Cyclin-Dependent Kinase 2–Dependent Phosphorylation of ATRIP Regulates the G2–M Checkpoint Response to DNA Damage

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Abstract
The ATR-ATRIP kinase complex regulates cellular responses to DNA damage and replication stress. Mass spectrometry was used to identify phosphorylation sites on ATR and ATRIP to understand how the kinase complex is regulated by post-translational modifications. Two novel phosphorylation sites on ATRIP were identified, S224 and S239. Phosphopeptide-specific antibodies to S224 indicate that it is phosphorylated in a cell cycle–dependent manner. S224 matches a consensus site for cyclin-dependent kinase (CDK) phosphorylation and is phosphorylated by CDK2-cyclin A in vitro. S224 phosphorylation in cells is sensitive to CDK2 inhibitors. Mutation of S224 to alanine causes a defect in the ATR-ATRIP–dependent maintenance of the G2-M checkpoint to ionizing and UV radiation. Thus, ATRIP is a CDK2 substrate, and CDK2-dependent phosphorylation of S224 regulates the ability of ATR-ATRIP to promote cell cycle arrest in response to DNA damage. [Cancer Res 2007;67(14):6685–90]

Introduction
The ATM (ataxia-telangiectasia mutated) and ATR (ATM and Rad3-related) kinases function at the apex of cell cycle checkpoint signaling pathways (1, 2). ATM and ATR share many biochemical and functional similarities. Both are large protein kinases with significant sequence homology and a strong preference to phosphorylate serine or threonine residues followed by glutamine. Both target an overlapping set of substrates that promote cell cycle checkpoints. However, ATR is essential for the viability of replicating human and mouse cells, whereas ATM is not (3–5). ATM functions primarily in response to double strand breaks. ATR is activated by double strand breaks but also during every S-phase to regulate the spatial and temporal firing of replication origins, the repair of damaged replication forks and to prevent the premature onset of mitosis (6, 7). ATM mutations, found in ~0.5% to 1% of the population, predispose carriers to developing cancer (8–11). People with mutations in both ATM alleles develop the neurodegenerative disease ataxia-telangiectasia. ATR mutations are rare and probably only compatible with viability when heterozygous or hypomorphic. One form of Seckel syndrome, a disease characterized by growth retardation, microcephaly, and mental retardation is caused by mutations in ATR (12).

ATR activation occurs primarily in S-phase due to replication stress induced by DNA-damaging agents or replication inhibitors. More specifically, ATR activation is stimulated when the replication machinery encounters a DNA lesion and becomes uncoupled (the helicase continues to unwind DNA, whereas the polymerase becomes stalled at the site of DNA damage; ref. 13).

One critical factor that promotes ATR activation is the accumulation of replication protein A (RPA)–coated ssDNA (7, 14, 15). At least two separate checkpoint complexes accumulate in distinct foci that colocalize with RPA. Rad17, a proliferating cell nuclear antigen–like clamp loader protein, is recruited to RPA-ssDNA and helps load the Rad9-Rad1-Hus1 checkpoint clamp at the junction of dsDNA and ssDNA (16–18). Independently, ATR is recruited by ATRIP, which binds the RPA-ssDNA that accumulates at DNA lesions (19–21). ATRIP-dependent localization of ATR to sites of DNA damage is not sufficient to activate the kinase. In vertebrates, the TopBP1 protein functions as an ATR-ATRIP activator (22). TopBP1 is an eight BRCT repeat protein that functions in both DNA replication and checkpoint activation (23).

ATRIP has at least three functional domains. An NH2-terminal domain of ATRIP is necessary for its stable association with RPA-ssDNA and promotes ATR-ATRIP localization to damage-induced nuclear foci (21, 24). A coiled-coil domain between amino acids 108 to 217 mediates ATRIP dimerization and is critical for ATR signaling (25, 26). The COOH terminus of ATRIP contains the ATR-interaction domain, and ATRIP binding to ATR is critical for the stability of both proteins (19, 21).

