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Mik1 levels accumulate in S phase and may mediate an intrinsic link between S phase and mitosis

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Two paradigms exist for maintaining order during cell-cycle progression: intrinsic controls, where passage through one part of the cell cycle directly affects the ability to execute another, and checkpoint controls, where external pathways impose order in response to aberrant structures. By studying the mitotic inhibitor Mik1, we have identified evidence for an intrinsic link between unperturbed S phase and mitosis. We propose a model in which S/M linkage can be generated by the production and stabilization of Mik1 protein during S phase. The production of Mik1 during unperturbed S phase is independent of the Rad3- and Cds1-dependent checkpoint controls. In response to perturbed S phase, Rad3-Cds1 checkpoint controls are required to maintain high levels of Mik1, probably indirectly by extending the S phase period, where Mik1 is stable. In addition, we find that Mik1 protein can be moderately induced in response to irradiation of G2 cells in a Chk1-dependent manner.

Ordered progression of the cell cycle is essential for genome integrity (1). Two mechanisms that maintain ordered progression of G1, S, G2, and M have been identified: intrinsic linkage of one event upon another and the imposition of order by active pathways known as checkpoint controls. An example of intrinsic linkage is the mechanism ensuring that replication origins fire only once per cell cycle (2). Replication origins have been broadly categorized into two states: prereplicative (G1) and postreplicative (G2, unable to support replication). Transition between the postreplicative (G2) and prereplicative (G1) states is controlled by cyclin-dependent kinase activity. Passage through, followed by exit from, mitosis (which requires low cyclin-dependent kinase activity) is necessary to license an origin. When an origin is replicated subsequently, it loses the license and becomes postreplicative. Thus, rereplication is prevented as a consequence of cell-cycle progression. An example of active imposition of order is the metaphase to anaphase checkpoint (3); chromosomes that are not bivalently attached to the spindle are actively detected, and a signal is generated to prevent anaphase. Loss of a checkpoint does not necessarily perturb the cell cycle; thus, checkpoint pathways have been defined by loss of function mutants (4).

In all eukaryotes, perturbations to DNA replication prevent the subsequent mitosis (5). Checkpoint controls required for this linkage have been identified. In the fission yeast Schizosaccharomyces pombe, treatment of cells with the S phase inhibitor hydroxyurea (HU) activates the Rad3-dependent checkpoint pathway (6, 7). Rad3 is required to phosphorylate and activate the Cds1 kinase (8). Cds1 may inhibit mitosis directly, and it also regulates DNA replication (8, 9). However, during unperturbed growth, Cds1 kinase is not active in S phase (8), suggesting that the Rad3-Cds1 checkpoint is not involved in linking mitosis to S phase unless replication is perturbed. By studying Mik1 (a p34Cdc2 inhibitor) during the unperturbed cell cycle and in response to HU treatment, we uncovered evidence that leads us to propose an intrinsic linkage between S phase and mitosis. In previous studies, this linkage has been obscured by the Rad3-Cds1 checkpoint, which prevents mitosis when S phase is perturbed. We also find that Mik1 can be moderately induced in G2 cells in a Chk1-dependent manner, independently of the Mik1 responses in S phase.

Materials and Methods

Genetics, Molecular Biology, and Cell Biology Techniques. Genetic procedures and fluorescence-activated cell sorter (FACS) analysis have been described (8, 10). FACS profiles of asynchronous cultures show the majority of cells in G2 (2n) and a population of ~10% within S phase, indicated by the 2–4n-shoulder (S. pombe undergoes replication while daughter cells are still attached). A truncated mik1 gene (lacking the first 974 bp of the ORF) was identified in a screen for multicopy suppressors of rad3+ HU sensitivity (11) at the semipermissive temperature (32°C). The plasmid did not affect cell size, and suppression was specific to the rad3+ allele.

