A role for the arginine methylation of Rad9 in checkpoint control and cellular sensitivity to DNA damage

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Received May 21, 2010; Revised November 18, 2010; Accepted November 22, 2010

ABSTRACT

The genome stability is maintained by coordinated action of DNA repairs and checkpoints, which delay progression through the cell cycle in response to DNA damage. Rad9 is conserved from yeast to human and functions in cell cycle checkpoint controls. Here, a regulatory mechanism for Rad9 function is reported. In this study Rad9 has been found to interact with and be methylated by protein arginine methyltransferase 5 (PRMT5). Arginine methylation of Rad9 plays a critical role in S/M and G2/M cell cycle checkpoints. The activation of the Rad9 downstream checkpoint effector Chk1 is impaired in cells only expressing a mutant Rad9 that cannot be methylated. Additionally, Rad9 methylation is also required for cellular resistance to DNA damaging stresses. In summary, we uncovered that arginine methylation is important for regulation of Rad9 function, and thus is a major element for maintaining genome integrity.

INTRODUCTION

DNA repair and cell cycle checkpoint cooperate in minimizing the DNA damage constantly caused by intra- and environmental genotoxic stresses and maintaining genomic integrity. Mutation in genes functioning in these two systems often leads to ‘mutator’ phenotype and enhances susceptibility to tumor development (1). Rad9 is conserved from yeast to human, and is critical for both DNA repair and cell cycle checkpoint control (2,3). Rad9 is required for homologous recombination, base excision and mismatch repairs (4–6), and for G2/M and S/M checkpoint activation (7,8).

Protein arginine methylation is a post-translational modification that results in symmetrical or asymmetrical dimethylarginines (9). Protein arginine methyltransferases (PRMTs) are classified as types I, II, III or IV enzymes. Types I, II and III PRMTs methylate terminal (or α) guanidino nitrogen atoms. Both type I and type II enzymes catalyze the formation of a mono-methylated (MMA) intermediate, subsequently type I PRMTs (PRMT1, 3, 4, 6 and 8) further catalyze the generation of asymmetrical double-methylated arginine (aDMA), whereas type II PRMTs (PRMT5, PRMT7 and FBXO11) catalyze the formation of symmetrical double-methylated arginine (sDMA) (10). Both type I and II enzymes regulate gene transcription via methylating histones, and other cellular activities through methylating non-histone proteins.

Several proteins involved in DNA repair (MRE11, p53, DNA polymerase β) have been shown to be regulated by arginine methylation (11). In this study, we identified a few Rad9-associated proteins by combining immunoprecipitation and mass spectroscopy, one of these proteins was PRMT5 (12–14). It is of interest that both human and mouse Rad9 contains a methylation consensus amino acid sequence RGRR. We found that Rad9 form a complex with PRMT5, and PRMT5 can methylate Rad9 at the RGRR sequence. The methylation is critical for cellular resistance to hydroxyurea, and for S/M and G2/M checkpoint activation.

MATERIALS AND METHODS

Mass spectrometry

HEK 293T cells stably expressing FL-hRad9 at a level close to the endogenous hRad9 expression level were used for affinity-immunoprecipitation of hRad9-interacting proteins. The procedure for identifying these
proteins by mass spectrometry has been described previously (13).

**Antibodies, immunoprecipitation and western blotting**

Anti-hRad9 polyclonal antibody was obtained by immunizing mice with purified MBP-hRad9 protein and anti-hRad9 monoclonal antibody (611324) was purchased from BD. Anti-PRMT5 rabbit polyclonal antibody (07-405) was from Millipore, and ab412 (anti-mono/dimethylarginine antibody) was from Abcam. Anti-FLAG M2 monoclonal antibody (F7425) and FLAG peptide were obtained from Sigma-Aldrich, and anti-HA antibody was obtained from Santa Cruz Technology. Phospho-Chk1 (P-Ser-345) and Chk1 (G-4) antibodies were purchased from Cell Signaling Technology and Santa Cruz Biotechnology, respectively. Immunoprecipitation and western blotting were performed as described previously (15).

