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Cdc25 Inhibited In Vivo and In Vitro by Checkpoint Kinases Cds1 and Chk1

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In the fission yeast *Schizosaccharomyces pombe*, the protein kinase Cds1 is activated by the S–M replication checkpoint that prevents mitosis when DNA is incompletely replicated. Cds1 is proposed to regulate Wee1 and Mik1, two tyrosine kinases that inhibit the mitotic kinase Cdc2. Here, we present evidence from in vivo and in vitro studies, which indicates that Cds1 also inhibits Cdc25, the phosphatase that activates Cdc2. In an in vivo assay that measures the rate at which Cdc25 catalyzes mitosis, Cds1 contributed to a mitotic delay imposed by the S–M replication checkpoint. Cds1 also inhibited Cdc25-dependent activation of Cdc2 in vitro. Chk1, a protein kinase that is required for the G_2–M damage checkpoint that prevents mitosis while DNA is being repaired, also inhibited Cdc25 in the in vitro assay. In vitro, Cds1 and Chk1 phosphorylated Cdc25 predominantly on serine-99. The Cdc25 alanine-99 mutation partially impaired the S–M replication and G_2–M damage checkpoints in vivo. Thus, Cds1 and Chk1 seem to act in different checkpoint responses to regulate Cdc25 by similar mechanisms.

INTRODUCTION

Mitotic DNA checkpoints ensure that chromosomes are properly replicated and repaired before nuclear division. Checkpoint failure increases genomic instability, leading to chromosome rearrangement, amplification, or loss. These events can lead to cell death or tumorigenesis. Thus, checkpoint mechanisms are a major priority of current cell cycle and cancer research.

Genetic studies of the fission yeast *Schizosaccharomyces pombe* have played a prominent role in the unraveling of replication and repair checkpoint mechanisms (Russell, 1998). These investigations have identified two structurally dissimilar protein kinases, Chk1 and Cds1, that link checkpoints to the mitotic cell-cycle control. Chk1 is essential for the G_2–M DNA damage checkpoint that prevents mitosis when DNA is being repaired (Walworth *et al.*, 1993). Mitosis is triggered by the cyclin-dependent kinase (CDK) Cdc2, which is inhibited by phosphorylation of tyrosine-15 that is catalyzed by the protein kinases Wee1 and Mik1. Cdc2 is activated by the protein phosphatase Cdc25 that dephosphorylates tyrosine-15. Phosphorylation of Cdc2 on tyrosine-15 is required for the G_2–M DNA damage checkpoint (Rhind *et al.*, 1997). Chk1 appears to inhibit Cdc25. This conclusion is based on in vivo studies that showed that the Chk1-dependent G_2–M DNA damage checkpoint inhibits dephosphorylation of Cdc2 (Furnari *et al.*, 1997; Rhind *et al.*, 1997). Chk1 and Cdc25 associate in vivo, a finding that indicated that Cdc25 is a direct substrate of Chk1 (Furnari *et al.*, 1997). This idea was supported by studies that showed that fission yeast, *Xenopus laevis*, and human Chk1 proteins phosphorylate Cdc25 in vitro on sites that are also phosphorylated in vivo (Peng *et al.*, 1997; Sanchez *et al.*, 1997; Kumagai *et al.*, 1998a; Zeng *et al.*, 1998). It was also reported that Chk1 phosphorylates Wee1 in vitro, but the physiological significance of this finding is uncertain (O'Connell *et al.*, 1997).

The mechanism by which Chk1 regulates Cdc25 is of substantial importance to checkpoint investigations. A clue was provided by the discovery that Chk1 phosphorylates human Cdc25C on serine-216, thereby creating a binding site for 14-3-3 proteins (Peng *et al.*, 1997; Sanchez *et al.*, 1997). Genetic studies of fission

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yeast have also suggested that Rad24, a 14-3-3 protein, is involved in the damage checkpoint (Ford *et al.*, 1994). Recent studies have indicated that Chk1 and Rad24 control the intracellular localization of Cdc25 (Lopez-Girona *et al.*, 1999). DNA damage caused the net nuclear export of Cdc25 by a process that requires Chk1 and Rad24. Cdc2/cyclin-B kinase is a nuclear protein in fission yeast (Booher *et al.*, 1989); thus, nuclear export of Cdc25 has the potential to inhibit the onset of mitosis. However, evidence of this mode of regulation does not exclude the possibility that Chk1 also regulates the phosphatase activity of Cdc25.

Chk1 regulation is a mystery. Chk1 is phosphorylated in response to damage, but the significance of this phosphorylation is unknown (Walworth and Bernards, 1996). It might indicate activation of Chk1 by an upstream regulatory kinase, autophosphorylation of activated Chk1, or even inhibition of Chk1 by a kinase that is part of a damage adaptation response. Chk1 phosphorylation requires a group of checkpoint Rad proteins (Walworth and Bernards, 1996). This group includes Rad3, a kinase related to human ATM/ATR and *Saccharomyces cerevisiae* Mec1/Tel1 (Bentley *et al.*, 1996). Checkpoint Rad proteins are required for both S–M replication and G_2–M damage checkpoints. Chk1 phosphorylation and the G_2–M damage checkpoint also require Crb2/Rhp9, a protein having a BRCT motif found in human p53 binding protein (53BP1), human BRCA1, and *S. cerevisiae* Rad9 (Saka *et al.*, 1997; Willson *et al.*, 1997).

Cds1, a second checkpoint kinase in fission yeast, seems to be specifically involved in the S–M replication checkpoint that prevents mitosis when DNA is incompletely replicated (Murakami and Okayama, 1995; Boddy *et al.*, 1998; Lindsay *et al.*, 1998). This checkpoint can be triggered by the drug hydroxyurea (HU), an inhibitor of ribonucleotide reductase, which causes depletion of deoxyribonucleotides. Cds1 is highly activated when HU-treated cells attempt DNA replication (Boddy *et al.*, 1998; Lindsay *et al.*, 1998) but not when DNA is damaged during G_2 (Lindsay *et al.*, 1998). Activation of Cds1 requires checkpoint Rad proteins (Boddy *et al.*, 1998; Lindsay *et al.*, 1998). Enforcement of the S–M replication checkpoint is dependent on inhibitory phosphorylation of Cdc2 on tyrosine-15 (Rhind and Russell, 1998a). Cds1 associates with and phosphorylates Wee1 in cell extracts (Boddy *et al.*, 1998). Cds1 is also required for the large accumulation of Mik1 that occurs in HU-treated cells (Boddy *et al.*, 1998). These findings suggest that Cds1 might enforce the S–M replication by increasing the activity of Wee1 and Mik1 kinases.

Chk1 is not normally required for the HU checkpoint, nor does HU induce Chk1 phosphorylation (Walworth *et al.*, 1993; Walworth and Bernards, 1996). However, in *cds1* mutants, Chk1 is essential for HU-induced checkpoint arrest (Boddy *et al.*, 1998; Lindsay *et al.*, 1998; Zeng *et al.*, 1998). Moreover, HU induces Chk1 phosphorylation in *cds1* cells (Lindsay *et al.*, 1998). One interpretation of these findings is that Cds1 stabilizes stalled replication forks, thereby preventing DNA damage and activating a Chk1-independent checkpoint that involves unknown effectors (Lindsay *et al.*, 1998). Alternatively, Cds1 might be a direct checkpoint effector that is the primary enforcer of the S–M replication checkpoint, with Chk1 becoming involved only in *cds1* mutants (Russell, 1998). A third possibility is that Cds1 and Chk1 jointly enforce the replication checkpoint, but for unknown reasons, HU does not lead to Chk1 phosphorylation if Cds1 function is intact (Boddy *et al.*, 1998; Russell, 1998).

Many questions concerning the S–M replication checkpoint remain to be answered. One group of questions involves regulation of Cdc25. We recently presented evidence that the rate of dephosphorylation of Cdc2 on tyrosine-15 is decreased in cells arrested in early S with HU, which indicates that Cdc25 might be inhibited by the S–M replication checkpoint (Rhind and Russell, 1998a). In this article, we report studies that substantiate this conclusion and implicate both Chk1 and Cds1 in this response. Other questions concern the mechanisms by which Cdc25 is regulated by the checkpoints. We recently reported that the damage checkpoint induces net nuclear export of Cdc25 (Lopez-Girona *et al.*, 1999), but whether checkpoint kinases also inhibit Cdc25 directly was left unexplored. Here we describe in vitro experiments that indicate that Cds1 and Chk1 are able to inhibit Cdc25 directly. In agreement with these findings, we also report that Cds1 and Chk1 phosphorylate Cdc25 to generate similar tryptic phosphopeptide maps. This discovery, which agrees with a recent study (Zeng *et al.*, 1998), further bolsters the notion that Cds1 and Chk1 are direct effectors of the S–M replication checkpoints. We report that the alanine-99 mutation of Cdc25, which eliminates the site of preferred phosphorylation by Cds1 and Chk1, partially abrogates the S–M replication and G_2–M damage checkpoints. Moreover, our studies indicate that the alanine-99 mutation seems to impair the S–M replication checkpoint-induced inhibition of Cdc25 in vivo. Together, these findings indicate that Cds1 and Chk1, two dissimilar protein kinases, enforce checkpoints by mechanisms that partially overlap.

