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***Mol. Cell. Biol.* 2005, 25(22):9910. DOI: 10.1128/MCB.25.22.9910-9919.2005.**

MOLECULAR AND CELLULAR BIOLOGY, Nov. 2005, p. 9910–9919 Vol. 25, No. 22
0270-7306/05/$08.00+0 doi:10.1128/MCB.25.22.9910–9919.2005

Protein Phosphatase 5 Is Required for ATR-Mediated Checkpoint Activation

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Received 30 December 2004/Returned for modification 24 January 2005/Accepted 8 August 2005

In response to DNA damage or replication stress, the protein kinase ATR is activated and subsequently transduces genotoxic signals to cell cycle control and DNA repair machinery through phosphorylation of a number of downstream substrates. Very little is known about the molecular mechanism by which ATR is activated in response to genotoxic insults. In this report, we demonstrate that protein phosphatase 5 (PP5) is required for the ATR-mediated checkpoint activation. PP5 forms a complex with ATR in a genotoxic stress-inducible manner. Interference with the expression or the activity of PP5 leads to impairment of the ATR-mediated phosphorylation of hRad17 and Chk1 after UV or hydroxyurea treatment. Similar results are obtained in ATM-deficient cells, suggesting that the observed defect in checkpoint signaling is the consequence of impaired functional interaction between ATR and PP5. In cells exposed to UV irradiation, PP5 is required to elicit an appropriate S-phase checkpoint response. In addition, loss of PP5 leads to premature mitosis after hydroxyurea treatment. Interestingly, reduced PP5 activity exerts differential effects on the formation of intranuclear foci by ATR and replication protein A, implicating a functional role for PP5 in a specific stage of the checkpoint signaling pathway. Taken together, our results suggest that PP5 plays a critical role in the ATR-mediated checkpoint activation.

Cellular responses to DNA damage are tightly controlled by a group of checkpoint proteins to ensure genomic integrity and stability (1, 46). Two closely related protein kinases, ATM (*a*taxia *t*elangiectasia *m*utated) and ATR (*AT*M and *R*ad3 related), transduce the checkpoint signals initiated by various forms of genotoxic stress, subsequently phosphorylating their substrates, leading to specific cellular responses, including cell cycle arrest, apoptosis, and DNA repair (1, 46). The functions of these two protein kinases are overlapping but not redundant in mammalian cells. ATM is mainly activated in the presence of DNA double-stranded breaks, whereas ATR responds to a wide variety of DNA damage and replication stress, such as UV irradiation or hydroxyurea (HU)-induced replication block (1, 30). Unlike ATM, deletion of the *ATR* gene in mice leads to embryonic lethality with chromosomal fragmentation in cultured blastocyst cells, suggesting an indispensable role for ATR during the normal cell cycle, possibly through monitoring DNA replication (12). ATR has been shown to phosphorylate a number of proteins, including Chk1, hRad17, Brca1, p53, and H2AX (5, 21, 27, 32, 33, 38, 45). Despite the identification of many substrates of ATR, how ATR becomes activated during a checkpoint response remains poorly understood.

ATR exists as a complex with a regulatory protein ATRIP (*ATR-i*nteracting *p*rotein) (16). Recent studies have shown that through the interaction with ATRIP, replication protein A (RPA)-coated single-stranded DNA (ssDNA) recruits the ATR complex to sites of DNA damage or a stalled replication fork and facilitates the recognition of ATR substrates for phosphorylation and initiation of checkpoint signaling (47). RPA is a heterotrimeric complex composed of the 70- (RPA1), 32- (RPA2), and 14- (RPA3) kDa subunits that is essential for DNA replication, recombination, and repair through its recognition and coating of ssDNA, a common structure generated at the sites of DNA damage or a stalled replication fork (22, 41). Depletion of RPA from *Xenopus laevis* extracts could prevent the binding of ATR to chromatin (24, 42). Inhibition of RPA expression in mammalian cells abrogates the ATR-mediated phosphorylation of Chk1 (37) and impairs the ability of ATR to form nuclear foci upon exposure to aphidicolin (17). Taken together, these results suggest that RPA may function as an upstream regulator of the ATR-mediated checkpoint signaling pathway. However, RPA-independent ATR activation has also been reported (11, 18). Thus, the exact function of RPA in ATR activation remains to be further explored.

Unlike ATR, the ATM kinase has been demonstrated to display a significantly higher catalytic activity, as reflected by the substantial increase in both the autophosphorylation on Ser1981 of ATM and the phosphorylation of its substrates in response to ionizing radiation (IR)-induced DNA damage (1, 4). A number of proteins have recently been implicated to play a role in the initial activation of the ATM kinase upon IR exposure, including the MRN complex, PP2A, and PP5 (3, 20, 25, 34). PP5 is a member of the serine/threonine phosphatase family that also includes PP1, PP2A, and PP2B. PP5 contains an N-terminal regulatory domain with three tetratricopeptide repeat (TPR) motifs and a C-terminal catalytic domain (14). Through the TPR domain, PP5 interacts with a number of

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proteins and has been reported to be involved in regulating various biological processes, including the activity of glucocorticoid receptor (13, 31), apoptosis (28), and cell growth (48). Our recent findings on the requirement of PP5 in the IR-induced activation of the ATM kinase defined a novel role for PP5 in the regulation of the ATM-mediated DNA damage checkpoint pathway (3).

Although the activity of the ATR-mediated checkpoint pathway has been considered to be regulated at the subcellular localization rather than its catalytic activity, the structural similarity and overlapping functions between the two checkpoint kinases prompted us to test whether PP5 could play a similar regulatory role for ATR as for ATM. In the present study, we demonstrate that PP5 forms an inducible complex with ATR in response to a variety of genotoxic insults. Down-regulation of PP5 protein expression level or overexpression of a dominant-negative PP5 mutant decreases the phosphorylation of the known ATR substrates, hRad17 and Chk1, in UV-irradiated or replication-stalled cells. Functionally, PP5 is required for the UV-induced replication checkpoint and the hydroxyurea-triggered S-M checkpoint, two S-phase checkpoint pathways mediated by ATR. Although the formation of genotoxic stress-induced ATR intranuclear foci is not changed in cells with PP5 suppression, the focus formation of RPA is significantly reduced. Together, our results suggest that PP5 plays a critical role in the regulation of ATR activity and place PP5 in a specific position in the ATR-mediated signaling cascade.

