Cdc2 tyrosine phosphorylation is required for the DNA damage checkpoint in fission yeast

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A common cellular response to DNA damage is cell cycle arrest. This checkpoint control has been the subject of intensive genetic investigation, but the biochemical mechanism that prevents mitosis following DNA damage is unknown. In *Schizosaccharomyces pombe*, as well as vertebrates, the timing of mitosis under normal circumstances is determined by the balance of kinases and phosphatases that regulate inhibitory phosphorylation of Cdc2. In *S. pombe*, the phosphorylation occurs on tyrosine-15. This method of mitotic control is also used in *S. pombe* to couple mitosis with completion of DNA replication, but the role of Cdc2 tyrosine phosphorylation in the Chk1 kinase-mediated DNA damage checkpoint has remained uncertain. We show that, in contrast to recent speculation, the G₂ DNA damage checkpoint arrest in *S. pombe* depends on the inhibitory tyrosine phosphorylation of Cdc2 carried out by the Wee1 and Mik1 kinases. Furthermore, the rate of Cdc2 tyrosine dephosphorylation is reduced by irradiation. This result implicates regulation of Cdc2 tyrosine dephosphorylation, mainly carried out by the Cdc25 tyrosine phosphatase, as an important part of the mechanism by which the DNA damage checkpoint induces Cdc2 inhibition and G₂ arrest.

[Key Words: *Schizosaccharomyces pombe*; mitotic control; DNA damage checkpoint; Cdc2; Tyrosine phosphorylation; Radiation]

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Cell cycle arrest in response to DNA damage is an important mechanism used to maintain genome integrity (Weinert and Hartwell 1988; Hartwell and Kastan 1994). In most cases, DNA damage leads to cell cycle arrest until the damage can be repaired or, in metazoan cells, programmed cell death can be activated. In the case of DNA damage during G₂, the cell cycle is arrested at the G₂-M transition point. For a normal mammalian or fission yeast cell cycle, the timing of the G₂-M transition is controlled by the inhibitory phosphorylation of Cdc2 (Russell and Nurse 1986, 1987; Gould and Nurse 1989; Krek and Nigg 1991; Norbury et al. 1991; McGowan and Russell 1993). In *Schizosaccharomyces pombe*, this phosphorylation occurs on tyrosine-15, whereas in mammalian cells the phosphorylation of both tyrosine-15 and threonine-14 play important roles in mitotic control. The tyrosine phosphorylation of Cdc2 is regulated by the balance of the activities of the tyrosine-15 kinases, encoded in *S. pombe* by *wee1*⁺ and *mik1*⁺ (Russell and Nurse 1987; Featherstone and Russell 1991; Lundgren et al. 1991; Parker et al. 1992; Lee et al. 1994), and phosphatases, the major one being encoded by *cdc25*⁺ (Russell and Nurse 1986; Millar et al. 1991). During G₂ in *S. pombe*, Cdc2 is bound to cyclin B and is phosphorylated

on threonine-167, but its activity remains low as a result of the phosphorylation of tyrosine-15, the only tyrosine residue of Cdc2 that is phosphorylated, and mitosis is prevented (Gould and Nurse 1989; Gould et al. 1991). At the G₂-M transition, Cdc2 is tyrosine dephosphorylated and activated, and mitosis ensues.

The G₂ DNA damage checkpoint has been investigated in a number of systems, but the mechanism of the arrest has remained unclear. In *Saccharomyces cerevisiae*, it has been shown that the checkpoint is independent of CDC28 tyrosine phosphorylation (Sorger and Murray 1992). This may reflect the fact that, in *S. cerevisiae*, CDC28 tyrosine phosphorylation does not play a central role in mitotic control (Amon et al. 1992; Sorger and Murray 1992). In mammalian cells there have been several studies that show a correlation between activation of the G₂ DNA damage checkpoint, tyrosine phosphorylation of Cdc2, and a failure to activate Cdc25C (O'Connor et al. 1994; Herzinger et al. 1995). However, it was not possible to determine whether these observations were the cause or effect of the checkpoint. Recently it has been shown that expression of an unphosphorylatable mutant of Cdc2, Cdc2-AF, in HeLa cells compromises the G₂ DNA damage checkpoint. Cdc2-AF cells irradiated during S phase fail to exhibit a normal G₂ delay (Jin et al. 1996). Cdc2-AF expression also prevents Cdc2 activation from being blocked by radiation during

