein, GST-Slp1, which were bound to glutathione–Sepharose 4B beads. The proteins bound to GST (lane 2) or to GST-slp1 (lane 3) were separated by polyacrylamide gel electrophoresis. (**D**) Each box represents a WD (Trp-Asp) repeat motif in Slp1. Truncated Slp1 fragments are shown below. The tester strain was transformed with pGAD-mad2 and pGBT8 carrying each of the truncated *slp1*$^+$ fragments. F6R bears F6 fragment in the reverse orientation. The transformants were streaked on the media containing (+) or lacking (−) histidine.

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resistant to the overproduction of Mad2. The domain of Slp1 important for binding to Mad2 (amino acid position 118 to 146) was mutagenized at random (*13*). Mutated *slp1* genes on an autonomously replicating plasmid were individually transformed into a wild-type strain with ADH-M2, a plasmid that allows the overexpression of *mad2*$^+$ from the alcohol dehydrogenase (ADH) gene promoter. When yeast cells were transformed with the wild-type *slp1*$^+$ gene on the autonomously replicating plasmid and with ADH-M2, the transforming efficiency was lower than that obtained by cotransformation with ADH-2M, a plasmid bearing the *mad2*$^+$ gene in the reverse orientation. In cells expressing *mad2*$^+$ from the ADH promoter and *slp1*$^+$ on the autonomously replicating plasmid, Mad2 probably exists in excess over Slp1 and inhibits the growth of the transformants. Three *slp1* mutants (*slp1-mr27*, *52*, and *63*) overcame the overexpression of *mad2*$^+$. When they were expressed along with ADH-M2, the transformation efficiency was comparable to that obtained when they were expressed with ADH-2M. Nucleotide sequence analysis revealed that the *slp1*$^+$ gene was mutated similarly in all the mutants at Ala-131 and Phe-132 (Fig. 4A). One of the mutant *slp1* genes, *slp1-mr63*, was used to replace a null allele of *slp1* in a diploid. The resulting heterozygous diploid (*+/slp1-mr63*) was resistant to the overexpression of *mad2*$^+$ (Fig. 4C), indicating that *slp1-mr63* can overcome the overexpression of *mad2*$^+$ in a dominant manner (*14*). The presence of *slp1-mr63* was determined by polymerase chain

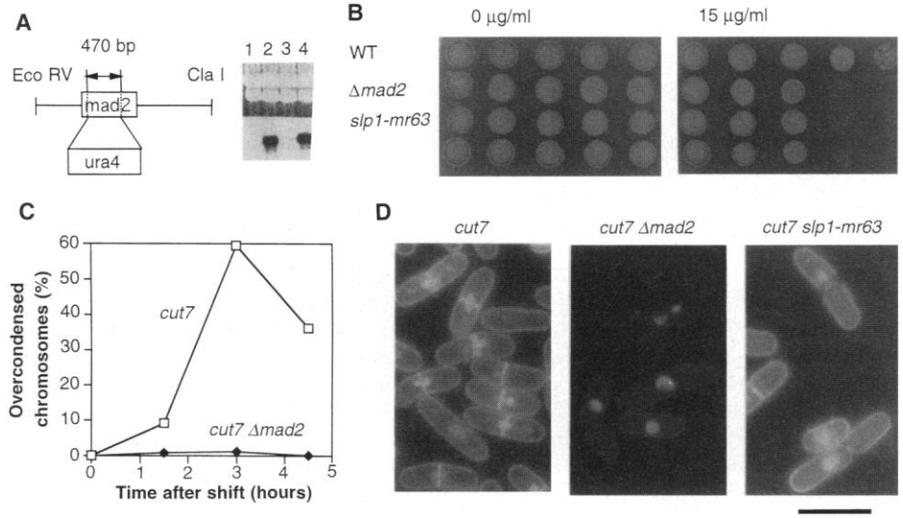
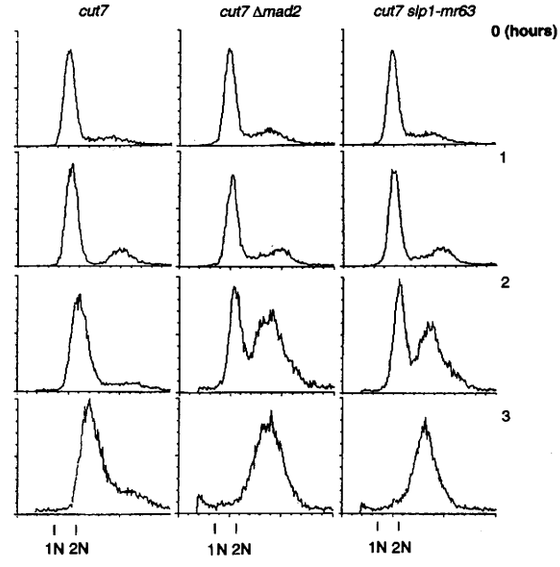


