The integrity of the human genome is preserved by signal transduction pathways called checkpoints, which delay progression through the cell cycle when DNA damage is present. Three checkpoint proteins, hRad9, hRad1, and hHus1, form a proliferating cell nuclear antigen-like, heterotrimeric complex that has been proposed to function in the initial detection of DNA structural abnormalities. hRad9 is highly modified by phosphorylation, in a constitutive manner and in response to both DNA damage and cell cycle position. Here we present evidence that Thr292 of hRad9 is subject to Cdc2-dependent phosphorylation in mitosis. Furthermore, our data are also consistent with four other hRad9 phosphorylation sites (Ser277, Ser288, Ser335, and Thr355) being regulated in part by Cdc2. We also identify Ser387 as a novel site of hRad9 constitutive phosphorylation and show that phosphorylation at Ser387 is a prerequisite for one form of DNA damage-induced hyperphosphorylation of hRad9. Characterization of nonphosphorylatable mutants has revealed that hRad9 phosphorylation plays a critical role in checkpoint signaling. 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 S phase.

Cell cycle checkpoints are signal transduction pathways that maintain the proper order of cell cycle events (1). Several checkpoints preserve the integrity of DNA by sensing genetic anomalies and delaying progression through the cell cycle so that enough time is provided for these anomalies to be corrected. The contribution of checkpoints to human health is illustrated by a growing list of checkpoint genes that are mutated in cancer and cancer predisposition syndromes (2–8).

The hRad9 protein is the human homologue of Schizosaccharomyces pombe Rad9, a member of the checkpoint Rad family of proteins. In fission yeast, the checkpoint rad genes (rad1+, rad3+, rad9+, rad17+, rad26+, and hus1+) are required for the S phase (DNA replication) and G2 (DNA damage) checkpoints (9–13). Yeasts lacking these genes fail to inactivate Cdc2 and enter premature, lethal mitosis when challenged with agents that inhibit DNA synthesis or damage DNA (14, 15). Like its yeast counterpart, hRad9 forms a ring-shaped, heterotrimeric complex with the hRad1 and hHus1 proteins (16–18). Each member of the hRad9-hRad1-hHus1 complex (also known as the 9-1-1 complex), shares sequence homology with PCNA,1 a homotrimer that encircles DNA and tethers DNA polymerase δ during DNA synthesis (19). PCNA is loaded onto DNA by the pentameric protein complex replication factor C (RFC), which is composed of one large subunit and four smaller subunits (20). In a manner analogous to PCNA and RFC, 9-1-1 is loaded onto DNA by a complex between hRad17 and the four smallest subunits of RFC (21). Since DNA damage induces hRad17-dependent association of 9-1-1 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 (22). Also involved in this recognition are two phosphatidylinositol 3-kinase-related kinases, ATM and ATR, that regulate several cell cycle transitions and are central components of the cell checkpoint machinery (23). Even though these kinases appear to respond to different types of DNA lesions, they share a long list of common checkpoint substrates, including hRad17 (24–26) and hRad9 (27). In fission yeast, Rad3 (which shares homology with both ATR and ATM) requires Rad9, Rad1, Hus1, and Rad17 to phosphorylate certain substrates (28). Similarly, in human cells, phosphorylation of hRad17 by ATR requires hHus1 (22). These findings support a model in which the 9-1-1 complex recruits substrates for ATM or ATR to sites of DNA damage or stalled replication forks (29).

In addition to interacting with hRad1 and hHus1, hRad9 also physically interacts with TopBP1, the human orthologue of Saccharomyces cerevisiae Dpb11, and Schizosaccharomyces pombe Cut5 (30). Each of these proteins contains multiple BRCA1 carboxyl-terminal (BRCT) domains, a putative protein-protein interaction motif common in cell cycle control and DNA repair. In addition to the checkpoint rad genes, cut5 is also required for slowing S phase and delaying mitosis when DNA replication is challenged by DNA damage (13, 31). A similar requirement also exists for DPB11 in budding yeast (32), and a physical interaction between Dpb11 and Ddc1 (the budding yeast orthologue of hRad9) may play a role in this response (33).

hRad9 is a unique member of the 9-1-1 complex in that it contains a C-terminal region (of about 110 amino acids) that does not share homology with PCNA and is not believed to be directly involved in association with hRad1 or hHus1. This region of the protein is both constitutively and transiently phosphorylated at several amino acid residues (34), and hence represents a potential regulatory region for the effector functions of 9-1-1. We have characterized the extensive phospho-

