ATM-dependent Phosphorylation of Human Rad9 Is Required for Ionizing Radiation-induced Checkpoint Activation

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ATM (ataxia-telangiectasia-mutated) is a Ser/Thr kinase involved in cell cycle checkpoints and DNA repair. Human Rad9 (hRad9) is the homologue of Schizosaccharomyces pombe Rad9 protein that plays a critical role in cell cycle checkpoint control. To examine the potential signaling pathway linking ATM and hRad9, we investigated the modification of hRad9 in response to DNA damage. Here we show that hRad9 protein is constitutively phosphorylated in undamaged cells and undergoes hyperphosphorylation upon treatment with ionizing radiation (IR), ultraviolet light (UV), and hydroxyurea (HU). Interestingly, hyperphosphorylation of hRad9 induced by IR is dependent on ATM. Ser272 of hRad9 is phosphorylated directly by ATM in vitro. Furthermore, hRad9 is phosphorylated on Ser372 in response to IR in vivo, and this modification is delayed in ATM-deficient cells. Expression of hRad9 S272A mutant protein in human lung fibroblast VA13 cells disturbs IR-induced G1/S checkpoint activation and increased cellular sensitivity to IR. Together, our results suggest that the ATM-mediated phosphorylation of hRad9 is required for IR-induced checkpoint activation.

In eukaryotic cells, DNA damage and stalled DNA replication forks activate evolutionarily conserved checkpoint pathways, resulting in a delay in cell cycle progression, initiation of DNA repair process, and transcriptional regulation of specific genes (for reviews, see Refs. 1–4). These checkpoint controls prevent damaged DNA from being replicated or distributed into daughter cells prior to the completion of DNA repair, thus helping to maintain genomic integrity. Failure of this cell cycle surveillance mechanism can cause genomic instability that eventually leads to cancer formation in mammals (5, 6).

The DNA damage checkpoint pathways are conserved among Saccharomyces cerevisiae, Schizosaccharomyces pombe, Drosophila melanogaster, Caenorhabditis elegans, and mammals (7–9). In S. pombe, the protein products of the six checkpoint rad genes (rad1, rad3, rad9, rad17, rad26, and hus1) play crucial roles in sensing changes in DNA structure. All of the six rad genes are required for checkpoint activation (for reviews, see Refs. 10–12). Genetic studies have placed the six checkpoint rad genes in the same signaling pathways that monitor DNA damage and stalled replication forks (13). Cells with mutations in any one of these genes are hypersensitive to both replication blocks- and DNA damage-causing agents in all cell cycle phases (14), suggesting that these genes are involved in all cell cycle checkpoints. Rad1 is a putative exonuclease (15) that forms a stable protein complex with Rad9 and Hus1 (16). Rad17 transiently interacts with the Rad1-Rad9-Hus1 complex (16, 17). There is a stable interaction between Rad3 and Rad26 (18). The finding that Rad3, but not other checkpoint Rad proteins, is required for DNA damage-induced phosphorylation of Rad26 suggests that the Rad3-Rad26 complex may be the “first sensor” of DNA damage (18). Importantly, all six checkpoint Rad proteins are required for DNA damage-induced phosphorylation of two downstream checkpoint protein kinases, Cds1 and Chk1 (19, 20). Phosphorylation of Cds1 correlates with its biological activity (19). These studies have provided a framework for understanding the cell cycle checkpoint signal pathways.

Mammalian counterparts of all the checkpoint rad genes except rad26 have been identified (21–27). The hRad9, hRad1, and hRad17 genes partially complement the DNA damage sensitivity of the counterpart yeast mutants (24, 27, 28), reinforcing the functional similarities between the human and yeast genes. Similar to their yeast homologues, hRad1, hRad9, and hHus1 form a stable complex (29–31). The exonuclease activity of both hRad9 and hRad1 has also been demonstrated (32, 33). The hRad9-hRad1-hHus1 complex may associate with chromatin upon DNA damage (34). Murine cells lacking Hus1 and nematode cells lacking MRT-2 (Rad1) have increased genomic instability and cellular sensitivity to DNA damage (35–37). To date, two related mammalian kinases, ATM (ataxia-telangiectasia mutated) and ATR (ataxia-telangiectasia and Rad3-related) have been shown to share structural and functional similarities with Rad3 in S. pombe (21). ATM was identified as the gene mutated in the ataxia-telangiectasia (A-T) patients (38). Cells established from A-T patients are hypersensitive to IR but not UV or HU. They are defective in multiple cell cycle checkpoints, including G1/S, G2/M. A-T cells exhibit radioresistant DNA synthesis, likely equivalent to the DNA damage-induced S phase checkpoint defect (reviewed in Ref. 39). Similarly, ATR regulates G1/S, G2/M checkpoint controls in response to DNA damage and a block in DNA replication, thus demonstrating functionally similarity to Rad3

