Protein phosphatase 2A interacts with Chk2 and regulates phosphorylation at Thr-68 after cisplatin treatment of human ovarian cancer cells

XIAOBING LIANG, EDDIE REED and JING JIE YU

Department of Biochemistry and Molecular Pharmacology, Mary Babb Randolph Cancer Center, Robert C. Byrd Health Sciences Center, West Virginia University, 1610-C Health Sciences South, Morgantown, WV 26506-9300, USA

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Abstract. High-fidelity maintenance of genomic integrity in eukaryotes is ensured by cell cycle checkpoints and DNA repair. The checkpoint kinase, Chk2, has been implicated in both of these responses. In response to DNA damage, Chk2 is initially phosphorylated at Thr-68, which leads to its full activation. The fully activated Chk2 then phosphorylates downstream substrates of cell cycle control. However, the mechanism of inactivation of Chk2 is still unknown. Protein phosphatase type 2A (PP2A) plays an essential role in cell cycle regulation and induction of G_2 arrest by a mechanism of phosphorylation/dephosphorylation with a variety of protein kinases. Data from our investigation provide evidence that, in response to cisplatin exposure, PP2A associates with Chk2 as a complex in cells and functions as a negative regulator of Chk2 activation by dephosphorylating p-Chk2. Results from immunostaining and coimmunoprecipitation demonstrate that Chk2 and PP2A can colocalize in cells, and the holoenzyme of PP2A (subunits A, B and C) coimmunoprecipitates with p-Chk2. Further, inhibition of PP2A by okadaic acid, an inhibitor of PP2A, and by small interfering RNA (siRNA) to PP2A results in enhanced Chk2 phosphorylation, implicating a direct enzyme-substrate relationship. An *in vitro* PP2A dephosphorylation assay shows that PP2A dephosphorylates p-Chk2 in a cell-free system. These findings suggest that the protein serine/threonine kinase, Chk2, is activated after cisplatin exposure and negatively regulated by a tightly associated protein serine/threonine phosphatase, PP2A.

Introduction

To protect genomic integrity and ensure survival, mammalian cells exposed to genotoxic stress halt cell cycle progression and result in apoptosis or DNA repair. Disruption of the cell cycle checkpoint/DNA-repair system leads to genomic instability and the development of cancer (1). Chk2, the mammalian homolog of the checkpoint kinases, Cds1 (*Schizosaccharomyces pombe*) and Rad53 (*Saccharomyces cerevisiae*), is a DNA damage-activated protein kinase (2). In response to IR and dsDNA breaks, Chk2 is initially phosphorylated at Thr-68, which then leads to further activation at Thr-383 and Thr-387, as well as autophosphorylation of Chk2 in a phosphothreonine 68-dependent manner (3-6). The fully activated Chk2 phosphorylates downstream substrates of cell cycle control (2-7). $Chk2^{-/-}$ ES cells are defective in maintaining IR-induced cell cycle arrest at G2 phase (8). Heterozygous germline mutations in the Chk2 gene have been identified in cancer predisposition syndrome Li-Fraumeni patients and other human cancers, suggesting that Chk2 plays a pivotal role in controlling activities of the cell cycle checkpoint (9,10).

The heterotrimeric protein, phosphatase type 2A (PP2A) is a major phosphatase involved in cell cycle checkpoints, regulation of signaling pathways and inactivation of a variety of kinases by a mechanism of phosphorylation/dephosphorylation of proteins (11,12). The holoenzyme of PP2A consists of a core AC heterodimer associated with a variety of regulatory B subunits (B=PR55, B'=B56 and B"=PR72) (13-15). These B subunits regulate the subcellular localization and substrate specificity of PP2A, and distinct PP2A heterotrimers can dephosphorylate different sites on the same substrate (16). In mammalian cells, PP2A is widely expressed and mutations in PP2A subunits are found in multiple cancers (17,18).

Our hypotheses were that the checkpoint kinase, Chk2, may be inactivated by PP2A and that the two proteins, serine/threonine kinase Chk2 and serine/threonine phosphatase PP2A, may directly interact in DNA damage-repair signaling. In this report, we provide evidence that PP2A associates with Chk2 as a complex and functions as a negative regulator of Chk2 signaling in response to cisplatin stimulation. This occurs via dephosphorylating p-Chk2 after cisplatin treatment in human ovarian cancer cells.

Materials and methods

Cell culture and treatment. The A2780 human ovarian cancer cell line was used in the current investigation. Cells were

Correspondence to: Dr Jing Jie Yu, Department of Biochemistry and Molecular Pharmacology, Mary Babb Randolph Cancer Center, Robert C. Byrd Health Sciences Center, West Virginia University, 1610-C Health Sciences South, Morgantown, WV 26506-9300, USA
E-mail: jyu@hsc.wvu.edu

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cultured in monolayer using RPMI-1640 media supplemented with 10% (v/v) fetal calf serum, 2 mM L-glutamine, 0.2 units/ml human insulin, 50 units/ml penicillin, 50 mg/ml streptomycin (Life Technologies, Inc., Gaithersburg, MD, USA). Cells were logarithmically grown at 37˚C in a humidified atmosphere consisting of 5% CO_2 and 95% air. Cells were routinely recovered from frozen stocks and allowed to grow until 85% confluence. For all experiments, cells were plated the day before exposure to treatment.

