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Cdc25 Mitotic Inducer Targeted by Chk1 DNA Damage Checkpoint Kinase

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Arrest of the cell cycle at the G_2 checkpoint, induced by DNA damage, requires inhibitory phosphorylation of the kinase Cdc2 in both fission yeast and human cells. The kinase Wee1 and the phosphatase Cdc25, which regulate Cdc2 phosphorylation, were evaluated as targets of Chk1, a kinase essential for the checkpoint. Fission yeast *cdc2-3w Δcdc25* cells, which express activated Cdc2 and lack Cdc25, were responsive to Wee1 but insensitive to Chk1 and irradiation. Expression of large amounts of Chk1 produced the same phenotype as did loss of the *cdc25* gene in *cdc2-3w* cells. Cdc25 associated with Chk1 in vivo and was phosphorylated when copurified in Chk1 complexes. These findings identify Cdc25, but not Wee1, as a target of the DNA damage checkpoint.

Eukaryotic cells have cell cycle checkpoints that arrest division in response to DNA damage (*1*, *2*). In the case of damage inflicted during the G_2 phase of the cell cycle (gap of time after DNA synthesis, but before mitosis), arrest occurs before the onset of mitosis. In metazoans, G_2 checkpoints are important for the maintenance of genome integrity, allowing time for the repair of damaged DNA, or, in the case of severe damage, for activation of programmed cell death. In haploid yeast the G_2 checkpoint is required for viability. Understanding how the checkpoint signal intersects with the central machinery controlling progression from the G_2 to the M phase is a major goal of current cell cycle studies.

In the fission yeast *Schizosaccharomyces pombe*, checkpoint arrest in the G_2 phase requires a large number of proteins, many of which are believed to play a direct role in DNA repair. These proteins include Rad3, a kinase related to the ATM protein that is defective in ataxia telangiectasia patients (*3*). Damaged DNA is presumed to activate Chk1, a protein kinase that is essential for the checkpoint arrest (*4*–*6*). The ultimate target of the checkpoint signal is believed to be Cdc2, the cyclin-dependent kinase that induces mitosis. In the normal cell cycle of fission yeast and mammalian cells, the timing of mitosis is determined by the inhibitory phosphorylation of Cdc2 (*7*). In fission yeast this phosphorylation occurs on Tyr^{15} and is catalyzed by the kinases Wee1 and Mik1, with Wee1 being the most active. Dephosphorylation of Tyr^{15} and consequent induction of mitosis is catalyzed by the phosphatase Cdc25. The induction of mitosis is thought to be facilitated by activation of Cdc25 and inhibition of Wee1 activity during the G_2-M transition. Initial studies suggested that the DNA damage checkpoint operated independently of tyrosine phosphorylation of Cdc2 (*8*, *9*), but a more recent analysis established that inhibitory phosphorylation of Tyr^{15} is essential for the checkpoint (*10*). These findings suggest several mechanisms for Chk1-mediated regulation of Cdc2, including the Chk1-dependent activation of Wee1 or inhibition of Cdc25 (*10*, *11*). We designed genetic and biochemical experiments to test these possibilities.

Cells of the genotype *wee1-50 Δmik1*, which lack Mik1 and express temperature-sensitive Wee1, undergo rapid dephosphorylation of Cdc2 on Tyr^{15} and induction of mitosis when shifted to the restrictive temperature (*12*). Irradiation of these cells before the temperature shift causes delay of dephosphorylation of Cdc2 and of entry into mitosis (*10*). Thus, irradiation induces a transient cell cycle arrest after inactivation of Tyr^{15} kinases, potentially by inhibition of Cdc25 activity. An experiment was performed to determine whether this delay was due to an authentic checkpoint mediated by Chk1 kinase. For this experiment we used $chk1^+$ and *Δchk1* alleles in a *wee1-50 Δmik1* background. Cells synchronized in early G_2 phase were exposed to gamma irradiation or mock irradiated and then shifted from 25° to 35°C. Irradiation caused a ~60-min delay of mitosis in the $chk1^+$ cells (Fig. 1). This delay is attributable to a reduction in the rate of dephosphorylation of Cdc2 on Tyr^{15} (*10*). In contrast, gamma irradiation did not delay mitosis in *Δchk1* cells (Fig. 1). Therefore, Chk1 is required for the irradiation-induced delay of mitosis observed after inactivation of Wee1 and Mik1 kinases.

