Polo-like Kinases Inhibited by Wortmannin

LABELING SITE AND DOWNSTREAM EFFECTS*

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Polo-like kinases play crucial roles throughout mitosis. We previously reported that wortmannin potently inhibits Polo-like kinase 1 (Plk1). In this study, we show that wortmannin also strongly inhibits Polo-like kinase 3 (Plk3). To further characterize this inhibition, we identified the sites of labeling on Plk1 and Plk3 targeted by AX7503, a tetramethylrhodamine-wortmannin conjugate. AX7503 labeling on Plk1 and Plk3 was found to occur on a conserved ATP binding site residue. In addition, we show that wortmannin inhibits Plk3 activity in live cells at concentrations commonly used to inhibit the more well known targets of wortmannin, the phosphoinositide 3-kinases. Importantly, we found that inhibition of Plk3 by wortmannin lead to a decrease in phosphorylation of p53 on serine 20 induced by DNA damage, demonstrating the effect of wortmannin on a downstream Plk3 target. Taken together, our results suggest that wortmannin can affect multiple functions of Plk3 in cell cycle progression and at the DNA damage checkpoint. The identification of the labeling sites of Plk1 and Plk3 by AX7503 may be useful in designing more effective compounds to target Polo-like kinases for cancer treatment and also may be useful for the structural study of Plk domains.

Polo-like kinases (Plks) are a conserved family of serine-threonine protein kinases that play many important roles during multiple stages of the cell cycle, especially in mitosis (1, 2). There are four Plk family members in mammals, namely Plk1, Plk2, Plk3, and Plk4 (3). Plk2 and Plk4 are less characterized than are Plk1 and Plk3. In human cells, the levels of Plk1 protein and its kinase activity reach their maximal level in mitosis (4). Plk1 exerts its multiple functions in mitosis, including the activation of cyclin B–Cdc2 (CDK1) (5–8), centrosome maturation (9–11), bipolar spindle assembly (11, 12), and breakdown of the nuclear membrane (13). Overexpression of Plk1 is observed in various human tumors and is a negative prognostic factor in patients suffering from diverse cancers (14–16).

Mammalian Plk3 was originally identified as an immediate-early response gene product (17, 18). Subsequent studies showed that this protein is functionally conserved and involved in the regulation of mitosis (19) and DNA damage responses (20, 21). On the other hand, Plk3 functions differently from Plk1 in human cells in terms of its regulation during the cell cycle and response to stimulation by growth factors and stresses (18–20). Plk3 is expressed throughout the cell cycle. The levels of Plk3 protein remain relatively constant during the cell cycle, and its kinase activity peaks during late S and G2 phases (22). Ectopic expression of Plk3 or its mutants perturbs microtubule integrity, resulting in dramatic morphological changes, G2/M arrest, and apoptosis (22, 23).

Wortmannin is a fungal metabolite that has been used widely as a potent, selective, and cell-permeable inhibitor of phosphoinositide (PI) 3-kinases (24, 25). Indeed, a Medline search of “wortmannin and PI 3-kinase” results in thousands of references. We previously reported that wortmannin potently inhibits Plk1 in addition to the PI 3-kinase superfamily (26). Unlike Polo-like kinases, which phosphorylate proteins, PI 3-kinases are lipid kinases that phosphorylate the 3′-hydroxyl position of the inositol head group of member phosphoinositides. PI 3-kinases and Polo-like kinases do not bear significant sequence similarity other than the basic requirements for being kinases. Therefore, it was surprising to find that wortmannin, in addition to inhibiting PI 3-kinases, is a potent inhibitor of Plk1.

Here, we show that wortmannin is also a potent inhibitor of Plk3. Using liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods (27, 28), we identified the sites of labeling on Plk1 and Plk3 labeled by AX7503, a tetramethylrhodamine-wortmannin conjugate. AX7503 labeling on Plk1 and Plk3 was found to occur on a conserved lysine residue in the ATP binding site. Additionally, we show that wortmannin inhibits Plk3 activity in an in vitro kinase assay and when incubated with intact cells. Importantly, we found that inhibition of Plk3 by wortmannin lead to a decrease in the Plk3-mediated downstream phosphorylation of p53 on serine 20 induced by DNA damage checkpoint.