MATERIALS AND METHODS

General Methods

General genetic and biochemical methods for the study of fission yeast have been described (Moreno *et al.*, 1991). Purification of glutathione *S*-transferase (GST) fusion proteins expressed in fission yeast and kinase assays were performed as described (Shiozaki and Russell, 1995). For the HU experiments, cells were synchronized by centrifugal elutriation at 25°C with a Beckman (Fullerton, CA) JE-5.0 rotor. Forty minutes after elutriation, one-half of the cultures were

treated with HU (12 mM) for the duration of the experiment. The *wee1-50* Δ*mik1* cultures were shifted to 35°C after the first mitosis (220–240 min after elutriation). For the bleomycin experiments, cells were synchronized by centrifugal elutriation at 25°C, and one-half of the cultures were treated with bleomycin sulfate (5 mU/ml) for the duration of the experiment. Cells were scored for progression through mitosis by microscopic observation as described (Rhind *et al.*, 1997).

Recombinant Baculovirus, Protein Purification, and Cdc25 Activity Assay

Recombinant virus encoding 6his–Cdc25 was generated using the Bac-to-Bac expression system from Life Technologies (Grand Island, NY) and was purified as described (Kumagai and Dunphy, 1995). 6his–Cdc25 purified on Ni^{2+}-nitrilotriacetic acid (NTA) beads from 10×10^6 Sf9 cells was washed three times with kinase assay buffer 10 (KAB10: 50 mM Tris, pH 7.4, and 10 mM $MgCl_2$). The beads were resuspended in 1 ml of kinase assay buffer 20 (KAB20: 50 mM Tris, pH 7.4, and 20 mM $MgCl_2$). Purification of GST–Chk1, GST–Cds1, and GST–Cds1KD expressed in 20 OD_{600} of fission yeast was performed as described (Shiozaki and Russell, 1995; Furnari *et al.*, 1997; Boddy *et al.*, 1998). Beads were washed three times with KAB10 before elution in 60 μl of KAB10 containing 250 mM NaCl and 10 mM glutathione for 2 min at room temperature. Eluted kinases were resuspended in 660 μl of KAB20 with 2 mM ATP. 6his–Cdc25 on Ni^{2+}-NTA beads or mock Ni^{2+}-NTA beads were aliquoted, and the supernatant was removed. Eluted kinases in KAB20 with 2 mM ATP were added to a final volume of 100 μl. Kinase reactions were performed at 30°C for 30 min. The Ni^{2+}-NTA bead complexes were washed three times with HEPES phosphatase assay buffer (HEPES-PAB: 50 mM HEPES, pH 7.4, 2 mM EGTA, and 0.05% β-mercaptoethanol) before the addition of 50 μl of Cdc2/cyclin-B complexes in imidazole-PAB (50 mM imidazole, pH 7.4, 10 mM EDTA, and 0.05% β-mercaptoethanol). For each reaction, Cdc2/cyclin-B was immunoprecipitated with 0.5 μl of α-cyclin-B1 antibody from 50 μg of protein extracted from thymidine-arrested HeLa cells (McGowan and Russell, 1995). Protein-A precipitates were washed three times with radioimmunoprecipitation assay buffer (McGowan and Russell, 1995) followed by three washes with imidazole-PAB. Cdc25 phosphatase reactions were performed at 30°C for 30 min. The Cdc2/cyclin-B complexes were then washed three times with KAB10. Histone H1 kinase assays were performed in 50 μl of KAB10 containing 40 μCi of [γ-^{32}P]ATP, 250 μM ATP, and 6 μg of histone H1 at 30°C for 15 min. After the incubation, 50 μl of Laemmli sample buffer was added, and the sample was boiled for 2 min. One-half of each reaction was resolved on a SDS-12% polyacrylamide gel. Dried gels were subjected to quantification (Molecular Dynamics Storm PhosphorImager, Sunnyvale, CA) and autoradiography.

Phosphorylation Site Mapping and In Vitro Mutagenesis

PCR was performed to generate fragments of Cdc25 encoding amino acids 1–56, 1–147, or 1–374 incorporating a 5′ *Nde*I site and a 3′ *Not*I site. These fragments were cloned into a modified pGEX vector and transformed into the *Escherichia coli* BL21 (DE3) expression host. Expression and purification of the GST fusion proteins were as described (Furnari *et al.*, 1993). Glutathione-Sepharose precipitates were washed three times with lysis buffer (0.5 ml of lysis buffer containing 50 mM Tris, pH 8.0, 150 mM NaCl, 5 mM EDTA, 10% glycerol, 0.1% Nonidet P-40, 50 mM NaF, 0.1 mM Na orthovanadate supplemented with 1 mM phenylmethylsulfonyl fluoride [PMSF], 1 μM microcystin-LR, and 5 μg/ml aprotinin, leupeptin, and pepstatin), followed by three washes with KAB10. The bound complexes were aliquoted and resuspended in 50 μl of KAB10 containing 40 μCi of [γ-^{32}P]ATP and 10 mM glutathione. Kinase reactions were incubated at 30°C for 30 min, and one-half of each reaction was resolved on a 12% SDS-polyacrylamide gel. Full-length phosphorylated proteins were isolated from the polyacrylamide gel and subjected to phosphoamino acid analysis and phosphopeptide mapping as described (Boyle *et al.*, 1991). Serine codons corresponding to residues 97 and 99 of Cdc25 were changed to alanine by the Altered Sites II in vitro mutagenesis system (Promega, Madison, WI). Plasmid pALTER-cdc25 (pBF180) was constructed by inserting a 1.74-kb *Eco*RI and *Pst*I fragment from pBF169 into pALTER-1. Plasmid pBF169 was made by PCR amplification of the *cdc25*$^+$ ORF using the primers 5′-CTCGTAAGAT-CTGAATTCAC-CATGGATTCT-CCGCTTTCTT-C-3′ and 5′-CAGCATGCGG-CCGCTTAACG-TCTGGGGAAG-CTAAC-3′, followed by restriction enzyme digestion with *Eco*RI and *Not*I and cloning into pFastBacHTa that had been digested with *Eco*RI and *Not*I. pBF180 was used as a template for the mutagenesis reaction. The primer sequences were as follows: for S97A, 5′-CGTACGCTCT-TTCGAGCTCT-TTCTTGTACT-GTAG-3′; for S99A, 5′-GCTCTTTCGA-TCTCTTGCTT-GTACTGTAGA-AACCC-3′; and for S97/99A, 5′-CGTACGCTCT-TTCGAGCTCT-TGCTTGTACT-GTAGAAACCC-3′. DNA sequence analysis confirmed the mutations. The alanine mutants from codons 1–147 were cloned into a modified pGEX vector by PCR amplification using the primers 5′-CGCGAATTCC-ATATGGATTC-TCCGCTTTCT-TCA-3′ and 5′-CAGTTGTATG-CGGCCGCTTA-GAA-ACACGTG-GGGAATCTTG-3′, followed by restriction enzyme digestion with *Nde*I and *Not*I and cloning into a modified pGEX that had been digested with *Nde*I and *Not*I, creating pBF218, pBF268, and pBF270, respectively. The S99A mutation was introduced into a genomic fragment containing the *cdc25* ORF by restriction enzyme digestion of the plasmid pALTER-cdc25 containing the S99A mutation with *Bam*HI and *Bgl*II and by cloning into p25SS (Millar *et al.*, 1991) that had been digested with *Bam*HI and *Bgl*II, creating pBF264. Plasmids p25SS and pBF264 were integrated in strain KZ1483 (*h*$^+$ *cdc25-22 ura4-D18 leu1-32*). This created strains in which *cdc25-22* was converted to *cdc25*$^+$ and the integrated plasmid contained the *cdc25-22* temperature-sensitive mutation. Additionally, the above plasmids were integrated in strain GL238 (*h*$^+$ *cdc25::LEU2 wee1-50 ura4-D18 leu1-32*) to create strains that contained either *cdc25*$^+$ or *cdc25-S99A*.

Yeast Strains

S. pombe strains of the following genotypes were used in this study: PR755, *wee1-50 mik1::ura4*$^+$; NB2238, *wee1-50 mik1::ura4*$^+$ *rad3::ura4*$^+$; NR1604, *wee1-50 mik1::ura4*$^+$ *chk1::ura4*$^+$; NB2239, *wee1-50 mik1::ura4*$^+$ *cds1::ura4*$^+$; BF2300, *wee1-50 mik1::ura4*$^+$ *chk1::ura4*$^+$ *cds1::ura4*$^+$; BF1921, *nmt:GST–chk1:leu1*$^+$; BF1916, *nmt:GST–cds1:leu1*$^+$; BF2301, *nmt:GST–cds1KD:leu1*$^+$; BF2302, *cdc25*$^+$*:cdc25-22:ura4*$^+$; BF2303, *cdc25-S99A:cdc25-22:ura4*$^+$; BF2338, *cdc25::LEU2:cdc25*$^+$*:ura4*$^+$ *wee1-50 mik1::ura4*$^+$; and BF2339, *cdc25::LEU2:cdc25-S99A:ura4*$^+$ *wee1-50 mik1::ura4*$^+$. All *nmt1* promoter constructs were integrated at the *leu1* locus.

