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# Phosphorylation of Chk1 by ATR Is Antagonized by a Chk1-Regulated Protein Phosphatase 2A Circuit†

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**In higher eukaryotic organisms, the checkpoint kinase 1 (Chk1) contributes essential functions to both cell cycle and checkpoint control. Chk1 executes these functions, in part, by targeting the Cdc25A protein phosphatase for ubiquitin-mediated proteolysis. In response to genotoxic stress, Chk1 is phosphorylated on serines 317 (S317) and 345 (S345) by the ataxia-telangiectasia-related (ATR) protein kinase. Phosphorylation of Chk1 on these C-terminal serine residues is used as an indicator of Chk1 activation in vivo. Here, we report that inhibition of Chk1 kinase activity paradoxically leads to the accumulation of S317- and S345-phosphorylated Chk1 in vivo and that ATR catalyzes Chk1 phosphorylation under these conditions. We demonstrate that Chk1 phosphorylation by ATR is antagonized by protein phosphatase 2A (PP2A). Importantly, dephosphorylation of Chk1 by PP2A is regulated, in part, by the kinase activity of Chk1. We propose that the ATR-Chk1-PP2A regulatory circuit functions to keep Chk1 in a low-activity state during an unperturbed cell division cycle but at the same time keeps Chk1 primed to respond rapidly in the event that cells encounter genotoxic stress.**

Reversible phosphorylation contributes to cellular homeostasis by regulating such diverse biological processes as trafficking, signaling, proliferation, and cell division (4, 10, 23, 33). In human cells, reversible phosphorylation relies on the antagonistic activities of over 500 protein kinases and approximately 180 protein phosphatases (4, 34). Checkpoint kinase 1 (Chk1) is a serine/threonine protein kinase that is also regulated by reversible phosphorylation. Chk1 was first identified in fission yeast as an essential component of the DNA damage checkpoint (3, 45). Although *Chk1* is not required for vegetative growth in fission yeast, it is an essential gene in mice (31, 44). A role for Chk1 in genome surveillance during DNA replication may explain why disruption of *Chk1* results in early embryonic lethality in mice.

In higher eukaryotic organisms, Chk1 regulates the timing and fidelity of cell cycle transitions, in part, by regulating the Cdc25A protein phosphatase (28, 41, 42, 51). Chk1-mediated phosphorylation of Cdc25A serves at least two functions. One is to target Cdc25A for ubiquitin-mediated proteolysis, and the second is to regulate interactions between Cdc25A and Cdk1/cyclin B1 complexes (8, 15, 18, 22, 42, 51). Cdc25A accumulates in cells treated with either Chk1 inhibitors or Chk1-specific small interfering RNAs (siRNAs) (42, 47, 51). Importantly, loss of Chk1 function not only results in Cdc25A accumulation but also causes cells to bypass the DNA damage and DNA replication checkpoints (42, 47, 51).

In response to DNA damage or replication stress, human

Chk1 becomes phosphorylated on two residues within its C terminus (serines 317 and 345) (31, 50). Studies conducted in *Xenopus* egg extracts and cultured human cells indicate that Chk1 is directly phosphorylated on these residues by the ataxia-telangiectasia-related (ATR) protein kinase (17, 21, 24, 31, 50), and C-terminal phosphorylation has been proposed to activate Chk1 by relieving autoinhibition (26, 37).

Three serine/threonine protein phosphatases have been implicated in dephosphorylating Chk1 on S317 and S345. In fission yeast, the type I protein phosphatase homolog Dis2 dephosphorylates S345 (13), whereas in human cells, the p53-regulated protein phosphatase Wip1 (PPM1D) dephosphorylates activated Chk1 on S345 in response to UV damage (32). Furthermore, protein phosphatase 2A (PP2A) can dephosphorylate *Xenopus* Chk1 on S344 (homologous to S345 of human Chk1) in extracts in vitro (38). Here we demonstrate that human Chk1 is maintained in a hypophosphorylated state during the S and G<sub>2</sub> phases of the cell division cycle through the antagonistic activities of ATR and PP2A.

## MATERIALS AND METHODS

**Cell lines.** HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (FBS) or bovine growth serum (HyClone), 100 U/ml penicillin and streptomycin, and 1 mM glutamine. AT221JE T cells expressing ataxia-telangiectasia mutated protein (ATM) (AT<sup>+</sup>) or not expressing ATM (AT<sup>-</sup>) (52) were cultured in DMEM supplemented with 10% fetal bovine serum, 100 U/ml of penicillin and streptomycin, 1 mM glutamine, and 100 µg/ml of hygromycin.

**Chemicals and drugs.** Hydroxyurea (HU), wortmannin, and okadaic acid (OA) were purchased from Sigma Chemical Co. VP-16, fostriecin, Gö6976, and SB-218078 were purchased from Calbiochem. UCN-01 (NSC 638850) was kindly provided by Jill Johnson (Drug Synthesis and Chemistry Branch, NCI, National Institutes of Health). Inhibitor 2 (I-2) was purchased from New England Biolabs.

**Plasmids.** The pEGFP-Chk1 wild-type (WT) construct was generated by inserting Chk1 cDNA at the XhoI site in pEGFP-C1 (Clontech). The pEGFP-Chk1 kinase-inactive mutant (D130A) was generated by using the pEGFP-Chk1 WT as a template; the mutation was introduced as described previously (50).

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† Supplemental material for this article may be found at <http://mcb.asm.org/>.

**Antibodies.** Chk1 was detected with rabbit polyclonal (FL-476; Santa Cruz Biotechnology) or mouse monoclonal (G-4; Santa Cruz Biotechnology) antibodies. Antibodies specific for phosphorylated Chk1, anti-phospho Akt, anti-Akt, and anti-phospho ATM/ATR substrate were purchased from Cell Signaling Technology. Other primary antibodies used were anti-Cdc25A (Ab-3; Neomarkers), anti-phospho Smc1 S966 (Novus Biologicals), anti-phospho Brca1 S1423 (Chemicon), anti-PP1 (Upstate), anti-PP2A (Upstate), anti-PP4 (recognizes PP2A and PP4) (Abcam), anti-PP6 (Exalpha Biologicals), anti- $\beta$ -catenin (BD Transduction), anti- $\gamma$ H2AX (Upstate), anti-ATR (Oncogene), and anti-p1981-ATM (Rockland Biochemicals). Bound primary antibodies were detected with either horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody (Jackson ImmunoResearch) or HRP-conjugated rat anti-rabbit antibody (Zymed), and proteins were visualized by chemiluminescence.

**Cell synchrony and in vivo treatment with chemical inhibitors.** HeLa cells were synchronized using a double-thymidine block-and-release protocol as described previously (8). Cells were released from the block and were incubated with dimethyl sulfoxide (DMSO), Gö6976, SB-218078, or UCN-01 as they entered different phases of the cell cycle. Cells were either analyzed by flow cytometry (11) or were lysed in MCLB (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, and 2 mM dithiothreitol [DTT]) containing 1 mM sodium orthovanadate, 10 mM  $\beta$ -glycerophosphate, 1  $\mu$ M microcystin, 2 mM phenylmethylsulfonyl fluoride (PMSF), 10  $\mu$ g/ml of aprotinin, and 5  $\mu$ g/ml of leupeptin and analyzed by Western blotting. Alternatively, asynchronously growing HeLa cells were incubated in culture medium containing either 20 mM HU, 20  $\mu$ M VP-16, 50  $\mu$ M wortmannin, 1  $\mu$ M OA, or 0.03 to 1  $\mu$ M Gö6976 for the times indicated in the figure legends and harvested in MCLB for Western blot analysis. In addition, AT+ and AT- cells were treated with or without 1  $\mu$ M Gö6976 for 1 or 2 h before being lysed in MCLB. For dephosphorylation experiments, phosphatase inhibitors were left out of the lysis buffer.