MATERIALS AND METHODS

Cell culture and antibodies. The human lung carcinoma A549, human cervix carcinoma HeLa, and HEK 293T cells were obtained from the American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS). The ATM-deficient mouse embryonic fibroblasts (MEFs) were cultured in DMEM-F12 with 15% FBS. The BJ human fibroblasts were maintained in DMEM containing 20% FBS. Phospho-specific antibody directed against hRad17 (pS635) has been previously characterized (5). The anti-phospho-Chk1 antibody (pSer345) was purchased from Cell Signaling. The anti-phospho-histone H3 (Ser10) polyclonal antibody was from Upstate Biotechnology. Affinity-purified antibodies against hRad17 (H300), Chk1 (G-4), ATR/FRP1 (C-19), and hemagglutinin (HA; Y-11) were purchased from Santa Cruz. The anti-ATR (Ab-2), anti-RPA2 (Ab-3), and anti-Rad9 antibodies were from Oncogene Research Products. The anti-FLAG M2 monoclonal antibody was from Sigma. The anti-PP5 antiserum has been previously described (3). The anti-PP2A (catalytic subunit) and anti-β-catenin antibodies were obtained from BD Transduction Laboratories. The polyclonal anti-ATRIP antibody and anti-ATR antibody were generously provided by David Cortez at Vanderbilt University and Randal S. Tibbetts at the University of Wisconsin—Madison, respectively.

Plasmids, oligonucleotides, and siRNA. PP5 constructs containing an N-terminal HA or C-terminal FLAG epitope have been described previously (3). The FLAG-ATR expression plasmid was kindly provided by Robert T. Abraham at the Burnham Institute. Control adenovirus or recombinant adenoviruses encoding FLAG-PP5^{WT} or FLAG-PP5^{MT} were generated and produced as previously described (3). Antisense oligonucleotides targeting PP5 (ISIS 15534 and ISIS 15521) were provided by ISIS pharmaceuticals and were transfected as described elsewhere (48). Control short interfering RNA (siRNA) duplex specific for green fluorescence protein and synthetic siRNA duplexes targeting *PP5* (PP5 siRNA, 5′-AACAUAUUCGAGCUCAACGGU-3′, or PP5 siRNA-2, 5′-AAGATCGT GAAGCAGAAGGCC-3′) were purchased from Dharmacon Research Inc. HeLa cells were transfected with 20 μM siRNA duplexes and oligofectamine (Invitrogen) and were analyzed 72 h after transfection. To stably knock down the expression of PP5 and ATR, gene-specific inserts were cloned into the mammalian expression vector pSUPER-Retro (pSR) according to the manufacturer's (OligoEngine) instructions. The retrovirus was produced in HEK 293T cells, and the virus-containing media were harvested for infection. The targeting sequence of PP5 for stable siRNA expression was 5′-CATATTCGAGCTCAACGGT-3′. The sequences of ATR for stable siRNA expression were 5′-AGCCACTTCTC AACATGAA-3′ and 5′-GTCAGCAGCTTTATCTGAA-3′.

Protein analysis, immunoprecipitation, and immunoblotting. To examine the interaction between ATR and PP5, HEK 293T cells were harvested with lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% NP-40, 1 mM dithiothreitol) supplemented with protease inhibitors (20 μg/ml leupeptin, 10 μg/ml pepstatin A, 10 μg/ml aprotonin) and phosphatase inhibitors (20 mM β-glycerophosphate, 50 nM microcystin-LR). The cleared lysates were immunoprecipitated with the indicated antibodies and protein A/G-Sepharose. The immunoprecipitates were washed three times with lysis buffer, solubilized with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer, electrophoresed, and analyzed by immunoblotting.

Inhibition of DNA synthesis assay. Forty-eight hours after siRNA transfection, HeLa cells were incubated with [^{14}C]thymidine (20 nCi/ml; NEN) for 24 h. The cells were then exposed to UV light. After 2 h, cells were pulse-labeled with [^{3}H]thymidine (2.5 μCi/ml; NEN) for 1 h. The cells were then harvested as described previously (15). The radioactivity was determined by liquid scintillation counting, and the relative DNA synthesis rate was calculated with the following equation: $([^{3}H]/[^{14}C])_{\text{after UV}}/([^{3}H]/[^{14}C])_{\text{before UV}}$. All samples were tested in triplicate, and consistent results were obtained among three independent experiments.

Immunofluorescence microscopy. Monolayer cells were fixed with 4% paraformaldehyde followed by permeabilization with 0.5% Triton X-100. After blocking with 3% bovine serum albumin, cells were then incubated with the indicated antibodies diluted according to the manufacturer's suggestions at 4°C overnight. Following three phosphate-buffered saline (PBS) washes, cells were incubated with secondary antibodies for 1 h. After washing with PBS, the cells were stained with Hoechst (Sigma). Samples were visualized on a Zeiss LSM410 confocal microscope. The anti-ATR/FRP1 (C-19) antibody from Santa Cruz and anti-RPA2 (Ab-3) antibody from Oncogene Research Products were used for immunofluorescence staining.

Flow cytometric analysis. For cell cycle analysis, cells were fixed with 70% ethanol and then incubated with RNase A (100 μg/ml) and propidium iodide (50 μg/ml) for 30 min at 37°C. Cell cycle distributions were analyzed by a flow cytometer. For detection of phosphorylated histone H3, cells were fixed in 70% ethanol and resuspended in 0.25% Triton X-100 in PBS. The cells were then incubated with the anti-phospho-histone H3 antibody and a fluorescein isothiocyanate-conjugated secondary antibody. After counterstaining with propidium iodide, the phospho-histone H3 fluorescence and DNA content were determined by a FACScan flow cytometer (BD Biosciences) and analyzed using CellQuest software.

RESULTS

Genotoxic stress-induced association between PP5 and ATR. Previous studies from our laboratory indicated that PP5 is required for ATM activation during the initial phase of checkpoint activation in response to DNA double-stranded breaks (3). Considering the structural and functional homology between ATM and ATR, we hypothesized that PP5 might be similarly involved in the activation of ATR. To test this, we examined whether PP5 interacts with ATR by performing co-immunoprecipitation assays in HEK 293T cells expressing epitope-tagged ATR and PP5. As shown in Fig. 1A, HA-tagged PP5 was readily detected in the anti-FLAG immunoprecipitates 30 min after treatment with the radiomimetic agent neocarzinostatin (NCS), UV irradiation, or the replication blocking agent HU. This result suggests that PP5 may form a complex with ATR in a genotoxic stress-inducible manner. Previously, we characterized a catalytically inactive form of PP5 that retained its ability to associate with ATM (3). To determine whether the same PP5 mutant could interact with ATR, we immunoprecipitated FLAG-tagged ATR from UV-treated HEK 293T cells that were transfected with HA-tagged wild-type or mutant PP5. As shown in Fig. 1B, both wild-type (PP5^{WT}) and mutant (PP5^{MT}) PP5 associated with ATR, suggesting that the PP5 mutant is likely to have a dominant inter-

