DNA Damage-Dependent and Independent Phosphorylation of the hRad9 Checkpoint Protein

Robert P. St.Onge1, Blair D.A. Besley2, Minwoo Park2, Richard Casselman, and Scott Davey1,2,3,4

Cancer Research Laboratories and the Departments of Pathology1, Biochemistry2, and Oncology3
Queen’s University
Kingston, Ontario, K7L 3N6
Canada

4To whom correspondence should be addressed:
Cancer Research Laboratories
Queen’s University
Kingston, Ontario, K7L 3N6
Canada
Tel: (613)-533-6923
Fax: (613)-533-6830
Email: sd13@post.queensu.ca

Running title: Complex Phosphorylation of hRad9
Summary

Cell cycle checkpoints are regulatory mechanisms that maintain genomic integrity by preventing cell cycle progression when genetic anomalies are present. The hRad9 protein is the human homologue of *Schizosaccharomyces pombe* Rad9, a checkpoint protein required for preventing the onset of mitosis if DNA damage is present or if DNA replication is incomplete. Genetic and biochemical analyses indicate that hRad9 is a component of the checkpoint response in humans, and has possible roles in regulating the cell cycle, apoptosis, and DNA repair. Previous studies have indicated that hRad9 is modified by phosphorylation, both in the absence of exogenous stress and in response to various genotoxins. In this study, we report the mapping of several sites of constitutive phosphorylation of hRad9 to [S/T]-P-X-[R/P] sequences near the C-terminus of the protein. We also demonstrate that a serine to alanine mutation at residue 272 abrogates an ionizing radiation (IR) induced phosphorylation of hRad9, and further show that phosphorylation at [S/T]-P sites is not a prerequisite for IR induced phosphorylation of serine 272. Finally, we report that hRad9 undergoes cell cycle regulated hyper-phosphorylation in G2/M that is enhanced by IR, but distinct from that on serine 272. Unlike the IR-induced phosphorylation at serine 272, this event is dependent on serine 277 and threonine 292, two C-terminal [S/T]-P sites in hRad9.
Introduction

An organism’s genome is under constant stress from a variety of endogenous and exogenous sources. While low frequencies of genetic mutation are tolerated, contributing to genetic diversity, high frequencies are harmful and can lead to cancer (1). At the cellular level, eukaryotes have evolved signal transduction pathways called checkpoints to cope with genetic insults (2-4). Checkpoints stall progression through the cell cycle, providing time for cellular responses, such as activation and re-localization of DNA repair enzymes to sites of DNA damage or transcriptional activation of specific genes. Checkpoint arrest can also lead to activation of apoptotic pathways perhaps under conditions when cell death is more beneficial to the organism as a whole than repair (reviewed in (5-7)).

The *hRad9* gene was first identified based on sequence homology to the *rad9*+ gene of the fission yeast *Schizosaccharomyces pombe* (8). In *S.pombe*, *rad9*+ is required for the S-phase and G2 checkpoints, which delay the onset of mitosis if DNA replication is incomplete, or if DNA damage is present, respectively (3,9-13). Five other *S.pombe* genes, *hus1*+, *rad1*+, *rad3*+, *rad17*+, and *rad26*+ are also required for this response (10-13) and are also, with the exception of *rad26*+, conserved in humans (14-17). Like their *S.pombe* orthologues, hRad9, hRad1, and hHus1, interact with each other in a stable complex (15,18-20), that has recently been dubbed the 9-1-1 complex (21). Structural homology between each member of the 9-1-1 complex and PCNA has led to the hypothesis that the 9-1-1 complex replaces replication associated PCNA-dependent functions during DNA repair (22-24). During DNA replication, the PCNA homotrimer forms a ring-like sliding clamp over DNA and acts to increase the processivity of DNA polymerase ∗ (25,26). The 9-1-1/PCNA model is supported by the observation that hRad9, hRad1, and hHus1 each interact with hRad17 (27) which
shares extensive homology to subunits of replication factor C, a protein required for loading PCNA onto DNA (reviewed in (28)). Furthermore, DNA damage induces not only the phosphorylation of hRad9 and hRad1, but also the association of 9-1-1 with chromatin (29). From this, an attractive model has emerged in which hRad17 dependent loading of 9-1-1 onto DNA, at sites of damage, could co-ordinate the multifaceted checkpoint response.

While these data suggest that hRad17 and 9-1-1 are early components of the checkpoint signaling cascade, whether they are responsible for the initial detection of DNA damage still remains unclear. In *S. pombe*, the Rad3 protein, a PI3 related kinase, phosphorylates Rad26 in response to DNA damage independently of the other checkpoint rads (30), suggesting that it is the initiator of the checkpoint signaling cascade. Two human homologues of Rad3, ATM and ATR, phosphorylate a wide variety of cellular proteins on [S/T]-Q sequences in response to DNA damage (31-38). Mutations in ATM result in the cancer predisposition syndrome, ataxia telangiectasia (39). Recently, hRad9 has been implicated as an ATM substrate (40). While the evidence from fission yeast indicate that hRad9 is fulfilling a role in the G2/M transition (11), ATM-dependent phosphorylation of hRad9 occurs regardless of cell cycle position and appears to be important for the G1 DNA damage checkpoint (40). hRad9, through interactions with the anti-apoptotic Bcl-2 and Bcl-xL proteins, can also promote apoptosis, and therefore, appears to have a multi-functional role in responding to genotoxins (41).

Previous studies have indicated that the hRad9 protein is extensively modified by phosphorylation under normal cellular conditions (19) and becomes hyper-phosphorylated in response to DNA damage at serine 272 (18,40). Here, we further the current understanding of hRad9 phosphorylation by mapping sites required for its
constitutive phosphorylation and by identifying of a novel, cell cycle regulated, ionizing radiation-induced phosphorylation event.

**Experimental Procedures**

*Plasmids* - The full length hRad9 cDNA was subcloned into unique XhoI and XbaI restriction sites of the pyDF31 mammalian expression vector. Protein expression from pyDF31 is driven by the strong constitutive SR alpha promoter composed of the SV40 early promoter and a segment of the LTR of Human T-Cell Leukemia Virus (42). All hRad9 point mutants were generated in pyDF31 using the Transformer Site-Directed Mutagenesis Kit (Clontech, Palo Alto, CA) according to the manufacturer’s instructions. Three selection primers were used to disrupt unique XhoI, NotI, and EagI restriction sites in pyDF31-hRad9. The sequence of these primers and the hRad9 mutagenic primers are shown in Table 1. Constructs with multiple point mutations were made by sequential mutagenesis reactions or concurrently by using multiple mutagenic primers in the same reaction. The presence of the desired base substitutions were confirmed by DNA sequencing using an automated sequencer (Cortec DNA Services Laboratory, Kingston ON, and Canadian Molecular Research Services, Ottawa ON).