Epitope Tagging of Mik1. A genomic 1,053-bp BamHI–HindIII fragment (648 bp of the mik1 ORF and 405 bp of 3’ untranslated region) was PCR amplified, and an NdeI site was created before the stop codon. Two MYC epitopes and 6× His residues (12) were inserted. The construct was integrated by using sup3-5 suppression of ade6-704 (13) and confirmed by Southern blotting. The tagged protein was shown to be functional by crossing mik1-MYC to wee1.50 and assaying viability at 36°C.

Preparation of Protein Extracts. Cells were disrupted with glass beads in a Ribolyser (Hybaid, Middlesex, U.K.) in 50 mM Tris/80 mM β-glycerolphosphate/250 mM NaCl/5 mM EDTA/50 mM NaF/0.1 mM sodium orthovanadate/1 mM DTT/15 mM dinitrophenyl phosphate/0.1% NP-40 adjusted to pH 7.5 and supplemented with a protease inhibitor mixture [AEBSF (4-(2-aminoethyl)benzenesulfonyl fluoride), leupeptin, aprotinin, and pepstatin at 10 μg/ml]. Extract was cleared (1,500 rpm for 2 min), and the supernatant (low spin) was centrifuged at 100,000 g for 1 h. The pellet was solubilized in SDS sample buffer and loaded onto 10% SDS gels. Mik1-MYC was detected with an anti-MYC monoclonal antibody (9E10, PharMingen), and the tagged protein was shown to be functional by crossing mik1-MYC to wee1.50 and assaying viability at 36°C.

Immunoblotting. Total protein (50 μg; Bradford) was boiled in SDS sample buffer and loaded onto 10% SDSPAGE and electroblotted to nitrocellulose. The membrane was probed with anti-MYC monoclonal antibody (9E10, PharMingen), and the tagged protein was shown to be functional by crossing mik1-MYC to wee1.50 and assaying viability at 36°C.

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Abbreviations: HU, hydroxyurea; FACS, fluorescence-activated cell sorter; DAPI, 4’,6-diamidino-2-phenylindole.

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(wt/vol) fat free milk powder/0.1% Tween 20. Membranes were incubated in Blotto containing 9E10 (dilution 1:1,000) or anti-HA monoclonal antibody (dilution 1:500), washed in Blotto, and incubated with peroxidase-conjugated secondary antibody (Dako, 1:5,000 dilution). Enhanced chemiluminescence (Amersham Pharmacia) was used for detection.

**Phosphatase Treatment.** Total protein (50 μg; high spin supernatant) was diluted in phosphatase buffer and incubated for 20 min at 0 or 30°C in the presence or absence of 2,000 units of λ-Phosphatase (BIOLABS, Northbrook, IL). Phosphatase inhibitors (sodium orthovanadate, Na2EDTA) were added to one sample.

**Elutriation.** Mid-log-phase cells (5 liters) were loaded on an elutriator (JE-5.0, Beckman Coulter). Small cells, those in G2, were collected, harvested, and resuspended in fresh medium (3 × 10^6 cells per ml). Septation was determined by fixing cells in methanol and staining with 4',6-diamidino-2-phenylindole (DAPI) and Calcofluor. Samples for protein extract were washed in ice-cold water, frozen in LN2, and stored at −80°C. For wee1-HA extracts, 50 μg (total protein) of high spin supernatant was Western blotted, and for mik1-MYC extracts, 50 μg (total protein) of high spin pellet was Western blotted.

**Immunofluorescent Microscopy.** Staining was performed as described (14). Cells were fixed in 3.7% (vol/vol) paraformaldehyde for 10 min and stained with primary antibody (9E10 at 1:50 or anti-Sad1 antibody at 1:500; ref. 15) and secondary antibody [FITC-conjugated anti-mouse (Dako) at 1:150 or CY3 conjugated anti-rabbit (The Jackson Laboratory) at 1:250].

**Northern Blotting.** Total RNA was isolated as described (16). Northern blot analysis and hybridization of membranes to single-stranded 32P-labeled RNA probes were performed as described (17). Single-stranded RNA probes specific for mik1 were transcribed from the pGEM3 vector (Promega) containing a 451-bp EcoR1–SalI fragment of mik1 ORF.

