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Analysis of Rad3 and Chk1 protein kinases defines different checkpoint responses

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Eukaryotic cells respond to DNA damage and S phase replication blocks by arresting cell-cycle progression through the DNA structure checkpoint pathways. In *Schizosaccharomyces pombe*, the Chk1 kinase is essential for mitotic arrest and is phosphorylated after DNA damage. During S phase, the Cds1 kinase is activated in response to DNA damage and DNA replication blocks. The response of both Chk1 and Cds1 requires the six 'checkpoint Rad' proteins (Rad1, Rad3, Rad9, Rad17, Rad26 and Hus1). We demonstrate that DNA damage-dependent phosphorylation of Chk1 is also cell-cycle specific, occurring primarily in late S phase and G_2, but not during M/G_1 or early S phase. We have also isolated and characterized a temperature-sensitive allele of *rad3*. Rad3 functions differently depending on which checkpoint pathway is activated. Following DNA damage, *rad3* is required to initiate but not maintain the Chk1 response. When DNA replication is inhibited, *rad3* is required for both initiation and maintenance of the Cds1 response. We have identified a strong genetic interaction between *rad3* and *cds1*, and biochemical evidence shows a physical interaction is possible between Rad3 and Cds1, and between Rad3 and Chk1 *in vitro*. Together, our results highlight the cell-cycle specificity of the DNA structure-dependent checkpoint response and identify distinct roles for Rad3 in the different checkpoint responses.

Keywords: ATM/ATR/cell-cycle checkpoints/Chk1/Rad3

Introduction

Throughout the cell cycle, checkpoint pathways monitor the status of the DNA, and arrest cell-cycle progression in response to DNA damage or inhibition of DNA replication (Hartwell and Weinert, 1989; Carr, 1995). The significance of the checkpoint response in maintaining genomic stability is highlighted by the evolutionary conservation of these pathways, as mammalian homologues of several yeast checkpoint genes, including those of *rad1* (h*Rad1*), *rad3* (*ATR/ATM*), *rad9* (h*Rad9*), *rad17* (h*Rad17*), *hus1* (h*Hus1*) and *chk1* (h*Chk1*), have been identified. Phenotypes have only been identified for mammalian homologues of the *rad3* gene (Savitsky *et al*., 1995; Bentley *et al*., 1996; Lieberman *et al*., 1996; Flaggs *et al*., 1997; Sanches *et al*., 1997; Cliby *et al*., 1998; Kostrub *et al*., 1998).

In fission yeast, Rad3 is one of six checkpoint Rad proteins required for both the DNA damage and DNA replication checkpoints (Bentley *et al*., 1996). However, these pathways can be differentiated by varying degrees of dependency on Chk1 kinase, which functions in a Rad3-dependent manner in response to DNA damage (Al-Khodairy *et al*., 1994; Ford *et al*., 1994; Walworth and Bernards, 1996). *chk1* null mutants are sensitive to DNA damage but not hydroxyurea treatment. Consistent with this, *chk1* null cells are unable to delay mitotic entry after DNA damage, but arrest normally in hydroxyurea. Chk1 is phosphorylated in response to DNA damage, although it is not known whether Chk1 phosphorylation corresponds to activation (Walworth and Bernards, 1996). Work in human cells suggests a model in which Chk1 targets the cell-cycle machinery by inhibiting the Cdc25 phosphatase (Peng *et al*., 1997; Sanches *et al*., 1997).

The Cds1 kinase is activated in response to both DNA damage and DNA replication blocks only during S phase (Lindsay *et al*., 1998). Similar to Chk1 phosphorylation, this response is dependent on the presence of the checkpoint Rad proteins, including Rad3. Cds1 is required for the delay of S phase progression in response to DNA damage, and is also required for cells to tolerate replication blocks imposed by hydroxyurea. Although neither *cds1* or *chk1* mutants show a clear mitotic arrest defect during incubation with hydroxyurea, a *cds1 chk1* double mutant is unable to delay mitosis during treatment, and the cells die with a classic 'cut' phenotype (Lindsay *et al*., 1998). Chk1, which is not normally phosphorylated during hydroxyurea treatment, becomes phosphorylated during hydroxyurea treatment in *cds1* null cells (Lindsay *et al*., 1998). This suggests that Cds1 and Chk1 are not simply functionally redundant, as has been suggested in a recent report by Boddy *et al*. (1998), but that in the absence of the Cds1 protein kinase, hydroxyurea causes DNA damage which is then capable of activating the Chk1 pathway. It is clear that Cds1 plays an important role in regulating DNA synthesis.

The observation that Cds1 activation is confined to S phase implies that the checkpoint response is regulated in a cell-cycle-dependent manner. Here we present evidence that the Chk1 response is also cell-cycle specific. Chk1 phosphorylation occurs during late S phase and G_2 in response to both UV and gamma irradiation. During G_1/early S phase and mitosis there is no significant change in the phosphorylation status of Chk1 in response to damage. As anticipated, the activation of the DNA damage checkpoint during S and G_2 required the presence of *rad3*.

We have also isolated and analysed a temperature-sensitive allele of *rad3* (*rad3ts*). We find that *rad3* function is different for the distinct checkpoint responses. After DNA damage, Rad3 is required to initiate mitotic arrest and phosphorylate Chk1. However, once initiated, these responses are maintained independently of Rad3. In response to DNA replication blocks, Rad3 is required for both initiation and maintenance of mitotic arrest and Cds1 activity. We have identified genetic interactions between *rad3* and *cds1*. Together with biochemical data, which demonstrate that Rad3 can both bind to and phosphorylate the Cds1 and Chk1 kinases *in vitro*, these results suggest that the link between Rad3 and the downstream kinases is direct. We discuss the possibility that these data reflect the formation of different Rad3 protein complexes.

Results

Cell-cycle specificity of Chk1 phosphorylation

G_2-delay in response to DNA damage requires the function of the Chk1 protein. Following DNA damage of asynchronous cells, Chk1 is phosphorylated and this modification can be detected as a decrease in mobility by SDS–PAGE. In order to establish whether the DNA damage checkpoint is regulated by the cell cycle, we synchronized HA-epitope tagged *chk1* cells (*chk1::HA*; Walworth and Bernards, 1996) using *cdc10* and *cdc25*. When incubated at the restrictive temperature, *cdc10* and *cdc25* arrest in the G_1 and G_2 phases of the cell cycle, respectively. When shifted back to the permissive temperature, cells will re-enter the cell cycle synchronously. *chk1::HA* cells were also arrested in early S phase using a hydroxyurea block. These cells proceeded through S phase synchronously when hydroxyurea was removed from the medium. Irradiation with both gamma (250 Gy) and UV (100 J/m^2) of arrested cells, and of cells at specific time points after release from arrest, showed that there was a significant increase in the amount of shifted (phosphorylated) Chk1 (compared with the total amount of non-shifted protein) during late S phase and G_2, but not during G_1/early S phase or mitosis (Figure 1A and B). These results were confirmed by irradiating (100 Gy) *cdc*$^+$ *chk1::HA* cells that had been synchronized by elutriation (Figure 1C). If Chk1 functions after irradiation in G_1/early S phase, these results indicate that this is distinct from its function during S phase and G_2. To rule out the possibility that the DNA content of the cells is responsible for these observations, we analysed *chk1::HA* diploid cells. These cells behaved in a similar manner to the *chk1::HA* haploid cells after gamma irradiation (data not shown).

Isolation and characterization of rad3ts mutant allele

Schizosaccharomyces pombe rad3 is required for all DNA structure-dependent checkpoints (Bentley *et al*., 1996). To understand the role of *rad3* we isolated a temperature-sensitive allele of *rad3* (*rad3ts*) by transforming a *rad3* null strain (*rad3.d*) with a randomly mutagenized genomic *rad3* plasmid. A total of 60 000 colonies were screened for sensitivity to hydroxyurea at 35°C but not at 27°C. One plasmid was chosen for further analysis. Gene replacement of the *rad3* deletion allele with the *rad3ts* allele confirmed that *rad3ts* gene conferred a clearly defined temperature-sensitive phenotype. Analysis of survival following either UV or hydroxyurea treatment, plus analysis of the checkpoint response to gamma-irradiation and hydroxyurea treatment, revealed that the *rad3ts* strain behaved like wild-type cells at the permissive temperature (27°C) but had a phenotype equivalent to the *rad3* null mutant at the restrictive temperature (35°C) (Figure 2A–C). Sequence analysis of the *rad3ts* plasmid revealed the presence of seven mutations, one of which was located at a conserved residue within the C-terminal kinase domain, in close proximity to the ATP-binding pocket (A2217V) (Figure 2D). We have confirmed that the A2217V mutation resulted in a similar phenotype to *rad3ts* by repeating the genomic integration using a *rad3* sequence containing only this point mutation (data not shown).