RESULTS

Evidence of Inhibition of Cdc25 by the S–M Replication Checkpoint

Our previous studies indicated that the S–M replication checkpoint inhibits the function of Cdc25 in vivo (Rhind and Russell, 1998a). These studies evaluated the activity of Cdc25 by analysis of a *wee1-50* Δ*mik1* strain that lacks Mik1 and expresses temperature-sensitive Wee1. Incubation of these cells at the restrictive temperature of 35°C eliminates all protein kinase activity that phosphorylates Cdc2 on tyrosine-15. Consequently, tyrosine-15 of Cdc2 is rapidly dephosphorylated, resulting in activation of Cdc2 and the induction of mitosis. Cdc25 activity determines the

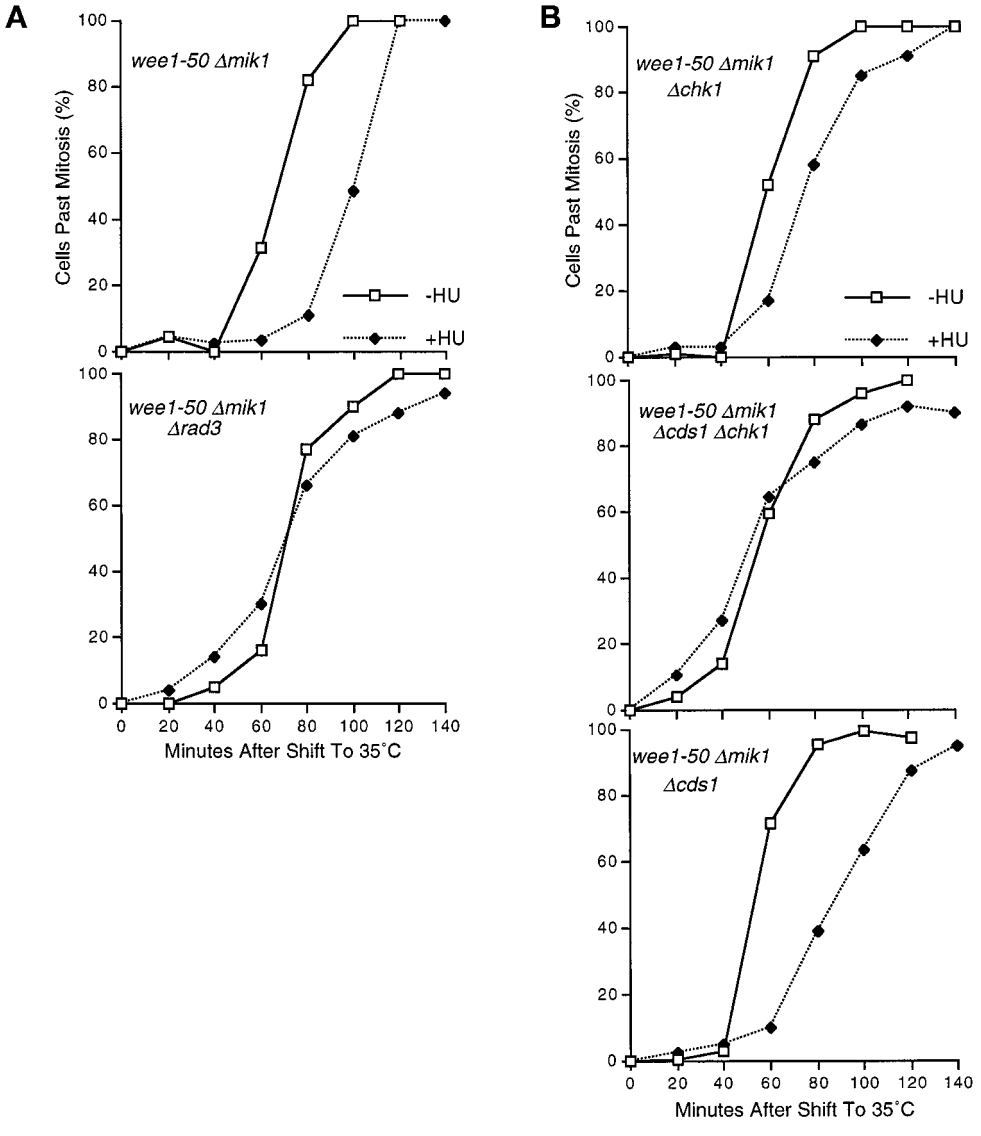

Figure 1. Rad3, Cds1, and Chk1 contribute to the HU-induced delay of mitosis after inactivation of Wee1 and Mik1. (A) Cells having *rad3*$^+$ or Δ*rad3* alleles in the *wee1-50* Δ*mik1* background were grown to midlog phase at the permissive temperature of 25°C and synchronized in early G_2 phase by centrifugal elutriation. HU was added at a concentration of 12 mM to one-half of each culture at 40 min. The culture temperature was shifted to the restrictive temperature of 35°C after the cells had divided once. HU caused a delay of ~40 min in the *rad3*$^+$ strain (top), whereas no delay was observed in the Δ*rad3* strain (bottom). The activity of Cdc25 determines the rate of mitotic onset after inactivation of Wee1 and Mik1. Rad3 is required for the replication checkpoint; thus these data indicate that the checkpoint inhibits the function of Cdc25 in vivo. (B) An experiment was performed to determine the contributions of Cds1 and Chk1 in the HU-induced checkpoint delay that is observed after inactivation of Wee1 and Mik1. The checkpoint delay was abolished in a Δ*cds1* Δ*chk1* background (middle). The duration of the delay was ~20 min in Δ*chk1* cells (top), compared with an ~40-min delay in a *cds1*$^+$ *chk1*$^+$ background. Thus, Cds1 contributes to the HU-induced inhibition of Cdc25 function in vivo. The delay appeared to be fully restored in the Δ*cds1* cells (bottom), which indicates that Chk1 makes a major contribution to the HU-induced mitotic delay. However, it is possible that the Δ*cds1* mutation leads to greater activation of Chk1, thereby appearing to exaggerate the contribution of Chk1 (Lindsay *et al.*, 1998).

rate of tyrosine-15 dephosphorylation and the induction of mitosis in this experiment (Lundgren *et al.*, 1991; Rhind *et al.*, 1997). Thus, measurement of the rate of mitosis after shift of *wee1-50* Δ*mik1* cells from 25 to 35°C provides an indirect measure of Cdc25 activity in vivo. In this assay, the rate of mitosis is delayed when cells are arrested in early S phase by treatment with HU before the temperature shift to 35°C (Rhind and Russell, 1998a). As stated above, these studies suggested that the S–M replication checkpoint inhibits Cdc25 function in vivo. Experiments were designed to test this hypothesis.

The first experiment determined whether Rad3 is required for the HU-induced delay of mitosis in the *wee1-50* Δ*mik1* assay. Rad3 is required for the S–M replication checkpoint and for the HU-induced activation of Cds1 (Enoch *et al.*, 1993; Boddy *et al.*, 1998; Lindsay *et al.*, 1998). Synchronous cultures of *rad3*$^+$ or Δ*rad3* cells in a *wee1-50* Δ*mik1* background were produced by centrifugal elutriation. Cells collected from the elutriation rotor were in G_2 phase. The cultures were incubated with or without HU at 25°C until the mock-treated cells completed mitosis and replicated DNA. The HU-treated cells also underwent mitosis but were unable to replicate DNA. These cultures were then shifted to a temperature of 35°C, which led to inactivation of Wee1 protein and consequent induction of mitosis (Figure 1A). In both the *rad3*$^+$ and Δ*rad3* cultures, most of the HU-treated cells exhibited a cut phenotype within 1 h of the shift of temperature to 35°C (our unpublished observations). This cut phenotype was typified by a nucleus that was bisected by the division plate or a single nucleus that was segregated to one side of the division plate. This phenotype

is diagnostic of mitotic catastrophe in which cells attempt mitosis before completing DNA replication (Russell and Nurse, 1986; Enoch and Nurse, 1990; Lundgren *et al.*, 1991). Importantly, in the *rad3*$^+$ cells, HU caused an ~40-min delay of mitosis upon a temperature shift from 25 to 35°C (Figure 1A). In contrast, HU did not delay mitosis in Δ*rad3* cells. Thus, an HU-activated and Rad3-dependent checkpoint delays mitosis upon inactivation of Wee1 and Mik1, which suggests strongly that Cdc25 is regulated by the S–M replication checkpoint.

Cds1 and Chk1 Contribute to the Apparent HU-induced Inhibition of Cdc25

Chk1 and Cds1 were proposed as effectors of the Rad3-dependent S–M replication checkpoint (Boddy *et al.*, 1998). Accordingly, we evaluated the respective contributions of Chk1 and Cds1 to the HU-induced checkpoint delay observed in the *wee1-50* Δ*mik1* temperature shift-up experiment. Relative to that in the *chk1*$^+$ *cds1*$^+$ control, the delay observed in the Δ*chk1* *cds1*$^+$ background was reduced by ~20 min, a decrease of ~50% (Figure 1B). These findings demonstrated that Chk1 contributes to the HU-induced mitotic delay in this assay system, which indicates that Chk1 is involved in the S–M replication checkpoint in a *cds1*$^+$ genetic background. The S–M replication checkpoint is abolished in a Δ*chk1* Δ*cds1* strain (Boddy *et al.*, 1998; Lindsay *et al.*, 1998; Zeng *et al.*, 1998). Thus, we expected the ~20-min HU-induced mitotic delay that remained in the *wee1-50* Δ*mik1* Δ*chk1* cells to depend on Cds1. Indeed, the HU-induced delay of mitosis after inactivation of Wee1 and Mik1 was abolished in a Δ*chk1* Δ*cds1* genetic background (Figure 1B). Thus, Cds1 is apparently able to contribute partially to the HU-induced inhibition of Cdc25 function in vivo.