Fig. 2. Disruption of *mad2*$^+$ and loss of spindle checkpoint function. (**A**) The *mad2*$^+$ gene was disrupted as shown. Total RNAs prepared from Ura$^-$ segregants (wild-type strains, lanes 2 and 4) and Ura$^+$ segregants (mad2 disruptants, lanes 1 and 3) were analyzed by Northern blotting with the *mad2*$^+$ cDNA as a probe. Each lane contained a similar amount of RNA. (**B**) Sensitivity to thiabendazole. Cell suspension of a wild-type strain Δ*mad2* and two independent isolates of the *slp1-mr63* mutant were serially diluted and spotted on the complete media containing thiabendazole at the concentrations indicated. (**C**) Percentages of cells exhibiting overcondensed chromosomes after the shift to the restrictive temperature. (**D**) Microscopy of *cut7*, *cut7* Δ*mad2*, and *cut7 slp1-mr63* at 3 hours after the shift. They were stained with 4,6-diamine-2-phenylindole (DAPI). Bar, 10 μm.



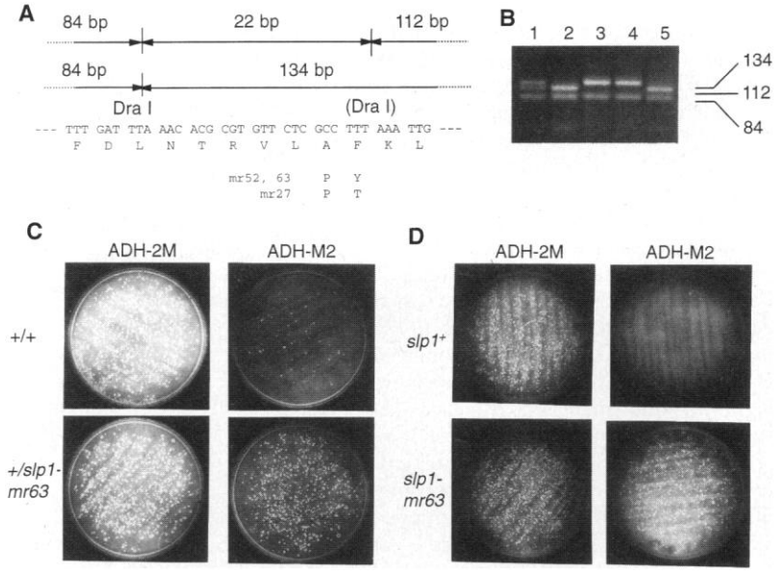


Fig. 3 (left). Loss of the spindle checkpoint in *cut7* mutant. Fluorescence-activated cell sorter (FACS) analysis of samples taken every hour after the shift to the restrictive temperature. The vertical axes indicate cell counts, and the horizontal axes, DNA content. **Fig. 4 (right).** Isolation of *slp1-mr63*. (**A**) Nucleotide and amino acid sequences of the region containing the mutation sites (Ala-131 and Phe-132). In the mutants, one of the Dra I sites (shown in parentheses) was disrupted. Dra I digestion of the PCR product yields 84-, 22-, and 112-bp fragments from a wild-type strain and 84- and 134-bp fragments from the mutant. Abbreviations for the amino acid residues are as follows: A, Ala; D, Asp; F, Phe; K, Lys; L, Leu; N, Asn; P, Pro; R, Arg; T, Thr; V, Val; and Y, Tyr. (**B**) The region shown in (A) was amplified by PCR and analyzed on a 2.5% agarose gel after Dra I digestion. Templates for PCR were genomic DNAs isolated from the heterozygous diploid (*+/slp1-mr63*, lane 1) and four segregants (lanes 2 to 5). The diploid yielded two wild-type strains (lanes 2 and 5) and two *slp1-mr63* mutants (lanes 3 and 4). (**C**) Growth of a wild-type diploid (+/+, top) and the heterozygous diploid (*+/slp1-mr63*, bottom). They were transformed with 0.5 μg of ADH-2M (left) or ADH-M2 (right). (**D**) Growth of a wild-type haploid (top) and a haploid carrying the *slp1-mr63* mutation (bottom). They were transformed with 0.5 μg of ADH-2M (left) or ADH-M2 (right).

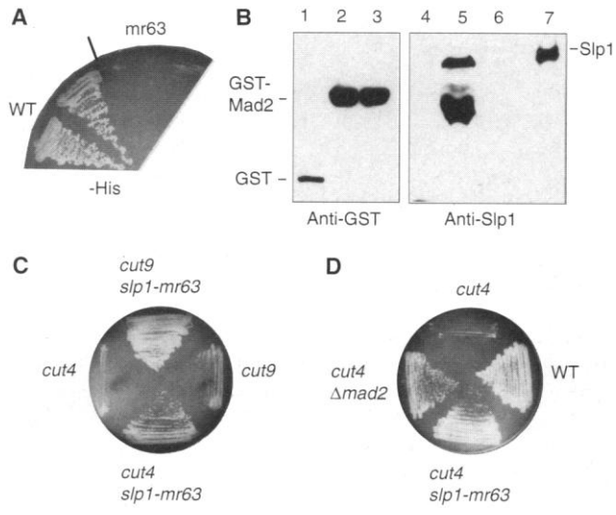


Fig. 5. Failure of Slp1-mr63 to bind to Mad2. (**A**) F1 fragment (Fig. 1D) of a wild-type (WT) or the *slp1-mr63* mutant (mr63) were tested for the ability to interact with Mad2 in the yeast two-hybrid system. (**B**) Binding of Slp1-mr63 to GST-Mad2. Cell extracts were prepared from the wild-type strain expressing GST (lanes 1 and 4) or GST-Mad2 (lanes 2 and 5). For lanes 3 and 6, cell extracts were prepared from an *slp1-mr63* mutant expressing GST-Mad2 and analyzed as described (*16*). Antibody to GST (Pharmacia) (lanes 1 to 3) or antibody to Slp1 (lanes 4 to 7) was used for protein immunoblotting. Bacterially expressed Slp1 was run in lane 7. Multiple bands (lane 5) probably represent the degradation products of Slp1. These bands, as well as the band of the intact Slp1, became more intense when *slp1*$^+$ was overexpressed. (**C**) Growth of the indicated strains transformed with pREP41-Mad2. The strains were streaked on minimum media (PMA) in the absence of thiamine to induce expression. (**D**) Growth of the indicated strains on complete media at 36°C.

reaction (PCR) followed by digestion with Dra I (*15*) (Fig. 4, A and B). Among the four haploid segregants produced meiotically from the heterozygous diploid, the Mad2-resistant phenotype gene segregated with *slp1-mr63* (Fig. 4D). These haploid cells grew normally, suggesting that *slp1-mr63* is otherwise biologically active.