**Gene subcloning**

pFLAG-CMV2-hRad9, pFLAG-CMV2-hRad1, pET32 (+)-hRad9 and pcDNA3-6HA-hRad9 plasmids have been described previously (16). Full-length human PRMT5 sequence was amplified by PCR using cDNA from HEK 293T cells and cloned into pFLAG-CMV2 and pcDNA3-6HA, respectively. pGEX-6P-1-PRMT5 was constructed by excising from pFLAG-CMV2-PRMT5 and ligating into the EcoRI site of pGEX-6P-1. PCR site-directed mutagenesis was performed using the Quick-Change method (Stratagene). pFLAG-CMV2-hRad9-3RK was constructed by overlap PCR using pFLAG-CMV2-hRad9. All constructs generated using PCR were confirmed by sequencing. The primers and restriction sites used are shown in Supplementary Table S1.

**RNA interference**

To generate the PRMT5 ShRNA vector, oligonucleotides (5’-GATCCCCGCGATATGATCGTTCAAGAGACAGACATTTATAGATGGCC-3’ and 3’-GGGCCGGTAGATATTTACAGCAAGTTCTCTGCTTGAAATATCACCAGAAAATTCGAG-5’) were designed to target PRMT5 nucleotides 1016–1034 (shown in boldface). Oligonucleotides were annealed and cloned into the Bgl II and Hind III sites of the pSUPERpuro vector. HCT116 cells were transfected with the pSUPERpuro PRMT5 vector or an empty pSUPERpuro vector. Cells were treated with 1 μg/ml puromycin for 3 days to eliminate cells without ShRNA.

**Cell culture**

Mouse ES cells, HeLa cells, HCT16 and HEK 293T cells were cultured according to previously published methods (7,13).

**Expression of wild-type and mutant hRad9 in mRad9−/− ES cells**

For R to A mutation analysis, ES cells were transfected using Lipofectamine with pZeoSV2-hRad9 or pZeoSV2-hRad9-3RA according to the manufacturer’s instructions (Invitrogen). Stable clones were selected in medium containing zeocin at a concentration of 100 μg/ml. Selected transfectants were subsequently cultured at 25 μg/ml zeocin to maintain the transfected genes within the cells. Multiple clones of each mutant expressing similar levels of protein were used for functional studies. For R to K mutation analysis, ES cells were transfected using Lipofectamine with pcDNA3.1-hygromycin (1 μg) and pFLAG-CMV2-hRad9-3RK (3 μg) or pFLAG-CMV2-hRad9 according to the manufacturer’s instructions (Invitrogen). Stable clones were selected in medium containing hygromycin at a concentration of 150 μg/ml. Selected cells were subsequently cultured at 50 μg/ml hygromycin to maintain the transfected genes within the cells. Multiple clones of each mutant expressing protein levels similar to the endogenous protein were used for functional studies.

**GST pull-down**

GST fusion proteins were expressed in cells of the *Escherichia coli* strain Rosetta (Invitrogen) using pGEX-6P-1 (GE Health). Purification of GST fusion proteins and *in vitro* GST pull-down test has been described previously (13).

**ES cell survival and cell cycle checkpoint assays**

Mouse ES cells were seeded in duplicate at designated numbers onto 60-mm gelatinized tissue culture dishes. Sensitivity to various doses of hydroxyurea (HU), and 60Co γ rays was tested using a previously published procedure (7). Assays for detecting G2/M checkpoint control and S/M checkpoint function of mouse ES cells were performed as described previously (7,13).

**In vitro methyltransferase assays**

HEK 293T cells were transfected with the plasmid pFLAG-CMV2-PRMT5, and FL-PRMT5 protein was immunoprecipitated with anti-FLAG agarose beads 24 h after transfection. Purified GST-hRad9 or GST-hRad9-3RA (2 μg) were incubated with immunoprecipitated FL-PRMT5 in the presence of 0.55 μCi of [methyl-3H] AdoMet (Amersham Biosciences) in 25 mM Tris–HCl at pH 7.5 in a final volume of 30 μl for 60 min at 37°C, histones (sigma) were introduced as a positive control. Reactions were stopped by adding 6 μl of 5× SDS–PAGE sample buffer, followed by heating at 100°C for 5 min. The samples were separated on 10% SDS–PAGE and stained with Coomassie blue. Destained gels were dried and exposed to X-ray film at −70°C for 14 days.

**RESULTS**

**hRad9 associates with PRMT5 in cells**

In the previous studies, using a strategy of immunoaffinity-Mass spectrometry, we found that hRad9 interacts with several proteins in HEK 293T cells, in which mismatch repair protein MLH1 was confirmed to be associated physically and functionally with hRad9 (13).
The analysis of Mass spectrometry shows that PRMT5, a key member of PRMTs (12–14), is also among the proteins associated with FL-hRad9 but not in the proteins associated with negative control FL-GFP (Figure 1A and Supplementary Table S2). Interestingly, hRad9 contains the glycine-arginine rich sequence (GAR) RGRR that exists in other proteins which have been shown in previous studies to be methylated by PRMTs (17). Therefore we chose to further characterize the interaction of hRad9 with PRMT5.

We used co-immunoprecipitation to confirm the interaction between hRad9 and PRMT5. The assay showed that overexpressed HA-tagged hRad9 (HA-hRad9) interacted with FLAG-tagged PRMT5 (FL-PRMT5) in HEK 293T cells, and did not interact with the negative control FL-GFP (Figure 1B). Importantly, endogenous PRMT5 and hRad9 were immunoprecipitated from HeLa cell extract by an anti-hRad9 polyclonal or anti-PRMT5 antibody, but not by pre-immune serum (Figure 1C-D). The data above indicate that hRad9 associates with PRMT5 in cells.

**hRad9 is arginine-methylated by PRMT5**

The above data have established that hRad9 interacts with PRMT5. Interestingly the hRad9 protein harbors a sequence RGRR (Figure 2A), the typical methylation target for PRMTs (18–21). The RGRR sequence is conserved among humans, monkeys, mice and rats, but only GRR is conserved in horses, calves and dogs, and the sequence is not conserved in frogs and other lower animals. Here we examined whether hRad9 is arginine-methylated. A pcDNA3-HA plasmid harboring wild-type hRad9 or mutated hRad9 in which Arg-172,174,175 were all mutated to Ala (hRad9-3RA) was transfected into HEK 293T cells. The transfected cell lysates were immunoprecipitated with anti-mono/dimethylarginine antibodies (ab412), the immunoprecipitated proteins were fractionated by SDS-PAGE and monitored with anti-HA antibody. As shown in Figure 2B, HA-hRad9 was clearly detected in proteins immunoprecipitated by ab412, while HA-hRad9-3RA could not be detected. These results indicate that hRad9 is methylated in vivo.

Having shown that hRad9 is methylated on the arginine residues in the arginine-rich motif, and that hRad9 interacts with PRMT5, we reasoned that PRMT5 might methylate hRad9. To test this hypothesis, an in vitro methylation assay was performed using [3H]-AdoMet as a methyl donor. GST-hRad9 (arrow indicated) was clearly methylated by FL-PRMT5 immunoprecipitated from HEK 293T cells overexpressing FL-PRMT5 (lane 2 in Figure 2C). As a positive control, core histones were also methylated by FL-PRMT5 (22) (lane 1 in Figure 2C).

![Figure 1](https://academic.oup.com/nar/article-abstract/39/11/4719/1135063)
Each of the three arginine residues in the arginine-rich stretch is a potential target of methyltransferase enzymes. To evaluate the arginine residues responsible for hRad9 methylation, we generated a series of mutants within the arginine-rich stretch by substituting the corresponding arginines with alanine. The mutated hRad9s were cloned into the pcDNA3-6HA plasmid and tested for their ability to serve as methyltransferase substrates in the in vitro assay system. None of the single arginine mutations or double-arginine mutations combinations significantly reduced hRad9 methylation, while the mutation of all three arginines abolished methylation of hRad9 (Figure 2D and E). We conclude that the three arginine residues (Arg172, Arg174 and Arg175) within the arginine-rich region of hRad9 are all methylated in vivo.

We have found that hRad9 is methylated in vivo and can be methylated by PRMT5 in vitro. To determine whether PRMT5 is the physiological enzyme methylating hRad9, we knocked down PRMT5 in HCT116 cells. Transfection of PRMT5 ShRNA reduced its protein level by ~80% (Figure 3A), and this lower level of PRMT5 correlated with a dramatic reduction in the hRad9 methylation level (Figure 3B), suggesting that PRMT5 is the main enzyme for hRad9 methylation in cells.

