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The human checkpoint Rad protein Rad17 is chromatin-associated throughout the cell cycle, localizes to DNA replication sites, and interacts with DNA polymerase ε

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ABSTRACT

The checkpoint Rad proteins Rad17, Rad9, Rad1, Hus1, ATR, and ATRIP become associated with chromatin in response to DNA damage caused by genotoxic agents and replication inhibitors, as well as during unperturbed DNA replication in S phase. Here we show that murine Rad17 is phosphorylated at two sites that were previously shown to be modified in response to DNA damage, independent of DNA damage and ATM, in proliferating tissue. In contrast to studies with *Xenopus laevis* extracts but similar to observations in *Schizosaccharomyces pombe*, the level of chromatin-bound hRad17 remains relatively constant during the cell cycle and does not change significantly in response to DNA damage or replication block. However, phosphorylated hRad17 preferentially associates with the sites of ongoing DNA replication and interacts with the DNA replication protein, DNA polymerase ε. These results provide a link between the DNA damage checkpoint machinery and the replication apparatus and suggest that hRad17 may play a role in monitoring the progress of DNA replication via its interaction with DNA polymerase ε.

INTRODUCTION

Damage to the genome, arising from replication errors and environmental factors, pose a grave threat to genomic stability and can ultimately lead to cancer formation. Cell cycle checkpoints prevent this damaged DNA from being replicated and passed on to future daughter cells (1,2). A group of six proteins called the checkpoint Rad proteins, first identified in *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*, mediate checkpoint activation, including the intra-S checkpoint and the S/M checkpoint, in response to DNA damage during DNA replication or incomplete replication, respectively (3,4). Human homologs of these checkpoint Rad proteins (hRad17, hRad9, hRad1, hHus1, ATRIP, and ATR) are required to activate cell cycle checkpoints through a signaling cascade to the downstream checkpoint effector Chk1 (5–11). Upon activation, Chk1 phosphorylates and thereby negatively regulates the phosphatase Cdc25 and activates the kinase Wee1 (12–16). Activation of Chk1 also results in the inactivation of Cdc2, which is required for the cell to progress through the G_2/M checkpoint (17,18). In mice, this checkpoint-signaling cascade appears to be required for embryogenesis. Cells derived from early embryos of $Atr^{-/-}$-, $Hus1^{-/-}$- and $Chk1^{-/-}$-deficient mice are sensitive to DNA damage, have an aberrant G_2/M checkpoint and exhibit spontaneous genomic instability (8,19–23).

Xenopus laevis egg extracts have been used to elucidate the role of checkpoint Rad proteins in the regulation of DNA replication and checkpoint activation during DNA synthesis. Initiation of DNA replication by the primase activity of DNA polymerase α is required for the chromatin association of *X.laevis* (Xl)ATR (24). Immunodepletion of XlATR, even in untreated extracts, abrogates the phosphorylation of Chk1 and the subsequent S/M replication checkpoint, resulting in a shortened cell cycle (25,26). Additionally, XlHus1 and XlRad17 are required for the S/M replication checkpoint and checkpoints activated in response to DNA damage or replication inhibitors (27–29). Unlike the replication-dependent loading of XlRad17, a significant portion of fission yeast *S.pombe* (Sp)Rad17 is bound to the chromatin throughout the cell cycle (30); however, there is a dynamic change in the amount of chromatin-bound SpRad17 in response to different genotoxic agents. Exposure to replication inhibitors results in the release of SpRad17 from the chromatin (31), whereas treatment with DNA-damaging agents causes an increase in chromatin-associated SpRad17 (30). Although these data suggest that the checkpoint Rad proteins function during S phase to monitor the progression of DNA replication and/or replication forks, it is not known how the checkpoint Rad proteins perform this monitoring function.

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Rad17 is closely related to the five replication factor C (RFC) subunits (32–35). The pentameric RFC complex loads proliferating cell nuclear antigen (PCNA) onto the DNA during replication. hRad17 replaces the large subunit of RFC, p140, in an alternative form of the clamp-loading complex that interacts with the PCNA-like heterotrimeric Rad9–Rad1–Hus1 (9-1-1) complex (36). Recent biochemical studies with the homologous complexes isolated from budding yeast have demonstrated that the alternative RFC-like complex linked with checkpoint activation has clamp-loading activity (37). In agreement with the functional interaction between the hRad17 clamp-loading complex and the 9-1-1 complex *in vitro*, DNA damage-dependent chromatin association of hRad9 requires hRad17 *in vivo* (7). Furthermore, phosphorylation of hRad17 by ATR on Ser635 and Ser645 in response to DNA damage and replication block stimulates the interaction between hRad17 and the 9-1-1 complex (38). Interestingly, hRad17 is also phosphorylated on these same two serine residues during unperturbed S phase, suggesting a role for hRad17 during DNA replication (6). In support of this idea, human cells engineered for conditional deletion of hRad17 alleles undergo endoreduplication after loss of hRad17 function (39). Recent reports have demonstrated that the checkpoint Rad protein hRad9 interacts with TopBP1, a DNA polymerase ε subunit, even in the absence of DNA damage (40). Additionally, hRad9 was shown to interact with PCNA (41,42). These observations suggest that the checkpoint Rad proteins may monitor DNA replication by interacting with the DNA replication machinery.