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G₂ (A. Blasina and C. McGowan, pers. comm.). These findings suggest that maintenance of the inhibitory phosphorylation of Cdc2 is important for the G₂ DNA damage checkpoint in mammalian cells, although there may also be other mechanisms that contribute to the checkpoint.

In *S. pombe*, a large number of genes involved in both the G₂ DNA damage checkpoint and the S-phase replication checkpoint have been identified. Many of these genes, including *rad1*, *rad3*, *rad9*, and *rad17*, are required for both the DNA damage checkpoint and the replication checkpoint that prevents mitosis in the presence of unreplicated DNA (al-Khodairy and Carr 1992). It is thought that these genes, which are collectively referred to as the checkpoint *rad* genes, are involved in a signal transduction pathway that results in the inhibition of mitosis. For the replication checkpoint, it is believed that mitosis is inhibited by maintaining Cdc2 in its tyrosine phosphorylated G₂ form (Enoch and Nurse 1990; Sorger and Murray 1992). In contrast, it has been proposed that the DNA damage checkpoint acts by a different mechanism, dependent on the protein kinase Chk1, that does not require Cdc2 tyrosine phosphorylation (Sheldrick and Carr 1993; Carr 1995). *chk1* is a checkpoint gene that acts downstream of the checkpoint *rad* genes and appears to be required only for the DNA damage checkpoint and not the replication checkpoint (Walworth et al. 1993; al-Khodairy et al. 1994; Walworth and Bernards 1996). It is thus conceivable that the checkpoint pathway diverges after the checkpoint *rad* genes, with the replication checkpoint signal leading to inhibition of mitosis by tyrosine phosphorylation of Cdc2, whereas the DNA damage signal is transduced by Chk1 to inhibit mitosis through a Cdc2 tyrosine phosphorylation-independent mechanism. We undertook to test this idea and found, on the contrary, that the Chk1 dependent DNA damage checkpoint acts to prevent mitosis via inhibitory tyrosine phosphorylation of Cdc2.

Results

Tyrosine phosphorylation of Cdc2 is maintained during a radiation-induced G₂ cell cycle arrest

Initially, we examined the tyrosine phosphorylation state of Cdc2 during a γ -radiation-induced cell cycle arrest. Centrifugal elutriation was used to generate a synchronous population of wild-type cells. These cells were irradiated early in G₂ with 100 Gy, enough to delay mitosis for ~1 hr relative to an unirradiated sample of the culture (Fig. 1A). Complexes between Cdc2 and Cdc13, which constitute the *S. pombe* mitotic CDK/cyclin B pair, were isolated by immunoprecipitation with α -Cdc13 antiserum. The immunoprecipitated Cdc2 was assayed for tyrosine phosphorylation by immunoblotting with monoclonal α -phosphotyrosine antibodies. As an internal control, the blot was reprobbed with monoclonal antibodies against the PSTAIR motif of Cdc2. During the period of the cell cycle arrest induced by γ -radiation, Cdc2 remained in its tyrosine phosphorylated interphase

Figure 1. A radiation-induced G₂ cell cycle delay correlates with the continued tyrosine phosphorylation of Cdc2. (A) Centrifugal elutriation was used to generate a synchronous population of wild-type cells (PR109) in early G₂ phase. Half of the culture of was irradiated with 100 Gy of γ -radiation. The subsequent cell cycle progression of the irradiated (●) and unirradiated (■) cultures was monitored by counting the percent of cells undergoing septation. (B) Cdc2 coimmunoprecipitated from samples of the same cultures with α -Cdc13 antibodies was analyzed by Western blotting with an anti-phosphotyrosine antibody. The blots were reprobbed with an anti-PSTAIR antibody to visualize the total amount of Cdc2 present in the immunoprecipitates.