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1 The abbreviations used are: PCNA, proliferating cell nuclear antigen; siRNA, small interfering RNA; GST, glutathione S-transferase; HU, hydroxyurea; IR, ionizing radiation.
rylation of hRad9 in this region and show that it is partially regulated by Cdc2. We also demonstrate that the C-terminal phosphorylation of hRad9 has roles in regulating both hRad9 interaction with TopBP1 and the cellular response to DNA damage in S phase.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfections—HeLa cells, fibroblasts derived from an ataxia telangiectasia patient (ATM-/-) (CRL-7201; ATCC, Manassas, VA), and IMR90 fibroblasts were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) at 37 °C in 5% CO2 atmosphere. hTERT-RPE1 cells, a human immortal pigment epithelial cell line that stably expresses the human telomerase reverse transcriptase subunit (BD Biosciences, Mississauga, Canada), were maintained as above, only in Dulbecco’s modified Eagle’s medium/F-12 medium (Invitrogen). Transient transfections were performed as described previously (34). The development of the stable HeLa Tet-Off cell line was performed according to the manufacturer’s instructions (BD Biosciences). This cell line was maintained in 200 μg/ml of Geneticin (G418) (Invitrogen). Transgene expression was limited by the addition of 1 μg/ml tetracycline to the culture medium. Small interfering RNA (siRNA) transfections were conducted using OligofectAMINE (Invitrogen), according to the manufacturer’s protocol. Briefly, siRNA transfections were carried out in six-well plates using 4 μl of OligofectAMINE reagent and 0.1 nmol of siRNA duplex per well. siRNAs targeting hRad9 and luciferase (GL2) were purchased commercially (Dharmacon Research, Lafayette, CO). The siRNA sequence targeting hRad9 was 5′-ACACCUUUUGGAGGCGGCUCCU-3′.

Drugs and Irradiation—Cells were irradiated using a 137Cs γ-irradiator at 0.78 grays/min. Thymidine (Sigma), hydroxyurea (Sigma), and nocodazole (Sigma) were typically administered for 18 h at concentrations of 2 μM, 1 mM, and 0.1 μg/ml, respectively.

Plasmids and Site-directed Mutagenesis—All hRad9 point mutants were generated using the Transformer site-directed mutagenesis kit (BD Biosciences) according to the manufacturer’s instructions. GST expression plasmids were generated by PCR subcloning segments of the hRad9 DNA (wild-type and point mutants) into the pGEX-2T vector. Antibodies—Rabbit polyclonal α-phospho-Ser272, α-phospho-Ser257, and α-phospho-Thr292 antibodies were raised against phospho-Ser272 (SDTDSHpSQDLGS), where pS represents phosphoserine), phospho-Ser257 (PVLAEDpSEGEG), and phospho-Thr292 (QLQAHSpTPHPDD; where pS represents phosphoserine), phospho-Thr292 (QLQAHSpTPHPDD; where pS represents phosphoserine), and phospho-Thr292 (QLQAHSpTPHPDD; where pS represents phosphoserine) antibodies were raised against phospho-Thr292 antibodies (upper panel) or phosphospecific antibodies directed against phosphorylated Thr292 of hRad9 (α-p292, lower panel). B, similar to A but involving transiently expressed hRad9 protein harboring at least three mutations (S328A, S336G, and S355A). The S277A and T292A mutations were introduced into this mutant background, as indicated above (+, wild type; −, alanine substitution).

Induced in logarithmically growing BL21 bacteria, with 0.1 mM isopropyl-1-thio-β-D-galactopyranoside for 3 h at 37 °C. GST fusion proteins were batch-purified from bacterial lysates with glutathione-Sepharose (Amersham Biosciences), Myc (9E10; Santa Cruz Biotechnology, Santa Cruz, CA), and Cdc2 (17) (Santa Cruz Biotechnology).

Flower Cytometry—HeLa cells were fixed in 50% ethanol in phosphate-buffered saline for at least 30 min on ice. Cells were then collected by centrifugation, resuspended in phosphate-buffered saline with 50 μg/ml PI and 0.1 mg/ml RNase A, and analyzed using a flow cytometer (Beckman/Coulter EPICS ALTRA, Mississauga, Canada). Antibodies were cleared of GST reactivity and then affinity-purified on a nickel column.

Phosphorylation of hRad9 at Thr292 during mitosis. A, HeLa cells, transiently expressing wild-type hRad9 or five hRad9 point mutants (indicated above), were grown in the presence (+) or absence (−) of 0.1 μg/ml microtubule inhibitor nocodazole (NOC) for 18 h. Mitotic (+NOC) and asynchronous (−NOC) cells were then lysed and immunoblotted with α-hRad9 antibodies (upper panel) or phosphospecific antibodies directed against phosphorylated Thr292 of hRad9 (α-p292, lower panel). B, similar to A but involving transiently expressed hRad9 protein harboring at least three mutations (S328A, S336G, and S355A). The S277A and T292A mutations were introduced into this mutant background, as indicated above (+, wild type; −, alanine substitution).