As noted above, there are differences in the regulation of Rad17 subnuclear localization among different eukaryotes. Therefore, we have examined the behavior of mammalian Rad17 during S phase. Here we show that mammalian Rad17 is phosphorylated during unperturbed S phase in replicating tissue in a DNA damage-independent and ATM-independent manner. We demonstrate that the level of chromatin-associated hRad17 remains constant throughout the cell cycle, in response to genotoxic agents, and regardless of phosphorylation status. Finally, we show that phosphorylated hRad17 localizes to sites of DNA replication and interacts with the DNA replication machinery.

MATERIALS AND METHODS

Collection of murine tissues samples

One-month-old wild-type ($Atm^{+/+}$) and ATM-deficient ($Atm^{-/-}$) mice were treated with 10 Gy ionizing radiation (IR) and then killed 1 h post-treatment. IR was performed using a ^{137}Cs γ-irradiator (Shepherd) at 2.08 Gy/min. Tissues were collected and frozen in liquid nitrogen. Extracts were prepared by grinding frozen tissues prior to resuspension in lysis 250 buffer (50 mM Tris pH 7.4, 250 mM NaCl, 2 mM EDTA and 1.0% NP-40) supplemented with a cocktail of protease and phosphatase inhibitors containing 10 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 100 mM sodium fluoride, 1 mM sodium orthovanidate, 1 mM benzamide and 1 mM β-glycerophosphate. Proteins were separated by SDS–PAGE and transferred to PVDF membranes (Millipore). Membranes were incubated with the indicated antibodies and antigen–antibody complexes were detected with an enhanced chemiluminescence kit (Amersham).

Antibodies

Mouse α-hRad17 (31E9) monoclonal antibodies have been described previously (35). Characterization of the rabbit α-hRad17 phospho-specific polyclonal antibodies has been reported previously (6). Mouse α-HA.11 (Babco), α-DNA polymerase ε (93H3A) (NeoMarkers), α-PCNA (PC10) (Santa Cruz), α-Flag (M2) (Sigma) and α-Mek2 (Cell Signaling) were purchased from commercial sources. Rabbit α-Orc2 was a kind gift from Dr Bruce Stillman.

Generation of cell lines

MCF-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with 15% fetal calf serum (FCS) (Gibco) and 1 mg/ml insulin (Sigma). To generate tetracycline-inducible stable cell lines, MCF-7 cells were transfected with the doxycycline repressible plasmid pUHG 15-1 and selected in 1 mg/ml neomycin for 2 weeks. Regulation of protein expression in selected cells was determined by transient transfection with the reporter plasmid pTRE-2dGFP (Clontech) in the presence or absence of doxycycline. Cells expressing N-terminal HA-tagged hRad17 were generated by co-transfecting each construct, at a 10:1 ratio with pPuro (Stratagene), followed by selection in 500 ng/ml puromycin (Sigma). Stable MCF-7 clones expressing the wild-type or the phosphorylation site mutant of hRad17 were maintained in DMEM supplemented with 15% FCS, 1 mg/ml insulin, 400 μg/ml G-418 (Gibco), 100 ng/ml puromycin and 1 mg/ml doxycycline (Sigma). Expression of wild-type and phosphorylation site mutants of HA-hRad17 was induced by removing doxycycline from the medium for 72 h. Expression of recombinant protein was determined by immunoblotting. Synchronized and unsynchronized T24 cell lines and HeLa cell lines were cultured as previously described (6,43).

Fluorescence-activated cell sorting

The human bladder carcinoma T24 cells were density arrested and released as described previously (6,43,44). After trypsinization, cells were harvested, washed with phosphate-buffered saline (PBS), resuspended in 70% ice-cold ethanol and then incubated at 4°C for 30 min. Cells were spun down, washed with PBS and resuspended in PBS containing 0.1% Triton X-100 and 200 μg/ml RNase A (Sigma) prior to incubation for 30 min at room temperature. Finally, cells were stained with 20 μg/ml propidium iodide and then analyzed using a FACScan (Becton-Dickinson).

DNA damage and replication inhibition

Hydroxyurea was added to cell culture medium at a final concentration of 1 mM for 24 h, aphidicolin at a final concentration of 5 μg/ml for 20 h and actinomycin D at a final concentration of 1 μg/ml for 6 h. Cell extracts were prepared from mock- or IR-treated cells 1 h post-treatment.