Analysis of rad3ts following DNA damage

Although it is known that *rad3* is required for the establishment of the DNA damage checkpoint response and for Chk1 phosphorylation, the temporal requirements for Rad3 are not known. When *rad3ts* cells were incubated at the restrictive temperature for 10 min prior to gamma or UV irradiation, cells behaved similarly to the *rad3.d* strain, with a significant decrease in cell survival and a complete absence of the DNA damage checkpoint (Figure 3A–C). In contrast, when *rad3ts* cells were shifted to the restrictive temperature, 5 or 10 min after irradiation with gamma or UV, the cells survived equally as well as wild-type cells and maintained a virtually intact checkpoint response (Figure 3A–C). In addition, both cell survival and the damage checkpoint response (after UV and gamma irradiation) were similar in cells shifted to the restrictive temperature at different time intervals following irradiation (Figure 3A–C). These results suggest that *rad3* is required to initiate the DNA damage checkpoint response, but that once activated, the response is maintained independently of *rad3*.

Phosphorylation of the Chk1 protein kinase

In wild-type cells there is a temporal correlation between mitotic delay and Chk1 phosphorylation, as determined by decreased mobility on SDS–PAGE (Walworth and Bernards, 1996). To investigate whether Chk1 phosphorylation correlates with our visual analysis of the checkpoint, we created a *rad3ts* strain containing the genomic *chk1::HA* locus described by Walworth and Bernards (1996). At the permissive temperature (27°C) for the *rad3ts* allele, a decrease in the mobility of Chk1 was observed in response to gamma and UV irradiation, indicating that Chk1 was phosphorylated as a result of checkpoint pathway activation (Figure 4; data not shown). At the restrictive temperature (35°C), these modifications were not observed (Figure 4; data not shown). If the *rad3ts chk1::HA* strain was shifted to the restrictive temperature 10 min prior to irradiation, the Chk1 protein was not modified (Figure 4; data not shown). In contrast, if the *rad3ts chk1::HA* cells were shifted to the restrictive temperature 10 min after irradiation, Chk1 was modified to similar levels as seen in wild-type cells (Figure 4; data not shown). These results are consistent with the physiological observations, and show that *rad3* is primarily required to initiate the DNA damage checkpoint response

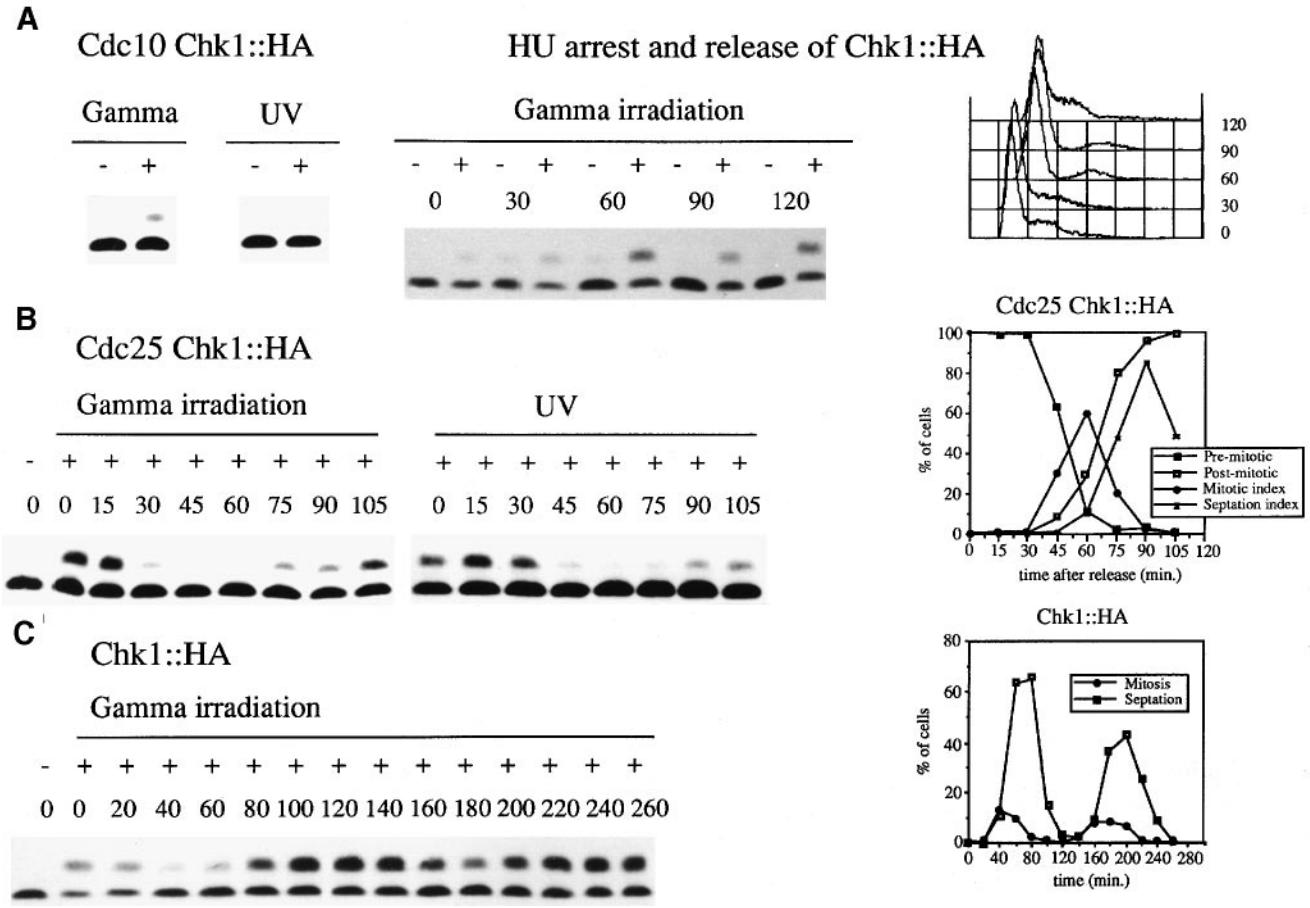

Fig. 1. Cell-cycle specificity of Chk1 phosphorylation. (**A**) *cdc10 chk1::HA* was arrested in G_1 by incubating at the restrictive temperature for 4 h. Aliquots were exposed to either ionising radiation (250 Gy) or UV (100 J/m^2). After 20 min recovery, cells were pelleted, and total protein extract was prepared and analysed by SDS–PAGE and Western blotting. *chk1::HA* cells were arrested in early S phase by incubation with 20 mM hydroxyurea for 3 h and released into cell cycle by removal of hydroxyurea with a wash with fresh medium. At t_0 and at 30 min intervals after release from the cell-cycle block, aliquots were exposed to ionising radiation (250 Gy). After 20 min recovery, the total protein extracts were prepared and analysed by SDS–PAGE and Western blotting. FACS analysis (right) at each timepoint shows the progress of bulk DNA synthesis. (**B**) *cdc25 chk1::HA* was synchronised in G_2 by incubating at the restrictive temperature for 4 h. Cells were irradiated at 15 min intervals following release and, after 20 min recovery, total protein extracts were prepared and analysed by SDS–PAGE and Western blotting. The cell-cycle stage of the culture was followed by Calcofluor and DAPI staining. A graph showing percentages of pre-mitotic (G_2), mitotic (separating nuclei), septated and post-mitotic (binucleate, septated and post-septated) cells at each timepoint is shown on the right. (**C**) *chk1::HA* was synchronized in G_2 by elutriation. Aliquots of cells were treated with ionising radiation (100 Gy) at 20 min intervals. Following 20 min recovery, total protein extracts were prepared and analysed by SDS–PAGE and Western blotting. Cell-cycle progression was followed using Calcofluor and DAPI staining, and a graph of the percentages of mitotic and septated cells at each timepoint is shown on the right.

and that once the checkpoint has been activated, this response is largely maintained independently of *rad3*.