**Results**

In a screen for multicopy suppressors of HU-induced lethality of a temperature-sensitive rad3 mutant (11), we identified a truncated mik1 ORF. Mik1 negatively regulates p34Cdc2 (18), which prompted an examination of Mik1 through the cell cycle and in response to checkpoint activation. A mik1-MYC strain, in which Mik1 is tagged and under control of its own promoter, was created. Mik1-MYC is largely present in the insoluble portion of cell extracts (Fig. 1A) and exists as multiple isoforms generated by phosphorylation (Fig. 1B). We find no evidence that these modifications change through the cycle or in response to checkpoint activation.

**Mik1 Protein Is S Phase Specific.** To determine the distribution of Mik1-MYC through the cell cycle, aliquots of a synchronous mik1-MYC culture were analyzed by Western blotting (Fig. 1C). Mik1-MYC peaks coincidently with the septation peak, a marker for S phase. We also synchronized a wee1-HA strain (19), because both Wee1 and Mik1 negatively regulate p34Cdc2. Wee1-HA levels are relatively constant throughout the cell cycle (Fig. 1D), modestly increasing during G2. These cells have a partial wee1 phenotype, possibly contributing to the short interval between the peaks of septation.

To ascertain whether the presence and phosphorylation of Mik1-MYC in S phase cells depends on Rad3 and Cds1, Mik1-MYC levels were analyzed in asynchronous rad3-d and cds1-d cultures. In both, Mik1-MYC levels increased in S phase and decreased in G2 (Fig. 2A). Thus, Rad3 and Cds1 proteins are not required for S phase Mik1-MYC accumulation. We also examined the cellular localization of Mik1-MYC by immunofluorescence microscopy. Mik1-MYC is localized to the nucleus in S phase (i.e., septated) cells but is not detected in the vast majority of mononuclear G2 cells (Fig. 2B).

**mik1 Transcript Is Up-Regulated in S Phase and Maintained in S Phase-Arrested Cells.** It has been reported (20) that Mik1 levels accumulate in a Rad3–Cds1-dependent manner in asynchronous cultures blocked in S phase by HU. We have confirmed this finding (Fig. 2C) and also show that the levels increase significantly above those seen in untreated S phase (Fig. 2D). The Mik1-MYC accumulation in the nucleus of all mononuclear S phase-arrested cells (Fig. 2E) is consistent with a Rad3–Cds1-dependent prolongation of S phase and subsequent increase in Mik1 levels. It has been reported that the mik1 transcript is not S phase-regulated (20). Our data suggested that mik1 transcript is up-regulated either in S phase cells or in response to HU treatment. To explore this suggestion, we synchronized mik1+, rad3-d mik1+, and cds1-d mik1+ cells for Northern analysis. Like the Mik1-MYC protein, mik1 transcript is up-regulated in unperturbed S phase in a Rad3–Cds1-independent manner (Fig. 3A), and Rad3 and Cds1 are required to maintain high levels of mik1 transcript when S phase is blocked by HU (Fig. 3B).

**Induction of Mik1 After Irradiation.** The mechanisms that ensure that mitosis is dependent on S phase completion share many
Total protein (pellet fraction; 50 or chk1-d rad monitore d Mik1-MYC protein (Fig. 4) be realized), recovery (designed to allow induced changes in protein levels to be studied).

These DNA damage responses regulate S phase progression by overlapping components with the DNA damage checkpoints. Thus, all cells are in or proceed into G1 and S with damaged DNA.

After 250 Gy of ionizing radiation (12.5 Gy/min) and 45-min recovery (designed to allow induced changes in protein levels to be realized), rad^+ cultures show a modest increase in Mik1-Myc levels. rad^3-d cultures do not show this increase. Unexpectedly, chk1-d cultures show a more pronounced increase in Mik1-Myc, whereas cds1-d cultures show a striking decline, below the level seen in the unirradiated cells. We postulated that these responses may be due to different damage-induced cell-cycle delay points in the different mutants. To address this possibility, we first determined the extent of S phase delay (intra-S phase checkpoint) for DNA damage (DAPI), Mik1-MYC (α-Myc), or the spindle pole marker Sad1 (α-Sad1; ref. 15). S indicates a septating S phase cell.