Interestingly, there was no apparent defect in the HU-induced mitotic delay when the *wee1-50* Δ*mik1* temperature shift-up experiment was performed in a *chk1*$^+$ Δ*cds1* background (Figure 1B). This result may be understood if loss of Cds1 is compensated by increased activity of Chk1, as indicated by enhanced HU-induced phosphorylation of Chk1 in Δ*cds1* cells (Lindsay *et al.*, 1998). Alternatively, it is possible that HU-induced activation of Chk1 is sufficient for maximal inhibition of Cdc25 regardless of the *cds1* genotype. Minimally, these data indicate that both Cds1 and Chk1 are able to contribute to the inhibition of Cdc25. Moreover, Chk1 is apparently a more effective inhibitor of Cdc25 function in vivo. This conclusion is based on the observations that Chk1 protein in Δ*cds1* cells was sufficient for a full ~40-min mitotic delay in the *wee1-50* Δ*mik1* temperature shift-up assay, whereas Cds1 protein in Δ*chk1* cells was not.

Cds1 and Chk1 Inhibit Cdc25 In Vitro

Our studies indicated that both Cds1 and Chk1 were involved in delaying mitosis after inactivation of Wee1 and Mik1 in HU-treated cells. These findings suggested that Cds1 and Chk1 might directly regulate Cdc25. An in vitro assay was developed to explore this possibility. This experiment used the following purified components: 1) Cds1 and Chk1 expressed as GST fusion proteins in fission yeast; 2) hexahistidine-tagged fission yeast Cdc25 protein expressed in insect cells; and 3) human Cdc2/cyclin-B complex immunoprecipitated with antibody to cyclin-B1 from HeLa cells arrested in early S phase by treatment with thymidine. Cdc25 activity was measured by its ability to activate Cdc2/cyclin-B in a H1 histone kinase assay (Figure 2A). Addition of increasing amounts of Cdc25 led to a concomitant increase in kinase activity of Cdc2/cyclin-B (Figure 2A). Pretreatment of Cdc25 with GST–Cds1 decreased the activation of Cdc2/cyclin-B, which indicates that Cds1 inhibited Cdc25 (Figure 2A). This effect was dependent on the dose of GST–Cds1. Virtually identical results were observed with assays involving GST–Chk1 (Figure 2A). In these experiments the H1 histone kinase activity was dependent on addition of Cdc2/cyclin-B complex (our unpublished observations). Insignificant H1 histone kinase activity was associated with the amounts of Cdc25, GST–Cds1, and GST–Chk1 used in these studies. Moreover, inhibition of Cdc25 by GST–Cds1 and GST–Chk1 was dependent on ATP (our unpublished observations). GST–Cds1KD, a kinase-inactive form of Cds1 that contains the D312E mutation (Lindsay *et al.*, 1998), was defective at inhibiting Cdc25 in the in vitro assay (Figure 2B). Coomassie blue staining confirmed that equal amounts of GST–Cds1 and GST–Cds1KD were used in these experiments (see Figure 3A). These data argue strongly that Cds1 and Chk1 directly inhibit the ability of Cdc25 to activate Cdc2/cyclin-B. This effect occurred without the addition of 14-3-3 proteins, although this fact does not exclude the involvement of 14-3-3 proteins in the inhibition of Cdc25 function in vivo (see DISCUSSION).

Cds1 and Chk1 Phosphorylate Cdc25 Primarily on Serine-99 In Vitro

These findings suggested that Cds1 and Chk1 might negatively regulate Cdc25 by a similar mechanism. Accordingly, studies were performed to map sites on Cdc25 that are phosphorylated by Chk1 and Cds1. These studies focused on the NH_2-terminal regulatory domain of Cdc25 (Millar *et al.*, 1991), because this region of human Cdc25C is phosphorylated by Chk1 (Peng *et al.*, 1997). GST–Cdc25$^{1–56}$, GST–Cdc25$^{1–147}$, and GST–Cdc25$^{1–374}$ were purified from bacteria and tested as substrates for GST–Cds1 and GST–Chk1 (Figure 3A). GST–Cdc25$^{1–56}$ was not phosphorylated by

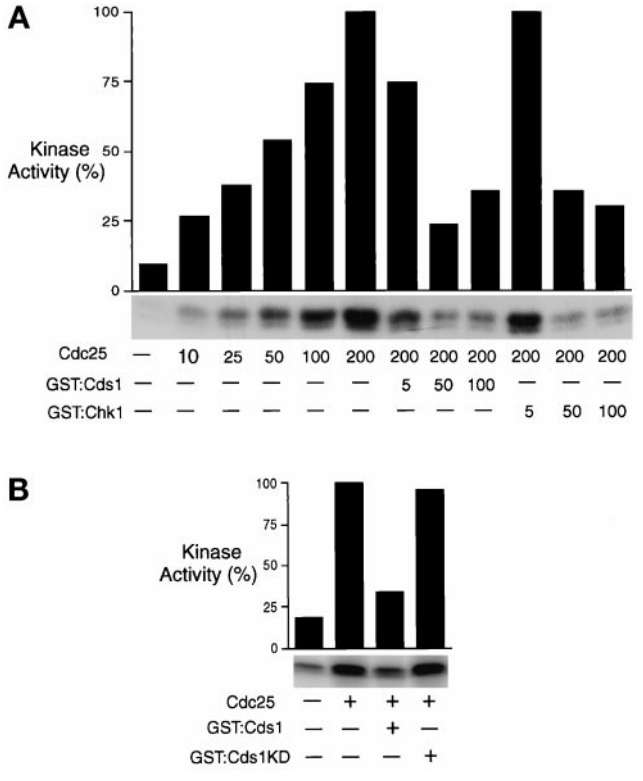

Figure 2. Cds1 and Chk1 inactivate Cdc25 in vitro. (A) Hexahistidine-tagged *S. pombe* Cdc25 was expressed and purified from insect cells and used to activate Cdc2/cyclin-B1 purified from HeLa cells arrested in early S phase with thymidine. Antibodies to human cyclin-B1 were used to purify Cdc2/cyclin-B1 that was assayed by its ability to transfer [γ-^{32}P]ATP to histone H1. Cdc25 was purified from 1×10^7 cells and suspended in 1 ml. Numbers in the Cdc25 row refer to the volume of Cdc25 preparation added to the reaction (see MATERIALS AND METHODS). Cdc25 caused a dose-dependent activation of Cdc2/cyclin-B. GST–Cds1 and GST–Chk1 were purified from fission yeast and used to phosphorylate Cdc25 before incubation with Cdc2/cyclin-B (see MATERIALS AND METHODS). Numbers in the GST–Cds1 and GST–Chk1 rows refer to the volume added of a 660-μl preparation made from 20 OD_{600} of cells. GST–Cds1 and GST–Chk1 caused a dose-dependent inhibition of Cdc25. The graph presents phosphorimager analysis of the autoradiogram shown below the graph. (B) The Cdc25 assay described above was repeated with GST–Cds1KD (GST–Cds1^{D312E}), a kinase-inactive form of Cds1. Relative to active GST–Cds1, GST–Cds1KD only modestly decreased activation of Cdc2/cyclin-B by Cdc25. Coomassie blue staining confirmed that equal amounts of GST–Cds1 and GST–Cds1KD were added to these reactions (see Figure 3).

GST–Chk1 and only weakly phosphorylated by GST–Cds1. In contrast, GST–Cdc25$^{1–147}$ and GST–Cdc25$^{1–374}$ were phosphorylated by both protein kinases. GST–Cds1KD failed to phosphorylate GST–Cdc25$^{1–147}$ or GST–Cdc25$^{1–374}$ (Figure 3A). Likewise, a kinase-inactive form of Chk1 failed to phosphorylate the GST–Cdc25 substrates (our unpublished observations). Two-dimensional tryptic phosphopeptide mapping of GST–Cdc25$^{1–374}$, phosphorylated by GST–Cds1 or GST–Chk1, revealed very similar patterns (Figure 3B). There were one major and several minor phosphopeptides. Additional minor phosphopeptides were detected upon longer exposure of the maps. A very similar pattern was observed in the maps of GST–Cdc25$^{1–147}$ (Figure 3B).

