Mutant alleles of *Schizosaccharomyces pombe rad9*⁺ alter hydroxyurea resistance, radioresistance and checkpoint control

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ABSTRACT

Schizosaccharomyces pombe rad9 mutations can render cells sensitive to hydroxyurea (HU), gamma-rays and UV light and eliminate associated checkpoint controls. *In vitro* mutagenesis was performed on *S.pombe rad9* and altered alleles were transplanted into the genome to ascertain the functional significance of five groups of evolutionarily conserved amino acids. Most targeted regions were changed to alanines, whereas *rad9-S3* encodes a protein devoid of 22 amino acids normally present in yeast but absent from mammalian Rad9 proteins. We examined whether these *rad9* alleles confer radiation and HU sensitivity and whether the sensitivities correlate with checkpoint control deficiencies. One *rad9* mutant allele was fully active, whereas four others demonstrated partial loss of function. *rad9-S1*, which contains alterations in a BH3-like domain, conferred HU resistance but increased sensitivity to gamma-rays and UV light, without affecting checkpoint controls. *rad9-S2* reduced gamma-ray sensitivity marginally, without altering other phenotypes. Two alleles, *rad9-S4* and *rad9-S5*, reduced HU sensitivity, radiosensitivity and caused aberrant checkpoint function. HU-induced checkpoint control could not be uncoupled from drug resistance. These results establish unique as well as overlapping functional domains within Rad9p and provide evidence that requirements of the protein for promoting resistance to radiation and HU are not identical.

INTRODUCTION

Cell cycle checkpoints are mechanisms that transiently delay cell cycle progression when DNA is damaged or DNA replication is incomplete (1–3). In the fission yeast *Schizosaccharomyces pombe*, a group of six such checkpoint control genes have been identified and include *rad1*⁺, *rad3*⁺, *rad9*⁺, *rad17*⁺, *rad26*⁺ and *hus1*⁺ (4–7). Mutations in any one of these genes render cells sensitive to gamma-rays, UV light or the DNA synthesis inhibitor hydroxyurea (HU) and eliminate the ability of cells to delay entry into mitosis after treatment with these agents. All

of these genes apparently link abnormal DNA structures to cell cycle control (8,9).

As for cell cycle-related genes in general, these checkpoint control genes are highly conserved throughout evolution. Human and mouse versions of several of the *S.pombe* genes have been isolated, providing strong evidence that checkpoint control mechanisms are also highly conserved (10–16). In mammals, these genes are thought to maintain genomic stability, especially in the presence of DNA damage (17,18). Therefore, when these genes are altered, genomic instability may occur and lead to cancer (19).

The biochemical activities of most of the checkpoint control gene products are not well established, although progress has been made towards learning more about their function. For example, examination of the structure of the protein encoded by human or *S.pombe rad9* reveals a BH3-like domain in the N-terminal region that can bind the anti-apoptotic proteins Bcl-2 and Bcl-X_L (20,21). Furthermore, overexpression of the gene from either organism in human cells can cause apoptosis in a BH3 domain-dependent manner. Both *S.pombe* and human versions of the protein can bind two other checkpoint control proteins, Hus1p (HUS1p) and Rad1p (RAD1p) (22–26). Human RAD9 protein binds HUS1 and RAD1 proteins at its C-terminal region, suggesting that RAD9 has at least two functional domains, one involved in apoptosis and the other in cell cycle checkpoint control (25). Interestingly, Rad1p, Rad9p and Hus1p show structural similarity with the DNA replication and repair protein PCNA, as judged by computer analysis, and may form a heterotrimer for function (26–29). In addition, Bessho and Sancar (30) found that the human RAD9 protein has an intrinsic 3'→5' exonuclease activity, despite the lack of obvious structural motifs usually indicative of this kind of function. Therefore, although it is clear that the fission yeast Rad9p protein plays an important role in promoting radioresistance, chemoresistance and controlling cell cycle progression when DNA damage is incurred or DNA replication is incomplete, the precise biochemical activities involved and the important, functionally-relevant structural features of this protein have not been established directly.

A structure–function analysis of Rad9p was used to identify domains of the protein important for activity and to learn more about the mechanistic relationships among Rad9p-mediated processes. *In vitro* mutagenesis was performed on five regions of the *S.pombe* protein (31,32) that are either conserved in cognate proteins from three other organisms, *Schizosaccharomyces*

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octosporus (33), *Homo sapiens* (13) and *Mus musculus* (34) or have other interesting features like similarity to an apoptosis-related BH3 domain (21). These altered forms of Rad9p were transplanted as a single copy into the *S.pombe* genome and assessed for the ability to promote resistance to gamma-rays, UV light and HU, as well as checkpoint controls. The results indicate the presence of multi-functional as well as DNA damaging agent response-specific domains in the Rad9p protein and are presented in the context of their implications for understanding cell survival-promoting mechanisms enacted after exposure to radiations or chemicals that damage DNA or block DNA replication.