RESULTS

hRad9 Is Phosphorylated at Thr292 during Mitosis—We had previously identified four amino acid residues in hRad9 that contribute to the constitutive phosphorylation of the protein (34). Phosphorylation at Ser277, Ser257, Ser336, and Thr292 results in a significant reduction in the electrophoretic mobility of hRad9 derived from undamaged, asynchronous cells. We had also previously shown that a T292A mutation prevented hyperphosphorylation of hRad9 in mitosis (34). To further characterize the nature of Thr292 phosphorylation, phosphospecific antibodies were raised against a phospho-Thr292 hRad9 pep-
The phosphorylation of hRad9 is partially dependent on Cdc2. To further characterize the mitotic phosphorylation of hRad9, we examined the mobility of endogenous hRad9 as cells entered and exited nocodazole-induced mitotic arrest. Consistent with our previous observations (34), the predominant phospho form of hRad9 in asynchronous cultures (hRad9α) migrated at an apparent molecular mass of ~60 kDa. The discrepancy between the apparent molecular mass of hRad9 and its predicted molecular mass of 43 kDa is largely due to phosphorylation at Ser277, Ser328, Ser336, and Thr355. Several less abundant, faster migrating phosphorylation intermediates (hRad9β forms) represent hRad9 protein that is only partially phosphorylated at these sites. When cells were blocked in mitosis by treatment with nocodazole, the abundance of both hRad9α and hRad9β forms was reduced, and the majority of hRad9 existed as an even slower migrating species, hRad9β (see Fig. 2A). When the mobility pattern of hRad9 was examined at increasing time points following the removal of nocodazole from the culture medium, the emergence of normal hRad9 mobility coincided with the exit of cells from mitosis, as defined by flow cytometry (Fig. 2A, 120 and 400 min).

The observations made in Figs. 1 and 2A, are consistent with each of Ser277, Ser328, Ser336, Thr355, and Thr392 being quantitatively phosphorylated during mitosis, although the vast majority of hRad9 is already phosphorylated at the four former residues prior to mitosis. Each of these five amino acids share the common consensus sequence ((S/T)PX(R/K)P) and are thus potential targets for cyclin-dependent kinases. For these reasons, we sought to determine whether Cdc2, the cyclin-dependent kinase that controls the G2/M transition, could phosphorylate Ser277, Thr355, Ser328, Ser336, and Thr392 in vitro. To this end, we purified a series of hRad9 C-terminal peptides as GST fusion proteins. Peptides were used as substrates for Cdc2, which was immunoprecipitated from in vivo and in vitro. Three hRad9 peptides (amino acids 266–301, 314–344, and 348–391) were used as GST fusion proteins. Peptides either contained wild-type (WT) hRad9 amino acid sequence or contained one or multiple mutations at Ser277, Thr355, Ser328, Ser336, and Thr392, as indicated above. The P3A 348–391 peptide contains the T355A mutation as well as the S375A and S380G mutations (two other C-terminal S-P sites). Purified peptides, as well as histone H1 and GST, were used as in vitro substrates for Cdc2 immunoprecipitated from mitotic or S phase HeLa cell extracts, as indicated on the right. Reactions were subjected to SDS-PAGE (12% acrylamide), and proteins were stained with Coomassie Brilliant Blue (a representative gel is shown in the top panel). Each gel was dried and exposed to x-ray film for 24 h. C, phosphorylation of hRad9 by Cdc2 in vivo. Asynchronous HeLa cells were left untreated (Asyn) or were treated with 0.1 μg/ml nocodazole (NOC) for 18 h, as indicated. These cells were grown in the presence (+) or absence (−) of a 100 μM concentration of the CDK inhibitor roscovitine (Rosc) from hour 14 to 18, as indicated. Soluble cell lysates were size-fractionated by SDS-PAGE (8%) and immunoblotted with antibodies directed against hRad9.

α-Cdc2 antibody. Phosphorylation of Ser277 was demonstrated by introduction of the S277A mutation in GST-hRad9266–301, which abolished phosphorylation of this protein by Cdc2. Nei-
ther the S277A mutant nor the S277A/T292A double mutant were phosphorylated at levels significantly above background, indicating that Thr<sup>272</sup> was not phosphorylated by Cdc2 in this assay. Phosphorylation of Ser<sup>272</sup> and Ser<sup>336</sup> was shown using GST-hRad9<sub>314-344</sub>. Whereas the S328A and S336G single mutants of GST-hRad9<sub>314-344</sub> were still modestly phosphorylated, the S328A/S336G double mutant showed only background levels of <sup>32</sup>P incorporation. Thr<sup>355</sup> was more efficiently phosphorylated than any of the other hRad9 sites (see GST-hRad9<sub>348-391</sub>). There was no appreciable difference in phosphorylation of T355A and P3A (T355A/S375A/S380G), indicating GST-hRad9<sub>314-344</sub> was not phosphorylated by Cdc2 in vitro.