Phosphoamino acid analysis revealed that serine was the major phosphoamino acid in GST–Cdc25$^{1–147}$ phosphorylated by GST–Cds1 or GST–Chk1 (Figure 4A). The major site of phosphorylation was further refined with a series of GST–Cdc25 fusion proteins spanning the 56–147 region of Cdc25. This analysis narrowed the major phosphorylation site to the 91–109 region (Figure 4B). Thus, two serine residues at positions 97 and 99 were considered likely sites of phosphorylation (Figure 4C). Analysis was performed with mutant forms of GST–Cdc25$^{1–147}$ that contained alanine at position 97 (S97A), 99 (S99A), or both 97 and 99 (S97/99A). This analysis revealed that phosphorylation was unaffected by S97A but abolished by S99A or S97/99A mutations (Figure 4B). Two-dimensional tryptic phosphopeptide mapping of the S99A mutation of GST–Cdc25$^{1–374}$ revealed that the mutation specifically eliminated the major phosphopeptide observed in Figure 3B (our unpublished observations). Thus, serine-99 was identified as the major site in the NH_2-terminal regulatory domain of Cdc25 that is phosphorylated by both Cds1 and Chk1.

Cdc25-S99A Mutation Impairs but Does Not Abolish the S–M Replication Checkpoint

The serine-99 codon was mutated to encode alanine in a cloned copy of *cdc25*. Plasmids containing *cdc25-S99A* or *cdc25*$^+$ were integrated in a temperature-sensitive *cdc25-22* background. The *cdc25-S99A* integrant divided at a slightly shorter length (10.4 ± 1.3 μm) compared with the *cdc25*$^+$ integrant (11.5 ± 0.8 μm), although these differences were apparently statistically insignificant. An experiment was performed to measure the effect of the *cdc25-S99A* mutation on the S–M replication checkpoint. In this experiment, the *cdc25*$^+$ and *cdc25-S99A* integrant strains were incubated in HU for 2 h before synchronization by centrifugal elutriation. This procedure selected small cells that were arrested in early S. These cells were then washed and resuspended in medium with or without HU. In this experiment the *cdc25*$^+$ and *cdc25-S99A* integrant cells that were resuspended in medium lacking HU underwent division at ~200 min after elutriation (Figure 5A). In the cultures that were maintained in HU, both the *cdc25*$^+$ and *cdc25-S99A* integrant cells remained arrested before mitosis for the entire 300-min duration of the experiment. These cells became elongated, indicative of an arrest at the S–M replication checkpoint. Thus, in this assay, the *cdc25-S99A* mutation did not seem to impair the S–M replication checkpoint.

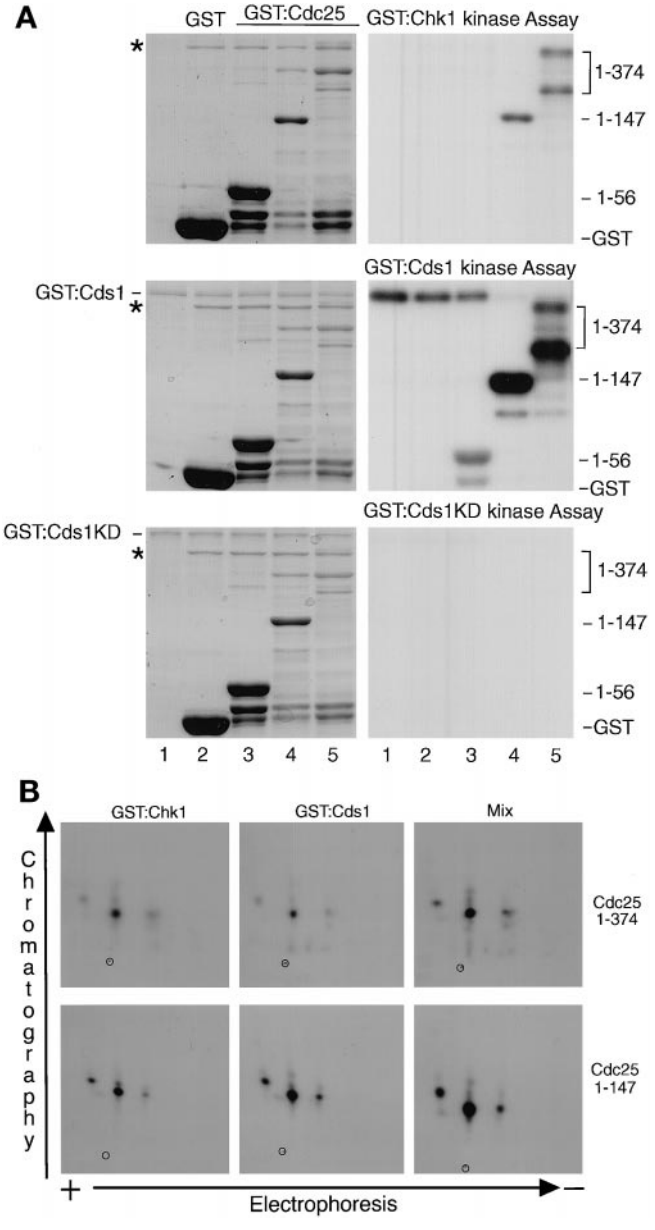

Figure 3. Cds1 and Chk1 phosphorylate the NH_2-terminal domain of Cdc25 to generate very similar phosphopeptide maps. (A) GST alone (lane 2) or GST fusion proteins containing amino acids 1–56, 1–147, or 1–374 of fission yeast Cdc25 (lanes 3–5, respectively) were expressed and purified from bacteria. The proteins were phosphorylated with GST–Chk1 or GST–Cds1 purified from fission yeast. Left, Coomassie blue stain analysis of protein gels is shown. Fusion proteins, degradation products, and GST–Cds1 are visible. There was insufficient GST–Chk1 to detect by Coomassie blue staining, but it was readily detected by immunoblotting (our unpublished observations). A major contaminating protein (*) from bacteria is indicated. Right, autoradiograms are shown. The 1–147 and 1–374 fusion proteins were effectively phosphorylated by the protein kinases, whereas unfused GST or the 1–56 fusion proteins were phosphorylated weakly or not at all. A 1–374 degradation product containing ~200 amino acids from Cdc25 was also phosphorylated. Phosphorylated GST–Cds1 was also detected, predominantly in lanes 1–3. These reactions did not contain a preferred substrate for GST–Cds1, perhaps accounting for the increased autophosphorylation in these samples. GST–Cds1KD (GST–$Cds1^{D312E}$), the kinase-inactive form of Cds1, was unable to phosphorylate itself or the GST–Cdc25 fusion proteins. Coomassie blue staining confirmed that equal amounts of GST–Cds1 and GST–Cds1KD were used in these studies. The same protein preparations were used for the experiment shown in Figure 2B. (B) Two-dimensional tryptic phosphopeptide maps of GST–$Cdc25^{1–374}$ and GST–$Cdc25^{1–147}$ phosphorylated by GST–Cds1 (middle) or GST–Chk1 (left) appear very similar. Experiments in which the Chk1 and Cds1 reactions of GST–$Cdc25^{1–374}$ or GST–$Cdc25^{1–147}$ (right) were mixed confirm the similarity of the maps.

Mik1 protein abundance undergoes a large increase in HU-arrested cells (Boddy *et al.*, 1998). This effect requires Rad3 and Cds1 and thus is presumably part of the S–M replication checkpoint response. The experiment shown in Figure 5A was repeated with *cdc25*$^+$ and *cdc25-S99A* integrants in a Δ*mik1* background. By itself, the Δ*mik1* mutation caused a partial checkpoint defect. Approximately 25% of the Δ*mik1 cdc25*$^+$ cells underwent mitosis during the 320-min course of the experiment (Figure 5B), whereas essentially none of the *cdc25*$^+$ or *cdc25-S99A* cells underwent mitosis during this period (Figure 5A). Interestingly, the checkpoint defect of Δ*mik1 cdc25-S99A* cells was increased relative to that of the Δ*mik1 cdc25*$^+$ cells (Figure 5B). Approximately 60% of the Δ*mik1 cdc25-S99A* cells underwent mitosis within 320 min. This finding suggested that the *cdc25-S99A* mutation caused a modest S–M replication checkpoint defect that was revealed in a Δ*mik1* background. However, the Δ*mik1* mutation clearly had a greater effect than did the *cdc25-S99A* mutation.

We also investigated the effect of the *cdc25-S99A* mutation in the *wee1-50* Δ*mik1* assay described in Figure 1. The *cdc25*$^+$ and *cdc25-S99A* plasmids were integrated into Δ*cdc25 wee1-50* Δ*mik1* cells. These cells were synchronized in early G_2 by centrifugal elutriation. The cultures were incubated with or without HU beginning 40 min after elutriation. The cultures were then shifted to 35°C shortly after the completion of mitosis. HU treatment caused an ~40-min delay of mitosis in the *cdc25*$^+$ integrant culture (Figure 5C), as observed with cells of the equivalent genotype shown in Figure 1. In contrast, HU treatment caused only an ~20-min delay of mitosis in the *cdc25-S99A* integrant strain culture (Figure 5C). These data suggest that the *cdc25-S99A* mutation impaired but did not abolish the regulation of Cdc25 by the S–M replication checkpoint.