One of the major functions of ATR signaling is to regulate cell cycle progression. This is done in part by regulating the activity of cyclin-dependent kinases (CDKs). Accumulating evidence indicates that the cell cycle and CDKs also regulate ATR. First, ATR is activated primarily during S-phase (27–29). Second, CDK activity is important to generate ssDNA by DNA end resection at double strand breaks (30, 31). The resection of the end to yield ssDNA promotes ATR activation (31–33). Third, CDKs phosphorylate the COOH terminus of Rad9, and this phosphorylation is important for checkpoint signaling (34). Fourth, inhibition of CDK activity can cause a loss of Chk1 expression in some cell types (35). Thus, CDK function may be both a target and regulator of ATR-dependent signaling.

We now report evidence that CDK2 directly phosphorylates the ATR-ATRIP complex. Using phosphopeptide-specific antibodies and mutational analysis, we have determined that CDK2-dependent ATRIP S224 phosphorylation is critical for proper checkpoint control in response to DNA damage. Thus, in addition to being a target for ATR-dependent checkpoint responses, CDK2 is also a direct regulator of the ATR-ATRIP checkpoint kinase complex.

Materials and Methods
Cell culture. HeLa and U2OS cells were grown in DMEM supplemented with 7.5% fetal bovine serum (FBS). RPE-KTERT cells were grown in DMEM/F12 supplemented with 7.5% FBS. Plasmid transfections were done with LipofectAMINE 2000 (Invitrogen). The small interfering RNA (siRNA)–resistant HA-ATRIP– and HA-ATRIP S224A–expressing U2OS cells were generated by retroviral infection and selection essentially as described...
The ATRIP siRNA and transfection methods were done with OligofectAMINE (Invitrogen) as described previously (21). HeLa cell synchronization was done with a double-thymidine block. RPE-hTERT cells were synchronized by growing cells at 100% confluence for 24 h. Trypsinization and plating at subconfluent densities released the cells into the cell cycle. Approximately 95% of cells were arrested with 2n DNA content in this procedure, and by 20 h after release, most of the cells have entered S-phase (36).

Antibodies and kinase inhibitors. The phosphorylated ATRIP S224 antibody was produced by Bethyl Laboratories. ATRIP-403 and ATRIP-N antibodies were described previously (3). Cyclin A and ATR antibodies were purchased from Santa Cruz Biotechnology. HA.11 antibody was purchased from Covance. All kinase inhibitors were purchased from Calbiochem.

Kinase assays. CDK2-cyclin A was purchased from New England Biolabs. About 10 units of kinase were used per reaction. Kinase assays were done in 30-μL reactions with ~0.2 μg of His-MBP-ATRIP substrate, 10 μM cold ATP, and 10 μCi of γ-32P-ATP (3000 Ci/mmol). His-MBP–tagged ATRIP substrate was purified from BL-21 codon plus cells using Ni chromatography with His-Select beads according to the manufacturer's (Sigma) instructions. Alternatively, HA-ATRIP-Flag-ATR complexes were purified from transiently transfected HEK293T cells using HA-agarose beads. HA-agarose beads were added to cell lysates created with TGN buffer [Tris (pH 8.0), 150 mM NaCl, 1.0% Tween 20, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 μg/ml aprotinin, 5 μg/ml leupeptin, 75 mM NaF, 20 mM/L β-glycerophosphate, 0.4 mM/L sodium vanadate, and 1 mM/L DTT]. After incubation for several hours, the beads were washed with TGN buffer and TGN buffer containing 500 mM/L LiCl. About 28 units of CDK2-cyclin A complexes were added directly to the HA-ATRIP-Flag-ATR complexes on the beads to perform the kinase reactions.