In Mik1-Myc, while the daughter cells are still attached via the septum, irradiating synchronous rad^+, and rad3^- cultures with and without prior irradiation before S phase (Fig. 4B). A rad3^-dependent delay of approximately 40 min is observed.

Next, we monitored Mik1-MYC protein levels in synchronous rad^+, rad3^-d, chk1^-d, and cds1^-d cells before and after 250 Gy of irradiation (plus 45 min of recovery time) every 20 min through the cell cycle (Fig. 5A). In rad^+ and cds1^-d cultures, cells irradiated in G2 do not enter mitosis or the subsequent S phase. These cultures show a slight increase in Mik1-Myc protein (i.e., rad^+ time 100; cds1^-d time 120). This increase suggested that a Cds1-independent G2-specific response affects Mik1-Myc. Because rad3^-d and chk1^-d cells continue to cycle through M and into the subsequent S phase after irradiation, we used cdc25^-22 arrested rad^+, cds1^-d, chk1^-d, and rad3^-d to study the Mik1-Myc response in G2 (Fig. 5B). In these cultures, cell-cycle progression after irradiation is prevented by continued incubation at the cdc25^-22 restrictive temperature. A Chk1-dependent increase in Mik1-Myc levels in G2 cells after irradiation is clear.

S. pombe cells undergo replication very soon after mitosis, while the daughter cells are still attached via the septum. Irradiating synchronous rad^+ or cds1^-d cells at the peak of septation (Fig. 5A, rad^+; times 20 and 40; cds1^-d, times 40 and 60) will divide the population into two: those cells that have not passed M and that arrest in G2 and those cells that have passed M and have therefore been irradiated in G1 or S phase. By comparing rad^+ (times 20 and 40) with cds1^-d (times 40 and 60), it is clear that irradiated G1/S phase cells require Cds1 to maintain significant levels of Mik1-Myc; however, these levels are already declining after 45 min of recovery, because the Rad3^- and Cds1-dependent intra-S phase checkpoint is brief after γ-irradiation (Fig. 4B). Irradiating chk1^-d cells at the peak of septation (times 40 and 60) does not separate the culture into two populations, because G2 cells can continue through mitosis. Thus, all cells are in or proceed into G1 and S with damaged DNA.

Fig. 2. (A) mik1-MYC cells (in a wild-type (wt), cds1^-d, or rad3^-d background) were synchronized in G2 by elutriation. Every 15 min, the septation index was monitored. At times indicated by 1 → 4, protein was extracted, and 50 μg (pellet fraction) was analyzed by Western blotting with 9E10. (B) mik1-MYC cells and untagged control cells were grown to mid-log phase (Left) or arrested in S phase by using 20 mM HU for 3.5 h. Cells were fixed and stained for DNA (DAPI), Mik1-MYC (α-Myc), or the spindle pole marker Sad1 (α-Sad1; ref. 15). S indicates a septating S phase cell. Within the unperturbed population, >90% of septated cells were stained. Less than 10% of unseptated cells were stained. (C) Asynchronous mik1-MYC cells (in wild-type, rad^-d, cds1^-d, or chk1^-d backgrounds) and an untagged control were grown to mid-log phase and arrested in S phase (20 mM HU; 3.5 h), and protein was extracted. Total protein (pellet fraction; 50 μg) was analyzed by Western blotting with 9E10. (D) mik1-MYC cells were synchronized in G2 by elutriation, and the culture was divided in two. HU (20 mM) was added to one half. Samples were taken for septation index every 15 min and for protein extraction and analysis (as above) at the times indicated by 1 → 4.
DNA and can activate the intra-S phase checkpoint. Consistent with this finding, we see more Mik1-Myc in cells irradiated at times 40 and 60. Similarly, cells irradiated in G2 (times 0, 20, 120, and 140) have high Mik1-Myc levels, because these cells proceed through mitosis and into S phase before harvesting. rad3-d cells are different from rad+ , cds1-d , or chk1-d , because cells irradiated in either G2 or S phase will continue to cycle. Furthermore, after 250 Gy, most rad3-d cells will die—many of them in S phase (7).

