DNA Replication Checkpoint Control Mediated by the Spindle Checkpoint Protein Mad2p in Fission Yeast*

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The relationship between the DNA replication and spindle checkpoints of the cell cycle is unclear, given that in most eukaryotes, spindle formation occurs only after DNA replication is complete. Fission yeast rad3 mutant cells, which are deficient in DNA replication checkpoint function, enter mitosis, and exit mitosis even when DNA replication is blocked. In contrast, the entry of cds1 mutant cells into mitosis is delayed by several hours when DNA replication is inhibited. We show here that this delay in mitotic entry in cds1 cells is due in part to activation of the spindle checkpoint protein Mad2p. In the presence of the DNA replication inhibitor hydroxyurea (HU), cds1 mad2 cells entered and progressed through mitosis earlier than did cds1 cells. Overexpression of Mad2p or inactivation of Slp1p, a regulator of the anaphase-promoting complex, also rescued the checkpoint defect of HU-treated rad3 cells. Rad3p was shown to be involved in the physical interaction between Mad2p and Slp1p in the presence of HU. These results suggested that Mad2p and Slp1p act downstream of Rad3p in the DNA replication checkpoint and that Mad2p is required for the DNA replication checkpoint when Cds1p is compromised.

Cell cycle checkpoints monitor cell cycle progression to ensure the integrity of the genome and the fidelity of sister chromatid separation (1–3). The DNA replication checkpoint blocks entry into mitosis if DNA replication is incomplete, whereas the spindle checkpoint delays sister chromatid separation until each and every kinetochore has achieved a bipolar attachment to the mitotic spindle apparatus. Cancer cells are checkpoint-defective and frequently have mutations in many of the components in the DNA replication and spindle checkpoints (4). Thus checkpoint failure contributes to cancer (5).

The relationship between the DNA replication checkpoint and the spindle checkpoint is largely unknown, given that spindle formation occurs only when cells are in M phase of the cell cycle in most eukaryotes (6). In fission yeast, like other eukaryotes, the spindle checkpoint therefore functions only after DNA replication is complete. In fission yeast two checkpoint kinases, Rad3p and Cds1p, are important for the DNA replication checkpoint (1–3). The spindle checkpoint requires the interaction of Mad2p with the anaphase-promoting complex (APC) (7, 8). APC is a multisubunit ubiquitin ligase, which targets the anaphase inhibitor securin and B-type cyclins for degradation, triggering chromosome segregation at the metaphase-anaphase transition (9, 10). APC inhibition during checkpoint activation is likely to occur through a direct interaction of Mad2p with the Slp1p/Cdc20/Fizzy/p55CDC protein (7, 8).

In fission yeast, whereas rad3 cells enter mitosis without delay when DNA replication is incomplete (1, 11), cds1 cells transiently arrest before an entry into mitosis under such conditions (12). The mechanism of this transient arrest in cds1 cells has remained unknown, although the kinase Chk1 has been implicated by the observation that the cds1 chk1 double-mutant cells enter mitosis without delay (13–15). We have now further characterized this delay in mitotic entry and progression in cds1 cells.

EXPERIMENTAL PROCEDURES

Yeast Strains—Complete medium (yeast extract plus supplements) and Edinburgh minimal medium were prepared as described (16). All strains of Schizosaccharomyces pombe were constructed by standard procedures (16). The strains used for the present study included HM4 (h·leu1-32 [wt]), HM701 (h·rad3-136 [rad3]), HM826 (h·cds1::ura4+ ura4-D18 leu1-32 [cds1]), HM849 (h·cds1::ura4 mad2::ura4 ura4-D18 leu1-32 [cds1 mad2]), HM1325 (h·rad3-136 mad2::GFP-kan ura4-D18 leu1-32 [rad3 Mad2-GFP]), HM1331 (h·cds1::ura4 mad2::GFP-kan ura4-D18 leu1-32 [cds1 Mad2-GFP]), HM1339 (h·mad2::ura4 ura4-D18 leu1-32 [mad2]), kindly provided by T. Matsumoto (7), HM1365 (h·rad3-136 mad2::ura4 ura4-D18 [rad3 mad2]), HM1371 (h·slp1-362 rad3-136 leu1-32 [slp1 rad3]), HM1373 (h·slp1-362 cds1::ura4 ura4-D18 leu1-32 [slp1 cds1]), HM1402 (h·slp1-362 leu1-32 [slp1]), kindly provided by T. Matsumoto (17), HM1450 (h·mad2-GFP-kan leu1-32 [wt Mad2-GFP]), kindly provided by T. Toda (18), HM1481 (h·sue2-GFP-kan leu1-32 [Sue2-GFP]), HM1570 (h·bub1::kan ura4-D18 leu1-32 [bub1]), HM1670 (h·bub1::kan cds1::ura4 ura4-D18 leu1-32 [bub1 cds1]), HM1700 (h·mad1::ura4 ura4-D18 leu1-32 [mad1]), kindly provided by T. Matsumoto (19, 20), HM2251 (h·cds1::mad1::ura4 ura4-D18 leu1-32 [mad1 cds1]), HM2255 (h·mad1::kan leu1-32 [mad1]), HM2256 (h·cds1::ura4 mad2::kan ura4-D18 leu1-32 [mad3 cds1]), and HM2284 (h·slp1-362 rad3-36 mad2::ura4 ura4-D18 leu1-32 [slp1 rad3 mad2]).

