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 G_2 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 G_1, S, G_2, 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 (G_1/early S, licensed for replication) and postreplicative (G_2, unable to support replication). Transition between the postreplicative (G_2) and prereplicative (G_1) 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 $p34^{Cdc2}$ 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 G_2 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 G_2 (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 $rad3^{ts}$ HU sensitivity (11) at the semipermissive temperature (32°C). The plasmid did not affect cell size, and suppression was specific to the $rad3^{ts}$ allele.

Epitope Tagging of Mik1. A genomic 1,053-bp *Bam*HI–*Hin*dIII fragment (648 bp of the *mik1* ORF and 405 bp of 3′ untranslated region) was PCR amplified, and an *Nde*I 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 4°C (14,000 rpm for 10 min on an Eppendorf centrifuge; high spin supernatant and insoluble pellet). The pellet was solubilized in the same buffer plus 2% (vol/vol) NP-40, and 50 μg of total protein was Western blotted. Mik1-MYC was detected with anti-MYC monoclonal antibody (9E10, PharMingen), and Wee1-HA was detected with anti-HA monoclonal antibody (Babco, Richmond, CA).

Immunoblotting. Total protein (50 μg; Bradford) was boiled in SDS sample buffer and loaded onto 10% (37.5:1 acrylamide/bisacrylamide; Ultrapure Protogel, National Diagnostics) SDS gels. Protein was transferred to PVDF-Plus membranes (Micron Separations, Westboro, MA) and blocked with Blotto [PBS/2.5%

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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-Protein Phosphatase (Biolabs, Northbrook, IL). Phosphatase inhibitors (sodium orthovanadate, Na_2EDTA) 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 G_2, 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 LN_2, 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 ^{32}P-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 *Eco*RI–*Sal*I fragment of *mik1* ORF.

Results

In a screen for multicopy suppressers of HU-induced lethality of a temperature-sensitive *rad3* mutant (11), we identified a truncated *mik1* ORF. Mik1 negatively regulates $p34^{Cdc2}$ (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. 1*A*) and exists as multiple isoforms generated by phosphorylation (Fig. 1*B*). 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. 1*C*). Mik1-MYC peaks coincidentally with the septation peak, a marker for S phase. We also synchronized a *wee1-HA* strain (19), because both Wee1 and Mik1 negatively regulate $p34^{Cdc2}$. Wee1-HA levels are relatively constant throughout the cell cycle (Fig. 1*D*), modestly increasing during G_2. 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 synchronous *rad3-d* and *cds1-d* cultures. In both, Mik1-MYC levels increased in S phase and decreased in G_2 (Fig. 2*A*). Thus, Rad3 and Cds1 proteins are not required for S phase Mik1-MYC accumulation. We also examined the cellular localization of Mik1-MYC by immunofluorescent 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 G_2 cells (Fig. 2*B*).

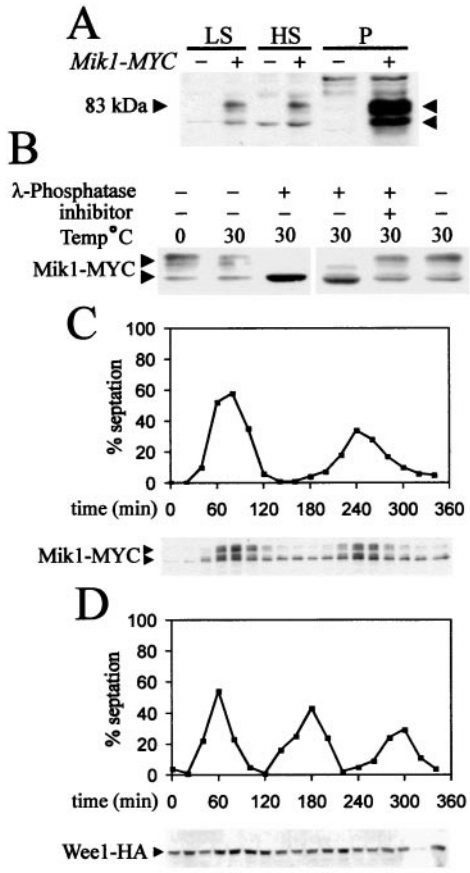

Fig. 1. (*A*) Equal amounts of total protein extracts from *mik1-MYC* (+) and untagged (−) cells grown to mid-log phase: low spin supernatant (LS), high spin supernatant (HS), and pellet (P). Arrowheads indicate bands specific to *mik1-MYC* extracts. (*B*) Mik1-MYC is resolved to a single form after phosphatase treatment. (*C*) *mik1-MYC* cells synchronized in G_2 by elutriation. Progression through the cell cycle was followed by the septation index. Protein extract was prepared every 20 min, and 50 μg of total protein (pellet fraction) was analyzed. (*D*) An HA-tagged Wee1 strain was treated similarly.