*Cell Culture and Transfections* - HeLa cells were maintained in Dulbecco’s Modified Eagle Medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% Fetal Bovine Serum (Invitrogen) at 37°C in 5% CO₂ atmosphere. The hTERT-RPE1 cell line, a human retinal pigment (RPE) cell line that stably expresses the human telomerase reverse transcriptase subunit (hTERT) (Clontech) was maintained as HeLa cells except in DMEM F12 media (Invitrogen). HeLa cells were transfected in 10cm or 6 well plates using 20:1 or 2:1 of a 2:1 molar ratio of DOPE (1,2-dioleoyl-
snnglycerophosphatidylethanol-amine) (Sigma, Oakville ON) and DDAB (dimethyldioctadecyl-ammonium bromide) (Sigma), respectively. The transfection reagent was mixed with 2\(\mu\)g DNA in 3.3ml DMEM (10cm plate) or 0.25\(\mu\)g in 600\(\mu\)l DMEM (6-well plate) and applied to cells for 4 hours at 37°C. The transfection solution was then replaced with DMEM plus 10% FBS, and cells were cultured for an additional 30 to 48 hours prior to lysis.

Cell Synchronization and Flow Cytometry - HeLa cells, cultured as described above, were synchronized in early S-phase by double thymidine block as previously described (43). Cells were cultured to a confluence of approximately 30% and treated with 2mM thymidine for 18 hours. After 18 hours, cells were released from thymidine for 8 hours, treated for an additional 18 hours, and then released for varying lengths of time. hTERT-RPE1 cells were synchronized in early S-phase using a single, 24 hour dose of 5mM thymidine. Mitotic HeLa cells were generated by treatment with 70ng/ml demicollcine (Sigma). Synchronized cell populations were followed by flow cytometry; cells were harvested, resuspended in 1ml of PBS +1% FBS, fixed by addition of 1ml of 100% ethanol, and stored at 4°C for at least 1 hour. After fixing, cells were washed twice in PBS, resuspended in 1ml of PBS + 1% FBS and 0.5mg/ml RNaseA, and incubated for 40 min at 37°C. Cells were then collected by centrifugation and resuspended in PBS + 50\(\mu\)g/ml propidium iodide and 0.1mg/ml RNaseA and analysed using a flow cytometer (Beckman/Coulter EPICS Elite, Mississauga, ON).

Calf Intestinal Phosphatase (CIP) Treatment - Cells were lysed in 1ml of NETN lysis buffer (250mM NaCl, 1mM EDTA, 20mM TRIS pH 8.0, 0.5% NP-40) supplemented with 1% Triton-X, 20\(\mu\)g/ml aprotinin, 4\(\mu\)g/ml leupeptin, 2mM sodium orthovanadate, 20mM 3-glycerophosphate, and 0.2mM sodium fluoride. Lysates were
incubated on ice for 30 minutes and centrifuged at 13000g. Supernatants were pre-cleared by the addition of 15:l of ∀-chicken IgY agarose (Promega, Madison WI) for 30 minutes at 4°C prior to immunoprecipitation. The immunoprecipitation was performed with 1:g of affinity purified ∀-hRad9 polyclonal antibodies ((19); RCH antibodies, Kingston ON) and 15:l of ∀-chicken IgY agarose (Promega) for 2 hours at 4°C. Immunoprecipitated proteins were washed four times with 1ml of cold PBS and resuspended in 50:l of 0.2 X NEB buffer 3 (New England Biolabs, Mississauga ON) and 1% SDS. Samples were boiled for 5 minutes and centrifuged at 6000g for 10 minutes. 20:l of supernatant was incubated in the presence of 30 U of calf intestinal phosphatase (Boehringer Manheim, Germany), in 0.2 X NEB buffer 3 in a final volume of 200:l for 30 minutes at 37°C. The reaction was stopped by the addition of 100:l of 3X SDS-PAGE sample buffer.

Metabolic Labelling - HeLa cells were transfected in a 6 well plate as described above. 24 hours after transfection, the cells were washed 3x with phosphate free DMEM (Gibco) then incubated for 1 hour in phosphate free DMEM + 10% dialyzed FBS (dialyzed against 150mM saline to deplete phosphate concentrations). Cells were labelled with 1.25ml of phosphate free DMEM containing 10% dialyzed FBS and 333:Ci 32P orthophosphoric acid (NEN, Boston, MA). Cells were further incubated at 37°C in 5% CO2 for 18 hours. Cells were washed 3x with DMEM and 3x with PBS and harvested with a cell scraper. Cells were collected by microcentrifugation at 500g for 2 minutes. The supernatant was removed and the cells lysed in 400:l NETN buffer containing, 0.4mM 4-(2-aminoethyl)benzenesulfonyl fluoride HCl (AEBSF), 20:g/ml aprotinin, 4:g/ml leupeptin, 0.7:g/ml pepstatin, 2mM Na3VO4, 20mM 3-glycerophosphate and 0.2mM NaF. Lysates were incubated on ice
for 1 hour, then centrifuged at 16000g for 20 min at 4°C. Soluble cell lysates were immunoprecipitated with antibodies directed against hRad9 essentially as described above. Agarose was washed 4x with NETN buffer and resuspended in 60:1 of 2x electrophoresis buffer and boiled for 5 min prior to SDS-PAGE (10% acrylamide). The gel was either transferred to nitrocellulose and immunoblotted with antibodies directed against hRad9 or dried using a gel slab dryer (BIORAD, Mississauga, ON). Protein quantification of the immunoblot was performed using AlphaEase software and a chemiimager (Alpha Innotech Corporation, San Leandro, CA). 32P quantification was performed using ImageQuant software and a phosphoimager (Molecular Dynamics, Sunnyvale, CA).

Immunoblotting - All samples were boiled for 5 minutes prior to electrophoresis through 8% or 10% acrylamide, as indicated. Proteins were transferred to nitrocellulose (Amersham Pharmacia, Baie d-Urfe, PQ) using a semi-dry transfer cell (BIO-RAD) or a wet transfer apparatus (Hoefer Scientific, San Francisco, CA). Membranes were blocked for 1 hour in 5% non-fat milk powder in PBS plus 0.1% Tween 20 and then incubated in affinity purified ∀-hRad9 polyclonal chicken antibodies, at 50ng/ml in PBS, for 1 to 18 hours at 4°C. Following extensive washing in PBS plus 0.1% Tween 20, blots were incubated in HRP conjugated ∀-chicken secondary antibody (Jackson Labs, Mississauga, ON) at a final dilution of 1 in 50,000 for 45 minutes at 4°C. Membranes washed and incubated in Western Blot Chemiluminescence Reagent Plus (NEN) prior to exposure to X-ray film (KODAK, Rochester, NY).

Results
hRAD9 is Constitutively Phosphorylated on Serine/Threonine-Proline Sequences - The hRad9 protein is constitutively phosphorylated in the absence of DNA damage (19), and becomes additionally phosphorylated when DNA damage is present (18). We have observed that under normal cellular conditions, (i.e. those where cells are not exposed to exogenous stresses) overexpressed hRad9 consists of four species that differ in migration rate on SDS-PAGE as visualized by immunoblot analysis. The slowest migrating of these species, which we have termed hRad9∀, co-migrates with the majority of endogenous hRad9 at an apparent molecular weight of approximately 60 kDa (Figure 1a). In vitro dephosphorylation of exogenous hRad9 causes each of its four migratory forms to collapse into a single band at about 45 kDa, which we have called hRad9∗ (Figure 1b). These data suggest that, relative to endogenous hRad9, a large portion of the overexpressed protein is only partially phosphorylated, and hence has various migratory forms. We have collectively designated all of these partially phosphorylated forms hRad9∃. Previous work in our lab has shown that deleting the C-terminus of exogenous hRad9 can reduce the number of differentially migrating species from four to one (our unpublished results), indicating that the C-terminus of hRad9 is required for constitutive phosphorylation of the protein.