This is consistent with our previous work in which we found no evidence for phosphorylation of these residues in vitro (34). Furthermore, there did not appear to be any significant phosphorylation of non-Cdk consensus sites in hRad9, thus underscoring the specificity of Cdc2 toward Ser<sup>277</sup>, Ser<sup>278</sup>, Ser<sup>328</sup>, and Thr<sup>355</sup> of hRad9 in this assay.

To determine whether Cdc2 phosphorylates hRad9 in vivo, we employed the Cdk inhibitor roscovitine. Roscovitine is a selective inhibitor of both Cdc2 and Cdk2 (35). When HeLa cells were arrested in mitosis with nocodazole and then treated with roscovitine (Ros) for 2 h, a drastic reduction in hRad9 phosphorylation was observed (Fig. 2C). Roscovitine had no effect however, on hRad9 phosphorylation in asynchronous cultures (Fig. 2C).

**hRad9 Is Constitutively Phosphorylated at Ser<sup>387</sup>**—Site-directed mutagenesis of potentially phosphorylated residues in the C terminus of hRad9 identified a mutant, S387A, which was not normally hyperphosphorylated in response to the DNA synthesis inhibitor hydroxyurea (HU). To characterize this residue, phosphospecific antibodies were raised against a phospho-Ser<sup>387</sup> hRad9 peptide. The quality of these antibodies was determined by testing their reactivity toward phosphorylated and dephosphorylated wild-type and S387A hRad9. Although transiently expressed wild-type and S387A hRad9 were indistinguishable in terms of their SDS-PAGE mobility, they were clearly distinct with regard to their detection when immunoblotted with α-phospho-Ser<sup>387</sup> (α-p387) antibodies (Fig. 3A). Whereas the α-p387 antibodies recognized all differentially migrating forms of wild-type hRad9, they were completely non-reactive toward the S387A mutant (Fig. 3A, right blot). In addition, the α-p387 antibodies did not detect wild type hRad9 that had been dephosphorylated with calf intestinal phosphatase. Thus, hRad9 is phosphorylated at Ser<sup>387</sup>, and the α-p387 antibodies are effective in specifically recognizing Ser<sup>387</sup>-phosphorylated hRad9.

To determine whether phosphorylation at Ser<sup>387</sup> of hRad9 was regulated in a cell cycle- or DNA damage-dependent manner, we collected HeLa cells that were in G<sub>1</sub>, S, G<sub>2</sub>, and M phases of the cell cycle, as we have previously described (34). HeLa cells were synchronized with a double thymidine block and then released and collected 2 h (S), 7 h (G<sub>1</sub>), and 11 h (G<sub>2</sub>) later. One hour prior to harvest, these cells were treated with 20 Gy of ionizing radiation (IR), as indicated. Cells were arrested in mitosis (M) by treating asynchronous cells with nocodazole. We also collected HeLa cells that were grown in the presence of HU for 18 h or were harvested 18 h after a 20-Gy dose of IR. At the time of harvest, these cells were arrested in early S phase and G<sub>2</sub>, respectively. In all cases, cell cycle position was confirmed using flow cytometry of propidium iodide-stained nuclei. Soluble cell lysates from these cells were subjected to immunoblotting with α-hRad9, α-p387, and phosphospecific antibodies directed against phosphorylated Ser<sup>272</sup> of hRad9 (α-p272), a site of DNA damage-dependent phosphorylation (27).

In agreement with several previous reports (16, 22, 27, 34, 36, 37), the constitutively phosphorylated hRad9 protein underwent hyperphosphorylation under various conditions (Fig. 3B, top panel). Ionizing radiation induced rapid phosphorylation of hRad9 at Ser<sup>277</sup> regardless of cell cycle position (27). Whereas Ser<sup>277</sup> phosphorylation did produce a subtle mobility shift, it was more easily visualized by immunoblotting with
α-p272 antibodies (Fig. 3B, middle panel). The α-p272 blot revealed that IR-induced phosphorylation at Ser272 was not only rapid but also transient and dissipated by 18 h following irradiation. The hRad9 protein from cells harvested 18 h after IR exhibited a second IR-induced mobility shift. This IR-induced modification, however, which persisted after phosphorylation at Ser272 had dissipated, was less rapidly induced and was not readily detectable 1 h after IR (Fig. 3B, top panel). The hRad9 protein also underwent similar mobility shifts in G2, in response to HU, and in mitosis.

