The budding yeast Rad9 checkpoint protein is subjected to Mec1/Tel1-dependent hyperphosphorylation and interacts with Rad53 after DNA damage

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The *Saccharomyces cerevisiae* RAD9 checkpoint gene is required for transient cell-cycle arrests and transcriptional induction of DNA repair genes in response to DNA damage. Polyclonal antibodies raised against the Rad9 protein recognized several polypeptides in asynchronous cultures, and in cells arrested in S or G₂/M phases while a single form was observed in G₁-arrested cells. Treatment with various DNA damaging agents, i.e. UV, ionizing radiation or methyl methane sulfonate, resulted in the appearance of hypermodified forms of the protein. All modifications detected during a normal cell cycle and after DNA damage were sensitive to phosphatase treatment, indicating that they resulted from phosphorylation. Damage-induced hyperphosphorylation of Rad9 correlated with checkpoint functions (cell-cycle arrest and transcriptional induction) and was cell-cycle stage- and progression-independent. In asynchronous cultures, Rad9 hyperphosphorylation was dependent on MEC1 and TEL1, homologues of the ATR and ATM genes. In G₁-arrested cells, damage-dependent hyperphosphorylation required functional MEC1 in addition to RAD17, RAD24, MEC3 and DDC1, demonstrating cell-cycle stage specificity of the checkpoint genes in this response to DNA damage. Analysis of checkpoint protein interactions after DNA damage revealed that Rad9 physically associates with Rad53.

Keywords: checkpoint/DNA damage/MEC1/phosphorylation/RAD9

Introduction

Cell-cycle progression in eukaryotes is temporarily arrested when DNA is damaged, replication is inhibited, or the mitotic spindle is incorrectly assembled. These transient delays, termed checkpoints, are believed to allow time for DNA repair, replication or proper chromosomal attachment to the spindle at metaphase (reviewed in Elledge, 1996; Rudner and Murray, 1996; Paulovich *et al.*, 1997b). Disruptions to checkpoint pathways are believed to be important at early stages of carcinogenesis as they result in increased mutagenesis and genomic instability (Hartwell and Kastan, 1994). The best evidence for a link

between checkpoints and cancer comes from studies of ATM, the gene mutated in the cancer-prone disease ataxia telangiectasia (reviewed in Morgan and Kastan, 1997), and the human homologue of the yeast spindle checkpoint gene, *BUB1*, which is mutated in certain cancers that display chromosomal instability (Cahill *et al.*, 1998).

The understanding of DNA damage-dependent checkpoint pathways is presently most advanced in the yeast model systems. In the budding yeast *Saccharomyces cerevisiae*, DNA damage causes cell-cycle arrests at the G₁/S and G₂/M transitions and a slowing of S phase (Weinert and Hartwell, 1988; Siede *et al.*, 1993; Paulovich and Hartwell, 1995). Genetic control of these cell-cycle delays was first demonstrated when the *RAD9* gene was shown to be required for the G₂/M arrest after irradiation with X-rays (Weinert and Hartwell, 1988). *RAD9* has also been shown to be required for the G₁/S arrest (Siede *et al.*, 1993), the intra S checkpoint (Paulovich *et al.*, 1997a), and a DNA damage-dependent arrest in mid-anaphase (Yang *et al.*, 1997). Genetic analysis has resulted in the identification of several genes in addition to *RAD9* that are required for all DNA damage checkpoints. These include *RAD53* (also termed *SPK1/MEC2/SAD1*), *MEC1* (*ESR1/SAD3*), *RAD17*, *RAD24*, *MEC3* and *DDC1* (Elledge, 1996; Longhese *et al.*, 1997). The essential genes *MEC1* and *RAD53* are believed to be central transducers of the DNA checkpoint pathway, and are also required for a checkpoint that prevents mitosis in the presence of unreplicated DNA, the S/M checkpoint (Elledge, 1996). The DNA damage checkpoint pathway also controls the transcriptional induction of a large regulon, the DNA damage regulon (DDR), of >15 genes with roles in DNA repair (Aboussekhras *et al.*, 1996; de la Torre-Ruiz *et al.*, 1998). Epistasis and overexpression analysis indicate that *RAD9* is in a distinct epistasis group from *RAD24*, *RAD17*, *MEC3* and *DDC1*, and that these two groups can be placed in separate but additive branches that converge on *MEC1* and *RAD53* (de la Torre-Ruiz *et al.*, 1998). *TEL1* is involved in telomere maintenance (Greenwell *et al.*, 1995) but also exhibits functional redundancy and sequence homology with *MEC1*. However, *tel1Δ* mutants are not checkpoint defective, although *mec1 tel1Δ* double mutants are more sensitive to DNA damage than a *mec1* mutant, and overexpression of *TEL1* can suppress some *mec1* defects (Morrow *et al.*, 1995; Sanchez *et al.*, 1996).

Significant progress has been made in identifying genes involved in checkpoint control and in the characterization of the downstream biological consequences of this pathway, but there is little biochemical information relating to the encoded products. *RAD53* encodes a protein kinase that is phosphorylated and activated by DNA damage or treatment with the ribonucleotide reductase inhibitor, hydroxyurea (HU) (Sanchez *et al.*, 1996; Sun *et al.*, 1996;

de la Torre-Ruiz *et al.*, 1998). These events are dependent on Mec1, a member of a kinase superfamily that includes the Atm and Atr proteins (Hunter, 1995). Atm, Atr and a *Schizosaccharomyces pombe* homologue, Rad3, have been shown to have an associated protein kinase activity (Bentley *et al.*, 1996; Keegan *et al.*, 1996; Jung *et al.*, 1997). *RAD9*, *RAD24*, *RAD17* and *MEC3* encode non-essential proteins that have been reported to be involved in processing single stranded subtelomeric DNA that accumulates in a *cdc13* mutant at the restrictive temperature (Lydall and Weinert, 1995). However, it is not known whether they are directly involved in DNA-damage recognition. Rad24 has some homology with members of the RFC family of proteins (Griffiths *et al.*, 1995) and the *Ustilago maydis* homologue of Rad17 has been reported to have 3'→5' exonuclease activity (Thelen *et al.*, 1994), suggesting that these proteins interact with DNA. Ddc1 is phosphorylated periodically during the cell cycle and becomes hyperphosphorylated by DNA damage; both of these events are at least partially dependent on *MEC3* (Longhese *et al.*, 1997).