With this in mind, we used site directed mutagenesis to identify amino acid residues required for the constitutive phosphorylation of hRad9. Potential phosphorylated residues near the C-terminus of the protein were converted to non-phosphorylatable amino acids. Mutants were expressed in HeLa cells following transient transfection, and screened for migratory shifts as detected by western analysis. The gross overexpression of protein by the strong SR-alpha promoter allowed us to distinguish plasmid derived hRad9 protein from the endogenous protein.
simply by limiting the exposure time to X-ray film. A trend between those mutants with altered western blot banding patterns soon emerged, as each of these mutants contained disrupted serine or threonine residues followed immediately by a proline (results summarized in Table 2). The [S/T]-P motif is the minimum consensus sequence for the cyclin dependent kinase (CDK) family of kinases (44). We therefore mutated all nine [S/T]-P sequences in hRad9, immunoprecipitated these proteins from transfected HeLa cells, and compared their banding pattern to the wild type protein (Figure 1c). Of the nine [S/T]-P mutants, four exhibit changes in hRad9 mobility on SDS-PAGE, suggesting that they are sites of phosphorylation. The S277A and S328A mutants exhibited complete loss of two bands each. The S336G mutation caused an increase in mobility of three of the four bands while the T355A mutation caused a slightly increased mobility in all four of the hRad9∀ and ∃ forms (Figure 1c; upper panel). When dephosphorylated by treatment with calf intestinal alkaline phosphatase, each protein migrated at about 45kDa (hRad9*) indicating that the mobility shifts in the upper panel are indeed the result of phosphorylation changes (Figure 1c; lower panel). Sequentially introducing these four mutations in combination leads to a reduction in the number of mobility forms and a progressive increase in the mobility of hRad9 (Figure 1d). While the P2A protein (S328A+S336G) migrated as a doublet, P3A (P2A+S277A) migrated as a single band with slightly less mobility than P4A (P3A+T355A). This subtle shift resulting from the T355A mutation is consistent with that observed in figure 1c, as well as figures 6a (panel 2) and 6b. P4A migrated with an approximate molecular weight of 45 kDa (Figure 1d), which is only slightly larger than the predicted molecular weight of 42.5 kDa for hRad9.
hRad9 is Constitutively Phosphorylated on Sites Other Than [S/T]-P

Sequences - The P4A mutant still exhibited a modest mobility shift when treated with alkaline phosphatase (Figure 2a). This suggested the existence of additional sites of constitutive phosphorylation in hRad9. To determine if these additional sites were [S/T]-P sites, whose phosphorylation produced little or no shift on a western blot, we mutated all nine [S/T]-P sites in hRad9 in combination. The migration of the P9A mutant (P4A + T60A, S160A, T292A, S375A, and S380G) was not readily distinguishable from that of the P4A mutant and was still sensitive to phosphatase treatment (Figure 2a). Therefore, despite being constitutively phosphorylated on at least four [S/T]-P sites, hRad9 is also phosphorylated on sites other than these sequences. We have called the form of hRad9, which completely lacks phosphorylation at [S/T]-P sites but still remains phosphorylated, hRad9.

To further characterize the constitutive phosphorylation of hRad9, HeLa cells were transfected with a series of hRad9 mutants or empty vector and metabolically labelled with 32P ortho-phosphoric acid. The hRad9 point mutants, P3A, P4A, P5A (P4A + T292A), P6A (P5A + S375A), and P9A were used in the transfection and following metabolic labelling, the hRad9 protein was immunoprecipitated from these cells. Immunoprecipitated protein was size-fractionated by SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted with antibodies directed against hRad9 (Figure 2b; left panel). The amount of hRad9 protein recovered from each immunoprecipitation was defined by densitometric analysis of the immunoblot using a chemiimager, and was used for data normalization as described below. A second, identical gel was also run, dried, and exposed to a phosphor screen and analyzed using a STORM phosphoimager (Figure 2b; right panel). In agreement with Figure 2a, each
hRad9 mutant, including P9A, was significantly phosphorylated confirming that the hRad9 protein is constitutively phosphorylated at sites other than [S/T]-P sites.

We went on to quantitate the $^{32}$P signal in Figure 2b using the ImageQuant software program (Figure 2c). Values were normalized to background $^{32}$P in each lane and to protein levels (which were all within 15%) determined above by spot densitometry of the hRad9 immunoblot. Since only the P3A mutant was wild-type at threonine 355, a site we knew to be constitutively phosphorylated (see Figure 1), we expected that P3A should have a stronger signal than each of the other four mutants, and it did. The remaining mutants exhibited smaller differences in relative $^{32}$P activity, which may be a reflection of confounding variables inherent to the experiment, such as protein normalization, rather than legitimate differences in phosphorylation.

$hRad9$ Phosphorylation on Serine 272 in Response to Ionizing Radiation Does not Require Constitutive Phosphorylation at [S/T]-P sites - ATM and ATR, two serine/threonine DNA damage responsive kinases that likely function in the same biochemical pathway as hRad9, have been demonstrated to phosphorylate target proteins at serine/threonine-glutamine ([S/T]-Q) sequences (33). Since serine 272 of hRad9 is followed immediately by a glutamine residue, we hypothesized that this amino acid was the site of the previously reported ionizing radiation (IR)-induced phosphorylation of hRad9 (18). In this regard, we observed that a serine to alanine mutation at serine 272 had no effect on constitutive phosphorylation but abrogated the ability of exogenously expressed hRad9 to become phosphorylated in response to ionizing radiation (Figure 3a). While sub-populations of both the $\forall$ and $\exists$ forms of wild-type hRad9 (WT) underwent a subtle mobility change when cells were treated
with 20 and 40 Gray doses of IR (hRad9∀ to hRad9( and hRad9∃ to hRad9∃()'), the
S272A mutant showed no changes in hRad9∀ or hRad9∃ mobility (Figure 3a; upper
panel). This shift was confirmed to be the result of phosphorylation, as CIP treatment
of these samples yielded co-migrating dephosphorylated proteins (hRad9*, Figure 3a;
lower panel). While the subtle nature of the mobility shift, combined with the
complex banding pattern of hRad9 has made this effect difficult to observe by
immunoblotting techniques, we have found this result to be reproducible and offer
further evidence in support of it in figures 3b and 3c. This observation also confirms a
recent report which demonstrated that ionizing radiation induced phosphorylation at
this residue, and that this phosphorylation was ATM-dependent (40). This report
however, like all previous studies demonstrating IR-induced phosphorylation of
hRad9, involved a constitutively phosphorylated protein (18,29). While the purpose of
this constitutive phosphorylation remains unknown, it may be potentiating some
aspect of hRad9's cellular activity. The observation in Figure 3a that the ∃ forms of
wildtype hRad9, like the ∀ form, shift subtly in response to IR, seems to indicate that
[S/T]-P phosphorylation of hRad9 is not required for IR-induced phosphorylation at
serine 272.

