RPA2 Is a Direct Downstream Target for ATR to Regulate the S-phase Checkpoint*§

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Upon DNA damage, replication is inhibited by the S-phase checkpoint. ATR (ataxia telangiectasia mutated- and Rad3-related) is specifically involved in the inhibition of replication initiation when cells are treated with DNA damage-inducing agents that stall replication forks, but the mechanism by which it acts to prevent replication is not yet fully understood. We observed that RPA2 is phosphorylated on chromatin in an ATR-dependent manner when replication forks are stalled. Mutation of the ATR-dependent phosphorylation sites in RPA2 leads to a defect in the down-regulation of DNA synthesis following treatment with UV radiation, although ATR activation is not affected. Threonine 21 and serine 33, two residues among several phosphorylation sites in the amino terminus of RPA2, are specifically required for the UV-induced, ATR-mediated inhibition of DNA replication. RPA2 mutant alleles containing phospho-mimetic mutations at ATR-dependent phosphorylation sites have an impaired ability to associate with replication centers, indicating that ATR phosphorylation of RPA2 directly affects the replication function of RPA. Our studies suggest that in response to UV-induced DNA damage, ATR rapidly phosphorylates RPA2, disrupting its association with replication centers in the S-phase and contributing to the inhibition of DNA replication.

Cell cycle checkpoints are surveillance mechanisms to monitor chromatin structures and arrest the cell cycle when DNA damage is detected (1, 2). Defects in checkpoint pathways lead to significant genome instability. Checkpoints are operated by DNA damage signal transduction pathways that send damage signals from sensor proteins through transducing proteins to downstream effector proteins, so that different cellular responses, including cell cycle arrest and damage repair, can be activated. The protein kinases, ataxia telangiectasia mutated (ATM), and ATM- and Rad3-related (ATR), are two essential transducing proteins required to mediate checkpoint responses (3).

The S-phase checkpoint inhibits ongoing DNA replication as soon as DNA lesions are detected, allowing time for DNA repair to take place before replication continues (4, 5). The ionizing radiation (IR)-induced S-phase checkpoint response is attenuated in ATM-deficient cells (6, 7), whereas ATR is required to inhibit replication in response to UV-induced DNA damage (8). In the IR-induced S-phase checkpoint, two parallel pathways mediated by Chk2-Cdc25A and Nbs1/Mre11/Rad50, respectively, have been described to mediate ATM-dependent replication inhibition (6). However, the detailed mechanisms of how ATR regulates its downstream effectors to control replication are not clear.

RPA is a heterotrimeric protein complex that binds specifically to single-stranded DNA (ssDNA) (9). It is composed of three subunits, RPA1 (70 kDa), RPA2 (32 kDa), and RPA3 (14 kDa), and plays multiple roles in DNA metabolism (10, 11). RPA is required for DNA replication initiation, as well as replication elongation (12). At the onset of DNA replication, RPA is loaded onto chromatin after the binding of Cdc45 to origins (13). RPA is needed for subsequent loading of DNA polymerase α and other replication proteins to initiate DNA replication (14). After DNA replication begins, RPA moves with replication forks, stabilizing ssDNA and assisting in DNA synthesis. In addition to its replication function, RPA is also known to play essential roles in damage repair and recombination (15–17). It has been suggested that RPA-bound ssDNA may be involved in the activation of ATR following DNA damage (18, 19). However, recent studies also indicate that RPA may not be absolutely required for ATR activation, and alternative pathways may exist (20). The interaction of RPA with ATRIP (ATR-interacting protein) is important for promoting recruitment of ATR/ATRIP to damage-induced foci, but may be dispensable for ATR activation to phosphorylate Chk1 (21, 22).

When cells enter S-phase, RPA is phosphorylated, primarily on the RPA2 subunit at the cyclin-dependent kinase sites (23–25). Upon DNA damage, additional sites on RPA2 are phosphorylated (26–28). In response to UV or hydroxyurea (HU) treatment specifically, seven additional residues clustered in the extreme amino terminus of RPA2 have been identified as potential sites of RPA2 phosphorylation (29, 30). In mammalian

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§ The abbreviations used are: ATM, ataxia telangiectasia mutated; ATR, ATM- and Rad3-related; IR, ionizing radiation; ssDNA, single-stranded DNA; shRNA, short hairpin RNA; HA, hemagglutinin; GST, glutathione S-transferase; UVDS, UV-resistant DNA synthesis; Gy, gray; DNA-PK, DNA-dependent protein kinase.
cells, the PI3-like kinases, including DNA-PK, ATM, and ATR, are implicated in the phosphorylation of RPA2 following DNA damage. DNA-PK plays an important role in IR-induced damage repair, particularly in nonhomologous end joining (31). DNA-PK interacts with RPA and phosphorylates RPA2 both in vivo and in vitro (28, 32–34). RPA2 phosphorylation induced by a high dose of IR also depends on ATM (27, 35). The involvement of ATR in RPA2 phosphorylation, however, is not quite clear. It has been reported that RPA2 is a good substrate of ATR in vitro (36), but whether ATR phosphorylates RPA2 in vivo and whether this phosphorylation affects RPA function following DNA damage are unknown. The observation that caffeine treatment does not further reduce RPA2 phosphorylation in ATM-deficient cells has led to the conclusion that ATR phosphorylation of RPA2 may not be relevant in vivo (37).

Here we show that RPA2 is phosphorylated on chromatin in an ATR-dependent manner when cells are treated with DNA damage-inducing agents that block replication. We demonstrate that not only does ATR phosphorylate RPA2 directly in vitro, but RPA2 phosphorylation on chromatin after UV or HU treatment in vivo is dependent on ATR protein levels and on its kinase activity. Mutations at the ATR-dependent phosphorylation sites of RPA2 specifically impair ATR-mediated replication inhibition following UV treatment. Analysis of phospho-mimetic mutants of RPA2 at ATR phosphorylation sites suggests that ATR-dependent RPA2 phosphorylation likely prevents RPA association with replication centers. These studies suggest that RPA2 is a physiologically relevant substrate of ATR in vivo and is a direct effector of ATR to inhibit replication following UV-induced DNA damage.

EXPERIMENTAL PROCEDURES

Cell Culture, Retroviral Infections, and shRNA—U2OS, 293T, GM847, or GM847/ATR-KD cells were cultured in Dulbecco’s modified Eagle’s medium containing 5% fetal bovine serum and 5% super calf serum. To induce expression of ATR-KD in the GM847 fibroblasts, 1 μg/ml of doxycycline was added to the media 24 h prior to chromatin isolation (38). HA-tagged RPA2 or mutant forms of RPA2 were introduced into U2OS cells by retroviral infection followed by selection of puromycin-resistant cells. Silencing of endogenous RPA2 in these cells was accomplished by two rounds of retroviral infection using pMKO vector (39) expressing the RPA2 shRNA target sequence, CCGAGUUUCACAAUCUGUGU, located in the 3'-UTR of the mRNA (40) followed by selection of G418- and hygromycin-resistant cells. Endogenous ATR, ATM, Chk1, and DNA-PK were silenced in U2OS cells by two rounds of retroviral infection using pMKO vector that expressed two different shRNA target sequences. The target sequences used for ATR silencing were AAGCAUGCUAGAUGUGCUAGA (41) and CGAGACUUCUGCGAUCUG. The target sequences used for ATM silencing were AACAUACUACUAAAGCAUU, and GCACGAGCUAGAUGCGCCU. The target sequences used to silence Chk1 were AACGCGUGGGCCUGAGCUGCC and AAGCAUCCAGAUGCUACCC. The target sequences used for DNA-PK silencing were GGCGCGUAUUGCGAAGAC and GAUCGCACCUCUACUGUGA (42).