form, even though the cells continued to grow past the size at which Cdc2 is normally dephosphorylated (Fig. 1B). Not until 180 min, as irradiated cells began to enter mitosis, did Cdc2 become tyrosine dephosphorylated. In addition, the α -PSTAIR signal decreased shortly after the dephosphorylation of Cdc2, as a result of the destruction of Cdc13 as cells exit mitosis (Moreno et al. 1989). The fact that the amount of coprecipitated Cdc2 does not decrease during the arrest shows that the checkpoint does not operate by destroying Cdc13 or by otherwise disrupting the Cdc2/Cdc13 complex. We have repeated this experiment to examine the tyrosine phosphorylation of total Cdc2 instead of Cdc13 bound Cdc2 and found identical results (Fig. 5C, below; data not shown). Thus in *S. pombe*, Cdc2 is maintained in its tyrosine phosphorylated form during a radiation-induced cell cycle arrest.

Cdc2 tyrosine-15 phosphorylation is required for the G₂ DNA damage checkpoint

To determine whether the observed correlation between radiation-induced cell cycle arrest and Cdc2 tyrosine phosphorylation reflects an important role for Cdc2 phosphorylation in the checkpoint, we examined the radiation response of cells expressing an unphosphorylat-

able form of Cdc2. Cdc2-Y15F has a phenylalanine in place of tyrosine-15, and is therefore not subject to regulation by tyrosine phosphorylation (Gould and Nurse 1989). Centrifugal elutriation was used to produce a synchronous G_2 culture of a *cdc2-Y15F* strain, carrying this dominant *cdc2-Y15F* allele integrated in a *cdc2⁺* background. One-half of this culture was exposed to γ -radiation for the duration of the experiment. The irradiated and unirradiated cultures proceeded through mitosis with indistinguishable kinetics (Fig. 2). Thus, in the absence of tyrosine phosphorylation of Cdc2, the DNA damage checkpoint is abolished.

Overproduction of Cdc25 overrides the G_2 DNA damage checkpoint

As an independent confirmation of the importance of Cdc2 tyrosine-15 phosphorylation in the DNA damage checkpoint, we examined the effect of overexpressing Cdc25, the major Cdc2 tyrosine-15 phosphatase, on the radiation response. If tyrosine phosphorylation of Cdc2 is necessary to prevent mitosis in response to DNA damage, then dephosphorylating Cdc2 by overexpressing Cdc25 should abrogate this radiation checkpoint, as it does the replication checkpoint (Enoch and Nurse 1990). To test this prediction, we used a strain carrying an integrated copy of *cdc25⁺* driven by the strong thiamine repressible *nmt1* promoter (Maundrell 1990) in a *cdc25⁺* background. The *nmt1-cdc25⁺* cells were grown in media lacking thiamine to induce overexpression of Cdc25, and then irradiated for 3 hr at 200 Gy/hr. Cdc25 overexpression overrides the DNA damage checkpoint, preventing cells from arresting in response to radiation (cf. Fig. 3A, left panels). The control *nmt1-cdc25⁺* cells not

Figure 2. Cdc2 tyrosine phosphorylation is required for a radiation-induced cell cycle arrest. Synchronous G_2 cultures of *cdc2-Y15F* (PR714) (solid symbols) or wild-type (PR109) (open symbols) cells were prepared by centrifugal elutriation. The cultures were then split and half was exposed to γ -radiation (circles). Every 20 min the cultures were examined microscopically to determine what percent of the cells had gone through mitosis. Note that the *cdc2-Y15F* cells are wee and therefore underwent mitosis early relative to the wild-type cells.