Cisplatin or CDDP (Sigma-Aldrich, St. Louis, MO) was prepared fresh daily by first dissolving it in phosphate-buffered saline (PBS) without Ca^{++} or Mg^{++} at a concentration of 1 mg per ml, and then further diluting it into pre-warmed media to achieve the IC_{50} dose of 5-day survival (3 μM) for A2780 cells. For the studies of cisplatin time-course, cells were allowed to grow for 24 h to reach 70-80% confluence. Cells were treated with cisplatin for 1 h at 3 μM. At the end of the 1-h exposure to cisplatin, cells were washed twice with phosphate-buffered saline without Ca^{++} or Mg^{++} and then incubated with fresh drug-free media for a specific period of time.

The reagent, okadaic acid (OA), was purchased from Sigma-Aldrich. In studies using inhibitor treatment, cells were pretreated for 30 min with different concentrations. Okadaic acid was prepared in DMSO and added to cells to achieve the respective dose and incubated for 30 min. Cisplatin was then added to the cells at a specific IC_{50} dose (3 μM) under continuous exposure to OA. At the end of the 1-h exposure to cisplatin, cells were washed twice with PBS and then incubated for 24 h with fresh medium containing OA inhibitor. The cells were then harvested for the indicated assays.

Immunostaining and microscopy. Cells were fixed for 10 min in PBS/3% paraformaldehyde/2% sucrose solution, followed by 5-min permeablization on ice in Triton buffer (0.5% Triton X-100 in 20 mM HEPES pH 7.4, 50 mM NaCl, 3 mM MgCl and 300 mM sucrose). Cells were blocked for 1 h in 1% BSA and incubated overnight with anti-p-Chk2 antibody or anti-PP2A antibody at 1:400 and at 4˚C. PP2A and p-Chk2 were visualized using secondary antibodies, fluorescein goat anti-rabbit conjugate for p-Chk2 and fluorescein goat anti-mouse IgG conjugate for PP2A (Molecular Probes, Invitrogen Life Technologies) at 1:200 for 1 h at 4˚C. All images were collected using an Olympus AX70 microscope with SPOT digital camera system (Opelco, Sterling, VA) and processed using Adobe Photoshop software.

Protein extraction, immunoprecipitation and Western blotting. Treated and untreated cells were extracted using whole cell lysis buffer [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, ß-glycerophosphate, 1 mM Na_3VO_4, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin and 5 μg/ml pepstatin] for 30 min before centrifugation at 14,000 rpm for 30 min at 4˚C. The supernatant was collected as whole cell lysate for Western blot analysis. Protein concentrations of extracts were determined using the Bio-Rad Protein Assay kit (Bio-Rad, Hercules, CA) with bovine serum albumin as standard.

For immunoprecipitation, 500 μg of lysates were incubated with 2-4 μg of primary Ab for 2 h at 4˚C and then with 100 μl of 25% (w/v) protein A/G agarose for 2 h at 4˚C. The immunoprecipitates were washed 3 times with lysis buffer and assay buffer and then resuspended in assay buffer.

The whole cell lysates or immunoprecipitates were separated on 12% SDS-polyacrylamide gels and transferred to PVDF membrane (Bio-Rad) using standard electrophoresis and electroblotting procedures. Pre-stained molecular weight markers were purchased from Invitrogen (Carlsbad, CA). To reduce nonspecific binding, blots were pre-incubated for 1 h in a blocking buffer (5% nonfat dry milk, 1X TBS, and 0.1% Tween-20). Membranes were then incubated with primary antibodies overnight at 4˚C. The primary antibodies applied were anti-Chk2 phosphothreonine-68, anti-Chk2 and PP2A-Bα/ß/γ antibodies (Santa Cruz, CA); PP2A-C subunit and PP2A-A subunit antibodies (Upstate Biotechnology, Lake Placid, NY); and HA-Tag monoclonal antibody (Cell Signaling, MA). To demonstrate the equal loading of each sample, membranes were re-probed with ß-actin using anti-ß-actin antibody (Sigma). The signals of immunoreactive proteins were visualized using horseradish peroxidase-conjugated sheep anti-mouse or donkey anti-rabbit antibodies (Amersham, UK) and enhanced using an Chemiluminescence ECL detection system (Amersham).

siRNA-mediated PP2A silencing assay. Small interfering RNAs (siRNAs) against the catalytic subunit of PP2A to the human PP2A (siRNA SMARTpool) were purchased from Upstate Biotechnology. The siRNA transfection reagent, Lipofectamine 2000 (Invitrogene), was used to transfect siRNA into A2780 cells at a final concentration of 100 nM. Negative non-specific siRNA was used as a control. Twenty-four hours after transfection, cells were treated with cisplatin for 1 h at 3 μM. At the end of the 1-h exposure to cisplatin, cells were washed twice and then incubated with fresh drug-free media for 24 h. The cells were harvested and the protein level of p-Chk2 was determined by Western blot analysis.