To address this directly, we tested the response to IR of hRad9 P9A, which
lacks all phosphorylatable [S/T]-P sites. The P9A hRad9 mutant was expressed in
cells that were subsequently irradiated or mock irradiated. P9A was
immunoprecipitated from these cells one hour later, treated with phosphatase as
indicated, size-fractionated by SDS-PAGE, and immunoblotted with antibodies
directed against hRad9 (figure 3b). In response to irradiation, a slower migrating form
of hRad9, (hRad9,(()) became readily apparent. Both hRad9, and hRad9,(()) increased
in mobility and co-migrated when treated with CIP (hRad9*) indicating that hRad9,(())
was in fact a phosphorylated form of hRad9. To confirm that the IR-induced phosphorylation of hRad9, was occurring at serine 272, the S272A mutation was introduced into the P9A mutant and these proteins were analyzed for mobility changes following exposure to low and high doses of IR (Figure 3c). Consistent with Figure 3b, the P9A mutant migrated as a doublet at all time points following 20 Gray of irradiation, although the slower migrating species was less abundant at 240 minutes post-IR. The formation of this doublet was not observed however, when the S272A mutation was introduced into the P9A mutant background (figure 3c; far right). At the lower dose of 4 Gray (Figure 3c; left panels) this mobility shift was much less pronounced and did not persist as long, indicating a dose dependent response typical of checkpoint control. Collectively, these results indicate that constitutive phosphorylation at [S/T]-P sites is not essential for the IR-induced phosphorylation of hRad9 at serine 272.

**hRad9 is Phosphorylated in a Cell Cycle Dependent Manner in HeLa Cells** - Given that hRad9 is phosphorylated on potential CDK consensus sites, we hypothesized that the attachment of these seemingly constitutive phosphate groups may be regulated in a cell cycle dependent manner. Therefore, a double thymidine block was used to generate synchronized cell populations, which were examined for differences in endogenous hRad9 phosphorylation by immunoblotting (Figure 4a; top). Cell cycle position was monitored by flow cytometry of propidium iodide stained nuclei (Figure 4a; bottom). Consistent with a recent report (40), hRad9∀, which we have concluded to be highly phosphorylated at C-terminal [S/T]-P sites, was observed in all phases of the cell cycle (Figure 4a). However, even slower migrating forms of hRad9, migrating at an apparent molecular weight of
approximately 65 kDa (hRad9Φ) appeared in samples collected during G2/M (Figure 4a; samples 7, 8, 9, and 10). We went on to demonstrate that this mobility shift was the result of a cell cycle specific phosphorylation event as the hRad9∀ and hRad9Φ bands in G2/M HeLa cells collapsed into a single faster migrating hRad9∗ band at 45 kDa (Figure 4b).

To address whether this phosphorylation change was occurring in G2, mitosis, or both, late S-phase HeLa cells were either irradiated with 4 Gray of IR to delay cells in G2, treated the microtubule inhibiting drug demicolcine to arrest cells in mitosis, or left untreated and allowed to cycle into G1. Consistent with Figure 4a, in the untreated cells, the hyper-phosphorylated hRad9Φ form(s) was present in G2 and M but not when cells had cycled into G1 (Figure 4c; first 4 lanes; 7, 8, 9, and 10h). If cells were treated with either a low dose of IR or demicolcine, cells did not progress into G1 and hRad9Φ persisted (Figure 4c; 10h time points). Taken together, these results indicate that in HeLa cells, endogenous hRad9 is hyper-phosphorylated in G2 and mitosis, on an amino acid residue(s) distinct from the constitutive phosphorylation sites we have identified.

Cell Cycle Dependent Phosphorylation of hRad9 is Enhanced by IR but is Distinct and Independent of Phosphorylation at Serine 272 of hRad9 - To confirm that the cell cycle regulated hyper-phosphorylation of hRad9 was distinct from that induced by high doses of ionizing radiation at serine 272, synchronized cells were irradiated with 20 Gray of IR at various stages of the cell cycle 30 minutes prior to their harvest. Consistent with Figure 3a, and the findings of Chen et al. (40), in Figure 5a, it was observed that endogenous hRad9 was shifted in a subtle manner in response to ionizing radiation regardless of cell cycle position (hRad9()). The appearance of
hRad9 occurred independently, and was distinct from hRad9Φ in G2/M cells (Figure 5a; 6 and 8 hour time points). Furthermore, the amount of hRad9Φ was modestly increased if cells were treated with ionizing radiation prior to harvest at time points 4, 6, 8, and to a lesser extent 10 when most cells were in G1.

To address whether the cell cycle regulated phosphorylation of hRad9 was cell type specific, occurring exclusively in HeLa cells, we repeated this experiment in hTERT-RPE1 cells (Figure 5b). These cells have an indefinite life span due to stable expression of the human telomerase reverse transcriptase subunit, but maintain the normal karyotype of primary epithelial cells. As in Figure 5a, hRad9 in the hTERT-RPE1 cells underwent a modest mobility shift in response to IR (hRad9Φ) at all stages of the cell cycle (Figure 5b). hRad9Φ in G2/M hTERT-RPE1 cells however, was much less prevalent in the absence of IR compared to that observed for the HeLa cell line. Nevertheless, upon irradiation the abundance of hRad9Φ increased significantly (Figure 5b; hours 12, 14, and 16), indicating that hRad9 undergoes cell cycle dependent IR-induced phosphorylation in both HeLa cells and hTERT-RPE1 cells.

Efficient Cell Cycle Regulated Hyper-phosphorylation of hRad9 Requires Serine 277 and Threonine 292 - To address whether the cell cycle regulated hyper-phosphorylation of hRad9 was dependent on phosphorylation at [S/T]-P sites, we expressed each of the nine single [S/T]-P mutants in HeLa cells and examined these mutants for the cell cycle dependent mobility changes we observed in the endogenous protein (Figures 4 and 5). The S272A ATM consensus site mutant was also included in this experiment. Transfected HeLa cells were arrested in mitosis by treatment with demicolcine, lysed, and then immunoblotted with antibodies directed against hRad9 (Figure 6a). The absence of signal in the mock-transfected lane confirmed that any
mobility changes were derived from the overexpressed protein and not endogenous hRad9. Propidium iodide staining was used to confirm the effectiveness of the demicollcine treatment. In each of the transfected cell populations, demicolcine treatment resulted in an increase in the number of cells in G2/M from 20-29% in the untreated, asynchronous cells, to 67-82% in the DC treated cells (our unpublished results). When cells were arrested in mitosis, all proteins exhibited a significant reduction in the abundance of the faster migrating hRad9∃ forms. Furthermore, the majority of the wild-type protein existed as two species which migrated slower through SDS-PAGE than the hRad9∀ form from asynchronous cells (figure 6a; upper panel). These species are similar to the hRad9Φ forms observed in the G2/M samples of figure 4a. The cell cycle dependent hyper-phosphorylation of hRad9 was also observed in the S272A mutant protein (figure 6a; lower panel) as well as each of the single [S/T]-P mutants with two notable exceptions. When the constitutively phosphorylated serine 277 is mutated to alanine, the efficiency of this mitotic hyper-phosphorylation is drastically reduced, as most of the protein co-migrates with the slowest migrating S277A form in the untreated cells. Mutational inactivation of threonine 292 results in the significant reduction of the fastest migrating hyper-phosphorylated species, and the complete loss of the slowest migrating hRad9Φ form (figure 6a; upper panel).

