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Rad9, an Evolutionarily Conserved Gene With Multiple Functions for Preserving Genomic Integrity

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Abstract The *Rad9* gene is evolutionarily conserved. Analysis of the gene from yeast, mouse and human reveal roles in multiple, fundamental biological processes primarily but not exclusively important for regulating genomic integrity. The encoded mammalian proteins participate in promoting resistance to DNA damage, cell cycle checkpoint control, DNA repair, and apoptosis. Other functions include a role in embryogenesis, the transactivation of multiple target genes, co-repression of androgen-induced transcription activity of the androgen receptor, a 3′-5′ exonuclease activity, and the regulation of ribonucleotide synthesis. Analyses of the functions of *Rad9*, and in particular its role in regulating and coordinating numerous fundamental biological activities, should not only provide information about the molecular mechanisms of several individual cellular processes, but might also lend insight into the more global control and coordination of what at least superficially present as independent pathways. J. Cell. Biochem. 97: 690–697, 2006.

Key words: Rad9; radiation; DNA damage; DNA repair; cell cycle checkpoint; apoptosis; genomic integrity

FISSION YEAST GENES THAT REGULATE RADIORESISTANCE

The isolation of *Schizosaccharomyces pombe* mutants sensitive to ionizing radiation and UV light, and their subsequent initial genetic analyses, lead to the conclusion that many loci in this fission yeast regulate radioresistance [Schupbach, 1971; Nasim and Smith, 1974; for reviews see Phipps et al., 1985; Lieberman et al., 1989; Lehmann, 1996]. Interestingly, mutations in four of these loci, *rad1* (*uvs1*), *rad3* (*uvs3*, *M-3*), *rad9* (*uvs9*), and *rad17* (W, *M-24*), caused extreme sensitivity to both UV and ionizing radiation, and were thus thought to play important roles in promoting radioresistance [Schupbach, 1971; Nasim and Smith, 1975]. Further analyses revealed that mutations in any one of these genes negate the ability of cells to delay cycling transiently in the G_2 phase of the cell cycle after exposure to ionizing radiation [Al-Khodairy and Carr, 1992; Rowley et al., 1992], an event thought normally to provide extra time for mending DNA lesions before entry into mitosis. Irradiated cells progressing through this phase of the cell cycle before essentially completing repair presumably have an increased risk of chromosome breakage or other deleterious events during cell division.

Of the known *S. pombe* checkpoint control gene group members, at least *rad3* and *rad9* not only regulate cell cycle progression after radiation exposure but additionally play a more direct role in repairing damaged DNA or at least have functions unrelated to cell cycle progression that modulate DNA damage resistance [Jimenez et al., 1992; Lieberman, 1995; Hang et al., 2000]. In support of this, extragenic suppressors of the radiation sensitivity of *rad9::ura4+* mutants function in cells with the *rad3-136* mutation as well and enhance resistance without restoring checkpoint control function [Lieberman, 1995]. Also, mutations in *rad9* can uncouple radioresistance from cell cycle checkpoint proficiency [Hang et al., 2000]. Furthermore, there is evidence that *rad9+*

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regulates UVDE, a UV endonuclease that participates in a secondary, alternative excision repair pathway [Davey et al., 1998]. It was demonstrated that the *rad9* encoded protein has a BH3-like domain and pro-apoptotic function when tested in human cells [Komatsu et al., 2000a]. Rad9 may participate in base excision repair in addition since the protein (along with Rad1 and Hus1) has the ability to bind the DNA glycosylase MYH [Chang and Lu, 2005]. *S. pombe* Rad9 protein can form a heterotrimer with Rad1 and Hus1 [Caspari et al., 2000; Venclovas and Thelen, 2000; Kaur et al., 2001; for review see Parrilla-Castellar et al., 2004; called the 9-1-1 complex, Burtelow et al., 2001], which resembles a PCNA-like sliding clamp. It is believed that, as part of this protein complex, Rad9 acts as a DNA damage sensor and is loaded around DNA via the action of a Rad17-containing clamp loader. However, whether Rad9 performs all of its functions solely as part of this complex is not fully established. Nevertheless, these results support multiple cell cycle dependent and independent functions for *rad9* in determining how cells respond to DNA damage.