Figure 3. (A) Cells overexpressing Cdc25 from the thiamine repressible *nmt1* promoter do not arrest in response γ -radiation. The cells (GL198) in the left panels were exposed to γ -radiation for 3 hr; those in the right panels were not. Cells in the top panels were grown for 23 hr in media lacking thiamine and therefore express high levels of Cdc25; those in the bottom panels were grown with thiamine and so do not overexpress Cdc25. Arrowheads show septa, which indicate cells that have just finished mitosis. The asymmetric septum in the top right panel is a common result of premature mitosis and is occasionally seen in cells overexpressing Cdc25. (B) Chk1 overexpression arrest requires tyrosine-15 phosphorylation of Cdc2. *cdc2-Y15F* (NR1775) or wild-type (BF1758) cells carrying an integrated *nmt1-GST-chk1⁺* fusion were grown in the absence (left panels) or presence (right panels), of thiamine for 18 hr. Similar results were obtained using an unintegrated *nmt1-chk1* fusion without the GST tag (data not shown). (A,B) Bar, $\sim 10 \mu\text{m}$.

overexpressing Cdc25 arrested within 1 hr of the initiation of irradiation, whereas the *nmt1-cdc25⁺* cells overexpressing Cdc25 continued to divide for >3 hr.

cdc2-Y15F cells are insensitive to Chk1 overproduction

The Chk1 kinase is required for the DNA damage checkpoint and is believed to be a downstream member of the checkpoint signal transduction pathway (Walworth and Bernards 1996). Overexpression of Chk1 from the *nmt1* promoter causes cells to arrest in G_2 and elongate (Ford et al. 1994; data not shown). It is thought that this phe-

Cdc-Y15 phosphorylation-dependent G₂ checkpoint

notype is attributable to inappropriate activation of the DNA damage checkpoint. To further investigate the mechanism of the Chk1-mediated checkpoint, we tested the requirement of Cdc2 tyrosine phosphorylation for the Chk1 overexpression arrest. Chk1 was overexpressed as a GST fusion protein from the *nmt1* promoter in both wild-type and *cdc2-Y15F* cells. Whereas the wild-type cells overexpressing Chk1 show a *cdc*-arrest phenotype, this phenotype is suppressed by *cdc2-Y15F* (Fig. 3B, cf. left panels). These results demonstrate that the Chk1 overexpression phenotype requires Cdc2 tyrosine phosphorylation.

Decrease in the rate of Cdc2 tyrosine dephosphorylation in response to DNA damage

The maintenance of Cdc2 tyrosine phosphorylation during a DNA damage-induced arrest could be a result of either an increase in the rate of phosphorylation, a decrease in the rate of dephosphorylation, or both. We investigated these possibilities by removing the Cdc2 tyrosine kinases during radiation-induced arrest. Wee1 and Mik1 are the two tyrosine kinases responsible for phosphorylating Cdc2 on tyrosine-15, with Wee1 having the major activity (Lundgren et al. 1991). *wee1-50 mik1Δ* double mutant cells, which have a temperature-sensitive *wee1* allele and lack a *mik1* gene, grow normally at the permissive temperature of 25°C, but when shifted to the restrictive temperature of 35°C, at which temperature Wee1-50 displays no measurable activity, they rapidly lose detectable Cdc2 tyrosine phosphorylation and enter mitosis regardless of size (Lundgren et al. 1991). We arrested a synchronous G₂ population of *wee1-50 mik1Δ* cells with γ-radiation at 25°C and then shifted half of the culture to 35°C, maintaining both cultures in the continued presence of radiation. Under these conditions, the cells at 25°C stayed arrested, whereas the cells at 35°C rapidly divided (Fig. 4). This result confirms the *cdc2-Y15F* results by demonstrating that the activities of Wee1 and Mik1, and thus presumably the tyrosine-15 phosphorylation of Cdc2, are required to maintain a cell cycle arrest in response to ionizing radiation. However, the irradiated *wee1-50 mik1Δ* cells shifted to 35°C did show a mitotic delay of ~40 min, relative to the unirradiated control (Fig. 4). Thus, even in the absence of Cdc2 tyrosine-15 kinase activity, the cell cycle can be delayed in response to DNA damage. This result shows that regulation of the Wee1 and Mik1 kinases is not absolutely required for a transient checkpoint delay. Because, as we have shown, tyrosine phosphorylation of Cdc2 is absolutely required for the DNA damage checkpoint, the ability of cells to delay mitosis in the absence of the Cdc2 tyrosine kinases implicates regulation of Cdc2 tyrosine dephosphorylation in the checkpoint mechanism.