Here we show that the Rad9 protein becomes phosphorylated when cells are arrested in the S and G₂/M phases of the cell cycle. In addition, it is hyperphosphorylated as a result of DNA damage. DNA damage-induced Rad9 phosphorylation occurs independently of cell-cycle position and does not require cell-cycle progression. This hyperphosphorylation occurs rapidly and persists throughout the G₂/M checkpoint. Moreover, DNA damage-dependent hyperphosphorylation of Rad9 correlates with induction of the DDR in a dose-dependent manner. This modification is dependent on an intact DNA damage-dependent checkpoint pathway in G₁-arrested cells, but in rapidly proliferating cells it only depends on *MEC1* and *TEL1*. Finally, after DNA damage, Rad9 physically interacts with phosphorylated Rad53, suggesting that this interaction is required for Rad9 function.

Results

Rad9 phosphorylation in the cell cycle and after DNA damage

We have raised polyclonal antibodies against the *S.cerevisiae* Rad9 protein produced in a bacterial expression system (see Materials and methods). Although *RAD9* encodes a protein with a predicted molecular weight of 148 kDa, immunoblot analysis of cell lysates from an asynchronous culture detected a broad band of polypeptides with mobilities ranging from ~190 to 220 kDa on SDS-polyacrylamide gels (Figure 1A). These polypeptides were absent in a *rad9Δ* strain and were detected in greater abundance in strains overexpressing Rad9 (data not shown), suggesting that they corresponded to various post-translational modifications at different points of the cell cycle. Accordingly, cells arrested in G₁ with the mating pheromone α -factor contained only the fastest migrating form(s), while cultures arrested in S phase with HU also exhibited several slower migrating forms (Figure 1A). Cells blocked in G₂/M with the microtubule inhibitor nocodazole displayed an increased accumulation of the slower migrating forms and the disappearance of the faster migrating forms (Figure 1A). The Rad9 forms detected in α -factor-, HU- and nocodazole-arrested cells co-migrated

Fig. 1. Rad9 is a phosphoprotein that becomes hyperphosphorylated after DNA damage. (A) Western blot analysis of total proteins from asynchronous log phase cultures (log), cells arrested in α -factor (α F), hydroxyurea (HU) or nocodazole (Noc) and UV irradiated log-phase cells (UV). (B) Western blot analysis of total proteins from log phase cells that were either untreated (-), UV irradiated (UV), MMS treated (MMS), or γ -ray irradiated (γ). (C) Western blot analysis of total protein extracts of hydroxyurea-arrested (HU), nocodazole-arrested (Noc) or UV irradiated (UV), exponentially growing asynchronous cells incubated in the presence (+) or absence (-) of λ protein phosphatase (λ PPase), and in the presence (+) or absence (-) of the phosphatase inhibitor, sodium orthovanadate (Na Vanadate). Cells were arrested in α -factor for 150 min, in hydroxyurea for 120 min and in nocodazole for 120 min. Irradiation was with 40 J/m² UV or 100 Gy γ -rays, MMS treatment was by addition of MMS to 0.01% which was left in the culture for the remainder of the experiment. Samples were collected 60 min after irradiation or MMS addition. Each lane corresponds to equivalent amounts (10 μ g) of total protein. Migration of the relevant molecular weight size standard is indicated on the left side.

with polypeptides present in asynchronous cultures (although the slowest migrating forms are much more easily detected after nocodazole arrest), suggesting that they did not result from the treatment leading to arrest. A more pronounced change in Rad9 migration through SDS-PAGE was observed when cells from an asynchronous culture were subjected to DNA damage by irradiation with ultraviolet light (UV). This treatment resulted in the appearance of Rad9 forms that migrated much more slowly than any of the forms seen in unirradiated cells, concomitant with the disappearance of some of the cell-cycle modified forms (Figure 1A). In order to determine whether these hypermodifications were specific to UV irradiation, we tested other DNA damaging agents (Figure 1B). Similar Rad9 modifications were detected after treatment with γ -rays, and methylmethane sulfonate (MMS)

Fig. 2. Time course analysis of Rad9 and Rad53 phosphorylation after UV irradiation of an asynchronously growing log-phase culture (time 0). Western blot of total proteins from cells collected at various times after UV irradiation (30 J/m²) and probed with anti-Rad9 (Rad9) or anti-Rad53 (Rad53) antibodies (upper panels). Equal amounts of total protein (10 µg) were loaded in each lane. Migration of the relevant molecular weight size markers is indicated along the left side. The lower panel shows the kinetics of accumulation of large budded cells (% LB cells) in the UV irradiated culture. These cells correspond to cells transiently arresting at the G₂/M checkpoint as determined by DAPI staining and FACS analysis, in addition to their large budded morphology, as previously described (de la Torre-Ruiz *et al.*, 1998).

(Figure 1B). Incubation of proteins from HU, nocodazole or UV-treated cells with protein phosphatase resulted in disappearance of all but the faster migrating forms of Rad9. This phosphorylation was prevented by addition of a phosphatase inhibitor (Figure 1C), indicating that the slower migrating forms of Rad9 in the cell cycle and after DNA damage are phosphorylated. The faster migrating form(s) of Rad9 resulting from phosphatase treatment and the Rad9 form(s) present in α -factor arrested cells co-migrated through SDS-PAGE substantially more slowly than the 148 kDa expected from the amino acid content of *RAD9*. This suggests that either its primary sequence results in anomalous migration through SDS-PAGE, or that an additional modification(s) is present.