MATERIALS AND METHODS

Yeast and bacterial strains and growth conditions

The *S.pombe* strains Sp348 (*h⁻ ade6-216 leu1-32 ura4-294*), Sp349 (*h⁻ rad9::ura4⁺ ade6-216 leu1-32 ura4-294*) and derivatives of the latter containing *rad9* alleles created as part of this study, in place of *rad9::ura4⁺*, were used in this investigation. Conditions for growth were as described previously (35–37). Cells were grown routinely in YEA liquid (YE supplemented with adenine at 75 µg/ml) or on YEA agar (YEA liquid with 2.0% agar). Minimal MB liquid medium supplemented with adenine and required amino acids at 75 µg/ml or solid MB (liquid MB with 2.0% agar) was also used as indicated. 5-fluoroorotic acid (5-FOA) was added at a concentration of 0.1% to media when needed to select *ura4⁻* cells from a *rad9::ura4⁺* population during transplacement of *rad9* mutant alleles into the genome (38). Plasmid DNA was introduced into *S.pombe* using a standard transformation protocol, as described by Okazaki *et al.* (39). Established transformation procedures (40) were used to introduce plasmids into *Escherichia coli* DH5α for DNA amplification and subsequent preparation. *Escherichia coli* were grown at 37°C in liquid or on agar YT medium, supplemented with ampicillin (100 µg/ml) as needed to select for cells harboring plasmids (40).

DNA manipulation and analysis

Standard procedures were used for preparing, digesting, subcloning and PCR amplifying DNA (40). Enzymes that

modify DNA were used according to the manufacturer's instructions (New England BioLabs, Beverly, MA).

The dideoxy chain termination method (41), in conjunction with DNA primers corresponding to vector sequences or internal regions of *rad9*, were used to determine the sequences of the gene. DNA sequences were translated and compared using the Genetics Computer Group (Madison, WI) programs PILEUP and PRETTY/CONSENSUS (42).

Site-directed mutagenesis

Targeted *in vitro* mutagenesis of *S.pombe rad9⁺* coding regions was performed by a two-step PCR-based procedure, essentially as described previously (43). For each construct, two primers were chosen that border the DNA fragment within which a mutation is to be created. These primers were designed so that the final amplified fragment will contain two unique restriction enzyme cutting sites, one upstream and the other downstream of the mutation. A third primer contains modified base pair(s) of interest in its central region, corresponding to the final mutation chosen for construction. The first PCR reaction produces a DNA fragment as a megaprimer by using one flanking primer and the base-modified primer. The megaprimer is then paired with another flanking primer in a second PCR reaction to make a mutant DNA fragment. The mutated *rad9* gene is made by replacing the normal fragment with the mutated fragment, using unique restriction sites and standard subcloning methodologies. To make *rad9* mutant genes, a 2.6 kb *rad9⁺*-containing genomic DNA fragment between unique restriction sites *Hind*III and *Eco*RV (32) was first blunt-ended, then subcloned into the *Xba*I site within pUC19. *In vitro* mutagenesis was verified by DNA sequence analysis. Table 1 describes the primers used and mutations constructed.

Transplacement of mutant alleles into the genome

Mutant alleles of *rad9* made in the genomic version of the gene by *in vitro* mutagenesis were isolated on a 1923 bp *Hae*III DNA fragment and used to transform *S.pombe rad9::ura4⁺* cells. Transformants were plated on medium containing 5-FOA to select for *Ura⁻* cells that had replaced the *rad9::ura4⁺* allele with the allele created *in vitro* (38). PCR as well as growth properties on medium containing or devoid of uracil were used to assess transplacement events.

Table 1. Primers used for site-directed mutagenesis to construct *rad9* mutant alleles

Allele	Flanking primers ^a	Restriction sites	Modified primers ^b
<i>rad9</i> -S1	U: 5'-TCG TTA TTC GTT CAG CAT TC-3'; D: 5'-ACA GAT AGT ATG GGC TTG AC-3'	<i>Pvu</i> II, <i>Sp</i> II	5'-AAT TTC CCA GTT GAC AGC AgC ATC GgC Tgc Agc Agc ATT TGT AAA GAT CCT TGC-3' (antisense)
<i>rad9</i> -S2	U: 5'-TCG TTA TTC GTT CAG CAT TC-3'; D: 5'-ACA GAT AGT ATG GGC TTG AC-3'	<i>Pvu</i> II, <i>Sp</i> II	5'-GT CAC CAT GCT AAA TCC TGc Cgc Agc AGc Agc TAA ACA TGT AAT CTC TAT C-3' (antisense)
<i>rad9</i> -S3	U: 5'-ACT AGG ATT TAG CAT GGT GAC-3'; D: 5'-AAT CGA GCT TCC CAA TGC CT-3'	<i>Sp</i> II, <i>Mlu</i> I	5'-CAC ATC TTT TCT GCT TGC AGA TCT AAA CAC AGA TAG TAT GGG-3' (antisense)
<i>rad9</i> -S4	U: 5'-ACT AGG ATT TAG CAT GGT GAC-3'; D: 5'-AAT CGA GCT TCC CAA TGC CT-3'	<i>Sp</i> II, <i>Mlu</i> I	5'-AGG ATT ATA TTT AAA TTC TTA gcC gcG CAC GlcA GcG ATT gcA gCA TAT AAA ATA TCA TAT GAA-3' (sense)
<i>rad9</i> -S5	U: 5'-AAG TTT CAC AGA AGA GGT CG-3'; D: 5'-AAT TTT GCA TTC AGA TGA AAA TGC-3'	<i>Mlu</i> I, <i>Dsa</i> I	5'-CTG CTA AAA TGA CGG CAG Cag cAg cTg CAC GTA GAG AAA GGG TAA CAG-3' (antisense)

^aU and D, sequences upstream and downstream from site-directed mutation, respectively.