These observations were confirmed using hRad9 mutant proteins harboring multiple mutations at [S/T]-P sites (figure 6b). When multiple constitutively phosphorylated [S/T]-P sites were disrupted but serine 277 was left intact (P2A and P2A+T355A), the protein was still efficiently hyper-phosphorylated when cells were mitotic. Upon introduction of the S277A mutation in these proteins (P3A and P4A), the efficiency of this hyper-phosphorylation was drastically reduced, just as it was for
the S277A single mutant. When the T292A mutation was introduced into the P3A and P4A proteins (P3A+T292A and P5A), the hyper-phosphorylation was no longer apparent. Not surprisingly, the same was observed for the P9A mutant which lacks all nine hRad9 [S/T]-P sites.

**Discussion**

We have mapped four amino acids that are constitutively phosphorylated in the hRad9 checkpoint protein. These amino acids (serine 277, serine 328, serine 336, and threonine 355), when converted to non-phosphorylatable residues alter the mobility of hRad9 in SDS-PAGE in a way which is consistent with decreased phosphorylation (Figure 1). Each of the four phosphorylated residues we have identified is followed immediately by proline in the primary amino acid sequence of hRad9. Based on this observation, the kinase(s) phosphorylating these sites in hRad9 could belong to the cyclin dependent kinase (CDK) family of kinases. Several CDKs recognise the [S/T]-P motif as a minimal consensus site in target substrates. The preferred CDK consensus site of [S/T]-P-X-[K/R] is only represented at serine 277 of hRad9 (S\textsuperscript{277}PER). A proline at position four, which some evidence indicates may be tolerated by CDKs (45), is found in each of the remaining three constitutive hRad9 sites we have identified (S\textsuperscript{328}PGP, S\textsuperscript{336}PGP, and T\textsuperscript{355}PPP). Threonine 292, which we conclude to be required for the cell cycle dependent phosphorylation of hRad9 represents the only other hRad9 [S/T]-P site with a proline at position four (T\textsuperscript{292}PHP). The four remaining [S/T]-P sequences in the protein (threonine 60, serine 160, serine 375, and serine 380) contain neither P, R, nor K at position 4 (Table 2). This, in addition to our observations that mutating these residues does not alter the mobility of hRad9 through SDS-PAGE (figures 1c, 2a, 6a, and 6b) or significantly reduce \(^{32}\text{P}\).
uptake in metabolically labelled cells (figure 2c) suggests that these four sites are not constitutively phosphorylated.

While we have termed the [S/T]-P phosphorylation sites constitutive, we can not rule out the possibility that phosphorylation at these sites is regulated in a complex manner. Several groups, using different cell lines and antibodies have observed, as we have, that endogenous hRad9 exists primarily as a single species migrating at 60kDa on a western blot (hRad9\(\forall\)). However, we have occasionally observed bands in the 45-60kDa range when studying endogenous hRad9 that correlate with the partially phosphorylated bands of the overexpressed protein (hRad9\(\exists\)). Even though we are able to limit the abundance of these bands by increasing the concentration of phosphatase inhibitors in our lysis buffer, or by lysing cells directly in SDS-PAGE sample buffer, we cannot rule out the possibility that constitutively phosphorylated hRad9 intermediates are physiologically significant. Importantly however, no consistent changes were observed in the 45-60kDa bands at different stages of the cell cycle (Figure 4a, Figure 5b and 5c), suggesting that phosphorylation at serine 277, serine 328, serine 336, and threonine 355 remains constant throughout the cell cycle.

Recently, a phosphospecific antibody directed against the serine 272 of hRad9 was used to demonstrate that IR-induced phosphorylation of hRad9 occurs at this site in vivo (40). We have confirmed these findings, using an independent method, by showing that a serine to alanine mutation at residue 272 abrogates hRad9 phosphorylation when asynchronous cells are given a high dose of IR (figure 3a). The observation made by Chen et al., that phosphorylation at this residue is dependent on ATM raises many interesting questions regarding the detection of IR-induced DNA damage and the initiation of the checkpoint response in general. In further agreement with the findings of Chen et al., is our observation that phosphorylation at serine 272
occurs independently of cell cycle position (Figure 3a and Figure 5a and 5b). Two lines of evidence indicate that this occurs independently of [S/T]-P phosphorylation as well. First, when cells are treated with high doses of IR, subtle migratory changes in both the ∀ and ∃ forms of overexpressed hRad9 are observed that are not observed in a S272A mutant (Figure 3a). Second, mutational inactivation of the [S/T]-P phosphorylation sites still yield a protein (hRad9,) that is capable of serine 272 phosphorylation in response to IR (Figure 3b and 3c).

We also report the identification a second hyper-phosphorylation event for hRad9 that is cell cycle regulated. We first identified these phospho-forms (hRad9Φ) in HeLa cells that had been synchronized in G2 or mitosis, and found that we could moderately increase their abundance by treating these cells with ionizing radiation. In contrast, we found that hTERT-RPE1 cells, a karyotypically normal human epithelial cell line, displayed practically no hRad9Φ in G2 and M unless cells were irradiated at this cell cycle position. The most plausible explanation for this inconsistency is that even in the absence of IR, the highly proliferative, cancerous HeLa cells contain sufficient endogenous DNA damage to trigger the checkpoint response. Hence, in the absence of IR this cell cycle regulated and DNA damage responsive phosphorylation event is readily detectable in HeLa cells. Based on our mutagenesis studies, we can conclude that the cell cycle regulated phosphorylation of hRad9 occurs at a site(s) other than those we have identified as constitutive phosphorylation sites (serine 277, serine 328, serine 336, or threonine 355), and does not occur on the IR-induced phosphorylation site at serine 272.

While phosphorylation at serine 272 is independent of cell cycle position and [S/T]-P phosphorylation, the cell cycle regulated phosphorylation of hRad9 is dependent on both cell cycle position and constitutive [S/T]-P phosphorylation.
Specifically, a S277A mutation, reduces not only the constitutive phosphorylation of hRad9, but also its ability to undergo cell cycle regulated hyper-phosphorylation when cells are arrested in mitosis (figure 6). Each of the other constitutive sites we have identified do not exhibit this behaviour as mutational inactivation of serine 328, serine 336, and threonine 355, singly or in combination still yield a protein capable of efficient cell cycle regulated hyper-phosphorylation (figure 6). Substituting threonine 292 with non-phosphorylatable alanine however, completely abrogates one of the G2/M hyper-phosphorylated species of hRad9 and reduces the abundance of the other (Figure 6). Interestingly, threonine 292 is the only [S/T]-P site that is conserved between hRad9 and S.pombe Rad9 (46). The complete loss of one of the hRad9Φ forms in a T292A mutant may indicate that threonine 292 is being phosphorylated in G2/M. An alternative explanation, is that threonine 292 is constitutively phosphorylated, and like serine 277, is a prerequisite for hyper-phosphorylation of hRad9. While the T292A mutation yields no detectable electrophoretic mobility change under normal conditions (figure 1c, 2a, 6a, and 6b) a modest decrease in $^{32}$P uptake was observed in $^{32}$P labelled cells (figure 2c; P4A compared to P5A, P6A, and P9A). Therefore, while the cell cycle regulated hyper-phosphorylation is clearly dependent on threonine 292, further experimentation will be required to fully resolve the nature of this dependency.