In contrast to these dynamic phosphorylation changes, the extent of Ser387 phosphate did not change significantly in response to cell cycle perturbation or DNA damage. In fact, the reactivity of the hRad9 protein to growth conditions known to induce DNA damage-dependent/Ser272-independent hyperphosphorylation and may also be a prerequisite for hyperphosphorylation of hRad9 under other conditions. To address this possibility, we examined the response of wild-type, S272A, and S387A hRad9 protein to growth conditions known to induce DNA damage-dependent/Ser272-independent hyperphosphorylation. These proteins were transiently expressed at low levels using the Tet-Off expression system and were Myc-tagged to distinguish them from endogenous (wild-type) hRad9. In these experiments, transient expression levels near those with homozygous mutations in ATM, with HU (Fig. 4A), and in mitosis (Fig. 3C). Thus, Ser387 represents a novel, ATM-independent, constitutive phosphorylation site in hRad9. Ser387 is distinct from previously identified constitutive sites in hRad9, however, in that it does not fit the (S/T)P consensus sequence seen in these other sites (Table I), and it is not phosphorylated by Cdc2 in vitro (Fig. 2B, peptide 348–391).

Ser387 Phosphorylation Regulates DNA Damage-dependent Hyperphosphorylation of hRad9—We next examined the dependence of DNA damage-induced hRad9 hyperphosphorylation events on hRad9 constitutive phosphorylation. As described previously (27) and as illustrated in Fig. 3B, hRad9 becomes hyperphosphorylated in a DNA damage-dependent manner at Ser272. We had previously shown that this process occurs independently of constitutive phosphorylation at (S/T)P sites (34). Using α-p272 antibodies, this observation was confirmed in Fig. 4A, since a P4A mutant (harboring the S277A, S328A, S336G, and T355A mutations) was still inducibly phosphorylated at Ser272 1 h after a 20-Gy dose of IR. Similarly, the S387A mutant was also inducibly phosphorylated at Ser272 at levels comparable with that of wild-type hRad9 (Fig. 4A), indicating that Ser387 phosphorylation is also not a prerequisite for DNA damage-induced phosphorylation at Ser272.

We had previously reported that hRad9 undergoes DNA damage-dependent hyperphosphorylation that is distinct from phosphorylation at Ser272 (34). This second hyperphosphorylation event was illustrated in Fig. 3B. When cells were subjected to DNA damage in late S phase/G2, treated with hydroxyurea, or harvested after extended periods of time following IR, hRad9 underwent a mobility shift that was similar to that observed in mitotic cells (Fig. 3B). This observation had initially led us to believe that this DNA damage-induced phosphorylation of hRad9 was occurring at Thr292 (the site of mitotic phosphorylation; Fig. 1). To address this directly, hRad9 protein was harvested from cells grown under each of the above conditions and immunoblotted with α-p292 antibodies. As shown in Fig. 4B, only hRad9 derived from mitotic cells was phosphorylated at Thr292. Thus, the location of Ser272-dependent, DNA damage-induced hRad9 phosphorylation remains unclear.

As mentioned earlier, our initial interest in Ser387 was sparked from the observation that a S387A mutant was not efficiently hyperphosphorylated in response to HU. Whereas this had initially led us to believe that Ser387 phosphorylation was induced by HU, our analysis with α-p387 antibodies (Fig. 3) has indicated this to be incorrect. We therefore reasoned that Ser387 phosphorylation was a prerequisite for HU-induced hyperphosphorylation and may also be a prerequisite for hyperphosphorylation of hRad9 under other conditions. To address this possibility, we examined the response of wild-type, S272A, and S387A hRad9 protein to growth conditions known to induce DNA damage-dependent/Ser272-independent hyperphosphorylation. These proteins were transiently expressed at low levels using the Tet-Off expression system and were Myc-tagged to distinguish them from endogenous (wild-type) hRad9. In these experiments, transient expression levels near those of endogenous hRad9 were necessary, since overexpressed hRad9 is not efficiently hyperphosphorylated in response to HU. Unlike wild-type and S272A hRad9, the S387A mutant was not efficiently hyperphosphorylated when HeLa cells were synchronized in G2, harvested 18 h after 20 Gy of IR, or treated with HU (Fig. 4C). We refer to this hyperphosphorylated form of hRad9 as hRad9α to distinguish it from mitotic hRad9 (hRad9µ), which is readily observed in the S387A mutant. As shown in Fig. 4D, neither the S272A nor the S387A mutation prevented mitotic phosphorylation of hRad9 at Thr292. A summary of the hRad9 phosphorylation sites described in this paper is given in Table I.