As hRad9 is critical for DNA damage repair and cell cycle control, we want to know whether DNA damage affects hRad9 methylation. To test this, HeLa cells were mock- or HU-treated for 24 h, then the cells were lysed and immunoprecipitated with ab412, and probed with anti-hRad9 monoclonal antibody. In HeLa cells, endogenously methylated hRad9 was increased after cells were treated with HU (Figure 3C), indicating that methylation of hRad9 is DNA damage dependent.

Methylation of hRad9 regulates cellular sensitivity to DNA damage.

It has been reported that phosphorylation of hRad9 at multiple amino acid residues influences cell sensitivity to the replication inhibitor HU, and S/M and G2/M checkpoint controls following genotoxin treatment (23–25). Since hRad9 is methylated on its arginine-rich motif, and methylation of hRad9 is DNA damage-dependent, we asked whether the arginine-rich domain is important for the known functions of hRad9 in DNA damage. We therefore established mRad9+/− ES cell clones stably expressing wild-type hRad9 and hRad9-3RA at levels equivalent to the endogenous mRad9 level in mRad9+/− cells (Supplementary Figure S2). First, we tested the influence of hRad9 methylation on cell survival against HU and γ rays. The mRad9+/− ES cells expressing hRad9-3RA were significantly more sensitive to HU than mRad9+/− cells and the mRad9+/− ES cells ectopically expressing hRad9. The hRad9-3RA-expressing mRad9+/− ES cells were equally sensitive to HU as the mRad9+/− ES cells at a low dose (100 μM), and were slightly more sensitive than the mRad9+/− ES cells at higher doses (250 and 500 μM) (Figure 4A). In contrast, mRad9+/− cells expressing hRad9-3RA exhibited equal sensitivity to γ rays at low doses (~6 Gy) and only moderately higher sensitivity to 8 Gy compared to mRad9+/− and mRad9−/− cells.
expressing wild-type hRad9 (Figure 4B). Collectively, our results indicate that hRad9 methylation plays important roles in the cellular response to HU, but a minor role in cellular resistance to ionizing radiation. Next, we performed a colony-formation assay to determine the effect of PRMT5 knock-down on cellular sensitivity to HU. HCT116 cells in which PRMT5 is knocked down are more sensitive than cells expressing control ShRNA (Figure 4C), suggesting that PRMT5 methylation of the three arginines in hRad9 is required for cellular resistance to DNA damage.

Loss of hRad9 methylation leads to S/M and G2/M checkpoint defects

It has been reported that hRad9 is critical in S/M and G2/M checkpoint controls (7,25). Here, we tested whether hRad9 methylation plays roles in these checkpoints. The classical cell cycle checkpoint analysis was introduced to test S/M and G2/M checkpoint controls (7,13). To test the G2/M cell cycle checkpoint, four types of cells (wild-type, mRad9<sup>−/−</sup>, mRad9<sup>−/−</sup> cells expressing wild-type hRad9 and mRad9<sup>−/−</sup> cells expressing hRad9-3RA) were mock irradiated or exposed to 6 Gy of γ rays. At various post-irradiation times, the cells were fixed and examined with flow cytometry. Another set of cells was treated with colcemid immediately after radiation exposure and harvested 12 h after irradiation. As shown in Figure 5A and B, at 8 h and 12 h after exposure to 6-Gy γ rays, more cells expressing hRad9-3RA accumulated in the G1 and S phase (arrow) than cells expressing wild-type hRad9, and difference between the two types of cells above in G1 phase is statistically significant (Figure 5B). mRad9<sup>−/−</sup> and mRad9<sup>−/−</sup> cells were used as negative and positive controls, respectively. A similar G2/M checkpoint deficient result of mRad9 knockout was reported previously by Hopkins et al. (7,26). As all the cells treated with colcemid were blocked in G2/M at 12 h after radiation exposure, these results suggest that unmethylatable hRad9-3RA leads to G2/M checkpoint deficiency.