Cell lysate preparation

Whole cell extracts were prepared by lysing cells in RIPA buffer (50 mM Tris, 150 mM NaCl, 1.0% NP-40, 0.5% DOC and 0.1% SDS) supplemented with protease and phosphatase

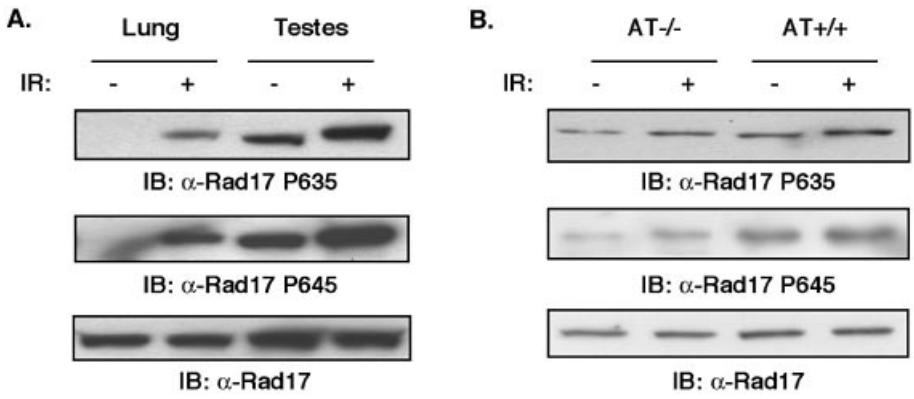

Figure 1. Phosphorylation of MmRad17 in replicating and quiescent mouse tissues. (**A**) Phosphorylation of MmRad17 in testis and lung tissues. Mice were mock-treated or treated with 10 Gy IR and killed 1 h post-treatment. Lysates were prepared from testes and lung tissues for western blotting analysis. (**B**) Phosphorylation of MmRad17 in *Atm*$^{-/-}$ mice. Mice were treated as described above.

inhibitors as above. Chromatin fractionation was performed using modifications to a previously described method (7,44,45). Briefly, equal numbers of synchronized or treated cells were washed twice in ice-cold PBS followed by incubation in buffer A (10 mM HEPES pH 7.9, 10 mM KCl, 1.5 mM MgCl, 0.34 M sucrose, 10% glycerol, 0.5% Triton X-100 and protease and phosphatase inhibitors) for 5 min. After low speed centrifugation, the supernatant cytoplasmic fraction (Cyto) was removed. The pellet containing nuclei was washed in buffer A and then lysed in buffer B (3 mM EDTA, 0.2 mM EGTA and protease and phosphatase inhibitors). Soluble nuclear proteins (Nuc Sol) were separated from the chromatin fraction (Chr) by low speed centrifugation. The chromatin-enriched pellet was washed in buffer B and resuspended in 2× SDS sample buffer and sonicated three times for 15 s.

Immunofluoresence

T24 cells were seeded onto 12 mm glass coverslips that had been pre-coated for 30 min with 0.1% gelatin and prepared for immunostaining as described previously (46). Briefly, cells were washed twice in cold PBS and then permeabilized by incubation in PBS containing 0.5% Triton X-100, 2 mM EDTA and 1% bovine serum albumin (BSA) for 15 min at 4°C. After washing with PBS, cells were fixed in 100% methanol at –20°C for 15 min. Cells were washed with PBS and then blocked by incubation with PBS containing 10% FCS for 2 h at room temperature. Antibodies were added and incubated in PBS containing 5% FCS for 2 h. Cells were washed five times in Tris-buffered saline with Tween-20 (TBST) for 10 min and then incubated with goat α-mouse conjugated with fluorescein isothiocyanate (Jackson Immunochemical), goat α-rabbit conjugated with rhodamine B isothiocyanate (Jackson Immunochemical) and 4′,6-diamidino-2-phenylindole (DAPI) for 1.5 h. After washing in TBST, coverslips were mounted on slides using Immunon mountant.

For bromodeoxyuridine (BrdU) and hRad17 co-localization, T24 cells were incubated in medium supplemented with 10 μM BrdU for 15 min prior to fixation. Cells were washed twice in cold PBS followed by permeabilization and fixation as above. After treatment with 4 N HCl containing 0.2% Triton X-100 for 10 min, cells were washed with PBS, then incubated with 50 mM glycine for 5 min, washed once with PBS and then blocked in PBS containing 10% FCS for 2 h at room temperature. Finally, cells were incubated with mouse α-BrdU (Becton Dickinson) and rabbit α-hRad17-phosphoSer645 in PBS containing 5% FCS for 2 h and then visualized as described above.