G_2–M Damage Checkpoint Diminished by the cdc25-S99A *Mutation*

The effect of the *cdc25-S99A* mutation on the G_2–M DNA damage checkpoint was also investigated. Initial studies in which cells were exposed to 100 Gy of

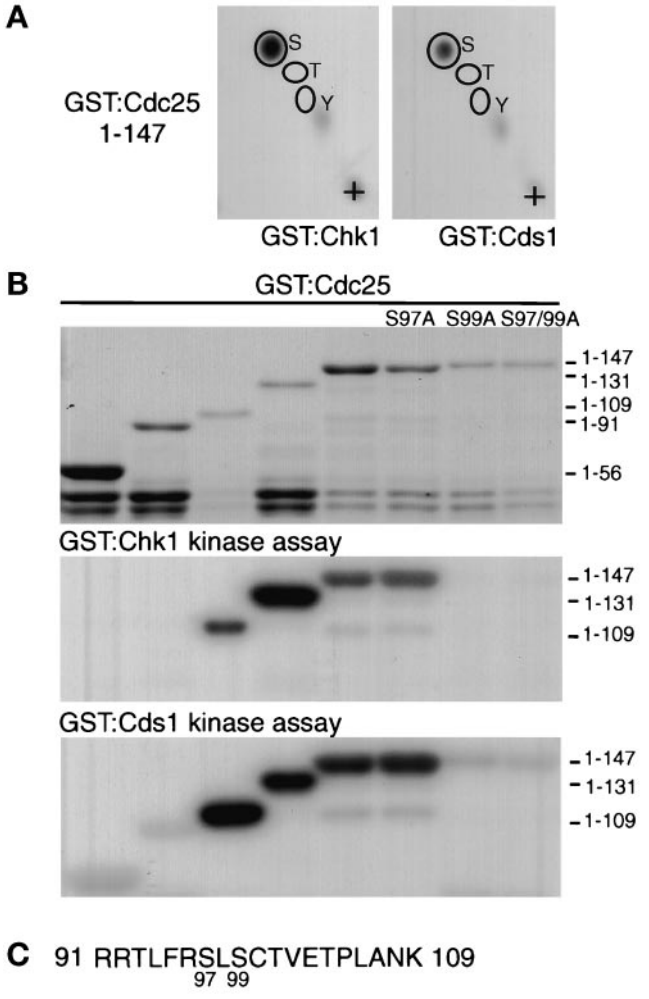

Figure 4. Cds1 and Chk1 phosphorylate serine-99 of Cdc25. (A) Two-dimensional phosphoamino acid analysis revealed that GST–Cds1 (right) and GST–Chk1 (left) phosphorylated GST–Cdc25$^{1-147}$ exclusively on serine. (B) Various regions of Cdc25 were expressed as GST fusion proteins in bacteria and tested as substrates for GST–Cds1 (bottom) or GST–Chk1 (middle). A Coomassie blue stain of the protein gel indicates the relative amounts and positions of fusion proteins and degradation products (top). Autoradiograms showed that the 1–109 construct was phosphorylated by both kinases, whereas the 1–91 construct was not phosphorylated. This result indicated that the major phosphorylation site is located in the 91–109 region of Cdc25. Mutation of serine-97 to alanine (S97A) did not change phosphorylation of the 1–147 construct, whereas the S99A and S97/99A forms of the 1–147 construct were not phosphorylated. Thus, serine-99 appears to be the major site of phosphorylation. (C) Sequence of the 91–109 region of Cdc25 is shown.

ionizing irradiation revealed that the DNA damage checkpoint was intact in the *cdc25-S99A* integrant (our unpublished observations). Therefore, a long-term DNA damage checkpoint experiment was performed in an attempt to reveal more subtle effects of the *cdc25-S99A* mutation. This type of experiment was most conveniently performed with the radiomimetic drug bleomycin, which causes double-strand breaks of DNA (Kostrub *et al.*, 1998). Cells were synchronized in early G_2 phase by centrifugal elutriation and then exposed to bleomycin. The bleomycin treatment induced an ~120-min mitotic delay in the *cdc25-S99A* integrant (Figure 6). However, the *cdc25-S99A* integrant also exhibited a substantial checkpoint defect. More than 80% of the *cdc25-S99A* cells completed mitosis within 300 min, compared with ~15% in the *cdc25*$^+$ control (Figure 6). Thus, the *cdc25-S99A* mutation partially impaired the G_2–M DNA damage checkpoint.

DISCUSSION

These studies have addressed mechanisms by which the protein kinases Cds1 and Chk1 enforce mitotic DNA checkpoints. Emphasis was placed on the S–M replication checkpoint elicited by HU. Our studies suggest that the S–M replication checkpoint inhibits the function of Cdc25 in vivo. This regulation seems to be mediated directly by Cds1 and Chk1, which are able to inhibit Cdc25 function in vitro. Remarkably, Chk1 and Cds1 phosphorylate the same preferred site on Cdc25 in vitro. These findings suggest a model in which Chk1 and Cds1 share overlapping responsibilities and mechanisms in the enforcement of the S–M replication checkpoint. These studies, coupled with recent findings implicating Cds1 in the regulation of Wee1 and Mik1 (Boddy *et al.*, 1998), suggest that the S–M replication checkpoint uses multiple processes to maintain Cdc2 in the tyrosine-15–phosphorylated state.

In Vivo Evidence of S–M Checkpoint Regulation of Cdc25 by Cds1 and Chk1

Our data indicate that the S–M replication checkpoint inhibits Cdc25 function in vivo. These studies used the *wee1-50* Δ*mik1* temperature shift-up assay to measure the rate at which Cdc25 induces the onset of mitosis. HU treatment causes an ~40-min delay of Cdc2 tyrosine-15 dephosphorylation and mitosis in this assay (Rhind and Russell, 1998a). This mitotic delay requires Rad3. Rad3 dependence implies that the S–M replication checkpoint inhibits Cdc25 function in vivo. Conceptually similar experiments provided some of the first evidence that the G_2–M damage checkpoint inhibits Cdc25 (Furnari *et al.*, 1997; Rhind *et al.*, 1997).

Rad3 is thought to regulate the activities of Cds1 and Chk1 (Walworth and Bernards, 1996; Boddy *et al.*, 1998; Lindsay *et al.*, 1998). Indeed, the HU-induced checkpoint arrest is abolished in *cds1 chk1* cells (Boddy *et al.*, 1998; Lindsay *et al.*, 1998; Zeng *et al.*, 1998). We observed that the HU-induced mitotic delay in the *wee1-50* Δ*mik1* temperature shift-up assay was correspondingly eliminated in a *cds1 chk1* background. Approximately one-half of the delay is retained in *chk1* cells, which indicates that Cds1 partially contributes to

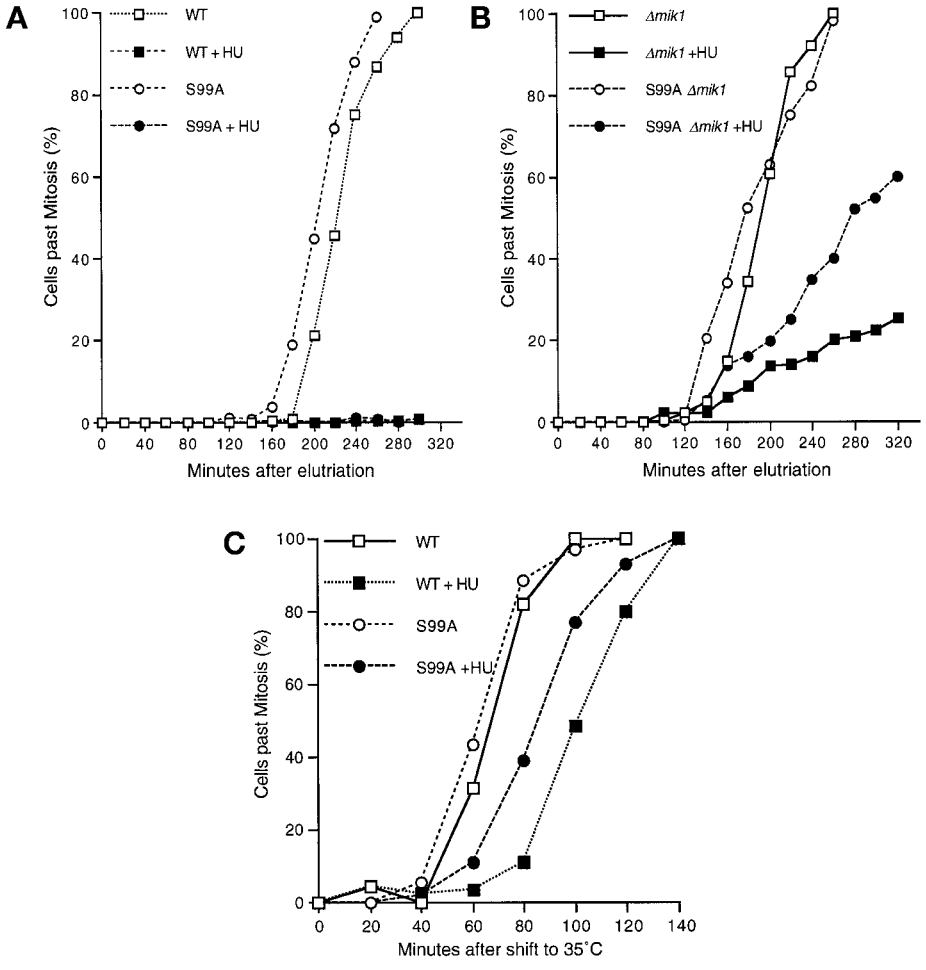

Figure 5. The *cdc25-S99A* mutation impairs the S–M checkpoint. (A and B) Plasmids containing *cdc25*$^+$ (WT) or *cdc25-S99A* (S99A) were integrated in *cdc25-22* or *cdc25-22 mik1::LEU2* cells. Synchronous cultures of cells in early S phase were produced by pretreatment with HU (12 mM) for 120 min at 25°C followed by centrifugal elutriation in the presence of HU. The cells were then washed and resuspended in medium with or without HU. Mock-treated cells underwent mitosis with similar kinetics. (A) The WT and S99A cells were unable to divide during the course of the experiment, indicative of a checkpoint arrest. (B) Approximately 25% of the Δ*mik1* cells underwent mitosis during the course of the experiment. This number was increased to ~60% in S99A Δ*mik1* cells. These findings indicate that the Δ*mik1* mutation impairs the checkpoint and that this effect is exacerbated by the S99A mutation. (C) An experiment was performed to determine the consequence of the S99A mutation in the HU-induced checkpoint delay that is observed after inactivation of Wee1 and Mik1. The *cdc25*$^+$ (WT) or *cdc25-S99A* (S99A) plasmids were integrated into Δ*cdc25 wee1-50* Δ*mik1* cells. The experiment was performed as described in Figure 1. HU caused a delay of ~40 min in the *cdc25*$^+$ strain, whereas only an ~20-min delay of mitosis was observed in the *cdc25-S99A* strain. These data suggest that the S99A mutation impaired but did not abolish the regulation of Cdc25 in response to HU.

the HU-induced inhibition of Cdc25 function in vivo. However, a full ~40-min mitotic delay is maintained in *cds1* cells. Thus, Chk1 is apparently able to effect maximum HU-induced inhibition of Cdc25 function in cells arrested with HU, at least in a *cds1* background. Whether Chk1 contributes as much in *cds1*$^+$ cells is uncertain, because loss of Cds1 activity may lead to enhanced function of Chk1 in HU-arrested cells (Lindsay *et al.*, 1998). However, one is left with the inescapable conclusion that Chk1 contributes at least one-half of the HU-induced mitotic delay in the *wee1-50* Δ*mik1* temperature shift-up assay. Cds1 is apparently unable to impose the maximum inhibition of Cdc25. These findings imply that Chk1 is a more effective inhibitor of Cdc25 function in vivo.

Direct Phosphorylation and Inhibition of Cdc25 by Cds1 and Chk1 In Vitro

In vitro studies also support the hypothesis that Cds1 and Chk1 inhibit Cdc25. These studies used a coupled assay system to measure the ability of Cds1 or Chk1 to inhibit activation of Cdc2/cyclin-B that is catalyzed by Cdc25. Cds1 caused a dose-dependent inhibition of

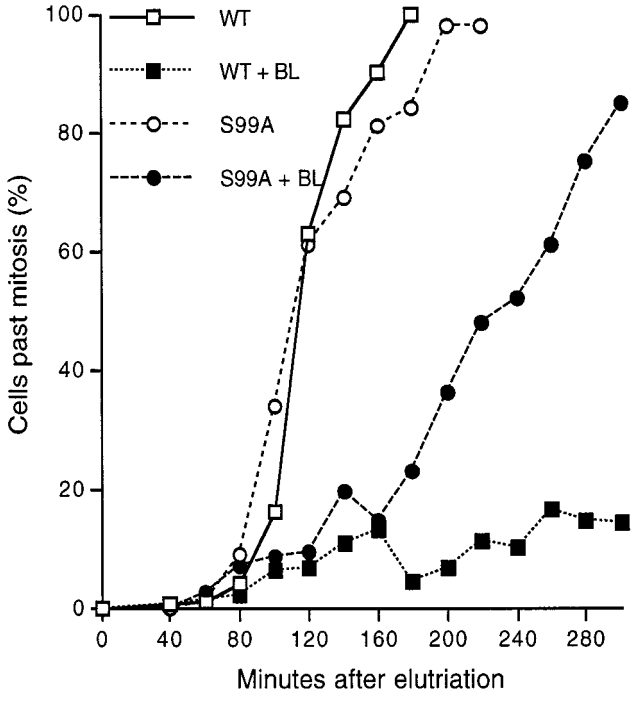

Figure 6. The *cdc25-S99A* mutation impairs the DNA damage checkpoint. Plasmids containing *cdc25*$^+$ (WT) or *cdc25-S99A* (S99A) were integrated in *cdc25-22* cells. Synchronous cultures of cells in early G_2 phase were produced by centrifugal elutriation and exposed to the radiomimetic drug bleomycin (BL) at a concentration of 5 mU/ml or mock treated. Mock-treated cells underwent mitosis with similar kinetics. Most of the WT cells (~80%) were unable to divide during the course of the experiment, indicative of a checkpoint arrest. In contrast, all but ~15% of the S99A mutant cells divided, indicative of a checkpoint failure.

Cdc25. These effects were dependent on the kinase activity of Cds1. Cdc25 was also inhibited by Chk1 in this assay. These findings agree with a recent study that showed that human Cds1, which is also known as Chk2 (Matsuoka *et al*., 1998), directly inhibits human Cdc25C in an in vitro assay (Blasina *et al*., 1999).

Phosphorylation site mapping studies performed in vitro also support the hypothesis that Cds1 and Chk1 inhibit Cdc25. These studies demonstrated that Cds1 and Chk1 phosphorylate the same preferred site in the NH_2-terminal regulatory domain of Cdc25, serine-99. These findings are in agreement with a recently published study that identified serine-99 as a site that is phosphorylated by Cds1 and Chk1 in vitro and that is phosphorylated in vivo (Zeng *et al*., 1998). These investigators also identified serine-192, serine-359, and apparently several other unidentified positions as sites that are phosphorylated by Cds1 and Chk1 in vitro. In our studies, the importance of serine-99 was confirmed with in vivo experiments that showed that the *cdc25-S99A* mutation causes partial defects of the S–M replication and G_2–M damage checkpoint. Zeng *et al*. (1998) also reported that expression of a mutant form of Cdc25 that has alanine substitutions at positions 99, 192, and 359 (*cdc25-S3*) partially impaired the S–M replication checkpoint.

Fission yeast, *Xenopus laevis*, and human Chk1 proteins phosphorylate Cdc25 at positions that are confirmed or potential binding sites for 14-3-3 proteins (Peng *et al*., 1997; Sanchez *et al*., 1997; Kumagai *et al*., 1998a,b; Zeng *et al*., 1998). This fact, coupled with studies of fission yeast mutants that are defective for the 14-3-3 protein encoded by *rad24*$^+$ (Ford *et al*., 1994), suggests that Chk1 might inhibit Cdc25 by inducing association with 14-3-3 proteins. Serine-99 of fission yeast Cdc25 is embedded in the sequence RSLpSCT. This sequence is similar to the preferred 14-3-3 binding motif RSXpSXP defined in two studies and to the RSPpSMP 14-3-3 protein binding region in human Cdc25C (Muslin *et al*., 1996; Peng *et al*., 1997; Yaffe *et al*., 1997). The fission yeast Cdc25 serine-99 motif lacks the proline at position +2, but recent studies have shown that not all 14-3-3 motifs have proline at this position (Liu *et al*., 1997; Zhang *et al*., 1997). Thus it is possible that phosphorylation of serine-99 causes 14-3-3 proteins to associate with Cdc25. Indeed, fission yeast Cdc25 associates with 14-3-3 proteins in vivo (Zeng *et al*., 1998; Lopez-Girona *et al*., 1999). Moreover, Cdc25 protein encoded by *cdc25-S3*, which includes the S99A mutation, exhibits reduced binding to 14-3-3 proteins in vivo (Zeng *et al*., 1998).

In our in vitro assays, we have not added 14-3-3 proteins, which indicates that 14-3-3 proteins are not essential for inhibition of Cdc25 by Chk1 or Cds1 in this in vitro assay system. However, a caveat to this conclusion is that 14-3-3 proteins are very abundant and sometimes contaminate protein preparations from eukaryotic cells. Unfortunately, active full-length fission yeast Cdc25 cannot be prepared from bacteria. Thus, at this point we cannot rigorously exclude the possibility that 14-3-3 proteins are contributing to the inhibition of Cdc25 in our assay system, although it seems very unlikely. However, the situation in vivo might be very different. Recent studies suggest that Chk1 and Rad24, a 14-3-3 protein in fission yeast, are involved in checkpoint-induced nuclear export of Cdc25 (Lopez-Girona *et al*., 1999). The mitotic Cdc2/cyclin-B kinase is located in the nucleus in fission yeast (Booher *et al*., 1989); thus, the nuclear export of Cdc25 that is induced by DNA damage would be expected to interfere with the onset of mitosis. These findings suggest that Chk1 and Rad24 may collaborate to both inhibit Cdc25 and reduce access to its substrate. It should also be recalled that protein phosphorylation can be a highly dynamic process in vivo, with protein phosphatases counterbalancing the activity of protein kinases. The 14-3-3 proteins might shield phosphorylated proteins from phosphatases, either by steric hindrance or by changing protein localization. Thus, in

vivo, 14-3-3 proteins might enhance the effect of Chk1 by decreasing the rate of Cdc25 dephosphorylation.

Chk1 and Cds1 Regulate Cdc25 by a Similar Mechanism

Perhaps the most remarkable finding of this study is that Chk1 and Cds1 phosphorylate the same major site on Cdc25, serine-99. This site seems to be physiologically important because expression of Cdc25-S99A causes checkpoint defects. Moreover, serine-99 was recently identified as an in vivo phosphorylation site on Cdc25 (Zeng *et al.*, 1998). Clearly, these findings indicate that Chk1 and Cds1 inhibit Cdc25 by very similar, if not identical, mechanisms. This finding is all the more remarkable for the fact that Chk1 and Cds1 are dissimilar protein kinases, having no apparent sequence homology other than the core consensus sequences of protein kinases (Walworth *et al.*, 1993; Murakami and Okayama, 1995). Fission yeast Chk1 has a large COOH-terminal regulatory domain that is highly conserved with human Chk1 but absent in Cds1. Cds1 has an NH_2-terminal forkhead-associated domain that is conserved with *S. cerevisiae* Rad53, a putative homologue of Cds1, but is not found in Chk1 (Hofmann and Bucher, 1995). It is unusual that unrelated protein kinases phosphorylate the same substrate on the same site.

Additional Sites of Phosphorylation of Cdc25 by Chk1 and Cds1

As mentioned above, a recent study identified serine-99 and several other positions as sites that are phosphorylated by Cds1 and Chk1 in vitro (Zeng *et al.*, 1998). In agreement with our analyses, these studies identified the phosphopeptide containing serine-99 as the most highly phosphorylated tryptic peptide. This was particularly true for Chk1. Serine-192, serine-359, and one or two other unidentified sites appeared to be phosphorylated at lower levels (Zeng *et al.*, 1998). Our mapping studies were performed with truncated forms of Cdc25 that did not extend beyond amino acid 374, which might account for the absence of a phosphopeptide containing serine-359 in our maps. Serine-192 appears to be a minor site (Zeng *et al.*, 1998). Zeng *et al.* (1998) performed checkpoint studies with a *cdc25* construct (*cdc25-S3*) that contained three mutations in which serine codons at positions 99, 192, and 359 were changed to alanine. Expression of *cdc25-S3* from an *nmt1* promoter construct caused a modest HU checkpoint defect, with ~20% of the cells exhibiting a cut phenotype when examined after 8 h in HU. In our studies, the *cdc25-S99A* mutation in a *mik1*$^+$ background caused no apparent S–M replication checkpoint defect during an ~6-h incubation in HU, although the time course of HU incubation was shorter. However, we have observed that the *cdc25-S99A* integrant strain is sensitive to chronic exposure of what is normally a sublethal amount of HU (our unpublished observations). These findings agree with the long-term HU studies performed by Zeng *et al.* (1998). Our studies shown in Figure 5C indicate that the *cdc25-S99A* mutation impairs but does not abolish the S–M replication checkpoint-induced inhibition of Cdc25 function in vivo. This result implies that phosphorylation of serine-99 is important for S–M replication checkpoint regulation of Cdc25, but apparently, other sites of phosphorylation are also involved in the inhibition of Cdc25. However, inhibition of Cdc25 probably only accounts for part of the S–M replication checkpoint, because other studies have shown that Mik1 and possibly Wee1 are positively regulated by the S–M replication checkpoint (Boddy *et al.*, 1998; Michael and Newport, 1998).

We have also shown that the *cdc25-S99A* mutation partially abrogates the G_2–M damage checkpoint, at least when assayed during long-term exposure to bleomycin. However, these cells relative to the mock-treated controls did exhibit a checkpoint delay of ~100 min. This result explains why checkpoint studies that involved a pulse of ionizing radiation that is sufficient to cause a mitotic delay of ~1 h did not reveal a checkpoint defect in *cdc25-S99A* cells (our unpublished observations). It remains to be determined whether the substantial G_2–M damage checkpoint delay that is present in *cdc25-S99A* cells involves phosphorylation of other sites on Cdc25.

Model for the S–M Replication Checkpoint

The results described in this report, together with previous studies, suggest the following model for the S–M replication checkpoint in fission yeast (Figure 7). The ultimate target of this checkpoint is the regulation of phosphorylation of Cdc2 on tyrosine-15, because this phosphorylation is required for the S–M replication checkpoint in fission yeast (Enoch and Nurse, 1990; Rhind and Russell, 1998a). Incompletely replicated DNA, which might be sensed as stalled replication forks, leads to the activation of Cds1 by a process that requires Rad3 and other checkpoint Rad proteins (Boddy *et al.*, 1998; Lindsay *et al.*, 1998). Cds1 inhibits Cdc25 by phosphorylation of serine-99 and other sites, induces the accumulation of Mik1 protein, and phosphorylates Wee1. Inhibition of Cdc25 and accumulation of Mik1 help to maintain phosphorylation of Cdc2 on tyrosine-15, thereby retaining Cdc2 in the inhibited state. The significance of phosphorylation of Wee1 by Cds1 remains to be established. HU might also cause small amounts of DNA damage, leading to activation of Chk1. This small amount of DNA damage is insufficient to cause a large amount of Chk1 phosphorylation. However, the fact that the Δ*chk1* mutation causes a partial defect in the *wee1-50* Δ*mik1* temperature

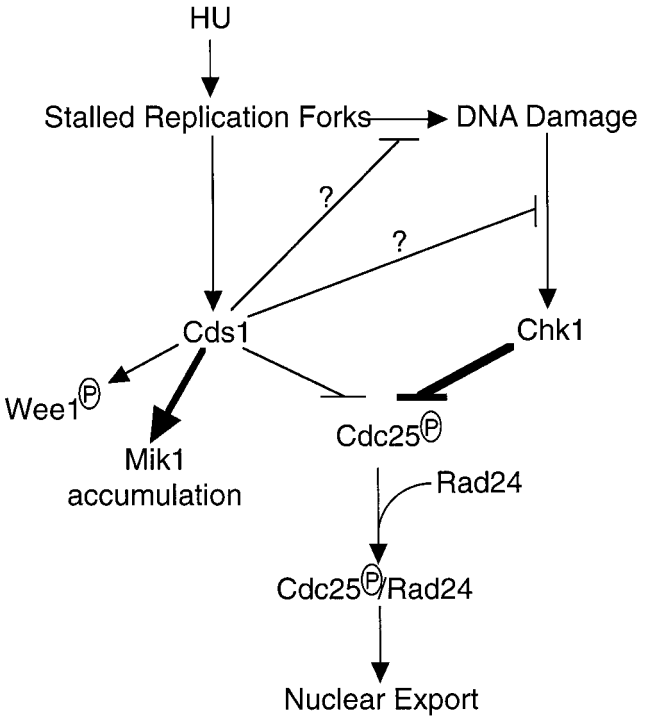

Figure 7. Model of replication and repair checkpoint pathways in fission yeast. See text for discussion of model.

shift-up assay suggests that Chk1 contributes to the S–M replication checkpoint arrest, at least in this genetic background. Damaged DNA, which might be sensed via the activity of repair enzymes, leads to phosphorylation of Chk1 by a process that requires Rad3 and the other checkpoint Rad proteins, as well as Crb2/Rhp9 (Walworth and Bernards, 1996; Saka *et al.*, 1997). Chk1 is presumably activated by phosphorylation, although this hypothesis remains to be proven. Chk1 targets Cdc25 (Furnari *et al.*, 1997; Peng *et al.*, 1997; Sanchez *et al.*, 1997), inhibiting Cdc25 by the same mechanism that is used by Cds1. This mechanism seems to involve direct inhibition of Cdc25 and net nuclear export of Cdc25 that is dependent on the 14-3-3 protein encoded by *rad24*$^+$ (Lopez-Girona *et al.*, 1999).

Evolutionary Conservation of Checkpoints

There is increasing evidence that indicates that mitotic DNA checkpoints in fission yeast are primarily conserved among eukaryotes. Inhibitory phosphorylation of Cdc2 catalyzed by Wee1 and Wee1-like kinases is important for damage and replication checkpoints in human cells (Jin *et al.*, 1996; Blasina *et al.*, 1997). Human AT cells, which are defective for the *ATM* gene that is related to fission yeast *rad3*, are profoundly defective in the damage checkpoint evoked by γ-irradiation (Hoekstra, 1997). Likewise, the human *CHK1* gene, cloned on the basis of its homology to fission yeast *chk1*$^+$, encodes a kinase that phosphorylates human Cdc25C on a site that seems to be important for checkpoint function in vivo (Flaggs *et al.*, 1997; Peng *et al.*, 1997; Sanchez *et al.*, 1997). *Grapes*, the *Drosophila* homologue of *chk1*$^+$, is also implicated in checkpoint control (Fogarty *et al.*, 1997; Sibon *et al.*, 1997). Recent studies have identified a *cds1* homologue in human cells (Matsuoka *et al.*, 1998; Blasina *et al.*, 1999). Other mammalian genes that are closely related to most of the fission yeast checkpoint Rad protein genes have recently been discovered (Rhind and Russell, 1998b). Investigations of these genes, guided by an understanding of checkpoint mechanisms in yeast, should continue to provide important insights into checkpoint controls in humans.

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