Antibodies and Immunoblot analysis—Immunoblot analysis was performed with rabbit polyclonal antibodies to Cdc2p phosphorylated on Tyr15 (1:750 dilution, Cell Signaling Technology), mouse monoclonal

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antibodies to Cdc13p (1:600 dilution, SP4, kindly provided by H. Yamano) (21), mouse monoclonal antibodies to green fluorescent protein (GFP) (1:1000 dilution, Roche Applied Science), rabbit polyclonal antibodies to Slp1p (1:30,000 dilution, kindly provided by T. Matsumoto) (7), and mouse monoclonal antibodies to α-tubulin (1:20,000 dilution, Sigma). Cell extracts (40 µg of protein) prepared as described previously (22) were fractionated by SDS-polyacrylamide gel electrophoresis on a 10% gel, and the separated proteins were trans-
ferred to an Immobilon-P membrane (Millipore). Immune complexes were detected with anti-mouse or anti-rabbit secondary antibodies (both 1:1000 dilution) and ECL reagents (Amersham Biosciences).

Immunoprecipitation—Extracts were prepared from frozen cells with glass beads in HB buffer (25 mM 3-morpholinopropanesulfonic acid, pH 7.2, 15 mM MgCl2, 15 mM ethyleneglycoltetraacetic acid, 1 mM dithiothreitol, 1% Triton X-100, 60 mM β-glycerophosphate, 15 mM p-nitrophenylphosphate, 0.1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, 40 μg/ml apronin) (22), and a soluble fraction was obtained by centrifugation at 13,000 rpm for 15 min at 4 °C. Monoclonal antibodies to GFP (3 μg/ml) and ECL reagents (Amersham Biosciences) were added to the soluble extract (1.5–2.0 mg of protein). After rotation for 2 h at 4 °C, the beads were washed, resuspended in 20 μl of HB buffer, and mixed with 5 μl of 5X Laemmli sample buffer. The samples were heated for 5 min at 100 °C and subjected to immunoblot analysis.

4,6-Diamidino-2-phenylindole (DAPI) and Flow Cytometric Analysis—For detection of the cut phenotype, cells were stained with DAPI as described previously (22). Flow cytometric analysis was performed as described previously (22).

Transformation—Transformation was performed as described (23). The pREP3X-mad2 vector was kindly provided by T. Matsumoto.