Immunoprecipitation

Cell lysates were prepared in lysis 250 buffer containing ethidium bromide at a final concentration of 20 μg/ml and supplemented with phosphatase and proteases inhibitors as above. The clarified lysates were incubated with the indicated antibodies followed by incubation with protein G–Sepharose beads for 2 h. Immunoprecipitates were washed four times in lysis 250 buffer and boiled in 2× SDS sample buffer. Proteins were separated by SDS–PAGE and transferred to PVDF membranes (Millipore).

In vitro hRad17 and DNA polymerase ε interaction

The pGEX4T-3 plasmid expressing full-length hRad17 as a GST fusion protein has been described (6). Digestion of this plasmid with EcoRV and SmaI followed by religation generated plasmid GST–hRad17$^{1–320}$ that encoded the N-terminal 320 residues of hRad17. Fragments of hRad17 cDNA encoding residues 319–670 and 491–670 were amplified by PCR and then subcloned into pGEX4T-3 to generate plasmids GST–hRad17$^{319–670}$ and GST–hRad17$^{419–670}$, which encode these C-terminal fragments of hRad17 as GST fusions proteins. GST fusion proteins were expressed and purified according to the manufacturer's protocol (Amersham). Full-length DNA polymerase ε cDNA, a gift from Dr Stuart Linn, was used as a template to synthesize ^{35}S-labeled DNA polymerase ε coupled by *in vitro* transcription–translation using the TNT T7 Quick Kit (Promega). For the GST pull-down assays, equal amounts of GST, GST–hRad17 or GST–hRad17 fragments bound to glutathione–Sepharose beads were incubated with labeled DNA polymerase ε in 50 mM Tris (pH 7.4), 120 mM NaCl, 2 mM EDTA, 0.1% NP-40 and 10% BSA for 2 h at 4°C. After extensive washing, bound proteins were released from the beads by boiling in 2× SDS sample buffer. Labeled DNA polymerase ε was visualized by fluorography after SDS–PAGE.