***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. 2*C*) and also show that the levels increase significantly above those seen in untreated S phase (Fig. 2*D*). The Mik1-MYC accumulation in the nucleus of all mononuclear S phase-arrested cells (Fig. 2*B*) 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. 3*A*), and Rad3 and Cds1 are required to maintain high levels of *mik1* transcript when S phase is blocked by HU (Fig. 3*B*).

Induction of Mik1 After Irradiation. The mechanisms that ensure that mitosis is dependent on S phase completion share many

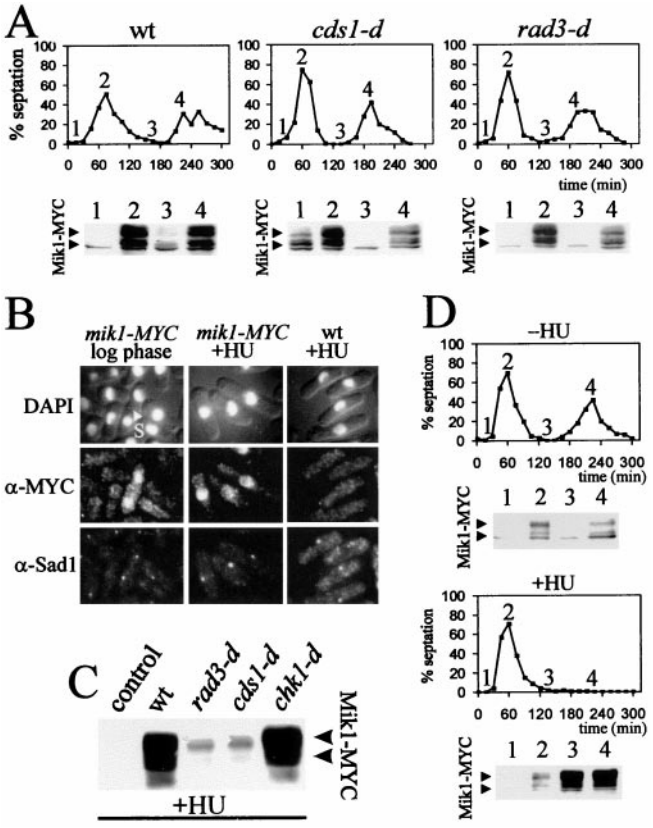

Fig. 2. (*A*) *mik1-MYC* cells [in a wild-type (wt), *cds1-d*, or *rad3-d* background] were synchronized in G_2 by elutriation. Every 15 min, the septation index was monitored. At times indicated by 1 → 4, protein was extracted, and 50 μg (pellet fraction) was analyzed by Western blotting with 9E10. (*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, *rad3-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 G_2 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.

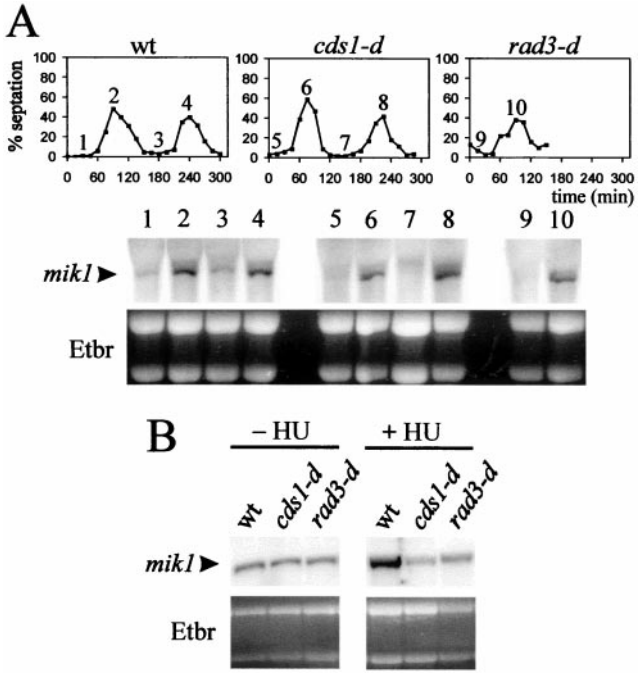

Fig. 3. (*A*) Wild-type (wt), *cds1-d*, or *rad3-d* cells were synchronized in G_2 by elutriation. Septation index was monitored every 15 min, and RNA was extracted at the times indicated by 1 → 10. Total RNA (10 μg) was analyzed by Northern blots probed with *mik1*. Loading control: ethidium bromide staining (*Bottom*). (*B*) Total RNA was isolated from wild-type, *cds1-d*, or *rad3-d* cells grown to mid-log phase or arrested in S phase by 20 mM HU (4 h).