Plasmids, Mutagenesis, and GST Protein Purification—To generate the serine to alanine mutations in RPA2, HA-tagged full-length RPA2 cloned into mammalian expression vector pcDNA3β was used as template for several rounds of site-directed mutagenesis (Stratagene). HA-tagged wild-type RPA2 and mutant forms generated by site-directed mutagenesis were cloned into the retroviral vector pBABEPuro. GST-tagged RPA2 fragments were made by cloning a PCR product generated using wild-type RPA2, or one of the mutant forms of RPA2 as a template, into pGEX4T-1 (Amersham Biosciences). GST fusion proteins were expressed in BL21 Escherichia coli cells after isopropyl 1-thio-β-D-galactopyranoside induction, purified on glutathione-Sepharose beads (Amersham Biosciences), and eluted with 15 mM glutathione in 50 mM Tris-HCl (pH 8.0). His-tagged RPA2 was constructed by cloning a PCR product made using pl11d-RPA, a generous gift from Marc Wold, as a template into pET28b (Novagen). His-tagged RPA containing phospho-mimetic mutations in RPA2 were generated by site-directed mutagenesis of pET28b-RPA. His-tagged RPA fusion proteins were expressed in BL21 E. coli cells after isopropyl 1-thio-β-D-galactopyranoside induction and purified on Ni-NTA resin (Qiagen).

Immunoprecipitation and Chromatin Isolation—Cells were lysed in NETN (150 mM NaCl, 1 mM EDTA, 20 mM Tris-Cl (pH 8.0), 0.5% Nonidet P-40) containing protease and phosphatase inhibitors. Immunoprecipitation was performed by incubating primary antibodies with cell lysates at 4 °C for 4 h, followed by the addition of secondary antibody (MP Biomedicals) and protein A-agarose (Calbiochem) for one additional hour. For Western blot analysis, proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes (Bio-Rad), and incubated overnight in primary antibodies followed by 1 h of incubation in horseradish peroxidase-conjugated secondary antibodies.

For chromatin isolation, cells were washed with PBS, trypsinized, collected, resuspended in CSK buffer (10 mM PIPES (pH 6.8), 100 mM NaCl, 300 mM sucrose, 3 mM MgCl₂, 1 mM EGTA, 50 mM sodium fluoride, 0.1 mM sodium orthovanadate, 0.1% Triton X-100, and protease inhibitors), and incubated at 4 °C for 5 min. Cytoplasmic proteins were separated from nuclei by low speed centrifugation at 1,300 × g for 5 min. Isolated nuclei were washed once in CSK buffer, lysed in solution B (3 mM EDTA, 0.2 mM EGTA, 1 mM DTT, and protease inhibitors). After centrifugation, the pellet was resuspended in CSK buffer and 2 × SDS loading buffer and boiled.

Pulldown Assays—His-tagged RPA wild type (RPA1-RPA2-RPA3 complex) or the RPA complex containing serine or threonine to aspartate mutations at specific residues in RPA2 were purified from E. coli on Ni-NTA resin (Qiagen). Primase subunits were expressed from baculoviruses infected either individually or in different combinations in insect cells. Insect cells were lysed 48 h after infection, and the lysates were combined with RPA-bound Ni-NTA at 4 °C for 4 h. After extensive washing, primase subunits retained on the RPA beads were analyzed by Western blot.

Immunofluorescence—For immunostaining analysis of retrovirally infected U2OS cells, cells grown directly on glass cover-slips were washed in PBS and then either fixed directly with 4%
formaldehyde in PBS (nonextracted) or extracted first with 0.5% Triton X-100 in PBS for 5 min (extracted), fixed with a modified Streck tissue fixative for 30 min at room temperature, and permeabilized for 15 min at room temperature as described previously (43, 44). Cells were blocked with 5% goat serum in PBS and then stained with primary antibodies (monoclonal anti-RPA1; polyclonal anti-HA) at 4 °C overnight. The following morning, the cells were washed and stained for 1 h at room temperature with rhodamine-conjugated anti-mouse and fluorescein isothiocyanate-conjugated anti-rabbit secondary antibodies (Jackson Immunoresearch). The frequency of cells with chromatin-bound HA-RPA2 was determined by counting the number of RPA1-positive staining cells with HA-RPA2 foci after extraction from three independent experiments. To detect damage foci, asynchronized cells were fixed by 70% methanol and 30% acetone at −20 °C for 15 min, followed by immunostaining analysis as described above.

Antibodies—The antibodies used in this study were purchased from Oncogene (ATM, RPA1, and RPA2), Bethyl Laboratories (RPA2-S4/8P and RPA2-S33P), Calbiochem (ATR), Covance (mono-HA), Cell Signaling Technology (pT68-Chk2), R&D Systems (pS317-Chk1), Santa Cruz Biotechnology (Chk1, Chk2, and Ku70), Serotec (DNA-PK), Neomarkers (primase p48 and p58), and Upstate (poly-HA). Monoclonal antibodies 9D5 (recognizing p68) and 1CT 102 and 2CT25 (recognizing p48 and p58), and Upstate (poly-HA) at 4 °C overnight. The following experiment was repeated three times, and the average number of surviving cells from each cell line was normalized to the average number of viable, uninfected U2OS cells that were plated as a control for each experiment.

RESULTS

ATR Kinase Activity Is Required for RPA2 Hyperphosphorylation on Chromatin in Response to UV or HU Treatment—Chromatin was isolated from U2OS cells after exposure to UV light or treatment with HU. Consistent with previous reports (18, 46), RPA1 and RPA2 were both recruited to the chromatin (Fig. 1A, compare the soluble versus the chromatin fractions), and several slower migrating forms of RPA2 appeared specifically on the chromatin after UV and HU treatments (Fig. 1A). To demonstrate conclusively that the slower migrating bands of RPA2 detectable after DNA damage were a result of hyperphosphorylation of the protein, chromatin isolated from U2OS cells exposed to UV light or HU was treated with a phosphatase. Following phosphatase treatment, the mobility of RPA2 reverted to that of RPA2 from mock-treated cells (Fig. 1B, and data not shown), indicating that all of the slower migrating forms of RPA2 detected upon UV or HU treatment are phosphorylated forms of RPA2. Notably, exposure to UV light and HU, two DNA damage-inducing agents that stall replication forks, significantly increased RPA recruitment to the chromatin and RPA2 hyperphosphorylation, and this recruitment and phosphorylation were not detectable after 20 Gy of IR treatment (Fig. 1A). Although RPA2 phosphorylation can be induced with an extremely high dose of IR (100 Gy (27, 32)), this result suggested that RPA2 hyperphosphorylation on chromatin is more prevalent when replication forks are stalled than when DNA double strand breaks are introduced.

Because ATR is activated in response to both UV light and HU, we investigated whether RPA recruitment to the chromatin or RPA2 phosphorylation depends on ATR kinase activity. Chromatin was isolated from an SV40-transformed fibroblast cell line GM847 or from GM847 cells expressing a doxycycline-inducible kinase-inactive allele of ATR ((GM847-KD) (47)) after cells were mock-treated, treated with HU, or exposed to UV light. Chk1 phosphorylation at Ser-317 after UV treatment was reduced when the overexpression of ATR-KD was induced (supplemental Fig. 1), suggesting that ATR activity was inhibited. Compared with GM847 cells that had normal ATR kinase activity, cells overexpressing the kinase-inactive allele of ATR...
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A. The soluble and the chromatin fractions were isolated from U2OS cells that were mock-treated (No), exposed to UV light (30 J/m², 1 h after), or IR (20 Gy, 30 min after) and immunoblotted for RPA1 and RPA2. The phosphorylated forms of RPA2 are indicated by RPA2-P, RPA2-S4/8P, or RPA2-S33P. Chromatin was isolated from U2OS cells that had been retrovirally infected with vector alone or with two shRNAs directed against ATR after mock treatment (bottom right). Chromatin was also treated with lambda phosphatase and then immunoblotted with an antibody to Ku70 as a loading control.