EXPERIMENTAL PROCEDURES

Reagents—Wortmannin, LY294002, and α-casein were purchased from Sigma. [γ-32P]ATP was from Amersham Biosciences. Recombinant Plk3 and Plk2 were from BPS Bioscience Inc. pCR259 vector was from Invitrogen. Anti-Plk3 antibody was from BD Pharmingen. Anti-His6 antibody was from Qia-
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gen. Anti-phosphorylated p53 (serine 20) was from Cell Signaling Technology. Anti-p53 antibody was from Santa Cruz Biotechnology.

Cell Culture—HeLa, Jurkat, and A549 cell lines were obtained from the American Type Culture Collection. The GM00637 cell line (human fibroblast) was originally obtained from the Coriell Institute for Medical Research. Cells were cultured in dishes in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and antibiotics (100 \( \mu \)g/ml penicillin and 50 \( \mu \)g/ml streptomycin sulfate) with 5% CO\(_2\). Jurkat cells were cultured in dishes in RPMI 1640 medium supplemented with 10% fetal bovine serum with 5% CO\(_2\). To obtain G\(_2\)/M-arrested cells, Jurkat cells were treated with 3 mM hydroxyurea for 15 h as described previously (26).

Transfection and Cell Treatment—HeLa and GM00637 cells were transfected with plasmid constructs for 16 h using the Lipofectamine method. HeLa cells were transfected with pCR259-Plk3-A, pCR259-Plk3K91R, or the empty vector, and the mutant K91R Plk3 construct were adjusted from HeLa cells transfected with empty vector, a wild-type Plk3 construct, and the empty vector (22). The transfected GM00637 cells were first mixed with 33 \( \mu \)g/ml leupeptin (19). The whole cell lysate was subjected to centrifugation and the supernatant was collected. The G\(_2\)/M-arrested cells, Jurkat cells were treated with 3 mM hydroxyurea for 60 min as described previously (26).

Preparation of Cell Lysates—HeLa and GM00637 cells were collected and lysed in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton 100, 1 mM EDTA, Na\(_3\)VO\(_4\), 1 mM phenylmethylsulfonyl fluoride, 10 \( \mu \)g/ml aprotinin, 10 \( \mu \)g/ml leupeptin) (19). The whole cell lysate was subjected to centrifugation and the supernatant was collected. The G\(_2\)/M-arrested Jurkat cells were collected and lysed as described previously (26).

Labeling and Detection of Plk3 with AX7503—Cell lysates from HeLa cells transfected with empty vector, a wild-type Plk3 construct, and the mutant K91R Plk3 construct were adjusted to 1.5 mM and then treated with AX7503 (75 nM) for 60 min. For labeling of recombinant Plk3, 1 \( \mu \)l (500 units/mg) of recombinant Plk3 was first mixed with 19 \( \mu \)l of 50 mM Hepes-NaOH (pH 7.4) containing 400 mM \( \beta \)-glycerol phosphate, 100 mM sodium pyrophosphate, and 500 mM sodium sulfate. The recombinant Plk3 was preincubated with or without wortmannin (0.5 \( \mu \)M) for 10 min and then treated with AX7503 (75 nM) for 60 min at room temperature. Reactions were quenched with 1 volume of standard 2\( \times \) SDS-PAGE loading buffer (reducing) and separated by SDS-PAGE. Proteins labeled with AX7503 were visualized in-gel with a flatbed laser scanner (Hitachi FM BioII).

Polo-like Kinase 3 Activity Assay—Plk3 kinase assays were performed essentially as described previously (20). For \textit{in vitro} kinase assay, 0.25 \( \mu \)l (31.25 milliunits) of recombinant Plk3 was first mixed with 33 \( \mu \)l of kinase buffer (10 mM Hepes-NaOH (pH 7.4), 10 \( \mu \)M MnCl\(_2\), 5 \( \mu \)M MgCl\(_2\)), 1 \( \mu \)l of 400 \( \mu \)M cold ATP, and wortmannin or LY294002 at the indicated concentration. The kinase reaction was initiated by the addition of [\( \gamma \text{-}^{32}\text{P}\)]ATP (2 \( \mu \)Ci) and 5 \( \mu \)l of 4 \( \mu \)g/ml a-casein. After incubation for 30 min at 37 °C, the reaction mixtures were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. The density of signals was measured with Chemilager\textsuperscript{TM} 5500 (Alpha Innotech) and analyzed by AlphaEaseFC software. The procedure of \textit{in vivo} kinase assay is the same as that of \textit{in vitro} kinase assay except that the recombinant Plk3 was replaced with Plk3 immunocomplex and the kinase reaction was set up in the absence of inhibitors. In brief, A549 cells were exposed to wortmannin at the indicated concentration for 30 min, washed, lysed, and subjected to immunoprecipitation with antibody to Plk3 (19). The resulting precipitates were resuspended in kinase buffer and subjected to kinase assay. A portion of SDS-PAGE gel from \textit{in vivo} kinase assay containing Plk3 protein was used for Western blot of Plk3. The density of Plk3 bands was used for normalization of Plk3 kinase activity.

Western Blot Analysis—Quenched AX7503 reactions were run on SDS-polyacrylamide gel electrophoresis followed by immunoblotting with antibodies to His\(_6\) or Plk3. For transfected GM00637 cells treated with or without wortmannin and H\(_2\)O\(_2\), equal amounts (40 \( \mu \)g) of protein lysates from the treated cells were analyzed by SDS-polyacrylamide gel electrophoresis followed by immunoblotting with antibodies to serine 20-phosphorylated p53. The same blots were also stripped and reprobed with antibody that recognizes p53 regardless of phosphorylation state. Signals were detected with horseradish peroxidase-conjugated goat secondary antibodies and enhanced chemiluminescence reagents as described previously (20).

Sample Preparation, Digestion, and Affinity Capture of AX7503-labeled Peptides—Soluble fractions from the G\(_2\)/M-arrested Jurkat cells or recombinant Plk3 were labeled with 250 mM AX7503 for 60 min. The AX7503-labeled recombinant Plk3 was then added to preboiled mouse brain soluble fraction (0.5 mg/ml). The samples were then denatured with urea, incubated with dithiothreitol and iodoacetamide, and digested with trypsin as described previously (27). After digestion, peptides were captured by agarose beads conjugated to monoclonal anti-tert-rhamethylrhodamine antibody. The enriched, labeled peptides were eluted from the capture beads with 50% acetonitrile, 0.1% trifluoroacetic acid as described previously (27).

Identification of AX7503 Labeling Sites on Plks—Peptides were analyzed in a combination system of a capillary HPLC Micro Autosampler (Agilent Technologies) and LTQ-linear ion trap mass spectrometer with nano-ESI source (Thermo Finnigan, San Jose, CA) and were identified with TurboSEQUEST (Thermo Finnigan). 5 \( \mu \)l of labeled tryptic peptides was injected through a micro autosampler, desalted, and concentrated on a peptide capTrap (Michrom Bioresearches, Auburn, CA). The tagged peptides were then separated in a 0.18 × 100-mm, C\(_{18}\), 5-mm reversed-phase column with a gradient of 5% acetonitrile, 0.1% formic acid to 50% acetonitrile, 0.1% formic acid for 2 h with a column flow of 1.5 ml/min and injected into the LTQ mass spectrometer. Data were acquired in data-dependent MS/MS on the top nine ions on a full scan in the range of 500 to 1800 \textit{m/z} with the following settings: spray voltage, 1.8 kV; capillary temperature, 200 °C; capillary voltage, 46 V; tube lens voltage, 120 V; relative collision energy, 35%.

LC-MS/MS data were searched against protein data bases by using the SEQUEST algorithm as described previously (27).

RESULTS

Inhibition of Polo-like Kinase 3 by Wortmannin—We recently showed that wortmannin is a potent inhibitor of Plk1 and AX7503, a tetramethylrhodamine-wortmannin conjugate, is
an activity-dependent probe for labeling Plk1 (26). To determine whether AX7503 could also label Plk3, another member of the Polo family, we incubated recombinant human His<sub>6</sub>-tagged Plk3 with AX7503 in the presence or absence of preincubation with wortmannin. As seen in Fig. 1, AX7503 strongly labeled Plk3. This labeling of Plk3 by AX7503 was totally blocked by preincubation of Plk3 with wortmannin. Western blot analysis using a monoclonal anti-His<sub>6</sub> antibody showed the presence of equivalent levels of His<sub>6</sub>-tagged Plk3 in both wortmannin-treated and untreated samples. These results indicated that AX7503 is an effective probe for Plk3 as well as for Plk1.

To determine the potency of wortmannin as an inhibitor of Plk3, we quantified competition between AX7503 and wortmannin for Plk3. As seen in Fig. 2A, wortmannin inhibited Plk3 reacting with AX7503 in a dose-dependent manner. By fitting the dose-response data in Fig. 2B, the IC<sub>50</sub> value of wortmannin for inhibiting labeling of Plk3 by AX7503 was determined to be 49 nM. We also determined the potency of LY294002 as an inhibitor of Plk3-AX7503 reactivity (Fig. 2B). LY294002 is a less potent noncovalent inhibitor of PI 3-kinases; it has been shown to inhibit Plk1 with a potency similar to that of PI 3-kinases (26). We found that LY294002 prevented Plk3 labeling with AX7503. The LY294002 IC<sub>50</sub> value for inhibiting Plk3-AX7503 reactivity was determined to be 3.0 μM. These results demonstrated that wortmannin and LY294002 inhibited Plk3-AX7503 reactivity with a potency similar to their inhibition of Plk1-AX7503 reactivity; their IC<sub>50</sub> values for inhibiting Plk1-AX7503 reactivity were 5.8 nM and 2.1 μM, respectively (26).

We previously reported that wortmannin and LY294002 were able to inhibit Plk1 activity in an in vitro substrate-based assay (26). To determine whether wortmannin and LY294002 could inhibit Plk3 substrate phosphorylation, we performed in vitro kinase assays using casein as a substrate. By fitting the dose-response data in Fig. 3, the in vitro kinase result showed that wortmannin could potently inhibit the activity of purified Plk3 with an IC<sub>50</sub> of 48 nM, consistent with the results obtained from the AX7503 reactivity assay (Fig. 2). As in the AX7503 assay, LY294002 was a much less potent inhibitor of Plk3 than wortmannin; in the substrate assay, the IC<sub>50</sub> value for inhibiting Plk3 was 88 μM.

Identification of AX7503 Labeling Sites on Plk1 and Plk3—To identify the site of labeling on Plks targeted by AX7503, we applied an LC-MS/MS platform (27, 28). The AX7503-labeled purified Plk3 was added to heat-denatured mouse brain proteome as carrier. The sample was denatured, reduced, alkylated, and digested as described previously (27, 28) and then analyzed by LC-MS/MS. For identification of the labeling site on Plk1, Jurkat cells were first chemically synchronized at G<sub>2</sub>/M, where the level and activity of Plk1 reach maximal during the cell cycle (4). Soluble protein extracts from the G<sub>2</sub>/M-arrested cells were labeled with AX7503, denatured, reduced, alkylated, and digested as described previously (27, 28) and then
analyzed by LC-MS/MS. Analysis of the labeling site of AX7503 on Plk3 by using LC-MS/MS and the SEQUEST search algorithms identified a single AX7503-modified peptide spanning cysteine 76 to arginine 97 (Fig. 4). Lysine 91 was identified to be the probe-labeled residue (Fig. 4). Similarly, analysis of the labeling site of AX7503 on Plk1 also identified a single AX7503-modified peptide spanning cysteine 67 to lysine 86 where lysine 82 was identified to be the probe-labeled residue (data not shown). Table 1 summarizes the sites of labeling of Plk1 and Plk3 by AX7503. As seen in Table 1, a single AX7503-modified peptide was identified for each enzyme, and MS/MS analysis identified a specific site of AX7503 labeling on each peptide (shown by asterisks in Table 1). Sequence alignment of human Polo subfamily members revealed that the labeling sites of AX7503 on Plk1 and Plk3 occurred on a conserved lysine residue in the ATP binding site (Fig. 5).

We next explored the impact on AX7503 reactivity of replacing lysine 91 with arginine. Mutation of lysine 91 has been shown to inactivate Plk3 kinase activity (21).