overlapping components with the DNA damage checkpoints. These DNA damage responses regulate S phase progression after damage (intra-S checkpoint; refs. 8 and 21) and prevent mitosis when DNA is damaged in G_2 (G_2/M checkpoint; refs. 4 and 6). To examine the effects of radiation on Mik1-MYC, we monitored Mik1-MYC protein (Fig. 4*A*) after irradiation of asynchronous *rad*$^+$, *rad3-d*, *chk1-d*, and *cds1-d* cultures.

After 250 Gy of ionizing radiation (12.5 Gy·min^{-1}) 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. *rad3-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) after 250 Gy of radiation. S phase progression after irradiation was monitored by FACS in synchronous *chk1*$^-$, *cds1*$^+$, and *rad3*$^-$ cultures with and without prior irradiation before S phase (Fig. 4*B*). 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. 5*A*). In *rad*$^+$ and *cds1-d* cultures, cells irradiated in G_2 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 G_2-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 G_2 (Fig. 5*B*). 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 G_2 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. 5*A*, *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 G_2 and those cells that have passed M and have therefore been irradiated in G_1 or S phase. By comparing *rad*$^+$ (times 20 and 40) with *cds1-d* (times 40 and 60), it is clear that irradiated G_1/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-radiation (Fig. 4*B*). Irradiating *chk1-d* cells at the peak of septation (times 40 and 60) does not separate the culture into two populations, because G_2 cells can continue through mitosis. Thus, all cells are in or proceed into G_1 and S with damaged

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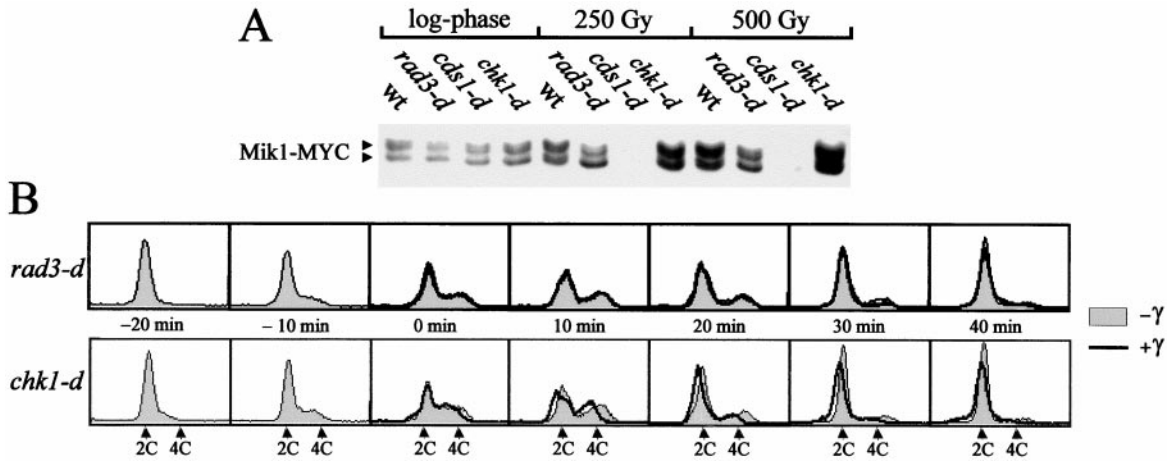

Fig. 4. (*A*) Wild-type (wt), *rad3-d*, *cds1-d*, and *chk1-d* cells were grown to mid-log phase and irradiated with either 250 or 500 Gy of ionizing radiation. After 45-min recovery, protein was extracted. Total protein (pellet fraction; 50 μg) was analyzed by Western blotting with 9E10. (*B*) FACS profiles of elutriation-synchronized *rad3-d* and *chk1-d* cells after irradiation (250 Gy between −20 and 0 min) during G_1/S. Both strains are defective for the G_2/M checkpoint and do not arrest mitosis when irradiated. The major difference between *rad3-d* and *chk1-d* is the lack of a Cds1 response. S phase is delayed by irradiation in *chk1-d* (*cds1*$^+$) cells but not in *rad3-d*.