B. Vector or ATRshRNA infected cells were mock-treated or treated with UV light (2 J/m², 1 h after) or HU (1 mM, 24 h after) and then immunoblotted for RPA2. An antibody to Ku70 was used as a loading control.

C. HU treatment: RPA2-P and RPA2-S33P were detected by anti-RPA2 antibody, and the reduction in serine 33 phosphorylation detected using a Ser-33 phospho-specific antibody (Fig. 1C). RPA recruitment to the chromatin was not affected by the overexpression of the kinase-inactive allele of ATR. In fact, there was a significant increase in the unphosphorylated chromatin-bound RPA2 in the GM847-KD cells presumably because of an inability to phosphorylate RPA2 following chromatin loading (Fig. 1C). This result suggests that ATR kinase activity is required for RPA2 hyperphosphorylation, but not for recruitment of RPA to the chromatin following UV and HU treatment.

To confirm this result, endogenous ATR expression was silenced by expressing two ATR-shRNA sequences from a retroviral vector (39). In the presence of the shRNAs directed against ATR, ATR protein levels were significantly reduced (Fig. 1D, top). In addition, both Chk1 phosphorylation and H2AX phosphorylation following UV light, two well characterized ATR-dependent phosphorylation events, were also reduced when compared with the levels of phosphorylation in the vector-infected cells (Fig. 1D, top and data not shown). To look at RPA2 hyperphosphorylation in these cells, the soluble and the chromatin fractions were isolated after exposure to UV or HU treatment. RPA recruitment to the chromatin was again not affected by the reduction in ATR expression (Fig. 1D, compare the soluble versus the chromatin fractions for RPA1 and RPA2), but RPA2 hyperphosphorylation was significantly reduced when ATR was silenced, and there was an accumulation of the chromatin-bound unphosphorylated form of RPA2 in response to both UV and HU treatment (Fig. 1D).

Among the multiple UV-inducible phosphorylation sites that have been mapped at the amino terminus of RPA2 (29, 48), threonine 21 (TQ) and serine 33 (SQ), but not serines 4 and 8 (non-SQ sites), are ATR consensus sites. Nevertheless, using phospho-specific antibodies to serine 33, or serines 4 and 8, we observed that both UV- and HU-induced phosphorylation at Ser-33, and at the Ser-4/Ser-8 sites, were reduced when ATR activity was inhibited (Fig. 1, C and D, bottom). Taken together, our results suggest that in response to UV light or HU, RPA is recruited to the chromatin
in an ATR-independent manner, and RPA2 is then phosphorylated at several sites in its amino terminus in an ATR-dependent manner.

**DNA-PK and ATM Do Not Significantly Contribute to UV- or HU-induced RPA2 Phosphorylation on Chromatin**—To determine the contribution of the other members of the PI3-like family of kinases to UV- or HU-induced RPA2 phosphorylation on chromatin, we silenced DNA-PK or ATM in U2OS cells by retroviral infection of two shRNAs directed against two different sites in the DNA-PK or in the ATM mRNA. Silencing the expression of DNA-PK or ATM by this method significantly reduced the levels of detectable protein (Fig. 2A). To confirm that ATM kinase activity was compromised, phosphorylation of Chk2, an ATM substrate, was analyzed after mock treatment or treatment with ionizing radiation (IR). Compared with the vector-infected cells, there was a significant reduction in Chk2 phosphorylation at threonine 68 after IR treatment in the cells expressing the ATM-shRNAs (Fig. 2A). To confirm that the DNA-PK shRNAs were effectively inhibiting the kinase activity of DNA-PK, the protein levels of c-Myc were analyzed. It has been demonstrated previously that DNA-PK activity is required for maintaining stable c-Myc [42]. Cells expressing the DNA-PK shRNAs had significantly reduced c-Myc protein levels when compared with vector-infected cells (Fig. 2A), suggesting that DNA-PK activity was effectively inhibited by the expression of the shRNAs. Cells expressing the ATM-shRNAs or the DNA-PK-shRNAs were mock-treated, exposed to UV light, or treated with HU alongside cells expressing two shRNAs directed against ATR. At specific times after treatment, chromatin was isolated from these cells, and the level of RPA2 phosphorylation was compared with cells infected with vector alone. Although silencing ATR had an obvious impact on RPA2 hyperphosphorylation, and a noticeable effect on phosphorylation of both serines 4/8 and serine 33 on chromatin following UV and HU treatment (Fig. 2B), a significant defect in RPA2 phosphorylation on the chromatin was not apparent when either ATM (Fig. 2C) or DNA-PK (Fig. 2E) was silenced, as judged by both RPA2 shift and by using phospho-specific antibodies. Although reduction of ATM protein levels had a small effect on RPA2 phosphorylation, specifically on phosphorylation of serine residue 33 in response to UV and HU treatment, this reduction was much less than when ATR protein levels were reduced.

Chromatin was also isolated from the ATM-deficient fibroblast cell line FT169 and its isogenic ATM-reconstituted derivative cell line YZ5 (49) after UV treatment. The expression of ATM was only detected in the YZ5-reconstituted cell line (Fig. 2D). Similar to the results using the ATM-shRNA-expressing cells, only a minor difference in the UV-induced phosphorylation of chromatin-bound RPA2 was observed in the ATM-deficient cells (FT169), relative to the reconstituted cell line (YZ5; Fig. 2D). From these observations, we conclude that UV- or HU-induced RPA2 phosphorylation on chromatin is primarily dependent on ATR, and DNA-PK and ATM do not significantly contribute to UV- or HU-induced phosphorylation of chromatin-bound RPA2.

**UV- or HU-induced Phosphorylation of Ser-4/8 or Ser-33 on RPA2 Does Not Depend on Phosphorylation of the Other Sites**—Among the mapped UV-inducible phosphorylation sites, Thr-21 (TQ) and Ser-33 (SQ) are consensus sites for ATR and the other members of the PI3-like kinase family, but Ser-4/8 and Ser-11, Ser-12, and Ser-13 are not (Fig. 3A). To examine the contribution of phosphorylation at these sites to the overall in vivo phosphorylation of RPA2 in response to UV light and HU, several residues in the amino terminus of RPA2 were mutated to alanine either individually or in combination with each other. HA-tagged copies of either wild-type RPA2 or the mutant alleles of RPA2 were retrovirally infected into U2OS cells, and chromatin was isolated from these cells following exposure to UV or HU treatment. Compared with wild-type RPA2, mutation of threonine 21 together with serine 33 reduced the intensity of the phosphorylated forms of RPA2—2-fold following UV light or HU (Fig. 3B). Notably, the unphosphorylated form of RPA2 (indicated as HA-RPA2) accumulated in the T21A/S33A mutant, but there was no obvious difference in the overall banding pattern of RPA2. Strikingly, when serines 4, 8, 11, 12, and 13 (non-SQ sites) were mutated to alanine residues, the slower migrating forms of RPA2 were almost completely abolished in response to both UV light and HU (Fig. 3B). When T21A/S33A was combined with S4A/S8A/S11A/S12A/S13A, the appearance of the slower migrating forms was not further reduced (Fig. 3B). These results suggest that the appearance of the phosphorylation-induced banding pattern of RPA2 requires phosphorylation at one or more of the serine 4, 8, 11, 12, and 13 sites.