CDK2-associated kinase activity was assayed in an immune complex kinase assay. Cells were lysed in 50 mM/L Tris (pH 8.0), 200 mM/L NaCl, 0.5% Igepal, 1 mM/L PMSF, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 75 mM/L NaF, 20 mM/L β-glycerophosphate, 0.4 mM/L sodium vanadate, and 1 mM/L DTT. Lysates were cleared by centrifugation and normalized for protein concentration. Immunoprecipitates for CDK2 were

![Figure 1. Identification of phosphorylation sites on ATRIP. A and B, ATRIP peptides were analyzed by LC-MS-MS using a Thermo LTQ linear ion trap mass spectrometer. MS/MS spectra of peptides containing phosphorylated S224 and S239 are shown with the corresponding b and y ions that allowed identification of the peptide with a mass shift of 80 Da corresponding to phosphorylation at the site indicated. C, sequence alignment of the ATRIP region containing S224 and S239.](image-url)
done with the M-20 CDK2 antibody (Santa Cruz Biotechnology) and protein-A-agarose. Immunoprecipitates were washed thrice in lysis buffer and twice in kinase buffer (50 mmol/L Tris, 10 mmol/L MgCl₂, 1 mmol/L DTT). Kinase buffer supplemented with GST-Rb (792–928) substrate, 20 μmol/L ATP, and 10 μCi of γ³²P-ATP (3,000 Ci/mmol) was added to the immunoprecipitates to start the reaction. The kinase reaction was stopped by the addition of SDS-sample buffer. After boiling, the samples were separated by SDS-PAGE; the gel was dried and exposed to X-ray film.

Mass spectrometry. Following affinity purification of ATR-ATRIP complexes and separation by SDS-PAGE, the ATRIP protein was digested in the gel with trypsin. The resulting peptides were extracted and analyzed by capillary liquid chromatography/electrospray ionization/mass spectrometry/mass spectrometry (LC-MS-MS) using a Thermo LTQ linear ion trap mass spectrometer. The data were analyzed with the SEQUEST (v. 27, rev. 12) and Pmod algorithms as previously described (37).

G₂-M checkpoint assay. The G₂-M checkpoint assay was done essentially as described (3) with the following modifications. UV treatment was conducted with a Stratalinker (Stratagene). IR treatment was done with a 137Cs irradiator with a dose rate of 1.4 Gy/min. Nocodazole was added immediately after irradiation to capture cells that entered mitosis. In some experiments, mitotic spreads were counted by microscopy following fixation with Carnoy’s fixative. Alternatively, mitotic cells were counted by flow cytometry following fixation in ethanol, permeabilization, and staining with phosphohistone H3 S10 antibody and propidium iodide.

Results
Identification of ATRIP post-translational modifications. To identify post-translational modifications on the ATR-ATRIP complex, the complex was purified from HeLa cells using affinity chromatography with an anti-ATRIP antibody. After separating the complex by SDS-PAGE, the ATR and ATRIP proteins were subjected to in gel digestion with trypsin and analyzed by reverse-phase liquid chromatography–coupled mass spectrometry. This method identified phosphorylation on ATRIP S224 and S239 (Fig. 1A and B). Several spectra were identified matching the S224 phosphopeptide including spectra that corresponded to neutral loss of the phosphate. In these cases, MS/MS/MS spectra were obtained to confirm the identity of the peptide. Both S224 and S239 are proline-directed phosphorylation sites that are conserved in many vertebrate species including Xenopus laevis (Fig. 1C). S224 in particular matches the consensus site (S/TPxK/R) for CDKs.

Figure 2. Characterization of an ATRIP phospho-Ser²²⁴ (P-S224)–specific antibody. HA immunoprecipitates from cell lysates containing HA-ATRIP, HA-ATRIP S224E, HA-ATRIP S224A or an empty vector control were separated by SDS-PAGE. Where indicated, the HA-ATRIP immunoprecipitate was treated with λ-phosphatase before separation. Blots were probed with the anti–P-S224 ATRIP or HA antibodies.