To confirm that Cdc2 tyrosine dephosphorylation is regulated by the DNA damage checkpoint, we directly monitored the level of Cdc2 tyrosine phosphorylation in irradiated cells after the elimination of Wee1 and Mik1. *wee1-50 mik1Δ* cells were synchronized, irradiated, and analyzed as the wild-type cells in Figure 1, except that

Figure 4. The Cdc2 tyrosine-15 kinases, Wee1 and Mik1, are required for a radiation-induced G₂ cell cycle arrest. Synchronous G₂ cultures of *wee1-50 mik1Δ* (PR754) or wild-type (PR109) cells were arrested by exposure to γ-radiation at the permissive temperature of 25°C for 60 min. Then half of both the irradiated and unirradiated cultures were shifted to the restrictive temperature of 35°C and radiation exposure was continued. Every 20 min the cultures were examined microscopically to determine what percent of the cells had gone through mitosis. For clarity, the data for the unirradiated *wee1-50 mik1Δ* culture at 25°C and both wild-type cultures at 25°C are not shown.

after exposure to radiation half of the culture was shifted to 35°C. As in the continuous exposure experiment (Fig. 4), the *wee1-50 mik1Δ* cells shifted to 35°C showed a radiation induced delay of mitosis (Fig. 5A). However, they also showed a corresponding radiation-induced delay in dephosphorylation of Cdc2 (Fig. 5B). Thus, radiation exposure causes Cdc2 to be maintained in its inactive tyrosine phosphorylated state even in the absence of active Cdc2 tyrosine kinases.

In Figure 5B, the α-PSTAIR signal decreased shortly after the dephosphorylation of Cdc2, as a result of the destruction of Cdc13 as cells exit mitosis (Moreno et al. 1989). To show more clearly that the observed decrease in the α-phosphotyrosine signal is attributable to dephosphorylation of Cdc2 and is independent of the decrease in the amount of immunoprecipitated Cdc2, we repeated the above experiment, but isolated total Cdc2 instead of that bound to Cdc13. As above, Cdc2 stayed tyrosine phosphorylated at its interphase level during the radiation arrest. It was then rapidly dephosphorylated as the cells began to enter mitosis (Fig. 5C).

Discussion

The role of Cdc2 tyrosine-15 phosphorylation in the G₂ DNA damage checkpoint

We have shown that the G₂ DNA damage checkpoint in *S. pombe* is completely dependent upon the inhibitory phosphorylation of Cdc2 on tyrosine-15. This is demonstrated by three independent methods of compromising

Figure 5. A radiation-induced cell cycle delay correlates with the continued tyrosine phosphorylation of Cdc2 even in the absence of the Cdc2 tyrosine kinases. (A) Half of a synchronous culture of *wee1-50 mik1Δ* cells (PR754) in early G₂ was irradiated (●) with 100 Gy of γ-radiation; (■) unirradiated cells. After 40 min the cultures were shifted to 35°C to inactivate Wee1. Subsequent cell cycle progression was monitored by counting the percent of cells undergoing septation. (B) Cdc2 coimmunoprecipitated from samples of the same cultures with α-Cdc13 antibodies was analyzed as in Fig. 1. All samples were analyzed on the same gel and data shown is from the same exposure. The background band at the bottom of the α-phosphotyrosine blots is a result of immunoglobulin light chain. (C) The experiment was repeated, but total Cdc2 was isolated by binding to Suc1-coated beads (Brizuela et al. 1987). The time at which half the cells had septated is circled. The irradiated 120-min sample was lost during preparation.