hRad9 Constitutive Phosphorylation Sites Regulate Association with TopBP1—hRad9 forms a ring-shaped, PCNA-like heterotrimeric complex with hHus1 and hRad1 (16–19). The C terminus of hRad9, which does not exhibit sequence or structural similarity to PCNA, has been shown to interact with the DNA damage-responsive protein TopBP1 (30). Using hRad9 C-terminal deletion mutants, we refined the region of hRad9 required for the interaction with TopBP1 to 17 amino acids at the extreme C terminus of hRad9. Because this region encompasses Ser387, we hypothesized that phosphorylation at Ser387 could be regulating the interaction between hRad9 and TopBP1. To test this, HeLa cells were transiently transfected with plasmids that overexpress wild-type, S272A, S387A, or P4A hRad9. hRad9-containing complexes were immunoprecipi-

Table I

| Amino acid | Context | Kinase | Regulation | Prior phosphorylation required | Migratory form |
|------------|---------|-------|------------|-------------------------------|---------------|
| Ser272     | SQ      | ATM, other | IR-induced (rapid and transient) | None | hRad9α |
| Ser277     | SPER    | Cdc2, other? | Constitutive/mitotic | None | hRad9α |
| Thr292     | TPHP    | Cdc2   | Mitotic    | None | hRad9α |
| Ser232     | SPGP    | Cdc2, other? | Constitutive/mitotic | None | hRad9α |
| Thr335     | TPHE    | Cdc2, other? | Constitutive/mitotic | None | hRad9α |
| Ser387     | EDSEGE  | ?      | Constitutive | None | hRad9α |

* Amino acid sequence surrounding phosphorylated residue (underlined).
* Ref. 27.
* hRad9α is phosphorylated at each of Ser272, Ser277, Ser336, Thr292, and Ser387.
* R. P. St. Onge, B. D. A. Besley, J. L. Pelley, and S. Davey, unpublished results.
* Greer, D., Besley, B. D. A., Kennedy, K., and Davey, S. (2003) Cancer Res., in press.
Fig. 4. Constitutive phosphorylation at Ser\textsuperscript{387} regulates DNA damage-induced hyperphosphorylation of hRad9. A. DNA damage-dependent hyperphosphorylation of hRad9 at Ser\textsuperscript{272} does not require prior phosphorylation at Ser\textsuperscript{387}. HeLa cells, transiently expressing wild-type, S272A, S387A, and P4A (S277A/S326A/S386D/T355A) hRad9 were lysed and immunoblotted with antibodies directed against hRad9 (top), phospho-Ser\textsuperscript{387} hRad9 (middle), or phospho-Ser\textsuperscript{387} hRad9 (bottom). One hour prior to lysis, cells were either mock-irradiated or irradiated with 20 Gy of IR, as indicated. B and C, DNA damage-dependent/Ser\textsuperscript{272}-independent hyperphosphorylation of hRad9 requires prior phosphorylation at Ser\textsuperscript{387}. B, HeLa cells were grown under the following conditions: Asyn, asynchronously growing, untreated culture; HU (18h), 0.1 mM hydroxyurea for 18 h; IR (18h), harvested 18 h after a 20-Gy dose of IR; G2 + IR, harvested 7 h after release from a thymidine block and 1 h after a 20-Gy dose of IR; NOC (18h), 0.1 μg/ml nocodazole for 18 h. Soluble cell extracts were immunoprecipitated with antibodies directed against hRad9. Immunoprecipitated hRad9 was then subjected immunoblotting with antibodies directed against hRad9 (α-hRad9; upper panel) and phosphospecific antibodies directed against phosphorylated Thr\textsuperscript{292} of hRad9 (α-p292; lower panel). C, Myc-tagged wild-type, S272A, and S387A hRad9 were transiently expressed at low levels, in the presence of 1 μg/ml tetracycline, using the Tet-Off expression system. Cells were grown under the conditions described for B (with the exception of nocodazole). Soluble cell lysates were immunoblotted with antibodies directed against the Myc epitope. The constitutively phosphorylated (hRad9\textsuperscript{ mutants} and hyperphosphorylated (hRad9\textsuperscript{ mutants}) forms of Myc-hRad9 are indicated. D, mitotic phosphorylation of hRad9 at Thr\textsuperscript{292} does not require Ser\textsuperscript{387}. HeLa cells, transiently expressing S272A or S387A hRad9 were grown in the presence (+) or absence (−) of 0.1 μg/ml nocodazole (NOC) for 18 h. Mitotic (+NOC) and asynchronous (−NOC) cells were then lysed and immunoblotted with α-hRad9 antibodies (upper panel) or phosphospecific antibodies directed against phosphorylated Thr\textsuperscript{292} of hRad9 (α-p292; lower panel).
cells overexpressing S387A and P4A exited G2/M at a reduced rate when compared with cells overexpressing wild-type or S272A hRad9 (Fig. 6B, 13-h time point). Thus, overexpression of hRad9 mutants that cannot be phosphorylated at Ser387 or constitutive (S/T)P sites results in prolonged G2/M arrest following IR, a defect that is also exhibited by cells with reduced hRad9 protein levels.