Perhaps of further interest is the observation that the hRad9∃ forms, whose abundance normally exceed that of the hRad9∀ form when the protein is overexpressed (see figure 1), are all but absent during a demecolcine induced mitotic arrest (figure 6a). This could be the result of destabilisation or further phosphorylation of hRad9∃ at the G2/M transition. Whether this has any relevance to the endogenous protein however, which exists predominantly in the hRad9∀ form, remains to be seen.
There has been much speculation recently that the association of hRad9 with hRad1 and hHus1 results in the formation of a ring-like heterotrimer that encircles the DNA double helix. While the crystal structure of this complex has yet to be solved, structural predictions using the primary amino acid sequence of these three proteins indicate similarity to the PCNA homotrimer, a ring-like complex that acts as a sliding clamp over DNA. In these modelling studies, the entire length of hRad1 and hHus1 are used but only the first 280 amino acids of hRad9 fit the predicted PCNA-like model (24). The SQ and [S/T]-P sites critical for hRad9 phosphorylation sites we have reported here are located at either the very end of this region (serine 272 and serine 277), or C-terminal to it (threonine 292, serine328, serine336, and threonine 355). Since some data suggest that phosphorylation at these residues may be important for 9-1-1 assembly (18,21), the C-terminus of hRad9 may be acting as a regulatory domain for assembly of this complex. Alternatively, the attachment of these phosphates could influence some other aspect of hRad9 function, such as its pro-apoptotic role.

While we have now identified two distinct forms of damage dependent hRad9 phosphorylation, mapped at least four sites of constitutive phosphorylation, and characterized the interdependence of these events, many questions remain regarding the nature and function of hRad9 phosphorylation. These include the location of the remaining constitutive sites of phosphorylation and the identification of proteins responsible for the addition and removal of phosphates. In addition, it remains unclear whether the cell cycle regulated phosphorylation of hRad9 occurs in G2 and persists through mitosis or whether these are two separate events. In any event, this is the first evidence linking the hRad9 protein to the G2/M transition, a transition in which the *S.pombe* Rad9 protein plays an instrumental regulatory role. Furthermore, the
discovery that hRad9 undergoes at least two distinct phosphorylation events in
response to IR raises other interesting questions. It is known hRad9’s hyper-
phosphorylation in response to IR occurs concurrently with its association with
chromatin (29), and though it has yet to be shown directly, there is likely an
interdependence between these two events. Which IR-induced phosphorylation of
hRad9 coincides with its association with DNA is currently an unresolved issue.

Acknowledgments

We thank Dr. David LeBrun and Dr. Susan Cole for helpful discussion. We
also thank Kathy Kennedy, Derek Schulze, and Maureen Rogers for technical
assistance and Marie Evangelista, Lee Fraser, and Deborah Greer for critically
reading the manuscript. This work was supported by the Canadian Institutes of Health
Research grant MOP-14352 and MOP-36526 and National Institutes of Health grant
E507940 (to SD). Flow cytometry was funded in part by the Canadian Institutes of
Health Research grant MT-7827. SD is a Cancer Care Ontario Scientist. RPS is the
recipient of a National Cancer Institute of Canada studentship. BDAB is the recipient
of a US Army Breast Cancer research studentship DAMD17-98-1-8080.

References

1. Loeb, L. A. (1991) Cancer Res 51, 3075-9
2. Hartwell, L. H., and Weinert, T. A. (1989) Science 246, 629-34
3. Weinert, T. A., and Hartwell, L. H. (1988) Science 241, 317-22
4. Hartwell, L. (1992) Cell 71, 543-6
5. Elledge, S. J. (1996) Science 274, 1664-72
6. Weinert, T. (1998) Curr Opin Genet Dev 8(2), 185-93
7. Zhou, B. B., and Elledge, S. J. (2000) *Nature* **408**(6811), 433-9.

8. Lieberman, H. B., Hopkins, K. M., Nass, M., Demetrick, D., and Davey, S. (1996) *Proc. Natl. Acad. Sci. USA* **93**, 13890-13895

9. Enoch, T., and Nurse, P. (1990) *Cell* **60**, 665-73

10. Enoch, T., Carr, A. M., and Nurse, P. (1992) *Genes Dev* **6**, 2035-46

11. al-Khodairy, F., and Carr, A. M. (1992) *EMBO J* **11**, 1343-50

12. al-Khodairy, F., Fotou, E., Sheldrick, K. S., Griffiths, D. J., Lehmann, A. R., and Carr, A. M. (1994) *Mol Biol Cell* **5**, 147-60

13. Rowley, R., Subramani, S., and Young, P. G. (1992) *EMBO J* **11**, 1335-42

14. Cimprich, K. A., Shin, R. B., Keith, C. T., and Schreiber, S. L. (1996) *Proc. Natl. Acad. Sci. USA* **93**, 2850-2855

15. Kostrub, C. F., Knudsen, K., Subramani, S., Enoch, T., Kostrub, C. F., Knudsen, K., Subramani, S., and Enoch, T. (1998) *EMBO JOURNAL* **17**(7), 2055-2066

16. Parker, A. E., Van de Weyer, I., Laus, M. C., Verhasselt, P., and Luyten, W. H. (1998) *J Biol Chem* **273**(29), 18340-6

17. Udell, C. M., Lee, S. K., and Davey, S. (1998) *Nucleic Acids Res.* **26**, 3971-3978

18. Volkmer, E., and Karnitz, L. M. (1999) *J Biol Chem* **274**(2), 567-70

19. St. Onge, R. P., Udell, C. M., Casselman, R., and Davey, S. (1999) *Mol Biol Cell* **10**(6), 1985-95

20. Hang, H., and Lieberman, H. B. (2000) *Genomics* **65**(1), 24-33.

21. Burtelow, M. A., Roos-Mattjus, P. M., Rauen, M., Babendure, J. R., and Karnitz, L. M. (2001) *J Biol Chem* **4**, 4
22. Caspari, T., Dahlen, M., Kanter-Smoler, G., Lindsay, H. D., Hofmann, K., Papadimitriou, K., Sunnerhagen, P., and Carr, A. M. (2000) *Mol Cell Biol* **20**(4), 1254-62.

23. Thelen, M. P., Venclovas, C., and Fidelis, K. (1999) *Cell* **96**(6), 769-70.

24. Venclovas, C., and Thelen, M. P. (2000) *Nucleic Acids Res* **28**(13), 2481-2493.

25. Bravo, R., Frank, R., Blundell, P. A., and Macdonald-Bravo, H. (1987) *Nature* **326**(6112), 515-7.

26. Krishna, T. S., Kong, X. P., Gary, S., Burgers, P. M., and Kuriyan, J. (1994) *Cell* **79**(7), 1233-43.

27. Rauen, M., Burtelow, M. A., Dufault, V. M., and Karnitz, L. M. (2000) *J Biol Chem*

28. Mossi, R., and Hubscher, U. (1998) *Eur J Biochem* **254**(2), 209-16.

29. Burtelow, M. A., Kaufmann, S. H., and Karnitz, L. M. (2000) *J Biol Chem*

30. Edwards, R. J., Bentley, N. J., and Carr, A. M. (1999) *Nat Cell Biol* **1**(7), 393-8.

31. Matsuoka, S., Rotman, G., Ogawa, A., Shiloh, Y., Tamai, K., and Elledge, S. J. (2000) *Proc Natl Acad Sci U S A* **97**(19), 10389-94.