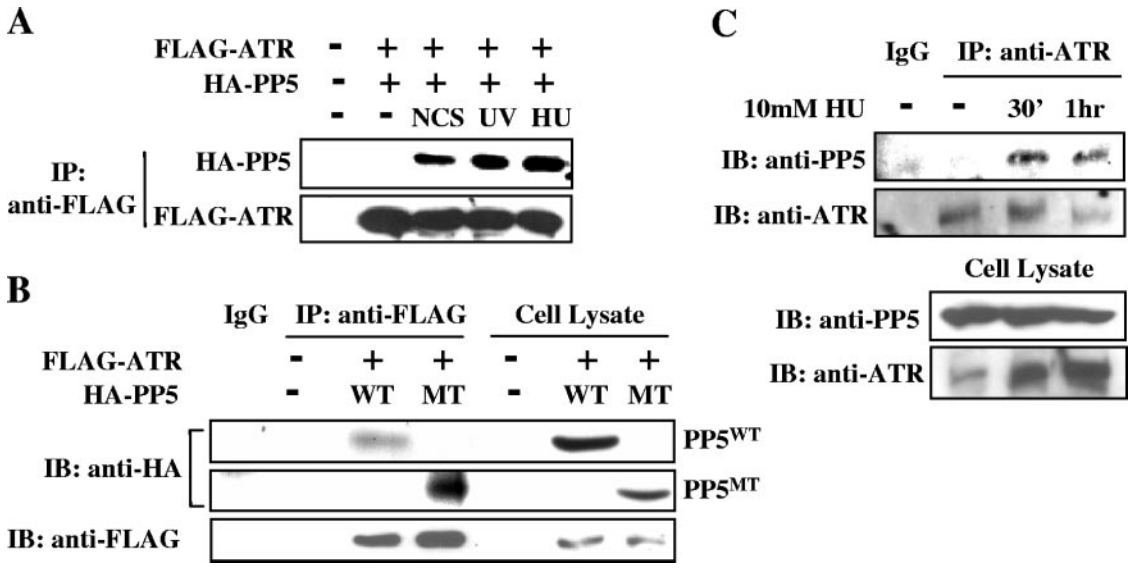

FIG. 1. Genotoxic stress-induced association of PP5 with ATR. (A) Ectopically expressed PP5 interacts with ATR in response to genotoxic stress. After transfection, HEK 293T cells were treated with 100 ng/ml NCS, 100 J/m^2 UV, or 10 mM HU for 30 min. Coimmunoprecipitated PP5 was detected by anti-FLAG immunoprecipitation (IP) followed by anti-HA immunoblotting (IB) (upper panel). (B) PP5^{MT} (a catalytically inactive form of PP5) remains capable of interacting with ATR. HEK 293T cells transfected with the indicated plasmids were treated with UV (100 J/m^2) for 1 hour. Cellular extracts were immunoprecipitated with anti-FLAG antibody and analyzed by immunoblotting with anti-HA antibody. (C) Association between endogenous PP5 and ATR induced by replication block. HEK 293T cells were left untreated or treated with 10 mM HU for 30 min or 1 hour. Cellular extracts were immunoprecipitated with anti-ATR antibody and analyzed by immunoblotting with anti-ATR or anti-PP5 antibody as indicated. Normal goat immunoglobulin G (IgG) was used as a negative control. The amounts of ATR and PP5 in total cell lysates were determined and are shown in the lower two panels.

fering effect on ATR in vivo. To determine the existence of a physical interaction between PP5 and ATR under physiological conditions, we examined the association between these two molecules in nontransfected HEK 293T cells. As the results shown in Fig. 1C indicate, endogenous PP5 was detected in the anti-ATR immunoprecipitates and, more importantly, their interaction was induced only after HU treatment. Taken together, our data indicate that PP5 and ATR form an inducible complex in response to genotoxic stress.

PP5 is required for the ATR-mediated phosphorylation of hRad17 and Chk1. To determine the functional significance of this inducible PP5-ATR interaction, we first examined the phosphorylation of hRad17 at the Ser635 residue and Chk1 at the Ser345 residue, as the phosphorylation of these two proteins following exposure to UV or HU is mediated primarily through ATR (5, 26, 27, 45). A549 cells were exposed to UV or NCS treatment 24 h after they were transfected with PP5 antisense or mismatch oligonucleotides, and PP5 expression levels were determined by immunoblotting. As shown in the fifth panel of Fig. 2A, transfection of antisense oligonucleotides almost completely eliminated the expression of PP5 protein. In mock-transfected cells, the phosphorylation of hRad17 at Ser635 and Chk1 at Ser345 was induced at 1 h after UV treatment. In contrast, UV-induced phosphorylation of these two proteins was substantially reduced in cells treated with PP5 antisense oligonucleotides (Fig. 2A, top and third panels). To determine the specificity of PP5 antisense oligonucleotides, we also examined the expression of PP2A, a related family member of PP5 that has been implicated in ATM activation (20). As shown in the bottom panel of Fig. 2A, transfection of antisense oligonucleotides had no effect on the expression of the catalytic subunit of PP2A. Because ATR is primarily responsible for the phosphorylation of hRad17 and Chk1 under such conditions, it is of importance to determine whether the expression level of ATR protein was altered in cells with PP5 depletion. Transfection of PP5 antisense oligonucleotides had minimal effects on the amount of ATR protein in total cell lysate (data not shown), suggesting that PP5 regulates ATR kinase activity rather than its expression. Interestingly, the level of the ATR-associating protein, ATRIP, was unchanged by PP5 inhibition (data not shown).

To confirm these initial observations, we examined whether expression of a catalytically inactive form of PP5 interferes with the phosphorylation of hRad17 and Chk1. This PP5 mutant retained its ability to interact with ATR (Fig. 1B); therefore, its expression is likely to have a dominant-negative effect on the activity of endogenous PP5. After infected with control or recombinant adenoviruses encoding either wild-type or mutant PP5, A549 cells were then left untreated or treated with UV or HU for 1 h. As shown in Fig. 2B, the genotoxic stress-induced phosphorylation of hRad17 and Chk1 was significantly increased in control cells or cells expressing wild-type PP5 (top and third panels). In contrast, expression of the PP5 mutant profoundly repressed the phosphorylation of these two proteins. The amounts of ectopically expressed PP5 proteins were determined and are shown in the fifth panel. The expression of both ATR and ATRIP remained the same in cells with ectopically expressed PP5 mutant compared to the control cells or wild-type PP5-expressing cells (data not shown). These findings further support the notion that PP5 is required for the phosphorylation of ATR substrates in UV- or HU-treated cells.