To test the effect of the Rad9 methylation on S/M checkpoint, the four types of cells used above were mock-treated or treated with 1 mM HU for 8 h to monitor S/M cell cycle checkpoint status. HU treatment activated S/M checkpoint and arrested mRad9<sup>+/+</sup> cells in the S phase, while cells with the Rad9 deletion exhibited S/M checkpoint deficiency and were not blocked in the S phase (4.99% cells entered the M phase without completing DNA replication). S/M checkpoint deficiency was also observed in mRad9<sup>−/−</sup> cells expressing hRad9-3RA; a higher percentage of cells (0.78%) expressing the mutant hRad9 entered the M phase without DNA replication than was the case for normal cells (0.26%) (Figure 5C). A statistical analysis shows that the difference is significant (Figure 5D), demonstrating that methylation of hRad9 plays an important role in S/M checkpoint control. The effect of mutation of the three arginine residues on the S/M checkpoint, although

Figure 3. Knock down of PRMT5 influences the arginine methylation of hRad9. (A) PRMT5 is knocked down in HCT 116 cells transiently expressing PRMT5 ShRNA. Levels of PRMT5 and GAPDH were assayed in HCT116 cells expressing PRMT5 ShRNA or control ShRNA. (B) Knockdown of PRMT5 reduces the arginine methylation of hRad9. HCT116 cells expressing PRMT5 ShRNA or control ShRNA were transfected with pFLAG-CMV2-hRad9 and arginine methylation of hRad9 was detected in these cells. HCT116 cells expressing FL-hRad9-3RA were used as a negative control. (C) Methylation of hRad9 is DNA damage dependent. HeLa cells were mock treated or treated with 0.5 mM or 1 mM HU for 24 h. Ten percent cells were lysed as input, the rest cells were lysed and immunoprecipitated with ab412 antibody and then immunoblotted with anti-hRad9 monoclonal antibody.
of four types of cells (activation, we treated whether hRad9 methylation also plays a role in Chk1 activation (25). In order to test whether hRad9 methylation is required for genotoxin-induced Chk1 activation (27,28), and that hRad9 phosphorylation is phosphorylation on Ser-345, which is essential for Chk1 activation (1,13,32,33), DNA repair (homologous recombination, base excision and mismatch repairs) as well as apoptosis (1,13,32,33), it is conceivable that the various functions of hRad9 are regulated through phosphorylation on multiple sites by multiple kinases. It is also not surprising that its activities leads to slight but statistically significant enhancement of G2/M checkpoint controls, and three different forms of sensitivity to DNA damage and Chk1 activation) are similar to those of hRad9-3RA. Therefore, we conclude that methylation, but not the charge, is important for Chk1 activation and resistance to DNA damage.

**DISCUSSION**

In this study we have documented that Rad9 is methylated on arginines of its RGRR amino acid sequence stretch by PRMT5 (Figure 2), and the methylation is critical for downstream signaling from hRad9 to Chk1 in response to genotoxic stresses. Our results on IR sensitivity (Figure 4B) and Chk1 phosphorylation (Figure 6A and B) indicate that Chk1 activation (a strong effect) by Rad9 is largely unrelated to IR sensitivity (a weak effect), consistent with a previous report (25).

Mutation of Arg-172,174,175 on hRad9 to Lys (hRad9-3RK) shows the similar phenotype as to Ala

As arginine carries positive charge and the charge of Arg 175 is completely conserved (Arg or Lys) across the species from tunicates to humans, suggesting the importance of this arginine on hRad9 function. To avoid changing the positive charge in the study of the methylation role on hRad9 function, we mutated the three Args to Lys and introduced the mutated hRad9 (hRad9-3RK) into mRad9−/− cells to study the known function of hRad9 in DNA damage resistance and cell cycle checkpoint activation. As seen in Supplementary Figure S4, the phenotypes (cell cycle checkpoint control, cell sensitivity to DNA damage and Chk1 activation) are similar to those of hRad9-3RA. Therefore, we conclude that methylation, but not the charge, is important for Chk1 activation and resistance to DNA damage.