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1 The abbreviations used are: ATM, ataxia-telangiectasia-mutated; ATR, ataxia-telangiectasia- and Rad3-related; HU, hydroxyurea; IR, ionizing radiation; hRad, the human Rad proteins; GST, glutathione S-transferase; CIAP, calf intestinal alkaline phosphatase; PAGE, polyacrylamide gel electrophoresis; Gy, gray(s); NBS, Nijmegen breakage syndrome; BrdUrd, bromodeoxyuridine; EGFP, enhanced green fluorescent protein.
in *S. pombe* (40–42). ATM, ATR, and Rad3 are members of a Ser/Thr kinase family that contains a serine/threonine kinase domain closely related to the phosphatidylinositol 3-kinase at the carboxy termini. ATM acts upstream of Chk2 (43), the human homologue of Cds1 in *S. pombe*. ATR operates upstream of Chk1 in response to UV (44). In mammalian cells, Chk1 and Chk2 phosphorylate Cdc25 phosphatase, resulting in the nuclear export of Cdc25 and inhibition of Cdc2 kinase activity (1, 45–47). Inactivation of Cdc2 eventually leads to cell cycle arrest at the G2/M boundary (48). Taken together, these studies highlight the evolutionary conservation of the checkpoint signaling pathways.

In addition to the genes described, ATM-mediated signaling pathways involve tumor suppressor genes such as p53, BRCA1, and *NBS1* (49–55). Molecular details of how ATM regulates these tumor suppressors have been described, but whether ATM regulates human checkpoint Rad proteins is unknown. Here, we investigate the regulation of hRad9 by phosphorylation. We demonstrate that ATM-mediated phosphorylation of hRad9 is critical for ionizing radiation-induced checkpoint activation.

**EXPERIMENTAL PROCEDURES**

**Antibodies**—Glutathione S-transferase (GST) and full-length hRad9 or hRad9224–395 fusion proteins were overexpressed in *Escherichia coli* and purified by affinity chromatography following the manufacturer’s instructions (Amersham Pharmacia Biotech). BALB/c mice were immunized with GST-hRad9 or GST-hRad9224–395 according to the standard procedure (56). The rabbit a-phospho-Ser272 antibodies were raised against the keyhole limpet hemocyanin-conjugated hRad9 peptide, CSDTDSHS(PO3)QDLG. The flow-through fractions of the antiserum in a CSDTDSHSGDLQ column were affinity-purified using the phosphopeptide column to eliminate antibodies reacting with the nonphosphorylated antigen peptide and nonspecific antigens. Antibodies against β-actin were from Sigma. Rabbit a-FLAG hRad9 polyclonal antibodies were purchased from Santa Cruz Biotechnology. α-ATM monoclonal antibodies were as described previously (57).