EVOLUTIONARY CONSERVATION OF *Rad9*

The *rad9* gene was first isolated from the fission yeast *S. pombe* by functional complementation of the radiosensitivity of corresponding mutant cells [Murray et al., 1991; Lieberman et al., 1992]. Subsequently, homology searches through Southern blotting under low stringency conditions, or more frequently via computer analysis, revealed homologs from many different species ranging from other yeasts to mammals. Table I lists some of the *Rad9* genes, in addition to the one from *S. pombe*, identified to date, characterized and published. The sequences of many other *Rad9* genes and encoded proteins, from organisms as diverse as frog and cow, have been contributed to computer databases, but more extensive, published characterizations are not yet available. Percent amino acid identity, or similarity and identity, in terms of physiochemical properties between the protein encoded by *S. pombe Rad9* and each of the orthologs are indicated in Table I. The human and mouse proteins are 82% identical and 88% similar/identical at the amino acid level [Hang et al., 1998].

Several functional domains of the human (hRAD9) protein have been identified. and many are present in Rad9 cognates from multiple species. For example, mouse and *S. pombe* Rad9, like hRAD9, have a BH3-like domain characteristic of pro-apoptotic proteins [Hang et al., 1998; Komatsu et al., 2000b]. Interestingly, this domain from human Rad9 can bind the anti-apoptotic human proteins Bcl-2 and Bcl-X_L, and is essential for the protein to induce apoptosis in human cells when ectopically expressed. The BH3 domain within *S. pombe* Rad9 protein can also bind human Bcl-X_L, and expression of this yeast *rad9* gene in human cell can induce programmed cell death [Komatsu et al., 2000a]. The human and *S. pombe* Rad9 proteins can form a heterotrimer complex with the cell cycle checkpoint proteins Hus1 and Rad1 from their respective organisms, and these complexes are thought to mediate the transient cell cycle delay observed after radiation exposure [for review see Parrilla-Castellar et al., 2004]. Thus, the abilities of Rad9 to mediate apoptosis, as well as to form the Rad9-Rad1-Hus1 heterotrimer and regulate cell progression have been retained during the evolution of yeast to human.

TABLE I. Orthologs of *Schizosaccharomyces pombe* Rad9 Protein

Organism	Gene	Homology of encoded protein to *S. pombe* Rad9	References
Homo sapiens	*hRAD9*	I = 25%, S + I = 46%	Lieberman et al. [1996]
Mus musculus	*Mrad9*	I = 27%, S + I = 48%	Hang et al. [1998]
Caenorhabditis elegans	*hpr-9*	I = 20%, S + I = 39%	Venclovas and Thelen [2000]
Drosophia melanogaster	*Rad9*	I = 21%, S + I = 42%	Dean et al. [1998]
Saccharomyces cerevisiae	*ddc1*	I = 21%, S + I = 46%	Longhese et al. [1997][a]
Schizosaccharomyces malidevorans	*rad9*	I = 100%, S + I = 100%	Lieberman and Hopkins [1994]
Schizosaccharomyces octosporus	*rad9*	I = 64%, S + I = 79%	Lieberman and Hopkins [1994]
Gallus domesticus	*Rad9*	I = 24%, S + I = 41%	Kobayashi et al. [2004]

I, percent identical amino acids; S + I, percent similar and identical amino acids; as determined by use of the default settings of blast provided by NCBI.

[a]The study describes the use of BESTFIT to align ddc1 with Rad9, and the results indicated that the two proteins were 20.6 identical and 45.9 similar/identical. Use of Clustal 1.8, with the default settings, indicated 17.4 identity, 32.6 similarity.

In addition to structural relatedness, often cross-species complementation can be demonstrated, whereby ectopic expression of the gene from one organism can at a minimum partially rescue mutant phenotypes of cells from another species devoid of the wild-type inherent gene. For example, *S. malidevorans* or *S. octosporus rad9* fully rescues the radiosensitivity of *S. pombe rad9::ura4+* cells [Lieberman and Hopkins, 1994]. Even more dramatically, ectopic expression of the human cognate, *hRAD9*, in the *S. pombe rad9* mutant can not only restore radioresistance but also complement the associated cell cycle checkpoint defects. Expression of the *hRAD9* cDNA in *Mrad9*$^{-/-}$ mouse ES cells rescues several defects, including sensitivity to radiation and hydroxyurea, deficiency in the maintenance of the radiation-induced delay in the G_2 phase of the cell cycle, and increased genomic instability in the form of high spontaneous frequencies of chromosome aberration. These results underscore the extent to which *Rad9* is conserved evolutionarily, at the structural level and at least partially in terms of function, and suggest that it performs important, fundamental biological activities.