Purification of recombinant HA-hChk2. A2780 cells were transfected with the plasmid, HA-hChk2 pEFBOS (provided by Dr Jann N. Sarkaria, Clinic of the Mayo Foundation, Rochester, MN), for the expression of HA-tagged wild-type human Chk2 (19). After 24-h transfection, cells were treated with cisplatin (3 μM) for 1 h and then continuously incubated with fresh media for 24 h. Cell lysate was obtained by lysing the cells in buffer [20 mM HEPES (pH 7.0), 1 mM DTT, 1 mM $MnCl_2$, 100 μg/ml BSA and 50 μM leupeptin]. After immunoprecipitation with HA-tagged monoclonal antibody (Cell Signal) and protein A/G-sepharose beads, HA-tagged Chk2 from the cell lysate was washed 3 times in lysis buffer and used for PP2A dephosphorylation assay *in vitro*.

Protein phosphatase activity assay. PP2A activity was measured using a Serine/Threonine Protein Phosphatase Assay kit (Upstate Biotechnology). According to the manufacturer's instructions, cells were extracted with lysis buffer consisting of 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, 10 μg/ml leupeptin, 10 μg/ml aprotinin and 1 mM PMSF. Cell lysates (500 μg) from cisplatin-treated cells were immunoprecipitated with 4 μg of PP2A catalytic subunit antibody overnight at 4˚C. At the end of incubation, 100 μl of

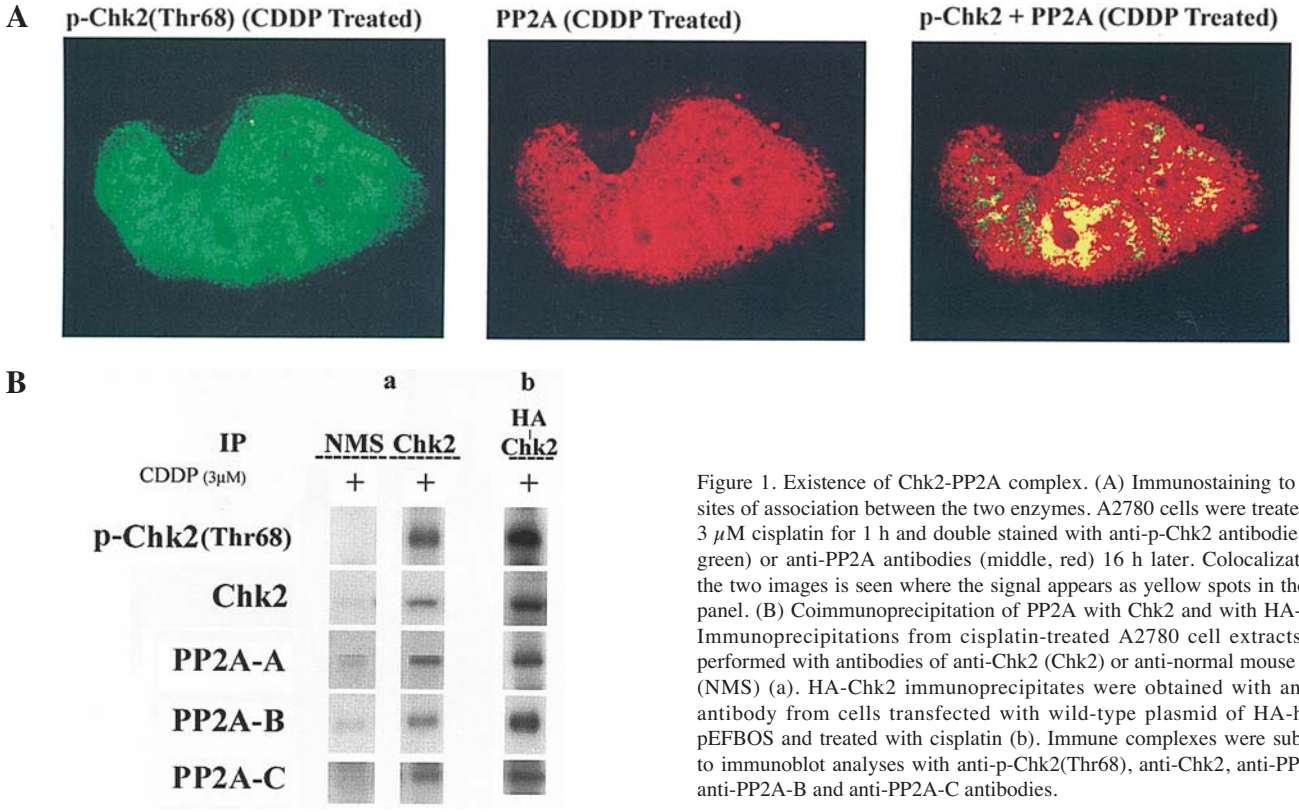

Figure 1. Existence of Chk2-PP2A complex. (A) Immunostaining to locate sites of association between the two enzymes. A2780 cells were treated with 3 μM cisplatin for 1 h and double stained with anti-p-Chk2 antibodies (left, green) or anti-PP2A antibodies (middle, red) 16 h later. Colocalization of the two images is seen where the signal appears as yellow spots in the right panel. (B) Coimmunoprecipitation of PP2A with Chk2 and with HA-Chk2. Immunoprecipitations from cisplatin-treated A2780 cell extracts were performed with antibodies of anti-Chk2 (Chk2) or anti-normal mouse serum (NMS) (a). HA-Chk2 immunoprecipitates were obtained with anti-HA antibody from cells transfected with wild-type plasmid of HA-hChk2 pEFBOS and treated with cisplatin (b). Immune complexes were subjected to immunoblot analyses with anti-p-Chk2(Thr68), anti-Chk2, anti-PP2A-A, anti-PP2A-B and anti-PP2A-C antibodies.