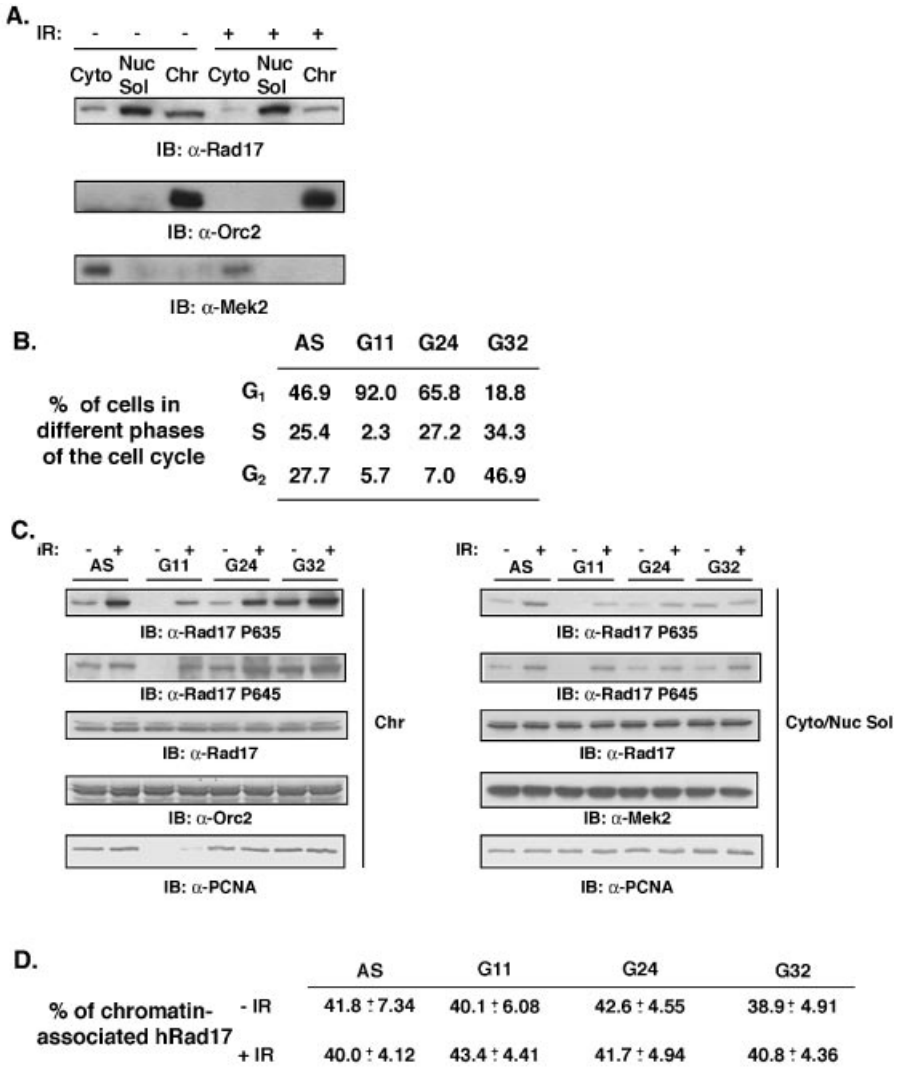

Figure 2. Chromatin association of hRad17 during the cell cycle and after IR. (**A**) Analysis of cytoplasmic, nuclear soluble and chromatin-bound proteins. Human T24 fibroblasts were mock-treated or treated with 10 Gy IR and harvested 1 h post-treatment. Cells were fractionated as described in Materials and Methods. After separation by SDS–PAGE, proteins in the cytoplasmic (Cyto), soluble nuclear (Nuc Sol) and chromatin-enriched (Chr) fractions were detected by immunoblotting with the indicated antibodies. Mek2 and Orc2 served as controls for the soluble and chromatin fractions, respectively. (**B**) Cell cycle distribution of T24 cells determined by FACS analysis. T24 cells were released from density arrest and harvested at the indicated time points, G11, G24 and G32, representing 11, 24 and 32 h after density release, respectively. (**C**) Chromatin association of hRad17 during the cell cycle. T24 cells were released from density arrest and harvested as above. Cells subjected to DNA damage were harvested 1 h post-treatment. The left panel represents the chromatin-enriched fraction (Chr) while the right panel corresponds to the soluble fractions (Cyto and Nuc Sol). Sample analysis was as described in (A). (**D**) Percentage of chromatin-associated hRad17 during the cell cycle. Levels of soluble and chromatin-associated hRad17 were determined by densitometry analysis. The average and standard deviation were determined from three separate experiments.

RESULTS

Mammalian Rad17 is phosphorylated in undamaged replicating tissue in an ATM-independent manner

We previously demonstrated that the two DNA damage-dependent phosphorylation sites of hRad17, Ser635 and Ser645, are also phosphorylated at the start of DNA replication in cultured synchronized cells (6). To demonstrate that these phosphorylation events were not caused by the synchronization protocol, we examined the phosphorylation status of murine Rad17 in replicating and non-replicating tissues. Phosphorylation on both Ser647 and Ser657 residues of *Mus musculus* (Mm)Rad17, corresponding to hRad17 Ser635 and Ser645, was observed in extracts from both undamaged and IR-treated testes (Fig. 1A). In contrast to replicating tissue, the same two residues were phosphorylated only after DNA damage in quiescent lung tissue. Since the kinase activity of ATR increases in response to S phase (25), and ATM and ATR are both involved in the activation of S phase-dependent checkpoints (47,48), we asked whether ATM influences the phosphorylation of mammalian Rad17 in replicating tissues after IR. The absence of ATM had little effect on the phosphorylation of MmRad17 in the presence or absence of DNA damage in testes (Fig. 1B). These results, coupled with our previous data demonstrating that ATM is not required for the ionizing irradiation-dependent phosphorylation of Rad17 in quiescent and cycling cells (6), indicates that