ROLE OF MAMMALIAN *Rad9* GENES IN DNA DAMAGE RESISTANCE AND GENOMIC INTEGRITY

Based on the sensitivity of *S. pombe rad9::ura4+* cells to radiations and chemicals that damage DNA or disrupt DNA replication [Murray et al., 1991; Lieberman et al., 1992], it would be predicted that the mammalian orthologs promote cell survival after exposure to such agents. Studies with human cells containing *hRAD9* siRNA or mouse ES cells devoid of wild-type *Mrad9* because of targeted gene deletion indicate that this is true. These Rad9-compromised cells are sensitive to DNA damage or replication inhibition [Hopkins et al., 2004; Loegering et al., 2004; Dang et al., 2005; Karnitz et al., 2005], indicating that the protein plays an important role in repairing, directly or indirectly, DNA lesions. These cells are also defective in aspects of cell cycle checkpoint control [Hirai and Wang, 2002; Hopkins et al., 2004; Loegering et al., 2004; Karnitz et al., 2005], similar to *rad9* mutant fission yeast cells, thus underscoring the evolutionary conservation and importance of this cell cycle function in the way mammals respond to DNA damage.

The DNA damage signaling mechanisms mediated by Rad9 have been investigated. Replication protein A can bind single stranded DNA at stalled replication forks. This facilitates the binding to DNA of a protein complex containing Rad17 and replication factor C small subunits 2–5, which in turn acts as a clamp loader and promotes binding of the preassembled Rad9-Rad1-Hus1 complex [St Onge et al., 1999; Volkmer and Karnitz, 1999; Burtelow et al., 2000; Hang and Lieberman, 2000; Lindsey-Boltz et al., 2001; Bermudez et al., 2003; Ellison and Stillman, 2003; Zou et al., 2003]. Independently, Atr associates with chromatin and the Rad9-Rad1-Hus1 complex, which enables Atr to phosphorylate and activate Chk1 as well as other substrates [Zou et al., 2002]. This activates the damage response in human cells caused by replication stress, and leads to the stabilization of replication forks as well as a delay in progression through S phase. Phosphorylation of hRad9 by Atm is important for ionizing radiation-induced delay in the G_1/S transition in humans [Chen et al., 2001], although this mechanism may be cell system dependent [Roos-Mattjus et al., 2003]. Nevertheless, it is clear that Rad9 protein can bind very rapidly to DNA double strand breaks [Greer et al., 2003], indicating participation in a very early response to DNA damage.

Aside from cell cycle checkpoint functions, there is mounting evidence that Rad9 also has a more direct role in repairing DNA lesions. It was already mentioned that *S. pombe* Rad9 can bind the base excision repair protein DNA glycosylase MYH [Chang and Lu, 2005]. Most recently, several studies indicate the ability of human Rad9 to bind multiple DNA repair proteins, primarily involved in base excision repair, and in some instances to regulate their activity [Wang et al., 2004b; Toueille et al., 2004; Friedrich-Heineken et al., 2005; Helt et al., 2005; Smirnova et al., 2005]. We found that *Mrad9*$^{-/-}$ mouse ES cells are deficient in base excision repair of some but not all DNA lesion substrate targets of this process, consistent with a direct role for Rad9 in DNA repair (A.S. Balajee, K.M. Hopkins, H.B. Lieberman, unpublished data).

The function of Rad9 is not limited to processing damage caused by exposure to exogenous agents. Mouse ES cells bearing *Mrad9*$^{-/-}$ show high spontaneous levels of chromosome and chromatid aberrations, as well as *hprt*

mutation, compared to isogenic $Mrad9^{+/+}$ or $Mrad9^{+/-}$ populations [Hopkins et al., 2004]. Likewise, human cells treated with *Rad9* siRNA demonstrate increased chromosome aberration frequency, compared to controls [Dang et al., 2005]. This indicates that the protein is important for maintaining genomic integrity, which is challenged routinely by the byproducts of normal metabolic processes. The function of Rad9 in base excision repair likely contributes to the role of the protein in maintaining genomic integrity. In addition, its role in facilitating normal progression through S phase via regulation of Chk1 and Cdc25A in the absence of DNA damage is important [Sorensen et al., 2004]. Studies with *S. pombe* indicate that the fission yeast Rad9 protein can associate with telomeres [Nakamura et al., 2002], and the mammalian orthologs likely have the same activity [Nabetani et al., 2004; T.K. Pandita, H.B. Lieberman, unpublished data]. This association suggests a role for Rad9 in telomere maintenance and stability, and would thus through this mechanism also serve to stabilize chromosomes in the presence of damage occurring spontaneously or after exposure to exogenous agents.