Because mutation of S4A/S8A/S11A/S12A/S13A almost completely abolished the slower migrating phosphorylated forms of RPA2 induced by UV or HU treatment (Fig. 3B), it raised a question as to whether phosphorylation at Thr-21/Ser-33 requires existing phosphorylation of serines 4, 8, 11, 12, and 13. If this is the case, loss of phosphorylation at Ser-4, Ser-8, Ser-11, Ser-12, and Ser-13 would prevent phosphorylation of Thr-21 or Ser-33 following DNA damage and would therefore almost completely abolish damage-induced RPA2 phosphorylation. To test whether phosphorylation of RPA2 at Ser-4/8 or Ser-33 depends upon phosphorylation of the other site, we mutated RPA2 at one of these sites and used phospho-specific antibodies to determine whether the other site was still phosphorylated after UV- or HU-induced DNA damage. HA-tagged wild-type RPA2 or RPA2 containing serine to alanine substitutions at residues 4, 8, 11, 12, and 13, or at threonine 21 and serine 33, or at all of these sites (S4A/S8A/S11A/S12A/S13A/T21A/S33A), were introduced into U2OS cells by retroviral infection. Endogenous RPA2 was silenced by two rounds of retroviral infection of an shRNA targeting the 3’-UTR of the RPA2 mRNA (40). Chromatin was isolated from these cell lines after cells were mock-treated, exposed to UV light, or treated with HU. Mutation of serines 4, 8, 11, 12, and 13 to alanine residues did not disrupt phosphorylation of RPA2 at serine 33 following UV or HU treatment (Fig. 3C, left). Likewise, substitution of alanine residues for threonine 21 and serine 33 of RPA2 did not disrupt phosphorylation of RPA2 at serines 4/8 in response to UV light or HU (Fig. 3C, right). Mutation of all of these sites (Ser-4, Ser-8, Ser-11, Ser-12, and Ser-13 and Thr-21/Ser-33, marked as 7xA mutant) completely abolished any detectable RPA2 phosphorylation at Ser-4/8
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A.

B.

C.

D.

E.
or Ser-33 following UV light and HU (Fig. 3C), confirming that silencing of endogenous RPA2 in these cell lines was efficient. These results suggest that phosphorylation of Ser-4/8 and phosphorylation of Ser-33 occur independently of one another in response to UV light and HU. When serines 4, 8, 11, 12, and 13 are mutated to alanines, loss of the damage-induced slower migrating form of RPA2 is not because of the fact that Ser-33 is not phosphorylated. Therefore, this dramatic change in the migration of RPA2 upon UV or HU treatment is likely because of a conformational change in the protein that is introduced upon phosphorylation of Ser-4, Ser-8, Ser-11, Ser-12, and Ser-13, whereas phosphorylation of Thr-21/Ser-33 has only a minor effect on its migration. This also indicates that the migration of RPA2 may not be proportional to the extent of RPA2 phosphorylation but may be more dependent upon which sites in RPA2 are actually phosphorylated.

**ATR Directly Phosphorylates Threonine 21 and Serine 33 of RPA2 in Vitro**—To determine which sites in the amino terminus of RPA2 can be phosphorylated directly by ATR in vitro, GST-tagged wild-type RPA2 consisting of amino acids 1–51 or mutant forms of this fragment containing serine to alanine substitutions at serine residues 4, 8, 11, 12, and 13, or at threonine 21 and serine 33 were purified from *E. coli* and used as substrates for an ATR in vitro kinase assay. Although mutation of serines 4, 8, 11, 12, and 13 had no effect on the in vitro phosphorylation of RPA2 by ATR, mutation of threonine 21 and serine 33 completely ablated phosphorylation by ATR in vitro (Fig. 4A). These experiments demonstrate that ATR can directly phosphorylate threonine 21 and serine 33 but not serines 4, 8, 11, 12, or 13 of RPA2 in vitro.

Downstream of the previously mapped UV-inducible phosphorylation sites in the amino terminus of RPA2, there are three additional SQ sites (Ser-52, Ser-72, and Ser-174). The contribution of these three sites to RPA2 phosphorylation following UV light or HU has not been described previously. To determine whether ATR could phosphorylate RPA2 at serines 52, 72, or 174 in vitro and to substantiate the idea that these SQ sites could contribute to RPA2 phosphorylation in vivo following DNA damage, GST-tagged RPA2 consisting of amino acids 1–188 or mutant forms of this fragment containing serine to alanine substitutions at serines 4, 8, 11, 12, and 13, threonine 21 and serine 33, or at all of these sites in addition to serines 52, 72, and 174 were purified and used as substrates for an ATR in vitro kinase assay. Using this larger GST-RPA2 protein as a substrate, mutation of threonine 21 and serine 33 again almost completely abolished the in vitro RPA2 phosphorylation by ATR (Fig. 4B), suggesting that Thr-21 and Ser-33 are the major ATR phospho-

*FIGURE 2. DNA-PK and ATM do not significantly contribute to HU- or UV-induced RPA2 phosphorylation.* A, U2OS cells that had been retrovirally infected with vector alone, or with two shRNAs directed against two different target sequences in ATM, were lysed, and the whole cell extracts were immunoblotted as indicated (1st panel). Vector-infected cells, or cells infected with two ATM shRNAs were mock-treated (–) or treated with ionizing radiation (2 Gy, 30 min after), lyzed, and immunoblotted with a phospho-specific antibody to Chk2 (Chk2-T68P) or an antibody against total cellular Chk2 (2nd panel). Cells infected with vector alone or with two shRNAs directed against DNA-PK were lysed, and the whole cell extracts were immunoblotted with the indicated antibodies (right two panels). B, chromatin was isolated from U2OS cells retrovirally infected with vector only or with two shRNAs directed against ATR after mock treatment (0), exposure to UV light (30 J/m², 2, 4, 6, and 8 h after), or treatment with HU (1 mm, 16, 18, and 24 h after). Chromatin was immunoblotted using antibodies to RPA1, RPA2, or two phospho-specific antibodies recognizing either phosphorylation of serine 33 of RPA2 (RPA2-S33P) or phosphorylation of serines 4/8 of RPA2 (RPA2-S4/8P). An antibody to Ku70 was used as a loading control. C, chromatin was isolated from U2OS cells retrovirally infected with vector only or with two shRNAs directed against ATM after mock treatment (0), exposure to UV light (30 J/m², 2, 4, 6, and 8 h after), or treatment with HU (1 mm, 16, 18, and 24 h after) and immunoblotted as indicated (top). D, chromatin was isolated from ATM-deficient cells (FT169) and cells reconstituted with wild-type ATM (YZ25) after mock treatment (0), or exposure to UV light (30 J/m², 2, 4, 6, and 8 h after) and immunoblotted with antibodies specific to RPA1, RPA2, phosphorylated RPA2, or Ku70 as a loading control (bottom). E, chromatin was isolated from U2OS cells retrovirally infected with vector only or with two shRNAs directed against DNA-PK after mock treatment (0), exposure to UV light (30 J/m², 2, 4, 6, and 8 h after), or treatment with HU (1 mm, 16, 18, and 24 h after) and immunoblotted as indicated.
Phosphorylation of RPA2 by ATR Is Required to Inhibit DNA Replication after Exposure to UV Light—It has been demonstrated that ATR is recruited to the chromatin specifically during S-phase (54) and plays important roles to maintain genomic integrity (55). ATR is not only required to maintain fork stability, and/or mediate damage repair during S-phase because chromosomal fragile sites are more unstable when ATR is compromised (41), but it is also involved in monitoring S-phase progression and activating the DNA damage-induced checkpoint when replication forks are arrested (3). In this regard, it has been described that following UV treatment, ATR is required to mediate the slowing down of DNA replication (8), but the mechanisms by which it does so are currently not well understood. We have shown that chromatin-bound RPA is a substrate of ATR when replication forks are stalled. This promoted us to examine whether RPA is an ATR effector protein mediating ATR-dependent damage responses to inhibit replication following UV treatment.