25% (w/v) protein A/G agarose was added, and incubation was continued for another 2 h at 4˚C. The immunoprecipitates were washed 3 times with lysis buffer and resuspended in 25 μl of assay buffer (50 mM Tris-HCl, pH 7.0 and 0.1 mM $CaCl_2$). The reaction of protein phosphatase activity assay was initiated by adding 5 μl of 1 μg/μl phosphopeptide substrate (200 mM, KRpTIRR) and incubating for 10 min at 37˚C. The reaction was then terminated by adding 100 μl of Malachite Green solution. Finally, the reaction mixture was spun down for 10 sec at 14,000 rpm. The absorbance of the supernatant was measured at 650 nm in a Spectra Max 384 microtiter plate reader (Molecular Devices Inc., Sunnyvale, CA). Phosphatase activity was calculated using a phosphate standard curve.

In vitro PP2A dephosphorylation assay. Whole cell lysates (40 μg) were prepared in PP2A reaction buffer [20 mM HEPES (pH 7.0), 1 mM dithiothreitol (DTT), 1 mM $MnCl_2$, 100 μg/ml BSA and 50 μM leupeptin] and incubated with PP2A enzyme 0.05 or 0.5 U (Upstate Biotechnology) for 30 min at 30˚C. IP-purified Chk2 and recombinant HA-hChk2 proteins were reacted with PP2A enzyme (0.05 or 0.5 unit) for 30 min at 30˚C in the presence or absence of OA. The reaction mixtures were separated on SDS-PAGE and blotted with p-Chk2 and Chk2 antibodies.

Densitometry and statistical analyses. Numerical values for the expression level of p-Chk2 (treated and untreated with cisplatin) in A2780 cells were calculated using IPLab-Gel software (Scanalytics, Inc., Fairfax, VA). For each sample, the densitometric readout value of p-Chk2 was corrected for the corresponding ß-actin value and presented as relative expression.

Results

PP2A associates with Chk2 after CDDP exposure. To determine whether Chk2 and PP2A enzymes exist as a complex in cells, we first performed immunostaining to locate sites of association between the two enzymes. Cisplatin-treated cells double stained with anti-p-Chk2 and anti-PP2A antibodies revealed overt dispersal of p-Chk2 (green dots) and PP2A (red dots). Colocalization of the two images is seen where the signal appears as yellow dots. (Fig. 1A). In the cisplatin-treated cells, p-Chk2 foci are significantly correlated with PP2A, indicating that the two enzymes are closely associated. We further immunoprecipitated Chk2 and HA-Chk2 from A2780 cells and analyzed the immune complex by immunoblotting with antibodies specific for anti-PP2A-A, anti-PP2A-B, anti-PP2A-C, anti-Chk2 and anti-p-Chk2(Thr68) antibody. As shown in Fig. 1B, subunits A, B and C of PP2A coimmunoprecipitated with Chk2 or HA-Chk2. The immune complexes of Chk2-PP2A from cisplatin-treated Chk2 immunoprecipitates were detected by anti-p-Chk2(Thr68), anti-Chk2, anti-PP2A-A, anti-PP2A-B and anti-PP2A-C antibodies, whereas the immunoprecipitates of normal mouse serum (NMS) failed to present the complex (Fig. 1Ba). The Chk2-PP2A association also presented in the HA-Chk2 immunoprecipitates of cells reconstituted with wild-type Chk2 and treated with cisplatin (Fig. 1B-b). These data suggest that a Chk2-PP2A holoenzyme complex appears in A2780 cells after cisplatin exposure.

Cisplatin induces increased Chk2 phosphorylation. The appearance of Chk2-PP2A complex after cisplatin treatment raises the possibility that phosphatase activity may regulate Chk2 signaling after cisplatin-induced DNA damage. To test