Correlation of DNA damage-dependent Rad9 phosphorylation with checkpoint functions

The major DNA damage checkpoint in *S.cerevisiae* occurs close to the G₂/M transition and is characterized by accumulation of large budded cells with a single 2C nucleus at the bud neck (Weinert and Hartwell, 1988). UV irradiation of cells from an asynchronous culture resulted in rapid hyperphosphorylation of Rad9, detected after just 10 min, which was maintained for 6–7 h after UV irradiation, at which point the majority of cells had exited the checkpoint (Figure 2). The levels of Rad9 appeared lower in the time points immediately after irradiation, presumably due to non-specific proteolysis as it was not always reproducible. There was a rapid decrease of cell-cycle modified forms of Rad9 upon UV irradiation which began to reappear after 120 min (Figure 2). The Rad53 protein kinase has previously been shown to be

Fig. 3. Damage-dependent transcriptional induction correlates with Rad9 hyperphosphorylation in a dose-dependent manner. (A) Western blot analysis of total proteins (10 µg) from cells arrested in G₁ with α -factor (α F) and incubated in the presence of the indicated concentrations of MMS (% MMS). Blots were probed with anti-Rad9 (Rad9) and anti-Rad53 (Rad53) antibodies. Migration of the relevant molecular weight standards is indicated. (B) Northern blot analysis of the DNA damage inducible *RNR1* gene. RNA was prepared from equivalent cell pellets to those used in part A. A *PRT1-H2A* probe was also used. The *PRT1* transcript is invariant under these conditions and was used as a loading control. *H2A* is not expressed in α -factor arrested cells confirming maintenance of the G₁ block. Furthermore, the cells remained unbudded for the duration of the experiment.

phosphorylated after DNA damage and during replication blocks with HU (Sanchez *et al.*, 1996; Sun *et al.*, 1996). We examined Rad53 in cells from the UV irradiated culture and found that the kinetics of phosphorylation were different to those of Rad9 (Figure 2). Rad53 phosphorylation also occurred rapidly after UV irradiation, however, there was a transient disappearance of unphosphorylated Rad53. At late time points Rad53 was primarily detected in two states: the fastest and slowest migrating forms, whereas the most slowly migrating forms of Rad9 were much less detectable. Thus, while the kinetics of Rad9 and Rad53 modification share some similarities there are also some differences perhaps indicative of distinct regulation.

Rad9 hyperphosphorylation not only coincided with checkpoint arrest, but also correlated with the DNA damage-dependent transcriptional response. When G₁-arrested cells were exposed to increasing concentrations of MMS, the extent of phosphorylation of both Rad9 and Rad53 correlated with *RNR1* induction in a dose-dependent manner (Figure 3A and B).

DNA damage-dependent Rad9 phosphorylation in G₁ and G₂/M arrested cells

Hyperphosphorylation of Rad9 was not simply a result of accumulation of cells at the G₂/M checkpoint, because it was also apparent after UV irradiation of mating pheromone-arrested cells in which the G₁ arrest was maintained throughout the experiment (Figure 4A). It also occurred in UV irradiated cells continuously arrested in G₂/M with nocodazole (Figure 4B). Similar to our previous observations in asynchronous cultures (Figure 2), phosphorylation of Rad53 in nocodazole-arrested UV irradiated cells was rapid and initially resulted in complete loss of

Fig. 4. DNA damage dependent hyperphosphorylation of Rad9 in G_1 and G_2/M arrested cells. (A) Western blot analysis of total proteins from cells arrested with α -factor, UV irradiated (40 J/m^2) and maintained in G_1 by continuous exposure to α -factor. Cells were collected prior to α -factor arrest (log), after α -factor arrest (0) and at the times indicated after irradiation. (B) Western blot analysis of total proteins from cells arrested with nocodazole, UV irradiated (40 J/m^2) and maintained in G_2/M by continuous exposure to nocodazole. Equal amounts of protein ($10 \mu\text{g}$) were loaded in each lane. Blots were probed with anti-Rad9 (Rad9) or anti-Rad53 (Rad53) antibodies. Migration of the relevant molecular weight standards is indicated.

the hypophosphorylated form (Figure 4B). These results indicate that phosphorylation of Rad9 and Rad53 in response to DNA damage can occur at any stage in the cell cycle.

DNA damage-dependent Rad9 phosphorylation in checkpoint mutants

RAD9, *RAD17*, *RAD24*, *MEC3* and *DDC1* are believed to function upstream of the central signal transducers *MEC1* and *RAD53* in the DNA damage response (Navas *et al.*, 1996; Sanchez *et al.*, 1996; Sun *et al.*, 1996; de la Torre-Ruiz *et al.*, 1998). We examined the phosphorylation state of Rad9 in various checkpoint mutant strains in order to determine whether these genes were involved in its DNA damage-dependent modification. Cells from asynchronous exponentially growing cultures of *rad17* Δ , *rad24* Δ , *mec3* Δ , *ddc1* Δ and a 'kinase-dead' allele of *RAD53* (*rad53K227A*) were treated with MMS and subjected to Western blot analysis. Rad9 hyperphosphorylation was indistinguishable from the wild-type in all mutants (Figure 5A). *DUN1* encodes a protein kinase required for damage-inducible transcription of the *RNR* genes (Zhou and Elledge, 1993), that may also have a minor role in G_2/M checkpoint arrest (Pati *et al.*, 1997). Rad9 hyperphosphorylation in a *dun1* Δ mutant was also indistinguishable from the wild type. When we examined Rad53 phosphorylation in these mutants we found that it was slightly impaired in the *rad17* Δ , *rad24* Δ , *mec3* Δ and *ddc1* Δ strains, relative to the wild-type strain (Figure 5A). This correlates with a partial checkpoint defect in these mutants (de la Torre-Ruiz *et al.*, 1998). Rad53 phosphorylation appeared to be unaffected in the *tel1* Δ and *dun1* Δ mutants (Figure 5A and B). Although Rad53 protein levels were substantially reduced

Fig. 5. Damage dependent phosphorylation of Rad9 in checkpoint mutants. (A) Western blot analysis of total protein extracts ($10 \mu\text{g}$) from MMS treated asynchronous log phase cultures of the strains indicated. Cells were collected prior to (0) and at the times indicated after addition of 0.01% MMS for each culture. (B) Western blot analysis of total protein extracts ($10 \mu\text{g}$) from untreated (-), UV irradiated (UV) or MMS-treated (MMS) asynchronous log phase cultures of the strains indicated (note that the *RAD53* single mutation is *rad53K227A* and the *RAD53 DUN1* double mutation is *rad53-11 dun1* Δ). Cells were collected prior to treatment with the DNA damaging agents (-) and 60 min after either UV irradiation (40 J/m^2) or addition of MMS (0.01%). Blots were probed with anti-Rad9 (Rad9) or anti-Rad53 (Rad53) antibodies. Migration of the relevant molecular weight markers is indicated. Note that in the experiment shown in (B) the MMS treatment of the *tel1* Δ strain was inefficient as indicated by the lower than expected levels of Rad9 and Rad53 phosphorylation.