^bLower and upper case letters, modified and unmodified nucleotides, respectively; sense and antisense, polarity of primer relative to coding DNA sequence of gene.

[illegible]

Figure 1. Comparison of the amino acid sequences of *S.pombe* Rad9p with related proteins from *S.octosporus* (33), *M.musculus* (34) and *H.sapiens* (13). S1–S5 show sites containing amino acids that are evolutionarily conserved and that have been targeted for mutagenesis. Amino acids altered within each site are underlined.

HU resistance and HU-induced checkpoint control

HU resistance was determined essentially as described previously (44). Cells were grown in liquid YEA, either containing 10 mM HU or devoid of the drug, and incubated at 30°C while shaking. Initially and at the indicated times, aliquots of cells were removed, diluted and plated on YEA agar. Plates were incubated at 30°C for 3–5 days, followed by tabulation of colony number. Resistance was expressed as colonies appearing on plates after the indicated HU treatment times, relative to the time just before the drug was added to media.

For detection of delays in cell cycle progression after exposure to HU, changes in the percentage of cells in a population bearing a septum (i.e. septation index) were monitored (44). Cells were synchronized in the late S or early G₂ phase of the cell cycle using a lactose gradient, as originally described by Mitchison and Carter (45) with modifications (44). An increase in septation frequency after HU treatment reflects progression past the HU-induced S phase checkpoint and subsequent entry into mitosis. After the addition of 10 mM HU to a culture of synchronously dividing, mid-logarithmic phase cells grown in YEA liquid, aliquots were removed at the indicated times and examined under the microscope at 150× magnification for cell number and at 600× magnification to monitor the percentage of the population containing septa.

Gamma-ray and UV resistance and radiation-induced checkpoint control

Procedures used to determine cell survival after exposure to gamma-rays or 254 nm UV light were as described previously (13,44). Cells in the mid-logarithmic phase of growth in YEA liquid medium were diluted, plated on YEA agar, then mock-

treated or exposed to the doses indicated. Plates were then incubated for 3–5 days at 30°C. Percentage survival was calculated relative to unirradiated controls.

Synchronized cells, as described for monitoring HU-induced checkpoint control, were used to assess changes in cell cycle progression caused by exposure to gamma-rays or UV light. For ionizing radiation, cells were exposed to 250 Gy of gamma-rays and septation index was examined at the times indicated post-irradiation. For UV, synchronized cells removed from the lactose gradient were washed and resuspended in H₂O, exposed to 50 J/m², resuspended in liquid YEA medium and monitored for changes in the percentage of cells in the population bearing a septum.

RESULTS

Construction of *rad9* mutant alleles

In vitro mutagenesis was performed to begin to assess the biological significance of specific amino acid sequences within the *S.pombe rad9⁺* gene product that are either conserved evolutionarily or exhibit other interesting features. Figure 1 illustrates a comparison of the amino acid sequences within Rad9p from *S.pombe* and related proteins from *S.octosporus*, *M.musculus* and *H.sapiens*. Based on this comparison, five sites (S) were targeted for analysis. S1, S2, S4 and S5 are sites that contain multiple amino acids present in those positions in all forms of the protein. These amino acids were changed to alanines, which are relatively small and uncharged. S2 is the most conserved target for study and consists of the sequence Asn-Ser-Ser-Arg-Ser. S3 is different since it consists of

22 amino acid residues present in the two yeast forms of Rad9p but absent from both mammalian cognates. To determine the biological significance of this region, the entire group of amino acids was deleted and the corresponding mutant allele encoding this protein is called *rad9-S3*. All of these mutant alleles of the *rad9⁺* gene were transplanted into the genome of *S.pombe* cells to analyze their function as a single copy gene substituting for the wild-type gene in its normal chromosomal location.

Sensitivity of cells to HU and HU-induced cell cycle checkpoint control

Schizosaccharomyces pombe rad9::ura4⁺ cells are very sensitive to the DNA synthesis inhibitor HU (4,7,44). To define regions of the protein important for mediating resistance to this drug, cells containing individual mutant *rad9* alleles were assessed for HU sensitivity (Fig. 2). *rad9-S1*, *rad9-S2* and *rad9-S3* conferred essentially wild-type levels of HU resistance upon *S.pombe* cells, suggesting that the altered sites within these versions of *rad9* are not important for promoting drug resistance. In contrast, cells expressing *rad9-S4* or *rad9-S5* demonstrated HU sensitivity at a level intermediate between those containing *rad9⁺* or *rad9::ura4⁺*, indicating that these sites are critical for resistance to the drug.