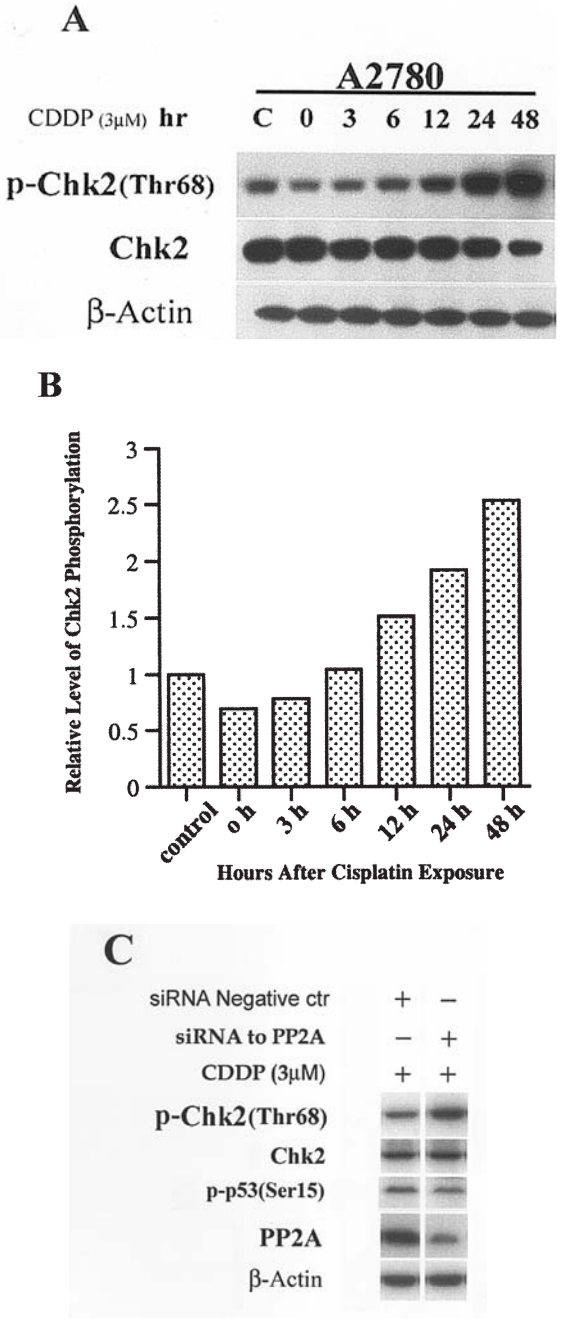

Figure 2. (A) Cisplatin-induced Chk2 phosphorylation at Thr-68. A2780 ovarian cancer cells were treated with cisplatin (CDDP) at the IC_{50} dose (3 μM) for 1 h. The drug was removed by washing cells with PBS. Cells were then incubated in fresh drug-free media for the indicated hours (0, 3, 6, 12, 24 and 48) until harvest. Proteins were extracted and separated on SDS-PAGE gels. Blots were probed with antibodies of anti-p-Chk2 or anti-Chk2, or anti-ß-actin as a control. (B) Quantification of p-Chk2 expression was determined using IPLab-Gel software. (C) siRNA-mediated PP2A silencing affects the p-Chk2 level in A2780 cells. Cells were transfected with siRNA to the catalytic subunit of PP2A for 24 h and treated with 3 μM cisplatin for 1 h. The extent of phosphorylated Chk2 and p53, and the protein level of Chk2, PP2A and ß-actin were estimated using antibodies against respective p-Chk2(Thr68), p-p53(Ser15), Chk2, PP2A and ß-actin.

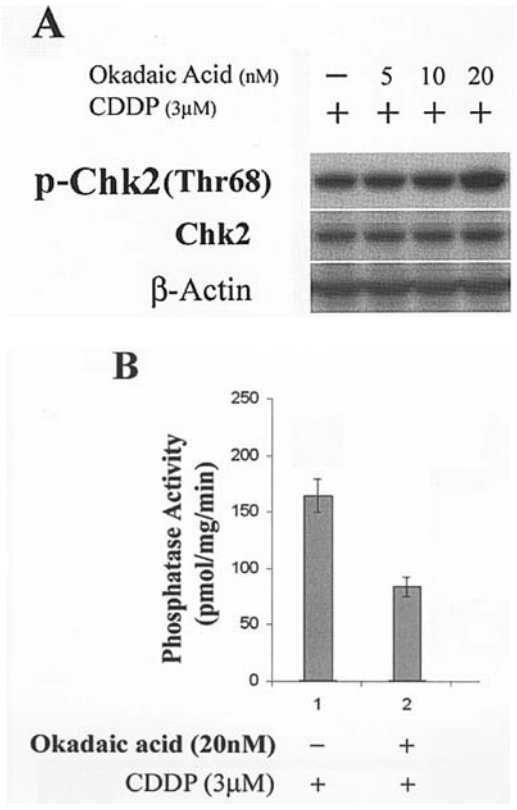

Figure 3. (A) Inhibition of PP2A activity by okadaic acid enhances Chk2 phosphorylation. A2780 cells were pretreated with concentrations of OA (0, 5, 10 and 20 nM) for 30 min and then treated with 3 μM cisplatin for 1 h under continuous exposure to OA. Cells were washed to remove cisplatin and re-incubated for 24 h in a replacement of fresh media containing OA. Proteins were prepared, separated and probed with antibodies of anti-p-Chk2, anti-Chk2, or anti-ß-actin. ß-actin served as a control. (B) Inhibitory effects of okadaic acid on protein phosphatase activity by protein phosphatase activity assay. Cells were pretreated with okadaic acid and then treated with cisplatin as described above. The protein phosphatase activity assay was performed following the manufacturer's instructions. Results are expressed as the means ± SD (n=3).

this, we first assessed the effect of cisplatin on Chk2 phosphorylation. Exposing cells to cisplatin for 1 h first resulted in a slight decrease 3 h after cisplatin removal and then an increase in the amount of phosphorylated Chk2 at Thr-68 in a time-dependent manner (Fig. 2A). Quantitative analysis of phosphorylated Chk2 expression demonstrated that the cisplatin-induced phosphothreonine Chk2 was doubled at 24 h compared to the control (Fig. 2B).