ROLE OF Rad9 IN APOPTOSIS

The proteins encoded by human and mouse Rad9 contain a BH3-like domain, typical of BH3-only, pro-apoptotic family members [Komatsu et al., 2000b]. Investigations with the hRad9 BH3 demonstrated the ability to bind anti-apoptotic proteins Bcl-2 and Bcl-X_L. Overexpression of *hRAD9* in a variety of human cell lines induced apoptosis, but partial deletion of the BH3 domain neutralized this pro-apoptotic activity. Likewise, co-overexpression of Bcl-2 or Bcl-X_L with *hRAD9* prevented apoptosis. Interestingly, as mentioned, *S. pombe* Rad9 protein also has a BH3-like domain and apoptosis-related features similar to those of hRad9 [Komatsu et al., 2000a].

Several models have been proposed to explain how hRad9 functions as a mediator of programmed cell death. Lee et al. [2003] provided evidence that DNA damage leads to cleavage of hRad9 by caspase 3. An N-terminal fragment of the protein then translocates from the nucleus to the cytoplasm, where it binds the anti-apoptotic protein Bcl-X_L and causes apoptosis. Interestingly the cleavage of hRad9 by caspase 3 is thought to disrupt the Rad9-Rad1-Hus1 complex, thus interfering with cell cycle checkpoint control and promoting apoptosis. The DNA damage-induced phosphorylation of hRad9 by the protein kinase c-Abl is important for binding of hRad9 to Bcl-X_L [Yoshida et al., 2002], while phosporylation by protein kinase C∂ (PKC∂) enhances the association of hRad9 and Bcl-2 [Yoshida et al., 2003].

Stress can also induce Atr to down regulate expression of *Frag1*, which leads to the release of Rad9 from damaged chromatin [Ishii et al., 2005]. This in turn allows Rad9 to associate with Bcl-2, again promoting apoptosis. Exactly how this set of molecular events coincides with the model proposed by Lee et al. [2003] needs to be addressed.

ADDITIONAL FUNCTIONS OF MAMMALIAN Rad9

Rad9 has multiple functions that impact on the way cells respond to DNA damage, caused either by exposure to exogenous agents or spontaneously via the action of normal cellular metabolism. The protein participates in cell cycle checkpoint control, DNA repair, apoptosis, and maintaining genomic integrity. hRad9 can, like p53, also serve as a transactivator of downstream target genes, some of which are likely essential for allowing the cell to handle damaged DNA [Lieberman and Yin, 2004; Yin et al., 2004]. However, recent studies indicate that *Rad9* is a genetic element with an even broader range of activities. In addition to checkpoint, apoptosis and genome stabilization functions, mouse and/or human Rad9 proteins demonstrate 3′–5′ exonuclease activity, which has yet to be assigned a biological role [Bessho and Sancar, 2000], the ability to repress the transactivity of androgen receptor induced by androgen-endrogen receptor binding in prostate cells [Hsu et al., 2004; Wang et al., 2004a], and the ability to stimulate the carbamoyl phosphate synthetase activity of CAD protein, required for de novo synthesis of pyrimidine nucleotides and cell growth, suggesting a role for Rad9 in ribonucleotide biosynthesis [Lindsey-Boltz et al., 2004]. Moreover, *Mrad9* is essential for embryonic development [Hopkins et al., 2004]. In addition, several studies suggest a link between alterations in Rad9 and cancer. Combined haploinsufficiency for Rad9 and Atm lead to increased susceptibility of mouse embryo

fibroblasts to radiation-induced transformation [Smilenov et al., 2005]. Alterations in levels of human Rad9 are linked to nonsmall cell lung carcinoma [Maniwa et al., 2005] and breast cancer [Cheng et al., 2005].

HOW CAN Rad9 PARTICIPATE IN A LARGE NUMBER OF DIVERSE ACTIVITIES?