Schizosaccharomyces pombe rad9⁺ cells transiently delay cycling in early S phase when treated with HU (4,7). Cells containing *rad9::ura4⁺* are devoid of this cell cycle checkpoint, which normally delays the onset of mitosis and promotes survival after drug treatment. Figure 3 illustrates the effects of expression of the *rad9* mutant alleles on this checkpoint in *S.pombe* cells. There is essentially a perfect correlation between the ability of mutant alleles to confer HU resistance (Fig. 2) and HU-induced checkpoint control. *rad9-S1*, *rad9-S2* and *rad9-S3* conferred the ability to delay cell cycle progression upon *S.pombe* cells after HU treatment, therefore indicating that the corresponding unaltered amino acids are not important for this function. Mutant cells expressing *rad9-S4* or *rad9-S5* were markedly aberrant in the HU-induced delay and were sensitive to HU. However, interestingly, cells containing either of these mutant alleles showed an intermediate delay response. Unlike the *rad9::ura4⁺* cell population, those containing *rad9-S4* or *rad9-S5* did not immediately accumulate septa at a high frequency, but instead showed a small increase then a reduction in septation index, like the *rad9⁺* strain, after HU treatment before this morphological trait appeared in a significant fraction of the population. These results indicate that the *rad9-S4* and *rad9-S5* mutant cells probably enter the checkpoint but fail to maintain the arrest. These results also imply that the wild-type versions of the mutagenized sites in *rad9-S4* and *rad9-S5* are very important for the DNA replication checkpoint. This relationship between drug resistance and induced cell cycle delays indicates that the two functions are encoded within the same regions of Rad9p. Furthermore, it suggests that Rad9p promotes HU resistance by controlling cell cycle progression. However, these results are also consistent with Rad9p being important for an early step in HU-induced signal transduction (i.e. increased transcription of genes encoding subunits of ribonucleotide reductase, for example), with the cell cycle delay response playing only a minimal role in mediating drug resistance. No difference in septation index was observed among the synchronized, untreated cell populations (data not shown).

Figure 2. Sensitivity of cells to HU. Survival was measured after incubation in medium containing 10 mM HU for the indicated times (see Materials and Methods). Cells contained *rad9⁺* (open circle), *rad9::ura4⁺* (closed circle) or the *rad9* alleles generated in this study. (A) *rad9-S1* (open triangle); (B) *rad9-S2* (open square); (C) *rad9-S3* (open diamond); (D) *rad9-S4* (closed triangle); (E) *rad9-S5* (closed square). Points represent the average of at least three independent experiments. Error bars indicate standard deviation.

Figure 3. HU-induced checkpoint control. Septation index, as a reflection of cell cycle progression, was measured after incubation of cells in medium containing 10 mM HU for the indicated times (see Materials and Methods). See Figure 2 legend for a description of symbols. Points represent the average of at least three independent experiments. Error bars indicate standard deviation.

Sensitivity of cells to gamma-rays and gamma-ray-induced checkpoint control

Schizosaccharomyces pombe cells containing *rad9::ura4⁺* are sensitive to gamma-rays (4,7,44). To define functional domains within Rad9p that promote ionizing radiation resistance, cells bearing individual *rad9* mutant alleles were assessed for survival after exposure to this DNA damaging agent. As shown in Figure 4, all of the mutant alleles adversely affected radio-resistance, but to different degrees. Cells expressing *rad9-S3* were marginally but significantly more gamma-ray-sensitive than the wild-type strain only at the highest dose examined, 1500 Gy. Therefore, the effect of this mutant allele on ionizing radiation resistance is minimal. Expression of *rad9-S1* and *rad9-S2*, on the other hand, made *S.pombe* cells gamma-ray-sensitive, relative to *rad9⁺* cells at all doses tested, although the mutants

Figure 4. Sensitivity of cells to gamma-rays. Survival was measured after exposure to the indicated doses of gamma-rays (see Materials and Methods). See Figure 2 legend for a description of symbols. Points represent the average of at least three independent experiments. Error bars indicate standard deviation.

Figure 5. Gamma-ray-induced checkpoint control. Septation index, as a reflection of cell cycle progression, was measured after exposure of cells to 250 Gy of gamma-rays (see Materials and Methods). See Figure 2 legend for a description of symbols. Points represent the average of at least three independent experiments. Error bars indicate standard deviation.

were never more than an order of magnitude more sensitive at any dose tested. Cells containing *rad9*-S4 or *rad9*-S5 were very sensitive to gamma-rays, relative to *rad9*⁺ cells. This indicates that all of the targeted groups of amino acids play a role in promoting resistance to this type of radiation, but some have a more critical function than others.

Gamma-ray-induced checkpoint control was also monitored in cells containing the *rad9* mutant alleles engineered *in vitro*, as another indicator of Rad9p function. As shown in Figure 5, *S.pombe* cells containing *rad9*-S1, *rad9*-S2 or *rad9*-S3 demonstrated cell cycle delays, as reflected by an altered septation index, after exposure to 250 Gy of gamma-rays that were indistinguishable from the response of *rad9*⁺ cells. The gamma-ray sensitivity of these cells (Fig. 4) was therefore not due to aberrant checkpoint control but rather to a defect in some other mechanism. In contrast, *rad9*-S4 and *rad9*-S5 mutant populations delayed the accumulation of cells bearing a septum up to 1 h after the initial treatment with gamma-rays.

Figure 6. Sensitivity of cells to UV light. Survival was measured after exposure to the indicated fluence of 254 nm UV light (see Materials and Methods). See Figure 2 legend for a description of symbols. Points represent the average of at least three independent experiments. Error bars indicate standard deviation.