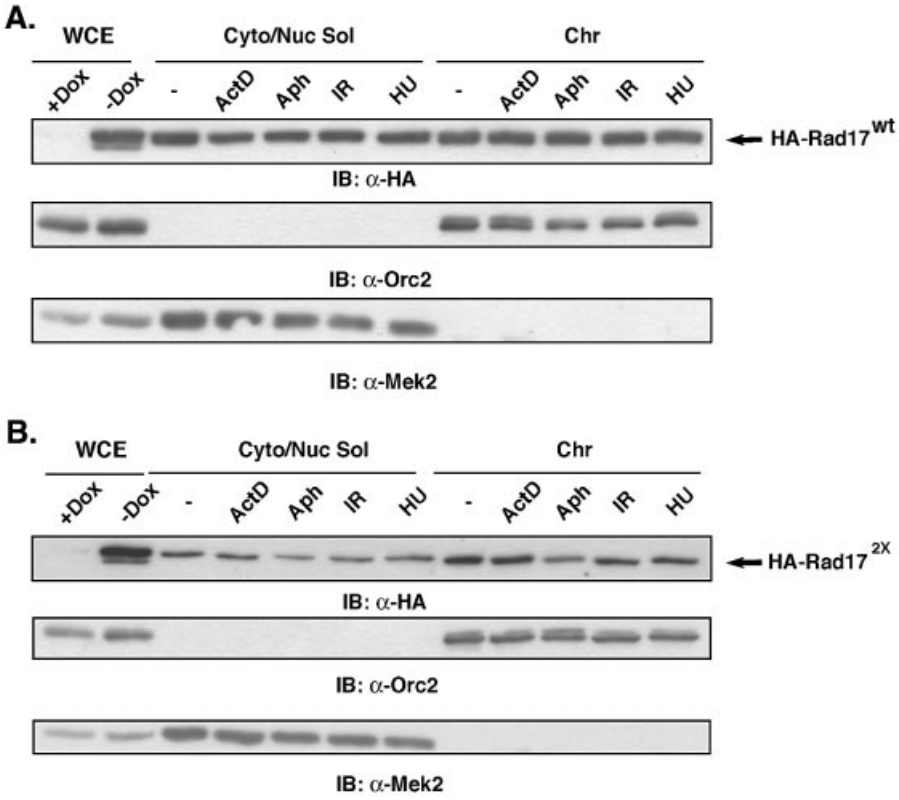

Figure 3. Replication inhibitors and phosphorylation status does not effect the chromatin association of hRad17. (**A**) Chromatin association of recombinant wild-type HA-tagged hRad17 in response to DNA damage and replication block. (**B**) Chromatin association of recombinant phosphomutant HA-tagged hRad17 in response to DNA damage and replication block. Cells expressing HA-tagged hRad17 were mock-treated, treated with 1 μg/ml actinomycin D for 6 h, 5 μg/ml aphidicolin for 20 h or 10 Gy IR or 1 mM hydroxyurea for 24 h. Cells lysates were fractionated as in Figure 2 and subjected to immunoblotting using the indicated antibodies. Doxycycline-treated and untreated whole cell extracts served as a control for loading and expression of HA-hRad17.

mammalian Rad17 is normally phosphorylated during replication in an ATM-independent manner and suggests that hRad17 monitors the progression of DNA replication in unperturbed cells.

Chromatin-associated levels of hRad17 remain constant throughout the cell cycle and in response to DNA damage

Using a fractionation procedure first described in the Stillman laboratory (45), we have examined whether the distribution of hRad17 between the nucleoplasmic and chromatin fractions changes either as a function of cell cycle progression or in response to DNA damage. In asynchronous cells, the majority of hRad17 is in the nucleoplasmic fraction, but an appreciable amount was present in the chromatin fraction (Fig. 2A). In agreement with data from the Elledge laboratory, the level of chromatin-associated hRad17 was not significantly altered by DNA damage (7).

To directly address whether hRad17 is shuttled on and off chromatin during the cell cycle, we density arrested, released and harvested human T24 cells at different phases of the cell cycle. The cell cycle distributions of these synchronized cells were determined by FACS analysis (Fig. 2B). As expected, the replication protein PCNA associates with chromatin at the start of S phase. In contrast to the S phase-dependent chromatin association of PCNA, we observed that ~35–45% of hRad17 is associated with chromatin throughout the cell cycle, as determined by densitometry (Fig. 2C and D). Thus, the behavior of hRad17 is different from that of XlRad17 (28), which associates with chromatin at the start of DNA replication, but similar to that of *S.pombe*, in which a fraction of Rad17 remains bound throughout the cell cycle (30). Furthermore, DNA damage did not affect the amount of hRad17 localized to the chromatin in specific cell cycle phases (Fig. 2C and data not shown). In contrast to the steady-state levels of chromatin-bound hRad17, there were significant changes in the phosphorylation of chromatin-bound hRad17 during cell cycle progression and in response to DNA damage. hRad17 Ser635 and Ser645 became phosphorylated at the start of S phase in undamaged cells and in IR-treated G_1 cells (Fig. 2C). These results demonstrate that a pool of hRad17 remains bound to the chromatin at all phases of the cell cycle and that the association of hRad17 with the chromatin is not regulated by DNA damage.

Replication inhibitors and phosphorylation status does not effect the chromatin association of hRad17

Next we wanted to determine if hRad17 disassociates from chromatin after treatment with replication inhibitors and, if so, whether phosphorylation of hRad17 is required for this release. To address these questions, we created a stable cell line expressing HA-tagged versions of the wild-type and a phosphorylation site mutant hRad17 from a tetracycline-regulated promoter. Unlike fission yeast (31), the replication

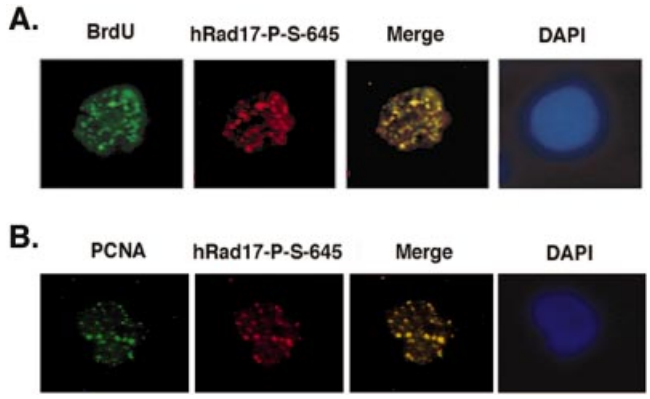

Figure 4. Co-localization of phosphorylated hRad17 with sites of active DNA replication in T24 cells. (**A**) Co-localization of hRad17-phosphoSer645 with sites of DNA synthesis. hRad17-phosphoSer645 (red fluorescence) and BrdU (green fluorescence) were detected by indirect immunofluorescence. DAPI staining serves a marker for nuclear staining (blue fluorescence, right panel). (**B**) Co-localization of hRad17-phosphoSer645 with PCNA. PCNA (green fluorescence), hRad17-phosphoSer645 and DNA were detected as above.