RESULTS

Role of Mad2p in the DNA Replication Checkpoint—We first examined the cell length in the DNA replication checkpoint mutant cells after 6 h with the ribonucleotide reductase inhibitor hydroxyurea (HU) treatment (Fig. 1, a and b). In fission yeast, premature entry into mitosis causes cells to divide in a reduced cell size, whereas activation of the DNA replication checkpoint prevents or delays entry into mitosis, resulting in elongated cells. HU at the concentrations used in this study has a limited half-life in fission yeast experiments because of the up-regulation of ribonucleotide reductase and the metabolism of HU itself. Therefore, we monitored the DNA contents using flow cytometric analysis. Exposure to HU inhibited the completion of DNA replication by at least 8 h as judged by flow cytometric analysis (Fig. 1c) and resulted in a marked increase of elongated cells in wild-type (wt), whereas with HU treatment the rad3 mutant cells were smaller than wt cells (Fig. 1, a and b, as reported previously (2, 3). HU treatment in cds1 mutant (cds1) cells resulted in an intermediate phenotype between rad3 and wt cells (Fig. 1, a and b) (12). We next monitored the cells for the “cut” phenotype, which is characterized by the septum either cleaving the single nucleus or dividing the cell giving rise to one daughter anucleate and is a marker for aberrant mitotic cells. In rad3 cells more than 70% of the cells showed the cut phenotype at 4 h after HU addition, whereas the appearance of the cut phenotype was delayed in cds1 cells (Fig. 1, a–c) (12). Because these DNA replication checkpoint mutant cells enter mitosis in the presence of unreplicated DNA, spindle formation would be defective in these cells. We next tested whether the spindle checkpoint proteins Mad1, Mad2, Mad3, and Bub1 are required for the DNA replication checkpoint (19, 20). After exposure to HU, mad1 (mad1), mad2 (mad2), mad3 (mad3), and bub1 mutant (bub1) cells showed elongated phenotype like wt cells (Fig. 1, a and b). However, the cut phenotype was apparent in a small proportion of mad2 cells after exposure to HU for 8 h suggesting that Mad2p plays a role, albeit a minor one, in the DNA replication checkpoint (Fig. 1c). In contrast, a proportion of this phenotype in mad1, mad3, and bub1 is similar to wt cells. To determine whether the spindle checkpoint proteins are required for the arrest in cds1 cells under such conditions, we constructed cds1 mad1, cds1 mad2, cds1 mad3, and cds1 bub1 double-mutant cells. We examined the cell length and the cut phenotype in these cells when DNA replication was incomplete with HU treatment (Fig. 1, a–c). In the presence of HU, cds1 mad2 cells were smaller than cds1 cells (Fig. 1, a and b), indicating that entry into mitosis was advanced in the double mutant. In addition, cds1 mad2 double-mutant cells manifested the cut phenotype earlier than cds1 cells but later than rad3 cells (Fig. 1c). These results suggested that Cds1p acts synergistically with Mad2p in the DNA replication checkpoint. However, mad1, mad3, or bub1 cells did not show a synergistic effect with cds1 mutant cells in DNA replication checkpoint function. These results suggested that Mad2 is specifically involved in the DNA replication checkpoint. We next tested whether Mad2p has an additional role in the DNA replication checkpoint that Rad3p does not have (Fig. 1, a–c). The rad3 mad2 double-mutant cells showed the cut phenotype at the same timing as rad3 cells, suggesting that Mad2p functions in the same pathway as Rad3p.

To confirm that the effect of the cds1 mad2 double mutant is because of the defect of cds1" and mad2" genes but not other genes, we introduced the cds1" and mad2" gene into the double mutant. As shown in Fig. 1c, the cut phenotype of the double mutant was complemented almost completely by cds1" and partially by mad2". Almost no cut phenotype at 8 h by cds1" may be because of the effect of cds1" overproduction or minimum medium in which the appearance of the cut phenotype is delayed.

HU, Ultraviolet (UV), and Methylmethan Sulfonate (MMS) Sensitivity—If Cds1p and Mad2p signaling pathways contributed independently to cell cycle arrest after treatment of cells
with HU, it would be expected that each pathway would also contribute independently to cell survival in the presence of HU. To test this prediction, we determined cell survival after plating serial dilutions of cells in the presence of HU (Fig. 2a). The sensitivity of \(\text{cds1}\) cells to HU was greater than that of \(\text{wt}\) or \(\text{mad2}\) cells (12). The HU sensitivity of \(\text{cds1 mad2}\) cells was in turn greater than that expected from an additive effect of the two individual mutant genes. The sensitivity of \(\text{rad3}\) cells was even greater than that of the \(\text{cds1 mad2}\) double mutant cells, possibly because of the additional roles of Rad3p in the DNA replication checkpoint. This observation was consistent with the kinetics of development of the cut phenotype and suggests that there is a critical period during which cells can be rescued from HU toxicity by activating the DNA replication checkpoint.
that Rad3p plays roles that are independent of Cds1p and Mad2p in HU resistance. Neither the cds1 or mad2 single mutants nor the cds1 mad2 double mutant showed an increased sensitivity to UV radiation (Fig. 2b). Similarly, neither the cds1 or mad2 single mutants nor the cds1 mad2 double mutant showed an increased sensitivity to 0.001% MMS, which induces DNA alkylation (Fig. 2c). At 0.005% MMS, however, the sensitivity of cds1 cells was greater than that of wt or mad2 cells (Fig. 2d). In addition, the MMS sensitivity of cds1mad2 cells was greater than that of the cds1 cells, which is similar to that found in HU. Higher concentrations of MMS can block DNA synthesis by stalling DNA replication forks (24). These results indicated that Cds1p and Mad2p act synergistically to promote cell survival when DNA replication is inhibited but that these proteins seem not to contribute to a tolerance to the DNA damage that does not affect DNA synthesis significantly.