Inhibition of PP2A by siRNA enhances Chk2 phosphorylation. To examine PP2A as a negative regulator of Chk2 phosphorylation, we used small interfering RNA (siRNA) to silence the expression of PP2A protein by RNA interference (RNAi) in A2780 cells. Cells transfected by specific siRNA to the catalytic subunit of human PP2A (containing 4 pooled SMART selected siRNA duplexes with 'UU' overhangs and a 5' phosphate on the antisense strand) and treated with cisplatin resulted in a hyperphosphorylated p-Chk2 at Thr-68 compared to the non-specific siRNA-treated control (Fig. 2C), indicating that the reduced amount of PP2A promoted 68-threonine phosphorylated Chk2.

PP2A modulation has no effect on p53 phosphorylation. To demonstrate that increased p-Chk2 in siRNA to PP2A assay is not induced by endogenous damage (cisplatin) but by the failure of PP2A in Chk2 dephosphorylation, we also examined the level of p53 phosphorylation after cisplatin exposure.

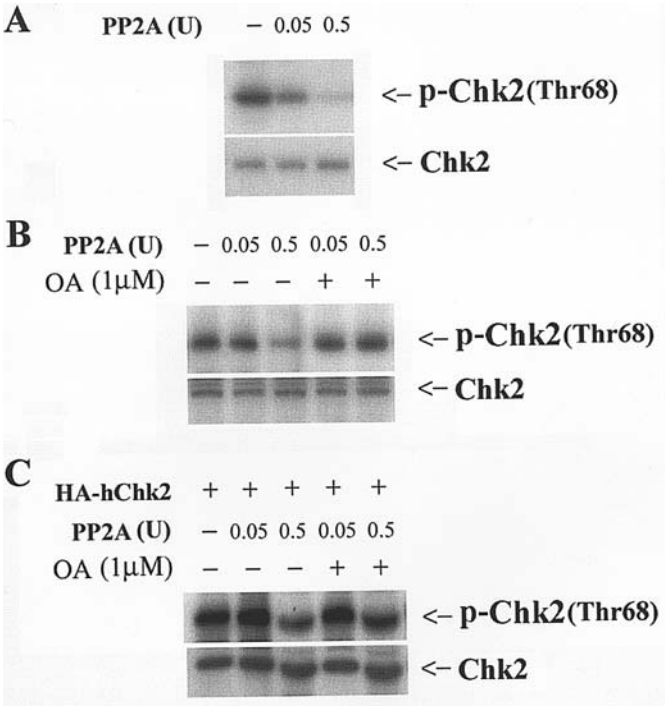

Figure 4. Direct dephosphorylation of p-Chk2 by PP2A *in vitro*. Chk2 was phosphorylated by cisplatin treatment to the A2780 cells. Forty μg of whole cell lysates (A), Chk2-immunoprecipitated protein (B), or HA-Chk2 immunoprecipitated protein (C) was incubated with 0.05 or 0.5 units of PP2A enzyme in the presence or absence of the phosphatase inhibitor OA (B and C), which was used to verify the specificity of the PP2A effect.

Jallepalli *et al* suggested that p53 is independent of Chk2 in the DNA-damage signaling pathway (21). As shown in Fig. 2C, p53 phosphorylation at Ser-15 in cells treated with cisplatin and siRNA to PP2A is not greater than the level of phosphorylated p53 in cells treated with cisplatin and non-specific siRNA control. This indicates that the increased level of p-Chk2 was due to the failure of PP2A to remove the phosphate on Thr-68.

Inhibition of PP2A by okadaic acid enhances Chk2 phosphorylation. We also assessed the effect of okadaic acid (OA), an inhibitor of PP2A (20), on Chk2 phosphorylation in these cells. Treatment of cells with OA (20 nM) resulted in increased abundance of phosphorylated Chk2 at Thr-68 (Fig. 3A), which was due to the PP2A inhibition to a 50% reduction of phosphatase activity (Fig. 3B), indicating that PP2A is involved in the Chk2 pathway as an inhibitory participant in the signaling process. These results demonstrate that PP2A has a strong effect on Chk2 after DNA damage induced by the genotoxic reagent, cisplatin, in these cells.

PP2A dephosphorylates Chk2 in vitro. To further determine whether PP2A acts directly on Chk2, we performed an *in vitro* dephosphorylation assay. PP2A enzyme was coincubated with either whole cell lysate (Fig. 4A), Chk2 (Fig. 4B), or recombinant HA-hChk2 (Fig. 4C). Anti-p-Chk2(Thr68) and anti-Chk2 antibodies were used to identify the coprecipitated protein complex. Increasing amounts of PP2A led to decreasing amounts of p-Chk2(Thr68) in all three circumstances. p-Chk2(Thr68) could be restored by inhibition of PP2A with okadaic acid (Fig. 4B and C).

Discussion

PP2A may exist *in vivo* in a dimeric (PP2AD) or trimeric (PP2AT) form. PP2AD is composed of a 36-kDa catalytic subunit (PP2AC) tightly bound to the 65-kDa regulatory (PR65/A) constant subunit. These two associated proteins form the core dimer, which can further interact with a third, variable regulatory B subunit or other regulatory proteins. Many B subunits are expressed during specific developmental stages and show tissue-specific expression. The variable regulatory subunits control the activity and specificity of the holoenzyme and target it to different cellular compartments (22). It is likely, however, that the physiological specificity of PP2A depends more on its localization than on contributions from the B-subunit because the AC catalytic complex of PP2A alone has phosphatase activity (23).