**Immunoblotting and Immunoprecipitation**—Cells were lysed in ice-cold lysis buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40) supplemented with protease inhibitors (10 μg ml−1 aprotinin, 1 mg ml−1 leupeptin, 10 μg ml−1 o-phenanthroline) and phosphatase inhibitors (100 mM NaF and 1 mM Na3VO4). Cell lysates containing 20–50 μg of protein, as determined by Bradford procedure (56), were subjected to SDS-7.5% polyacrylamide gel electrophoresis (SDS-PAGE) followed by immunoblotting with mouse anti-ATM antibody (3E8). Recombinant FLAG-His-ATM was immunoprecipitated from detergent lysates of the human bladder carcinoma T24 cells with anti-FLAG M2 mouse monoclonal antibody (Sigma). The mass analyses were conducted as described previously (58). Briefly, the ATM protein in the protein G-Sepharose beads was incubated with GST-hRad9 fusion protein (2–10 μg) in kinase buffer (50 mM Hepes, pH 7.5, 1.5 mM MgCl2, 10% glycerol, 1 mM dithiothreitol, 4 mM MnCl2, 6 mM MgCl2, 100 μM NaVO4, 20 μM ATP, and 10 μCi of [γ-32P]ATP) at 30 °C for 30 min. The reaction was stopped by addition of 4× SDS loading buffer. Proteins were separated by SDS-PAGE and analyzed after Coomassie Blue staining and autoradiography.

**Plasmid Construction**—pcMV2-FLAG-hRad9 was constructed by subcloning the full-length hRad9 fragment generated by polymerase chain reaction using pHRAD9-1 cDNA as template (24) into a BamHI-XbaI-linearized pFLAG-CMV-2 (Kodak) vector. The hRAD9 EcoRI-XhoI fragment from pGEX-5X-3-hRad9 was subcloned into pGEX-4T-1 to generate pGEX-4T-1-hRad9224–395. Both pGEX-5X-3-hRad9 and pGEX-4T-1-hRad9224–395 were used to produce GST fusion proteins for immunization. pcMV-FLAG-His-ATMWT and pcMV-FLAG-His-ATM60270A expression vectors were as described previously (55). pEGFP-N1 was purchased from CLONTECH, Inc.

**Cell Culture, Transfection, and Treatments for DNA Damage Induction**—EB5 (ATM-deficient) and Y5T (ATM-complemented) cell lines were kindly provided by Y. Shiloh. NBS1-LBI cells are SV40 T antigen-immortalized fibroblasts established from NBS patients (59). All other cell lines were from the American Type Culture Collection. Cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (Life Technologies, Inc.). For cell cycle studies, human bladder carcinoma T24 cells were synchronized by density arrest. Extracts were prepared from cells at different cell cycle stages as described previously (60). Transfections were performed using LipofectAMINE (Life Technologies, Inc.) according to the manufacturer’s instructions. IR treatment was performed using a 170 kV Cs γ-iradiator (Shepherd) at 2.55 Gy/min. UV irradiation (as described previously) using UV Stratosource (Cytotoxic Research) was added to cell culture medium at a final concentration of 1 or 3 μM for 24 h. Extracts were prepared from mock- or radiation-treated cells 1 h post-treatment unless otherwise indicated.

**DNA Damage-induced G1/S Checkpoint**—Cells co-transfected with pEGFP and pcMV2-FLAG-hRad9 or pcMV2-FLAG-hRad9WT/DOPE (1:1 ratio of EGFP to FLAG-hRad9) were exposed to 20 Gy of γ-rays. After IR treatment, cells were immediately incubated in fresh culture medium containing 10 μM BrdUrd for 2 h followed by immunostaining with α-BrdUrd monoclonal antibody (Becton-Dickinson). The percentage of EGFP/BrdUrd double-positive cells among the EGFP-positive population before and after IR was determined (55).

**Cellular Sensitivity to Ionizing Radiation**—Cells were co-transfected with pEGFP and pcMV2-FLAG-hRad9 or pcMV2-FLAG-hRad9K772A as described above. Cells were exposed to 2 Gy of γ-irradiation 36 h post-transfection. The viable EGFP-positive cells in a defined area were counted before and 72 h after IR treatment as described previously (55).