These findings implicate Cdc25 as a potential target of Chk1 regulation, but they do not indicate whether Wee1 may also be regulated by Chk1. We used a *cdc2-3w Δcdc25* strain to address this question. The

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cdc2-3w mutation is a dominant active allele that relieves the requirement for Cdc25 but leaves Cdc2 responsive to Wee1. The latter point is evident from the observations that *cdc2-3w wee1-50* cells undergo lethal premature mitosis when incubated at 35°C and that *cdc2-3w* cells are sensitive to increased *wee1*$^+$ gene dosage (*13*, *14*). In addition, elimination of the kinase Nim1, an inhibitor of Wee1 (*15*–*18*), causes a large increase in the size of *cdc2-3w* Δ*cdc25* cells, indicative of a G_2 delay resulting from increased Wee1 activity (*19*).

If Chk1 activates Wee1, then *cdc2-3w* Δ*cdc25* cells should undergo an irradiation-induced checkpoint delay or arrest. However, whereas wild-type cells underwent cell cycle arrest in response to gamma irradiation, *cdc2-3w* Δ*cdc25* cells did not (Fig. 2A). The irradiated *cdc2-3w* Δ*cdc25* cells underwent division at a size equivalent to the mock-irradiated *cdc2-3w* Δ*cdc25* cells. Expression of large amounts of a fusion protein of glutathione-S-transferase (GST) with Chk1 induces G_2 arrest, presumably by inappropriate activation of the checkpoint signal (*5*, *10*, *20*). Whereas wild-type cells underwent cell cycle arrest in response to GST-Chk1 expression, *cdc2-3w* Δ*cdc25* cells were insensitive (Fig. 2B). The *cdc2-3w* Δ*cdc25* cells that expressed GST-Chk1 continued to divide at a rate and cell size that was equivalent to that of *cdc2-3w* Δ*cdc25* cells in which GST-Chk1 expression was repressed.

These studies indicate that the DNA damage checkpoint does not activate Wee1 because *cdc2-3w* Δ*cdc25* cells respond to increased Wee1 activity but are insensitive to irradiation and expression of GST-Chk1. This conclusion is consistent with the fact that *wee1*$^-$ cells undergo cell cycle arrest in response to irradiation (*8*). Moreover, GST-Chk1 expression in Δ*wee1* cells led to the appearance of elongated cells, although the response of Δ*wee1* cells was delayed relative to that of wild-type cells (Fig. 2B). This delay may account for the previous failure to observe an effect of Chk1 overexpression in Δ*wee1* cells (*11*).

Although the *cdc2-3w* allele bypasses the requirement for Cdc25, *cdc2-3w* cells remain responsive to changes in Cdc25 activity. This conclusion is derived from the observation that *cdc2-3w* cells divide at a length of ~9 μm, whereas *cdc2-3w* Δ*cdc25* cells divide at ~17 μm (*13*). If Chk1 primarily acts by inhibiting Cdc25, then expression of large amounts of GST-Chk1 in a *cdc2-3w* background should result in the same phenotype as mutational inactivation of Cdc25. As predicted by this hypothesis, *cdc2-3w* cells that overexpressed Chk1 underwent division at a size that was very similar to that of *cdc2-3w* Δ*cdc25* cells (Fig. 2B).

We tested whether the checkpoint mechanism might involve a close interaction between Cdc25 and Chk1. Lysates from cells expressing functional GST-

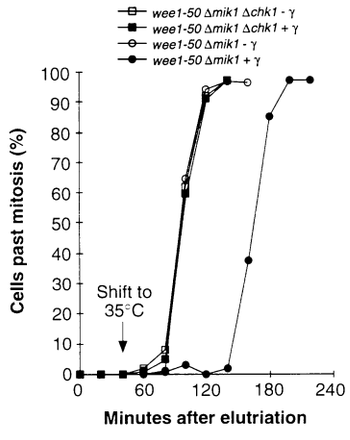

Fig. 1. Requirement of Chk1 for the radiation-induced delay of mitosis that occurs after inactivation of Wee1 and Mik1 kinases. Synchronous cultures of *wee1-50* Δ*mik1* (PR754) or *wee1-50* Δ*mik1* Δ*chk1* (NR1604) cells in early G_2 phase were split and irradiated with 100 Gy of gamma radiation (+γ) or mock irradiated (−γ). The cultures were then shifted to 35°C to inactivate Wee1-50 protein. Cell cycle progression was monitored by counting the percent of cells undergoing septation (*24*).