Analysis of rad3ts in response to replication blocks

rad3 is required for the response to DNA replication blocks and for the activation of the Cds1 protein kinase during S phase, in response to both DNA replication blocks and DNA damage (Lindsay *et al*., 1998). Analysis of the *rad3ts* strain indicated that at 27°C, survival of hydroxyurea exposure and activation of the replication checkpoint was similar to wild-type cells, while at 35°C it was similar to *rad3.d* cells (Figure 2A). Interestingly, the checkpoint response established by the *rad3ts* strain at the permissive temperature could not be maintained at the restrictive temperature and cells entered a catastrophic mitosis in a synchronous manner following a temperature shift to 35°C (Figures 2C and 5). A 60 min delay was observed between the time of temperature shift and subsequent entry into mitosis. This was observed even when cells had been incubated at the permissive temperature with hydroxyurea for a prolonged period.

Cds1 protein kinase activity

Cds1 activation is dependent on the 'checkpoint *rad*' genes. Cds1 is essential for the delay to S phase progression in response to DNA damage and is also essential for tolerance to replication blocks imposed by incubation with hydroxyurea (Murakami and Okayama, 1995; Lindsay *et al*., 1998). To determine whether the loss of cell-cycle arrest observed in *rad3ts* cultures shifted to the restrictive temperature was related to the activity of the Cds1 protein kinase, we assayed for Cds1 protein kinase activity on the non-physiological substrate, myelin basic protein (MBP). After shifting to the restrictive temperature, Cds1 protein kinase activity was reasonably high between 30 and 60 min, but beyond this time a decay was observed and Cds1 kinase activity was almost non-detectable (Figure 5B). Thus, there is a strong correlation between

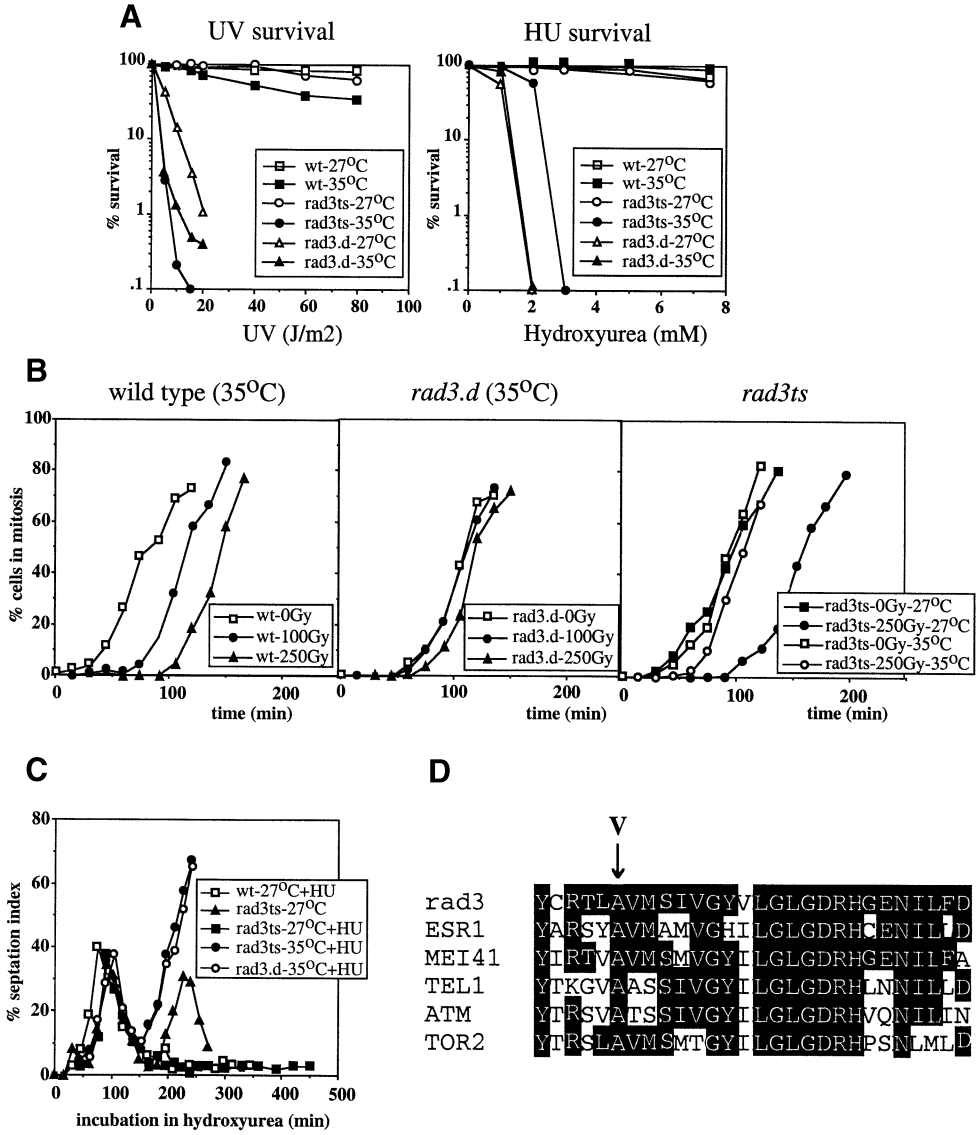

Fig. 2. Isolation and characterization of a *rad3ts* mutant. (**A**) Cell survival following UV irradiation, or incubation with hydroxyurea at 27°C and 35°C. wt, wild-type cells. (**B** and **C**) Checkpoint response after ionizing radiation (B) or after incubation with hydroxyurea (C). Wild-type (wt), *rad3.d* and *rad3ts* cells were synchronized in G_2 using lactose gradients and gamma treated at either 27°C or 35°C. Cell-cycle progression was monitored by Calcofluor and DAPI staining. (**D**) Sequence alignment of Rad3 and of Rad3-related proteins for the region containing the mutation (A2217V) which results in the *rad3ts* phenotype.

the decrease in Cds1 kinase activity and subsequent entry into a catastrophic mitosis.

Taken together these results suggest that *rad3* is required to both initiate and maintain the checkpoint response to DNA replication blocks. The temporal correlation between the decrease in Cds1 protein kinase activity and the time which cells enter a catastrophic mitosis suggests a functional dependency.

Multi-copy suppressor screen for rad3ts hydroxyurea survival

The creation of the *rad3ts* allele gave us a tool to identify genes which genetically interacted with *rad3*. Screens were initiated to identify multi-copy suppressors of the sensitivity of the *rad3ts* mutant to hydroxyurea at the semi-permissive temperature (32°C). Of the 150 multi-copy suppressors isolated, 40 were characterized as strong suppressors of the *rad3ts* hydroxyurea sensitivity. Sequence and Southern-blot analysis revealed that 36 of these were plasmids containing the *rnr3* gene, which encodes the catalytic subunit of ribonucleotide reductase and functions to maintain nucleotide pools. Given that hydroxyurea reduces nucleotide pools by inhibiting ribonucleotide reductase, it is perhaps not surprising that multiple clones of *rnr3* were isolated. This presumably represents a classic bypass of the requirement for *rad3* function, since cells do not elongate under these circumstances (data not shown).