the ability of cells to phosphorylate Cdc2. First, a substitution of a phenylalanine for tyrosine-15 in Cdc2, so that the protein can no longer be tyrosine phosphorylated, completely abolishes the checkpoint (Fig. 2). Second, overexpression of Cdc25, the major Cdc2 tyrosine-15 phosphatase, likewise drives cells through the checkpoint (Fig. 3A). Finally, inactivation of the Wee1 and Mik1, the two Cdc2 tyrosine kinases, after the check-

point is established causes the checkpoint to fail and cells to re-enter the cell cycle (Fig. 4). These results are consistent with the simple model that the G₂ DNA damage checkpoint operates by maintaining Cdc2 in its tyrosine-phosphorylated G₂ form while the DNA damage signal persists, thus preventing mitosis. We cannot exclude more complicated models that postulate the existence of some other form of negative regulation of Cdc2 that is effective only in concert with tyrosine phosphorylation of Cdc2. However, given the fact that tyrosine phosphorylation of Cdc2 is both necessary and sufficient to arrest cells in G₂, such models are not required (Russell and Nurse 1987; Aligue et al. 1994; this study).

There has been speculation that the *S. pombe* G₂ DNA damage checkpoint may be independent of Cdc2 tyrosine-15 phosphorylation (Sheldrick and Carr 1993; Carr 1995). This hypothesis is based largely on the observation that *wee1-50 mik1Δ* cells show a cell cycle delay in response to radiation at the restrictive temperature. The fact that the kinases are not required for a short cell cycle delay was interpreted to demonstrate that tyrosine phosphorylation of Cdc2 was not required for the delay. As we have shown in Figures 4 and 5, *wee1-50 mik1Δ* cells do show a transient cell cycle delay in response to radiation at the restrictive temperature. However, the tyrosine-15 kinase activity of Wee1 and Mik1 is only half of the equilibrium that maintains the phosphorylation of Cdc2. Even in the absence of these kinases, the DNA damage checkpoint reduces the rate of tyrosine dephosphorylation of Cdc2, maintaining Cdc2 in its tyrosine phosphorylated state and leading to a transient cell cycle delay (Fig. 5B). These results explain the previous observations with the *wee1-50 mik1Δ* strain (Sheldrick and Carr 1993) and reconcile them with the absolute requirement of Cdc2 tyrosine-15 phosphorylation in the G₂ DNA damage checkpoint.

The role of the Cdc2 tyrosine phosphatase and kinases in the G₂ DNA damage checkpoint

The central role for Cdc2 tyrosine-15 phosphorylation in the G₂ DNA damage checkpoint raises the question of how this phosphorylation is regulated. Although we cannot directly answer this question, our results suggest that rate of tyrosine dephosphorylation of Cdc2 plays an important role in the mechanism of the checkpoint. When Wee1 and Mik1, the kinases that phosphorylate Cdc2 on tyrosine-15, are inactivated, there is still a radiation-induced delay of Cdc2 tyrosine dephosphorylation (Fig. 5B). This delay can be attributed only to a change in the rate of dephosphorylation of Cdc2. This delayed dephosphorylation of Cdc2 is presumably a result of a reduction in the activity of Cdc25, the major Cdc2 tyrosine-15 phosphatase in fission yeast (Millar et al. 1991, 1992). The reduction of the Cdc25 activity could be through direct down-regulation of enzyme-specific activity or abundance, or through changes in the accessibility of Cdc2. The mitotic induction process is

thought to involve a positive feedback loop in which Cdc2 catalyzes directly or indirectly the activation of Cdc25 via phosphorylation (Dunphy 1994; Kumagai and Dunphy 1996). In *S. pombe*, the activations of Cdc2 and Cdc25 that occur at the G₂-M transition are mutually dependent, observations consistent with a Cdc2-Cdc25 positive feedback loop (Gould and Nurse 1989; Kovelman and Russell 1996). Therefore, one possible mechanism for the inhibition of Cdc2 tyrosine dephosphorylation is the interruption of this Cdc25 activation loop.