To test the biological function of ATR-mediated phosphorylation of RPA2, we used the U2OS cell lines expressing wild-type RPA2 or the RPA2-(7xA)-mutant (S4A/S8A/S11A/S12A/S13A/T21A/S33A) where endogenous RPA2 was silenced by shRNAs targeting the 3'UTR. Following HU treatment, no detectable RPA2 phosphorylation was evident in the cell line expressing the RPA2-(7xA)-mutant when phospho-specific
ATR Phosphorylates RPA2 to Mediate the S-phase Checkpoint

![Diagram](image)

**FIGURE 4. ATR directly phosphorylates threonine 21 and serine 33 of RPA2.** A. FLAG-tagged ATR transiently transfected into 293T cells was immunoprecipitated and incubated in the presence of [γ-32P]ATP with glutathione-eluted GST-RPA2 (amino acids 1–51) wild type, one of the indicated RPA2 mutant proteins (amino acids 1–51), containing alanine mutations at the specified residues, or as a positive control, glutathione-eluted GST-Chk2 (amino acids 1–92) all of which had been purified from *E. coli* on glutathione-Sepharose (top, right). The Coomassie-stained gel shows the relative amount of protein added to the *in vitro* kinase reactions (bottom right). The Western blot shows the whole cell extract (WCE) and immunoprecipitate (IP) from cells transfected with FLAG-tagged ATR (top, left). B. FLAG-tagged ATR transiently transfected into 293T cells was immunoprecipitated and incubated in the presence of [γ-32P]ATP with glutathione-eluted GST-RPA2 (amino acids 1–188), wild type, one of the indicated RPA2 mutant proteins (amino acids 1–188), containing alanine mutations at the specified residues, or as a positive control glutathione-eluted GST-Chk2 (amino acids 1–92), all of which had been purified from *E. coli* on glutathione-Sepharose (left). The Coomassie-stained gel shows the relative amount of protein added to the *in vitro* kinase reactions (right). C. HA-tagged Chk1 transiently transfected into 293T cells was immunoprecipitated and then incubated in the presence of [γ-32P]ATP with glutathione-eluted GST-Cdc25C (amino acids 200–256), GST-RPA2 (amino acids 1–51), or GST alone (top, right). The Coomassie-stained gel shows the relative amount of protein added to the *in vitro* kinase reactions (bottom right). The Western blot shows the whole cell extract (WCE) and immunoprecipitate (IP) from cells transfected with HA-Chk1 (top left).

antibodies recognizing Ser-4/8 or Ser-33 were used (Fig. 5A), suggesting that endogenous RPA2 was efficiently inactivated by expression of the shRNAs. The effectiveness of the RPA2 shRNAs was supported by the fact that their expression caused significant cell death in U2OS cells (Fig. 5C). Like wild-type RPA2, the RPA2-(7xA)-mutant co-immunoprecipitated RPA1, indicating that substitution of the serine residues in the amino terminus of the protein did not affect the interaction of RPA2 with RPA1 (Fig. 5B). Notably, there was no obvious growth defect in this cell line (data not shown), and the lethality caused by the expression of the RPA2-shRNA was suppressed by both retrovirally expressing either wild-type RPA2 or the RPA2-(7xA)-mutant (Fig. 5C). Furthermore, the RPA2-(7xA)-mutant cell line (with endogenous RPA2 silenced) resumed replication with a rate similar to the cell line expressing wild-type RPA2 after release from HU arrest (Fig. 5D), and immunostaining analysis demonstrated that the RPA2-(7xA)-mutant, like wild-type RPA2, co-localized with proliferating cell nuclear antigen in S-phase foci indicating that the RPA2-(7xA)-mutant did not have a defect in migrating to replication centers in S-phase (data not shown). The percentage of S-phase cells judged by DNA damage, we examined the rate of DNA synthesis in U2OS cells expressing wild-type RPA2 or the RPA2-(7xA)-mutant (with endogenous RPA2 silenced) following exposure to UV radiation. Although the rate of DNA synthesis was down-regulated following UV light in cells infected with vector only (endogenous RPA2 was not silenced) or with wild-type RPA2 (with endogenous RPA2 silenced), cells expressing the RPA2-(7xA)-mutant (with endogenous RPA2 silenced) inhibited DNA synthesis to a significantly lesser extent (*p* < 0.01; Fig. 6A). Likewise, inactivation of ATR, but not DNA-PK, compromised the down-regulation of DNA synthesis after UV light (*p* < 0.005 for ATR shRNA; Fig. 6B). Notably, although the RPA2-(7xA)-mutant shows a clear defect in the inhibition of DNA replication after UV treatment, the effect is less prominent than the effect observed in the cell line expressing the ATR shRNAs. This suggests that other pathway(s), parallel to the RPA pathway, exist downstream of ATR and are also required to mediate the S-phase checkpoint. In this regard, it has also been demonstrated that MCM proteins are directly phosphorylated by ATM/ATR (58) and may also be involved in mediating the ATR-dependent S-phase checkpoint following UV light.
Although multiple pathways may be involved, our studies demonstrate that RPA is a critical target of ATR in the UV-induced S-phase checkpoint response.

We showed that phosphorylation of multiple sites in the amino terminus of RPA2 is ATR-dependent. Although Thr-21 and Ser-33 were directly phosphorylated by ATR, serines 4, 8,
FIGURE 6. ATR-dependent phosphorylation of RPA2 is required to inhibit DNA replication after exposure to UV light, without affecting checkpoint activation. A, the rate of DNA synthesis following exposure to UV light (10 J/m²) was determined in U2OS cells retrovirally infected with vector alone when endogenous RPA2 was not silenced, with HA-tagged RPA2 when endogenous RPA2 was silenced, or with the HA-tagged RPA2-(7xA)-mutant when endogenous RPA2 was silenced. These rates were expressed as percentages by normalizing to the appropriate mock-treated controls. The average of three independent experiments is shown in the graph with error bars indicating the standard deviation from the average. The difference between the rates of DNA synthesis after UV light in the cells expressing the RPA2-(7xA)-mutant protein compared with cells expressing the wild-type RPA2 protein is statistically significant (p < 0.01). B, rate of DNA synthesis following exposure to UV light (10 J/m²) was determined in U2OS cells retrovirally infected twice with vector alone, with two shRNAs directed against ATR, or with two shRNAs directed against DNA-PK. These rates were expressed as percentages by normalizing to the appropriate mock-treated controls. The average of three independent experiments is shown in the graph with error bars indicating the standard deviation from the average. The difference between the rates of DNA synthesis after UV light in the cells expressing the ATR shRNAs compared with cells expressing vector alone is statistically significant (p < 0.005). C, the rate of DNA synthesis following exposure to UV light (10 J/m²) was determined in U2OS cells retrovirally infected with HA-tagged RPA2, with the RPA2-T21A/S33A mutant allele, or with the RPA2-(7xA)-mutant, when endogenous RPA2 was silenced in each of these cell lines (left), or in U2OS cells retrovirally infected with vector alone when endogenous RPA2 was silenced at normal cellular levels, with HA-tagged RPA2 when endogenous RPA2 was silenced, or with the RPA2-S4A/S8A/S11A/S12A/S13A mutant allele when endogenous RPA2 was silenced (right). These rates were expressed as percentages by normalizing to the appropriate mock-treated controls. The averages from three independent experiments for each cell line are shown in the graphs with error bars indicating the standard deviation from the average. The reduction of DNA synthesis after UV light in the RPA2-S4A/S8A/S11A/S12A/S13A cells is not significantly different from the wild-type RPA2 cells (p > 0.1), whereas the reduction of DNA synthesis after UV light in the RPA2-T21A/S33A cells is significantly less than in the wild-type RPA2 cells (p < 0.005). D, U2OS cells retrovirally expressing HA-tagged RPA2 or RPA2-(7xA)-mutant, where endogenous RPA2 was silenced, were mock-treated (−), exposed to UV light (2 J/m², 1 h after), IR (2 Gy, 1 h after), or treated with HU (0.1 mM, 2 h after) and then lysed and immunoblotted with the indicated antibodies.
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11, 12, and 13 were not direct ATR substrates. We determined whether the two groups of phosphorylation sites, either direct or indirect substrates of ATR, were required for ATR-mediated down-regulation of DNA replication following UV light. To do this, the rate of DNA synthesis was determined in U2OS cells expressing RPA2 containing serine to alanine mutations at residues 4, 8, 11, 12, and 13, and in cells expressing RPA2 with threonine 21 and serine 33 mutated to alanine, when endogenous RPA2 was silenced (Fig. 6C). Mutation of threonine 21 and serine 33 resulted in significant UV-resistant DNA synthesis ($p < 0.005$; Fig. 6C, left), although slightly less than the RPA2-7xa-mutant (S4A/S8A/S11A/S12A/S13A/T21A/S33A), but mutation of serines 4, 8, 11, 12, and 13 alone did not affect the down-regulation of DNA synthesis following UV light (Fig. 6C, right). Therefore, phosphorylation of threonine 21 and/or serine 33 of RPA2 is more critical than phosphorylation of serines 4, 8, 11, 12, and 13 for the inhibition of DNA replication following UV light.