in the *rad53K227A* strain, damage-dependent phosphorylation of Rad9 was not affected, despite the pronounced checkpoint defect of this strain. Similar results were obtained when UV irradiation was used as the DNA damaging agent (data not shown). In contrast with the other checkpoint mutants, the damage-inducible phosphorylation of Rad9 was less pronounced in a *mec1-1* mutant and was completely absent in a *mec1-1 tel1* Δ double mutant after MMS or UV treatment (Figure 5B). Although greatly reduced relative to that observed in wild-type cells and in the other checkpoint mutants, some Rad53 phosphorylation after UV irradiation could be reproducibly detected in the *mec1-1* strain (Figure 5B). The Rad9 phosphorylation deficiency after DNA damage in *mec1-1* cells was more pronounced when cells were grown at lower temperatures or in a poor carbon source. Under these conditions no phosphorylation of Rad53 could be detected (data not shown). Rad9 hyperphosphorylation was not affected in *tel1* Δ cells consistent with normal checkpoint function in this mutant. Thus, *MEC1* and *TEL1* are both required for hyperphosphorylation of Rad9 after DNA damage, but the role of *TEL1* is likely to be less important, perhaps only contributing to Rad9 hyperphosphorylation in the absence of *MEC1* and/or when growth conditions are optimal. We also tested a *rad53-11 dun1* Δ double mutant and found that although the amount of Rad53 protein in this strain was severely reduced, as in the *rad53K227A* strain, Rad9 hyperphosphorylation was not (Figure 5B). Interestingly, cell-cycle-specific phosphorylation of Rad9 prior to DNA damage was essentially

Fig. 6. G₁-arrested cells require *MEC1* and the *RAD24* epistasis group for Rad9 hyperphosphorylation after DNA damage. (A) Western blot analysis of total proteins (10 µg) from the strains indicated arrested in G₁ with α -factor, UV irradiated and maintained in G₁. UV irradiated (+) or non-irradiated (-) G₁ arrested wild-type (WT) cells are indicated. A non-irradiated asynchronous log phase (log) control is also shown. (B) Western blot analysis of total proteins (10 µg) from the strains indicated arrested in S phase with hydroxyurea, UV irradiated and maintained in S phase in the continuous presence of HU. (C) Western blot analysis of total proteins (10 µg) from the strains indicated arrested in G₂ phase with nocodazole, UV irradiated and maintained in G₂ phase in the continuous presence of nocodazole. For both (A) and (B), cells were UV irradiated in saline with 40 J/m² and maintained in YPD for an additional 30 min prior to harvesting. For (C) cells were harvested 45 min after irradiation. Blots were probed sequentially with anti-Rad9 (Rad9) and anti-Rad53 (Rad53) antibodies. The strains examined are indicated at the top of each set of panels. Note that for parts (A) and (B) the *RAD53* mutation used is *rad53K227A*, and for (C) it is *rad53-11*. Migration of the relevant molecular weight markers are indicated.

identical to the wild-type in all of the mutants tested, indicating that the checkpoint pathway is unlikely to control this modification (Figure 5A and B; J.E. Vialard, unpublished data).

RAD9, *RAD17*, *RAD24*, *MEC3* and *DDC1* are required for all DNA damage checkpoints, although they only have a minor role in the intra-S checkpoint (Navas *et al.*, 1996; Paulovich *et al.*, 1997a; de la Torre-Ruiz *et al.*, 1998). Therefore, we decided to look at the effect of DNA damage on Rad9 phosphorylation in G₁-arrested cells (Figure 6A). Under these conditions, Rad9 hyperphosphorylation was not only dependent on *MEC1*, but also on the *RAD17*, *RAD24*, *MEC3* and *DDC1* genes. Once

more, mutation of *RAD53* had no effect on damage-inducible phosphorylation of Rad9. Similarly, Rad9 modification was unaffected in a *tel1*Δ mutant. The different requirements of the various checkpoint genes in asynchronous cells and cells arrested in G₁ for Rad9 hyperphosphorylation suggest that there is cell-cycle stage specificity. Thus, alternative pathways resulting in Rad9 hyperphosphorylation must operate outside of G₁ that are redundant with the pathway defined by the *RAD24* epistasis group. In an attempt to further dissect this cell-cycle specificity, cultures of the various checkpoint mutants were arrested in S-phase with HU and then subjected to UV irradiation (Figure 6B). Rad9 hyperphosphorylation after UV irradiation was detected in all known mutants of the *RAD24* epistasis group and was indistinguishable from the wild type. Thus, these genes are not required for this modification in HU-arrested cells. As previously reported, after HU treatment alone some Rad53 phosphorylation occurred in wild-type cells and in the *rad17*Δ, *rad24*Δ, *mec3*Δ and *ddc1*Δ mutants (Figure 6B; Sanchez *et al.*, 1996; Sun *et al.*, 1996). However, UV irradiation after HU-arrest resulted in more extensive Rad53 phosphorylation in the wild-type and *RAD24* epistasis group mutants (Figure 6B). Interestingly, in *mec1-1* and *rad53K227A* cells, Rad9 was hyperphosphorylated by HU treatment alone, perhaps indicating the presence of DNA damage. Although less apparent than in the *RAD24* epistasis group mutants, residual Rad53 phosphorylation was also detected in the *mec1-1* mutant after HU treatment, but increased phosphorylation resulting from UV treatment was not observed (Figure 6B). Similarly, although Rad53 is difficult to detect in the *rad53K227A* mutant, phosphorylation occurred after HU treatment, but no increase was observed after UV irradiation.