**hRad9 methylation is required for genotoxin-induced Chk1 activation**

It has been reported that both replication inhibitors and γ-rays irradiation trigger ATR-dependent Chk1 phosphorylation on Ser-345, which is essential for Chk1 activation (27,28), and that hRad9 phosphorylation is required for Chk1 activation (25). In order to test whether hRad9 methylation also plays a role in Chk1 activation, we treated mRad9+/+, mRad9−/−, mRad9−/− expressing wild-type hRad9, and mRad9−/− expressing hRad9-3RA with 1 mM HU for 12 h or 10 Gy of γ rays. Both treatments resulted in Chk1 Ser-345 phosphorylation in mRad9+/+ cells and mRad9−/− cells which express wild-type hRad9. However, the phosphorylation induced was eliminated in mRad9−/− cells and dramatically reduced in mRad9−/− cells expressing hRad9-3RA (Figure 6A and B), indicating that hRad9 methylation on the arginine-rich motif is required for downstream signaling from hRad9 to Chk1 in response to genotoxic stresses. Our results on IR sensitivity (Figure 4B) and Chk1 phosphorylation (Figure 6A and B) indicate that Chk1 activation (a strong effect) by Rad9 is largely unrelated to IR sensitivity (a weak effect), consistent with a previous report (25).

**DISCUSSION**

In this study we have documented that Rad9 is methylated on arginines of its RGRR amino acid sequence stretch by PRMT5 (Figure 2), and the methylation is critical for cell sensitivity to HU (Figure 4A), and for S/M and G2/M (Figure 5) cell cycle checkpoint activation.

Human hRad9 is highly phosphorylated constitutively and inductively (29). Both Tyr28 and Ser387 are required for checkpoint activation (26). Tyr28 phosphorylation is carried out by c-Abl tyrosine kinase in response to DNA damage, and required for the interaction between hRad9 and Bcl-2 (30). hRad9 Ser272 was reported to be phosphorylated by ATM and it was shown that the overexpression of hRad9 (Ser272Ala) sensitized cells to γ rays slightly. However, a more detailed characterization later did not confirm the sensitization by the mutation. The Ser328 of hRad9 can be phosphorylated by Tousled-like kinase TLKB and the Ser328Ala mutation leads to slight but statistically significant enhancement of sensitivity to γ rays (31). hRad9 being involved in S/M and G2/M checkpoint controls, and three different forms of DNA repair (homologous recombination, base excision and mismatch repairs) as well as apoptosis (1,13,32,33), it is conceivable that the various functions of hRad9 are regulated through phosphorylation on multiple sites by multiple kinases. It is also not surprising that its activities
are modulated by other post-translational modifications such as methylation shown in this study.

Cellular sensitivity to HU is most severely affected by mutating the three arginines in RGRR methylation site into alanines (Figure 4A), but only moderately changed cellular sensitivity to γ rays at high dose (Figure 4B). Similar phenotypes were also observed when all the eight phosphorylation sites of the hRad9 C-terminus are altered (25). The hRad9 C-terminus stretches out of the ring formed by Rad9, Rad1 and Hus1. The RGRR methylation site (amino acids 172–175) is located on the ring. The hRad9 Tyr28 is also situated on the 9-1-1 ring (Figure 6D) and critical for HU induced S/M checkpoint activation. That the three separate sites are all needed in response to HU treatment suggests that different portions of hRad9 coordinate the events critical for managing the disturbance caused by HU. In contrast to the response to HU treatment, the mutations on the C-terminus and RGRR cause very slight changes in cellular resistance to γ rays irradiation is not overlapped with those of the response to HU treatment. The γ rays cause DNA double-strand breaks, and two studies demonstrated that Rad9 and its partner Hus1 in the 9-1-1 complex repaired double-strand breaks by homologous recombination (4,34). Further study will be needed to map the region(s) of the Rad9 protein that is responsible for the recombin-ation repair function.

To understand the functional mechanism of Rad9 methylation in cell cycle checkpoint activation and DNA damage response, we have investigated the roles of Rad9 methylation in the formation of the 9-1-1 complex, DNA damage induced Rad9 association onto chromatin, but Rad9 with mutations on all the three arginines in the RGRR sequence showed no effect on the 9-1-1 complex formation (Supplementary Figure S1) and DNA damage induced Rad9 association onto chromatin (Supplementary Figure S3). It is worth noting that we used overexpressed, instead of endogenous, Rad1 and Hus1 to test the effect of the arginines methylation on the 9-1-1 complex formation.