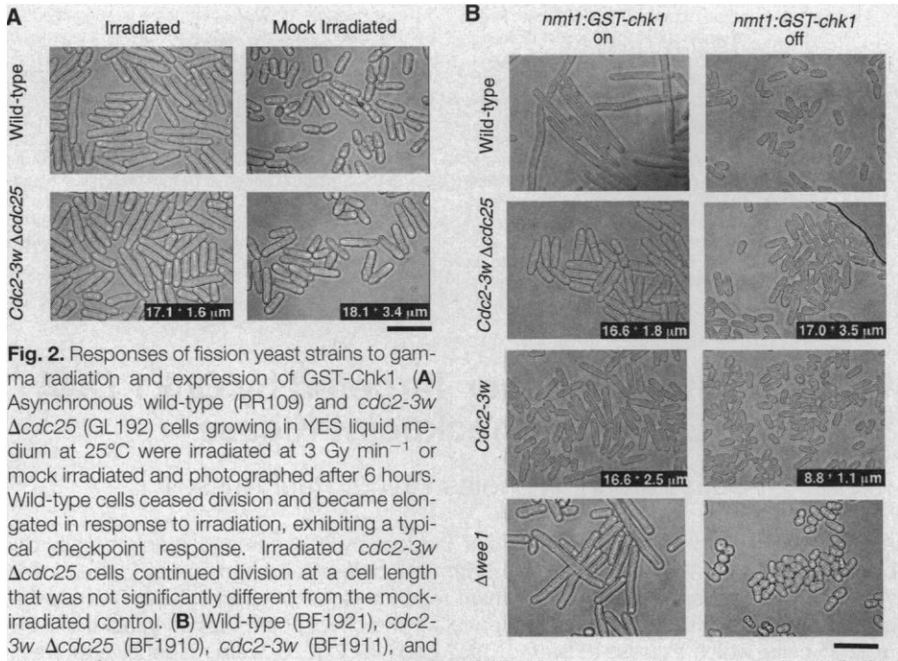

Fig. 2. Responses of fission yeast strains to gamma radiation and expression of GST-Chk1. (**A**) Asynchronous wild-type (PR109) and *cdc2-3w* Δ*cdc25* (GL192) cells growing in YES liquid medium at 25°C were irradiated at 3 Gy min^{-1} or mock irradiated and photographed after 6 hours. Wild-type cells ceased division and became elongated in response to irradiation, exhibiting a typical checkpoint response. Irradiated *cdc2-3w* Δ*cdc25* cells continued division at a cell length that was not significantly different from the mock-irradiated control. (**B**) Wild-type (BF1921), *cdc2-3w* Δ*cdc25* (BF1910), *cdc2-3w* (BF1911), and Δ*wee1* (NR1970) strains having an integrated *nmt1:GST-chk1*$^+$ construct were grown in media that derepressed (EMM2, *nmt1:GST-chk1* on) or repressed (EMM2+B1, *nmt1:GST-chk1* off) expression of GST-Chk1 from the thiamine-repressible *nmt1* promoter (*25*). Cells were photographed after 16 hours (27 hours in the case of NR1970) at 30°C. Expression of GST-Chk1 caused cell cycle arrest in wild-type cells, whereas *cdc2-3w* Δ*cdc25* cells continued to divide. Cells of the genotype *cdc2-3w* became moderately elongated in response to GST-Chk1 expression, dividing at a size similar to that of *cdc2-3w* Δ*cdc25* cells. Elongated Δ*wee1* cells first appeared at ~22 hours after incubation in derepressing medium. Numbers in panels refer to cell length at division. Scale bars represent 22 μm.

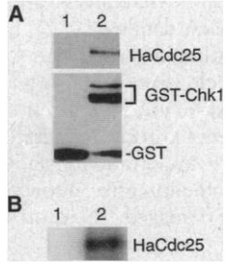

Fig. 3. Association of Cdc25 with GST-Chk1 in vivo and phosphorylation of Cdc25 in GST-Chk1 complexes in vitro. (**A**) GST or GST-Chk1 were expressed in cells in which genomic *cdc25*$^+$ was replaced with *cdc25:6HA*, a functional *cdc25* construct containing a sequence encoding six tandem copies of the hemagglutinin (HA) epitope (*26*). GST and GST-Chk1 were purified by GSH-Sepharose chromatography. Immunoblotting with antibody to GST (bottom panel) detected GST as a ~27-kD protein (lane 1), whereas GST-Chk1 was detected as a full-length ~80-kD fusion protein together with ~65-, ~63-, and ~27-kD presumptive degradation products (lane 2). Immunoblotting with antibody to HA (top panel) detected HaCdc25 (gene product of *cdc25:6HA*) in association with GST-Chk1. (**B**) Incubation of GST and GST-Chk1 complexes with [γ-^{32}P]ATP followed by immunoprecipitation with antibody to Cdc25 showed that Ha-Cdc25 became phosphorylated in the GST-Chk1 complexes (lane 2), whereas no Ha-Cdc25 was detected in association with GST (lane 1) (*27*).