Our findings do not exclude the possibility that the checkpoint mechanism also involves up-regulation of Wee1 and/or Mik1 following DNA damage. Because *wee1-50 mik1Δ* cells are able to arrest at the permissive temperature, Mik1 alone cannot be required for the arrest. Likewise, *wee1Δ* cells also exhibit a cell cycle arrest under the same conditions (data not shown), in agreement with previous results (Barbet and Carr 1993). Thus, Wee1 and Mik1 provide redundant activities in the G₂ DNA damage checkpoint. However, it remains to be seen whether Wee1 and Mik1 simply serve in their regular G₂ role to phosphorylate Cdc2 or whether the activities of these kinases are up-regulated in response to DNA damage.

The role of Cdc2 tyrosine phosphorylation in other species

In view of the high degree of conservation of mitotic controls among eukaryotic species, it is reasonable to speculate that the checkpoint mechanisms regulating mitosis would also be conserved (Nurse 1990; Dunphy 1994). This is, however, not the case for *S. cerevisiae*, where tyrosine phosphorylation of CDC28 does not play a major role in mitotic control or in the DNA damage checkpoint (Amon et al. 1992; Sorger and Murray 1992). The situation also seems to be more complicated in mammalian cells. It has not been possible to test directly whether Wee1-like kinases are required for the DNA damage G₂ checkpoint in mammals, although maintenance of Cdc2 tyrosine phosphorylation and a failure to activate Cdc25C tyrosine phosphatase are correlated with G₂ arrest induced by DNA damage (O'Connor et al. 1994; Herzinger et al. 1995). Furthermore, expression of the unphosphorylatable Cdc2-AF mutant in HeLa cells

prevents Cdc2 activation from being blocked by the checkpoint and greatly reduces the G₂ delay in response to radiation (Jin et al. 1996; A. Blasina and C. McGowan, pers. comm.). This demonstrates that tyrosine phosphorylation of Cdc2 is an important part of the G₂ DNA damage checkpoint in mammals. However, Cdc2-AF expression does not abolish the checkpoint completely. Instead, cells go into a delayed or abnormal mitosis (Jin et al. 1996; A. Blasina and C. McGowan, pers. comm.). This may reflect the fact that mammalian cells appear to have multiple controls over the initiation of mitosis, one involving Cdc2 and another involving the NIMA protein kinase (Fry and Nigg 1995; Lu and Hunter 1995). Thus it is possible that tyrosine phosphorylation of Cdc2 is responsible for restraining Cdc2 activity in response to DNA damage and that another checkpoint mechanism regulates other mitotic controls. The obvious conclusion is that there is a correlation between the importance of Cdc2 tyrosine phosphorylation in normal mitotic control and its role in the G₂ DNA damage checkpoint. This correlation implies that the G₂ DNA damage checkpoint in various organisms relies on whatever mechanism controls the timing of normal mitoses. By feeding in upstream of the normal mitotic controls, the DNA damage checkpoint is able to utilize efficiently the regulator machinery that is already in place.

Materials and methods

General methods for studying fission yeast were performed as described (Moreno et al. 1991). Unless otherwise stated all strains (Table 1) were grown in yeast extract-glucose (YES) media at 25°C. Synchronous cultures were prepared by centrifugal elutriation with a Beckman JE-5.0 elutriation rotor (Creanor and Mitchison 1979). Cells were irradiated with γ-radiation from a ¹³⁷Cs source at 3.3 Gy/min at room temperature, which ranged from 23°C to 25°C. For the *wee1-50 mik1Δ* temperature shift irradiation experiment, after 60 min of irradiation at room temperature, half of each of the irradiated and unirradiated cultures were shifted 35°C. To continue radiation exposure at 35°C, the 35°C irradiated culture was alternatively irradiated for 20 min and then incubated at 35°C for 20 min. The temperature of the culture varied between 32°C and 35°C. The 25°C irradiated culture was irradiated in the same manner and varied between 24°C and 25°C. The number of cell having passed mitosis was determined by the number of cells having begun or finished septation divided by the total number of cells.