32. Cortez, D., Wang, Y., Qin, J., and Elledge, S. J. (1999) *Science* **286**(5442), 1162-6.

33. Kim, S. T., Lim, D. S., Canman, C. E., and Kastan, M. B. (1999) *J Biol Chem* **274**(53), 37538-43.

34. Banin, S., Moyal, L., Shieh, S., Taya, Y., Anderson, C. W., Chessa, L., Smorodinsky, N. I., Prives, C., Reiss, Y., Shiloh, Y., and Ziv, Y. (1998) *Science* **281**(5383), 1674-7.
35. Canman, C. E., Lim, D. S., Cimprich, K. A., Taya, Y., Tamai, K., Sakaguchi, K., Appella, E., Kastan, M. B., and Siliciano, J. D. (1998) *Science* **281**(5383), 1677-9.

36. Tibbetts, R. S., Cortez, D., Brumbaugh, K. M., Scully, R., Livingston, D., Elledge, S. J., and Abraham, R. T. (2000) *Genes Dev* **14**(23), 2989-3002.

37. Chen, J. (2000) *Cancer Res* **60**(18), 5037-9.

38. Tibbetts, R. S., Brumbaugh, K. M., Williams, J. M., Sarkaria, J. N., Cliby, W. A., Shieh, S. Y., Taya, Y., Prives, C., and Abraham, R. T. (1999) *Genes Dev* **13**(2), 152-7.

39. Savitsky, K., Bar-Shira, A., Gilad, S., Rotman, G., Ziv, Y., Vanagaite, L., Tagle, D. A., Smith, S., Uziel, T., Sfez, S., Ashkenazi, M., Pecker, I., Frydman, M., Harnik, R., Patanjali, S. R., Simmons, A., Clines, G. A., Sartiel, A., Gatta, R. A., Chessa, L., Sandal, O., Lavin, M. F., Jaspers, N. G. J., Taylor, A. M. R., Arlett, C. F., Miki, T., Weissman, S. M., Lovett, M., Collins, F. S., and Shiloh, Y. (1995) *Science* **268**, 1749-53.

40. Chen, M. J., Lin, Y. T., Lieberman, H. B., Chen, G., and Lee, E. Y. (2001) *J Biol Chem* **276**(19), 16580-6.

41. Komatsu, K., Miyashita, T., Hang, H., Hopkins, K. M., Zheng, W., Cuddeback, S., Yamada, M., Lieberman, H. B., and Wang, H. G. (2000) *Nat Cell Biol* **2**(1), 1-6.

42. Takebe, Y., Seiki, M., Fujisawa, J., Hoy, P., Yokota, K., Arai, K., Yoshida, M., and Arai, N. (1988) *Mol Cell Biol* **8**(1), 466-72.

43. Fang, G., Yu, H., and Kirschner, M. W. (1998) *Mol Cell* **2**(2), 163-71.

44. Endicott, J. A., Noble, M. E., and Tucker, J. A. (1999) *Curr Opin Struct Biol* **9**(6), 738-44.
Figure Legends

**Figure 1.** hRad9 is constitutively phosphorylated on [S/T]-P sequences. (A) Exogenous, overexpressed hRad9 from transiently transfected HeLa cells (+OP) or endogenous hRad9 from untransfected HeLa cells (-OP) was immunoprecipitated with polyclonal antibodies directed against hRad9. Immunoprecipitated proteins were subjected to SDS-PAGE (8%) and immunoblotted with antibodies directed against hRad9. (B) hRad9 overexpressed in HeLa cells was immunoprecipitated with antibodies directed against hRad9 and then incubated in the presence (+CIP) or absence (no CIP) of calf intestinal phosphatase prior to SDS-PAGE (8%) and immunoblotting. The +CIP was diluted prior to electrophoresis for presentation purposes. (C) Each of the nine serine and threonine amino acids that are followed immediately by proline in the hRad9 amino acid sequence were converted to non-phosphorylatable alanine or glycine residues (as indicated) by site directed mutagenesis. Plasmids encoding wild type hRad9 and each mutant protein were used to transfect HeLa cells. Cellular proteins were immunoprecipitated and incubated in the presence (lower panel) or absence (upper panel) of CIP. Proteins were then separated by SDS-PAGE (8%) and immunoblotted with antibodies directed against hRad9. (D) The four mutations that caused mobility shifts in A were introduced to the hRad9 cDNA sequentially in combination. Wild type, P2A (S328A+S336G), P3A (P2A+S277A), and P4A (P3A+T355A), proteins were expressed in HeLa cells and immunoblotted as before.

**Figure 2.** hRad9 is constitutively phosphorylated at sites other than [S/T]-P sequences. (A) P4A, and P9A forms of hRad9 were expressed in HeLa cells by transient transfection and immunoprecipitated with antibodies directed against hRad9. Immunoprecipitated proteins were left untreated (-), or treated with CIP (+), size
fractionated by SDS-PAGE (8%) and immunoblotted with antibodies directed against hRad9. (B) hRad9 protein was immunoprecipitated from P3A, P4A, P5A, P6A, P9A, and empty vector transfected HeLa cells that were metabolically labelled with inorganic $^{32}$P for 18 hours prior to lysis. A portion of each immunoprecipitated, radiolabelled protein was size fractionated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with antibodies against hRad9 (left panel). A second, identical gel was dried and exposed to a phosphoimager screen for 3 days (right panel). (C) Quantification of the $^{32}$P signal from the right panel of Figure 2b as determined by the Image Quant software program. Values were normalized $^{32}$P background in each lane and to the chemiluminescence signal from the left panel of Figure 2b.

**Figure 3.** Ionizing radiation induced phosphorylation at serine 272 of hRad9 does not require constitutive phosphorylation at [S/T]-P sequences. (A) Wild type hRad9 and S272A, harboring a serine to alanine mutation at the putative ATM phosphorylation site, were expressed in HeLa cells which were exposed 0, 20, and 40 gray of ionizing radiation, as indicated. One hour later cell lysates were immunoprecipitated and left untreated (upper panel) or treated with CIP (lower panel) prior to SDS-PAGE (8%) and immunoblotting with antibodies directed against hRad9. (B) The P9A protein was immunoprecipitated from cells one hour after irradiation with 20 Gray, as indicated above. Immunoprecipitated hRad9 was then treated with calf intestinal phosphatase (CIP), as indicated, size fractionated by SDS-PAGE (8%), and immunoblotted as before (C) The S272A mutation was introduced into the mutant described previously. P9A and P9A + S272A were expressed in HeLa cells, treated with 0 (-), 4 (left panel only) or 20 gray (right panel only) of ionizing radiation and harvested at 30, 60, or 240 minutes later, as indicated. Lysates were immunoblotted with antibodies directed against hRad9.