Chk1 fusion protein or unfused GST were analyzed by glutathione (GSH)-Sepharose precipitation followed by immunoblotting. GST-Chk1 precipitated with Cdc25, whereas no Cdc25 was detected in association with GST (Fig. 3A). Incubation of GST-Chk1 with associated Cdc25 in the presence of [γ-^{32}P]adenosine triphosphate (ATP) resulted in phosphorylation of Cdc25 (Fig. 3B), suggesting that Cdc25 may be a direct substrate of Chk1 kinase. Chk1 protein purified from an insect cell expression system phosphorylated Cdc25 in vitro (*21*).

Activation of the DNA damage checkpoint requires Rad3, a kinase related to the ATM protein that is defective in ataxia telangiectasia patients (*3*). DNA damage leads to increased phosphorylation of Chk1 by a Rad3-dependent process, suggesting that Chk1 may be activated by phosphorylation (*5*). Our studies identify Cdc25 as a key, possibly direct, target of Chk1. In addition, these findings exclude Wee1 as an important Chk1 substrate. Therefore, we propose that Rad3-dependent activation of Chk1 leads to negative regulation of Cdc25 (Fig. 4). This negative regulation may occur by direct inhibition of Cdc25 activity, prevention of the activation of Cdc25 that occurs at the G_2-M transition, or interference in the interaction between Cdc25 and Cdc2. Inhibitory phosphorylation of Cdc2 is crucial for G_2 DNA damage arrest in mammalian cells (*22, 23*). In these cells it is not known whether this arrest is brought about by inhibition of Cdc2 dephosphorylation, nor is it known if mammals have a Chk1 homolog. However, in view of the striking degree of homology of mitotic control mechanisms in fission yeast and mammals, we expect that the *S. pombe* checkpoint control will serve as a useful paradigm for investigating the DNA damage checkpoint mechanism in more complex organisms.

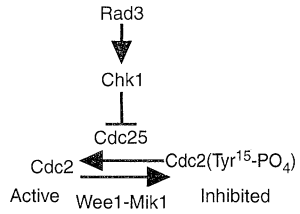

Fig. 4. Model of the DNA damage checkpoint mechanism in fission yeast. Rad3 and Chk1 kinases are required for the checkpoint. Chk1 undergoes a Rad3-dependent phosphorylation in irradiated cells, and Chk1 overexpression induces cell cycle arrest by a Rad3-independent mechanism (*20*), indicating that Chk1 activation is regulated by Rad3, perhaps by direct phosphorylation. Chk1 inhibits Cdc25 and thereby prevents Cdc2 Tyr^{15} dephosphorylation.

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27. Purification of GST proteins expressed in fission yeast, immunoblotting, protein kinase assays, and immunoprecipitation were performed as described [K. Shiozaki and P. Russell, *Nature* **378**, 739 (1995); *Genes Dev.* **10**, 2276 (1996); S. Moreno, P. Nurse, P. Russell, *Nature* **344**, 549 (1990)]. Control experiments with *cdc2-3w* Δ*cdc25* cells that expressed GST-Chk1 confirmed that Cdc25 was specifically associated with GST-Chk1 and phosphorylated in association with GST-Chk1. Analysis of the GST-Chk1 kinase reaction mixture before immunoprecipitation with antibody to Cdc25 revealed proteins migrating with the predicted size of GST-Chk1 and Cdc25 as the major ^{32}P-labeled protein.

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Conservation of the Chk1 Checkpoint Pathway in Mammals: Linkage of DNA Damage to Cdk Regulation Through Cdc25

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In response to DNA damage, mammalian cells prevent cell cycle progression through the control of critical cell cycle regulators. A human gene was identified that encodes the protein Chk1, a homolog of the *Schizosaccharomyces pombe* Chk1 protein kinase, which is required for the DNA damage checkpoint. Human Chk1 protein was modified in response to DNA damage. In vitro Chk1 bound to and phosphorylated the dual-specificity protein phosphatases Cdc25A, Cdc25B, and Cdc25C, which control cell cycle transitions by dephosphorylating cyclin-dependent kinases. Chk1 phosphorylates Cdc25C on serine-216. As shown in an accompanying paper by Peng *et al.* in this issue, serine-216 phosphorylation creates a binding site for 14-3-3 protein and inhibits function of the phosphatase. These results suggest a model whereby in response to DNA damage, Chk1 phosphorylates and inhibits Cdc25C, thus preventing activation of the Cdc2–cyclin B complex and mitotic entry.

Cell cycle checkpoints are regulatory pathways that control the order and timing of cell cycle transitions and ensure that critical events such as DNA replication and chromosome segregation are completed with high fidelity. In response to DNA