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 G_2 (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 G_2 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 G_2/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 p34^{cdc2} Y15 residue (22). Dephosphorylation 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 p34^{Cdc2} 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 p34^{Cdc2}.

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 p34^{Cdc2}, 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 p34^{Cdc2} activator and/or activating the p34^{Cdc2} inhibitors; Cds1 has been proposed to phosphorylate directly both Wee1, an inhibitor of p34^{Cdc2} (20), and Cdc25, the activator of p34^{Cdc2} (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

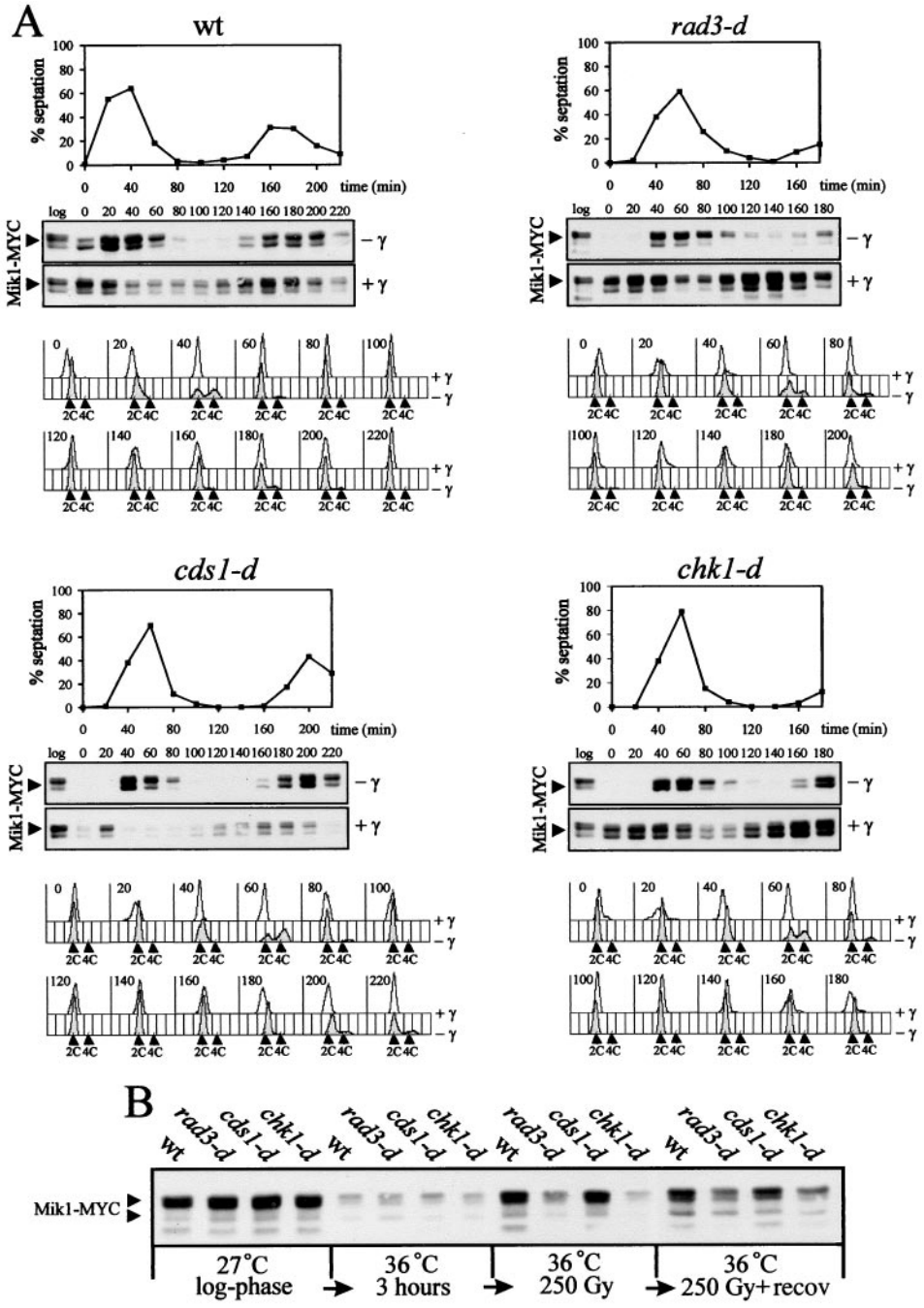

Fig. 5. (*A*) *mik1-MYC* cells [in wild-type (wt), *rad3-d*, *cds1-d*, *or chk1-d* background] were synchronized in G_2 by elutriation, and aliquots were collected every 20 min. For each time point, an aliquot was assayed for septation index, Mik1-Myc levels (50 μg of total protein, pellet fraction), and DNA content (FACS). A second aliquot was treated with 250 Gy of irradiation, allowed to recover (for 45 min at 30°C) and similarly assayed for Mik1-Myc levels and DNA content. (*B*) *cdc25.22 mik1-MYC* cells (in wild-type, *rad3-d*, *cds1-d*, *or chk1-d* background) were grown to log phase, shifted to 36°C (for 3 h), irradiated (250 Gy), and allowed to recover for 40 min. At each stage, samples were removed; protein was extracted; and 50 μg (pellet fraction) was analyzed for Mik1-MYC.

permissive temperature and in *mik1-d* at longer times of incubation in HU (25).

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. 4*B*). This delay is brief

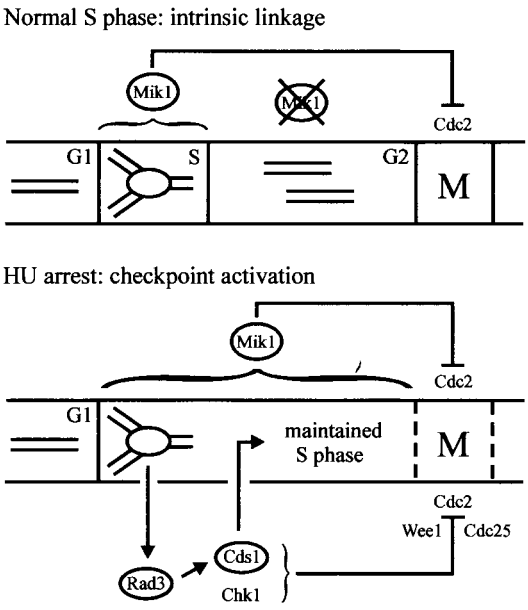

Fig. 6. A model indicating two mechanisms linking S phase and mitosis. (Normal S phase, *Upper*) Intrinsic: Mik1 is synthesized and stable only in S phase. (*Lower*) Checkpoint control: The Rad3-Cds1 checkpoint maintains HU-treated cells within S phase, as well as directly inhibiting mitosis. HU-arrested cells therefore indirectly require Rad3-Cds1 to accumulate Mik1.