PP2A has been reported to form stable complexes with various serine/threonine protein kinases, such as casein kinase 2α, Ca^{2+}-calmodulin-dependent protein kinase IV (CaMKIV), and p21-activated kinases (PAK1 and PAK3) (24-26). Some are inactivated by PP2A (27). By immunoprecipitation and immunostaining, we observed a direct enzyme-substrate relationship between Chk2 and PP2A. Since most antisubunit antibodies do not quantitatively immunoprecipitate PP2A heterotrimers, we prepared cell extracts from A2780 cells expressing HA-tagged wild-type Chk2, and performed immunoprecipitation using the anti-HA antibody. A, B and C subunits of PP2A were detected in the immunoprecipitates prepared by anti-Chk2 and anti-HA. The B56 subunit was not found in the immunoprecipitates of our investigation (data not shown). The interaction of PP2A with Chk2 appears to be specific and functional because the dephosphorylation of Chk2 *in vitro* by the purified PP2A enzyme was concentration-dependent, and the dephosphorylation of Chk2 was blocked by OA, an inhibitor of PP2A.

We have demonstrated that OA treatment enhanced the phosphorylation of Chk2 at Thr-68 after cisplatin treatment (Fig. 3A). OA is the cell-permeant inhibitor of PP1 and PP2A, and inhibits PP2A (50% inhibitory dose IC_{50}, 1-10 nM) more potently than PP1 (IC_{50}, 100 nM to 1 μM) (28). As 20 nM OA promoted the phosphorylation of Chk2, PP2A was thought to be more important in phospho-Thr-68 of Chk2. To further confirm the possibility that PP2A dephosphorylates Chk2, we used small interfering RNA (siRNA) to silence the expression of PP2A protein in A2780 cells. siRNA to PP2A reduced the protein amount of PP2A and promoted 68 threonine-phosphorylated Chk2 induced by cisplatin (Fig. 2C).

Our observations indicate that using specific siRNA to PP2A inhibited PP2A activity of p-Chk2 dephosphorylation, and resulted in an increase of phosphate on threonine-68 of Chk2. To address the possibility that the increased level of p-Chk2 might be caused by an endogenous damage signal that increases the rate of phosphorylation of Thr-68, rather than the failure of PP2A to remove the phosphate on Thr-68, we performed additional experiments. Experiments using p53 showed that the phosphorylation level of other ATM/ATR substrates is not increased in response to DNA damage under the same conditions for p-Chk2 assessment (in siRNA to PP2A assay). Of note, p53 has been reported to be an important

substrate of the ATM/ATR-dependent pathway in response to DNA damage (1).

In conclusion, we propose that PP2A has an important regulatory function on Chk2, which is to dephosphorylate Chk2 at Thr68 as needed in response to cellular exposure to DNA damaging agents. Dozier and colleagues, using the yeast two-hybrid screen technique, demonstrated similar findings that B' gamma containing a PP2A holoenzyme associates with Chk2 and that B' gamma3 is a potent Chk2 substrate (29). Data generated from our series of experiments that included stimulation and suppression of the two enzymes, *in vitro* and *in vivo*, provide strong evidence that functional protein ser/thr phosphatase 2A (PP2A) interacts with Chk2 and functions as a negative regulator of this checkpoint kinase by dephosphorylating phosphor-Thr-68. These findings are important with respect to understanding the role that Chk2 plays in controlling cell cycle checkpoints and DNA damage/repair pathways.