Cdc2p Tyr15 Phosphorylation and Cdc13p Level in HU—The DNA replication checkpoint maintains the phosphorylation of Cdc2p on Tyr15 (25). The spindle checkpoint inhibits the activity of the APC and thereby prevents cyclin degradation (10). We therefore monitored the phosphorylation of Cdc2p on Tyr15 and the abundance of the major B-type cyclin, Cdc13p, in the various mutant yeast cells after exposure to HU (Fig. 3). Both Cdc2p Tyr15 phosphorylation and the abundance of Cdc13p increased in wt and mad2 cells. In cds1 cells both Cdc2p Tyr15 phosphorylation and the abundance of Cdc13p increased in wt and mad2 cells. In the various mutant yeast cells after exposure to HU (Fig. 3). Both Cdc2p Tyr15 phosphorylation and the abundance of Cdc13p were transiently increased and then decreased in HU. Both Cdc2p Tyr15 phosphorylation and the abundance of Cdc13p were not increased in rad3 or cds1 mad2 cells. These results suggested that either Cds1p or Mad2p is required for the maintenance of Cdc2p Tyr15 phosphorylation and Cdc13p abundance in HU-treated cells.

Mad2p Level and Its Interaction with Slp1p in HU—We next examined the effects of HU exposure of the abundance of Mad2p in the various mutant cells (Fig. 4, a and b). For these experiments, we used yeast strains in which Mad2p was tagged with GFP. Treatment of the exponential cultures of wt and rad3 mutant cells with HU resulted in a slight reduction of Mad2p. In contrast, the exposure to HU did not vary the amount of Mad2p in cds1 cells, implicating that Cds1p is involved in the maintenance of Mad2 in HU.

The impairment of spindle formation results in the direct binding of Mad2p to the APC regulator Slp1p and consequent inhibition of APC activity (7). To determine the precise role of Rad3p, we examined the physical interaction between Mad2p and Slp1p (Fig. 4, c and d). Because the Mad2p level in HU at...
4 h is similar to that without HU, we monitored the Slp1p level associated with Mad2p. We immunoprecipitated Mad2p-GFP from cells before or 4 h after exposure to HU and then subjected the resulting precipitates to immunoblot analysis with antibodies to Slp1p. The amount of Slp1p in the cell extracts and the supernatants was almost constant regardless of HU treatment in wt, cds1, and rad3 cells. Slp1p coprecipitated with Mad2p regardless of HU treatment in wt and cds1 cells. In contrast, in rad3 cells, the amount of Slp1p that coprecipitated with Mad2p in the absence of HU treatment was reduced, compared with the amount apparent for wt cells, and was decreased further after exposure to HU probably because of the accumulation of rad3 cells in mitosis. These data suggested that Rad3p is required for the interaction between Mad2p and Slp1p. To confirm that Slp1p specifically interacts with Mad2p, we immunoprecipitated Suc22p-GFP and treated it similarly to Mad2p-GFP (Fig. 4c). No significant interaction between Suc22p and Slp1p was observed, indicating that the anti-GFP antibody did not interact nonspecifically with Slp1p.

**Genetic Interactions among Rad3p, Mad2p, and Slp1p**—To determine whether Slp1p acts downstream of Rad3p, we examined the effect of inactivation of Slp1p on the DNA replication checkpoint defect in rad3 cells (Fig. 5). We synchronized rad3 cells containing a temperature-sensitive slp1 mutation in the G1 phase of the cell cycle by nitrogen deprivation and then released them into medium containing HU at the restrictive temperature for the slp1 mutation. All of the cells tested recovered from G1 arrest as judged by flow cytometric analysis. The development of the cut phenotype was markedly inhibited by the inactivation of Slp1p in rad3 cells (Fig. 5a). The inactivation of Slp1p slightly increased the survival of rad3 cells after exposure to HU (Fig. 5b). In addition, slp1Δ rad3Δ mad2Δ cells showed a similar cut phenotype and viability to slp1Δ rad3Δ cells, suggesting that Rad3p and Mad2p function in the same pathway. In contrast, the cut phenotype was not manifested in cds1 cells probably because of the complete block of DNA replication and the survival of cds1 cells was not suppressed by the Slp1p inactivation. These results thus suggested that Rad3p, but not Cds1p, acts upstream of Slp1p.