It has been described that after RPA2 is phosphorylated, the interaction of RPA with Rad9/Rad1/Hus1 is increased (56). This suggests that the initial phosphorylation of RPA2 may be involved in amplifying checkpoint signals by regulating the Rad9/Rad1/Hus1 pathway. Therefore, the requirement of ATR-mediated RPA2 phosphorylation in the inhibition of replication could be due to loss of checkpoint activation when ATR-mediated RPA2 phosphorylation is impaired. To determine whether phosphorylation of RPA2 by ATR plays any role in checkpoint activation following RPA recruitment to the chromatin, cell lines expressing wild-type RPA2 or the RPA2-7xa-mutant where endogenous RPA2 was silenced were treated with a low concentration of HU or exposed to low doses of UV light or IR. Checkpoint activation was determined by using phospho-specific antibodies to Chk1 and Chk2, two kinases downstream of ATR and ATM, respectively. Disruption of RPA2 phosphorylation had no significant effect on either Chk1 phosphorylation following UV light or HU or Chk2 phosphorylation following IR (Fig. 6D). These data suggest that RPA2 phosphorylation does not affect ATR-mediated activation of the checkpoint response following UV or HU treatment. Thus, the loss of UV-induced replication inhibition in the presence of the RPA2 phospho-mutants is likely because of a defect in inhibiting DNA replication directly. Taken together, our studies suggest that ATR-mediated phosphorylation at the amino terminus of RPA2 is required to prevent DNA replication after UV-induced S-phase checkpoint activation in mammalian cells.

Phosphorylation of RPA2 by ATR May Prevent RPA Localization to Replication Centers—To explore the possible mechanism by which ATR-mediated RPA2 phosphorylation inhibits DNA replication, we generated phospho-mimetic mutants of RPA2 by replacing serines 4, 8, 11, 12, and 13, threonine 21, and serine 33, or all of these residues with aspartate residues. Although an aspartate residue is not the same as a phosphorylated serine or threonine, it has been shown in many cases that the replacement of serine/threonine with aspartate can functionally substitute for phosphorylation events (60–63). It has been described that RPA2 phospho-mimetic mutants with aspartate substitutions at both the cyclin-Cdk2 phosphorylation sites and at the damage-inducible phosphorylation sites fail to localize to DNA replication centers (40), suggesting that phosphorylation of RPA2 may play an important role in regulating DNA replication. In this study, we specifically examined RPA2 mutants containing serine or threonine to aspartate substitutions at the ATR direct or indirect phosphorylation sites for their ability to localize to replication centers.

HA-tagged wild-type RPA2 or RPA2 phospho-mimetic mutants, including S4D/S8D/S11D/S12D/S13D, T21D/S33D, or S4D/S8D/S11D/S12D/S13D/T21D/S33D, were retrovirally expressed in U2OS cells at similar levels (Fig. 7B). Because RPA forms S-phase-specific foci at replication centers that can be visualized after nonionic detergent extraction prior to fixation (64), we examined the localization of HA-tagged wild-type RPA2 and the phospho-mimetic RPA2 mutants to replication centers that were marked by endogenous RPA1 foci. Endogenous RPA1 immunostaining after extraction was used to indicate S-phase cells and S-phase-specific RPA foci (Fig. 7A). Immunostaining of nonextracted cells showed that HA-tagged wild-type RPA2 and all of the phospho-mimetic RPA2 mutants were expressed in the nucleus in every cell following retroviral infection and selection (Fig. 7A and data not shown).

After extraction, cells retrovirally expressing wild-type HA-RPA2 formed RPA2 S-phase foci (detected by an anti-HA antibody) that were visually similar to endogenous RPA1 (Fig. 7A), whereas the HA-tagged RPA2 phospho-mutants exhibited significant defects when different sites were mutated. The RPA2 mutant containing aspartate residues at all of the ATR-dependent phosphorylation sites (S4D/S8D/S11D/S12D/S13D/T21D/S33D) did not form any S-phase foci indicating a complete inability of the protein to migrate into replication centers that were marked by RPA1 immunostaining. The T21D/S33D and the S4D/S8D/S11D/S12D/S13D mutants formed foci only in a fraction of cells containing RPA1 foci, with a more dramatic decrease in the T21D/S33D mutant (Fig. 7C, left). Moreover, among the cells containing foci in T21D/S33D and S4D/S8D/S11D/S12D/S13D mutant cell lines, a substantial number of cells had faint foci (Fig. 7A, white arrows), whereas all of the cells containing wild-type HA-RPA2 had bright foci. There was a significant difference in the percentage of S-phase cells containing normal, bright HA-RPA2 foci relative to the overall number of S-phase cells containing foci in the T21D/S33D and S4D/S8D/S11D/S12D/S13D mutant cell lines (Fig. 7C, right). Although a large percentage of the cells expressing the S4D/S8D/S11D/S12D/S13D mutant had foci that looked similar to the wild-type HA-RPA2 foci and were bright staining, only a small fraction of the cells in the T21D/S33D mutant had foci that were bright.

These results suggest that the RPA2 phospho-mimetic mutations at ATR-dependent phosphorylation sites prevent RPA2 from localizing to replication centers. Phosphorylation at the ATR-direct sites, Thr-21 and Ser-33, may more strongly inhibit RPA2 S-phase foci formation than phosphorylation at the ATR-indirect sites Ser-4, Ser-8, Ser-11, Ser-12, and Ser-13. The effect of phosphorylation at serines 4, 8, 11, 12, and 13 is also significant, however, because these phospho-mimetic mutations do have a notable defect in foci formation and also further enhance the phenotype of the T21D/S33D mutant as demon-
strated in the S4D/S8D/S11D/S12D/S13D/T21D/S33D mutant. Collectively, these studies suggest that the inhibition of DNA replication by ATR-dependent phosphorylation of RPA2 is likely a result of the prevention of RPA association with replication centers. The relative minor effect of phosphorylation at serines 4, 8, 11, 12, and 13 on replication may be masked by the phosphorylation at Thr-21/Ser-33, and by other ATR downstream pathway(s), and thus the UVDS phenotype is not observed in the S4A/S8A/S11A/S12A/S13A mutant cell line.