**RESULTS**

**Human Rad9 Is a Phosphoprotein**—In the Western blotting analysis using mouse a-hRad9 antibodies, a single protein band with a molecular mass of about 60 kDa was detected in extracts prepared from human bladder carcinoma T24 cells (Fig. 1A). A protein band of similar size was also detected after immunoprecipitation with rabbit a-hRad9 antibodies followed by Western blotting analysis using mouse a-hRad9 antisera (Fig. 1B). Upon IR, this protein was detected as a slower migrating band. Together, these results confirm that the 60-kDa protein is hRad9. Consistent with a previous report, the molecular mass of the endogenous hRad9 protein is much bigger than its predicted mass of 45 kDa, suggesting that hRad9 might undergo extensive post-translational modifications (31). To investigate the nature of modification of hRad9, extracts prepared from T24 cells pulse-labeled with [32P]orthophosphate acid were subjected to immunoprecipitation using mouse a-hRad9 polyclonal antibodies. The detection of a labeled 60-kDa protein that disappears following CIAP treatment indicates that hRad9 is a phosphoprotein in undamaged cells (Fig. 1C).
2A, lanes 2 and 3). To further characterize the phosphorylation modification, hRad9 was immunoprecipitated from unlabeled cell extracts and incubated with CIAP. Upon treatment with CIAP, 60-kDa hRad9 was converted to faster migrating bands of either 45 or 55 kDa, depending on the amount of CIAP used in the reaction (Fig. 2B, lanes 2 and 4). In the presence of the CIAP inhibitor β-glycerophosphate, the alternation in the hRad9 electrophoretic mobility was abolished (Fig. 2B, lane 5). These results demonstrate that the anomalous electrophoretic mobility of endogenous hRad9 is due to phosphorylation. Similar to a previous report (30), IR treatment resulted in the appearance of a slow migrating form of hRad9 that could also be converted to a 45- or 55-kDa band by CIAP treatment (Fig. 2B, lanes 6 and 7), indicating that IR resulted in hyperphosphorylation of hRad9. Taken together, these results demonstrate that endogenous hRad9 is a heavily phosphorylated protein that undergoes hyperphosphorylation after IR.

**IR-induced Hyperphosphorylation of hRad9 Is Cell Cycle-Independent**—In *S. pombe*, the checkpoint rad genes are required for the activation of all checkpoints upon DNA damage. We examined whether IR-induced hyperphosphorylation of hRad9 occurs in all cell cycle stages. Following synchronization of T24 cells by density arrest (60), extracts were prepared from cells in G1, S, or G2/M, and the proteins were fractionated by SDS-PAGE. The electrophoretic mobility of the hRad9 protein was constant throughout the cell cycle (Fig. 3A, lanes 1, 3, and 5 and data not shown). The IR-induced mobility shift of hRad9 was observed at all cell cycle phases (Fig. 3A, lanes 2, 4, and 6). Unlike its counterpart DDC1 in *S. cerevisiae* (61), no mobility change of hRad9 was observed in the S phase of untreated cells. Since not all phosphorylation events lead to protein mobility shifts, it remains to be established whether there are cell cycle-specific modifications of hRad9 in mammalian cells. In *S. pombe*, the checkpoint Rad proteins are required for IR-, UV-, as well as replication block-induced checkpoint activation. We tested whether hyperphosphorylation of hRad9 occurs upon different treatments. Similar to the IR-induced response, UV and HU also caused hyperphosphorylation of hRad9 (Fig. 3B). The IR-, UV-, and HU-induced hyperphosphorylation of hRad9 was also detected in several other human cell lines (data not shown). Collectively, these results demonstrate that hRad9 hyperphosphorylation occurs in response both to different types of DNA damage and to blockage of DNA replication, supporting the notion that there may be several upstream pathways that trigger hyperphosphorylation of hRad9.

**IR-induced Hyperphosphorylation of hRad9 Is Dependent on ATM**—Since the ATM kinase is activated, and hRad9 is hyperphosphorylated upon IR, we tested whether the two proteins act in the same signaling pathways. Treatment of A-T cells with 10-Gy irradiation did not induce hyperphosphorylation of hRad9. Conversely, hRad9 was hyperphosphorylated, in a dosage-dependent manner, in A-T cells complemented with wild-type ATM cDNA (YZ5 cells, Fig. 4A). In contrast to the IR-induced response,
treatment with HU resulted in the hyperphosphorylation of hRad9 in A-T cells (Fig. 4B), indicating IR-induced, but not HU-induced, hyperphosphorylation of hRad9 is ATM-dependent. Since the NBS shares similar clinical and cellular phenotypes with the A-T disorder, we examined whether hyperphosphorylation of hRad9 is defective in NBS1 cells. IR-, UV-, and HU-induced hyperphosphorylations were normal in NBS cells (Fig. 4C), demonstrating that NBS1 is not required for the hyperphosphorylation of hRad9.