**Treatment of cell lysates with inhibitors.** HeLa cell cultures were lysed in MCLB. Clarified lysates were collected at two time points with an initial aliquot collected immediately after lysis and kept on ice. The remaining fractions were incubated with the different phosphatase inhibitors or Gö6976 for 30 min at 30°C. The concentrations of inhibitors were as follows: 1  $\mu$ M OA, 0.5  $\mu$ M fostriecin, 1  $\mu$ M I-2, and 1  $\mu$ M Gö6976.

**Chk1 dephosphorylation in vivo.** HeLa cells were treated with 20 mM HU for 2 h to increase phosphorylated Chk1, washed twice with phosphate-buffered saline (PBS), and cultured in complete DMEM. Samples were collected at different time points after HU removal in the absence or presence of chemical inhibitors. Phosphorylation of Chk1 was assessed by Western blotting.

**RNAi treatment.** The knockdown of ATR was accomplished with an ATR-specific siRNA with the sequence 5'-AACUGACUCACGCCAACCCUC-3'. Approximately  $2 \times 10^5$  HeLa cells were seeded per well in six-well dishes the day before transfection. Transfection was performed using Oligofectamine (Invitrogen) according to the manufacturer's instructions. The final siRNA concentration was 100 nM. Forty-eight hours posttransfection, cells were treated with or without 1  $\mu$ M Gö6976 for 2 h and harvested in MCLB. Lysates were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose. The resulting membranes were blotted with the indicated antibodies. Proteins were visualized and quantified using the ECL+ enhanced chemiluminescence reagent (Amersham), which contains a chemifluorescence component for quantification on a Molecular Dynamics Storm Imager (Molecular Biosystems, Piscataway, NJ). To knockdown PP2A and PP1, approximately  $2 \times 10^5$  HeLa cells were seeded per well in six-well dishes. The next day, cells were transfected with Smartpool siRNA (Dharmacon) against the specific phosphatases using Dharmafect 2 reagent (Dharmacon) according to the manufacturer's instructions. The final siRNA concentration was 100 nM. Forty-eight hours later, cells were treated with or without 1  $\mu$ M Gö6976 for 1 h and harvested in MCLB. Lysates were analyzed by immunoblotting or were used to dephosphorylate ectopic green fluorescent protein (GFP)-Chk1 (see below). The Smartpool reagents were the scrambled control (D00121002), PP1 (M00892700), and PP2A (M00359800 and M00359902).

**Chk1 dephosphorylation in vitro.** To generate substrate for phosphatase reactions, approximately  $2 \times 10^6$  HeLa cells per 100-mm dish were seeded and transfected the next day with 10  $\mu$ g of pEGFP-Chk1 WT using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. One day after transfection, cells were treated with 20 mM HU for 2 h to induce phosphorylation of GFP-Chk1. Cells were lysed in PBS lysis buffer (137 mM NaCl, 2.7 mM KCl, 4.3 mM  $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ , 1.4 mM  $\text{KH}_2\text{PO}_4$ , 0.5% Nonidet P-40, and 2 mM DTT) containing 2 mM phenylmethylsulfonyl fluoride (PMSF), 10  $\mu$ g/ml of aprotinin, and 5  $\mu$ g/ml of leupeptin. Phosphorylated GFP-Chk1 was immunoprecipitated using 20  $\mu$ l of prewashed anti-GFP beads (MBL International) at 4°C for 2 h. Immunoprecipitated GFP-Chk1 was washed five times with PBS lysis

buffer, twice with LiCl buffer (0.5 M LiCl, 50 mM Tris, pH 8), and twice with phosphatase buffer (50 mM Tris, pH 7, 0.1 mM  $\text{CaCl}_2$ , 1 mM  $\text{MnCl}_2$ ) and resuspended in phosphatase buffer. For dephosphorylation in siRNA-treated cell lysates, 20  $\mu$ l of GFP-Chk1 substrate was added to 200  $\mu$ g of lysate in MCLB buffer. Dephosphorylation reactions were performed at 30°C for 30 min. For in vitro phosphatase reactions, 20  $\mu$ l of GFP-Chk1 substrate was incubated with 100 ng of purified PP1 or PP2A (Sigma-Aldrich Co.) for 30 min at 30°C in the presence or absence of chemical inhibitors. Dephosphorylation of GFP-Chk1 was analyzed by Western blotting.

**Flow cytometry.** HeLa cells were harvested by trypsinization and collected by centrifugation. Cells were washed once with PBS and fixed in 5 ml of 70% ethanol at 4°C. Cells were washed once with PBS-1% bovine serum albumin (BSA) and then incubated with 1 ml of PBS-1% BSA containing 30  $\mu$ g/ml propidium iodide (PI) and 0.25 mg/ml RNase A for 1 h at room temperature. Cells were analyzed for DNA content by flow cytometry using a FACS Calibur fluorescence-activated cell sorter (BD Biosciences). For experiments using pEGFP plasmids, only GFP-positive cells were analyzed for DNA content. To gate on GFP-positive cells, untransfected cells stained with PI were analyzed first and located at less than  $10^1$  on the x axis (FL-1 channel) in histograms. Transfected cells were then analyzed and DNA content was determined on cell populations that located greater than  $10^1$  on the x axis in histograms. The data were analyzed using CellQuest analysis software (BD Biosciences).

**Indirect immunofluorescence.** HeLa cells were cultured on glass coverslips and treated as indicated in the figure legends. Cells were fixed in ice-cold 100% methanol at -20°C for 15 min and permeabilized with ice-cold acetone for 30 s. Cells were incubated in blocking solution (PBS-1% BSA) for 1 h at room temperature and then incubated with anti- $\gamma$ H2AX at 1:400 for 2 h at room temperature. Cells were washed five times in washing solution (PBS-1% BSA), incubated in fluorescein isothiocyanate (FITC)-labeled goat anti-mouse secondary antibody (Molecular Probes) for 1 h at room temperature, and then washed five more times. Images were obtained using a confocal laser-scanning microscope (MRC 1024; Bio-Rad).

## RESULTS

**Chk1 inhibitors induce Chk1 phosphorylation on S317 and S345.** Genotoxic stress activates a protein kinase-signaling cascade that initiates with the ATM and ATR protein kinases and ultimately signals to the Chk1 protein kinase (25, 40). ATR directly phosphorylates Chk1 and, depending on the type of genotoxic stress encountered, Chk1 phosphorylation by ATR is either ATM dependent or independent (1, 24, 31, 50). Chk1 residues phosphorylated by ATM/ATR include S317 and S345, and phosphorylation of Chk1 on these C-terminal residues is used to monitor the activation state of Chk1 in vivo (31, 50). Thus, it was surprising to observe that incubation of cells with three different Chk1 inhibitors, UCN-01 (7, 20), Gö6976 (29), and SB-218078 (12), caused Chk1 to accumulate in an S317/S345-phosphorylated (presumably active) form (Fig. 1). The accumulation of Cdc25A in cells treated with each Chk1 inhibitor indicated that the inhibitors blocked Chk1 function in vivo (Fig. 1A and B). The phosphorylation of Chk1 on S317 and S345 was observed within 15 min of drug addition and peaked after 90 min in asynchronously growing cells (Fig. 1A). The ability of Chk1 inhibitors to induce Chk1 phosphorylation was greatest in the S and G<sub>2</sub> phases of the cell cycle (Fig. 1C). Importantly, Gö6976 does not detectably alter the cell cycle distribution of treated cells, demonstrating that the alterations in Chk1 phosphorylation reported in this study are not due to indirect effects of Chk1 inhibitors on the cell division cycle (see Table S1 in the supplemental material).