However, 1–2 h post-treatment, these mutant populations showed a significant increase in the frequency of septum-bearing cells. After an initial small increase in septation index for *rad9*⁺ cells, the numbers remained low until 2–3 h post-irradiation. Therefore, *rad9*-S4 and *rad9*-S5 mutant populations demonstrated an aberrant cell cycle delay response to gamma-ray exposure that was significant but not as severe as the defect observed in the *rad9::ura4*⁺ cell population. These results suggest that after gamma-ray exposure, the *rad9*-S4 and *rad9*-S5 mutant cells may initiate but have trouble maintaining the checkpoint.

The ability of *rad9* mutant alleles to confer HU resistance versus gamma-ray resistance upon *S.pombe* cells did not correlate completely. For example, *rad9*-S1, *rad9*-S2 and *rad9*-S3 cells demonstrated wild-type HU resistance, but varying degrees of ionizing radiation sensitivity. Also, cells containing *rad9*-S4 or *rad9*-S5 were sensitive to both agents. Therefore, Rad9p has functional domains that are either unique or overlapping with respect to maintaining cell survival after exposure to HU or gamma-rays.

Sensitivity of cells to UV light and UV-induced checkpoint control

Schizosaccharomyces pombe rad9::ura4⁺ cells are also sensitive to UV light, relative to cells containing *rad9*⁺ (4,7,44). The UV sensitivity of cells housing the series of *rad9* alleles made as part of this study was determined to identify amino acids important for modulating resistance to this type of non-ionizing radiation. As illustrated in Figure 6, the patterns of UV resistance were almost identical to those achieved after analysis of gamma-ray resistance (Fig. 4). Mutant alleles *rad9*-S4 and *rad9*-S5 made cells the most sensitive to UV light. *rad9*-S2 marginally decreased UV resistance, relative to *rad9*⁺ cells, but the differences are statistically insignificant. This was judged by calculating standard deviations, as illustrated in Figure 6, as well as determining *P* values [i.e. when comparing UV sensitivity of *rad9*⁺ versus *rad9*-S2 cell survival at 50 J/m² (*P* = 0.68), 100 J/m² (*P* = 0.85) or 150 J/m² (*P* = 0.12)]. *rad9*-S3 conferred essentially wild-type levels of UV resistance upon cells, whereas *rad9*-S1 made cells given 100 or 150 J/m² of UV an

Table 2. Ability of *rad9* alleles to function in *S. pombe* cells^a

<i>rad9</i> allele	Gamma-ray resistance	UV light resistance	HU resistance	HU, Gamma-ray, or UV-induced checkpoint
<i>rad9</i> ⁺	++++	++++	++++	++++
<i>rad9::ura4</i> ⁺	—	—	—	—
<i>rad9</i> -S1	+++	+++	++++	++++
<i>rad9</i> -S2	+++	++++	++++	++++
<i>rad9</i> -S3	++++	++++	++++	++++
<i>rad9</i> -S4	+	+	++	++
<i>rad9</i> -S5	+	+	++	++

^a++++, wild-type ability; —, *rad9::ura4*⁺ ability; +++, ++, +, intermediate ability whereby the more '+' the greater the ability.

Figure 7. UV-induced checkpoint control. Septation index, as a reflection of cell cycle progression, was measured after exposure of cells to 50 J/m² of UV light (see Materials and Methods). See Figure 2 legend for a description of symbols. Points represent the average of at least three independent experiments. Error bars indicate standard deviation.

order of magnitude more sensitive than the *rad9*⁺ strain. These results indicate that many groups of amino acids within the protein are important for enhancing cell survival after UV as well as gamma-ray exposure. In addition, the sequences important for conferring UV or HU resistance often but do not always overlap.

Schizosaccharomyces pombe rad9::ura4⁺ cells are unable to delay progression into mitosis after exposure to UV light. The ability of 50 J/m² of UV to induce this delay response in cells containing the mutant alleles of *rad9* made in this study was examined. As illustrated in Figure 7, UV-irradiated *rad9*-S1, *rad9*-S2 and *rad9*-S3 cells show a septation index pattern similar to *rad9*⁺ cells post-UV exposure, indicating that they are not defective in this checkpoint response. However, mutants containing *rad9*-S4 or *rad9*-S5 show a delay profile intermediate between those observed for *rad9*⁺ and *rad9::ura4*⁺ cells, suggesting that the corresponding amino acids found in the wild-type Rad9 protein are important for UV-induced cell cycle checkpoint control. As for treatment with HU or gamma-rays, *rad9*-S4 or *rad9*-S5 mutants exposed to UV appear to initiate but fail to maintain a cell cycle delay.

Table 2 summarizes the effects of the *S. pombe rad9* mutant alleles on radioresistance, HU resistance and the associated,

inducible cell cycle delays. Included are data for cells containing *rad9*⁺ and *rad9::ura4*⁺ to serve as controls.

Visualization of DAPI-stained cells exposed to HU, gamma-rays or UV

To better understand the effects of HU, gamma-rays or UV on cell cycle progression, we examined *S. pombe rad9*⁺, *rad9::ura4*⁺, *rad9*-S1, *rad9*-S2, *rad9*-S3, *rad9*-S4 and *rad9*-S5 cells before or after exposure to these agents by DAPI-staining and fluorescence microscopy. The results are shown in Figure 8. However, since *rad9*-S1, *rad9*-S2 and *rad9*-S3 cells were indistinguishable morphologically from those containing *rad9*⁺ at all the times and conditions indicated in Figure 8, except that initially they were somewhat smaller, only cells with the wild-type allele are presented to represent this group.

