Multiple Functions of Rad9 for Preserving Genomic Integrity

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Abstract: DNA-damage checkpoints sense and respond to genomic damage. Human Rad9 (hRad9), an evolutionarily conserved gene with multiple functions for preserving genomic integrity, plays multiple roles in fundamental biological processes, including the regulation of the DNA damage response, cell cycle checkpoint control, DNA repair, apoptosis, transcriptional regulation, exonuclease activity, ribonucleotide synthesis and embryogenesis. This review examines work that provides significant insight into the molecular mechanisms of several individual cellular processes which might be beneficial for developing novel therapeutic approaches to cancerous diseases with genomic instability.

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INTRODUCTION

The human genome is exposed to variety of genotoxins which can elicit unscheduled replication. After DNA is damaged or replication is perturbed, cells respond by activation of evolutionarily conserved signal transduction pathways that delay cell cycle progression and induce repair of the damaged DNA. These signal transduction pathways include protein sensors that recognize aberrant DNA structures and activate kinases, thereby inducing phosphorylation cascades that ultimately lead to cell cycle arrest and DNA repair [1-4]. Failure of this cell cycle surveillance mechanism can cause genomic instability that eventually leads to cancer formation in mammals [5, 6].

hRad9 protein is the human homologue of *Schizosaccharomyces pombe* Rad9. *Rad9* is a member of the checkpoint *rad* genes (*rad1+*, *rad3+*, *rad9+*, *rad17+*, *rad26+* and *hus1+*) that are required for the S phase (DNA replication) and G2 (DNA mitosis) checkpoints [7-9]. Multifunctional participation of hRad9 has been demonstrated in the DNA damage sensor as the 9-1-1 complex not only in checkpoint signaling, but also in DNA repair *via* DNA polymerase β [10] or flap endonuclease 1 (FEN1) [11], and in apoptosis *via* potential binding to Bcl-2 or Bcl-xl [12, 13].

In this article, we note that hRad9 takes many roles including the regulation of the DNA damage response, cell cycle checkpoint control, DNA repair, apoptosis, transcriptional regulation, exonuclease activity, ribonucleotide synthesis and embryogenesis. Thus hRad9 preserves genomic integrity in many ways (Fig. (1)).

DNA DAMAGE SENSOR

Cell cycle checkpoints are signal transduction pathways that maintain the proper order of cell cycle events. As was

shown previously, γ -H2AX is an important marker for detection of double-strand breaks [14] and has been used for evaluating DNA repair dynamics [15]. In mammalian cells, a signal initiated by two phosphatidylinositol 3-kinase-related kinases (PIKK), ataxia-telangiectasia mutated (Atm) and ataxia-telangiectasia mutated and Rad3-related (Atr), plays a central role in the checkpoint signaling pathways [16]. Atm and Atr are activated by genotoxins and phosphorylate downstream signaling proteins, including Chk1 and Chk2, two protein kinases that regulate checkpoint responses [17-19].

Like its yeast counterpart, hRad9 forms a ring-shaped, heterotrimeric complex with the hRad1 and hHus1 proteins [20, 21]. Each member of the hRad9-hRad1-hHus1 complex (known as the 9-1-1 complex) shares sequence homology with proliferating cell nuclear antigen (PCNA), a homotrimer that encircles DNA and tethers DNA polymerase δ during DNA synthesis [21-25]. Hus1 and Rad1 are involved in checkpoint activation [21-27]. PCNA is loaded onto DNA by pentameric protein complex replication factor C (Rfc) [28], which is composed of one large subunit and four smaller subunits. Previous study indicates that the 9-1-1 complex is loaded onto DNA by a complex between hRad17 and the four small subunits of Rfc [26] and forms a DNA-damage focus, which acts to repair damage [26, 27, 29]. Since DNA damage induces hRad17-dependent association of 9-1-1 complex with chromatin, the 9-1-1 complex is believed to be involved in the direct recognition of DNA lesions during the initial stages of the checkpoint response. The 9-1-1 complex may associate with chromatin after DNA damage to transduce signals for DNA damage-activated checkpoint signaling pathways [27]. The C-terminal region of the hRad9 protein acts to transport the 9-1-1 complex into the nucleus [29]. hRad9 and Atm rapidly colocalize to regions containing DNA double-strand breaks after DNA damage [30, 31], and Atm can phosphorylate Rad9 [32]. hRad9 is phosphorylated directly by Atm at Ser-272 during ionizing radiation (IR)-induced G1/S checkpoint activation

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Fig. (1). Multiple functions of hRad9 in DNA damage response. After DNA is damaged by exogenous genotoxins, unscheduled replication etc, the damage is sensed with Atr/Atm or foci of γ -H2AX and 9-1-1 complex. Rad9 regulates the *P21* expression, supposed to be involved in G1/S checkpoint. The phosphorylation of C-terminus of Rad9 plays a role in the activation of Chk1, and the activation of Chk1 induces the stimulation of the G2/M checkpoint. Under the checkpoint activation, Rad9 is involved in DNA repair through DNA polymerase β activity and ribonucleotide synthesis. A termination of the Chk1 arrest may elicit the induction of apoptosis [49], or Rad9 participates in induction of apoptosis interacting with Bcl-2 and Bcl-xL.

[32]. The other phosphorylation sites of the C-terminal region of hRad9 are also essential for Chk1 activation following hydroxyurea (HU), IR, and UV treatment [33], although the constitutive phosphorylation of hRad9 does not influence the stability of the 9-1-1 complex [33, 34].