**ATR mediates Chk1 phosphorylation in presence of Chk1 inhibitors.** Several approaches were taken to determine if ATM and/or ATR was required for Chk1 phosphorylation under these conditions (Fig. 2). The ability of the Chk1 inhibitor Gö6976 to induce phosphorylation of Chk1 on S345 was

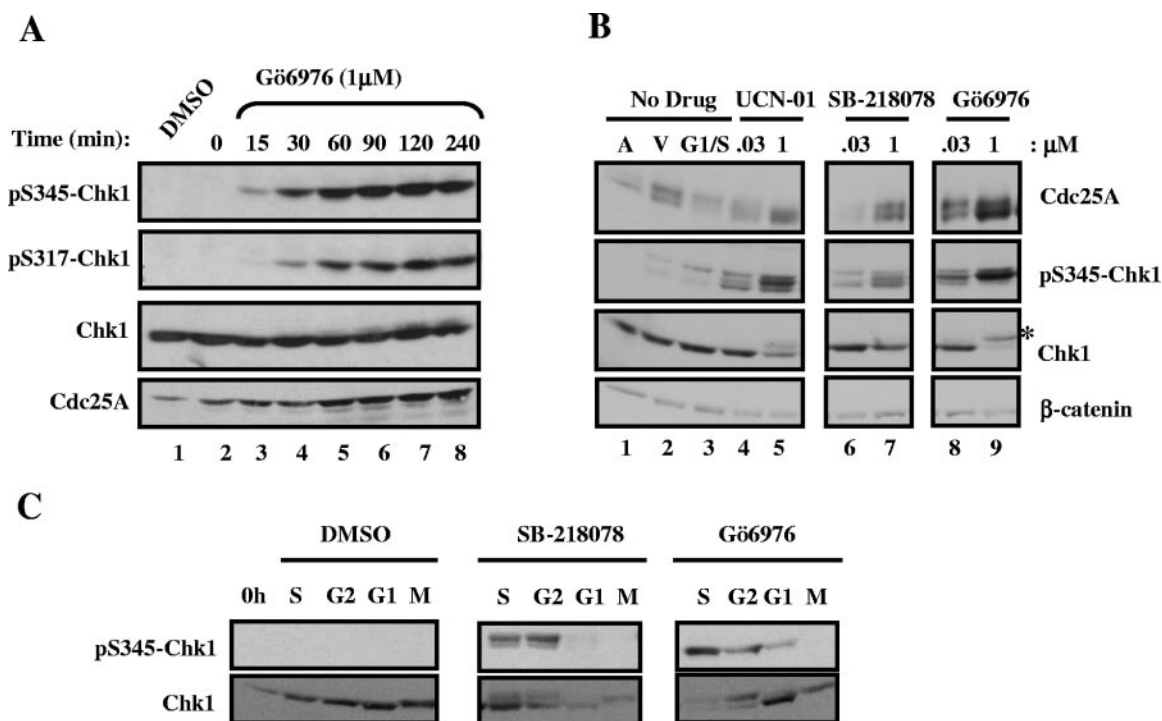


FIG. 1. Chk1 inhibitors induce Chk1 phosphorylation on S317 and S345 in vivo. (A) Asynchronously growing HeLa cells were treated with either vehicle (DMSO) or 1  $\mu$ M Gö6976 for the indicated times. Western blotting was performed with the indicated antibodies. (B) HeLa cells, synchronized by a double-thymidine-block protocol, were released into S phase and treated with different concentrations of either UCN-01, SB-218078, or Gö6976 for 1.5 h. Total lysates were probed with antibodies specific for Cdc25A, phosphorylated Chk1 (pS345), total Chk1, and  $\beta$ -catenin. Controls included asynchronously growing cells (Asy), cells treated with the vehicle DMSO (V), and cells collected at 0 h before release (G<sub>1</sub>/S). The asterisk indicates phosphorylated Chk1. (C) HeLa cells synchronized by a double-thymidine-block protocol were released and harvested at the indicated times: 1.5 h (S phase), 3 h 45 min (G<sub>2</sub> phase), and 10 h 50 min (G<sub>1</sub> phase). In addition, nocadazole was added for 10 h 50 min after release to collect M-phase cells. DMSO, SB-218078, or Gö6976 (5  $\mu$ M) was added 1.5 h prior to harvest of cells at each cell cycle stage. Total lysates were prepared and analyzed by Western blotting.

reduced in cells pretreated with wortmannin (Fig. 2A, lane 4), a phosphatidylinositol 3-kinase inhibitor that inhibits ATM, DNA protein kinase, and ATR (39). Gö6976 also effectively induced Chk1 S345 phosphorylation in cells lacking functional ATM (Fig. 2B, lanes 5 and 6), demonstrating that ATM is not necessary for induction of Chk1 phosphorylation in vivo by Gö6976. Finally, partial knockdown of ATR reduced Chk1 S345 phosphorylation by 40% in Gö6976-treated cells (Fig. 2C, lane 4). Taken together, these results indicate that ATR contributes to Chk1 phosphorylation in cells treated with Chk1 inhibitors.

**Chk1 inhibitors do not detectably activate ATR or induce  $\gamma$ H2AX foci.** One explanation to account for these results is that Chk1 inhibitors directly or indirectly activate ATR to promote Chk1 phosphorylation in vivo. To test this possibility, the phosphorylation statuses of general and specific ATR substrates were monitored in cells treated with Gö6976 (Fig. 3). As positive controls, cells were treated with VP-16 to activate ATM or with HU to activate ATR. As seen in Fig. 3A (lane 2), Gö6976 did not induce phosphorylation of substrates seen in cells with activated ATM (lane 3) or ATR (lane 4). Nor did Gö6976 treatment induce significant phosphorylation of the ATM/ATR substrates Brca1 (Fig. 3B) and Smc1 (Fig. 3C). Finally, phosphorylation of ATM on S1981 (5) was not observed in Gö6976-treated cells (Fig. 3C).

A previous study conducted with U2OS cells reported that Chk1 inhibition activates a DNA damage checkpoint response, as indicated by phosphorylation of the histone variant H2AX, and this, in turn, induced Chk1 phosphorylation (43). Thus, we monitored for the appearance of phosphorylated H2AX ( $\gamma$ H2AX) at discrete nuclear foci in Gö6976-treated cells because this is one of the earliest events observed in cells with DNA double-strand breaks (DSBs). As seen in Fig. 3D,  $\gamma$ H2AX foci were readily detected in cells treated with VP-16 or HU. In contrast, incubation of cells with Gö6976 for 1 or 2 h did not increase the number of  $\gamma$ H2AX foci relative to control cells. However, an increase in the number of  $\gamma$ H2AX foci was observed in cells treated with Gö6976 for longer periods (6 h). Thus, the rapid phosphorylation of Chk1 on S317 and S345 following Chk1 inhibition is not accounted for by activation of a DNA damage checkpoint response in HeLa cells.