Inhibition of RPA localization to replication centers by phospho-mimetic events suggests that damage-induced RPA2 phosphorylation by ATR may prevent RPA association with the rest of the cellular replication machinery. It has been described that RPA associates with DNA polymeraseα/primase, and RPA

FIGURE 7. Phospho-mimetic mutations at threonine 21 and serine 33 of RPA2 result in a significant decrease in the formation of S-phase-specific foci. A, U2OS cells retrovirally expressing HA-tagged RPA2 (top left), HA-RPA2-S4D/S8D/S11D/S12D/S13D (top right), HA-RPA2-T21D/S33D (bottom left), or HA-RPA2-S4D/S8D/S11D/S12D/S13D/T21D/S33D (bottom right) were fixed directly with 4% formaldehyde in PBS (nonextracted) or extracted with 0.5% Triton X-100 first and then fixed to visualize S-phase specific foci (extracted). Cells were immunostained with an anti-RPA1 antibody, an anti-HA antibody to specifically recognize endogenous RPA1 or HA-tagged wild-type RPA2 or mutant forms of RPA2. B, U2OS cells retrovirally expressing HA-RPA2 or one of the indicated HA-tagged RPA2 phospho-mimetic mutant alleles were lysed. The whole cell extracts were immunoblotted with an anti-HA antibody, an anti-RPA1 antibody, and an anti-Ku70 antibody. C, the frequency of chromatin-bound HA-RPA2 after extraction (showing S-phase-specific foci) was determined by counting the number of RPA1-positive staining cells with HA-RPA2 foci (left panel). Six hundred cells were counted for each cell line in three independent experiments. The average percentage of cells with HA-RPA2 foci is shown in the graph with error bars indicating the standard deviation from the average. The difference in the number of S-phase cells (cells with RPA1 foci) containing HA-RPA2 foci in the cell line expressing the HA-RPA2-S4D/S8D/S11D/S12D/S13D mutant compared with cells expressing the HA-RPA2-T21D/S33D mutant is statistically significant (p < 0.01; left graph). From these experiments, there was a noticeable difference in the number of cells containing bright staining HA-RPA2 foci, compared with cells containing faint staining HA-RPA2 foci among all of the HA-RPA2-positive staining cells (both bright and faint) was determined in U2OS cells expressing HA-tagged wild-type RPA2 or phospho-mimetic RPA2 mutants S4D/S8D/S11D/S12D/S13D, T21D/S33D, and all D) and was graphed in C (right panel). One hundred cells were counted for each cell line in three independent experiments, and the average percentage of cells with bright HA-RPA2 foci was graphed. Error bars indicate the standard deviation from the average. Examples of cells with faint staining chromatin-bound HA-RPA2 foci, as compared with bright staining HA-RPA2 foci, are indicated with arrows in the pictures at the top.
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His-RPA wild-type and the phospo-mimetic mutants (S4D/ S8D/S11D/S12D/S13D, T21D/S33D, or all Asp) were expressed and purified from *E. coli*. The interaction of His-RPA, or the His- RPA-phospho-mimetic mutants with primase p48/p58, was analyzed in *vitro*. As shown in Fig. 8B, similar amounts of p48 or p48/p58 interacted with His-RPA-wild type, and with each of the RPA2 phospho-mimetic mutants, including S4D/S8D/ S11D/S12D/S13D, T21D/S33D, and S4D/S8D/S11D/S12D/ S13D/T21D/S33D. These data suggest that at least in *vitro*, phospho-mimetic mutations in RPA2 at the ATR-dependent phosphorylation sites do not alter RPA association with primase. The failure of the phospho-mimetic mutants in forming S-phase specific foci is therefore likely not caused by their impaired interaction with polymerase a/primase.

DISCUSSION

Upon DNA damage, the S-phase checkpoint is activated to suppress DNA replication. Although the mechanism of ATM- mediated inhibition of replication following IR has been studied intensely, the downstream effectors of ATR in the S-phase checkpoint remains elusive. Our studies demonstrate that when replication forks encounter DNA damage, RPA is loaded onto chromatin, and RPA2 is then phosphorylated by ATR. Mutating ATR-dependent phosphorylation sites on RPA2 prevents the inhibition of DNA synthesis following UV-induced DNA damage, suggesting that ATR-mediated RPA2 phosphorylation is essential for the suppression of DNA replication following UV light.

RPA2 phosphorylation on chromatin is mediated predominantly by ATR after UV or HU treatment—RPA2 phosphorylation can be triggered by many different kinds of DNA damage-inducing agents. However, significant RPA2 phosphorylation seems associated specifically with replication fork arrest and chromatin-associated RPA2. Treatment with agents such as HU, UV light, as well as camptothecin, which arrest replication forks, induce dramatic phosphorylation of RPA2 (see Refs. 26, 28, and this study), whereas only minor RPA2 phosphorylation is detectable when cells are treated with IR at a dose of 20 Gy (Fig. 1A and data not shown). In fact, RPA2 phosphorylation is apparent only after cells are treated with extremely high doses (50–100 Gy) of IR (27, 32). When ATR is silenced, or when the kinase activity of ATR is compromised, RPA2 phosphorylation on the chromatin is impaired, but RPA chromatin loading remains unchanged, suggesting that RPA chromatin loading occurs prior to RPA2 phosphorylation. An accumulation of ssDNA in S-phase is likely one of the major signals to trigger RPA2 phosphorylation by promoting RPA chromatin binding. HU, UV light, and camptothecin arrest replication forks in S-phase and generate a significant amount of ssDNA, which promotes RPA chromatin loading. In contrast, IR primarily generates double strand breaks, and it may require a high dose to produce enough ssDNA for a significant amount of RPA to be loaded onto chromatin. Alternatively, high doses of IR may activate different mechanisms such as the ATM- and/or DNA-PK-mediated damage response pathways to phosphorylate RPA2.

Multiple checkpoint kinases have been described to be involved in RPA2 phosphorylation, likely depending on the
activation of specific damage-induced signal transduction pathways. In budding yeast, both Mec1 (ATR homolog) and Tel1 (ATM homolog) are important for RPA phosphorylation upon DNA damage (57, 68). RPA2 phosphorylation induced by a high dose of IR depends on ATM and DNA-PK in mammalian cells (27, 32). The requirement for a high dose of IR to phosphorylate RPA2 suggests that both ATM and DNA-PK may not be the primary pathways required for this phosphorylation. Our studies show that UV- and HU-induced RPA2 phosphorylation on chromatin is mainly mediated by ATR. Inhibition of ATR activity by shRNA or overexpression of ATR-KD significantly reduced RPA2 phosphorylation following UV and HU treatment, whereas RPA2 phosphorylation was not changed when DNA-PK was inhibited by the expression of two shRNAs, and only a very minor decrease in chromatin-bound RPA2 phosphorylation was detected when ATM was silenced. Previously, it was described that RPA2 phosphorylation after UV light requires ATM (48), which is different from our conclusion. This is likely because we analyzed RPA2 phosphorylation on the chromatin in our study, where the majority of RPA2 is rapidly phosphorylated after UV light (within 1 h after treatment). Other studies have used cell lysates or nuclear extracts to analyze RPA2 phosphorylation after DNA damage. Although RPA2 phosphorylation occurs predominantly on chromatin after UV and HU treatment, some soluble RPA2 might also be phosphorylated, and ATM may contribute more significantly to the phosphorylation of soluble RPA2. We find that on chromatin, ATM seems to contribute only slightly to RPA2 phosphorylation after UV light, and its contribution is most obvious at later times after the induction of DNA damage. Therefore, ATM likely participates in maintaining RPA2 phosphorylation, but it does not significantly contribute to the rapid phosphorylation of chromatin-bound RPA2 after UV treatment. From our observations, we conclude that the main pathway to phosphorylate chromatin-bound RPA2 after UV or HU treatment is mediated by ATR.