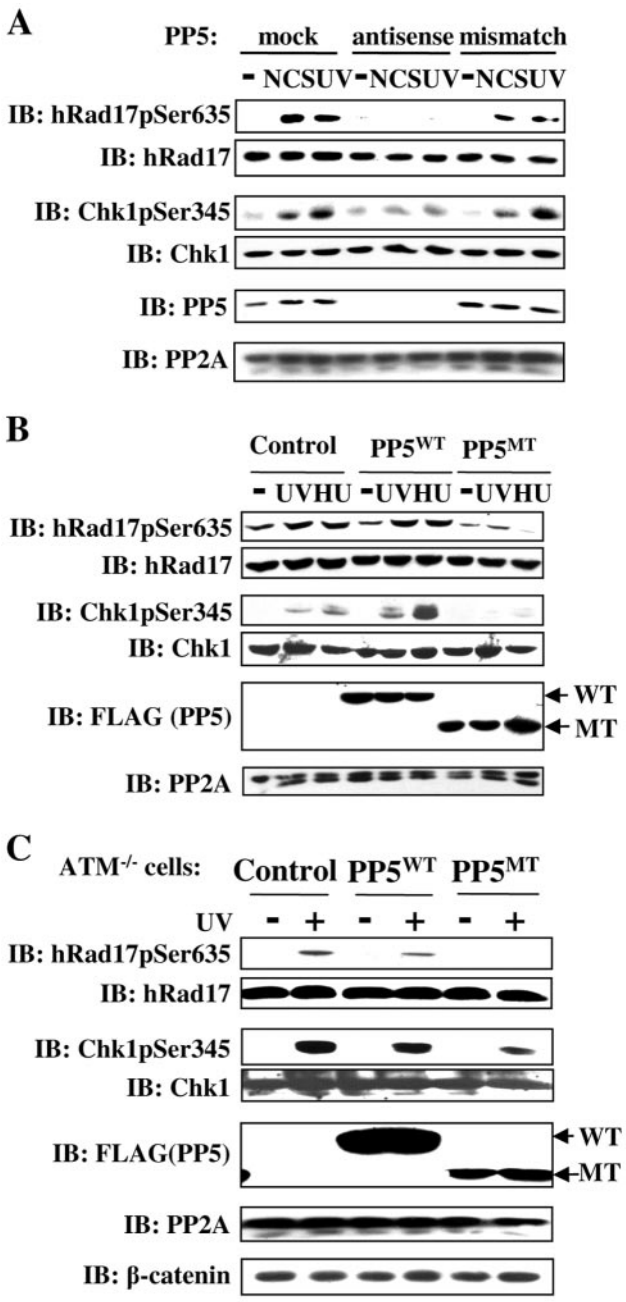

FIG. 2. Reduction in PP5 expression or activity results in an impairment of UV- or replication stress-induced phosphorylation of hRad17 and Chk1. (A) Decreased PP5 expression attenuates DNA damage-induced phosphorylation of hRad17 and Chk1. After transfection with PP5 antisense or mismatch oligonucleotides, A549 cells were exposed to 100 ng/ml NCS or UV (100 J/m^2) and harvested 1 hour after the treatment. Amounts of hRad17pSer635, total hRad17, Chk1pSer345, total Chk1, PP5, and PP2A (catalytic subunit) were determined by immunoblotting (IB) with the respective antibodies and are shown in the indicated panels. (B) Expression of a catalytically inactive form of PP5 reduces ATR-mediated phosphorylation of hRad17 and Chk1. After infection with the indicated adenoviruses, A549 cells were exposed to 100 J/m^2 UV or 10 mM HU and then incubated for 1 hour. Cell lysates were immunoblotted as indicated. (C) UV-induced phosphorylation of hRad17 and Chk1 is abrogated in ATM$^{-/-}$ MEFs expressing the PP5 mutant. After infection, ATM$^{-/-}$ MEFs were exposed to 100 J/m^2 of UV and harvested 1 hour later. Anti-β-catenin antibody was used as the loading control.

It is worth noting that treatment of cells with NCS also induced the phosphorylation of hRad17 at Ser635 and Chk1 at Ser345 (Fig. 2A, top and third panels). Moreover, the phosphorylation of these two molecules was decreased in PP5 antisense-transfected cells following NCS treatment. The NCS-induced DNA double-stranded breaks primarily activate the ATM kinase, although ATR functions at the late stage of the checkpoint (2). Considering the overlapping substrate specificity between ATM and ATR and our recent finding that PP5 regulates the kinase activity of ATM (3), we next determined whether ATR but not ATM is mainly involved in the UV- or replication stress-induced phosphorylation of hRad17 and Chk1 observed under our assaying conditions. To rule out a possible role for ATM in this process, ATM-deficient MEFs were infected with control or recombinant adenoviruses expressing wild-type or mutant PP5. Immunoblotting analysis revealed that the phosphorylation of hRad17 at Ser635 and Chk1 at Ser345 was stimulated by UV treatment in control cells (Fig. 2C), consistent with previous observations that in cells damaged by genotoxic insults other than IR, Chk1 and hRad17 are phosphorylated primarily by ATR (5, 26, 27, 45). More importantly, expression of the dominant interfering PP5 mutant in ATM$^{-/-}$ MEFs significantly inhibited the UV-induced hRad17 and Chk1 phosphorylation, suggesting that PP5 could specifically target the ATR-mediated checkpoint pathway. Collectively, these results indicate that interference with PP5 activity leads to impairment of the ability of ATR to phosphorylate its substrates in UV-irradiated or replication-stalled cells.