Analysis of the remaining four strong suppressors identified the *cds1* gene. The genetic interaction between *rad3* and *cds1* was found to be specific to the *rad3ts* allele, and specific to the semi-permissive conditions at the expression levels used (multi-copy genomic fragment under its own promoter). *cds1* was neither able to suppress the hydroxyurea sensitivity of the *rad3.d* mutant (Figure 6A) or the hydroxyurea sensitivity of the *rad3ts* mutant

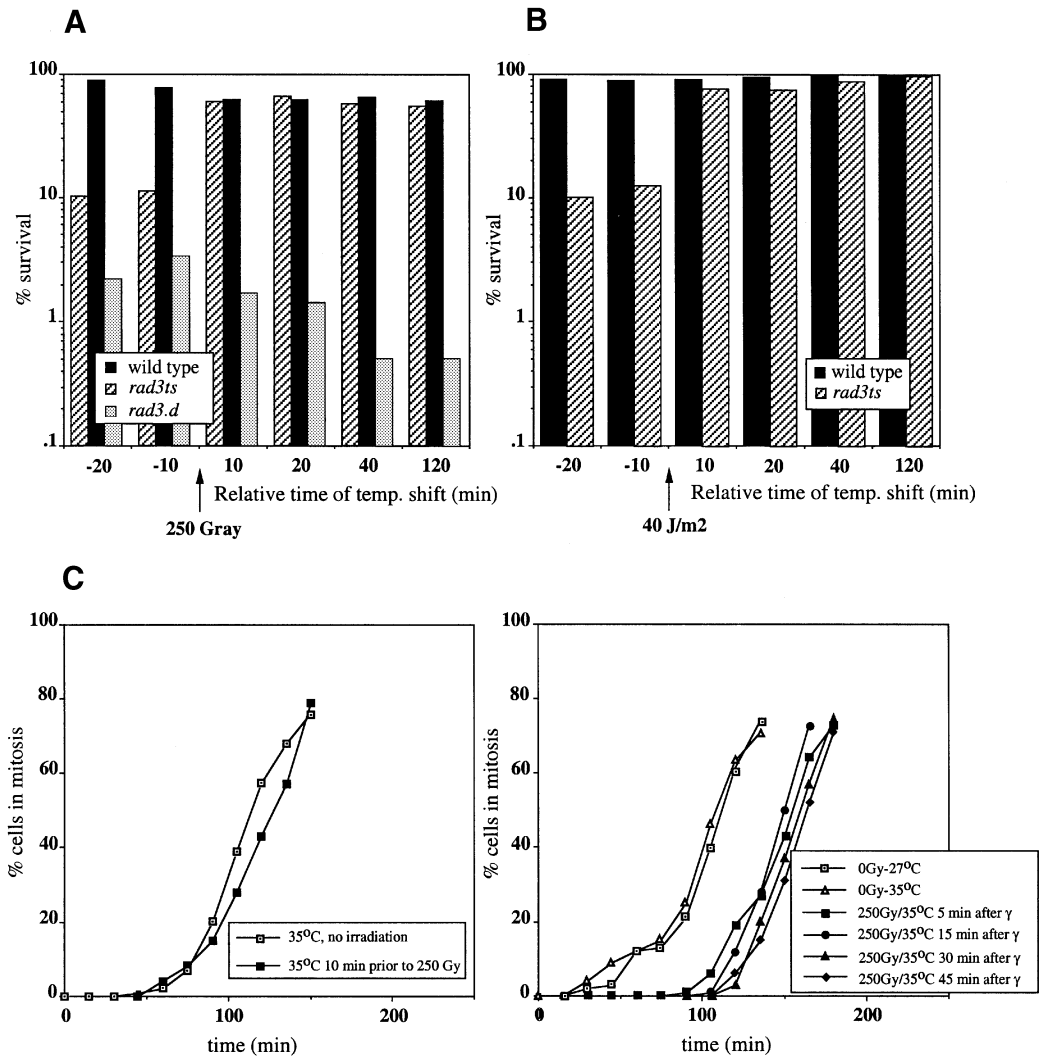

Fig. 3. Analysis of *rad3ts* following DNA damage. Cell survival following (**A**) 250 Gy ionising or (**B**) 40 J/m^2 UV irradiation. Asynchronous cells were shifted from the permissive to the restrictive temperature for *rad3ts* either before (–20, –10 min) or after (10, 20, 40 and 120 min) treatment. Plates were incubated at the restrictive temperature for 3 days. Survival is expressed as a percentage of colonies on unirradiated plates. (**C**) G_2/mitotic checkpoint response after gamma irradiation. *rad3ts* cells were synchronized in G_2 using lactose gradients, and gamma irradiated. Cell-cycle progression was monitored by Calcofluor and DAPI staining. Left panel: cells were shifted from 27°C to 35°C (restrictive temperature) 10 min prior to gamma irradiation. Right panel: cells were shifted from 27°C to 35°C at the times indicated following irradiation.

at the restrictive temperature of 35°C (data not shown). These results represent a genetic interaction between *rad3* and *cds1*: *cds1* suppression requires the Rad3 protein in a partially active form, suggesting a close link exists between these proteins.

Chk1 is a multi-copy suppressor of rad3ts UV sensitivity

Suppression of the sensitivity of the *rad3ts* mutant to replication blocks by the downstream kinase Cds1, suggested that the Chk1 kinase might suppress the sensitivity of *rad3ts* to DNA damage in a manner specific to the *rad3ts* allele. Analysis of survival to DNA damage of *rad3ts* and *rad3* null cells over-expressing *chk1* demonstrated that *chk1* was able to suppress the UV sensitivity of both the *rad3ts* and *rad3.d* mutants at the semi-permissive temperature for the *rad3ts* allele (Figure 6B). *chk1* over-expression is known to delay the cell cycle independently of *rad3* (Ford *et al*., 1994) and even a moderate cell-cycle delay has been previously shown to partially rescue survival of checkpoint *rad* mutants (Al-Khodairy and Carr, 1992). Thus, a genetic interaction, if it exists, might be masked by non-specific effects.

Rad3 interacts with Cds1 and Chk1

To explore the interactions identified between *rad3* and *cds1*, and possibly between *rad3* and *chk1*, we asked whether the corresponding proteins interact biochemically. Co-immunoprecipitation experiments were performed using a genomic *myc*-tagged allele of *rad3* (described elsewhere) but neither Cds1 nor Chk1 proteins at wild-type levels or when over-expressed using a plasmid-borne attenuated *nmt1* promoter could be detected (IP of myc-tagged Rad3 requires cell lysis in mild detergent). However, when glutathione *S*-transferase (GST)-tagged Chk1 or Cds1 were co-overexpressed with myc-tagged Rad3, Rad3 was identified in both Chk1 and Cds1 precipitates (Figure 6C). When both HA-tagged Rad3 and myc-tagged Chk1 were overexpressed in wild-type cells (we estimate the level of over-expression to be ~10- to 15-fold), Chk1

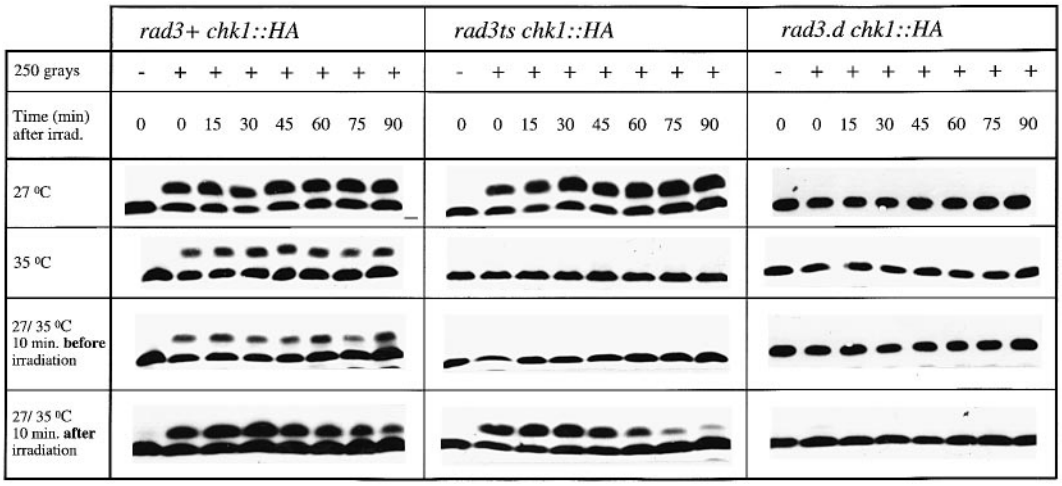

Fig. 4. Rad3 is required to initiate the DNA damage checkpoint. *rad*$^{+}$ chk1::HA, *rad3ts chk1::HA* and *rad3.d chk1::HA* cells were irradiated at t_0 with 250 Gy of ionizing radiation. Total protein was prepared from unirradiated control cells and from cells immediately after irradiation (t_0) and at 15 min intervals thereafter. The phosphorylation status of Chk1 was investigated by SDS–PAGE and Western blot analysis. The experiment was performed either at 27°C (permissive for *rad3ts*) or 35°C (restrictive for *rad3ts*), and separately with a temperature shift from 27 to 35°C either 10 min before or 10 min after irradiation.