**ATR Directly or Indirectly Phosphorylates RPA2 at Its Amino Terminus**—Several sites at the amino terminus of RPA2 are phosphorylated upon DNA damage. We have shown that loss of phosphorylation at serines 4, 8, 11, 12, and 13 results in the loss of the appearance of the slowest migrating form of RPA2 following UV light or HU, whereas loss of phosphorylation at Thr-21/Ser-33 results in a slight change of the overall banding pattern. This suggests that phosphorylation at different sites of RPA2 causes different conformational changes in the protein that are responsible for the changes in its migration. We also showed that phosphorylation of Thr-21/Ser-33, as well as Ser-4/8, and potentially Ser-11, Ser-12, and Ser-13, is dependent on ATR following UV light and HU. However, in vitro kinase assays indicate that ATR can directly phosphorylate Thr-21 and Ser-33 in vitro, but Ser-4, Ser-8, Ser-11, Ser-12, and Ser-13 are not direct substrates of ATR. By mutating either Thr-21/Ser-33 or Ser-4, Ser-8, Ser-11, Ser-12, and Ser-13, we demonstrated that phosphorylation of serine 33 and that of serines 4/8 are independent events. Therefore, the dependence of phosphorylation of serines 4/8 of RPA2 on ATR is not because of a requirement for Thr-21/Ser-33 phosphorylation by ATR. Currently, the kinases that phosphorylate Ser-4, Ser-8, Ser-11, Ser-12, or Ser-13 are not clear. The Ser-4, Ser-8, Ser-11, Ser-12, and Ser-13 sites are not Chk1 consensus sites, and both in vivo and in vitro studies ruled out the possibility that Chk1 is the downstream kinase to phosphorylate these sites when ATR is activated. Although the identity of the kinases that phosphorylate Ser-4 and Ser-8 is not clear, these kinase(s) are activated through the ATR pathway upon DNA damage. Identifying these kinases will be of great interest in the future to clarify the damage signaling pathways downstream of ATR, and to help contribute to the understanding of how ATR and its downstream kinases act together to regulate RPA function upon DNA damage.

**Loss of ATR-mediated Phosphorylation of RPA2 Leads to UVDS Phenotype**—This study shows that mutation of RPA2 at ATR-dependent phosphorylation sites compromises the inhibition of DNA replication following UV treatment and identifies one pathway by which ATR acts to inhibit replication when DNA is damaged. The defect in the inhibition of DNA synthesis that we observed in the RPA2 phospho-mutants is less dramatic than what we observed in the ATR-deficient cells. This is consistent with the idea that ATR may target more than one replication protein at replication forks to mediate the suppression of DNA replication when DNA is damaged. It has been described that MCM proteins are substrates of ATR in response to UV light and HU (58), and MCM phosphorylation may also be involved in ATR-mediated replication inhibition.

The UVDS phenotype of the RPA2 phospho-mutants is not attributable to the impairment of checkpoint activation following UV treatment. It was described that hyperphosphorylation of RPA2 stimulated the interaction between RPA and Rad9 (59), suggesting that phosphorylation of RPA2 by ATR may be involved in amplifying the checkpoint signal. Our studies show that cells expressing the RPA2 phospho-mutant exhibit similar levels of Chk1 phosphorylation as cells expressing wild-type RPA2 (when endogenous RPA2 is silenced), suggesting that checkpoint activation occurs normally even when RPA2 cannot be phosphorylated by ATR. RPA2 phosphorylation may stabilize the interaction of RPA with Rad9, but it is not required for assisting in checkpoint activation. Therefore, the UVDS phenotype of the RPA2 phosphorylation mutants is caused by a defect downstream of checkpoint activation and is likely because of a direct suppression of the replication function of RPA.

**ATR-mediated RPA2 Phosphorylation May Inhibit RPA to Localize into Replication Centers**—What is the mechanism by which ATR-mediated RPA2 phosphorylation inhibits DNA replication? It has been described previously that mimicking phosphorylation by replacing serine and/or threonine residues with aspartate residues at multiple cyclin-dependent and damage-inducible phosphorylation sites in the amino-terminal domain of RPA2 specifically prevents RPA from associating with replication centers (40). This suggests that phosphorylation of RPA2 could prevent RPA from associating with the replication machinery. We specifically examined RPA2 phospho-mimetic mutants containing aspartate residues at the ATR-dependent phosphorylation sites in RPA2. The mutant containing aspartate substitutions at all of the damage-inducible phosphorylation sites (S4D/S8D/S11D/S12D/S13D/T21D/S33D) of RPA2 prevented RPA2 from forming S-phase-specific
foci. The T21D/S33D mutant also dramatically reduced the percentage of cells with normal bright HA-RPA2 foci, whereas the S4D/S8D/S11D/S12D/S13D mutant had a more minor defect in forming normal S-phase foci. This is consistent with the observation that the RPA2 phospho-mutant containing serine to alanine mutations at all of the ATR-dependent phosphorylation sites (Ser-44/Ser-8/Ser-11/Ser-13/Thr-21/Ser-33), and the mutant with alanine substitutions at the Thr-21/Ser-33 sites exhibit a defect in the S-phase checkpoint following UV light, whereas the S4A/S8A/S11A/S12A/S13A mutant does not. Although we do not detect a defect in the inhibition of DNA synthesis after UV light in cells expressing the S4A/S8A/S11A/S12A/S13A mutant, the phospho-mimetic mutant with mutations at these sites does display a minor defect in migrating to S-phase foci. Furthermore, the S4D/S8D/S11D/S12D/S13D mutations enhanced the defect observed in the formation of S-phase foci when combined with phospho-mimetic mutations at the Thr-21/Ser-33 sites. This suggests that phosphorylation of Ser-4, Ser-8, Ser-11, Ser-12, and Ser-13 also has some impact on the regulation of the replication function of RPA, although this mutant does not show an obvious UVDS phenotype. This is likely caused by the minor contribution of phosphorylation at serines 4, 8, 11, 12, and 13 to the prevention of RPA S-phase foci formation, and the redundancy of Thr-21/Ser-33 phosphorylation with other ATR-dependent downstream pathway(s) in the regulation of the S-phase checkpoint.

The lack of association of RPA2 phospho-mimetic mutants with replication centers could be due to a loss of an interaction between RPA and other components of the replication machinery. It has been described that mitotic-dependent RPA2 phosphorylation impairs the interaction between RPA and DNA polymerase/primase binding (66). However, our studies showed that the RPA complex, containing either wild-type RPA2 or RPA2 with phospho-mimetic mutations at ATR-dependent phosphorylation sites, interacts with primase. This suggests that the association of RPA with primase may not be influenced by ATR-dependent phosphorylation of RPA2. It also implies that the regulation of RPA replication function in the cell cycle and upon DNA damage is not identical. It still remains possible that the interaction between RPA and other components of the replication machinery may be affected by ATR-dependent RPA2 phosphorylation. RPA interacts with multiple replication proteins in addition to primase, such as replication factor C, and DNA polymerases α and δ (13, 14). An alternative possibility is that the ATR-dependent RPA2 phosphorylation may prevent RPA loading during DNA replication. It has been proposed that RPA is actively loaded onto ssDNA during DNA replication by the replication machinery (40). This is supported by the recent findings that SV40T unwinds DNA and simultaneously assists in RPA loading through its direct interaction with RPA.3 In cellular replication, we can envision that the MCM helicase complex and other associated proteins may play similar roles in assisting RPA loading. Phosphorylation of RPA2 at ATR-dependent sites may cause a conformational change in the RPA complex resulting in an alteration of chromatin loading by the replication machinery.

Collectively, our studies suggest that RPA2 is phosphorylated on chromatin by ATR when replication forks are arrested. RPA2 hyperphosphorylation may reduce the access of RPA to sites of replication initiation, and/or to established forks, thereby inhibiting replication. Although RPA2 phosphorylation is an important event downstream of ATR to mediate replication inhibition following DNA damage, it remains to be investigated whether RPA2 phosphorylation plays other roles in maintaining genome stability. Phosphorylation of RPA2 may also contribute to stimulating DNA repair and maintaining fork stability. Through ATR-mediated RPA2 phosphorylation, either direct or indirect, different functions of RPA may be coordinated so that DNA replication and repair can be coupled with DNA damage.

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