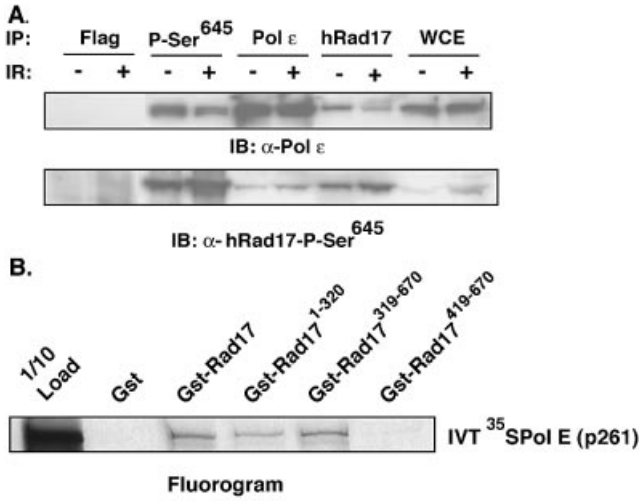

Figure 5. Interaction between hRad17 and DNA polymerase ε. (**A**) Co-immunoprecipitation of phosphorylated hRad17 and DNA polymerase ε. Extracts from mock-treated or irradiated HeLa cells were prepared as described in Materials and Methods. After immunoprecipitation with α-hRad17-phosphoSer645, α-DNA polymerase ε or α-hRad17 antibodies, proteins in the immunoprecipitates were separated by SDS–PAGE and immunoblotted with α-hRad17-phosphoSer645 or α-DNA polymerase ε antibodies. Whole cell extracts served as a positive control and immunoprecipitation with α-Flag antibodies served as a negative control. (**B**) Interaction between ^{35}S-labeled *in vitro* transcribed/translated DNA polymerase ε and GST–hRad17. *In vitro* transcribed/translated DNA polymerase ε was incubated with equal amounts of GST–full-length hRad17–, GST–N-hRad17$^{1–320}$–, GST–C-hRad17$^{319–670}$– or GST–hRad17$^{419–670}$–Sepharose beads and then separated by SDS–PAGE followed by fluorography. One-tenth load of ^{35}S-labeled *in vitro* transcribed/translated DNA polymerase ε served as a control.

inhibitors hydroxyurea, aphidicolin and actinomycin D did not disrupt the chromatin association of hRad17 (Fig. 3A and B). Furthermore, the identical behavior of the phosphorylation site mutant form of hRad17 indicates that phosphorylation of hRad17 is not required for either the initial association with chromatin or in maintaining the interaction between hRad17 and chromatin after DNA damage or replication block (Fig. 3A and B).

Chromatin-associated and phosphorylated hRad17 co-localizes to sites of DNA replication

Since hRad17 is phosphorylated at the beginning of S phase, we examined whether phosphorylated hRad17 associates with the replication machinery or sites of DNA replication by immunocytochemistry. Prior to fixation, we used a protein extraction method first described in Aboussekhra *et al*. to remove cytoplasmic and soluble nuclear proteins (46). Immunostaining of the fixed T24 cells revealed that phosphorylated hRad17 is extraction resistant and co-localizes with sites of active DNA replication as determined by co-localization with α-BrdU and α-PCNA (Fig. 4A and B). Thus, phosphorylated hRad17 is localized at the sites of ongoing DNA replication.

Human Rad17 interacts with the replication protein DNA polymerase ε

Since phosphorylated hRad17 co-localizes with sites of DNA replication, we examined whether the phosphorylated hRad17 associates with replication proteins. Using lysates from untreated and IR-treated cells, the catalytic subunit of DNA polymerase ε was co-immunoprecipitated by the hRad17 phospho-specific antibody (Fig. 5A). These antibodies have been extensively characterized and shown to exclusively recognize phosphorylated Rad17 (6). In reciprocal experiments, phosphorylated hRad17 was immunoprecipitated by the α-DNA polymerase ε antibody (Fig. 5A). This appears to reflect a specific association between hRad17 and DNA polymerase ε because α-Flag antibodies were unable to immunoprecipitate either DNA polymerase ε or hRad17 and another replication protein, PCNA, was not co-immunoprecipitated by the hRad17 phospho-specific antibody (data not shown).