was identified in Rad3 immunoprecipitates (Figure 6D). Under equivalent conditions Cds1 was not identified in Rad3 immunoprecipitates. These data suggest that Rad3 may interact with Cds1 and Chk1 in a transient manner which can only be detected by immunoprecipitation following over-expression. To address whether Cds1 and Rad3 are found in a common complex in the soluble material (equivalent to that used for IP), we examined the size distribution of these two proteins using Superdex 200 column chromatography. In soluble extracts, Rad3 and Cds1 did not elute in common fractions (Figure 6F and G). We do not consider that the interactions detected when the proteins are over-expressed are artefactual, since interactions between Rad3 and several other checkpoint proteins such as Rad1 and Rad17 were not identified using equivalent conditions (data not shown).

Rad3 phosphorylates Cds1 and Chk1 in vitro

Genetic suppression of the *rad3.ts* mutant strain by *cds1* suggests that the kinase encoded by this gene is able to amplify the presumably weakened checkpoint signal transmitted by the defective form of the Rad3 protein, and therefore maintain the checkpoint response. In addition to the genetic interaction, Rad3-dependent activation (Cds1) and modification (Cds1 and Chk1) suggest that these kinases may be substrates for Rad3. The potential for interactions identified between Rad3–Cds1 and Rad3–Chk1 would be consistent with a kinase–substrate relationship between Rad3 and these downstream kinases. To investigate this we tested kinase dead (KD) Cds1 and Chk1 as *in vitro* substrates for Rad3, immunoprecipitated from cells overexpressing *rad3*. Although the Rad3 protein has relatively weak *in vitro* kinase activity and does not phosphorylate a number of non-physiological substrates (Bentley *et al*., 1996), immunoprecipitated Rad3 was shown to phosphorylate Cds1-KD and Chk1-KD substrates (Figure 6E). Control experiments using immunoprecipitated Rad3.KD indicate that Cds1 and Chk1 phosphorylation was dependent on the integrity of the Rad3 kinase. In these assays Chk1 is not converted to a lower mobility form. This may be because a kinase dead form of Chk1 was used and it has been reported that Chk1 kinase activity is required to see the mobility change (Walworth and Bernards, 1996). These results show that the Rad3 kinase is capable of phosphorylating both Cds1 and Chk1 protein kinases *in vitro*, although it does not establish that they are direct targets *in vivo*. The inability to detect Rad3 kinase activity using a variety of non-physiological substrates, such as MBP, lends support to a biological significance for the phosphorylation of Cds1 and Chk1 by Rad3. Further analysis to establish whether or not this phosphorylation resulted in the activation of Cds1 and Chk1 by Rad3 proved inconclusive. This might be expected, given the complexity of the checkpoint response and that additional factors may be required for the activation of Rad3 substrates, which were not present in the *in vitro* conditions used.

Discussion

Two main points emerge from our analysis of Rad3 and Chk1: first, the response of Chk1 to DNA damage is specific to the later stages of the cell cycle (post DNA replication). Secondly, the function of Rad3 in activating the Cds1 (S phase specific) and Chk1 (late S/G_2 phase specific) responses is distinct. For the Chk1 response, Rad3 is required to initiate but not to maintain mitotic delay. For the Cds1 response, Rad3 is required for both initiation and maintenance. We also present genetic and biochemical data that are consistent with a direct interaction between Rad3 and these two downstream kinases.

Cell-cycle specificity of checkpoint responses

The *chk1* null mutant is sensitive to DNA damaging agents and is unable to delay mitotic entry in response to DNA damage (Walworth *et al*., 1993; Al-Khodairy *et al*., 1994). Chk1 is phosphorylated after DNA damage, and there is a temporal correlation between Chk1 phosphorylation and the activation of the checkpoint response. Analysis in human cells suggests Chk1 prevents entry into mitosis

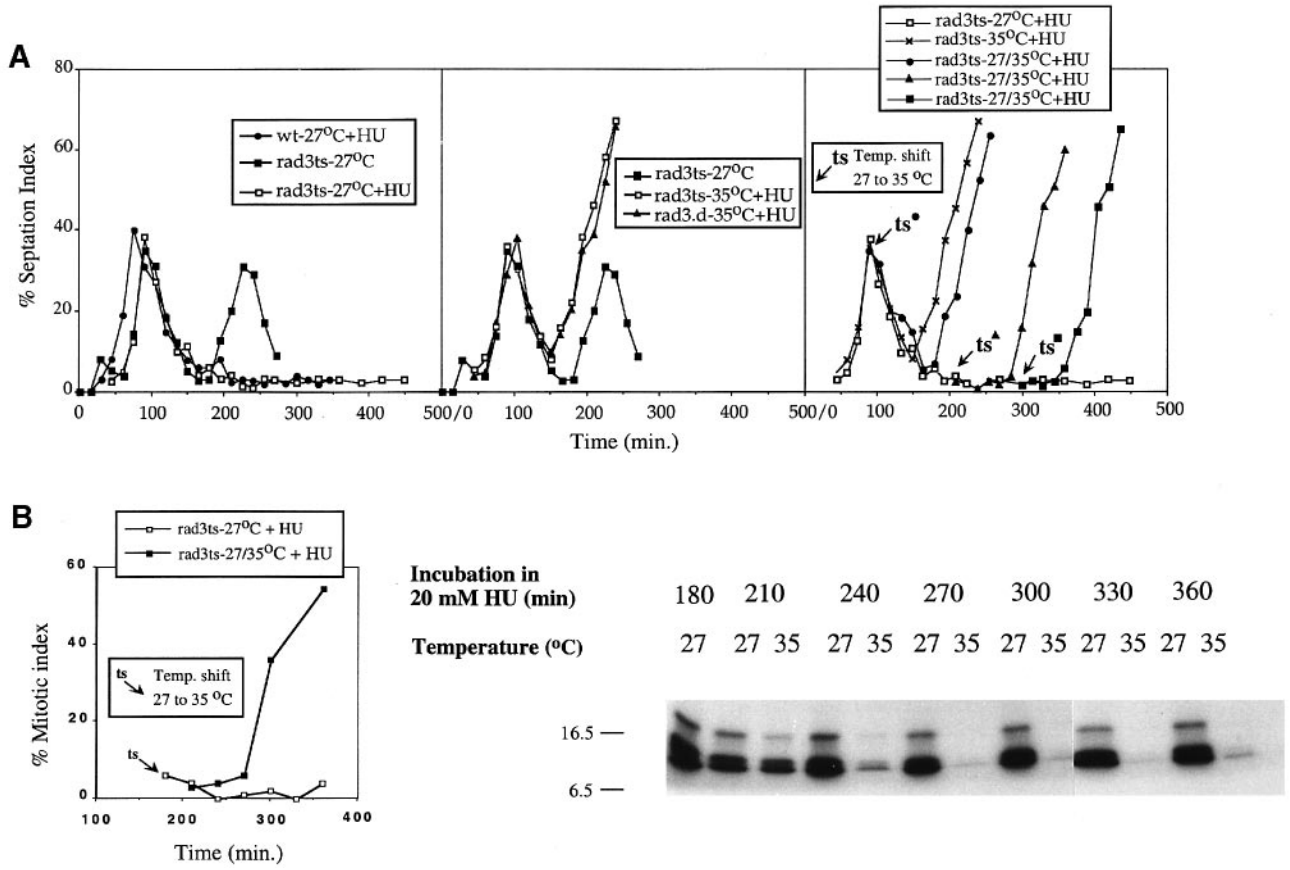

Fig. 5. The role of rad3 in response to replication blocks. (**A**) Wild-type, *rad3.d* and *rad3ts* cells were synchronized in G_2 using lactose gradients, and released into medium containing 20 mM hydroxyurea at the permissive temperature (27°C). At specific time intervals an aliquot of cells were removed and shifted to the restrictive temperature (35°C). Cell-cycle progression was monitored at regular intervals by fluorescent microscopy after staining with Calcofluor and DAPI. Septation index is shown. Note that some of the results shown form part of Figure 1 and are shown here for clarity. (**B**) Release of cell-cycle arrest correlates with a decrease in Cds1 kinase activity. An asynchronous culture of *rad3ts* cells was synchronized in S phase by incubation in 20 mM hydroxyurea for 3 h at the permissive temperature (27°C). The culture was divided into two; one half remained at 27°C while the other half was shifted to 35°C (restrictive for *rad3ts*). Left panel: at 30 min intervals, cell-cycle progression was monitored by DAPI and Calcofluor staining. Right panel: at 30 min intervals, 10 ml samples were taken, protein extracts prepared and IP kinase activity for Cds1 was measured using MBP.