By carefully examining the data, we draw the following conclusions: (i) irradiating cells in different stages of the cycle produces different results, even when a single end point is monitored, and (ii) the Chk1-dependent G2/M checkpoint has a very modest effect on Mik1 protein levels that could be easily obscured by the more robust Cds1-dependent intra-S phase checkpoint.

Discussion

In fission yeast, the timing of mitosis is controlled through the phosphorylation status of the p34Cdc2 Y15 residue (22). De-phosphorylation of Y15 by the Cdc25 phosphatase promotes mitosis. Phosphorylation of Y15 by either the Wee1 or Mik1 kinases inhibits mitosis (23). Exactly how the DNA-integrity checkpoints interact with the regulators of p34Cdc2 is not clear. Analysis of the genetic and biochemical data concerning how the S/M checkpoint and the DNA damage checkpoint interact with these regulators suggests that there are overlapping pathways leading to the inactivation of p34Cdc2.

The Role of Mik1 in Linking S Phase to Mitosis. Gain of Cdc25 function significantly abrogates the HU-induced checkpoint (7), and in vitro phosphorylation data are consistent with Cdc25 being a target of the Rad3-Cds1 checkpoint (24). Loss of function mutants in wee1 show no evidence of mitosis when replication is inhibited, but mik1 loss of function results in a partial yet significant mitotic arrest defect (25, 26). Wee1 and Mik1 share a redundant essential function, because deletion of both wee1 and mik1 in the same cell causes a lethal mitotic catastrophe phenotype (18). However, although a double mutant of wee1–50 (a temperature-sensitive allele of wee1) and mik1-d is inviable at 35.5°C, it is viable at 27°C. Interestingly, and importantly for our interpretation, these cells have a complete defect in mitotic arrest (25) when S phase is inhibited by HU at the permissive temperature (27°C). These data clearly implicate an overlapping function of the Wee1 and Mik1 kinases in control of mitosis during DNA replication arrest. Furthermore, deletion of both wee1 and cdc25 results in cells that are still able to produce a mitotic delay when exposed to HU, again suggesting a role for Mik1 (7).

Our analysis of Mik1 helps to explain the complexity of the genetic data and has led us to develop a model in which two distinct mechanisms can control mitosis during DNA replication (Fig. 6). The first control is intrinsic; Mik1, an inhibitor of mitotic p34Cdc2 , is produced and stabilized during S phase, thus preventing S phase cells from entering mitosis. At the completion of S phase, Mik1 is presumably degraded. Such regulation would establish a situation wherein cells in S phase are intrinsically unable to attempt mitosis.

The second control is the active imposition of a Rad3- and Cds1-dependent S/M checkpoint. This checkpoint is engaged only when replication problems arise and is not active in unperturbed S phase (8). The Rad3-Cds1 checkpoint has at least two effects. First, it maintains cells in S phase (8), which may well be sufficient to ensure that Mik1 levels accumulate and remain high, although direct effects on mik1 transcription or protein stability are not excluded. The second effect of Rad3-Cds1 also produces mitotic delay. This delay may be achieved directly by inhibiting the p34Cdc2 activator and/or activating the p34Cdc2 inhibitors; Cds1 has been proposed to phosphorylate directly both Wee1, an inhibitor of p34Cdc2 (20), and Cdc25, the activator of p34Cdc2 (24). The genetic evidence also suggests Rad3-Cds1 checkpoints target both Wee1 and Cdc25; up-regulated Cdc25 abrogates the checkpoint, and in the absence of Mik1, the wee1–50 mutation is defective for HU-induced mitotic delay even at the permissive temperature of 27°C (when its function in the size control mechanism remains intact; ref. 25).