Figure 3. Regulation of ATRIP S224 phosphorylation by CDK2. A, cells were treated with 5 Gy of ionizing radiation, 50 J/m² UV light, or 1.5 μmol/L of aphidicolin. Cells were harvested 1, 2, or 14 h after treatment, respectively. Cell lysates were separated by SDS-PAGE and blotted with ATRIP-P-S224 or antibodies to the N-terminus of ATRIP. B, HeLa cells were synchronized by double-thymidine block and released. Every 2 h after release, cells were harvested for analysis. Immunoblotting was done with ATRIP P-S224, ATRIP, cyclin A, and ATR antibodies. In addition, CDK2 was immunoprecipitated, and its kinase activity toward an Rb substrate was measured using an in vitro kinase assay (³²P-Rb). Asynch, asynchronous cells. C, RPE-1/HTERT cells were arrested in G₀ by contact inhibition for 48 h (0 h time point) and then released into S phase (20 h time point). Cell lysates were harvested and immunoblotted with antibodies to ATRIP or ATRIP P-S224. D, HeLa cells were treated with the following inhibitors for 14 h: roscovitine (25 and 50 μmol/L), U0126 (25 μmol/L), 10 μmol/L rapamycin (Rap), 20 μmol/L wortmannin (Wort), 10 μmol/L 5,6-dichlorobenzimidazole riboside (DRB), 10 μmol/L H-7 dihydrochloride (H-7). Cells lysates were separated by SDS-PAGE and immunoblotted with ATRIP P-S224 or ATRIP antibodies.
Regulation of S224 phosphorylation. A phosphopeptide-specific antibody to S224 was generated by immunizing rabbits with the S224 phosphopeptide followed by affinity purification. The antibody recognizes wild-type ATRIP (Fig. 2). However, it does not recognize ATRIP that has been dephosphorylated with E-phosphatase or ATRIP containing a mutation at the S224 site (Fig. 2). These data confirm that ATRIP is phosphorylated on S224 in cells.

We next examined if S224 phosphorylation is regulated in response to DNA damage. Cells were treated with ionizing radiation (IR), UV radiation, or aphidicolin. Immunoblotting with the ATRIP P-S224 antibody indicates that none of these treatments significantly alters the phosphorylation status of S224 (Fig. 3A). Because S224 matches a consensus site for CDK phosphorylation, we examined whether its phosphorylation is regulated through the cell cycle. Cells were synchronized by double-thymidine block, released, and harvested every 2 h. Flow-cytometric analysis of DNA content indicated that the cells completed S-phase by ~8 h after the release (data not shown). We also monitored cell cycle progression by blotting with cyclin A and performing CDK2 immunoprecipitation-kinase assays using Rb as a substrate. These analyses confirmed that the synchronized cells contained high CDK activity levels until 8 h after release (Fig. 3B). ATRIP S224 phosphorylation is high at the double-thymidine block (early S-phase, time = 0), peaked around 4 to 6 h after release corresponding to the peak of CDK2 activity, and decreased at the 10-h time point as cyclin A and CDK2 activity also decreased. In a second synchronization strategy, we found that S224 is not phosphorylated in RPE-hTERT cells arrested in G0 by

**Figure 4.** CDK2-cyclin A phosphorylates ATRIP S224. A, recombinant His-MBP-ATRIP or His-MBP-ATRIP S224A were purified from bacterial cells and added to an in vitro kinase assay with recombinant CDK2-cyclin A. Following incubation, the kinase reaction was separated on SDS-PAGE and stained. The autoradiogram showing the amount of 32P incorporated into the ATRIP proteins and a Coomassie blue stain of the gel demonstrating that equal amounts of substrate were added to each reaction is shown. A reaction in which no substrate was added is included as a control (−). B, wild-type or mutant HA-ATRIP-Flag-ATR complexes were purified from transiently transfected HEK293T cells with HA-agarose beads. Kinase reactions were done with recombinant CDK2-cyclin A complexes. Reactions were separated by SDS-PAGE. Equivalent gels were stained with Coomassie blue and exposed to film or immunoblotted with antibodies to ATRIP and ATR to visualize the amount of the ATR-ATRIP complex in each reaction.