**Figure 4.** Constitutively phosphorylated hRad9 is hyper-phosphorylated in G2 and M phases of HeLa cells. HeLa cells were synchronized in early S-phase using a double thymidine block, released and harvested 0, 2, 6, 7, 8, 9, 10, and 12 hours later. Cells from each time point were either lysed, size-fractionated by SDS-PAGE (10%) and immunoblotted with antibodies against hRad9 (top), or stained with propidium iodide to measure DNA content prior to analysis by flow cytometry (bottom). (B) S-phase and G2/M HeLa cells, harvested 2 and 8 hours after release from a double thymidine block respectively, were lysed and the hRad9 immunoprecipitated. hRad9 protein from each time point was then treated with CIP in the presence or absence of the phosphatase inhibitor $\exists$-glycerophosphate ($\exists$-GP). Proteins were separated by SDS-PAGE (10%) and immunoblotted as above. (C) Late S-phase HeLa cells, generated from a single thymidine block and release, were left untreated (-), treated with 4 Gray of IR to delay cells in G2 (+ 4 Gy), or 1.7 $\mu$g/ml of the microtubule inhibitor demicollcine (+ DC) to arrest cells in mitosis. Cells were harvested 7, 8, 9, and 10 hours after thymidine release (1.5, 2.5, 3.5, and 4.5 hours after IR and demicolcine administration) and used for immunoblotting (top) and flow cytometry (bottom) as in A.

**Figure 5.** Phosphorylation of hRad9 in G2/M is enhanced by IR but is distinct from phosphorylation at serine 272. (A) HeLa cells, synchronized with a double thymidine block and release, were analyzed by immunoblotting with antibodies directed against hRad9. At each time point, cells were either left as is, or irradiated
with 20 Gray 30 minutes prior to harvest, as indicated. Cells from each time point were also followed by flow cytometry (bottom). (B) Similar to A only using hTERT-RPE1 cells that were synchronized with a single thymidine block and release.

**Figure 6.** **Cell cycle regulated phosphorylation of hRad9 is dependent on threonine 292.** (A) HeLa cells, mock or transiently transfected with each of the single [S/T]-P hRad9 mutants or the S272A ATM consensus site mutant were either left untreated, or treated with demicolcine for 18 hours prior to harvest. Cells were subjected to SDS-PAGE (8%) followed by immunoblotting for hRad9 (upper) or flow cytometry analysis to confirm mitotic arrest (lower). (B) Similar to A only using mutants with multiple [S/T]-P disruptions. (P2A = S328A + S336G, P3A = P2A + S277A; P4A = P3A + T355A; P5A = P4A + T292A; P9A = P5A + T60A, S160A, S375A, and S380G)
### Table 1
Sequence of selected oligonucleotides used for hRad9 site-directed mutagenesis

| Oligonucleotide  | Sequence (5’ to 3’)                      |
|------------------|------------------------------------------|
| Selection Primer #1 | GCTCTAGCCCTGGAGATGAAGTGC                  |
| Selection Primer #2 | CAAGTAGCGGCCGGTAATTCCTGATTG              |
| Selection Primer #3 | GACAAGTAGCGGCAGGTAATTCCTGAT              |
| T60A              | ATACCAAGGCAGCGCCCCCTTGTCAGGC             |
| S160A             | TGTTCTGCCCTTCGCTCCTGCACCTGGCT            |
| S277A             | CAGGACCTGGGCAGCCCCAGAGCGTCA              |
| T292A             | CAGGCCTACAGCGCAAGCCCACCACCGGA            |
| S328A             | TCCATTTCCCTTGACCTGGCCGCCCCCA             |
| S336G             | AGCCCCCAAGGGCCGGCGCTCCCA                 |
| T355A             | CAGTG CCTTGCGGCTCCCCACCCCAA              |
| S375A             | GCCCCTGTACGCGCCCCAGGGCCCCCGCCGTCCA      |
| S380G             | CCCAGGGCCCGGCCCTGTGCTGGCG                |
| S272A             | CCGACTCGCACGCCAGGACCTGGG                |

### Table 2
Summary of characterized hRad9 phosphorylation mutants transiently expressed in HeLa cells

| Amino Acid | Context | Immunoblot Shift |
|------------|---------|------------------|
| Serine 380 | GPSPVL  | no               |
| Serine 375 | VRSPQG  | no               |
| Serine 368 | FGSILA  | no               |
| Serine 363 | FRSLEF  | no               |
| Threonine 355 | PGTPPP | yes             |
| Threonine 351 | PSTVPG | no               |
| Serine 350 | EPSTVP  | no               |
| Serine 341 | PHSEE   | no               |
| Serine 336 | PKSPGP  | yes              |
| Serine 328 | SLSPGP  | yes              |
| Tyrosine 306 | DSYMIA | no               |
| Threonine 292 | HSTPHP | no               |
| Serine 277 | LGSPER  | yes              |
| Serine 272 | SHSQDL  | yes (IR induced) |
| Serine 160 | PFSPAL  | no               |
| Threonine 60 | AATPGQ  | no               |
Figure 1

A. $\text{OP} \quad \text{OP}$

B. $\text{CIP} \quad +\text{CIP}$

C. $\text{MOCK} \quad \text{WT} \quad \text{T60A} \quad \text{S160A} \quad \text{S277A} \quad \text{T292A} \quad \text{S328A} \quad \text{S336G} \quad \text{T355A} \quad \text{S375A} \quad \text{S380G}$

D. $\text{MOCK} \quad \text{WT} \quad \text{P2A} \quad \text{P3A} \quad \text{P4A}$
Figure 4

A.

Hours: 0 2 6 7 8 9 10 12

- hRad9σ
- hRad9α

B.

Hours: 2 8 2 8 2 8 2 8

CIP: - - + + + +

β-GP: - - - - + +

IgG

- hRad9σ
- hRad9α
- hRad9δ

C.

Hours: 7 8 9 10 + 4 Gy + DC

- hRad9σ
- hRad9α

7h 8h 9h 10h

Count

+4Gy

+DC

DNA (PI)
Figure 5

A.

| Hrs:  | 0   | 2   | 4   | 6   | 8   | 10  | 12  |
|-------|-----|-----|-----|-----|-----|-----|-----|
| IR:   | -   | +   | -   | +   | -   | +   | -   |

DNA (PI)

B.

| Hrs:  | 0   | 4   | 8   | 10  | 12  | 14  | 16  | 18  |
|-------|-----|-----|-----|-----|-----|-----|-----|-----|
| IR:   | -   | +   | -   | +   | -   | +   | -   | +   |

DNA (PI)
Figure 6

A. MOCK  WT  T60A  S160A  S277A  T292A
DC: -  +  -  +  -  +  -  +  -  +

62-  48-

S328A  S336G  T355A  S375A  S380G  S272A
DC: -  +  -  +  -  +  -  +  -  +

62-  48-

B. P2A  P2A+  T355A  P3A  P4A  P3A+  T292A  P5A  P9A
DC: -  +  -  +  -  +  -  +  -  +

62-  48-
DNA damage-dependent and -independent phosphorylation of the hRad9 checkpoint protein

Robert P. St.Onge, Blair D.A. Besley, Minwoo Park, Richard Casselman and Scott Davey

J. Biol. Chem. published online September 10, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M105152200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts