Arsenic Trioxide Augments Chk2/p53-mediated Apoptosis by Inhibiting Oncogenic Wip1 Phosphatase*§

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The oncogenic Wip1 phosphatase (PPM1D) is induced upon DNA damage in a p53-dependent manner and is required for inactivation or suppression of DNA damage-induced cell cycle checkpoint arrest and of apoptosis by dephosphorylating and inactivating phosphorylated Chk2, Chk1, and ATM kinases. It has been reported that arsenic trioxide (ATO), a potent cancer chemotherapeutic agent, in particular for acute promyelocytic leukemia, activates the Chk2/p53 pathway, leading to apoptosis. ATO is also known to activate the p38 MAPK/p53 pathway. Here we show that phosphatase activities of purified Wip1 toward phosphorylated Chk2 and p38 in vitro are inhibited by ATO in a dose-dependent manner. Furthermore, DNA damage-induced phosphorylation of Chk2 and p38 in cultured cells is suppressed by ectopic expression of Wip1, and this Wip1-mediated suppression can be restored by the presence of ATO. We also show that treatment of acute promyelocytic leukemia cells with ATO resulted in induction of phosphorylation and activation of Chk2 and p38 MAPK, which are required for ATO-induced apoptosis. Importantly, this ATO-induced activation of Chk2/p53 and p38 MAPK/p53 apoptotic pathways can be enhanced by siRNA-mediated suppression of Wip1 expression, further indicating that ATO inhibits Wip1 phosphatase in vivo. These results exemplify that Wip1 is a direct molecular target of ATO.

Arsenic trioxide (As$_2$O$_3$; ATO)$^3$ has been used therapeutically for several thousand years as a part of traditional Chinese medicine, and it is widely used as an effective anticancer drug for acute promyelocytic leukemia (APL) (1–3). Most APL cases are characterized by a specific chromosomal translocation t(15; 17), which results in the rearrangement of the PML (for promyelocytic leukemia) gene and retinoic acid receptor (RAR$\alpha$) gene and the expression of PML–RAR$\alpha$ fusion protein. The PML–RAR$\alpha$ fusion protein functions as a constitutive transcriptional silencer in the retinoic acid signaling pathway, thereby inducing a differentiation block (4). All-trans-retinoic acid (ATRA), another drug for APL, directly targets the PML–RAR$\alpha$ fusion protein and can induce clinical complete remission in APL patients by degradation of the PML–RAR$\alpha$ fusion protein and a differentiation process; however, these patients often relapse and are resistant to further treatment with ATRA (5). ATO also degrades the PML–RAR$\alpha$ fusion protein and induces differentiation with lower concentration, but unlike ATRA, ATO induces a potent apoptosis of the APL cells with higher concentration via a variety of mechanisms, which appear to be independent of the presence of the PML–RAR$\alpha$ fusion protein (6, 7). It has been well demonstrated that ATO induces high complete remission rates in relapsed APL patients resistant to ATRA as well as in primary APL patients (around 85–90%) (8). ATO-induced apoptosis is thought to play a major role in the therapeutic effects of ATO (7). ATO is of potential therapeutic value for the treatment of other hematologic malignancies and solid tumors in addition to APL (9).

Arsenic predominantly exists in two oxidation states, As(III) (e.g. As$_2$O$_3$; ATO) and As(V) (e.g. As$_2$O$_5$; arsenic pentoxide; APO). Although high concentrations of As(V) have been shown to substitute for phosphate in enzyme-catalyzed reactions, arsenic toxicity is postulated to be primarily due to the binding of As(III) to target molecules, including sulphydryl-containing enzymes (10–12). Among these enzymes, protein phosphatases can be targets of As(III). For example, phenylarsine oxide and arsenite were shown to inhibit CD45 tyrosine phosphatase and dual specificity c-Jun N-terminal kinase phosphatase, respectively (13, 14). ATO-induced apoptosis is associated with

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$^3$ The abbreviations used are: ATO, arsenic trioxide; ATM, ataxia telangiectasia-mutated; ATR, ATM- and Rad3-related; ATRA, all-trans-retinoic acid; APL, acute promyelocytic leukemia; APO, arsenic pentoxide; ER, estrogen receptor; GST, glutathione S-transferase; HA, hemagglutinin; MAPK, mitogen-activated protein kinase(s); MBP, maltose-binding protein; WCL, whole cell lysate; WT, wild type; ROS, reactive oxygen species; FCS, fetal calf serum; 4-OHT, 4-hydroxytamoxifen; NAC, N-acetyl-L-cysteine; siRNA, small interfering RNA; PAG, polyacrylamide gel; PVDF, polyvinylidene difluoride; PBS, phosphate-buffered saline; DAPI, 4′,6-diamidino-2-phenylindole; DK, dead kinase.
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the generation of reactive oxygen species (ROS) with subsequent accumulation of H$_2$O$_2$ in several experimental models (15, 16). ATO-induced apoptosis is inhibited when cells are treated with various antioxidants, free radical scavengers, or inhibitors of ROS-producing enzymes (15). Recently, TrxR (thioredoxin reductase), one of the critical regulators of the cellular redox environment, was identified as a molecular target of ATO (17). However, other targets of ATO are not known, and molecular mechanisms of ATO-induced apoptosis remain elusive.

It has been well appreciated that genotoxic stresses activate cell cycle checkpoint machinery to maintain genomic integrity (18, 19). Following DNA damage, ataxia telangiectasia-mutated (ATM) and/or ATM and Rad3-related (ATR) kinases are activated rapidly, and these kinases then phosphorylate and activate checkpoint kinases, Chk1 and Chk2, thereby amplifying the DNA damage signal to execute cell cycle checkpoint regulation (20–24). It has been reported that ATO induces DNA damage and p53 accumulation through an ATM-dependent pathway and that ATO-induced apoptosis is required for a pathway composed of ATR, PML, Chk2, and p53 (25, 26).

Mitogen-activated protein kinases (MAPK) are a family of enzymes that transduce signals from the cell membrane to the cell interior in response to a wide range of stimuli and modulate several important biological functions, including gene expression, mitosis, proliferation, motility, and apoptosis (27, 28). It has been shown that p38 MAPK stimulates p53 in response to various stress agents, including UV irradiation and anticancer drugs, and regulates both activation of p53-mediated transcription and p53-dependent apoptosis (29, 30). ATO is known to activate p38 MAPK in various neoplastic cell lines, suggesting a role for this pathway in the regulation of ATO-induced responses in malignant cells (31–35).

In mammals, the PP2C (protein phosphatase 2C) family of protein phosphatases consists of at least seven distinct isoforms and has been implicated in stress response signaling (36–38). Among the members of the PP2C family protein phosphatases, Wip1 (PPM1D) possesses unique biological characteristics. Wip1 is induced by DNA damage in a p53-dependent manner and inhibits UV irradiation-induced activation of p38 MAPK by dephosphorylating Thr$^{180}$ in p38, thereby inhibiting the function of p53 (38, 39). It has also been reported that Wip1 dephosphorylates the two ATM/ATR (ATM and Rad-3-related) targets, Chk1 and p53 (40, 41), thereby suppressing intra-S and G$_2$/M checkpoint regulations. Recently, we have shown that Wip1 inactivates Chk2 kinase by dephosphorylating threonine 68 (Thr$^{68}$) in activated Chk2, and suppresses Chk2-mediated apoptosis (42). Furthermore, it has been shown that the Wip1 (PPM1D) gene is amplified or overexpressed in various human cancers, including breast cancers (43–46), and that overexpression of Wip1 (PPM1D) cooperates with the oncogenes Ras, Myc, and Neu1 to transform wild-type mouse embryonic fibroblasts (43), suggesting that Wip1 is an oncogenic protein. In addition, a recent study using Wip1-deficient mice has revealed that blocking its function results in enhanced apoptosis in Ras- and Erbb2-induced breast tumors and impairs tumor formation (47–49). However, the roles of the Wip1 phosphatase in ATO-induced cellular responses remain obscure.

In this study, we examined the effect(s) of ATO on APL cells, in particular the roles of Chk2, p38 MAPK, and Wip1 in ATO-induced apoptosis. We show that both Chk2 and p38 MAPK are phosphorylated following ATO stimulation, but not APO or ATRA stimulation, of APL cells in a dose-dependent manner. Inhibition of either Chk2 or p38 MAPK resulted in suppression of ATO-induced apoptosis, whereas inhibition of Wip1 resulted in enhancement of ATO-induced apoptosis, indicating that these proteins are involved critically in ATO-induced apoptosis. Furthermore, we found that ATO inhibits phosphatase activities of Wip1, but not kinase activities of Chk2 and p38 MAPK, in vitro, and that Wip1-mediated suppression of phosphorylation of Chk2 and p38 MAPK is inhibited by ATO treatment in vivo. These results support our notion that ATO induces apoptosis by activating Chk2/p53 and p38/p53 pathways, which is enhanced by direct inhibition of Wip1 by ATO.

EXPERIMENTAL PROCEDURES

Cells, Antibodies, and Chemical Reagents—NB4 and HL-60 cells were maintained in RPMI1640 (Nissui) supplemented with 10% (v/v) fetal calf serum (FCS), HEK293T (293T), MCF7, and HeLa cells were maintained in Dulbecco’s modified Eagle’s medium (Nissui) supplemented with 10% (v/v) FCS. Transient cDNA transfection was performed using the calcium phosphate method (50) or Lipofectamine reagent (Invitrogen) according to the manufacturer’s instructions. Rabbit polyclonal anti-Chk2 and anti-Wip1 antibodies were prepared as described previously (42). Antibodies against FLAG (M2; Sigma), HA (16B12; Babco), β-actin (AC-15; Sigma), phospho-Chk2 (Thr$^{68}$) (Cell Signaling), p38 (Cell Signaling), phospho-p38 (Thr$^{180}$/Ty$^{182}$) (Cell Signaling), p53 (DO1; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), phospho-p53 (Ser$^{46}$ and Ser$^{96}$) (Cell Signaling), PML (Santa Cruz Biotechnology), and Wip1 (WC10; Trevigen) were purchased commercially. Alexa Fluor 546 (goat anti-mouse IgG, red) and Alexa Fluor 488 (goat anti-rabbit IgG, green) were from Invitrogen. ATO and APO were purchased from Wako. SB203580, ATRA, 4-hydroxytamoxifen (4OHT), and N-acetyl-L-cysteine (NAC) were from Sigma.

Plasmid Constructions—Mammalian expression plasmids pEBG-Chk2 wild type (WT) (encoding glutathione S-transferase (GST)-HA-Chk2 (WT)), pcDNA-FLAG-Wip1 (WT), and pcDNA-FLAG-Wip1 (D/A) were constructed as described previously (42). CDNA for Wip1-(1–516)-estrogen receptor (ER) fusion protein was isolated from pECE-HA-Wip1-(1–516)-ER (51) and subcloned into pcDNA vector to generate pcDNA-Wip1-(1–516)-ER. pEBG-p38 (encoding GST-p38 MAPK) was constructed as described (38). Prokaryotic expression vectors were constructed in pGEX (GE Healthcare) or pMAL-C2 (New England Biolabs) as described previously (38, 42, 51).

siRNA—The siRNA duplexes were 21 base pairs, including a 2-base nucleotide overhang synthesized by RNAI Co., Ltd. or Dharmacon Research. The sequence of the Chk2 siRNA (number 1 and 2) and Wip1 siRNA (number 1 and 2) oligonucleotides were GAACUUGAGGCAAGAACCUCU (Chk2 siRNA 1), CGCCGUCUUUUGAAUAACAAUU (Chk2 siRNA 2), UUG-GCCUUGUGCCUACAUUU (Wip1 siRNA 1), and GCCU-
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UUCUCGUUGUCACCdTdT (Wip1 siRNA 2). The control siRNA oligonucleotide used was GUACCGACGUCAUCCGAUC. Cells were transfected with siRNA duplexes by electroporation. Forty-eight hours after transfection, cells were treated with ATO and harvested 24 h after ATO treatment.

**Preparation of Cell Lysates and Coimmunoprecipitation/Immunoblot Analyses**—The cells were solubilized with lysis buffer (50 mM Tris-HCl (pH 7.4), 0.5% (v/v) Nonidet P-40 (Nonidet P-40), 150 mM NaCl, 5 mM EDTA, 50 mM NaF, 1 mM Na3VO4, 1 mM phenylmethysulfonyl fluoride, 10 µg/ml leupeptin, and 10 µg/ml aprotinin) and whole cell lysates (WCLs) were prepared by centrifugation at 12,000 × g for 15 min at 4 °C. WCLs were precleared with protein A-Sepharose (GE Healthcare) for 1 h at 4 °C. The precleared supernatants were then coimmunoprecipitated with glutathione-Sepharose beads (GE Healthcare) or with antibodies conjugated to protein A-Sepharose beads for 2 h at 4 °C. The immunoprecipitates were washed five times with lysis buffer and eluted with Laemmli sample buffer. Proteins either from the coimmunoprecipitation analyses or WCLs were separated by SDS-PAGE (10% PAG) and transferred onto PVDF membrane filters (Immobilon; Millipore). The membranes were immunoblotted with the respective antibodies, and bound antibodies were visualized with horseradish peroxidase-conjugated antibodies against mouse or rabbit IgGs (Bio-Rad) using chemiluminescence reagents (Western Lightning; PerkinElmer Life Sciences).

**Immunofluorescence Analyses**—NB4 cells were washed twice in RPMI1640 without serum, resuspended in 100 µl of RPMI1640, and attached on coverslips. 293T and HeLa cells were cultured on coverslips coated with rat tail collagen. Fixation, permeabilization, blocking, and staining were performed essentially according to the manufacturer’s instructions. For phospho-Chk2 (Thr68) and Wip1 staining, cells on coverslips were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 15 min at room temperature, washed once with TBS (50 mM Tris-HCl (pH 7.4), 150 mM NaCl), and permeabilized with TBS containing 0.2% (v/v) Triton X-100 for 5 min at room temperature. Cells were then washed three times with TBS and washed once with TBS containing freshly prepared 1 mg/ml sodium borohydride to quench reactions. After blocking with PBS containing 10% (v/v) FCS, 10 mg/ml bovine serum albumin, and 0.2 mg/ml NaN3, for 1 h at room temperature, cells were washed with PBS and incubated with the respective primary antibodies, rabbit polyclonal anti-phospho-Chk2 (Thr68) (1:100) and/or mouse monoclonal anti-Wip1 antibodies (WC10; 1:100), in PBS containing 10 mg/ml bovine serum albumin overnight at 4 °C. Cells were washed twice with PBS and then incubated with the respective secondary antibodies, Alexa Fluor 546 (goat anti-mouse IgG; 1:500) and/or Alexa Fluor 488 (goat anti-rabbit IgG; 1:200), in PBS containing 0.3% (v/v) Triton X-100 and 0.5 µg/ml DAPI for 60 min at room temperature. After they were washed three times with PBS, cells were mounted with Pristine Mount (Research Genetics) and analyzed with an inverted confocal laser microscope (Zeiss).

**Expression and Purification of GST and MBP Fusion Proteins**—GST, GST-Wip1 (WT), GST-Cdc25C (aa 200–256), GST-ATF2 (aa 1–103), and MBP-Chk2 (WT) were expressed in *Escherichia coli* BL21, respectively, and were extracted with PBS containing 1% (v/v) Triton X-100, 1 mM EDTA, 1 mM phenylmethysulfonyl fluoride, 10 µg/ml leupeptin, and 10 µg/ml aprotinin. GST and GST fusion proteins were isolated with glutathione-Sepharose beads. Maltose-binding protein (MBP) fusion proteins were isolated with amylose resin beads (New England Biolabs). GST or MBP fusion proteins were eluted from beads by incubating with 25 mM glutathione (reduced) or 10 mM maltose, respectively, and further purified by gel filtration chromatography using Nick columns (GE Healthcare).

To prepare phosphorylated GST-HA-Chk2 (WT or dead kinase (DK)) or phosphorylated GST-p38, 293T cells transfected with the respective expression vectors, encoding GST-HA-Chk2 (WT or DK) or GST-p38, were exposed to γ-irradiation (10 grays) or UV irradiation (50 J/m2), respectively, and were cultured for 1 h prior to harvest. Phosphorylated GST-HA-Chk2 (WT or DK) or phosphorylated GST-p38 in 293T were extracted with lysis buffer (50 mM Tris-HCl (pH7.4), 0.5% (v/v) Triton X-100, 150 mM NaCl, 5 mM EDTA, 50 mM NaF, 1 mM Na3VO4, 1 mM phenylmethysulfonyl fluoride, 10 µg/ml leupeptin, and 10 µg/ml aprotinin) and were purified as described above.

**In Vitro Kinase Assays**—*In vitro* kinase assays were performed essentially as described previously (42). For *in vitro* kinase assays of Chk2, 1 µg of GST-HA-Chk2 (WT) or GST-HA-Chk2 (DK) was suspended in 30 µl of kinase buffer (10 mM Hepes (pH 7.5), 5 mM MgCl2, and 2 mM dithiothreitol) containing 1 µg of GST-Cdc25C and 15 µCi of [γ-32P]ATP (6000 Ci/mmol; GE Healthcare). For *in vitro* kinase assays of p38, 1 µg of GST-p38 was suspended in 30 µl of kinase buffer containing 1 µg of GST-ATF2 and 15 µCi of [γ-32P]ATP. Samples were incubated in the absence or presence of ATO (1 mM) for 30 min at 30 °C, and the reactions were terminated by the addition of Laemmli sample buffer, separated by SDS-PAGE (12% PAG), and subjected to autoradiography.

**In Vitro Phosphatase Assays**—*In vitro* phosphatase assays were performed essentially as described previously (42). Puri-
fied phosphorylated GST-HA-Chk2 (1 μg) or phosphorylated GST-p38 (1 μg) was suspended in phosphatase buffer (50 mM Tris-HCl (pH 7.5), 30 mM MgCl₂, 1 mg/ml bovine serum albumin, 0.05% 2-mercaptoethanol). In vitro phosphatase reactions were initiated by the addition of purified GST-Wip1 (WT) or GST as a control in the presence of the indicated concentrations of ATO or APO and allowed to incubate for 3 h at 30 °C. The reactions were terminated by the addition of Laemmli sample buffer and subjected to SDS-PAGE (10% PAG) followed by immunoblot analyses.

Steady-state Kinetic Assays—32P-Labeled Chk2 proteins were prepared by self-phosphorylation of GST-HA-Chk2 in vitro. The reaction mixture contained 10 μg of GST-HA-Chk2 and was suspended in 400 μl of kinase buffer (10 mM Hepes (pH 7.5), 30 mM MgCl₂, 1 mg/ml bovine serum albumin, 0.05% 2-mercaptoethanol) for 30 min at 30 °C. The reactions were initiated by the addition of 32P-labeled GST-HA-Chk2. The mixtures (100 μl) were incubated for 5 min at 30 °C. The reactions were terminated by the addition of a 5% activated charcoal suspension (500 μl). The addition of the charcoal suspension to the reaction mixtures quenched the phosphatase activity immediately. The activated charcoal absorbed 32P-labeled GST-HA-Chk2 but not the released phosphate. Therefore, after charcoal particles were removed by centrifugation, aliquots of the supernatant (100 μl) were counted for radioactivity in 2 ml of scintillation fluid.

To determine the modes of inhibition of Wip1 phosphatase by ATO, the initial velocities were measured at various concentrations of a substrate ([S]) (53). Kinetic behavior was approximated as a Michaelis-Menten system, and Lineweaver-Burk plots were derived from data. To calculate Kᵢ values, data were approximated by the formula for generic inhibition, \( V_{\text{max}} = \frac{V_{\text{max}}}{1 + [I]/Kᵢ} \), where [I] represents concentration of inhibitor. All assays were performed in triplicate, and data are presented as means ± S.D.

Induction of Nuclear Localization of Wip1-ER Fusion Protein with 4OHT—293T cells were transfected with pcDNA-Wip1-(1–516)-ER, encoding Wip1-ER fusion protein, that translocates from the cytoplasm to the nuclei in response to ligand (51). The ER portion of this fusion protein was mutated so that it specifically responds to 4OHT but not to estrogen (54). Twenty-four hours after transfection, cells were treated with 4OHT (1 μM) or an equivalent amount of vehicle (ethanol) for 4 h and were subjected to immunofluorescence analyses or preparation of cell lysates for immunoblot analyses.

Apoptosis Assays—Twenty-four hours after treatment with ATO at the indicated concentrations, NB4 and HL-60 cells were washed twice in RPMI1640, resuspended in 100 μl of RPMI1640, and allowed to attach onto coverslips. MCF7 cells were cultured on coverslips. Cells on coverslips were fixed with 4% paraformaldehyde plus PBS for 15 min at room temperature and then permeabilized with PBS containing 0.1% (v/v) Triton X-100 for 15 min at room temperature, and their nuclei were stained with DAPI. The fractions of apoptotic cells were determined by dividing the number of cells that exhibit apoptotic morphology (round cell shape with condensed nucleus) by the total number of cells observed. The assays were performed in triplicates, and in each assay at least 500 cells were counted.

RESULTS

Activation of Chk2 and p38 MAPK in an APL Cell Line following Treatment with ATO—It has been reported that ATO induces activation of Chk2 and p38 MAPK in various types of cells (26, 31–35). To verify that ATO can induce activation of Chk2 and p38 MAPK in APL cells, we monitored the phosphorylation status of Chk2 and p38 MAPK in an APL cell line, NB4 cells, following treatment with ATO, APO, or ATRA at the indicated concentrations. Extents of Thr68 phosphorylation in Chk2 and of Thr180 and Tyr182 phosphorylation in p38 MAPK, which are reliable biochemical markers for activation of Chk2 and p38 MAPK, respectively (55, 56), were monitored by using the respective phosphorylation site-specific antibodies (see “Experimental Procedures”). As shown in Fig. 1A, treatment of NB4 cells with ATO, but not APO, resulted in drastic increases in phosphorylation of Thr68 in Chk2 and of Thr180/Tyr182 in p38 MAPK in dose-dependent manners, indicating that ATO indeed induces activation of Chk2 and p38 MAPK. Similar results were obtained when a leukemia cell line, HL-60 cells, and a breast cancer cell line, MCF7 cells, were treated with ATO (data not shown). In accordance with the result, treatment of NB4 cells with ATO, but not APO, induced reproducible phosphorylation of Ser20 and Ser46 in p53 (Fig. 1A), well established p53 phosphorylation sites by activated Chk2 and p38 MAPK, respectively (20, 29). In addition, ATRA, another drug for therapy of APL patients, failed to induce the phosphorylation of Chk2, p38 MAPK, and p53 in NB4 cells (Fig. 1A).

These results indicate that ATO, but not APO or ATRA, induces activation of Chk2/p53 and p38 MAPK/p53 pathways.

It has been shown that the Thr⁶⁸-phosphorylated Chk2 forms distinct nuclear foci in response to γ-irradiation (ionizing radiation-induced nuclear foci) (57). Thus, we examined whether or not these nuclear foci of phosphorylated Chk2 can be induced by ATO treatment of NB4 cells. To this end, NB4 cells treated with ATO at the indicated concentrations for 24 h were subjected to immunofluorescence analyses using anti-phospho-Chk2 (Thr⁶⁸) antibodies, as indicated under “Experimental Procedures.” As shown in Fig. 1B, nuclear foci formation of Thr⁶⁸-phosphorylated Chk2 was induced by ATO in NB4 cells in a dose-dependent manner. The result suggests that, like γ-irradiation, ATO induces DNA damage, resulting in drastic nuclear foci formation of Thr⁶⁸-phosphorylated Chk2.

It has been shown that phosphorylation of Chk2 and p38 MAPK can be induced within 1 h in response to various stimuli, including γ-irradiation, UV irradiation, hydrogen peroxide, and anticancer reagents (57, 58). We thus examined the kinetics of phosphorylation of Chk2 and p38 MAPK following treatment of NB4 cells with ATO (10 μM). Interestingly, phosphorylation of Chk2 and p38 MAPK was slightly induced at 12 h after ATO treatment and increased thereafter (data not
Chk2 (Fig. 1C). Interestingly, APO, which failed to induce Chk2 phosphorylation, could induce degradation of PML-RARα in NB4 cells. Considering that both ATO and APO could induce degradation of PML-RARα in NB4 cells and that ATO, but not APO, could induce phosphorylation of Chk2, p38 MAPK, and p53 in NB4 cells, it is conceivable that activation of Chk2/p53 and p38 MAPK/p53 pathways by ATO is not necessarily attributable to degradation of PML-RARα.

**Activation of Chk2 and p38 MAPK by ATO-induced Oxidative Stress**—We next examined whether or not ATO can directly activate Chk2. To this end, GST-HA-Chk2 (WT) and GST-HA-Chk2 (DK) purified from 293T cells expressing the respective fusion proteins were subjected to *in vitro* kinase assays using GST-Cdc25C (aa 200–256) as a substrate in the absence or presence of ATO (see “Experimental Procedures”). As expected, purified GST-HA-Chk2 (DK) did not exhibit any detectable kinase activities toward GST-Cdc25C, irrespective of the absence or presence of ATO (1 mM) (Fig. 2A, compare *lanes* 5 and 6). Apparent kinase activities of purified GST-HA-Chk2 (WT) were unaffected even in the presence of a higher concentration of ATO (1 mM) (Fig. 2A, compare *lanes* 3 and 4).

We also examined the direct effect of ATO on kinase activities of p38 MAPK. The active form of GST-p38 MAPK was purified from UV-irradiated 293T cells expressing GST-p38 MAPK and was subjected to *in vitro* kinase assays using GST-ATF2 (aa 1–103) as a substrate in the absence or presence of ATO (see “Experimental Procedures”). As shown, kinase activities of GST-p38 MAPK toward GST-ATF2 were completely inhibited by an inhibitor of p38 MAPK, SB203580 (10 μM) (Fig. 2B, compare *lanes* 3 and 5). However, apparent kinase activities of GST-p38 MAPK toward GST-ATF2 were scarcely affected in the presence of a higher concentration of ATO (1 mM) (Fig. 2B, compare *lanes* 3 and 4). These results indicate that activation of Chk2 and p38 MAPK in NB4 cells following ATO treatment (Fig. 1A) is not due to direct effects of ATO on kinase activities of Chk2 and p38 MAPK.

Considering previous reports showing that ATO induces production of intracellular hydrogen peroxide in NB4 cells and that ATO-induced apoptosis of NB4 cells was inhibited by the presence of an antioxidant, NAC (16, 59), it is conceivable that ATO elicits oxidative stress, thereby activating Chk2 and p38 MAPK. We therefore investigated the effect of NAC on ATO-induced activation of Chk2 and p38 MAPK in NB4 cells. As shown, ATO-induced activation of Chk2 and p38 MAPK in NB4 cells was inhibited by NAC (10 μM) as assessed by immunoblot analysis with phosphorylation site-specific antibodies (Fig. 2C, compare *lanes* 2 and 4). The results indicate that activation of both Chk2 and p38 MAPK pathways are mediated by intracellular oxidative stress signaling elicited by ATO treatment.

**Involvement of both Chk2 and p38 MAPK in ATO-induced Apoptosis**—We then examined the effect of ATO on apoptosis of NB4 cells. NB4 cells were untreated (mock-treated) or treated with either ATO or APO at the indicated concentrations for 24 h, and apoptotic cells were scored as described under “Experimental Procedures.” As shown in Fig. 3A, enhanced apoptosis of NB4 cells was observed when the cells were treated with ATO, but not APO, in a dose-dependent manner.

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### FIGURE 1. ATO Induces Activation of Chk2 and p38 MAPK in an APL cell line, NB4. A, ATO-induced phosphorylation of Chk2, p38 MAPK, and p53 in NB4 cells. NB4 cells were treated with ATO, APO, or ATRA at the indicated concentrations for 24 h. WCLs were subjected to SDS-PAGE (10% PAG), transferred onto a PVDF membrane, and then immunoblotted with anti-phospho-Chk2 (Thr68), anti-Chk2, anti-phospho-p38 MAPK (Thr180/Tyr182), anti-p38 MAPK, anti-phospho-p53 (Ser20), anti-phospho-p53 (Ser392), or anti-p53 antibodies, respectively, as indicated. B, ATO-induced nuclear foci formation of Chk2 (Thr68)-irradiation fusion protein was degraded at 24 h. Consistent with previous reports showing that ATO induces phosphorylation of Chk2 and expression levels of PML-RARα following treatment of NB4 cells with ATO, phosphorylated Chk2 in NB4 cells following treatment of NB4 cells with ATO. Considering that both ATO and APO could induce degradation of PML-RARα in NB4 cells and that ATO, but not APO, could induce phosphorylation of Chk2, p38 MAPK, and p53 in NB4 cells, it is conceivable that activation of Chk2/p53 and p38 MAPK/p53 pathways by ATO is not necessarily attributable to degradation of PML-RARα.
manner. Similar results were obtained when HL-60 and MCF7 cells were treated with ATO or APO (supplemental Figs. S1A and S2A).

It has been reported that thymocytes from Chk2−/− mice were less sensitive to ATO-induced apoptosis compared with those from wild-type mice, indicating a role of Chk2 in the ATO-induced apoptosis (26). Thus, we examined whether or not Chk2 is required for ATO-induced apoptosis of NB4 cells. To this end, we performed siRNA-mediated knockdown of Chk2 in NB4 cells. Transfection of NB4 cells with Chk2 siRNA (number 1 or 2) resulted in a marked suppression of Chk2 expression in the cells (Fig. 3B (inset)). After transfection of NB4 cells with either control siRNA or Chk2 siRNA (number 1 or 2), the respective cells were untreated (mock-treated) or treated with ATO for 24 h, and apoptosis assays were performed as described under “Experimental Procedures.” In the absence of ATO, transfection of cells with control siRNA or Chk2 siRNA (number 1 or 2) by itself resulted in somewhat
Enhanced basal levels of apoptosis when compared with untransfected cells (Fig. 3, A and B). As shown in Fig. 3B, ATO-induced apoptosis was markedly inhibited by suppressed expression of Chk2, indicating that Chk2 is required for ATO-induced apoptosis of NB4 cells. Similar results were obtained when HL-60 and MCF7 cells, transfected with control siRNA or Chk2 siRNA (number 1 or 2), were subjected to apoptosis assays following ATO treatment (supplemental Figs. S1B and S2B).

Activation of p38 MAPK has been shown to associate with ATO-induced apoptosis of human leukemic cell lines, U937 and K562 cells (31, 32, 35). To determine whether or not p38 MAPK is required for ATO-induced apoptosis of NB4 cells, the cells were untreated or treated with ATO (10 μM) in the absence (DMSO alone) or presence of the p38 MAPK inhibitor SB203580 (10 μM), and 24 h after incubation, apoptotic cells were scored (see “Experimental Procedures”). Although SB203580 alone failed to affect the basal level of apoptosis, ATO-induced apoptosis was markedly inhibited by the presence of SB203580 (Fig. 3C), indicating that the activity of p38 MAPK is required for ATO-induced apoptosis of NB4 cells. Similar results were obtained when HL-60 and MCF7 cells were subjected to apoptosis assays following ATO treatment in the absence or presence of SB203580 (supplemental Figs. S1C and S2C).

Involvement of Wip1 in ATO-induced Apoptosis—It has been shown that Wip1, induced upon DNA damage in a p53-dependent manner, acts as a negative regulator of Chk2 and p38 MAPK by dephosphorylating phosphorylated Thr68 and Thr180 in Chk2 and p38 MAPK, respectively, during DNA damage response (38, 42, 60) and that Wip1 antagonizes Chk2-mediated apoptosis (42, 51). These findings led us to test whether or not Wip1 is involved in ATO-induced activation of Chk2 and p38 MAPK. To this end, we examined the effect of siRNA-mediated suppression of Wip1 expression on ATO-induced activation of Chk2 and p38 MAPK in NB4 cells. Two days after transfection of NB4 cells with either control siRNA or Wip1 siRNA (number 1 or 2), the respective cells were cultured in the absence or presence of ATO (5 μM) for 24 h. Transfection of NB4 cells with Wip1 siRNA (number 1 or 2) resulted in a drastic suppression of Wip1 expression in the cells, as assessed by anti-Wip1 immunoblot analysis (Fig. 4A). As shown, suppressed expression of Wip1 resulted in an enhanced phosphorylation of p38 MAPK but not Chk2 in the absence of ATO (Fig. 4A, compare lanes 1–3 in the first and third panels). Importantly, sup-
pressed expression of Wip1 further augmented ATO-induced phosphorylation of both Chk2 and p38 MAPK in NB4 cells (Fig. 4A, compare lanes 4–6 in the first and third panels), indicating that Wip1 suppresses ATO-induced activation of both Chk2 and p38 MAPK in NB4 cells. We next examined the effect of suppressed expression of Wip1 on the ATO-induced apoptosis. After transfection of NB4 cells with either control siRNA or Wip1 siRNA (number 1 or 2), the respective cells were cultured in the absence or presence of ATO (5 μM) for 24 h, and apoptosis assays were performed as described under “Experimental Procedures.” As shown in Fig. 4A, suppressed expression of Wip1 further augmented ATO-induced apoptosis of NB4 cells, indicating that Wip1 is involved in ATO-induced apoptosis. Similar results were obtained when HL-60 and MCF7 cells, transfected with control siRNA or Wip1 siRNA (number 1 or 2), were subjected to apoptosis assays following ATO treatment (supplemental Figs. S1D and S2D).

We next examined whether or not ATO can directly affect the dephosphorylation of the phosphorylated Thr^68 in Chk2 by Wip1. For this purpose, purified GST-Wip1 (WT) was subjected to in vitro phosphatase assays using phosphorylated GST-HA-Chk2 as a substrate in the presence of ATO or APO at the indicated concentrations, and the levels of Thr^68 phosphorylation in Chk2 were monitored by immunoblot analysis with anti-phospho-Chk2 (Thr^68) (see “Experimental Procedures”). As shown in Fig. 4B, GST-Wip1, but not GST, dephosphorylated the phosphorylated-Thr^68 in Chk2 in vitro (lanes 1 and 2). Interestingly, ATO, but not APO, suppressed the dephosphorylation of Chk2 by Wip1 in a dose-dependent manner (lanes 2–7). To examine the effect of ATO on dephosphorylation of the phosphorylated Thr^{180}/Tyr^{182} in p38 MAPK by Wip1 in vitro, purified GST-Wip1 (WT) was subjected to in vitro phosphatase assays using phosphorylated GST-HA-p38 MAPK as a substrate in the presence of ATO or APO at indicated concentrations, and the levels of Thr^{180}/Tyr^{182} phosphorylation in p38 MAPK were monitored by immunoblot analysis with anti-phospho-p38 MAPK (Thr^{180}/Tyr^{182}) (see “Experimental Procedures”). As shown in Fig. 4C, GST-Wip1, but not GST, dephosphorylated the phosphorylated Thr^{180}/Tyr^{182} in p38 MAPK in vitro (lanes 1 and 2).
ATO, but not APO, suppressed the dephosphorylation of p38 MAPK by Wip1 in a dose-dependent manner (lanes 2 and 5). Taken together, these results indicate that ATO directly inhibits phosphatase activities of Wip1 toward the phosphorylated Thr\textsuperscript{68} in Chk2 and phosphorylated Thr\textsuperscript{180}/Tyr\textsuperscript{182} in p38 MAPK, respectively.

To understand the molecular mechanism of Wip1 inhibition by ATO, we examined whether ATO can affect the interaction between Wip1 and Chk2. To this end, we performed in vitro binding analyses using MBP-Chk2 (WT) and GST-Wip1 (WT) purified from *E. coli*, respectively, in the presence of ATO at the indicated concentrations. MBP-Chk2 (WT) associated specifically with GST-Wip1 (WT), but not with GST, and the interaction between Wip1 and Chk2 was unaffected by the presence of ATO (supplemental Fig. S3). To determine the modes of inhibition of Wip1 phosphatase activity by ATO, the initial velocities of Wip1-mediated dephosphorylation reactions in the absence or presence of ATO at various concentrations of substrate were measured. Lineweaver-Burk plot analysis revealed that ATO acts as a noncompetitive mechanism for Wip1 (supplemental Fig. S4). A calculated *K*\textsubscript{i} value of ATO for Wip1 was ~25 \(\mu\text{M}\). It has been proposed that protein sulfhydryl (-SH) groups can be targeted by ATO. However, PP2C family protein phosphatases do not possess cysteine residues within their catalytic sites. Thus, it is possible that ATO targets cysteine residues outside the catalytic site of Wip1, affecting its catalytic activity.

**ATO Inhibits Wip1 Phosphatase Activity in Vivo** —We examined whether or not ATO inhibits phosphatase activity of Wip1 in vivo. For this purpose, we took advantage of a Wip1-ER fusion protein (Wip1-(1–516)-ER) that translocates from the cytoplasm to the nucleus in response to 4OHT (51). Wip1-(1–516)-ER were expressed transiently in 293T cells along with HA-Chk2 (WT), and transfected cells were treated with ATO or APO at the indicated concentrations in the absence or presence of 4OHT for 4 h. Subsequently, the levels of Thr\textsuperscript{68} phosphorylation in Chk2 were monitored by immunoblot analysis with anti-phospho-Chk2 (Thr\textsuperscript{68}) (see “Experimental Procedures”). As shown in Fig. 5A, HA-Chk2 (WT), expressed in 293T cells along with Wip1-(1–516)-ER, was heavily phosphorylated on Thr\textsuperscript{68} in the absence of 4OHT (lane 1), and this Thr\textsuperscript{68} phosphorylation in Chk2 was inhibited drastically in the presence of 4OHT (lane 3). The levels of Thr\textsuperscript{68} phosphorylation in Chk2 were unaltered in cells expressing HA-Chk2 (WT) alone with or without 4OHT (lanes 8 and 9), indicating that this 4OHT-induced inhibition of Thr\textsuperscript{68} phosphorylation in Chk2 is mediated by Wip1-(1–516)-ER localized in the nuclei. Interestingly, in the presence of 4OHT, ATO, but not APO, suppressed the inhibition of Thr\textsuperscript{68} phosphorylation in Chk2 by Wip1-(1–516)-ER in a dose-dependent manner (lanes 3–7). Furthermore, in the absence of 4OHT, the levels of Thr\textsuperscript{68} phosphorylation in Chk2 were unaffected by ATO treatment (lanes 1 and 2). Taken together, these results indicate that ATO inhibits phosphatase activity of Wip1 in the nuclei, resulting in Thr\textsuperscript{68} phosphorylation in Chk2.

We have previously shown that ectopic expression of Wip1-(1–516)-ER resulted in decreased numbers of γ-irradiation-induced phospho-Chk2 foci in a 4OHT-dependent manner (51). Thus, we examined the effect of ATO on the inhibition of nuclear foci formation of phospho-Chk2 by Wip1-(1–516)-ER, localized in the nuclei. To this end, Wip1-(1–516)-ER were expressed transiently in 293T cells, and transfected cells were treated with ATO at the indicated concentrations in the absence or presence of 4OHT for 3 h. Subsequently, cells were nonirradiated (−IR) or exposed to γ-irradiation (10 grays; +IR) and further cultured for 1 h. The nuclear foci of phospho-Chk2 were monitored by immunofluorescence analysis using anti-phospho-Chk2 (Thr\textsuperscript{68}) antibody (see “Experimental Procedures”). It was found that in the absence of 4OHT, the Wip1-(1–516)-ER fusion proteins were sequestered in the cytoplasm (Fig. 5B, top). However, Wip1-(1–516)-ER proteins were found exclusively in the nuclei following treatment of cells with 4OHT (1 \(\mu\text{M}\)) (Fig. 5B, middle and bottom). The nuclear foci of phospho-Chk2 were found in irradiated (+IR) but not nonirradiated (−IR) cells in the absence of 4OHT (Fig. 5B, top). Ectopic expression of Wip1-(1–516)-ER resulted in decreased numbers of IR-induced phospho-Chk2 foci in a 4OHT-dependent manner (Fig. 5B, middle and bottom). Importantly, the inhibition of IR-induced phospho-Chk2 foci by Wip1-(1–516)-ER, localized in the nuclei (+4OHT), was abrogated by the presence of ATO (Fig. 5B, bottom).

FIGURE 5. Wip1-mediated suppression of Thr\textsuperscript{68} phosphorylation in Chk2 is inhibited by treatment of NB4 cells with ATO. A, inhibition of Wip1-mediated suppression of Thr\textsuperscript{68} phosphorylation in Chk2 by ATO but not APO. 293T cells, expressing HA-Chk2 (WT) proteins along with or without the Wip1/estrogen receptor (ER) chimeric protein (Wip1-(1–516)-ER), were treated with the indicated concentrations of ATO or APO in the absence (ethanol alone) or presence of 4OHT (1 \(\mu\text{M}\)) for 4 h. WCLs were subjected to SDS-PAGE (10% PAGE), transferred onto a PVDF membrane, and then immunoblotted with anti-phospho-Chk2 (Thr\textsuperscript{68}), anti-HA, or anti-Wip1 (WC10) antibodies, respectively, as indicated. B, inhibition of Wip1-mediated suppression of nuclear foci formation of Thr\textsuperscript{68}-phosphorylated Chk2 by ATO. 293T cells, expressing Wip1-(1–516)-ER proteins were found exclusively in the nuclei following treatment of cells with 4OHT (1 \(\mu\text{M}\)) (Fig. 5B, middle and bottom). The nuclear foci of phospho-Chk2 were found in irradiated (+IR) but not nonirradiated (−IR) cells in the absence of 4OHT (Fig. 5B, top). Ectopic expression of Wip1-(1–516)-ER resulted in decreased numbers of IR-induced phospho-Chk2 foci in a 4OHT-dependent manner (Fig. 5B, middle and bottom). Importantly, the inhibition of IR-induced phospho-Chk2 foci by Wip1-(1–516)-ER, localized in the nuclei (+4OHT), was abrogated by the presence of ATO (Fig. 5B, bottom).
We next examined the effect of ATO on dephosphorylation of Thr<sup>180</sup>/Tyr<sup>182</sup> phosphorylation in p38 MAPK by Wip1 in vitro. To this end, Wip1 (WT) proteins were expressed transiently in 293T cells along with HA-p38 MAPK (WT), and transfected cells were treated with either ATO or APO at the indicated concentrations, followed by UV irradiation (50 J/m²), or left nonirradiated. Four hours after irradiation, HA-p38 MAPK proteins were immunoprecipitated with anti-HA antibody, and the levels of Thr<sup>180</sup>/Tyr<sup>182</sup> phosphorylation in p38 MAPK were monitored by immunoblot analysis with anti-phospho-p38 MAPK (Thr<sup>180</sup>/Tyr<sup>182</sup>) (see “Experimental Procedures”). As shown in Fig. 5C, HA-p38 MAPK was heavily phosphorylated on Thr<sup>180</sup>/Tyr<sup>182</sup> in UV-irradiated cells (lane 5), and this Thr<sup>180</sup>/Tyr<sup>182</sup> phosphorylation in p38 MAPK was inhibited in the presence of Wip1 (WT) (lane 7). Interestingly, ATO, but not APO, suppressed the inhibition of Thr<sup>180</sup>/Tyr<sup>182</sup> phosphorylation in p38 MAPK by Wip1 (WT) in a dose-dependent manner (lanes 7–11). Furthermore, in the absence of Wip1 (WT), the levels of Thr<sup>180</sup>/Tyr<sup>182</sup> phosphorylation in p38 MAPK were unaffected by ATO treatment (lanes 3 and 4). These results indicate that ATO inhibits phosphatase activity of Wip1, resulting in Thr<sup>180</sup>/Tyr<sup>182</sup> phosphorylation in p38 MAPK.

It has been shown that the nuclear accumulation of phosphorylated endogenous p38 MAPK is inhibited by ectopic expression of Wip1 (WT) in UV-irradiated HeLa cells (38). Thus, we examined the effect of ATO on the inhibition of nuclear accumulation of phospho-p38 MAPK by Wip1 (WT). To this end, Wip1 (WT) proteins were expressed transiently in HeLa cells, and transfected cells were treated with ATO at the indicated concentrations, followed by UV irradiation (50 J/m²) or left nonirradiated. Four hours after irradiation, the nuclear accumulation of phospho-p38 MAPK was monitored by immunofluorescence analysis using anti-phospho-p38 MAPK (Thr<sup>180</sup>/Tyr<sup>182</sup>) antibody (see “Experimental Procedures”). As shown in Fig. 5D, the phosphorylated p38 MAPK was primarily localized in the nuclei following UV irradiation. However, nuclear accumulation of the phospho-p38 MAPK was markedly inhibited in the cells expressing FLAG-Wip1 (WT) protein. Importantly, the inhibition of nuclear accumulation of UV-induced phospho-p38 MAPK by Wip1 (WT) was abrogated by the presence of ATO (Fig. 5D, bottom).

DISCUSSION

It has been well established that ATO, an effective drug for the treatment of APL, can induce apoptosis of APL cells in vitro and in vivo. In the present study, we show that ATO induces phosphorylation of both Chk2 and p38 MAPK and a nuclear foci formation of phosphorylated Chk2 (Fig. 1). Activation of Chk2 and p38 MAPK is mediated by ATO-induced oxidative stress but not by a direct effect of ATO on kinase activity of Chk2 and p38 MAPK (Fig. 2). We also show that both Chk2 and p38 MAPK are required for ATO-induced apoptosis (Fig. 3). Intriguingly, we found that ATO inhibits phosphatase activity of Wip1 directly in vitro and enhances phosphorylation of Chk2 and p38 MAPK by inhibiting Wip1 in vivo (Figs. 4 and 5). Wip1 is also involved in apoptosis of APL cells in response to ATO (Fig. 4). These results indicate that Wip1 is one of the critical target molecules of ATO and that ATO can enhance the activities of both Chk2 and p38 MAPK by inhibiting Wip1, in addition to its effect on Chk2 and p38 MAPK via oxidative stress signaling.

APL is characterized by the chromosome translocation generating the PML-RARα fusion gene, which plays a role in the disease. ATO as well as ATRA can induce degradation of the PML-RARα fusion protein, which is thought to be one of the mechanisms underlying therapeutic effects of ATO on APL. We show that APO also induces PML-RARα degradation; however, APO fails to induce activation of Chk2 and p38 MAPK and to induce apoptosis (Figs. 1 and 3), indicating that ATO-induced apoptosis of APL is not necessarily attributable to PML-RARα degradation by ATO. It has been reported that NB4 cells resistant to ATO-induced apoptosis remain sensitive to ATO-induced PML-RARα degradation (61). In addition, ATO, but not APO, has been shown to induce apoptosis of non-APL malignant cells (9). Therefore, it is likely that activation of Chk2 and p38 MAPK by ATO-induced oxidative stress and inhibition of Wip1 is required for a therapeutic effect of ATO on APL.

At present, it is unclear why APL cells are sensitive to ATO. In this respect, it is of importance to note that Wip1 has been considered to be an oncogenic protein on the basis of the findings that the gene encoding Wip1 (PPM1D) is amplified in human malignancies, including breast cancers (43–45), and that inactivation of Wip1 inhibits mammary tumorigenesis (47, 48). Thus, it can be envisaged that ATO may also be a therapeutic agent for tumors, such as breast cancers, overexpressing Wip1 in addition to APL. Further study will be required to clarify this issue.

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