**Figure 5.** ATRIP S224 is required for ATR-ATRIP-dependent G2-M checkpoint responses to DNA damage. A, U2OS cell expressing siRNA-resistant cDNAs for HA-ATRIP (WT), HA-ATRIP S224A, or no cDNA (Vec) were transfected with nonspecific (NS) or ATRIP (A4) siRNA. Cells were harvested 3 d after transfection and analyzed for ATRIP expression by immunoblotting. B and C, 3 d after transfection with ATRIP siRNA, U2OS cells containing wild-type ATRIP, ATRIP S224A, or vector were treated with 25 J/m² UV radiation (B) or 4 Gy of IR (C). Nocodazole was added to the culture media to trap cells in mitosis. In (B), cells were harvested and fixed with Carnoy’s fixative, and mitotic spreads were analyzed 8 h after exposure to UV. The percentage of mitotic cells was calculated based on counting at least 600 cells. Bars, SE. In (C), cells were harvested at the indicated time points following IR, fixed with ethanol, permeabilized, and stained with anti–phospho-histone H3 S10 antibody and propidium iodide. The percentage of mitotic cells was determined by flow cytometry.
contact inhibition, but becomes phosphorylated as cells are stimulated to re-enter the cell cycle (Fig. 3C). Thus, ATRIP S224 phosphorylation correlates with CDK2 activity in cells.

To examine whether CDK2 is responsible for ATRIP S224 phosphorylation, we used the CDK2 inhibitor roscovitine. We found a marked sensitivity of S224 phosphorylation to roscovitine treatment (Fig. 3D). Roscovitine is relatively specific to CDK2, although at higher concentrations, it also inhibits extracellular signal-regulated kinase (ERK) kinases. To rule out ERK and other kinases, we used a panel of kinase inhibitors, including the mitogen-activated protein/ERK kinase inhibitor U0126. S224 phosphorylation is not sensitive to any other kinase inhibitor tested (Fig. 3D). In addition, we also confirmed that S224 phosphorylation does not correlate with ERK activation after serum stimulation of cells (data not shown). These data suggest that ATRIP S224 is a CDK2 phosphorylation site in cells.

Finally, we examined whether the cyclin A-CDK2 kinase could directly phosphorylate recombinant ATRIP. To perform this assay, full-length His-ATRIP or His-ATRIP S224A were purified from bacteria. Wild-type ATRIP is a substrate for cyclin A-CDK2 in vitro (Fig. 4A). ATRIP S224A is also phosphorylated by CDK2 but to a much lower extent, indicating that S224 is the major in vitro CDK phosphorylation site on ATRIP. We also examined whether cyclin A-CDK2 could phosphorylate ATRIP when it is complexed with ATR. HA-ATRIP and Flag-ATR were coexpressed in HEK293T cells and purified using HA-immunoaffinity chromatography. The ATRIP-ATR complexes bound to the HA-agarose beads were used directly in the cyclin A-CDK2 kinase reaction. Mg was used as the divalent cation in these reactions. ATR has negligible kinase activity in vitro under these conditions, so no ATRIP phosphorylation is visible when cyclin A-CDK2 is omitted from the reaction (Fig. 4B, lane 3). Wild-type ATRIP is efficiently phosphorylated by CDK2 under these conditions. However, the ATRIP S224A mutant is a poor substrate (Fig. 4B). Mutation of S239 to alanine only slightly reduced phosphorylation of ATRIP. Thus, the major site of cyclin A-CDK2 phosphorylation on ATRIP is S224.

**S224 is important for G2-M checkpoint responses to DNA damage.** To examine the functional significance of S224 phosphorylation, we generated cell lines that express HA-ATRIP or HA-ATRIP S224A by retroviral infection. The ATRIP cDNAs also contain wobble base pair mutations, making them insensitive to RNAi depletion using a specific ATRIP siRNA (21). Transfection of the ATRIP siRNA silences endogenous ATRIP expression in these cells by at least 90% without altering the expression of exogenous HA-ATRIP (Fig. 5A). We found no defects in the localization of ATRIP S224A to damage-induced foci in these cells, and the protein bound to ATR as efficiently as wild-type ATRIP (data not shown). However, we did observe a significant functional defect in cells expressing ATRIP S224A. In particular, whereas cells expressing wild-type ATRIP efficiently arrest at the G2-M checkpoint following UV or ionizing radiation, cells expressing ATRIP S224A had significant defects in maintaining this checkpoint response (Fig. 5B and C).