One of the conundrums relating to mik1 is the lack of a clear phenotype when mik1 is deleted. In rapidly proliferating cultures, S. pombe cells complete S phase over 60 min before they attempt mitosis. Thus, deletion of Mik1 might not be expected to reveal mitotic phenotypes under standard laboratory conditions. In response to S phase delay, the overlapping Wee1/Cdc25 functions (induced through the Rad3-Cds1 checkpoint) obscure the majority of the phenotype. However, a clear mitotic delay defect is revealed in wee1–50 mik1-d double mutants at the

![Fig. 4.](image-url)
Radiation-Dependent Induction of Mik1. When asynchronous cells are irradiated, a modest increase in Mik1-MYC level is observed. This up-regulation is significantly reduced in rad3-d cells and increased in chk1-d cells. Most strikingly, Mik1-MYC levels rapidly decline to almost undetectable levels when cds1-d cells are irradiated, a dramatic phenotype not seen in rad3-d cells. Based on an analysis of synchronous cultures, our explanation for these observations is as follows.

First, in response to ionizing radiation, there is a brief but significant delay to S phase. This delay may be confined to early-S phase cells, because we see no significant shoulders in FACS profiles after irradiation of synchronized cells proceeding through S phase. A previous report (27) concluded that there was no intra-S phase checkpoint in response to γ-irradiation. However, we had previously reported that γ-irradiation results in activation of the Cds1 checkpoint kinase in S phase (8), and in this study, we have shown a Rad3-dependent but Chk1-independent (and thus presumably Cds1-dependent) delay to S phase in response to γ-irradiation (Fig. 4B). This delay is brief.
through mitosis and enter S phase, whereas irradiated M/G1/S phase cells will rapidly pass through S phase and accumulate in G2. Because all cells are thus in G2 (this profile contrasts with wild-type cells, where the M/G1/S phase cells in the population are delayed in S phase by the Rad3-Cds1-dependent intra-S phase checkpoint), little Mik1 is present. The continued presence of Mik1 in rad3-d cells after irradiation can be explained by the fact that rad3-d cells, in contrast to cds1-d cells, continue to cycle through mitosis, and thus cells can continue to enter S phase, where Mik1 is synthesized and stabilized in a Rad3-Cds1-independent manner.

Our data further show that, during the G2 phase, synchronous rad+ cells increase Mik1-MYC protein levels from almost undetectable levels to detectable levels in response to irradiation (Figs. 4 and 5B). By artificially blocking cells in G2 by using the cdc25.22 temperature-sensitive mutation, we have been able to determine satisfactorily that this effect depends on Chk1 and not Cds1. Importantly, the Chk1-dependent induction of Mik1 in G2 cells is very modest compared with the levels seen in normal S phase and HU-arrested cells. Deletion of mik1 does not result in significant radiation sensitivity or in any obvious effect on the γ- or UV-radiation-induced delay to mitosis seen in G2 cells (ref. 26 and data not shown), indicating that Mik1 induction does not have a major role in the G2/M checkpoint.

The targets of the Chk1-dependent G2/M checkpoint remain elusive but have been reported to include regulation of Cdc25 (31) and Wee1 (32). Deletion of wee1 or abrogation of wee1 function in mik1-d cells does not abrogate the G2/M checkpoint (25, 33), suggesting that Cdc25 may be a major target. However, the cdc2.3w mutant, which harbors a mutation in cdc2 making p34Cdc2 function largely independent of Cdc25 activity, maintains normal DNA damage sensitivity and mitotic delay after irradiation (6). This observation suggests that Cdc25 is not the sole target of the G2/M checkpoint. Taken together, these data suggest multiple effects of the Chk1-dependent response, including increased Mik1 stability that helps phosphorylate p34Cdc2 and keep it inactive.

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