Figure 5. Deficiency of hRad9 methylation leads to S/M and G2/M checkpoint control defects. (A) The lack of hRad9 methylation affects ionizing radiation-induced G2 arrest. The four types of mouse ES cells (mRad9+/+, mRad9+/−, mRad9−/−, cells expressing wild-type hRad9 and mRad9−/− cells expressing hRad9-3RA, respectively) were mock-treated or irradiated with 6 Gy of γ rays in the absence or presence of colcemid. Regions of the profiles corresponding to G1, S or G2/M are delineated above the first row of graphs, and the ratio of cells in G1, S or G2/M phase were shown. (B) Statistics analysis of the relative cell number in G1 phase out of three independent experiments in (A). Double asterisks indicate extremely significant difference (P < 0.01) and asterisk indicates significant (P < 0.05). (C) Lack of hRad9-methylation results in the S/M checkpoint control defect. The four types of cells were treated or mock-treated with 1 mM HU for 8h. Cells were collected and labeled with the mitotic marker phosphor-histone H3 antibody, stained with propidium iodide, and analyzed by flow cytometry. Staining intensity for PI (x-axis) is plotted versus that for phosphor-histone H3 (y-axis). The cells in the boxed region are premature mitotic cells. Numbers above the box are the percentage of the cells boxed in the total cells. (D) Statistics analysis of relative premature mitotic cells derived from three independent experiments described in (C). In (B) and (D), Double asterisks indicate extremely significant difference (P < 0.01) and asterisk indicates significance (P < 0.05).
because proper anti-Rad1 and anti-Hus1 are not available, thus small effect of the Rad9 methylation on the 9-1-1 complex formation is still possible.

We noticed that knocking down PRMT5 did sensitize HCT116 to HU but the sensitized extent was less than the mutations of the arginines on Rad9 using mouse ES cells (Figure 4A and C). We also found that knocking down PRMT5 did not impair S/M checkpoint activation in HCT116 cells (data not shown). These differences may reflect the differences of these two cell types. Indeed, untreated HCT116 cells were much more resistant to HU than wild-type mouse ES cells (Figure 4A and C).

A large body of evidence demonstrates that the phosphorylation/dephosphorylation of proteins functioning in cell cycle checkpoint controls and DNA damage repair plays an essential role in orchestrating molecular events required for maintaining genome integrity (35,36). Arginine methylation of histones has been demonstrated to be critical in the regulation of transcription induction/repression and chromatin remodeling, and arginine methylation of other cellular proteins is also emerging to play important roles in other cellular events including DNA damage repair and cell cycle checkpoint controls (10,37). DNA polymerase β methylation by PRMT6 strongly stimulates the activity of this enzyme and is required for efficient DNA base excision repair (22). MRE11 methylation by PRMT1 and p53 methylation by PRMT5 play important roles in G1/S and intra-S phase checkpoint controls, respectively (18,38). In this study we have documented that Rad9 is methylated on the arginines in its RGRR amino acid sequence stretch by PRMT5, and that this methylation is critical for cellular resistance to DNA damage caused by HU, and for S/M and G2/M cell cycle checkpoints activation. Therefore, protein arginine methylation is important for the activation of at least four of the five major cell cycle checkpoints; the role of arginine methylation in spindle checkpoint control remains to be tested. Although the DNA repairs behind the hRad9 methylation-conferred cellular resistance to HU treatment have not been identified, hRad9 has been shown to play important roles in DNA base excision, mismatch and homologous recombination repairs, and these repairs have been shown to be at least partially responsible for resistance to HU treatment (1,39). In summary (Figure 6C), in addition to protein phosphorylation, protein arginine methylation has emerged as another major factor critical for cell cycle checkpoint controls and DNA damage repairs, and thus for maintaining genome integrity.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

FUNDING

National Natural Science Foundation of China 30530180, National Protein Project of Ministry of Science and Technology 2006CB910902, Knowledge Innovation Program of Chinese Academy of Sciences KSCX2-YW-R63, National Science and Technology Special Project of Major New Drugs Creation 2009ZX09501-025.

Conflict of interest statement. None declared.

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