The above data suggest that Rad9 hyperphosphorylation is dependent on the genes of the *RAD24* epistasis group in G₁- but not S-arrested cells. To further test this cell-cycle dependency, we examined this modification in cells arrested in G₂/M with nocodazole (Figure 6C). Hyperphosphorylation of Rad9 after UV irradiation was normal in all the single mutants tested but once more was absent in *mec1-1 tel1*Δ cells. Thus, this modification in nocodazole-arrested cells is regulated in a similar manner to both HU-arrested cells and asynchronously growing cells (compare Figure 5 with Figure 6). However, unlike HU-blocked cells, Rad53 phosphorylation after UV irradiation in nocodazole-arrested cells was much reduced. Furthermore, in nocodazole-arrested cells hyperphosphorylation of Rad9 was relatively normal in *mec1-1* cells, as was observed in asynchronous exponentially growing cells, and in HU-arrested cells, whereas phosphorylation of Rad53 after damage was significantly reduced in *mec1-1* cells under all conditions tested. Thus, a major difference in the regulation of phosphorylation of these two proteins after DNA damage occurs in G₁-arrested *mec1-1* cells. Rad9 hyperphosphorylation is only Mec1-dependent in G₁-arrested cells, whereas DNA damage-dependent phosphorylation of Rad53 is almost completely eliminated by mutation of *MEC1* under all conditions tested. In summary, our data demonstrate a role for *MEC1* and *TEL1* in the DNA damage-dependent hyperphosphorylation of Rad9 at most cell-cycle stages. However, in G₁-arrested cells

Fig. 7. Co-fractionation of phosphorylated Rad53 with hyperphosphorylated Rad9. Proteins extracts from either tagged (10HisRAD9) or untagged (RAD9) strains were prepared after either mock treatment (–UV) or UV irradiation (+UV) and were bound to Ni^{2+} -agarose beads, washed with lysis buffer containing 20 mM imidazole, and bound proteins eluted with lysis buffer containing 250 mM imidazole as indicated. Aliquots from various fractions were run on 6.5% SDS–PAGE gels, blotted and probed with immune sera specific to the proteins indicated.

these modifications are *TEL1*-independent, but are dependent on *RAD17*, *RAD24*, *MEC3* and *DDC1*.

Rad9 interacts with Rad53 after DNA damage

In a preliminary attempt to address the functional significance of Rad9 hyperphosphorylation, we examined whether other checkpoint proteins physically interacted with Rad9 after DNA damage. A tagged version of Rad9 was generated in which chromosomal *RAD9* was replaced by a version of *RAD9* containing 10 histidine codons at its N-terminus. The *10HisRAD9* gene is under the control of the normal *RAD9* promoter and the tag does not detectably interfere with *RAD9* function or cell growth (data not shown). *10HisRAD9* cells were either UV irradiated or mock treated, and Rad9 was purified by affinity chromatography on Ni^{2+} agarose (Figure 7). This protocol reproducibly resulted in most of Rad9 being in the hyperphosphorylated form and all the detectable Rad53 being in its most modified form after UV irradiation. We could find no evidence of Rad24, Rad17 or Mec3 co-fractionating with 10HisRad9 in extracts from either UV-irradiated or mock-treated cells (C.S.Gilbert and C.M.Green, unpublished results). However, in irradiated extracts a small fraction of the phosphorylated form of Rad53 reproducibly co-purified with 10HisRad9. This co-purification is not due to nonspecific binding of Rad53, as it was not detected when untagged extracts were used. Furthermore, we did not find any evidence for hypophosphorylated Rad53 co-fractionating with cell-cycle modified Rad9 in the mock treated extracts, even after a 10-fold overloading of the elution fractions. Thus, DNA damage-dependent hyperphosphorylation of Rad9 appears to allow interaction between a portion of phosphorylated Rad53 and either Rad9 directly, or a Rad9 containing complex.

Discussion

Rad9 phosphorylation is regulated by cell-cycle progression and DNA damage

The *S.cerevisiae* *RAD9* gene is required for efficient checkpoint function in response to DNA damage. Genetic

analysis of the DNA damage checkpoint pathway suggests that *RAD9* functions upstream of the central transducing components *MEC1* and *RAD53* (Navas *et al.*, 1996; de la Torre-Ruiz *et al.*, 1998), and may be directly involved in sensing or processing DNA damaging lesions (Lydall and Weinert, 1995). Although it has not been shown to have a direct role in the cell cycle, a possible function in monitoring endogenously occurring DNA damage is suggested by the increased chromosomal instability observed in *rad9* mutants (Weinert and Hartwell, 1990). Consistent with such a role, we have observed that Rad9 is phosphorylated in the S and G₂ phases of the cell cycle. However, cell cycle phosphorylation may correspond to inactivating or attenuating modifications that prevent Rad9 from improperly recognising replication structures as DNA damage.

In this study we have focused on the effect of DNA damage on the Rad9 protein. Treatment with various types of DNA damaging agents results in a rapid hyperphosphorylation of Rad9 that persists until the cellular responses to DNA damage are completed. Lack of damaging agent specificity is consistent with the frequently made observation that arrest and survival phenotypes are also independent of damaging agent. This is also true for transcriptional induction of the DDR (our unpublished data, but see Figure 4 for MMS-specific transcription). The damage-specific hyperphosphorylated forms of Rad9 migrate through denaturing polyacrylamide gels much more slowly than the most modified forms detected in asynchronously growing cells or after an extended G₂/M arrest. Interestingly, there is loss of at least some of the cell-cycle phosphorylated forms after DNA damage, perhaps indicating that some of the cell-cycle modifications may be removed to allow the DNA damage-specific function of Rad9.

The kinetics of Rad9 hyperphosphorylation after DNA damage follows the time the population of cells spend in the G₂/M checkpoint. This is consistent with either a continuous requirement for Rad9 throughout the checkpoint response, or with a requirement for it to be inactivated immediately after DNA damage has been sensed and the checkpoint response initiated. Interestingly, the transcrip-

tional response to DNA damage, which is also dependent on Rad9 (Aboussekhra *et al.*, 1996; Kiser and Weinert, 1996; Navas *et al.*, 1996; de la Torre-Ruiz *et al.*, 1998), is more transient in nature (Aboussekhra *et al.*, 1996; de la Torre-Ruiz *et al.*, 1998).