**DISCUSSION**

We have characterized the extensive constitutive phosphorylation at the C terminus of the hRad9 checkpoint protein. We find that Thr292 is phosphorylated exclusively in mitosis, in a manner that is consistent with it being an *in vivo* substrate of Cdc2. Four other amino acids in hRad9 (Ser277, Ser283, Ser306, and Thr355) that contribute to the constitutive phosphorylation of the protein and share a common consensus sequence with Thr292 (S/T)PXR/R may also be subject to phosphorylation by Cdc2. We have also identified a fifth constitutive site of hRad9 phosphorylation at Ser387. Analysis of hRad9 phosphorylation mutants has indicated that interdependencies exist between distinct hRad9 phosphorylation events. Perhaps more importantly, however, these phosphates appear to be of critical importance for hRad9 association with TopBP1. A P4A mutant, harboring the S277A, S328A, S336G, and T355A mutations, displays reduced affinity for TopBP1 relative to wild-type hRad9, and S387A displays no detectable interaction with TopBP1. Transient expression of the S387A and P4A mutants also disrupts cell cycle progression of cells irradiated during S phase.

ATM phosphorylates hRad9 at Ser272 in response to ionizing radiation (27). hRad9 also undergoes a second DNA damage–regulated hyperphosphorylation that occurs on an amino acid residue other than Ser272 (Fig. 4C). Whereas both of these events are dependent on DNA damage, they differ significantly in their regulation in a number of ways. Ser272 phosphorylation is response to IR occurs rapidly (27) (Fig. 3A), independently of cell cycle position (27) (Fig. 3A), independently of constitutive phosphorylation at Cdk sites and Ser387 (Fig. 4A), and is not.

![Fig. 5. The S387A and P4A mutants display reduced affinity for TopBP1.](image)

**Fig. 5.** The S387A and P4A mutants display reduced affinity for TopBP1. A, wild-type (WT), S272A, S387A, or P4A (S277A/S328A/S336G/T355A) hRad9 proteins were transiently overexpressed in HeLa cells. hRad9 protein was immunoprecipitated from cell extracts using antibodies directed against hRad9. hRad9 containing immune complexes and an aliquot of the corresponding soluble transfected cell lysates were immunoblotted with α-TopBP1, α-hRad9, α-p387, α-hUs1, or α-hRad1 antibodies, as indicated on the left. B, similar to A except that proteins were expressed with an N-terminal Myc tag and immunoprecipitated with antibodies directed against the Myc epitope. Immune complexes were immunoblotted with α-TopBP1, α-Myc, or α-p387 antibodies, as indicated.

![Fig. 6. Prolonged G2/M arrest following IR in cells with reduced hRad9 protein and cells overexpressing the P4A and S387A hRad9 mutants.](image)

**Fig. 6.** Prolonged G2/M arrest following IR in cells with reduced hRad9 protein and cells overexpressing the P4A and S387A hRad9 mutants. A, prolonged G2/M arrest of cells in response to siRNA-mediated reduction of hRad9 protein. Cells transfected with nothing (mock), or small interfering RNA targeting hRad9 (hRad9 siRNA) or luciferase (Luc siRNA), were analyzed for their ability to recover from a 20-Gy dose of IR. Cells were harvested, fixed, and stained with propidium iodide (PI) at increasing periods of time after irradiation (*x* axis). The percentage of cells exiting IR-induced G2/M arrest is represented on the *y* axis. Reduction of hRad9 protein levels by hRad9 siRNA at time points 16, 18, and 20 h was confirmed by immunoblotting with α-hRad9 and α-PCNA (control) antibodies. B, HeLa cells transiently transfected with plasmids encoding wild-type (WT), S272A, S387A, or P4A (S277A/S328A/S336G/T355A) hRad9 were blocked in early S phase with thymidine. 15 min after the removal of thymidine, cells were irradiated with 10 Gy of IR and then fixed 9, 13, and 19 h later (*x* axis). Fixed cells were stained with propidium iodide and analyzed with a flow cytometer. Error bars indicate the S.D. from three independent experiments.
limited when hRad9 is overexpressed (Fig. 4A). Ser272-independent IR-induced phosphorylation, on the other hand occurs less rapidly (Fig. 3B), requires prior phosphorylation at Ser387 (Fig. 4C), and does not occur efficiently when hRad9 is overexpressed. The fact that a similar Ser272-dependent hRad9 mobility shift is observed in cells that are exposed to hydroxyurea or synchronized in G2 (Fig. 4C) suggests that this event is also cell cycle-dependent and may be a response to DNA damage detected during S phase. Others have reported that hyperphosphorylation of hRad9 under similar conditions requires the activity of phosphatidylinositol 3-kinase-related kinases (37). Further experimentation will be required to determine whether this modification is the same as the one reported here.