EXONUCLEASE ACTIVITY

Recombinant hRad9 has recently been shown to possess 3'-5' exonuclease activity, suggesting that exonucleolytic processing of primary DNA lesions by hRad9 may contribute to DNA damage checkpoint response in humans [35], although the exact mechanism remains to be investigated further.

CELL CYCLE REGULATION

Genotoxic stress induces stabilization and transient accumulation of wild-type p53 protein in mammalian cells, leading to increased expression of p53 down-stream genes such as *P21/WAF1* [36, 37]. The G1/S checkpoint is activated immediately after DNA damage, and inhibits DNA-damaged cells from entering the S phase [30, 38]. The phosphorylation of hRad9 is essential for Chk1 activation at S to G2 checkpoints following HU, IR, and UV treatment [33], and Chk1 is phosphorylated at two residues (Ser-317 and Ser-345) located in a Ser/Thr-Gln-rich domain [39]. Thus the phosphorylation of hRad9 is required for S-phase and G2/M

checkpoint activation [33], although a potential transactivating property of Rad9 to *P21* promoter has been shown, which suggests that p21 is involved also in the G1/S checkpoint [40].

DNA SYNTHESIS (WITH DNA POLYMERASE β)

The 9-1-1 complex can physically interact with DNA polymerase β *in vitro*, and the 9-1-1 complex has a stimulatory effect on DNA polymerase β activity, suggesting the possibility that the 9-1-1 complex might attract DNA polymerase β to DNA damage sites, thus directly connecting checkpoints and DNA repair [10]. Also, 9-1-1 is a damage-specific activator of FEN1 [41]. Although each member of the 9-1-1 complex shares sequence homology with PCNA, PCNA and the 9-1-1 complex can independently bind to and activate FEN1 [11].

RIBONUCLEOTIDE SYNTHESIS

Immunoprecipitated Rad9 complex contains a 240 kDa protein that has been identified as carbamoyl phosphate synthetase/aspartate transcarbamoylase/dihydroorotase (CAD), a multienzymatic protein required for the *de novo* synthesis of pyrimidine nucleotides and cell growth. Further investigation revealed that only free Rad9, but not Rad9 within the 9-1-1 complex, binds to CAD. These findings suggest that Rad9 may play a role in ribonucleotide biosynthesis [42].

APOPTOSIS (Bcl-2, Bcl-xL)

The *Schizosaccharomyces pombe* Rad9 (SpRad9) protein contains a group of amino acids with similarities to the Bcl-2 homology 3 death domain, which is required for SpRad9 interaction with human Bcl-2 and apoptosis induction in human cells [12]. hRad9 interacts with the anti-apoptotic Bcl-2-family proteins Bcl-2 and Bcl-xL, and induces apoptosis [13].

TRANSCRIPTIONAL REGULATION

Rad9 can control expression of a number of genes that might be involved in developmental processes. Rad9 may control embryonic development through regulation at the transcriptional level specifically of target genes critical for this process [40].

EMBRYOGENESIS

Mouse Rad9 (-/-) embryo fibroblasts were not viable. mRad9 is a key mammalian genetic element of pathways that regulate the cellular response to DNA damage, maintenance of genomic integrity, and proper embryonic development [43].

PHOSPHORYLATION OF hRad9

hRad9 is highly modified by phosphorylation in a constitutive manner in response to both DNA damage and cell cycle position, and this phosphorylation plays a critical role in checkpoint signaling [44]. hRad9 has multiple functions in DNA damage response through the involvement of its phosphorylations, including checkpoint activation and damage repair [32, 33, 44]. IR induces Atm-dependent phosphorylation of hRad9 at least at Ser-272, which was detected in an *in vitro* study [33]. Atm-mediated phosphorylation of hRad9 is required for IR-induced G1/S checkpoint activation [32]. Thr-292 of hRad9 is subject to Cdc2-dependent phosphorylation in mitosis. Furthermore, four other hRad9 phosphorylation sites (Ser-277, Ser-328, Ser-336, and Thr-355) are regulated in part by Cdc2. Phosphorylation at Ser-387 is a constitutive, prerequisite form of DNA damage-induced hyperphosphorylation of hRad9. Overexpression of these mutants blocks the interaction between hRad9 and the DNA damage-responsive protein TopBP1 and impairs the cellular response to DNA damage during the S phase [44]. hRad9 mutants lacking a Ser-272 phosphorylation site, which is phosphorylated in response to genotoxins, had no effect on survival or checkpoint activation in mRad9 (-/-) mouse ES cells [33], whereas the maintenance of basal phosphorylation of the C-terminus appears to be essential for hRad9-mediated Chk1 activation following HU, IR, and UV treatment [33]. Consistent with a role for Chk1 in S-phase arrest, HU- and UV-induced S-phase arrest was abrogated in hRad9 phosphorylation mutants [33]. The exact roles of hRad9 phosphorylation in the DNA damage network remain to be investigated.

CONCLUSION

The disruption of these functions contributes to the accumulation of genomic instability and characterizes cancerous cells [2, 45, 46]. Recent studies of tumors indicate that

the *RAD9* gene is located in the chromosomal region of 11q13, which is amplified and frequently overexpresses both mRNA and protein in breast and lung cancer [47, 48]; the up-regulations correlate with tumor size and local recurrence [47]; and silencing *RAD9* expression by RNA interference inhibits its proliferation *in vitro* [47], leading to unscheduled replications after DNA damage. Such conditions contribute to pathology in carcinogenesis or tumor progression.

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