following DNA damage by targeting the cell-cycle machinery through inhibition of the Cdc25 phosphatase (Sanches *et al*., 1997). This is consistent with data from *S.pombe* (Furnari *et al.*, 1997), although it has also been reported that Wee1 is a downstream target of Chk1 (O'Connell *et al*., 1997). The precise nature of the interactions between Chk1 and the cell-cycle machinery thus awaits identification of the *in vivo* targets.

Our data provide evidence that activation of the DNA damage checkpoint is cell-cycle specific. Chk1 is phosphorylated in response to damage during late S phase and G_2. During G_1/early S phase and mitosis there was no significant modification of Chk1 in comparison with the total amount of non-shifted Chk1 protein. *chk1::HA* diploid cultures had a similar Chk1 phosphorylation profile in response to both UV and gamma irradiation, indicating that the cell-cycle specificity identified for the DNA damage checkpoint is not caused simply by the DNA content of the cell. It is known that the Chk1 protein kinase is required to prevent catastrophic mitosis in *cdc10* arrested cells (G_1 phase cells), which suggested a role for the Chk1 protein kinase in G_1 (Carr *et al*., 1995). From our data it is clear that if the Chk1 protein kinase performs different roles (or performs the same role in a different manner) during specific stages of the cell cycle, the onset of S phase and the beginning of DNA replication are required for the switch from the G_1-form of response to the G_2-form of response, which is seen following DNA damage.

Interestingly, a very weak modification of Chk1 protein in non-irradiated cells can be seen at t_{30} and t_{60} (S phase), but not at t_0, t_{90} and t_{120} after the release of the hydroxyurea block. This suggests that problems are caused by incubation of cells in hydroxyurea, but that these only cause modification of the Chk1 protein kinase once cells progress through S phase.

Cell-cycle- and checkpoint-specific roles of Rad3

Characterization of the *rad3ts* mutant showed a null phenotype at the restrictive temperature (35°C) and a wild-type phenotype at the permissive temperature (27°C). We have used this allele to investigate the temporal requirement for *rad3* in the checkpoint responses. *rad3* is primarily required to initiate the DNA damage checkpoint. Once activated, this checkpoint is maintained largely independently of *rad3*. When the *rad3ts* strain was incubated at the restrictive temperature 10 min prior to UV or gamma irradiation, the DNA damage checkpoint was

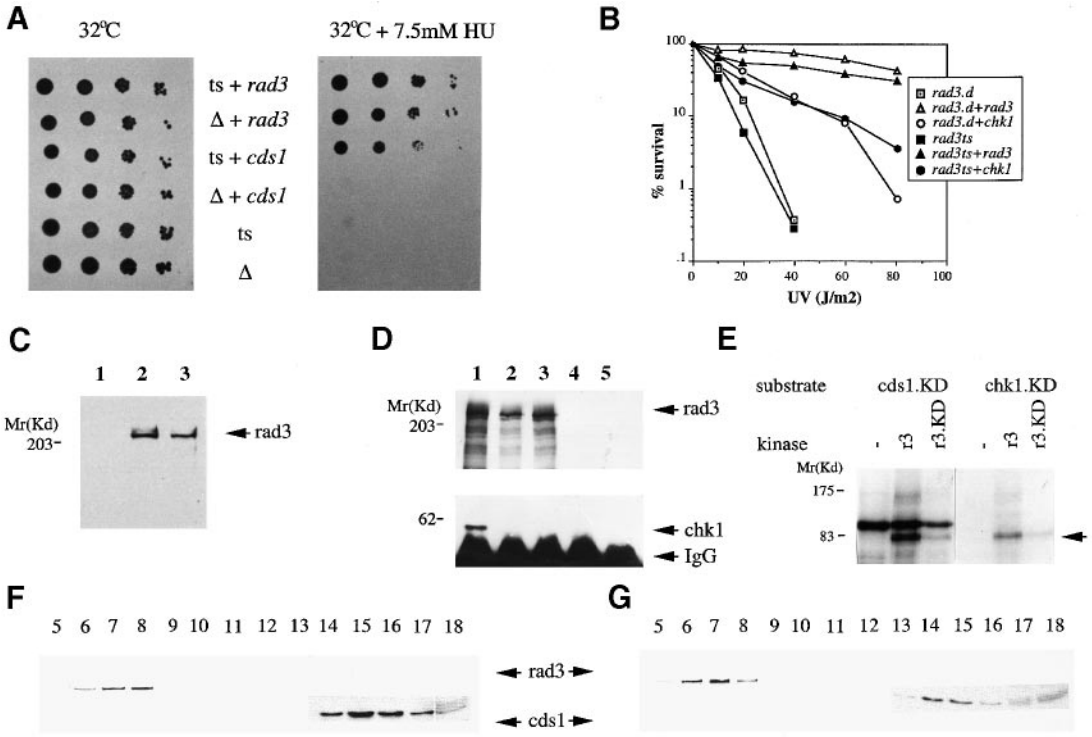

Fig. 6. Interactions between Rad3 and downstream kinases. Characterization of genetic suppressers of *rad3ts*. (**A**) *rad3ts* and *rad3.d* mutants were transformed with plasmids containing either the genomic *rad3* or *cds1* gene, or with the vector alone. Serial dilutions of each strain were inoculated onto plates with or without 7.5 mM hydroxyurea, and incubated at 32°C (semi-permissive for *rad3ts*). (**B**) *rad3ts* and *rad3.d* mutants were transformed with plasmids containing the *rad3* and *chk1* genes, respectively, or vector alone, and percentage survival was determined following increasing doses of UV irradiation at 32°C. (**C**) GST pull-downs from wild-type cells transformed with plasmids expressing Rep1GST/myc-tagged Rad3 (lane 1), Rep1GST-Chk1/myc-Rad3 (lane 2) and Rep1GST-Cds1/myc-Rad3 (lane 3). Total protein extracts were incubated with glutathione–Sepharose 4B beads (Pharmacia), and the presence of Rad3 in Chk1 and Cds1 precipitates (lanes 2 and 3, respectively) detected using an anti-myc monoclonal antibody. (**D**) Immunoprecipitation from wild-type cells transformed with attenuated *nmt1* (41/42) promoter plasmids expressing HA-Rad3/myc-Chk1 (lane 1), HA-Rad3/myc epitope only (lane 2), HA-Rad3 only (lane 3), HA epitope only/myc-Chk1 (lane 4) and myc-Chk1 only (lane 5). Total protein extracts were immunoprecipitated with anti-HA antibody, and the presence of Chk1 in Rad3 precipitates (lane 1) was detected by Western analysis using an anti-myc monoclonal antibody. (**E**) IP kinase activity of Rad3 and Rad3.KD on Cds1.KD and Chk1.KD substrates. The arrow indicates the position of the phosphorylated substrates, Cds1.KD and Chk1.KD, respectively. (**F**) Size fractionation of total soluble protein extract from mid-log phase cells with a myc-tagged *rad3* gene, integrated at the *rad3* locus and under the control of its own promoter. Rad3 is detected in fractions 5–8 by SDS–PAGE and Western blotting using an anti-myc monoclonal antibody. Cds1 is similarly detected in fractions 13–18 using a Cds1 polyclonal sera. (**G**) Size fractionation of extract from the strain used in (F) following 500 Gy ionising radiation. No variation in profile is evident for either Rad3 or Cds1.

defective (determined by cell survival, cell-cycle arrest and Chk1 phosphorylation). When the *rad3ts* strain was shifted to the restrictive temperature, 5 or 10 min after irradiation, the cells behaved in a similar manner to wild-type cells.

The difference in phenotypes between cells shifted to the restrictive temperature 10 min before or 10 min after UV and gamma irradiation, and the fact that cells shifted to the restrictive temperature at different time points post-irradiation show a similar survival and duration of cell-cycle arrest, indicates that the Rad3ts protein is not simply being inactivated slowly. The similar response of a temperature-sensitive allele of another DNA damage checkpoint gene, *rad4/cut5* (unpublished data; McFarlane *et al*., 1997), suggests that these results reflect the overall organization of the DNA damage checkpoint and are not caused by an unusual feature of the *rad3ts* allele itself.

In contrast to the DNA damage checkpoint role, Rad3 is required to both activate and maintain the response to replication blocks. When *rad3ts* cells were incubated with hydroxyurea at the permissive temperature (27°C), the checkpoint response activated could not be maintained when cells were shifted to the restrictive temperature (35°C). The synchronous entry into mitosis observed at the restrictive temperature suggests that the Rad3ts protein is not slowly inactivated. Interestingly, a 60 min delay was observed between the shift to the restrictive temperature and entry into mitosis. The kinetics of this delay correlated with a decrease of Cds1 protein kinase activity, which was observed between 30 and 60 min.

The *cds1* gene was isolated four times from a screen for multi-copy suppressors of the hydroxyurea sensitivity of *rad3ts* cells at the semi-permissive temperature (32°C). This suppression was specific to the *rad3ts* allele and did not occur at the restrictive temperature, suggesting a close association between *rad3* and *cds1*. Attempts were made to detect a physical interaction between these two proteins at physiological levels. These demonstrated that Rad3 and Cds1 are not associated in soluble extracts. However, under conditions in which both Cds1 and Rad3 were moderately overexpressed (we estimate ~10- to 15-fold over wild-type levels), Rad3 was reproducibly found in Cds1 precipitates. Furthermore, Rad3 could phosphorylate Cds1 *in vitro*. Together, these data indicate that Rad3 is capable of forming a kinase–substrate interaction with Cds1, and that the connection between these kinases is

Table I. Strains used in this study

Strain	Genotype	Mating type	Reference
rad+	*ura4.D18 leu1.32 ade6.704*	h^-	Murray *et al*. (1992)
rad3ts	*ura4.D18 leu1.32 ade6.704*	h^-	this study
rad3.d	*ura4.D18 leu1.32 ade6.704 rad3::ura4*$^+$	h^-	Bentley *et al*. (1996)
chk1::HA	*ura4.D18 leu1.32 ade6.704*	h^-	Walworth and Bernards (1996)
rad3ts chk1::HA	*ura4.D18 leu1.32 ade6.704*	h^+	this study
rad3.d chk1::HA	*ura4.D18 leu1.32 ade6.704 rad3::ura4*$^+$	h^-	this study
cds1.d	*ura4.D18 leu1.32 ade6.704 cds1::ura4*$^+$	h^-	Murakami and Okayama (1995)
chk1.d	*ura4.D18 leu1.32 ade6.704 chk1::ura4*$^+$	h^-	Al-Khodairy *et al*. (1994)
cdc10.129 chk1::HA	*ura4.D18 ade6.704*	h^-	this study
cdc25.22 chk1::HA	*ade6.704*	h^-	this study

direct and does not require additional factors. It should also be remembered that co-precipitation and co-size fractionation experiments only address the state of interactions in soluble material. It remains possible that a weak but temporally long-lasting interaction may occur in conjunction with DNA and DNA associated proteins that is not accessible to such techniques.

Using similar genetic and biochemical tools, we also asked whether Chk1 and Rad3 could associate. When overexpressed, the *chk1* gene is able to partially suppress the UV sensitivity of the *rad3ts* mutant at the restrictive temperature. Yet, since *chk1* is also able to suppress the UV sensitivity of the *rad3* null mutant to equivalent levels as the *rad3ts* allele, the specificity of this suppression is unclear, especially when it is considered that *chk1* overexpression delays cell-cycle progression independently of *rad3* (Ford *et al*., 1994). The over-expression of *chk1* and *rad3* in wild-type cells, and subsequent precipitation of the Chk1 protein with Rad3 and vice versa, supported an interaction between Rad3 and Chk1, although this interaction did not occur at physiological levels of protein. Rad3 was able to phosphorylate Chk1 *in vitro*. Combined with the genetic data available, this suggests a direct link between Rad3 and Chk1.

A model for the cell-cycle specificity of checkpoint responses

Cds1 activation is S phase specific and Chk1 phosphorylation is late S/G_2-phase specific. The role of Rad3 in these two events is also distinct. Since the Rad3 protein is required for all DNA structure dependent checkpoint responses, a model that could account for the cell-cycle specificity would be the formation and/or utilization of different populations of Rad3 protein complexes during the cell cycle. At different stages of the cycle, Rad3 could be associated with different proteins (e.g. Cds1 and Chk1) and these might modulate its function.

The different temporal requirements for Rad3 in the Chk1 and Cds1 responses probably reflects the different functions of the two checkpoints. DNA damage appears to be rapidly recognized by the checkpoint machinery and signalled to Chk1, which then maintains the checkpoint for a period of time consistent with the phosphorylation status of Chk1. Thus, Chk1 dephosphorylation may be the key event in determining the duration of the delay. Such a model would define a period for repair which would be based on the initial intensity of the damage. In this way, the damage checkpoint would be independent of the DNA or DNA–protein structures which are generated by subsequent repair. Unlike the damage checkpoint, which is a response to sudden events, the DNA replication checkpoint monitors an ongoing process. The associated DNA and DNA protein structures would therefore be persistent. The replication checkpoint would be expected to respond to release from the replication block quite rapidly. Thus, the constant requirement for Rad3 is consistent with the continuous monitoring of a stable structure until the situation is resolved.

Conclusion

In humans, different complexes are likely to be required during different stages of the cell cycle. The two Rad3 related proteins ATM and ATR appear to share responsibility for the DNA structure checkpoints (Savitsky *et al*., 1995; Cliby *et al*., 1998). ATR and ATM complexes might be expected to monitor a number of distinct DNA structures, and the occurrence of these protein complexes might be associated with passage through G_1, S and G_2 phases of the cell cycle. Our analysis of the different modes of action for Rad3 in fission yeast and the differential phosphorylation of Chk1 during the cell cycle may therefore help to provide insight into the cellular mechanisms that maintain genomic integrity in humans.

Materials and methods

Strains

The strains used in this study are listed in Table I.

Genetic and cell biology techniques

Double mutants were created by standard genetic techniques (Gutz *et al*., 1974). Cell survival analysis and checkpoint measurements following DNA damage and replication blocks were performed using methods described previously (Al-Khodairy *et al*., 1994). Cells were synchronized in G_2 using centrifugal force [lactose gradients or elutriation (Edwards and Carr, 1997)], or a 4 h temperature shift from 27 to 35°C of the temperature-sensitive mutant strain *cdc25.22*. Cells were synchronized in G_1/S phase by either a 4 h temperature shift from 27 to 35°C of the temperature-sensitive mutant strain *cdc10.129*, or by hydroxyurea-induced DNA replication block (20 mM) and release by washing cells in fresh medium.

We noticed that, possibly due to the centrifugal stress cells suffer during the elutriation procedure, there is a decrease of the total amount of Chk1 protein in the first two time-points after harvesting (this effect appears to be reproducible), and that a high degree of synchrony is required to detect a clear absence of Chk1 protein modification after DNA damage during mitosis and G_1/early S phase because these periods are relatively short.