Finally, we examined the effect of overexpression of Mad2p on the development of the cut phenotype in HU-treated rad3 cells (Fig. 6). The ectopic expression of Mad2p in a wt background has previously been shown to result in metaphase arrest (26). Overexpression of mad2Δ in rad3 cells reduced the proportion of cells that developed the cut phenotype after exposure to HU, although mad2Δ was not as effective in this regard as cds1Δ. These results suggested that Mad2p acts downstream of Rad3p in the DNA replication checkpoint.

**Discussion**

A failure to complete DNA replication in cds1 cells treated with HU results in a delay in the entry into mitosis (12). In addition to Chk1p (13–15), Mad2p is required for the transient cell cycle arrest in cds1 cells, because cds1Δ mad2Δ double-mutant cells enter and progress through mitosis earlier than cds1Δ cells do after treatment with HU. These observations further suggest that Mad2p and Cds1p act in a synergetic manner in the DNA replication checkpoint. It is thus possible that HU treatment induces a spindle defect in cds1 cells and that this defect is detected by Mad2p. Nevertheless, other spindle checkpoint proteins Bub1p, Mad1p, and Mad3p did not show a synergetic effect with Cds1p in DNA replication checkpoint function. It is unlikely that Mad2p detects the spindle defect alone. Given that Mad2p forms a complex with Mad1p and Mad3p (19, 20) and that Mad1p and Mad3p do not contribute to the DNA replication checkpoint, Mad2p may form distinct complexes in response to different perturbations of the cell cycle. Together, these observations suggested that Mad2p is required specifically among spindle checkpoint proteins for DNA replication checkpoint function.

The observation that a small proportion of mad2Δ cells developed the cut phenotype after prolonged exposure to HU further supports a role for Mad2p in the DNA replication checkpoint. Mad2p associates specifically with proteins that interact with DNA polymerase ζ in human cells (27). Thus, it is possible that in addition to monitoring the status of the spindle, Mad2p checks the progress of DNA replication by interacting with proteins that contribute to this process.

**How is Mad2p regulated in the DNA replication checkpoint?** Mad2p directly binds to Slp1p (also known as Cdc20 or Fizzy) and thereby inhibits APC activity (7, 8). We have now shown that in the presence of HU the abundance of Mad2p is maintained and Mad2p binds to Slp1p, at least in part, in a Rad3p-dependent manner. The DNA replication checkpoint defect in rad3 cells was also rescued by overexpression of Mad2p or by inactivation of Slp1p. Furthermore, the major B-type cyclin Cdc13p was degraded in both rad3Δ and cds1Δ mad2Δ cells in the presence of HU. On the basis of these observations and the results of previous studies (28), we propose the following model. The failure to complete DNA replication is associated with the generation of replication bubbles and replication intermediates that are maintained by the DNA replication initiation proteins Orp1p and Cdc15p. These structures generate the DNA replication checkpoint signal, which then activates Rad3p. The signal is then transmitted to Cds1p, which is indirectly required for the maintenance of the phosphorylation of Cdc2p on Tyr15. If the operation of this system is impaired, Rad3p activates Chk1p and Mad2p. Chk1p inhibits Cdc25p leading to the phosphorylation of Cdc2p on Tyr15, whereas Mad2p prevents B-type cyclin proteolysis by binding to Slp1p. However, given that Mad2p and Cds1p both appear to play a role in the DNA replication checkpoint before spindle formation, it is likely that Mad2p not only inhibits Slp1p function but also affects the phosphorylation of Cdc2p on Tyr15 by an unknown mechanism. The relationship between Mad2p and Chk1p is largely unknown at present, although both are required for the DNA replication checkpoint when Cds1p is impaired. It is likely that Mad2p and Chk1p act in the same pathway for the DNA replication checkpoint.

**In budding yeast, the DNA replication checkpoint arrests cells in a metaphase-like state with spindle formation rather than in interphase (29), as in most other eukaryotes, including fission yeast.** Consistent with this observation, Mad1p and Mad2p were recently shown to be required for the DNA replication checkpoint in budding yeast (30). These findings are also consistent with the notion that unreplicated chromosomes lacking tension trigger activation of the spindle checkpoint (31, 32). Spindle checkpoint proteins thus monitor a wider spectrum of chromosome abnormalities than previously recognized, and the role of Mad2p in the DNA replication checkpoint is apparently conserved between budding and fission yeasts, although the fundamental mechanisms of its action differ. Homologs of Rad3p, Cds1p, Mad2p, and Slp1p are present in higher eukaryotes, and their functional roles are generally conserved (33, 34). All of these proteins are required for the maintenance of genomic stability. It will thus be important to establish whether similar checkpoint controls operate in multicellular eukaryotes.

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