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References

1. Zhou BB and Elledge SJ: The DNA damage response: putting checkpoints in perspective. Nature 408: 433-439, 2000.
2. Matsuoka S, Huang M and Elledge SJ: Linkage of ATM to cell cycle regulation by the Chk2 protein kinase. Science 282: 1893-1897, 1998.
3. Melchionna R, Chen XB, Blasina A and McGowan CH: Threonine 68 is required for radiation-induced phosphorylation and activation of Cds1. Nat Cell Biol 2: 762-765, 2000.
4. Ahn JY, Schwarz JK, Piwnica-Worms H and Canman CE: Threonine 68 phosphorylation by *ataxia telangiectasia* mutated is required for efficient activation of Chk2 in response to ionizing radiation. Cancer Res 60: 5934-5936, 2000.
5. Lee CH and Chung JH: The hCds1 (Chk2)-FHA domain is essential for a chain of phosphorylation events on hCds1 that is induced by ionizing radiation. J Biol Chem 276: 30537-30541, 2001.
6. Ahn JY, Li X, Davis HL and Canman CE: Phosphorylation of threonine 68 promotes oligomerization and autophosphorylation of the Chk2 protein kinase via the forkhead-associated domain. J Biol Chem 277: 19389-19395, 2002.
7. Falck J, Mailand N, Syljuasen RG, Bartek J and Lukas J: The ATM-Chk2-Cdc25A checkpoint pathway guards against radioresistant DNA synthesis. Nature 410: 842-847, 2001.
8. Hirao A, Kong YY, Matsuoka S, Wakeham A, Ruland J, Yoshida H, Liu D, Elledge SJ and Mak TW: DNA damage-induced activation of p53 by the checkpoint kinase Chk2. Science 287: 1824-1827, 2000.
9. Bell DW, Varley JM, Szydlo TE, Kang DH, Wahrer DC, Shannon KE, Lubratovich M, Verselis SJ, Isselbacher KJ, Fraumeni JF, Birch JM, Li FP, Garber JE and Haber DA: Heterozygous germ line hCHK2 mutations in Li-Fraumeni syndrome. Science 286: 2528-2531, 1999.
10. Miller CW, Ikezoe T, Krug U, Hofmann WK, Tavor S, Vegesna V, Tsukasaki K, Takeuchi S and Koeffler HP: Mutations of the CHK2 gene are found in some osteosarcomas, but are rare in breast, lung, and ovarian tumors. Genes Chromosomes Cancer 33: 17-21, 2002.
11. Kawabe T, Muslin AJ and Korsmeyer SJ: HOX11 interacts with protein phosphatases PP2A and PP1 and disrupts a G2/M cell-cycle checkpoint. Nature 385: 454-458, 1997.
12. Sathyanarayanan S, Zheng X, Xiao R and Sehgal A: Post-translational regulation of *Drosophila* PERIOD protein by protein phosphatase 2A. Cell 116: 603-615, 2004.
13. Virshup DM: Protein phosphatase 2A: a panoply of enzymes. Curr Opin Cell Biol 12: 180-185, 2000.
14. Janssens V and Goris J: Protein phosphatase 2A: a highly regulated family of serine/threonine phosphatases implicated in cell growth and signaling. Biochem J 353: 417-439, 2001.
15. Usui H, Imazu M, Maeta K, Tsukamoto H, Azuma K and Takeda M: Three distinct forms of type 2A protein phosphatase in human erythrocyte cytosol. J Biol Chem 263: 3752-3761, 1988.
16. Seeling JM, Miller JR, Gil R, Moon RT, White R and Virshup DM: Regulation of beta-catenin signaling by the B56 subunit of protein phosphatase 2A. Science 283: 2089-2091, 1999.
17. Kitagawa Y, Tahira T, Ikeda I, Kikuchi K, Tsuiki S, Sugimura T and Nagao M: Molecular cloning of cDNA for the catalytic subunit of rat liver type 2A protein phosphatase, and detection of high levels of expression of the gene in normal and cancer cells. Biochim Biophys Acta 951: 123-129, 1988.
18. Wang SS, Esplin ED, Li JL, Huang L, Gazdar A, Minna J and Evans GA: Alterations of the PPP2R1B gene in human lung and colon cancer. Science 282: 284-287, 1998.
19. Busby EC, Leistritz DF, Abraham RT, Karnitz LM and Sarkaria JN: The radiosensitizing agent 7-hydroxystaurosporine (UCN-01) inhibits the DNA damage checkpoint kinase hChk1. Cancer Res 60: 2108-2112, 2000.
20. Haystead TA, Sim AT, Carling D, Honnor RC, Tsukitani Y, Cohen P and Hardie DG: Effects of the tumour promoter okadaic acid on intracellular protein phosphorylation and metabolism. Nature 337: 78-81, 1989.
21. Jallepalli PV, Lengauer C, Vogelstein B and Bunz F: The Chk2 tumor suppressor is not required for p53 responses in human cancer cells. J Biol Chem 278: 20475-20479, 2003.
22. Lechward K, Awotunde OS, Swiatek W and Muszynska G: Protein phosphatase 2A: variety of forms and diversity of functions. Acta Biochim Pol 48: 921-933, 2001.
23. Mumby MC and Walter G: Protein serine/threonine phosphatases: structure, regulation, and functions in cell growth. Physiol Rev 73: 673-699, 1993.
24. Heriche JK, Lebrin F, Rabilloud T, Leroy D, Chambaz EM and Goldberg Y: Regulation of protein phosphatase 2A by direct interaction with casein kinase 2alpha. Science 276: 952-955, 1997.
25. Westphal RS, Anderson KA, Means AR and Wadzinski BE: A signaling complex of Ca^{2+}-calmodulin-dependent protein kinase IV and protein phosphatase 2A. Science 280: 1258-1261, 1998.
26. Westphal RS, Coffee Jr RL, Marotta A, Pelech SL and Wadzinski BE: Identification of kinase-phosphatase signaling modules composed of p70 S6 kinase-protein phosphatase 2A (PP2A) and p21-activated kinase-PP2A. J Biol Chem 274: 687-692, 1999.
27. Millward TA, Zolnierowicz S and Hemmings BA: Regulation of protein kinase cascades by protein phosphatase 2A. Trends Biochem Sci 24: 186-191, 1999.
28. Gupta V, Ogawa AK, Du X, Houk KN and Armstrong RW: A model for binding of structurally diverse natural product inhibitors of protein phosphatases PP1 and PP2A. J Med Chem 40: 3199-3206, 1997.
29. Dozier C, Bonyadi M, Baricault L, Tonasso L and Darbon JM: Regulation of Chk2 phosphorylation by interaction with protein phosphatase 2A via its B' regulatory subunit. Biol Cell 96: 509-517, 2004.