A role for PP5 in S-phase checkpoints. In response to UV irradiation, eukaryotic cells activate the replication checkpoint to slow down DNA synthesis (1, 7). Accumulating evidence indicates that ATR is intimately linked to this process (29). To determine whether PP5 was involved in the ATR-mediated replication checkpoint, we suppressed PP5 expression in HeLa cells by transfection with siRNA specific for PP5. As shown in Fig. 3A, introduction of synthetic siRNA duplexes led to a reduction of more than 80% of PP5 protein. We next confirmed that inhibition of PP5 expression resulted in the abrogation of UV- or HU-induced Chk1 phosphorylation (data not shown). In addition, treatment of HeLa cells with another synthetic siRNA duplex (PP5 siRNA-2) with lower knockdown efficiency also reduced the HU- or UV-induced phosphorylation of Chk1 to a lesser extent (data not shown). In subsequent experiments, we determined the effect of reduced PP5 expression on the UV-induced replication checkpoint activation. At 72 h after HeLa cells were transfected with control or PP5 siRNA duplexes, the cells were left untreated or exposed to UV irradiation and the DNA synthesis rates were then determined by a [^{3}H]thymidine incorporation assay. As expected, control cells showed a dosage-dependent inhibition of DNA synthesis following UV treatment (Fig. 3B; 45% and 29% of that prior to UV treatment, respectively). In contrast, cells treated with PP5 siRNA had a modest decrease in DNA synthesis (Fig. 3B; 58% and 44%, respectively), suggesting that the ability of those cells to suppress DNA synthesis is compromised. To rule out the possibility that the observed UV-resistant DNA synthesis is due to altered cell cycle by PP5 inhibition, we next determined the cell cycle distribution of transfected HeLa cells. Although loss of functional PP5 sensi-

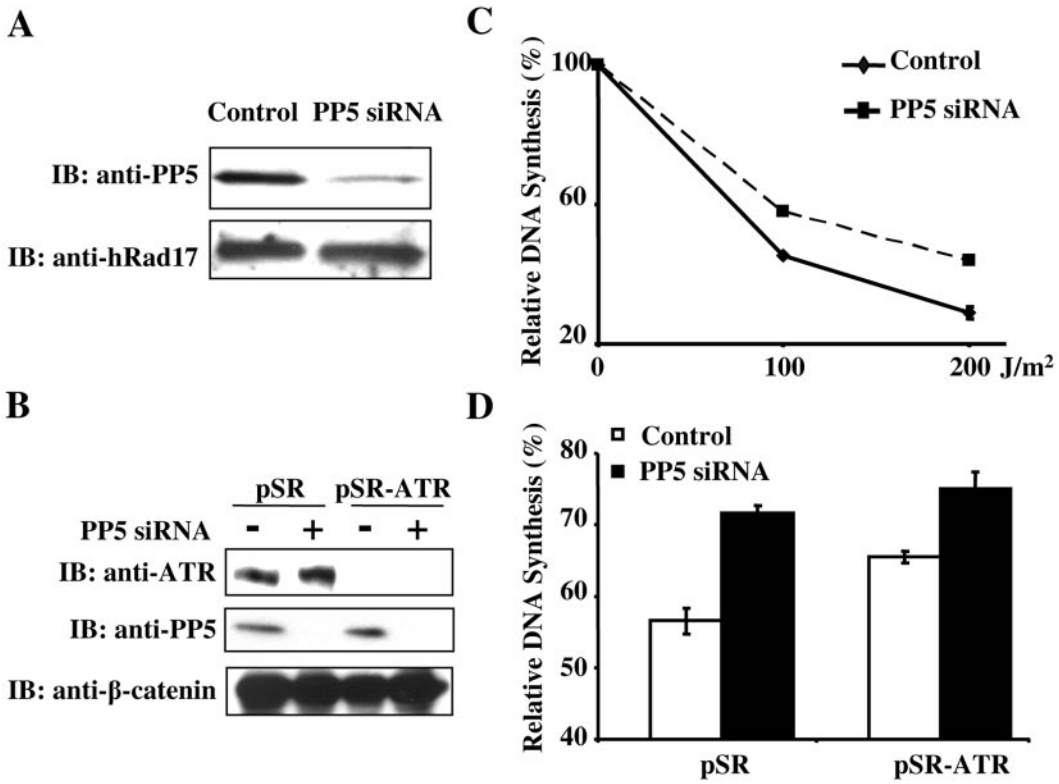

FIG. 3. PP5 is involved in the UV-induced replication checkpoint. (A) Suppression of PP5 expression by synthetic siRNA duplexes. HeLa cells were transfected with control green fluorescent protein siRNA or siRNA specific for PP5. Seventy-two hours after transfection, cells were harvested and subjected to SDS-PAGE and immunoblotting (IB) using antibody against PP5. The expression level of hRad17 is shown as the loading control. (B) Involvement of PP5 in the UV-induced replication checkpoint. At 72 h after transfection, HeLa cells were exposed to 100 J/m^2 or 200 J/m^2 UV light and incubated for another 3 hours. DNA synthesis rates were determined as described in Materials and Methods. (C) Combined depletion of PP5 and ATR. HeLa cells were infected with retroviruses containing empty vector (pSR) or siRNA sequences targeting ATR (pSR-ATR). After 2 weeks of puromycin selection, HeLa cells were transfected with control or synthetic PP5 siRNA duplexes. The expression levels of ATR and PP5 were examined by immunoblotting. (D) UV-induced inhibition of DNA synthesis in cells with PP5, ATR, or combined PP5 and ATR depletion. HeLa cell lines were irradiated with 100 J/m^2 UV, and the DNA synthesis rates were determined 3 hours later. All samples were tested in triplicate, and consistent results were obtained from three independent experiments. The relative rates of DNA synthesis prior to DNA damage were similar in control and PP5 knockdown cells.

tized cells to apoptosis, there was little or no defect in S-phase progression in PP5-depleted cells or cells expressing the PP5 mutant (data not shown).

To further explore the role of PP5 in the ATR-mediated replication checkpoint, we examined the DNA synthesis profile in cells with combined PP5 and ATR depletion. Retroviruses encoding empty vector (pSR) or ATR siRNA (pSR-ATR) were produced and used to infect HeLa cells for stable knockdown. As shown in Fig. 3C (top panel), the expression of ATR protein was significantly reduced in cells expressing ATR siRNA. It is of interest that after 2 weeks' selection, the cells with stable ATR knockdown were viable and had a similar cell cycle distribution profile and DNA synthesis rate as those of control cells (data not shown). We then transfected those HeLa cells with siRNA duplexes targeting PP5, irradiated them with UV light, and analyzed the DNA synthesis rate by the [^{3}H]thymidine incorporation assay. In cells with no ATR and PP5 down-regulation, DNA synthesis was inhibited to 56% of the untreated cells by UV irradiation (Fig. 3D). However, depletion of PP5 or ATR led to elevated levels of DNA synthesis after UV treatment (72% and 65%, respectively). Interestingly, combined knockdown of PP5 and ATR increased the DNA synthesis rate to 75% following UV exposure. Because of the inherent limitation associated with siRNA-based experiments, we cannot rule out the possibility that DNA synthesis was only partially resistant to UV irradiation due to the residual amount of ATR left in ATR knockdown cells. Under such a condition, addition of PP5 depletion led to further DNA synthesis resistance by targeting the ATR-mediated checkpoint pathway. However, it is possible that interaction with ATR is not the only mechanism by which PP5 regulates the replication checkpoint. Together, our results suggest that PP5 is involved in the UV-induced checkpoint activation, a process controlled primarily by ATR.