All of the cell populations examined looked similar just before treatment, except *S. pombe rad9*⁺ cells were somewhat larger (Fig. 8A, E, I and M). After 6 h in 10 mM HU, *rad9*⁺ cells appear elongated, their DNA looks intact and cells with a 'cut' phenotype, wherein a septum bisects the nucleus, are not observed. In contrast, *rad9::ura4*⁺ cells are smaller, some appear to have divided and their DNA is often not distributed equally between progeny and many 'cuts' are present. At this time after HU treatment the septation index of *rad9*-S4 and *rad9*-S5 cells reach the level found in the *rad9::ura4*⁺ population (Fig. 3D and E). Interestingly, *rad9*-S5 cells at this 6 h point rarely demonstrate 'cuts' and their DNA appears intact. Also, they are very elongated, thus retaining these morphological features of a cell cycle delay despite the dramatic increase in the frequency of septum appearing in the population and indicative of a failure to express checkpoint control. Therefore, the *rad9*-S5 mutation splits expression of at least two phenotypes associated with HU-induced checkpoint control, cell elongation and septum formation. Cells containing *rad9*-S4, on the other hand, demonstrate a more dramatic mutant phenotype. Although they appear somewhat more elongated than *rad9::ura4*⁺ cells treated the same way, the *rad9*-S4 population is, in general, morphologically similar to the *rad9::ura4*⁺ cells since they have a high frequency of 'cuts' and often demonstrate an unequal distribution of DNA between putative progeny. After 3 h post-treatment with HU, when the septation index of *rad9*-S4 and *rad9*-S5 are equivalent to the *rad9*⁺ strain (Fig. 3D and E), their morphology appears wild-type (data not shown).

Figure 8. Visualization of DAPI-stained cells. Cells were treated as indicated in Materials and Methods and in Figures 3, 5 and 7, to prepare for the analysis of septation index as a measure of checkpoint control. Then, aliquots of cells were placed on glass slides, heat-fixed, stained by adding a drop of DAPI solution (1.5 $\mu\text{g/ml}$) and visualized by fluorescence microscopy. Strains: *rad9*⁺, (A, B, C and D); *rad9::ura4*⁺, (E, F, G and H); *rad9-S4*, (I, J, K and L); *rad9-S5*, (M, N, O and P). Just before treatment, A, E, I, M; 6 h in 10 mM HU, B, F, J, N; 2 h after 250 Gy gamma-rays, C, G, K, O; 3 h after 50 J/m² of UV, D, H, L, P. Several panels are composites of multiple fields. *S.pombe rad9-S1*, *rad9-S2* and *rad9-S3* cells were slightly smaller on average but otherwise indistinguishable from the *rad9*⁺ population under all conditions indicated.

Cells were also examined 2 h after exposure to 250 Gy of gamma-rays (Fig. 8C, G, K and O), when the septation index of the *rad9-S4* and *rad9-S5* populations reached the level demonstrated by *rad9::ura4*⁺ cells (Fig. 5D and E). *rad9*⁺ cells contained no 'cuts' at this time. Cells containing *rad9::ura4*⁺ were not elongated, on average relative to untreated cells and frequently had septum bisecting their nuclei. Cells containing the *rad9-S5* allele looked similar to those bearing *rad9*⁺. *rad9-S4* mutant cells were longer than those containing *rad9::ura4*⁺ and they often demonstrated an asymmetrical distribution of DNA or DNA streaks along their length. Therefore, as for HU treatment, the *rad9-S4* and *rad9-S5* mutations split checkpoint-related phenotypes, namely the delay in septum formation and cell elongation. However, the dissociation was more dramatic for cells containing *rad9-S5*. At 1 h post-treatment, when the *rad9::ura4*⁺ cell population has a high septation index and all other cells have a reduction in the frequency of septated cells (Fig. 5D and E), the morphology of *rad9*⁺, *rad9-S4* and *rad9-S5* cells are the same (data not shown). Furthermore, at 6 h post-irradiation, *rad9*⁺ cells are very elongated and their DNA appears intact, much like *rad9-S3* and *rad9-S5* cells, while *rad9::ura4*⁺ cells are small and many 'cuts' are observed (data not shown). At this time, *rad9-S1* and *rad9-S2* appear normal but are not as elongated as the *rad9*⁺ cells. Furthermore, defects in the *rad9-S4* population become more exaggerated since 'cuts' appear and cells do not demonstrate wild-type levels of elongation. Therefore, for gamma-ray exposure, defects in *rad9-S1*, *rad9-S2* and *rad9-S4* are observed.

Cells were stained with DAPI and visualized by fluorescence microscopy also after exposure to 50 J/m² of UV light (Fig. 8D, H, L and P). Three hours after treatment, *rad9*⁺ cells were not appreciably elongated, nor were 'cuts' visible. The *rad9::ura4*⁺ cell population demonstrated a high frequency of smeared DNA that was unequally distributed throughout individual cells. At this time, the septation index for *rad9-S4* and *rad9-S5* approached the level observed for *rad9::ura4*⁺ cells (Fig. 7D and E). However, *rad9-S5* cells were morphologically similar to the wild-type strain, indicating that this mutant allele retains some but not all functions related to the UV-induced cell cycle checkpoint (i.e. septum formation is not delayed but DNA remains intact). *rad9-S4* cells appeared similar to the *rad9::ura4*⁺ mutant population. At 2 h post-UV exposure, when the septation index for *rad9-S4* and *rad9-S5* cells is reduced to normal levels and the frequency of septated cells in the *rad9::ura4*⁺ population is increasing (Fig. 7D and E), the morphology of *rad9*⁺, *rad9-S4* and *rad9-S5* cells is essentially identical (data not shown). Interestingly, after 5 h post-UV treatment, *rad9-S5* cells were elongated, like *rad9*⁺ cells, but *rad9-S4* did not (data not shown).