DNA damage results in hyperphosphorylation of Rad9 by DNA damage in cells arrested in either G₁ or G₂/M, indicating that cell cycling is not required for initiating the signal. This is not the case with damage-dependent phosphorylation of the Ddc1 checkpoint protein which only becomes phosphorylated after damage when G₁-arrested cells are allowed to progress through the cell cycle (Longhese *et al.*, 1997). These observations suggest that the damage sensing mechanism that transmits the checkpoint signal to Ddc1 must differ from the one that functions through Rad9. However, mutation in any of *RAD17*, *RAD24* or *MEC3* is sufficient to severely reduce transcriptional induction of the DDR in G₁-arrested cells, suggesting that these genes do not require cell cycling to sense DNA damage (de la Torre-Ruiz *et al.*, 1998).

While studying the correlation between Rad9 hyperphosphorylation (and Rad53 phosphorylation) and the transcriptional response to MMS treatment, we noticed that asynchronous exponentially growing cells were more sensitive to MMS treatment than G₁-arrested cells. Both Rad9 and Rad53 were more dramatically modified by 0.01% MMS treatment in exponential cultures (Figure 5B) than in G₁-arrested cultures (Figure 4A and data not shown). This effect appears to be specific to MMS (our unpublished data) and may be related to S phase-specific mechanisms, perhaps replication itself, capable of sensing MMS-specific damage that do not operate in G₁- or G₂/M-arrested cells.

Damage-induced Rad9 phosphorylation is MEC1 and TEL1 dependent

DNA-damage signals emanating from *RAD9* and the *RAD24* epistasis group of genes are thought to be transduced to a central component of the checkpoint pathway, *MEC1*, which in turn activates *RAD53* resulting in cell-cycle arrest and transcriptional induction of DNA repair genes. Rad9 hyperphosphorylation resulting from DNA damage was not detectably affected in asynchronously growing cells harbouring a 'kinase-dead' allele of *RAD53*, nor in a *rad53-11 dun1Δ* double mutant (we tested this strain because there may be some functional redundancy between these two genes; Pati *et al.*, 1997). Rad9 hyperphosphorylation was also unaffected in asynchronous cultures of strains harbouring null mutations in each of the *RAD24* epistasis group of genes. However, *mec1-1 tel1Δ* cells are completely devoid of detectable Rad9 hyperphosphorylation after DNA damage in asynchronously growing cells. The *mec1-1* mutant is only partially defective in Rad9 hyperphosphorylation, although it seems to have a more severe defect in Rad53 phosphorylation. *MEC1* and *TEL1* have some overlapping functions, as sequence analysis suggests that they encode related kinases and overexpression of *TEL1* can complement deficiencies in *MEC1* (Morrow *et al.*, 1995; Sanchez *et al.*, 1996). A similar dependence on *MEC1* (and a minor role for *TEL1*) has been observed in damage-dependent Rpa2 phosphorylation (Brush *et al.*, 1996). Pds1, an effector of G₂/M arrest is also phosphorylated

after DNA damage in a *MEC1* dependent manner (Cohen-Fix and Koshland, 1997). Pds1 modification is *RAD9*-dependent whereas Rpa2 phosphorylation is not, indicating subtle differences in the regulation of *MEC1*-dependent phosphorylation. Unlike *RPA2* and *PDS1*, which are believed to function downstream of *MEC1* (Brush *et al.*, 1996; Yamamoto *et al.*, 1996; Cohen-Fix and Koshland, 1997), genetic studies suggest that *RAD9* (and the *RAD24* epistasis group) functions upstream of *MEC1* in the DNA damage checkpoint pathway (Elledge, 1996; de la Torre-Ruiz *et al.*, 1998). If this model is correct then it predicts that Rad9 hyperphosphorylation should correspond to feedback regulation Rad9 function. Some support for feedback regulation comes from comparing the kinetics of Rad9 with Rad53 phosphorylation after DNA damage (Figure 2). Both are phosphorylated rapidly, but whereas Rad53 is completely modified, the peak of Rad9 hyperphosphorylation appears to occur much later, at which point a proportion of Rad53 is already returning to the hypophosphorylated state. Alternative models in which Mec1 functions upstream of Rad9, or where Rad9 is a cofactor for Mec1 function, cannot be excluded. All these models are compatible with genetic data demonstrating that overproduction of Rad9 cannot overcome defects observed in *mec1-1* cells (de la Torre-Ruiz *et al.*, 1998). Distinguishing between these models will require either the development of biochemical assays for Rad9 function or detailed phosphopeptide mapping followed by analysis of specific site-directed mutants.

We have attempted to address the functional significance of the Mec1/Tel1-regulated hyperphosphorylation of Rad9 after DNA damage by assessing whether some of the known checkpoint components are recruited to a Rad9 containing complex after DNA damage. Using Ni²⁺ affinity chromatography, and antibodies specific to Rad24, Rad17, Mec3 and Rad53 we have demonstrated that after DNA damage, phosphorylated Rad53 and histidine tagged Rad9 physically interact. However, we can find no evidence for any physical interaction between Rad9 and Rad24, Rad17 or Mec3 under any conditions examined. This latter point is consistent with epistasis and overexpression analyses which place *RAD9* and *RAD24/RAD17/MEC3* into two functionally distinct subgroups (Lydall and Weinert, 1995; Longhese *et al.*, 1997; de la Torre-Ruiz *et al.*, 1998). During preparation of this manuscript, a study using Rad53-GST pull-down and co-immunoprecipitation assays demonstrated interaction between plasmid-expressed Rad9 and Rad53 (Sun *et al.*, 1998). Our studies confirm this interaction using UV irradiation and direct biochemical fractionation of normally expressed Rad9 and Rad53. Furthermore, our study suggests that only a fraction of the total phosphorylated Rad53 is bound to the hyperphosphorylated Rad9, indicating that this interaction may be relatively transient and/or weak.