Our results also demonstrate that the constitutive phosphorylation of hRad9 at (S/T)PXR/R sites and Ser387 is dispensable for interaction with hHus1 and hRad1 (Fig. 5A). These results are in agreement with a previous report, which demonstrated that constitutive phosphorylation of hRad9 does not influence the stability of the 9-1-1 complex (38). In contrast, a recent report has suggested that hRad9 phosphorylation is important for 9-1-1 stability (39). Analysis of a hRad9 mutant, in which phosphorylation is completely ablated, will be required to fully reconcile these seemingly conflicting results.

We have shown, however, that hRad9 interaction with TopBP1 is partially compromised in the P4A mutant and undetectable in the S387A mutant. Since the majority of cellular hRad9 exists as part of the 9-1-1 complex (40), our data are consistent with hRad9 phosphorylation regulating protein interactions with the 9-1-1 complex rather than interactions between 9-1-1 members.

Depletion of hRad9 protein using hRad9-directed siRNA results in a prolonged accumulation of cells in G2/M following irradiation (Fig. 6A). Furthermore, overexpressing hRad9 mutant proteins that cannot be phosphorylated at Ser387 or (S/T)PXR/R constitutive sites (P4A) produces a similar, albeit less pronounced, effect (Fig. 6B). Prolonged G2/M accumulation following irradiation is a common phenotype of S phase checkpoint-defective cells, such as those lacking ATM, BRCA1, Nbs1, and Smc1 (41, 42). It has been proposed to be a compensatory mechanism that provides cells that failed to appropriately arrest DNA synthesis extra time to deal with replicated damaged DNA (41). Therefore, S phase checkpoint dysfunction may be a plausible explanation for the cell cycle defects associated with hRad9 protein depletion and S387A or P4A mutant overexpression.

The fact that these mutants are also defective in their ability to interact with TopBP1 further suggests that the hRad9/TopBP1 interaction plays a functionally significant role in the S phase checkpoint. This is supported by work in S. cerevisiae, which indicates an important role for the Dpb11/Ddc1 interaction in responding to genotoxins (33). The abrogation of DNA damage-dependent, Ser272-independent phosphorylation of hRad9 (Fig. 4C) may also be a contributing factor to the prolonged G2/M phenotype. Mapping the site(s) of this phosphorylation will be required to fully resolve this possibility.

Finally, the data reported here suggest that hRad9 is regulated in part by Cdc2, a kinase generally thought to represent the ultimate target of hRad9-associated signaling pathways. Whereas the maintenance of basal phosphorylation of the hRad9 C terminus appears to be essential for hRad9-mediated DNA damage response, the hyperphosphorylation of hRad9 at Thr292 in mitosis is particularly intriguing and could potentially represent a means of resetting hRad9 activity for future cell cycles. This fact that this phosphorylation is exclusively a mitotic event is consistent with Thr292 being an in vivo substrate of Cdc2. Whereas Thr292 is the only amino acid in hRad9 with the (S/T)PXR/R consensus sequence that was not phosphorylated by Cdc2 in vitro (Fig. 2B), this is probably due to the dependence of Thr292 phosphorylation on prior phosphorylation at Ser272 (Fig. 1, A and B). The extent to which Ser272, Ser387, and Thr292 are phosphorylated outside of mitosis suggests that these sites may be regulated by other kinases in addition to Cdc2. In fact, we have observed that Cdk2 can also phosphorylate each of these four residues in vitro. In addition, protein kinase Cδ has recently been shown to directly regulate hRad9 phosphorylation, although the location of protein kinase Cδ-mediated phosphorylation in hRad9 remains unclear (39).

The non-PCNA-like, C-terminal region of hRad9 represents a potential regulatory domain for the effector functions of the 9-1-1 complex. Its extensive phosphorylation may enable hRad9 and 9-1-1 to coordinate multiple functions during the DNA damage response. The complex nature of hRad9 phosphorylation, however, as illustrated by the large number of phosphorylation sites and the interdependencies between these sites, has made deciphering the precise role of individual phosphates a formidable task. The results presented here advance the current understanding of how hRad9 phosphorylation contributes to checkpoint signaling.

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