During S phase, upon exposure to various genotoxic stresses, cells also trigger another checkpoint, S-M checkpoint, to prevent the onset of mitosis before DNA replication is complete (7). It has been found that the ATR-Chk1 pathway is essential for the activation of this checkpoint (10, 43, 44). Our observation that PP5 was required for the phosphorylation of Chk1 after UV or HU treatment has prompted us to determine whether PP5 plays an important role in regulating the ATR/

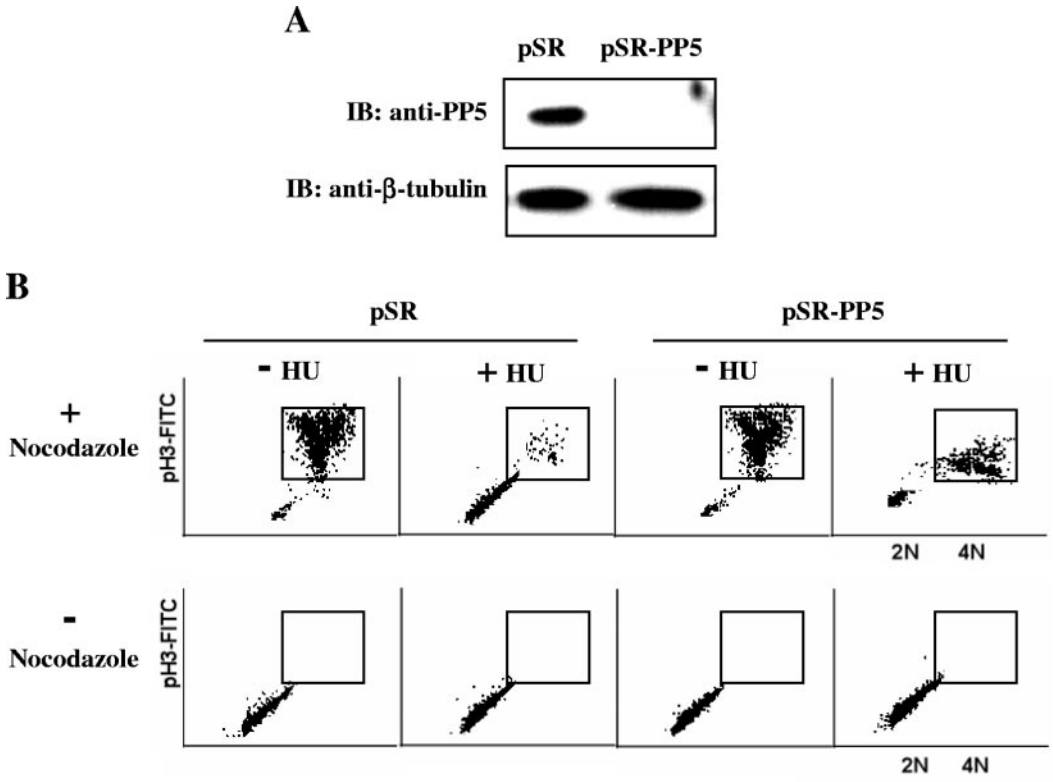

FIG. 4. PP5 is required for the ATR-mediated S-M checkpoint. (A) Stable down-regulation of PP5 by retrovirus-delivered siRNA. Human diploid BJ fibroblasts were infected with control virus (pSR) or retrovirus expressing PP5 siRNA (pSR-PP5). Two weeks after selection, the cells were harvested and subjected to SDS-PAGE and immunoblotting (IB) with anti-PP5 antibody. The expression level of β-tubulin is shown as the loading control. (B) HU-induced S-M checkpoint is abrogated in PP5 knockdown cells. After serum starvation, BJ cells were allowed to return to normal culture medium and synchronized in G_1/S phase. The cells were then left untreated or treated with 10 mM HU and 0.5 μg/ml nocodazole as indicated for 20 hours, followed by flow cytometric analysis after staining with anti-phospho-histone H3 antibody. The open boxes contained cells positive for phospho-histone H3.

Chk1-mediated S-M checkpoint. First, we stably knocked down the expression of PP5 in human diploid BJ fibroblasts through retrovirus-delivered siRNA (see Fig. 5A, below) and examined the association between ATR and ATRIP in the PP5 knockdown cells. No disruption of ATR-ATRIP interaction was observed after suppression of PP5 (data not shown). We next examined the percentage of cells that prematurely entered mitosis after HU treatment using phospho-specific histone H3 (Ser10) antibody as a marker for mitotic cells (40). The BJ fibroblasts were first synchronized to G_1/S phase through serum starvation and subsequent serum stimulation. The cells were then treated with HU to inhibit DNA synthesis together with a microtubule-disrupting agent, nocodazole, to trap mitotic cells. As shown in Fig. 4B, both the control cells (pSR) and PP5 knockdown cells (pSR-PP5) accumulated in mitosis in the absence of HU treatment, as indicated by the majority of cells stained positive for phospho-histone H3 at residue serine 10 after 20 hours of nocodazole treatment (83% and 79%, respectively). After HU treatment, very few (~16%) control cells were mitotic, indicating a functional S-M checkpoint in these cells. In contrast, a significant portion (~58%) of cells with stable PP5 knockdown were stained positive with the phospho-histone H3 antibody, suggesting that they entered into mitosis prematurely. Together, our results found that PP5 is required for the ATR-mediated S-M checkpoint, suggesting that PP5 resides in the same signaling pathway as ATR.

Effects of PP5 on genotoxic stress-induced nuclear focus formation of ATR and RPA. It has been suggested that in response to DNA damage or replication stress, ATR is redistributed into intranuclear foci, where it is localized at sites of DNA damage or a stalled replication fork and gains access to its substrates (33). To determine whether the activity of PP5 is required for the redistribution of ATR and thus the accessibility of ATR to its substrates, we examined the formation of nuclear foci by ATR in cells with PP5 down-regulation. After transfection with PP5 siRNA, HeLa cells were treated with HU or UV followed by 5-hour incubation. Cells were then fixed and stained with specific antibodies. As shown in Fig. 5A, prior to HU or UV treatment, ATR was localized within the nucleus in a uniform, diffused pattern. Consistent with previous reports, distinct nuclear ATR foci were observed in control cells after treatment. There were about <10% of cells that stained positive with ATR foci in HU-treated cells and 20 to 30% of those in UV-irradiated cells (Fig. 5B). More importantly, inhibition of PP5 expression by siRNA failed to cause a decrease in the ATR focus formation induced by HU or UV (Fig. 5A and B). In a parallel experiment, the protein expression levels of PP5 in control cells or cells transfected with PP5

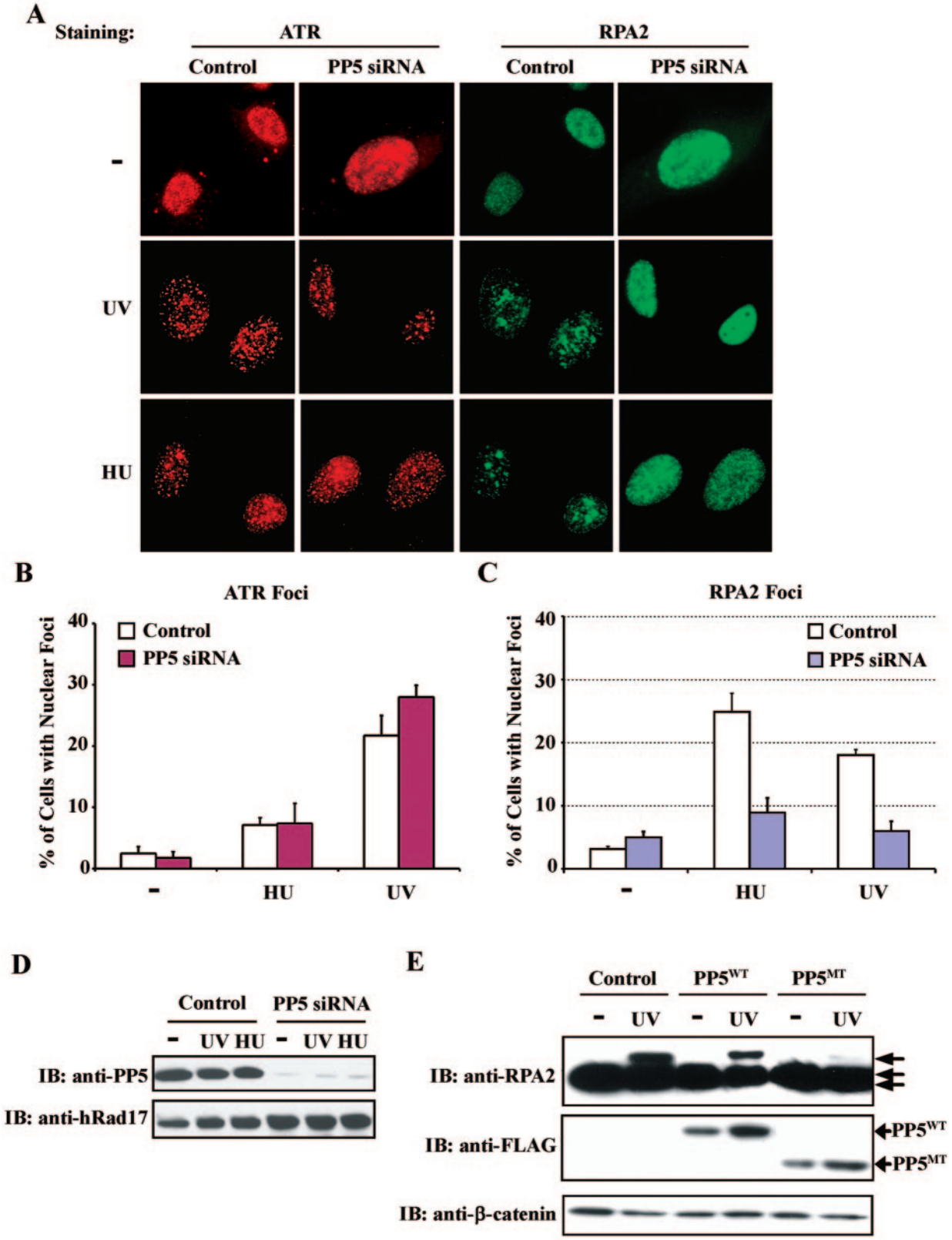

FIG. 5. Differential requirements of PP5 in nuclear focus formation by ATR and RPA in response to genotoxic stress. (A) PP5 is required for the nuclear focus formation of RPA but not ATR. HeLa cells were transfected with control or PP5 siRNA for 72 hours. Five hours after HU (10 mM) or UV (100 J/m^2) exposure, cells were fixed and costained with anti-ATR antibody and anti-RPA2 antibody. The localization of ATR and RPA was observed under immunofluorescence microscopy. The percentages of cells with ATR foci (B) or RPA foci (C) were determined from a total of over 200 cells and graphed (mean ± standard deviation from at least three experiments). (D) Suppression of PP5 expression in

siRNA were determined by immunoblotting (Fig. 5D), since the available anti-PP5 antibodies were unable to detect the protein in the nucleus by immunofluorescence microscopy. Consistent with the above observation, ectopic expression of the catalytically inactive PP5 mutant had no significant effect on ATR focus formation (data not shown). However, under the same assaying conditions, phosphorylation of hRad17 and Chk1 was clearly diminished (data not shown). These results suggest that despite the critical role of PP5 in the ATR-mediated phosphorylation of downstream target proteins, PP5 is not required for the recruitment of ATR to sites of DNA damage or a stalled replication fork.

It has been shown that RPA colocalizes with ATR to nuclear foci after IR or replication stress and is required for ATR focus formation (17, 47). However, other studies suggested that the genotoxic stress-induced intranuclear translocation of RPA was an active process regulated by the kinase activity of ATR (6). To further explore the role of PP5 in the ATR-mediated checkpoint activation, we next examined the localization of RPA in the same cells shown in Fig. 5A. Antibody against RPA2, the second largest subunit of the heterotrimeric RPA protein complex, was used to monitor the potential changes in RPA localization. As expected, exposure of control cells to HU or UV treatment resulted in an increase of cells with RPA foci in the nucleus (Fig. 5A). Notably, RPA foci were formed in about 24% of the cells after HU treatment and 18% of the cells following UV irradiation (Fig. 5C). In contrast, we observed a significant reduction of RPA foci in cells treated with PP5 siRNA (Fig. 5A and C), with 9% for HU and 6% for UV treatment of total cells, respectively, whereas the level of ATR focus formation remained unchanged. Taken together, these results suggest that PP5 is required for the nuclear focus formation of RPA in response to genotoxic stress, but not for that of ATR.