Table 1. *S. pombe* strains used in this study

Strain	Genotype ^a	Reference
PR109	<i>h⁻</i>	laboratory stock
PR714	<i>h⁻ cdc2⁺:cdc2-Y15F:LEU2</i>	K. Gould (unpubl.)
GL198	<i>h⁻ cdc25⁺:nmt1-cdc25:ura4⁺</i>	G. Lenaers and P. Russell (unpubl.)
OM1603	<i>h⁺ ura4-294</i>	laboratory stock
BF1758	<i>h⁺ ura4-294 leu1-32:nmt1-GST-chk1:leu1⁺</i>	this study
NR1757	<i>h⁻ ura4-294 leu1-32:nmt1-GST-chk1:leu1⁺ cdc2⁺:cdc2-Y15F:LEU2</i>	this study
PR754	<i>h⁻ wee1-50 mik1::ura4⁺</i>	laboratory stock

^aAll strains are *leu1-32 ura4-D18* unless otherwise noted.

For *nmt1* expression experiments, cells were grown in Edinburgh minimal media 2 (EMM2), supplemented with leucine, uracil, adenine, and histidine, with or without 5 µg/ml thiamine. For the Cdc25 overexpression experiments, cells were grown for 20 hr without thiamine and then irradiated for 3 hr in the same media. For the Chk1 overexpression experiments, cells were grown without thiamine for 18 hr. In both cases, cells were photographed using phase contrast optics.

Immunoprecipitations and Western blots

For each sample, 10 OD₆₀₀ units of cells were harvested by centrifugation, washed once in ice-cold stop buffer (150 mM NaCl, 50 mM NaF, 10 mM EDTA, 1 mM NaN₃), and frozen as cell pellets at -70°C. All steps in the immunoprecipitations were done at 4°C, using standard procedures (Harlow and Lane 1988). Cells were thawed in 200 µl of lysis buffer (150 mM NaCl, 50 mM Tris at pH 8.0, 50 mM NaF, 5 mM EDTA, 1 mM NaVO₄, 1 mM PMSF, 10% glycerol, 1% NP-40, and 5 µg/ml each of aprotinin, leupeptin, and pepstatin), broken by vortexing with glass beads, and centrifuged to prepare a cleared cell extract. The protein concentrations of the supernatants were measured at 280 nm and normalized with lysis buffer. Cdc13 was immunoprecipitated using the polyclonal rabbit serum E7 bound to protein A-Sepharose. The Sepharose beads were washed 3 times with lysis buffer and then boiled in SDS-PAGE sample loading buffer. Samples were electrophoresed on a 6%–12% polyacrylamide gradient gel and transferred to Immobilon (Millipore) with a semi-dry blotting apparatus. The blots were probed with a mouse monoclonal antibody against phosphotyrosine (Upstate Biotech), then stripped and reprobed with a mouse monoclonal antibody against the PSTAIR peptide. In both cases, the primary antibody was detected using an HRP-conjugated anti-mouse IgG antibodies (Promega) and Luminol reagents (Pierce). For the Suc1 precipitations, Sepharose-coupled Suc1 was substituted for protein A-Sepharose-bound α-Cdc13 antibodies (Brizuela et al. 1987).

Construction of the *nmt1*-GST-*chk1* fusion

The *nmt1*-GST-*chk1* fusion (pBF147) was constructed by inserting the coding region of GST flanked with *Nde*I sites into the *Nde*I site of the pRep1 based *nmt1*-*chk1* expression plasmid (pBF146) (Maundrell 1993). The activity of the GST-Chk1 fusion was confirmed by overexpression in a wild-type background. For the construction of the integrating *nmt1*-GST-*chk1* fusion vector (pBF161), the *Pst*I-*Sst*I fragment from pBF147 was ligated into the *Pst*I and *Sst*I sites of pJK148 (Keeney and Boeke 1994). Integration was targeted to the *leu1*-32 locus in OM1603.

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