Next, we performed *in vitro* binding assays to determine if there is an interaction between these proteins. Full-length *in vitro* transcribed/translated DNA polymerase ε interacted with GST–hRad17 but not GST alone (Fig. 5B). To determine which region of hRad17 interacts with DNA polymerase ε, we generated a GST–N-terminal fragment of hRad17 containing amino acids 1–320 and two GST–C-terminal fragments of hRad17 containing amino acids 319–670 and 419–670, respectively. Similar to the full-length GST–hRad17, both the N-terminal fragment of hRad17$^{1–320}$ and the large C-terminal fragment of hRad17$^{319–670}$ specifically interact with *in vitro* transcribed/translated DNA polymerase ε (Fig. 5B). In contrast, the small C-terminal fragment, encoding amino acids 419–670 of hRad17, did not interact with DNA polymerase ε *in vitro* (Fig. 5B). Together these results demonstrate that hRad17 associates with DNA polymerase ε in cell extracts and suggests that this association is mediated by a direct interaction between DNA polymerase ε and the N- and C-terminal regions of hRad17.

DISCUSSION

Although phosphorylation of hRad17 is required for DNA damage-induced checkpoint activation (6,38), the same residues are also phosphorylated in unperturbed S phase proliferating cells *in vitro* and *in vivo* (Fig. 1) (6). This

phosphorylation pattern is similar to the S phase-dependent checkpoint-signaling pathway observed in both human and *X.laevis*, in which Chk1 is also phosphorylated by ATR in the absence of exogenous DNA damage during DNA replication (25,49). The simplest explanation for these signaling events is that low levels of DNA damage occur during DNA replication and activate the cell cycle checkpoint-signaling cascade. However, DNA damage-dependent checkpoint activation appears unlikely, as hRad9 does not exhibit the characteristic DNA damage-induced mobility shift caused by phosphorylation in the absence of exogenous DNA-damaging agents during S phase (42,50). Thus, it appears that hRad17 phosphorylation occurs as a normal consequence of DNA replication and may reflect the participation of hRad17 in a function associated with DNA replication, in addition to the DNA damage-induced checkpoint pathway.

Here we have shown that ~40% of hRad17 is constitutively chromatin-associated independent of cell cycle phase or the start of DNA replication. This behavior is similar to that observed in *S.pombe* but differs from the observations in *X.laevis* (28,30). Several lines of evidence indicate that phosphorylation of hRad17 does not affect its chromatin association. First, the amount of chromatin-associated hRad17 remains relatively unchanged throughout the cell cycle or in response to genotoxic stress. Second, both the wild-type and phosphorylation site mutant forms of hRad17 associate with chromatin before and after treatment with genotoxic agents (Fig. 3). This further demonstrates that ATR-dependent phosphorylation is not required for Rad17 association with chromatin and is consistent with the observation that the lack of DNA damage- and replication block-induced phosphorylation of hRad17 in Hus1$^{-/-}$ cells does not affect the chromatin association of hRad17 (7).

Interestingly, we observed that phosphorylated hRad17 co-localizes to sites of ongoing DNA replication as determined by co-immunostaining with α-BrdU and α-PCNA antibodies (Fig. 4A and B). These data, coupled with the recent finding of endoreduplication in the absence of hRad17, strongly support the notion that hRad17 has a key role in the regulation of DNA replication and suggest that chromatin-associated, phosphorylated hRad17 may serve as a sensor of DNA replication progression (39). In support of this replication surveillance model, we found that the phosphorylated form of hRad17 associates with DNA polymerase ε, a DNA replication enzyme that has also been implicated in cell cycle checkpoints (51,52). Although it is not known whether hRad17 phosphorylation regulates its interaction with DNA polymerase ε, this interaction provides a physical link between checkpoint and DNA replication proteins. In addition to our results linking DNA polymerase ε with the hRad17 clamp-loading complex, there is a conserved and direct interaction between TopBP1 in humans (Dpb11 in budding yeast), a subunit of DNA polymerase ε and hRad9 (Ddc1 in budding yeast) that occurs in the absence of DNA damage (40,53). Thus, DNA polymerase ε interacts with both the checkpoint clamp-loader and the checkpoint clamp complex, providing a possible molecular mechanism for the proposed checkpoint activities of DNA polymerase ε and the participation of hRad17 in the regulation of DNA replication.

Even though phosphorylated hRad17 interacts with the replication machinery and localizes to sites of DNA replication during unperturbed S phase, the possibility remains that hRad17 may only localize to sites of stalled forks or where DNA damage is sensed during S phase, because not all α-phospho-hRad17 and α-BrdU or α-PCNA foci overlap (Fig. 4A and B). A possible role for phosphorylated hRad17 is that it may monitor replication fork stability and sense replication blocks during unperturbed replication. Alternatively, it may inhibit DNA reduplication, likely through its interactions with components of the replication machinery. Although the effect of hRad17 phosphorylation on DNA replication remains to be elucidated, our research provides evidence that phosphorylated hRad17 links the checkpoint Rad proteins and the replication machinery.

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