Isolation of a thermosensitive allele of rad3

The complete *rad3* gene, cloned as a *Sac*I–*Pst*I fragment in the leucine selectable expression vector pAL, was mutagenized with hydroxylamine

(Humphreys *et al*., 1976). Mutagenized plasmids were transformed into *rad3.d* and the resulting colonies selected on medium lacking leucine. To identify thermosensitive alleles of the *rad3* gene, 60 000 colonies were screened by replica plating onto plates containing either 7.5 or 10 mM hydroxyurea at both 27°C and 35°C, respectively (Martinho and Carr, 1998). Twenty colonies which were able to grow at 27°C, which possessed an elongated cellular phenotype and which died at 35°C with a 'cut' phenotype were selected. Further characterization yielded a single colony with a well-defined phenotype under these conditions. To generate a *rad3ts* strain, the plasmid from this colony was isolated and the *Sac*I–*Pst*I fragment encoding the mutagenized *rad3* gene transformed into the *rad3.d* using FOA selection (Grimm *et al*., 1988). Genomic integration and replacement of the *ura4*$^+$ marker in the *rad3.d* strain by the mutated *rad3* clone was confirmed by Southern blotting. Sequence analysis was used to identify the mutations present in the *rad3* clone. The causative mutation of the temperature-sensitive phenotype (A2217V) was confirmed by genomic integration.

Isolation of multi-copy suppressors of rad3ts

Unless stated otherwise, the semi-permissive temperature used was 32°C and the restrictive temperature 35°C. The *rad3ts* strain was transformed with the pURSP1 and pURSP2 *S.pombe* genomic libraries (Barbet *et al*., 1992), and 50 000 colonies were screened by replica plating onto plates containing 4, 5 and 7.5 mM hydroxyurea at both the semi-permissive and restrictive temperature, respectively (Martinho and Carr, 1998). Approximately 150 colonies, which were able to grow at the semi-permissive temperature but not at the restrictive temperature in the presence of hydroxyurea, were isolated and characterized further. To identify the suppressers of the *rad3ts* hydroxyurea sensitivity, the library plasmids from these clones were recovered and analysed by restriction, Southern blot and sequence analyses. Analysis revealed multiple isolates of the *rnr3* gene and the isolation of four *cds1* clones. To characterize further *cds1* suppression, *cds1* under the control of its own promoter or the inducible *nmt1* promoter, was transformed into both the *rad3ts* and *rad3.d* strains. The resulting transformants were serially diluted (10^7–10^4 cells/ml) and 2 µl were spotted onto plates with or without hydroxyurea (7.5 mM); plates were then incubated at the semi-permissive temperature. To analyse the suppression of *rad3* by *chk1*, the *chk1* gene under the control of the inducible *nmt1* promoter (Rep41) was transformed into both the *rad3ts* and *rad3.d* strains, which were assayed for survival with increasing doses of UV radiation (Edwards and Carr, 1997).

Cds1 kinase assays

Protein extraction and Cds1 kinase assays were performed as previously described (Lindsay *et al*., 1998). One milligram of total protein was immunoprecipitated using an affinity purified Cds1 kinase polyclonal antibody and protein A beads. The beads were washed three times with kinase buffer prior to incubation with 5 µl myelin basic protein (1 mg/ml of stock), 1 µl 2 mM ATP and 5 µCi [γ-^{32}P]ATP (ICN) at 30°C for 15 min. The reaction was stopped by the addition of 20 µl of 2× sodium dodecyl sulfate (SDS) sample buffer, and Cds1 kinase activity was analysed by SDS–PAGE.

Immunoprecipitation with Rad3

Wild-type cells were transformed with either Rep1GST*cds1* or Rep1GST*chk1* and *myc*-tagged *rad3*, or with Rep41 and/or Rep42 plasmids (enabled *nmt1* inducible expression) containing HA-tagged *rad3* or *myc*-tagged *chk1*, respectively (Griffiths *et al*., 1995; Bentley *et al*., 1996). The tagged proteins were expressed by growing in media lacking thiamine for 18 h (Maundrell, 1990). Yeast cells were lysed and 1 mg total protein was immunoprecipitated using appropriate antibodies, as described previously (Bentley *et al*., 1996). The immunoprecipitates were run on 8% polyacrylamide gels. Anti c-Myc (PharMingen) and anti-HA (Babco) antibodies, at dilutions of 1:1000, were used to probe the subsequent Western blots. Chemiluminescent detection of horse radish peroxidase (HRP)-conjugated secondary antibodies was carried out using ECL (Amersham).

Size fractionation

Extracts were prepared from a strain containing the *rad3* gene tagged at the genomic locus with the *myc* epitope as follows: mid-log phase cells were harvested, washed in ice-cold water, washed again in ice-cold buffer B (50 mM NaH_2PO_4 /Na_2HPO_4 pH 7.0, 150 mM NaCl, 0.1% NP-40, 10% glycerol, 0.5 mM DTT, 5 mM EGTA, 60 mM β-glycerophosphate, 0.1 mM NaF, 1 mM Na-orthovanadate) and resuspended in 1 ml buffer B plus protease inhibitors (BCL complete protease inhibitor cocktail). Cells were frozen in liquid nitrogen and ground to a fine powder using a mortar and pestle. Thawed powder was cleared by centrifugation (14 K in a microfuge 2× 5 min) and 200 µl of supernatant loaded onto a Superdex 200 column (Pharmacia HR10/30). Fractions (0.5 ml) were collected and aliquots analysed by SDS–PAGE and Western blotting with anti-Myc monoclonal or anti-Cds1 serum (Lindsay *et al*., 1998).

Kinase assays with Rad3

rad3.d was transformed with the *nmt1* inducible expression vector containing either Rep41HA-*rad3* or Rep41HA-*rad3.KD*, and the corresponding tagged proteins were induced in medium lacking thiamine. Protein extracts were prepared as described previously and 1 mg total protein immunoprecipitated with 1 ug anti-HA antibody (Babco).

KD mutations were introduced into the *cds1* and *chk1* genes by site-directed mutagenesis. A D318E mutation was introduced into the *cds1* gene using the oligonucleotide GCCAAGCCAAATTCAGATATTTTAAGATGG, and a D155E mutation introduced into the *chk1* gene using the oligonucleotide AGATGCAAAGCCGAATTCTGAAATCTTTAAG. Both mutations disrupt the conserved DFG motif in the kinase domain. The mutations were confirmed by sequence analysis. Cds1.KD and Chk1.KD protein kinase substrates were prepared by transforming *cds1.d* and *chk1.d* mutant strains with inducible plasmids containing GST-tagged *cds1.KD* and *chk1.KD* genes, Rep1GST*cds1.KD* and Rep1GST*chk1.KD*, respectively. GST-fusion proteins were expressed by growing in medium lacking thiamine for 18 h. The cells were disrupted in GST buffer (50 mM Tris pH 7.5, 80 mM β-glycerophosphate, 250 mM NaCl, 15 mM nitrophenylphosphate, 50 mM NaF, 5 mM EDTA, 1 mM DTT, 0.1% NP-40 and supplemented with protease inhibitors, AEBSF, leupeptin, aprotinin, pepstatin, bestatin, and E-64, all at 10 µg/ml final concentration) with glass beads (BDH) using a mikrodismembranator (Braun) for 3× 1 min at 2000 r.p.m. Protein extracts were cleared in a microfuge at 14 000 r.p.m. for 5 min at 4°C. 1 mg total protein was mixed with glutathione–agarose beads (Sigma) for 1 h at 4°C, the beads were washed (×4) with GST buffer at 4°C, and the bound GST fusion proteins eluted in batch with 50 mM Tris (pH 8.0) containing 10 mM glutathione. The eluates were analysed by SDS–PAGE and then frozen as aliquots in liquid nitrogen.

Rad3 or Rad3.KD proteins were immunoprecipitated as described above. The beads were washed three times with lysis buffer and once with kinase buffer (10 mM HEPES pH 7.5, 75 mM KCl, 5 mM $MgCl_2$, 0.5 mM EDTA, 1 mM DTT). Twenty microlitres of bead slurry was incubated with 10 µl 2× kinase buffer, 5 µl substrate and 10 µCi of [γ-^{32}P]ATP (ICN) at 30°C for 15 min. The reaction was stopped by the addition of 20 µl 2× SDS sample buffer and samples were analysed by SDS–PAGE.

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