In contrast to rapidly growing asynchronous cells and cells arrested with either HU or nocodazole, damage-induced Rad9 hyperphosphorylation in G₁-arrested cells is dependent not only on *MEC1* (with no obvious requirement for *TEL1*), but also the *RAD24* epistasis group. This observation suggests the existence of additional pathways functioning outside G₁ that do not require the *RAD24* epistasis group. Nevertheless, it results in *MEC1/TEL1*-dependent Rad9 hyperphosphorylation. An S-phase-

specific pathway which responds to DNA damage and results in Rad53 phosphorylation (Navas *et al.*, 1996) is one candidate for this additional signal in asynchronous cells. In support of this possibility, Rad9 hyperphosphorylation after DNA damage was detected in *RAD24* epistasis group defective cells, blocked in S phase with HU (Figure 6). The appearance of Rad9 phosphorylated forms in HU-arrested *mec1-1* and *rad53K227A* cells, similar to those seen after DNA damage, suggests that these mutant cells are responding to DNA damage, possibly caused by the HU arrest itself. *MEC1* and *RAD53* are required for cell-cycle arrest in the presence of HU, and mutants in these genes undergo mitosis improperly (Allen *et al.*, 1994; Weinert *et al.*, 1994). Defects in replication arrest or in preventing mitosis under these conditions may cause DNA damage that in turn results in *RAD9* hyperphosphorylation via a Mec1-independent process. Further evidence that Rad9 hyperphosphorylation outside of G₁ can be independent of the *RAD24* epistasis group comes from examination of nocodazole-arrested cells. As with exponentially growing cells, the only checkpoint pathway dependency we observed for this phenotype in nocodazole-arrested cells was with *mec1-1 tel1Δ* cells.

The differences in DNA damage-dependent phosphorylation of Rad9 and Rad53 are interesting. Rad53 phosphorylation is much more dependent on *MEC1* than is Rad9 hyperphosphorylation. With the exception of G₁-arrested cells, Rad9 hyperphosphorylation requires either one of *MEC1* or *TEL1*. This raises the interesting speculation that Rad9 is a good target for the *TEL1* pathway (at least in the absence of *MEC1* function) whereas Rad53 is not. Interestingly, *rad9Δ* mutant cells have shorter telomeres than wild-type cells (unpublished observations). Furthermore, as Rad9 hyperphosphorylation is dependent on *MEC1* but not *TEL1* in G₁-arrested cells, either *TEL1* is not active in such cells or the hypophosphorylated G₁ form of Rad9 is not efficiently recognized by the *TEL1* pathway.

Implications from other systems

The only recognizable motifs in the Rad9 protein are tandem repeats of a C-terminal motif, the BRCT domain. This motif has been detected in various proteins with roles in DNA damage responses (Bork *et al.*, 1997; Callebaut and Morion, 1997) and has been shown to be involved in protein-protein interactions (Critchlow *et al.*, 1997; Nash *et al.*, 1997). In the fission yeast, the Crb2/Rhp9 protein contains two C-terminal BRCT repeats that are most closely related to the repeats found in Rad9 (Willson *et al.*, 1997; Saka *et al.*, 1998). Although sequence homology between Rad9 and Crb2/Rhp9 is minimal outside of the BRCT repeats, Crb2/Rhp9 is required for the DNA damage checkpoint but not the HU-dependent checkpoint, is modified during the cell cycle, and becomes hypermodified after DNA damage in a checkpoint pathway-dependent manner (Willson *et al.*, 1997; Saka *et al.*, 1998). These data suggest that fission yeast Crb2/Rhp9 might be functionally equivalent to budding yeast Rad9. BRCT motifs were originally defined in the human Brca1 protein (Koonin *et al.*, 1996), which is often mutated in familial breast and ovarian cancers (Futreal *et al.*, 1994; Miki *et al.*, 1994). Brca1 is regulated similarly to Rad9: it is phosphorylated in cells blocked in S phase and in

mitosis, and becomes hyperphosphorylated in the presence of DNA damage (Ruffner and Verma, 1997; Scully *et al.*, 1997). However, despite the similarities between Brca1 and Rad9 it is by no means clear that they are functional equivalents. Further examination of the roles of these proteins in the cellular responses to DNA damage will be required to establish their exact functional relationships.

Materials and methods

Yeast strains

All yeast strains used in this work are isogenic to W303-1a (*MATa ade2-1; trp1-1; leu2-3,112; his3-11,15; ura3*). The *rad17Δ*, *rad24Δ* and *mec3Δ* strains have been previously described (de la Torre-Ruiz *et al.*, 1998). The *ddc1Δ* strain (YLL244) was a gift from Maria Pia Longhese (Longhese *et al.*, 1997). The *mec1-1* and *tel1Δ* strains, termed N1-3A and N1-8A respectively, were obtained as *CDC*⁺ segregants from a cross between RGY39 (a W303 derivative of genotype: *Mata; tel1::URA3; mec1-1; ade2-1; trp1-1; leu2-3,112; his3-11,15; ura3; cdc13* and/or *cdc15*) and W303-1b. The *tel1Δ* mutation was scored by following the *URA3* marker and *mec1-1* by following HU and MMS sensitivity. The *rad53-11* and *dun1Δ* strains, termed N2-16C and N2-16B respectively, were obtained as *CDC*⁺ segregants from a cross between RGY204 (a W303 derivative of genotype: *Mata; dun1::HIS3; rad53-11; ade2-1; trp1-1; leu2-3,112; his3-11,15; ura3; cdc13* and/or *cdc15*; plus plasmid p2μTRP1RNR1) and W303-1b. The *dun1Δ* mutation was scored by following the *HIS3* marker and *rad53-11* by following HU and MMS sensitivity. The *mec1-1* and *rad53-11* strains were further confirmed by complementation. RGY39 and RGY204 were gifts from David Lydall and the *rad53K227A* strain was a gift from Marco Foiani.

Rad9 expression and purification from bacteria for production of polyclonal antibodies

The entire *RAD9* open reading frame was amplified by polymerase chain reaction (PCR) using primers corresponding to 5' (CGCGGATCCATGT-CAGGCCAGTTAGTTC) and 3' (CGCGGATCCTCTAACCTCAGAA-ATAGTGTTG) sequences of the open reading frame flanked by *Bam*HI restriction sites (shown in bold). The resulting PCR product was digested with *Bam*HI and *Sac*I endonucleases, and the resulting 2020 bp fragment corresponding to the N-terminal half of the Rad9 protein was cloned directionally into a *Bam*HI- and *Sac*I-digested pET21a bacterial plasmid (Novogen). This resulted in fusion of a T7 epitope to the N-terminal and six histidines to the C-terminus of the partial Rad9 sequence. This protein fusion was expressed in BL21(DE3) cells by IPTG induction and purified from bacteria by Ni²⁺-NTA column chromatography under denaturing conditions as recommended by the manufacturer (Qiagen). The purified product was used to immunise rabbits using standard procedures.