ATM Phosphorylates Ser272 of hRad9 in Vitro—To extend the above findings, we explored the possibility that ATM phosphorylates hRad9 in vitro. Human Rad9 protein contains one SQ site, the preferred target site for the ATM kinase (62). GST fused to hRad9 residues 255–295 (GST-hRad9255–295) encompassing the Ser272 site was readily phosphorylated by ATM immunoprecipitated from T24 cells (Fig. 5B, lane 2). Interestingly, ATM immunoprecipitated from irradiated T24 cells exhibited enhanced phosphorylation of GST-hRad9255–295 (Fig. 5B, lane 3). Substitution of Ser272 to alanine abolished phosphorylation (Fig. 5B, lanes 5 and 6). In contrast, GST-hRad9255–295 was not phosphorylated by ATR under similar conditions (Fig. 5C). To further confirm the kinase-substrate relationship between ATM and GST-hRad9255–295, recombinant FLAG-tagged wild-type ATM (pCMV-Flag-ATM WT) and kinase-inactive ATM (pCMV-Flag-ATM S223A) were overexpressed in 293 cells. The immunoprecipitated recombinant ATM was used as the kinase source for the in vitro kinase assays. Wild-type recombinant ATM phosphorylated the Ser272 site of hRad9 (Fig. 5D, lane 1), whereas the kinase-inactive form of ATM did not (Fig. 5D, lane 2), providing further evidence that Ser272 of hRad9 is phosphorylated by ATM but not by a contaminating kinase. Examination of other hRad9 fragments revealed that full-length (Fig. 5E), NH2-terminal (1–223aa), and COOH-terminal (224–391aa) fragments of hRad9 (data not shown) could all be phosphorylated by both ATM and ATR in vitro, indicating that there are likely to be additional phosphorylation sites for these two kinases. Taken together, these results demonstrate that Ser272 of hRad9 is a substrate site specifically for ATM but not for ATR in vitro.

IR-induced Phosphorylation of Ser272 of hRad9 in Vivo and Delayed Phosphorylation of Ser272 in A-T Cells—To further examine phosphorylation of hRad9 on Ser272 in vivo, we used affinity-purified anti-phospho-Ser272 antibodies for immunoprecipitation. These antibodies immunoprecipitate hRad9 protein only after IR treatment in YZ5 and T24 cells (Fig. 6A and data not shown). The hRad9 with p-Ser272 was detected 1 h post-IR, and the amount of p-Ser272 was increased at 4 h post-IR. hRad9 levels remained constant following IR (Fig. 6A, middle panel). In contrast, IR-induced phosphorylation of Ser272 was delayed in ATM-deficient cells. The level of p-Ser272 at 8 h post-IR was similar to that of YZ5 cells 1 h post-IR (Fig. 6B). This result suggests that ATM phosphorylates Ser272 of hRad9 in response to IR in vivo. In the absence of ATM, another kinase may phosphorylate Ser272 in response to IR, but in a delayed manner.

Expression of the hRad9S272A Mutant Protein Disturbs G1/S Checkpoint Activation and Sensitizes Cells to IR—We next examined the effect of phosphorylation of the hRad9 Ser272 residue on checkpoint activation. Similar levels of recombinant hRad9 were detected in cells transfected with either pCMV2-FLAG-hRad9WT or pCMV2-FLAG-hRad9S272A (Fig. 7A). The effects of phosphorylation of Ser272 of hRad9 on G1/S checkpoint activation was determined by co-transfecting human lung fibroblast VA13 cells with EGFP, and hRad9WT or hRad9S272A. In comparison with wild-type pCMV2-FLAG-hRad9WT, expression of pCMV2-FLAG-hRad9S272A resulted in defective checkpoint activation (Fig. 7B). Since uptake of BrdUrd was scored in these assays, the experiment mainly measured G1/S checkpoint and may not reflect intra-S-delay. The effects of hRad9S272A expression on cellular sensitivity to IR were examined. VA13 cells co-transfected with EGFP and wild-type hRad9 or mutant hRad9 (hRad9S272A) were exposed to 5 Gy of...
Together, these results suggest that phosphorylation of Ser272 affected cells is due to checkpoint or/and repair defect. Taken (64). As yet, it is unclear whether radiosensitivity of the trans- because prolonged overexpression of hRad9 induces apoptosis. These assays were performed using transient transfections, B viable EGFP-positive cells were scored 72 h after IR (Fig. 7). The substrates were as in B. The arrow indicates unknown protein in the ATR inmunoprecipitants. D, kinase assays using the recombinant ATM protein. 293 cells transiently transfected with FLAG-tagged wild-type (ATMWT) or kinase-inactive mutant ATM (ATM D2870A) were exposed to 10-Gy γ-irradiation or mock-treated. Recombinant FLAG-ATM was immunoprecipitated 1 h post-treatment using α-FLAG antibodies. IP-FLAG-ATM WT and IP-FLAG-ATM D2870A were used as the kinase source, and the substrates were as in B. E, IP-ATM and IP-A. TR kinase assays using GST-fused full-length hRad9 as substrate. Arrowheads indicate IgG. p531–106 serves as a positive control.

γ-rays. Transfection with hRad9 S272A significantly increased cellular sensitivity to IR compared with wild-type controls when viable EGFP-positive cells were scored 72 h after IR (Fig. 7B). These assays were performed using transient transfections, because prolonged overexpression of hRad9 induces apoptosis (64). As yet, it is unclear whether radiosensitivity of the transfected cells is due to checkpoint or/and repair defect. Taken together, these results suggest that phosphorylation of Ser 72 of hRad9 is required for cell cycle checkpoint activation in response to IR.

DISCUSSION

Here we demonstrated that the anomalous molecular mass of endogenous hRad9 is due to basal phosphorylation. This basal phosphorylation is likely to occur at multiple sites, since the 60-kDa form of hRad9 could be converted to 45 or 55 kDa molecular mass, depending on the amounts of phosphatase used in CIAP treatment. While the functional significance of the extensive phosphorylation of hRad9 is unknown, it is plausible that the basal phosphorylation of hRad9 is either required for its interaction with HsU1 and/or hRad1 to form a stable complex. Alternatively, phosphorylation may be required for the stabilization of hRad9 within the cell (31).

Several observations described here lead to our conclusion that ATM phosphorylates Ser72 of hRad9 in response to IR. First, ATM specifically phosphorylated Ser72 in vitro, but not other serines around it. Second, IR-induced hyperphosphorylation of hRad9 is diminished in A-T cells, and hyperphosphorylation of hRad9 is restored by the re-introduction of ATM DNA into A-T cells. Third, IR-induced phosphorylation of hRad9 on Ser272 was detected in vivo, and ATM-deficient cells had delayed phosphorylation of Ser72. Fourth, transient expression of hRad9 S272A resulted in the defective G1/S checkpoint activation upon DNA damage and increased cellular sensitivity to IR, suggesting that the hRad9 S272A mutant may act in a dominant-negative manner. The finding that hyperphosphorylation of hRad9 in response to IR is ATM-dependent is consistent with the observation in yeast species that DNA damage-induced phosphorylation of DDC1 or Rad9 is dependent on MEC1 or Rad3, respectively (8, 61). While we demonstrated that phosphorylation of Ser72 of hRad9 is required for activation of the DNA damage-induced G1/S phase checkpoint, the mechanism by which hyperphosphorylation of hRad9 activates downstream effectors remains to be investigated.

Hyperphosphorylation of hRad9 was also observed upon UV and HU treatment. It is likely that ATR mediates the UV- and HU-induced hyperphosphorylation of hRad9. In this regard, we found that ATR did phosphorylate full-length hRad9 and NH2-terminal (amino acids 1–223) and COOH-terminal (amino acids 224–391) hRad9 in vitro (Fig. 5E and data not shown). Interestingly, Ser72 was not a favorable substrate site for ATR in vitro, suggesting that there may be unique phosphorylation sites for ATM and ATR in the hRad9 protein. Studies using oriented peptide libraries in the kinase assay have shown that some sequences are preferentially phosphorylated by one kinase but not by the other (63). Interestingly, delayed phosphorylation is also seen in p53 and NBS1 in A-T cells (55, 65–67).
The requirement of all other checkpoint Rad proteins for DNA was due to their presence in large protein complexes.