compared with that in cells damaged with UV or methyl methanesulfonate (8, 21, 27). A comparatively brief γ-induced delay is consistent with the data in previous reports (8, 27) and with the vast literature on mammalian cells. These reports show that different DNA-damaging agents affect replication in distinct ways (28–30): γ-irradiation inhibits the initiation step of replication, whereas UV and methyl methanesulfonate activate additional responses, probably affecting the elongation stage of replication.

Second, because *cds1-d* cells have a normal G_2/M DNA damage checkpoint but no intra-S phase checkpoints, irradiation of asynchronous cells will result in the rapid accumulation of all cells in G_2; irradiated G_2 cells in the population will not pass through mitosis and enter S phase, whereas irradiated M/G_1/S phase cells will rapidly pass through S phase and accumulate in G_2. Because all cells are thus in G_2 (this profile contrasts with wild-type cells, where the M/G_1/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 G_2 phase, synchronous rad^+ cells increase Mik1-MYC protein levels from almost undetectable levels to detectable levels in response to irradiation (Figs. 4 and 5*B*). By artificially blocking cells in G_2 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 G_2 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 G_2 cells (ref. 26 and data not shown), indicating that Mik1 induction does not have a major role in the G_2/M checkpoint.

The targets of the Chk1-dependent G_2/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 G_2/M checkpoint (25, 33), suggesting that Cdc25 may be a major target. However, the *cdc2.3w* mutant, which harbors a mutation in *cdc2* making $p34^{Cdc2}$ 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 G_2/M checkpoint. Taken together, these data suggest multiple effects of the Chk1-dependent response, including increased Mik1 stability that helps phosphorylate $p34^{Cdc2}$ and keep it inactive.

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