The *slp1-mr63* mutation abolished binding to Mad2 in the yeast two-hybrid system (Fig. 5A) and in vivo. Mutant Slp1 protein did not precipitate with GST-Mad2 in fission yeast cell extracts, whereas wild-type Slp1 did (*16*, *17*) (Fig. 5B). We also made cells with both the *slp1-mr63* and *cut7* mutations. At the restrictive temperature, the double mutant (*slp1-mr63 cut7*) showed no delay of chromosome decondensation in response to the *cut7*-induced defect (Fig. 2D). Like Δ*mad2 cut7*, the *slp1-mr63 cut7* double mutant initiated the next round of DNA replication before the completion of the spindle assembly (Fig. 3). Furthermore, both Δ*mad2* and *slp1-mr63* were hypersensitive to thiabendazole, an inhibitor of the spindle assembly (Fig. 2B). The *slp1-mr63* mutant behaved indistinguishably from Δ*mad2*. These results indicate that Slp1 is the primary target of Mad2 and that *slp1-mr63*, defective in recognizing the signal from Mad2, abrogates the function of the spindle checkpoint.

Proteins with the destruction box, such as cyclins, Pds1, Cut2, and Ase1 (*18*), are degraded by ubiquitin-dependent proteolysis at anaphase. These proteins participate in regulating the timing of exit from mitosis or of initiation of sister chromatid separation. They are ubiquitinated by the anaphase-promoting complex (APC), which exerts the driving force to initiate anaphase (*19*). Fission yeast *cut9*$^+$ (*20*) and *cut4*$^+$ (*21*) encode components of the APC. The *cut9* and *cut4* mutants are hypersensitive to overexpression of *mad2*$^+$. When *mad2*$^+$ is overexpressed in amounts not toxic to wild-type strains, these mutants are unable to grow at the permissive temperature (*3*) (Fig. 5C). Mad2 probably inhibits the activity of APC indirectly. The *cut9* and *cut4* mutants are no longer hypersensitive to the overexpression of *mad2*$^+$ in the background of *slp1-mr63* (Fig. 5C). In addition, *slp1-mr63*, as well as Δ*mad2*, partially suppressed the *cut4* mutation. At the restrictive temperature, 36°C, the *cut4 slp1-mr63* mutant and *cut4* Δ*mad2* mutant formed colonies (Fig. 5D) at a relatively slow rate. Thus, loss of function of the spindle checkpoint appears to remove a negative constraint on APC. The *cut9* mutant is synthetically lethal in the background of a recessive mutation, *slp1-362* (*4*). Taken together, the genetic link between Mad2, Slp1, and APC suggests that the three components may act in a single sequential pathway. We propose that fission yeast Slp1 is the effector molecule that receives an anaphase-inhibitory signal from the Mad2-dependent spindle checkpoint.

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9. The NH_2-terminal 289 amino acids region of *slp1*$^+$ was used as bait. Among the ~5 × 10^5 transformants screened, 12 were selected. The subsequent analysis of the cDNA inserts revealed that all inserts contain the coding region of the *mad2*$^+$ gene.
10. GST-Slp1 and GST were purified with the glutathione–Sepharose 4B beads (GSH) as recommended by the manufacturer (Pharmacia). An in vitro–translated Mad2 protein was obtained with the use of reticulocyte lysate (Promega).
11. The *mad2*$^+$ gene was isolated from a fission yeast genomic DNA library, and the coding region was localized in 2.75 kb. An Sph I fragment containing the *ura4*$^+$ gene was inserted between two Sph I sites that were generated at ~70 bp and 540 bp downstream of the putative translation initiation site of the gene.
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13. A region of Slp1 (amino acid position 118 to 146) was subjected to site-directed mutagenesis with degenerate oligonucleotides. About 600 mutated *slp1* genes on an autonomously replicating plasmid (pSP1) were individually transformed into a tester strain (*h*$^-$ *leu1-32 ura4-d18*) with ADH-M2. A detailed protocol will be available upon request.
14. ADH-2M yielded ~1000 transformants for both the wild-type and the heterozygous diploids. ADH-M2 yielded 10 to 20 transformants for the wild-type diploid. These transformants were either stable integrants or spontaneous mutants unable to express *mad2*$^+$.
15. A pair of oligonucleotides, GGGCATATGTTTGTAAATTCTATCAGCAGTGACGTT and GGGCATATGAGTGTTAAAGCGTCGTTTAGCCGGTGT, were used for PCR.
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17. GST and GST-Mad2 were expressed and the binding assay was carried out as described (*16*).
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