Co-immunoprecipitation of hRad9 by co-immunoprecipitation. The absence of this evidence in vivo, we were unable to show interaction between ATM and hRad9 upon IR. YZ5 cells (A-T cells complemented with ATM cDNA) phosphorylated Ser272 of hRad9 in the absence of ATM. Recent studies showed that ATR or another kinase may phosphorylate Ser272 of hRad9 after IR in A-T cells. Immunoblotting with mouse anti-hRad9 antibodies. Middle panel shows direct Western blotting with anti-hRad9 antibodies, and bottom panel shows direct Western blotting with β-actin antibodies. B. phosphorylation of Ser272 of hRad9 after IR in A-T cells. Immunoblotting followed by Western blotting or direct Western blotting of Rad9 as described in A.

Using α-phosphopeptide antibodies, we showed that Ser272 of hRad9 was phosphorylated in vivo upon DNA damage (Fig. 6). IR-induced phosphorylation of Ser272 was delayed in ATM-deficient cells, suggesting that ATM or another kinase may phosphorylate Ser272 of hRad9 in the absence of ATM. Recent studies showed that Hus1-deficient mouse cells are sensitive to UV but not IR, while C. elegans mrt2 (S. pombe rad1+ homologue) are sensitive to IR but less sensitive to UV (36, 37), suggesting that members of the checkpoint Rad complex may be selectively activated by a specific type of DNA damage. In addition, our results on hRad9, together with other’s results on p53, also indicate that different sites of the same protein could be targeted by different upstream kinases in response to DNA damage or stalled DNA replication forks (68–71). Thus, multiple levels of regulation may provide fine tuning of cellular responses to different DNA structure changes.

Although hRad9 is a direct substrate of ATM in vitro and in vivo, we were unable to show interaction between ATM and hRad9 by co-immunoprecipitation. The absence of this evidence may reflect a transient interaction or inefficient co-immunoprecipitation due to the presence of large protein complexes. The requirement of all other checkpoint Rad proteins for DNA damage-induced phosphorylation of DDC1/Rad9 (72) suggests that the Rad protein complexes, not the DDC1/Rad9 itself, are likely to be required for the interaction between MEC1/Rad3 and DDC1/Rad9 in yeast species. Interestingly, in our 32P labeling experiment (Fig. 2A) and GST-hRad9 pull-down assay (data not shown), additional proteins were readily detectable, suggesting that hRad9 is likely to be present in multiple protein complexes.

DNA damage-induced hyperphosphorylation of hRad9 appears to be normal in NBS cells. There are two plausible explanations. Human Rad9 may operate upstream of NBS1,
alternatively, hRad9 may function separately from NBS1 (Fig. 8). The checkpoint Rad proteins are hypothesized to play the roles of sensors in recognizing DNA structure changes (9). Other events, including DNA repair, transcription, and apoptosis, are likely downstream cellular processes following the binding of these sensors to damaged DNA (73–75). If this is true, it is most likely that hRad9 acts upstream of NBS1. Further studies will be required to clarify the relationship between Rad9 and NBS1.

Our studies provide the first demonstration that the mere presence of the hRad9 protein may not be sufficient to activate checkpoint controls. Instead, phosphorylation of Ser\textsuperscript{272} of hRad9 per se is required to activate the cellular response to IR.

Given that the main functions of the six checkpoint Rad proteins are conserved among yeast and mammals, we anticipate that human checkpoint Rad proteins, including ATM, hRad1, hRad9, hHus1, hRad17, and ATR, may form a multiprotein scaffold that is critical for the activation of the cellular response to DNA damage. The integrity and the tight coordination among these checkpoint Rad proteins underlie their roles in maintaining genomic stability and in preventing cancer development.

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Hypermethylation of hRad9 in Checkpoint Activation

16586
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