**Chk1 inhibitors impair Chk1 dephosphorylation in vivo but not in vitro.** Given that Chk1 inhibitors do not induce significant ATR activation in HeLa cells, we next asked whether they impair Chk1 dephosphorylation. Cells were treated with HU to enrich for phosphorylated Chk1. HU was then removed, and Chk1 dephosphorylation on S317 and S345 was monitored in vivo. As seen in Fig. 4A, dephosphorylation of S317 and S345 occurred in a time-dependent manner in control cells treated with DMSO (lanes 3 and 4). Importantly, incubation of cells

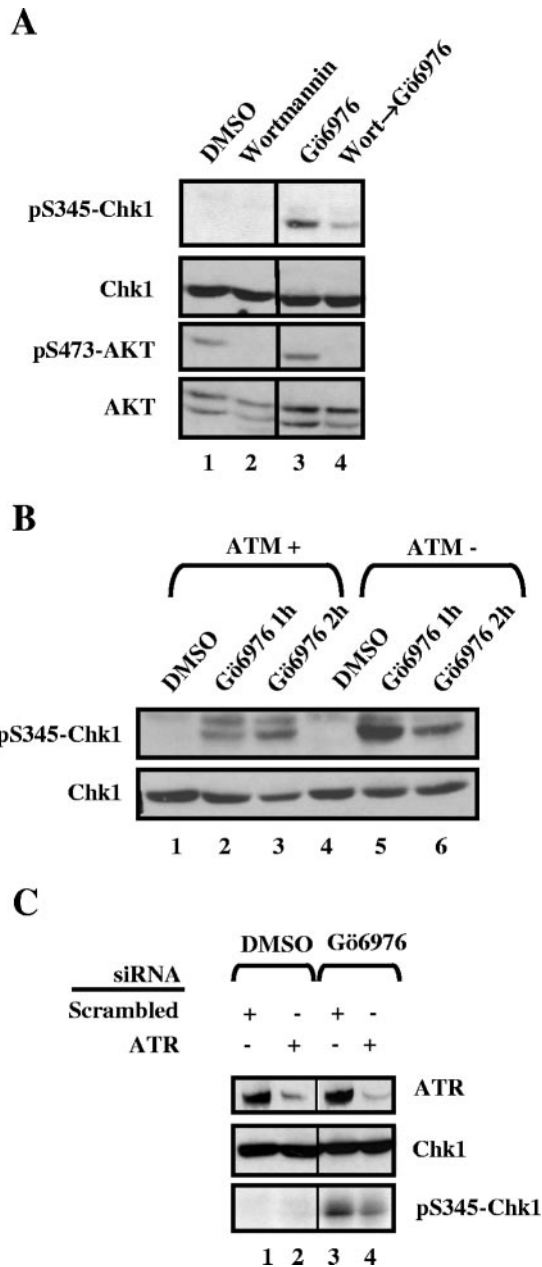


FIG. 2. Gö6976-induced phosphorylation of Chk1 is mediated by ATR. (A) Asynchronously growing HeLa cells were treated with vehicle (DMSO), 50  $\mu$ M wortmannin for 2 h, or 1  $\mu$ M Gö6976 for 1 h. HeLa cells were also pretreated with wortmannin for 2 h and then treated with Gö6976 for 1 h. As a positive control for wortmannin, inhibition of Akt phosphorylation was analyzed. Western blotting was performed with the indicated antibodies. (B) Cells expressing functional ATM (ATM+) or cells lacking functional ATM (ATM-) were incubated with 1  $\mu$ M Gö6976 for 1 h or 2 h. Western blotting was performed with the indicated antibodies. (C) HeLa cells were transfected with 100 nM ATR-specific siRNAs or scrambled siRNAs. After 48 h, cells were treated with DMSO or 1  $\mu$ M Gö6976 for 1 h and then harvested for Western blotting with the indicated antibodies.

with the Chk1 inhibitor Gö6976 subsequent to HU removal impaired S317 and S345 dephosphorylation (lanes 5 and 6) as did incubation with the phosphatase inhibitor OA (lanes 7 and 8). Chk1 phosphorylation was maximally inhibited in vivo at

Gö6976 concentrations between 0.3 and 1  $\mu$ M (see Fig. S1A in the supplemental material).

Interestingly, treatment of cells with OA alone (Fig. 4A, lane 11), like Gö6976 (lane 10), resulted in the accumulation of phosphorylated Chk1 in vivo.

To determine whether Gö6976 functions to inhibit the phosphatase or phosphatases that dephosphorylate Chk1 on S317 and S345, the dephosphorylation of Chk1 was monitored in cell lysates in vitro (Fig. 4B). Lysates were incubated with DMSO, Gö6976, or OA, and the phosphorylation status of endogenous Chk1 was monitored after a 30-min incubation period. OA at a concentration of 10 nM effectively blocked Chk1 dephosphorylation in vitro (lane 8). In contrast, Gö6976 did not prevent Chk1 dephosphorylation in vitro even at concentrations of 2  $\mu$ M (lane 6). These results demonstrate that the Chk1 inhibitors do not directly inhibit the phosphatase(s) responsible for dephosphorylating Chk1 on S317 and S345 and that the Chk1 phosphatase(s) may be OA sensitive.

**Chk1 kinase activity positively regulates Chk1 dephosphorylation.** Taken together, these results demonstrate that the Chk1 inhibitors do not function by directly stimulating Chk1 phosphorylation or by directly inhibiting Chk1 dephosphorylation. Another model that accounts for the data is that Chk1 is continually being phosphorylated on S317 and S345 in vivo and at the same time Chk1 promotes its own dephosphorylation. In this case, inhibition of Chk1 kinase activity would result in a net reduction in Chk1 dephosphorylation and by default a net increase in phosphorylated Chk1. This model predicts that levels of phosphorylated kinase-inactive Chk1 would be higher than levels of phosphorylated kinase-active Chk1 because kinase-inactive Chk1 would be incapable of stimulating its own dephosphorylation. As seen in Fig. 5A, kinase-inactive Chk1 (lane 6) was more highly phosphorylated on S345 than was wild-type Chk1 (lane 1) under basal conditions. This model also predicts that dephosphorylation of kinase-inactive Chk1 might occur more slowly than dephosphorylation of wild-type Chk1 in vivo. To test this, cells expressing wild-type or kinase-inactive GFP-Chk1 were treated with HU to induce phosphorylation of Chk1. HU was then removed to allow Chk1 dephosphorylation to occur in vivo. The phosphorylation status of Chk1 was monitored at various times after HU removal. As seen in Fig. 5A, dephosphorylation of wild-type Chk1 (lanes 2 to 5) occurred more quickly than did dephosphorylation of kinase-inactive Chk1 (lanes 7 to 10). FACS analysis revealed that the cell cycle distributions of cells expressing kinase-active and -inactive forms of Chk1 were similar (Fig. 5B). Thus, the differences in phosphorylation observed between kinase-active and -inactive forms of Chk1 cannot be attributed to cell cycle differences. These results are consistent with a model whereby Chk1 dephosphorylation is regulated, at least in part, by Chk1 kinase activity.