In summary, the results in Figures 3, 5, 7 and 8, as well as additional observations of DAPI-stained cells after treatment, indicate that *rad9-S1*, *rad9-S2* and *rad9-S3* retain checkpoint control activity induced by HU or UV, but the former two show some defects at 6 h after treatment with gamma-rays. However, *rad9-S4* and *rad9-S5* likely initiate cell cycle arrest in response to all three agents but fail to maintain the checkpoint.

Furthermore, *rad9*-S5 in particular, and to some extent also *rad9*-S4, retain a subset of checkpoint-related activities, in contrast to *rad9::ura4⁺* cells where all such functions are lost.

DISCUSSION

The *rad9* gene of the fission yeast *S.pombe* participates in mechanisms that promote radioresistance or HU resistance and mediate the associated checkpoint controls. However, little is understood about the domains of Rad9p important for its known biological functions. Therefore, structure–function studies were performed with this fission yeast gene to identify amino acid sequences of the protein critical for its activities. Five independent regions were targeted for *in vitro* mutagenesis studies. The choice was based on the evolutionary conservation of the positions of the amino acids within *S.pombe* Rad9p (31,32) relative to the cognate proteins from *S.octosporus* (33), *H.sapiens* (13) and *M.musculus* (34) demonstrated previously to be capable of at least partially complementing *rad9::ura4⁺* mutant phenotypes. Genes encoding proteins structurally similar to *S.pombe* Rad9p have also been identified from *Arabidopsis thaliana* (27), *Caenorhabditis elegans* (27), *Drosophila melanogaster* (46) and *Saccharomyces cerevisiae* (47) but functional equivalence has not yet been demonstrated. Nevertheless, each of the regions chosen for this investigation is at least partially conserved in most of these other putative versions of Rad9. Interestingly, *S.pombe rad9*-S2 retains all tested functions expected of *rad9⁺*, except demonstrates only a marginal although statistically significant reduction in gamma-ray resistance [as judged by standard deviation calculations illustrated in Figure 4 and *P* values when comparing the survival of *rad9⁺* versus *rad9*-S2 cells especially after 500 Gy (*P* = 0.01) or 1000 Gy (*P* = 0.02) exposure doses] and does not elongate as markedly as wild-type cells 6 h post-irradiation. These results indicate that, although the S2 region of the protein is conserved evolutionarily, its functional significance is not dramatic or obvious. However, the need for this region in mediating Rad9p activities not tested or as of yet undiscovered biological roles for the protein remain possible.

Recently, it was found that the human homolog of *S.pombe* Rad9p, as well as the yeast protein, have a BH3-like domain at amino acids 16–30 (20,21). In addition, both proteins can bind the human anti-apoptotic proteins Bcl-2 and Bcl-X_L and can cause apoptosis in human cells when overproduced if the BH3 domain is intact. *S.pombe rad9*-S1 mutant cells have alanine substitutions in amino acids 20–25, which presumably alter the structure of the BH3 domain. The mutant is sensitive to gamma-rays and UV light, but demonstrates wild-type levels of HU resistance and nearly normal checkpoint control responses. These results suggest that the BH3 domain is important only for the response of *S.pombe* cells to radiation exposure and functions independently of checkpoint control mechanisms. These results are also consistent with previous findings that the C-terminal region of at least human RAD9 protein can bind two other checkpoint control proteins, HUS1 and RAD1, suggesting that the N-terminal part of the human protein (and likely also the yeast protein) participates in apoptosis and the C-terminal portion regulates cell cycle progression delay after DNA damage is incurred (25). However, the BH3 domain in the *S.pombe* protein is probably involved in an apoptotic-like response in yeast that differs

from the mechanism described in mammalian cells since disruption of the structure of this domain would be expected to increase not decrease radioresistance if the programmed cell death response was eliminated. Therefore, the precise biological and biochemical function of the BH3 domain in *S.pombe* contributes to radioresistance but the mechanism involved needs further clarification.

Kostrub *et al.* (22) showed that *S.pombe* Hus1p and Rad1p form a stable complex that depends upon the presence of Rad9p. In addition, Caspari *et al.* (26) demonstrated that all three proteins associate physically. Since the C-terminal region of the human RAD9 protein can bind human HUS1 and RAD1 (25), it is likely that the same region in *S.pombe* Rad9p participates in its association with yeast Hus1p and Rad1p. Interestingly, *S.pombe* cells containing *rad9*-S4 and *rad9*-S5, which have alterations in evolutionarily conserved amino acids in the C-terminal region of the protein, are sensitive to gamma-rays, UV light and HU and show aberrant cell cycle delay responses after exposure to any of these agents. These results suggest that the alterations within Rad9p encoded by *rad9*-S4 or *rad9*-S5 neutralize the ability of the protein to bind the other two checkpoint control proteins, Hus1p and Rad1p, and might explain why corresponding mutant cells lost the ability to delay cell cycle progression after exposure to gamma-rays, UV light or HU.