Cell-cycle arrests and DNA damage treatments

Cells grown to log phase in YPD medium were blocked in G₁ by addition of 20 μg/ml of α-factor for 150 min. S-phase blocks were accomplished by addition of 0.2 M HU to log phase cultures for 120 min. Cells were blocked in G₂ by addition of 5 mg/ml nocodazole for 120 min. Efficiency of arrest was verified by the absence of budded cells (<1%) in α-factor treated cultures and absence of unbudded cells (<5%) in HU and nocodazole-treated cultures. Damage treatments were performed as follows: γ-irradiation was carried out on log phase cultures in YPD medium as previously described (Aboussekhra *et al.*, 1996). MMS was added directly to log phase or arrested cultures. For UV irradiation experiments, cells were washed and resuspended in saline at a density of 0.5 × 10⁷ cells/ml, irradiated under a UV germicidal lamp (260 nm) and subsequently resuspended in fresh YPD medium. For cultures arrested prior to irradiation the final YPD medium contained the arresting agent at the same concentration as the starting culture. Large scale UV irradiation of cells: cells were grown to mid-log phase in 5 l of YPD, harvested and washed with saline. The cells were split into two, whereupon one half was UV irradiated (60 J/m² in a total volume of 800 ml of saline) and the other half was mock treated. The treated cells were pelleted and resuspended in 400 ml of prewarmed (30°C) YEPD and allowed to grow at 30°C for a further 45 min.

Yeast extract preparation and immunoblot analysis

Yeast extracts were prepared by glass bead beating in 20% trichloroacetic acid (TCA), washing the glass beads in 5% TCA and combining the

wash with the lysate. The protein suspension was pelleted, resuspended in 1× Laemmli loading buffer (pH 8.0), boiled for 2 min, pelleted, and the supernatant retained as whole-cell extract. Native yeast protein extracts were prepared for chromatography as follows: cell pellets were washed with de-ionised sterile water and the compacted cell pellet passed through a syringe into liquid nitrogen. The frozen cell pellet was ground in the continuous presence of dry ice in a coffee grinder until at least 70% of the cells were lysed as judged by microscopy. A volume of frozen 2× lysis buffer (100 mM HEPES/KOH pH 7.5, 600 mM potassium acetate, 10 mM magnesium acetate, 1 mM EDTA, 20% glycerol, 8 mM β-mercaptoethanol, 5mM imidazole, 0.1 mM phenyl methyl sulfonate fluoride, 0.6 mM leupeptin, 2 mM pepstatin A, 2 mM benzamidine) equal to that of the starting cell pellet was added and mixed with the lysed cells by brief grinding. The dry ice was allowed to sublime and the thawed extracts clarified by spinning at 20 000 r.p.m. for 20 min in an SS34 rotor (Sorvall), aliquoted and stored at −80°C. For Western blotting, proteins were separated on 6.5% SDS–PAGE containing an acrylamide to bis-acrylamide ratio of 80:1 (to improve resolution of phosphorylated forms of Rad9) and transferred to nitrocellulose membranes (Amersham) by semi-dry electroblotting (Bio-Rad). Polyclonal antiserum directed against the Rad9 protein was incubated with the nitrocellulose membranes at a 1:10 000 dilution in PBS containing 0.2% Tween-20 and 0.5% milk for 2–16 h. Secondary horse radish peroxidase, conjugated anti-rabbit antibody (Sigma) was incubated with the membranes for 30 min to 1 h at a 1:10 000 dilution and the blot revealed by chemiluminescence (Amersham). Rad53 immunoblots were carried out using the same conditions as described for detection of the Rad9 protein on filters previously probed with anti-Rad9 immune serum. The generation of anti-Rad53 immune sera has been previously described (de la Torre-Ruiz *et al.*, 1998).

Dephosphorylation assay

Whole-cell extracts were prepared by glass bead beating in a non-denaturing lysis buffer (150 mM KCl, 50 mM Tris–HCl pH 8.0, 20% glycerol, 0.1 mM phenyl methyl sulfonate fluoride, 0.6 mM leupeptin, 2 mM pepstatin A, 2 mM benzamidine). The dephosphorylation assays were carried out by incubating 10 µg of whole-cell extract in 1× λ protein phosphatase buffer (50 mM Tris–HCl pH 7.5, 0.1 mM EDTA, 5 mM DTT, 2 mM MnCl₂) containing 80 U of λ protein phosphatase (New England Biolabs) for 20 min at 30°C. Some reactions contained the phosphatase inhibitor sodium orthovanadate (Sigma) at 2.5 mM final concentration. Phosphatase reactions were stopped by adding an equal volume of 2× Laemmli sample buffer (125 mM Tris–HCl pH 6.8, 4% SDS, 10% β-mercaptoethanol, 20% glycerol, 0.002% bromophenol blue) and incubation at 95°C for 2 min.

Northern blots

Preparation of RNA, probes and Northern blotting have been described elsewhere (Aboussekhra *et al.*, 1996).

Ni²⁺ agarose chromatography

Fifteen milligrams of extract in lysis buffer plus 5 mM imidazole was bound to 100 µL of Ni²⁺-NTA superflow agarose beads (Qiagen) at 4°C for 1 h with constant rotation. The extract plus beads were loaded onto a small disposable column and unbound proteins were allowed to gravity flow through the column. After extensive washing (40 column volumes, of which the first three were allowed to equilibrate with the beads for 15 min before collection) with lysis buffer containing 20 mM imidazole, proteins were eluted with lysis buffer containing 250 mM imidazole in eight, one column volume steps (the second, third and fourth column volumes were allowed to equilibrate with the beads for 15 min each prior to collection).

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