**PP2A dephosphorylates Chk1 in vivo and in vitro.** Enhanced phosphorylation of Chk1 was observed in cells treated with OA (Fig. 4A) and OA efficiently blocked Chk1 dephosphorylation in extracts incubated in vitro (Fig. 4B). These findings identify the PPP family of protein phosphatases as potential Chk1 regulators because this family is sensitive to inhibition by OA (23). The dephosphorylation of Chk1 on S317 was monitored in extracts supplemented with additional phosphatase inhibitors in order to aid in the identification of cellular phosphatases

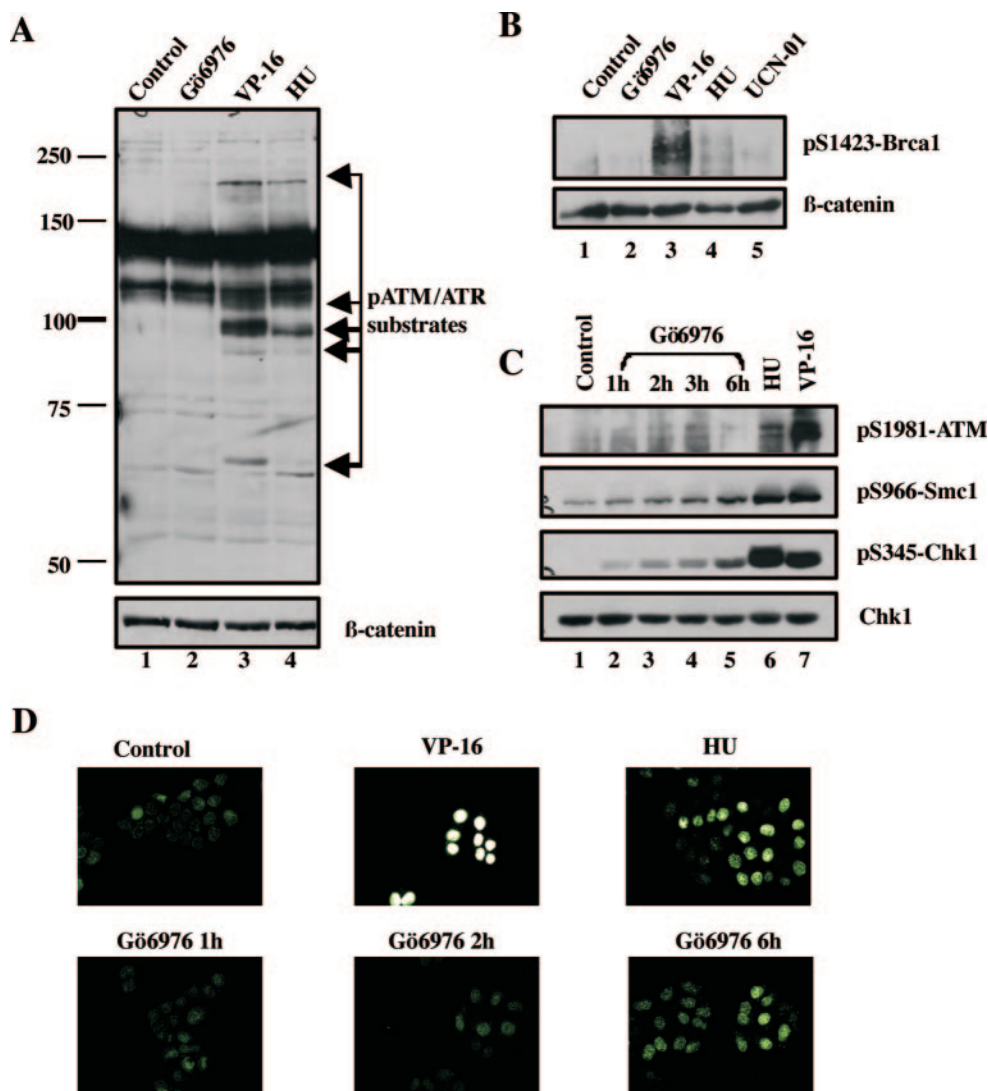


FIG. 3. Gö6976 does not induce global phosphorylation of ATR targets or  $\gamma$ H2AX foci. HeLa cells were untreated (control) or were incubated with 20 mM HU (2 h), 20  $\mu$ M VP-16 (4 h), 1  $\mu$ M Gö6976 (1 h), or 1  $\mu$ M UCN-01 (2 h). Cell lysates were resolved by SDS-PAGE and subjected to Western blotting with antibodies that recognize phosphorylated substrates of ATM/ATR (A) or phosphorylated Brca1 (B).  $\beta$ -Catenin antibodies were used to verify equal loading. (C) HeLa cells were treated as in panels A and B, except that cells were incubated with Gö6976 for 1 h, 2 h, 3 h, and 6 h before harvest. Samples were analyzed by Western blotting for pS1981-ATM, pS966-Smc1, pS345-Chk1, and Chk1. (D) HeLa cells were treated as in panel C and analyzed by indirect immunofluorescence for  $\gamma$ H2AX foci.

responsible for Chk1 dephosphorylation (Fig. 6A). Chk1 dephosphorylation occurred normally in extracts containing DMSO (lane 3), Gö6976 (lane 4), or the PP1-specific inhibitor I-2 (lane 7). These results demonstrate that PP1 is not required for Chk1 dephosphorylation in this assay. In contrast, fostriecin (lane 6), like OA (lane 5), impaired Chk1 dephosphorylation in vitro. Fostriecin is a potent inhibitor of PP2A, PP4, and possibly PP6; is a poor inhibitor of PP1; and does not inhibit PP5 (6, 30, 53). Taken together, these results suggest that PP2A, PP4, and PP6 may contribute to Chk1 dephosphorylation in vivo.

Next, the consequences of reducing levels of specific protein phosphatases on Chk1 phosphorylation in vivo were assessed (Fig. 6B). If Chk1 is undergoing a continual cycle of phosphorylation/dephosphorylation, then enhanced Chk1 phosphoryla-

tion should be observed in vivo if the appropriate phosphatase is knocked down. As seen in Fig. 6B, siRNAs specific for individual protein phosphatases significantly reduced levels of PP1 and PP2A without affecting levels of PP4 or PP6 (Fig. 6B and C). Importantly, enhanced phosphorylation of Chk1 on S317 and S345 was observed in PP2A-deficient cells in both the absence and presence of Gö6976 (Fig. 6B, lanes 3 and 6). In contrast, knockdown of PP1 did not cause Chk1 to accumulate in a phosphorylated form (Fig. 6B, lanes 2 and 5), confirming that PP1 does not contribute to S317 and S345 dephosphorylation in vivo. Interestingly, despite the higher levels of phosphorylated Chk1 in PP2A-deficient cells (Fig. 6B, lanes 3 and 6), overall levels of Chk1 were reduced in these cells. To test if phosphorylation impaired Chk1 recognition by Chk1 antibodies, samples were treated with  $\lambda$  phosphatase to dephosphor-

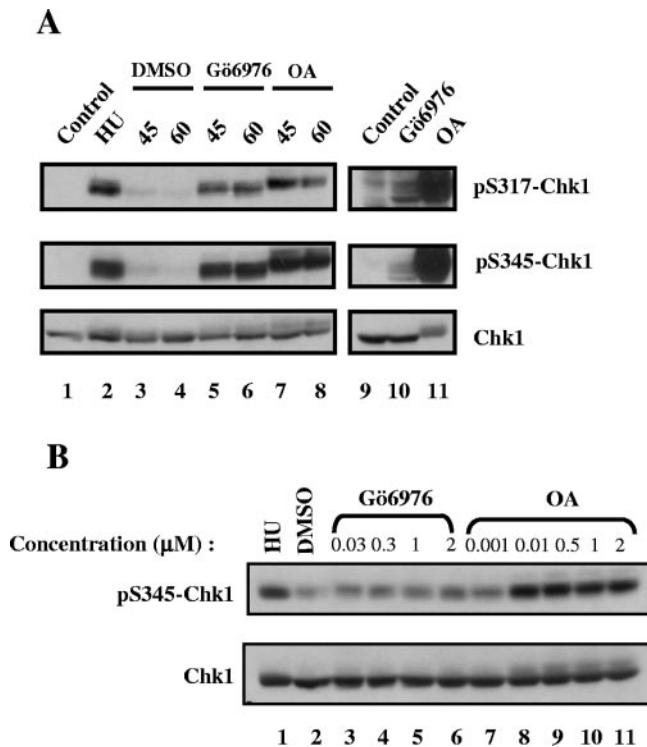
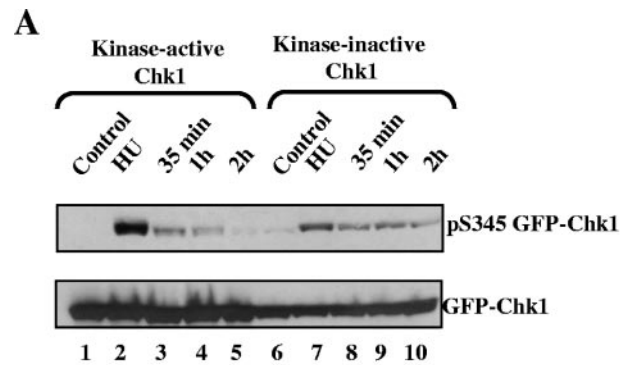


FIG. 4. OA impairs Chk1 dephosphorylation in vivo and in vitro. (A) HeLa cells were untreated (lane 1) or were incubated with 20 mM HU for 2 h to generate phosphorylated Chk1 (lanes 2 to 8). The culture medium was then removed, and cells were incubated in complete medium containing DMSO, 1 μM Gö6976, or 1 μM OA. Chk1 phosphorylation was monitored at 45 min and 60 min after HU removal by Western blot analysis. Alternatively, HeLa cells were incubated with 1 μM Gö6976 or 1 μM OA for 1 h followed by Western blot analysis (lanes 10 and 11). (B) HeLa cells were incubated with 20 mM HU for 2 h to generate phosphorylated Chk1. Cells were washed, and lysates were prepared and either analyzed directly by SDS-PAGE and Western blotting (lane 1) or were incubated in vitro in the presence of DMSO (lane 2) or the indicated concentrations of Gö6976 (lanes 3 to 6) or OA (lanes 7 to 11) for 30 min at 30°C. Reaction products were resolved by SDS-PAGE, and endogenous Chk1 was monitored by Western blotting.

ylate Chk1. Immunoblotting revealed that phosphorylated and dephosphorylated Chk1 reacted equivalently with Chk1 antibodies, allowing us to conclude that samples deficient in PP2A had lower overall levels of Chk1 (data not shown). A recent report by Zhang et al. (49) indicates that S345-phosphorylated Chk1 is targeted for ubiquitin-mediated proteolysis, and this could account for the reduced levels of Chk1 observed in PP2A-deficient cells (49).

The level of increased Chk1 phosphorylation in cells treated with the PP2A siRNA (Fig. 6B, lane 3) was less than that in Gö6976-treated cells (Fig. 6B, lane 4). If Gö6976 acts solely by disrupting the Chk1-PP2A regulatory circuit, then the effects of PP2A siRNA and Gö6976 on Chk1 phosphorylation should be similar. The differences observed in Fig. 6B can be accounted for in a couple of ways. Since PP2A acts catalytically, the reduction in PP2A protein achieved by siRNA treatment may not translate into a corresponding reduction in PP2A enzymatic activity. Thus, there may not be a direct correlation between loss of PP2A protein and increase in phospho-Chk1.



**B**

		Control	HU	Recovery from HU		
				35 m	1 h	2 h
				Kinase Active	G1	68 %
	S	13 %	12 %	12 %	13 %	12 %
	G2/M	19 %	16 %	15 %	15 %	14 %
Kinase Inactive	G1	66 %	72 %	73 %	74 %	76 %
	S	14 %	12 %	11 %	11 %	10 %
	G2/M	20 %	16 %	16 %	15 %	14 %

FIG. 5. Kinase-inactive Chk1 exhibits higher basal phosphorylation and slower kinetics of dephosphorylation. HeLa cells were transiently transfected with kinase-active GFP-Chk1 or kinase-inactive GFP-Chk1. Twenty-four hours posttransfection, cells were left untreated (lanes 1 and 6) or were incubated with HU for 2 h. HU was removed, and cells were harvested immediately (lanes 2 and 7) or were cultured in complete medium for the indicated times. (A) A fraction of the cells were lysed and analyzed by Western blotting with the indicated antibodies. (B) The remaining cells were stained for DNA content, and GFP-positive cells were analyzed by flow cytometry. The percentages of cells in each phase of the cell cycle are indicated.

Another explanation to account for this result is that PP2A may not be the only phosphatase that contributes to Chk1 dephosphorylation. For example, we have not ruled out a role for PP4 or PP6—both are OA and fostriecin sensitive and both share regulatory subunits with PP2A. In addition, PPM1D is known to dephosphorylate Chk1 (32). Thus, levels of phosphorylated Chk1 would be higher in Gö6976-treated cells than in PP2A-siRNA-treated cells if Gö6976 treatment affected the ability of phosphatases in addition to PP2A to dephosphorylate Chk1.

Next, lysates prepared from PP2A-deficient cells were tested for their ability to dephosphorylate Chk1 in vitro (Fig. 6C). Dephosphorylation of Chk1 on S317 and S345 was observed in lysates prepared from cells treated with scrambled siRNAs (lane 2). In contrast, Chk1 remained stably phosphorylated on S317 and S345 when incubated in OA-treated lysates (lane 1) and PP2A-deficient lysates (lane 3). Finally, purified PP2A was tested for its ability to directly dephosphorylate Chk1 on S317 and S345 in vitro. As seen in Fig. 6D, PP2A (lane 3) but not PP1 (lane 2) was able to dephosphorylate Chk1 in vitro. OA (lane 5) but not Gö6976 (lane 4) inhibited Chk1 dephosphorylation by PP2A in vitro, supporting the conclusion that Gö6976 is not a phosphatase inhibitor.



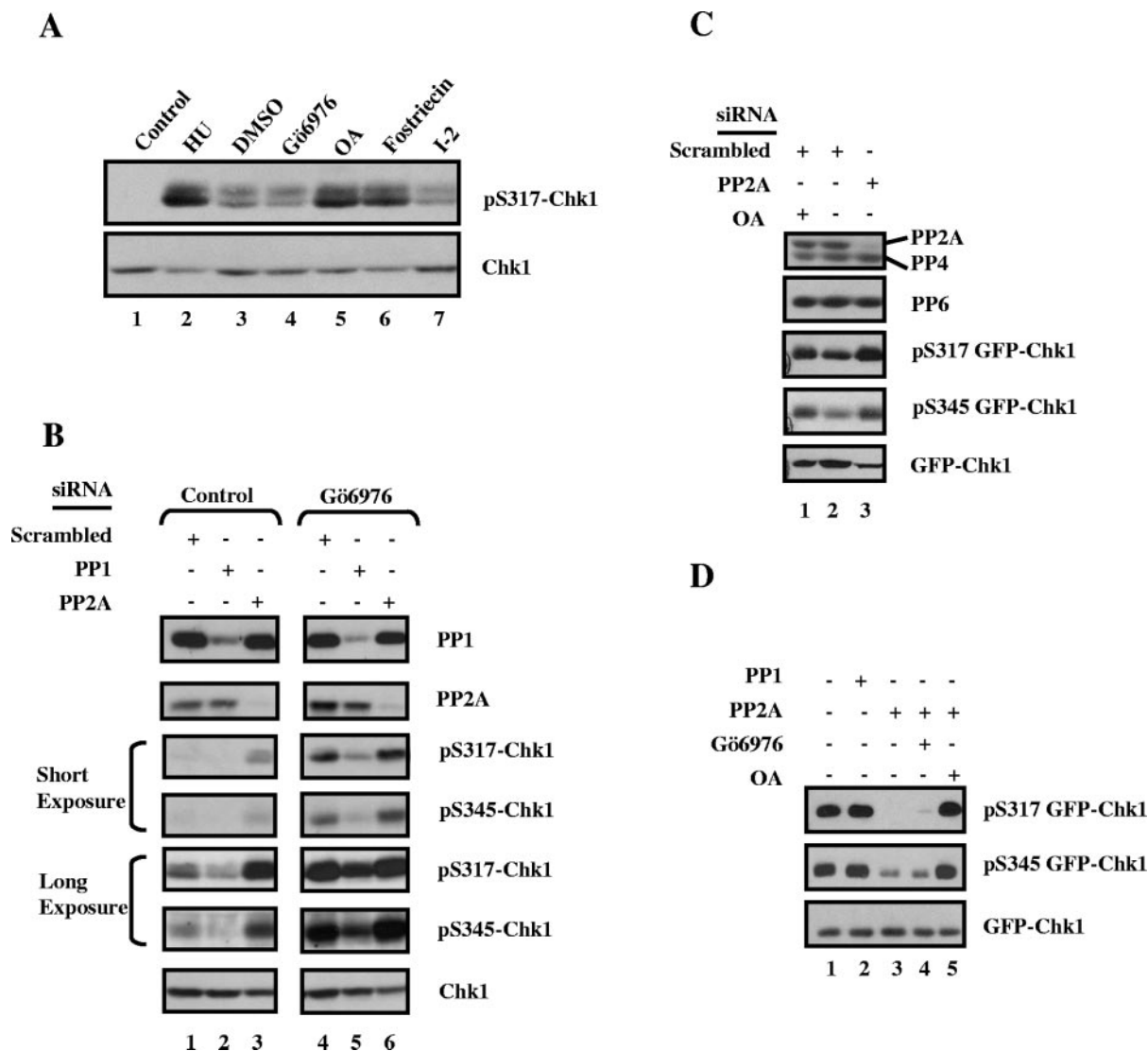


FIG. 6. PP2A dephosphorylates Chk1 on S317 and S345. (A) HeLa cells were untreated (lane 1) or were incubated in the presence of HU for 2 h to induce Chk1 phosphorylation. Lysates derived from HU-treated cells were harvested immediately (lane 2) or were incubated in the presence of DMSO (lane 3), 1  $\mu$ M Gö6976 (lane 4), 1  $\mu$ M OA (lane 5), 0.5  $\mu$ M fostriecin (lane 6), or 1  $\mu$ M I-2 (lane 7) for 30 min at 30°C (lanes 3 to 7). Phosphorylation of Chk1 was assayed by immunoblotting. (B) Targeted knockdown of specific protein phosphatases was accomplished by transfecting HeLa cells with the indicated siRNAs. After 48 h, cells were either untreated (lanes 1 to 3) or were incubated with 1  $\mu$ M Gö6976 for 1 h (lanes 4 to 6). Cell lysates were prepared and analyzed for phosphorylated Chk1, total Chk1, PP1, and PP2A by Western blotting using the Femto maximum sensitivity substrate (Pierce). Short (5 s) and long (15 s) exposures of the Western blot are shown. (C) HeLa cells were transfected with scrambled or PP2A-specific siRNAs. Lysates were prepared 48 h later and incubated *in vitro* with phosphorylated GFP-Chk1 for 30 min at 30°C in the presence or absence of OA. Western blotting was performed with the indicated antibodies. (D) Purified PP1 or PP2A was incubated with phosphorylated GFP-Chk1 immunoprecipitated from HU-treated cells for 30 min at 30°C in the presence or absence of 1  $\mu$ M Gö6976 or 1  $\mu$ M OA. Reaction products were resolved by SDS-PAGE and analyzed by Western blotting.

**DISCUSSION**

Phosphorylation of Chk1 on S317 and/or S345 has been the gold standard in the checkpoint field for monitoring the activation state of Chk1 *in vivo* (31, 50). Here we report that S317/S345-phosphorylated Chk1 (the presumably “active” form of Chk1) paradoxically accumulates in cells treated with Chk1 inhibitors. The stabilization and accumulation of Cdc25A were observed in cells treated with each Chk1 inhibitor, confirming that they were effective at blocking Chk1 ac-

tivity *in vivo*. Furthermore, we demonstrated that phosphorylation of Chk1 under these conditions requires ATR.

One might predict that Chk1 inhibition would elicit a checkpoint response leading to activation of ATM/ATR, and this, in turn, would lead to enhanced Chk1 phosphorylation on S317 and S345. However, Chk1 phosphorylation in response to Chk1 inhibition was detected within 15 min of treatment with Chk1 inhibitors (Fig. 1A), yet DNA damage, as measured by the appearance of  $\gamma$ H2AX foci, was not detected until after 2 h of Gö6976 treatment (Fig. 3D). In addition, phosphorylation

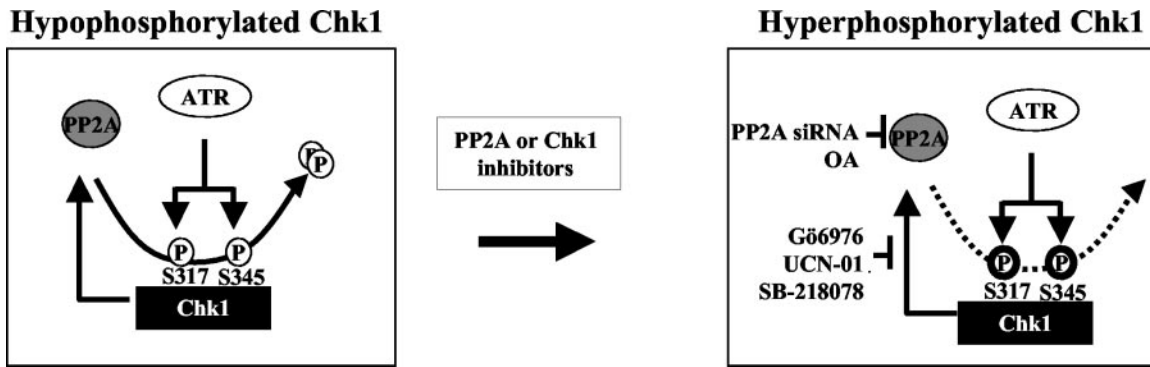


FIG. 7. The ATR-Chk1-PP2A regulatory circuit. During an unperturbed cell division cycle, Chk1 is continually being phosphorylated on S317 and S345 by ATR. However, phosphorylated Chk1 does not accumulate to any significant extent because PP2A continually dephosphorylates Chk1 on S317 and S345. Importantly, the ability of PP2A to dephosphorylate Chk1 depends, in part, on Chk1 kinase activity as chemical inhibition of Chk1 promotes the accumulation of phosphorylated Chk1 in vivo. Thus, Chk1 promotes its own dephosphorylation during an unperturbed cell division cycle.

of ATM/ATR targets was not readily detected in cells treated with Chk1 inhibitors (Fig. 3). These results led us to investigate the contribution made by protein phosphatases to Chk1 regulation. Several interesting observations relevant to Chk1 dephosphorylation were made. First, phosphorylated Chk1 accumulated in cells treated with OA while both OA and fostriecin but not I-2 blocked Chk1 dephosphorylation in cell extracts. Second, S317/S345-phosphorylated Chk1 accumulated in cells where PP2A levels were reduced by siRNA treatment and PP2A was able to directly dephosphorylate Chk1 on S317 and S345 in vitro. Taken together, these findings argue that PP2A regulates Chk1 in vivo. Dis2, the fission yeast homolog of PP1, dephosphorylates S345 in fission yeast Chk1 (13) and PP1 dephosphorylates a human Chk1 S345 phosphopeptide in vitro (32), suggesting that PP1 might regulate both fission yeast and human Chk1. The following lines of evidence suggest that this is not the case: I-2 failed to block human Chk1 dephosphorylation in vitro (Fig. 6A), PP1 knockdown resulted in a decrease in levels of phosphorylated Chk1 in vivo (Fig. 6B), and PP1 was incapable of dephosphorylating full-length human Chk1 on S317 and S345 in vitro (Fig. 6D). Interestingly, PPM1D (or Wip1) has recently been shown to dephosphorylate Chk1 on S345 and to a lesser extent on S317 (32). PPM1D is a p53 transcriptional target gene that is insensitive to OA. Thus, in response to DNA damage, p53 induces PPM1D, which in turn may function to return Chk1 to a hypophosphorylated state as cells recover from checkpoint arrest. Our study indicates that PP2A has a role distinct from that of PPM1D in that it facilitates Chk1 dephosphorylation in the absence of genotoxic stress.

Given that ATR was not detectably activated in HeLa cells treated with Chk1 inhibitors, another model to explain the accumulation of phosphorylated Chk1 under conditions of Chk1 inhibition would be if Chk1 inhibitors also functioned as PP2A inhibitors. However, unlike OA, Chk1 inhibitors did not block Chk1 dephosphorylation in cell extracts or block the ability of PP2A to dephosphorylate Chk1 in vitro. A third important observation made in this study is that kinase-inactive Chk1 is phosphorylated on S317 and S345 under basal, unstressed conditions and its dephosphorylation occurs more slowly than does that of wild-type Chk1. Taken together, these

results suggest a regulatory circuit whereby ATR continually phosphorylates Chk1 on S317 and S345 and phosphorylated “active” Chk1 in turn, either directly or indirectly, stimulates PP2A to dephosphorylate S317 and S345 (Fig. 7). In this model, kinase-inactive Chk1 would be unable to stimulate its own dephosphorylation and therefore would be expected to accumulate in a more highly phosphorylated form in vivo. We conclude that Chk1 inhibitors disrupt the ATR/Chk1/PP2A feedback loop by blocking PP2A “activation” by Chk1 allowing the ATR/Chk1 phosphorylation circuit to dominate and likewise, phosphatase inhibitors like OA and fostriecin block the enzymatic activity of PP2A, thereby enabling phosphorylated Chk1 to accumulate. Future studies will focus on how Chk1 kinase activity contributes to Chk1 dephosphorylation in vivo. Possibilities include direct activation of PP2A by Chk1 or enhanced recognition of phosphorylated Chk1 by PP2A. In addition, it will also be important to understand how checkpoint activation pushes the equilibrium in favor of the ATR/Chk1 arm of the pathway and away from the Chk1/PP2A arm of the pathway.

Interestingly, treatment of cells with increasing doses of OA (see Fig. S1A in the supplemental material) or with a single dose of OA for increasing periods of time (see Fig. S1B in the supplemental material) caused phosphorylated Chk1 to accumulate to higher levels than in cells treated with Gö6976 and to even higher levels than that of the initial phospho-Chk1 at the start of the reaction. This suggests that, in vivo, OA stimulates both the kinase and phosphatase arms of the pathway (i.e., inhibits dephosphorylation of Chk1 and stimulates phosphorylation of Chk1). In contrast, treatment of cells with increasing doses of Gö6976 (see Fig. S1A in the supplemental material) or with a single dose of Gö6976 for increasing periods of time (see Fig. S1B in the supplemental material) blocked dephosphorylation of Chk1, but did not stimulate Chk1 phosphorylation above the initial phospho-Chk1 level at the start of the reaction. This result coupled to the observation that Gö6976 does not activate ATR, strongly argues that Gö6976 regulates the phosphatase rather than the kinase arm of the pathway. How does OA regulate the ATR-Chk1 arm of the pathway? Chowdhury et al. (9) demonstrated that DNA DSB repair and checkpoint recovery in vivo require dephos-

phorylation of  $\gamma$ H2AX by PP2A. OA blocks  $\gamma$ H2AX dephosphorylation, thereby sustaining/enhancing the DNA damage signaling pathway. These results predict that if HU-treated cells are incubated with OA subsequent to HU removal, these cells would fail to repair DNA DSBs and sustained incubation in the presence of OA might lead to enhanced DNA damage and further activation of checkpoint pathways. If this were the case, phosphorylated Chk1 would be expected to accumulate to higher levels than in cells treated with G $\ddot{6}$ 976 alone because OA would not only block Chk1 dephosphorylation by PP2A, it would also stimulate Chk1 phosphorylation by ATR.

Recent studies have uncovered important roles for serine/threonine protein phosphatases in regulating checkpoint responses. In addition to Chk1 (this study), PP2A regulates ATM (19, 38), Chk2 (14), and  $\gamma$ H2AX (9, 27). PP5 regulates ATM, ATR, and DNA protein kinase signaling (2, 46, 48). Recently the Pph3 phosphatase complex has been shown to dephosphorylate  $\gamma$ H2AX in budding yeast (27). PPM1D dephosphorylates Chk1, Chk2, and p53 (16, 32), and PP1 regulates fission yeast Chk1 (13) and *Xenopus* Cdc25 (35, 36). Interestingly, OA was shown to induce the autophosphorylation of ATM on serine 1981 in unirradiated cells, indicating that PP2A functions during a normal cell division cycle to counteract the autoactivation tendencies of ATM (19). Thus, PP2A functions to maintain both ATM and Chk1 in a low-activity state during an unperturbed cell division cycle. In the case of ATM, PP2A counteracts ATM autophosphorylation, whereas in the case of Chk1, PP2A counteracts ATR-mediated phosphorylation. The net effect is to prevent ATM and Chk1 from relaying checkpoint signals when cells are not experiencing genotoxic stress.

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