Multiple functional domains in the human Rad9 protein have been identified, thus revealing structure-function relationships. For examples, amino acid sequences critical for apoptosis [Komatsu et al., 2000b], the 3′–5′ exonuclease activity [Bessho and Sancar, 2000], and nuclear localization [Hirai and Wang, 2002] have been described. However, regions of the protein responsible for each known activity have not been defined completely. Moreover, it is unlikely that each Rad9 molecule participates in all of the known activities of the protein simultaneously. Therefore, an important question concerns a detailed explanation of the mechanisms that allow the protein to mediate one function versus another. Phosphorylation status must be considered. Rad9 protein is phosphorylated at many sites in cells not challenged by exogenous DNA damaging agents, but the protein becomes hyperphosphorylated after exposure to radiation [St Onge et al., 1999, 2001; Volkmer and Karnitz, 1999; Chen et al., 2001]. Several of the specific phosphorylated amino acids have been identified, but functional significance has been assigned unequivocally to only a few [Chen et al., 2001; Roos-Mattjus et al., 2002, 2003; Yoshida et al., 2002, 2003; St Onge et al., 2003]. Other types of post-translational processing events are likely also important for determining which activities Rad9 will perform. For example, as mentioned previously, cleavage by caspase 3 appears critical for the role of hRad9 in programmed cell death [Lee et al., 2003].

HUMAN AND MOUSE PARALOGS OF Rad9

Homology searches revealed that mammalian *Rad9* is part of a gene family, thus adding another level of complexity that must be considered when elucidating the function of the gene. Paralogs of *Rad9*, from human (*hRAD9B*) and mouse (*Mrad9B*), were identified (Hopkins et al., 2003; Dufault et al., 2003; see Table II), but interestingly no cognate was found in yeast, at least after a computer search of genomic DNA databases (To search for yeast cognates, Blast BLOSUM62 default settings at the *Saccharomyces cerevisiae* Genome Database web site [www.yeastgenome.org] and at the *S. pombe* Genome Center site [www.sanger.ac.uk/Projects/S_pombe] were used.). While *hRAD9*/*Mrad9* message is essentially in all tissues, the paralogs are expressed primarily although not exclusively in testis. The human paralog protein, hRad9B, resides in the nucleus, and post-translational processing appears minimal. As for hRad9, the human paralog can physically interact with human Hus1, Rad1, and Rad9 proteins, thus suggesting that an alternative or perhaps even competing 9-1-1 complex can form. A paralog of human Hus1 has also been identified [Hang et al., 2002]. However, since that paralog, called Hus1B, directly interacts with Rad1, but not Rad9 or Hus1, whereas Hus1 can bind Rad1 and Rad9 to form the classic 9-1-1 complex, Hus1B cannot simply substitute for Hus1. Analyses of the role of Rad9 alone, as part of a 9-1-1 complex, or in relation to Rad9B and Hus1B should thus yield important information.

TABLE II. Comparison of hRad9, hRad9B, Mrad9, and Mrad9B Proteins*

Protein comparison	Identical amino acids (%)	Similar + identical amino acids (%)
hRad9 versus hRad9B	35	55
Mrad9 versus Mrad9B	35	50
hRad9 versus Mrad9	81	86
hRad9B versus Mrad9B	63	76

*The default settings for Blast on NCBI's web page were used to determine levels of homologies.

FUTURE STUDIES AND DIRECTIONS

Understanding how cells maintain their genomic integrity, in the presence of exogenous DNA damaging agents or even in their absence when normal metabolic processes can generate damage, impacts on many important issues. The mechanisms involved determine whether DNA lesions are properly repaired or potentially lead to deleterious effects, such as cancer, mutation or death. Rad9 plays multiple roles in helping cells cope with damage, and is likely pivotal in determining whether such cells survive or undergo apoptosis. Interestingly, the protein has several other functions that at

least superficially appear important for a variety of basic biological processes unrelated to the maintenance of genomic integrity. Whether these functions are truly independent, or not only regulated but also coordinated through Rad9, remains to be determined. In addition, it is not clear whether each Rad9 molecule can participate at any given time simultaneously in many biological processes such as cell cycle control, DNA repair, apoptosis, and transactivaton or co-repression of transcription. It seems unlikely, and as such the molecular mechanisms that regulate the ability of Rad9 to mediate one process versus another at any given time need to be addressed. Alternative post-translational processing, subcellular localization or recruitment to different protein complexes could contribute. Determining all the biochemical activities of Rad9 and assigning those activities to specific biological functions will be important. Given the range of processes regulated by Rad9, an understanding of its function and regulation will bear on numerous significant topics as diverse as health risk assessment after exposure to DNA damaging agents, carcinogenesis, embryogenesis, and the maintenance of prostate function. The ability of a single protein to regulate so many processes suggests a more global level of coordination and molecular linkage of fundamental cellular mechanisms. What was once thought to be multiple, essentially independent pathways now appear connected. Future studies are likely to reveal this global regulatory theme repeated as investigations of Rad9 and other proteins advance.

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