Previously, it has been shown that upon DNA damage RPA2 is phosphorylated by the phosphoinositide 3-kinase related protein kinases (PIKK) and the kinases both colocalizes and interacts with RPA at the sites of damage (9). Interestingly, in HeLa cells with stable knockdown of ATR, we observed a decreased phosphorylation of RPA2 following UV treatment (data not shown), suggesting that ATR is the kinase for the UV-induced RPA2 phosphorylation. To further explore the molecular mechanism underlying the differential effects of PP5 on ATR and RPA localization, we next determined the ATR-dependent phosphorylation of RPA in cells with impaired function of PP5. A549 cells were infected with control or recombinant adenoviruses encoding wild-type or mutant PP5. Three hours after exposure to UV light, the phosphorylation of RPA2 was determined and is shown in Fig. 5E. In control cells or cells expressing wild-type PP5, UV irradiation resulted in a strong phosphorylation of RPA2, as indicated by the slowest mobility shift band. However, the hyperphosphorylation of RPA2 was barely detectable in cells overexpressing the PP5 mutant, suggesting that the catalytic activity of PP5 is required for RPA phosphorylation. Together, our results indicated that PP5 may regulate the nuclear focus formation of RPA through modulation of its phosphorylation by ATR.

DISCUSSION

The present findings demonstrated a regulatory link between PP5 and ATR. Reduced expression of PP5 or interference of PP5 activity substantially decreased genotoxic stress-induced phosphorylation of certain known ATR substrates and impaired S-phase checkpoint responses without affecting the redistribution of ATR to nuclear foci. Furthermore, functional PP5 was required for the nuclear focus formation and hyperphosphorylation of RPA. Taken together with previous reports that ATR regulates the localization as well as phosphorylation of RPA (6, 8), our results suggest an important role of PP5 in the activation of the ATR-mediated checkpoint pathway.

The precise mechanism by which ATR becomes activated by DNA damage or replication stress has remained one of the main questions unresolved in checkpoint signaling. Accumulating evidence has suggested that RPA-coated ssDNA is required for the recruitment of the ATR-ATRIP complex to sites of DNA damage and facilitates the recognition of ATR substrates, implicating RPA as an upstream regulator of the ATR kinase (47). However, additional evidence suggests a much more complicated role of RPA in the ATR-mediated checkpoint activation. Upon DNA damage, RPA is phosphorylated by PIKK, and the hyperphosphorylation event has been proposed to redirect RPA activity from DNA replication to DNA repair (reviewed by Binz et al. [9]), as indicated by the observation that a hyperphosphorylation-mimetic mutant of RPA2 was unable to associate with replication centers but competent to associate with DNA damage foci (35). In our current studies, we have found that the UV-induced hyperphosphorylation of RPA2 was dependent on the presence of ATR, suggesting that ATR functions as the checkpoint kinase to modulate RPA activity. It is possible that RPA plays dual roles during the process of ATR activation, initially recruiting the ATR-ATRIP complex to the sites of DNA damage and accumulating in the damage-induced nuclear foci after being phosphorylated by ATR to facilitate further checkpoint activation and DNA repair (9). More intriguingly, studies of the localization of RPA in the presence of an ATR kinase-inactive mutant have suggested that the nuclear focus formation of RPA is an active process regulated by ATR (6). Thus, it is tempting to speculate that the phosphorylation of RPA by ATR is required for its nuclear focus formation. Accordingly, our observation of the diminished nuclear focus formation of RPA in PP5 knockdown cells may suggest a role of PP5 in regulating the kinase activity of ATR, similar to the role of PP5 as a critical modulator of ATM activation (3).

Unlike ATM, it has been difficult to detect increased activity

siRNA-transfected cells. In a parallel experiment, 5 hours after treatment, the cells were harvested and subjected to SDS-PAGE and immunoblotting (IB) with anti-PP5 antibody. The expression level of hRad17 is shown as the loading control. (E) Inhibition of UV-induced RPA2 phosphorylation by the PP5 mutant. A549 cells were infected with control or recombinant adenoviruses encoding wild-type or mutant PP5. Twenty-four hours after infection, cell were exposed to 100 J/m^2 UV irradiation and harvested 3 hours later. The cell lysates were subjected to 12% SDS-PAGE, and the phosphorylation of RPA2 was determined by the slow migrating bands on the immunoblot.

of immunoprecipitated ATR after DNA damage (1), although a few recent papers have observed ATR activation in vitro (23, 36). Indeed, when we performed an ATR immuno-complex kinase assay using glutathione S-transferase–hRad17 as the substrate, no increase of ATR kinase activity was observed after UV treatment (data not shown). Moreover, down-regulation of PP5 appeared to have no effect on the kinase activity of ATR under the same in vitro assay conditions (data not shown). However, these negative results may be the consequence of technical difficulties in performing the in vitro ATR kinase assay and could not be used to rule out the possibility that ATR is subject to activation by genotoxic stress signals with PP5 functioning as a modulator to increase the catalytic activity of the ATR kinase. It is important to note that the nuclear focus formation of ATR was reported to depend on its catalytic activity (6), a notion that is apparently inconsistent with our observation that the loss of PP5 function exerted an insignificant effect on the localization of ATR. However, it is possible that instead of directly targeting the enzymatic activity, PP5 may regulate the ATR kinase through modulating its substrate accessibility.

In addition to the previous and the present reports on the involvement of PP5 in regulating the activity of ATM and ATR, PP5 has recently been shown to interact and regulate the activity of DNA-dependent protein kinase through dephosphorylation of specific sites (39). ATM, ATR, and DNA-dependent protein kinase all belong to the same PIKK family, which shares a certain degree of structural similarity and conserved modes of recruitment to DNA damage sites, as reported recently (19). The involvement of PP5 with these three kinases suggests the existence of a common regulatory mechanism. Unlike most of the protein serine/threonine phosphatases, whose substrate specificities are controlled by the presence of various regulatory subunits, PP5 is regulated by protein-protein interaction through its N-terminal TPR motifs. How PP5 recognizes and differentiates members of the PIKK family currently remains unknown, and further characterization of the functions of PP5 is needed to provide a better understanding of the mechanism underlying the regulation of genotoxic stress-induced checkpoint pathways.

ACKNOWLEDGMENTS

This research was supported by an NIH grant (CA93676) and grants from the A-T Children's Project to X.F.W. J.Z. was supported by a Department of Defense Breast Cancer Predoctoral Fellowship (DAMD 17-02-1-0379). A.A. was supported by a postdoctoral training grant from the Department of Defense Breast Cancer Research Program (DAMD17-00-1-0228).

We thank Robert T. Abraham for reagents and helpful advice, Randal S. Tibbetts for his assistance in our in vitro ATR kinase assay and ATR immunofluorescence staining, David Cortez for anti-ATRIP antibodies, Lee Zou (Massachusetts General Hospital, Boston, Mass.) for helpful discussions, Nu Zhang (Duke University, Durham, N.C.) for assistance in flow cytometric analysis, Yong Yu for technical help, and members of the Wang laboratory for their support and valuable scientific discussions.

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