The checkpoint defects mediated by *rad9*-S4 and *rad9*-S5 are unique. Mutant cells containing either of these alleles appear to initiate cell cycle delays in response to HU, gamma-rays or UV, but fail to maintain the checkpoints. Furthermore, both mutant alleles, but especially *rad9*-S5, retain the ability to elongate in response to these agents although they are unable to delay septum formation. Since the more extreme *rad9::ura4⁺* mutation completely eliminates all inducible checkpoint related phenotypes, including a delay in septum formation and in segregation of DNA to progeny cells, as well as cell elongation, the results suggest that the Rad9 protein has separate domains that control and maintain subsets of checkpoint related functions.

Several groups recently used primarily computational analyses to predict that Rad9 protein, as well as the products of *hus1* and *rad1* contain structural features found within domains of PCNA, a protein that functions as a sliding clamp to bring DNA polymerase to its template (26–29). These investigators suggest that Rad1p/Rad9p/Hus1p form a heterotrimeric ring structure, like PCNA, for function. All of the *rad9* mutations made as part of this study are found within strand or helix domains of PCNA predicted by one or more versions of the published models. Therefore, the functional significance of those domains, as indicated by the phenotypes mediated by the different *rad9* alleles, has been tested. The information gained by analyzing these and other *rad9* mutant alleles, as well as mutations in *hus1* and *rad1*, should help validate the model predicting similarities between PCNA and this group of checkpoint control proteins and aid in defining their biochemical function.

A comparison of Rad9p from humans and mice versus fission yeasts reveals that a 22 amino acid long block of residues is not present in the mammalian versions of the protein. To assess the functional significance of this region in *S.pombe* Rad9p, the coding sequences for these amino acids were deleted, resulting in the construction of *rad9*-S3. *Schizosaccharomyces pombe* cells containing *rad9*-S3 demon-

strated essentially wild-type resistance to gamma-rays, UV light and HU and no defect in checkpoint control. One possible explanation is that this region does not have important functional significance, consistent with its absence in the mammalian proteins. Alternatively, this region might participate in a process in *S.pombe* that was not examined or has not yet been identified.

None of the *rad9* mutant alleles examined dissociate HU resistance from HU-induced checkpoint control. Although a more exhaustive search for such alleles might uncover one with this property, this type of allele is either rare or cannot be generated since the array of mutants already constructed and analyzed split other phenotypes examined. Site-directed mutagenesis studies of *S.pombe rad1* also demonstrated a good correlation between HU resistance and DNA replication checkpoint proficiency (48). Interestingly, extragenic suppressors of *S.pombe rad9::ura4⁺*, first isolated by a screen for the acquisition of increased radioresistance, demonstrated enhanced HU resistance in the absence of HU-induced checkpoint control (44). Also, *S.cerevisiae rad52* mutants can exhibit HU sensitivity, indicating that recombinational repair proteins could function in resistance to this drug without involving checkpoint control (49). These results suggest that cellular mechanisms of HU resistance include, but are not limited to, the induction of changes in cell cycle progression. However, the inability of any *rad9* allele to dissociate HU resistance from the DNA replication checkpoint suggests that Rad9p mediates resistance to this drug primarily through regulation of the cell cycle. Alternatively, these results are also consistent with the control by Rad9p of HU resistance through a signal transduction event that is coordinately regulated with cell cycle progression.

Interestingly, gamma-ray resistance was not completely dissociated from UV resistance by any of the *rad9* mutant alleles. To date, no such allele separating UV from gamma-ray resistance has been identified for any of the known checkpoint control genes, suggesting that *rad9* may play a similar role in promoting resistance to these two DNA damaging agents.

Recently, Bessho and Sancar (30) reported that the human RAD9 protein has 3'→5' exonuclease activity possibly needed for processing a primary DNA lesion and contributing to the DNA damage checkpoint response. Even though no motifs characteristic of this type of activity are apparent in the protein, this exonuclease function was localized to amino acids 51–91. *In vitro* mutagenesis was not performed on the similar region of *S.pombe* Rad9p as part of the current study. However, it would be important to test the biochemical and biological significance of that region in the yeast Rad9p and determine how it functions in relation to the previously described activities of the protein.

The evolutionary conservation of *S.pombe rad9⁺* (13,33,34), as well as several other checkpoint control related genes (10–12,14–16,50,51), suggests that mechanisms regulating cell cycle progression and promoting cell survival after the acquisition of DNA damage or a block in DNA replication are also conserved. This implies that checkpoint control processes are fundamentally important for maintaining genomic integrity. The findings presented in this study indicate that Rad9p contains functional domains that regulate several activities associated with the protein and other regions that are activity-specific. These studies provide the foundation for future investigations focused on understanding the precise role of

Rad9p in the cellular response to radiation and chemical exposure and, in addition, will be valuable in terms of defining the functional significance of physical interactions between Rad9p domains and other proteins. These results are also important because *rad9* cognates are found in mammals and defective checkpoint control mechanisms are linked to carcinogenesis (19).

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