Following UV treatment, the ATRIP S224A expressing cells are nearly as defective as cells lacking ATRIP (Fig. 5B). In response to IR, the ATRIP S224A expressing cells are intermediate in the G2-M checkpoint response in comparison to cells expressing no ATRIP or wild-type ATRIP (Fig. 5C). In this assay, cells are placed in nocodazole media immediately after irradiation to trap cells escaping the G2 checkpoint in mitosis. All three cell lines have indistinguishable growth curves, cell cycle profiles, and percentages of mitotic cells (~1.7%) before irradiation (data not shown). When treated with nocodazole for 12 h in the absence of DNA damage, all three cell lines contain ~45% mitotic cells. Following irradiation, there are no significant differences between any of the cell types at early time points; however, by 8 h after IR, 11.6% of ATRIP-depleted cells have entered mitosis. At this time point, complementation with wild-type ATRIP reduces the mitotic percentage to 5.0%, whereas complementation with the ATRIP S224A mutant only reduces it to 9.5%. By 16 h after irradiation, 34% of ATRIP-depleted cells have entered mitosis compared with 13% of wild-type ATRIP and 24% of ATRIP S224A–expressing cells. Thus, phosphorylation of ATRIP S224 is important for the ATR-ATRIP–dependent maintenance of the G2 checkpoint arrest in response to DNA damage.

**Discussion**

Cyclin-dependent kinases are major targets of the ATR/ATM-dependent checkpoint responses to DNA damage. Inhibition of CDK activity promotes cell cycle arrest and provides time for DNA repair. However, it is becoming increasingly clear that the cell cycle and CDK activity actually regulate many aspects of the DNA damage response (27–31, 33–35). We have now defined a requirement for CDK-dependent phosphorylation of ATRIP to promote the G2-M checkpoint response. Thus, there is a regulatory loop between CDK and ATR-ATRIP.

ATRIP S224 phosphorylation correlates with cyclin A levels and CDK2 activity, indicating that it is regulated in a cell-cycle–dependent manner. However, some S224 phosphorylation is detectable even in G1-phase cells. S224 phosphorylation decays slowly after roscovitine inhibition of CDK2 activity, indicating that S224 dephosphorylation is relatively slow. Furthermore, S224 phosphorylation is not altered when cells are treated with DNA-damaging agents. These observations suggest that CDK-dependent phosphorylation of ATRIP is probably not used as rapid means of turning on or off the ATR-ATRIP kinase. Instead, S224 phosphorylation may be important to potentiate specific ATR activities.

The mechanism by which ATRIP S224 phosphorylation regulates ATR-ATRIP checkpoint responses is unclear. S224 phosphorylation is not required for ATRIP binding to RPA, localization to sites of DNA damage, or binding to ATR (data not shown). In addition, ATR-ATRIP complexes containing the S224A ATRIP mutant have similar kinase activities in vitro as wild-type complexes (data not shown). One possibility is S224 phosphorylation creates a binding site for another protein involved in the G2-M checkpoint response.

S224 is likely not the only CDK site on the ATR-ATRIP complex. Indeed, we found that mutation of S224 to alanine did not completely abolish CDK2 phosphorylation of recombinant ATRIP in vitro. The identity of other sites is unclear; S239 is a candidate, although it does not seem to be a major site in vitro for cyclin A-CDK2. We did observe S239 phosphorylation in our mass spectrometry analysis, suggesting that it is phosphorylated in cells. Unfortunately, we have yet to successfully generate a phosphopeptide-specific antibody to this site, so we are unable to determine whether it is a CDK phosphorylation site in cells.

In summary, these results indicate that CDK-dependent phosphorylation of the ATR-ATRIP complex regulates its ability to promote the G2-M checkpoint in response to DNA damage. This regulatory mechanism may provide a means of potentiating the ATR response to DNA damage during S and G2 phases when ATR
function is essential. Because both CDK and ATR activity are required to regulate S-phase in the absence of DNA damage, it will be interesting to determine whether direct phosphorylation of ATRIP by CDK2 influences replication control mechanisms.

Acknowledgments


References

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