### Table 1

| Enzyme | Labeled peptide | Labeling site | Residue |
|--------|-----------------|---------------|---------|
| Plk1   | CFESDADTFTGPKAG*KVIPQSR | Lys-82 ATP binding | |
| Plk3   | CYEATDTGSAYAV*KVIPQSR | Lys-91 ATP binding | |

*An asterisk follows the amino acid labeled by AX7503.*
PLK1 59–LGGGFAPCPEDADKPEFPG1V9KPS
PLK2 88–LGGGFAPCTEVDLLTNKYAKTK19H6
PLK3 68–LGGGFAPCEAWEKTETGSCAVV9VIB6
PLK4 18–LGGGFAPCVYPAESIGHSCLEVNL9KDNK

FIGURE 5. Sequence alignment of human Polo subfamily members in the local region surrounding their ATP binding site (marked by an asterisk).

Western blot analysis with a monoclonal Plk3 antibody confirmed that the expression of Plk3 did not change upon treating cells with wortmannin (data not shown). These results indicate that wortmannin inhibits both Plk3 and Plk1 in experiments where its use is intended to inhibit PI 3-kinases.

Inhibition of Plk3-mediated Phosphorylation of p53 by Wortmannin—We next conducted experiments to determine the impact of wortmannin treatment on a representative Plk3 biological function, the response to a DNA damage checkpoint. Plk3 was transfected into GM00637 cells for 16 h followed by treatment with or without wortmannin (1 μM) prior to H_{2}O_{2} treatment. As expected, H_{2}O_{2} significantly increased the level of serine 20-phosphorylated p53 in the Plk3-transfected cells (Fig. 8) (20). However, preincubation with wortmannin at 1 μM, which inhibited 75% of Plk3 activity in A549 cells, largely impaired the up-regulation of p53 serine 20 phosphorylation (Figs. 7 and 8). This result indicated that wortmannin was able to suppress cellular Plk3 activity and, consequently, decrease the downstream p53 phosphorylation induced by the DNA damage checkpoint.

DISCUSSION

In this study, we show that wortmannin covalently labels Plk1 and Plk3 by targeting conserved lysine residues in their ATP binding sites. Wortmannin inhibits Plk1 and Plk3 with a potency similar to its inhibition of PI 3-kinases (Figs. 2 and 3) (26). Wortmannin was found to inhibit Plk3 activity within cells, and it suppressed the Plk3-mediated phosphorylation of p53 on the serine 20 residue induced by H_{2}O_{2}, demonstrating an effect of wortmannin on a downstream Plk target.

The Plk family consists of four members, Plk1–Plk4. Because the kinase domains of Plks are highly conserved (29% amino acid identity for all four enzymes, 48% amino acid identity among Plk1, Plk2, and Plk3), our results suggest that wortmannin should also inhibit Plk2 and Plk4. Indeed, preliminary experiments indicate that AX7503 labels recombinant Plk2 (data not shown). This labeling was strongly inhibited by prior incubation of Plk2 with 1 μM wortmannin (data not shown), suggesting that it is specific.
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Because wortmannin has been used extensively to experimentally inhibit PI 3-kinases, it is of interest to know whether Plks are also inhibited by wortmannin in vivo. Our current and previous studies have shown that wortmannin significantly inhibits Plk1 and Plk3 activity at the concentrations (50–500 nM) of wortmannin commonly used for inhibiting PI 3-kinases in live cells (26). PI 3-kinases play a central role in many physiological and pathological processes related to cancer, inflammation, immunology, and cardiovascular disease (31). However, our data suggest that some of the pharmacological effects of wortmannin, originally assumed to reflect the inhibition of PI 3-kinases, might be because of the inactivation of Plks. Moreover, wortmannin is also used to inhibit PIKKs such as ATM in live cells at higher concentrations (micromolar) of wortmannin because of the reduced potency of wortmannin in inhibiting members of these kinases (32). PIKKs play central roles in stress-induced signaling pathways (33, 34). However, our results suggest that wortmannin should lead to complete inhibition of Plk1 and Plk3 at the concentrations that are used to inhibit PIKKs. Therefore, some effects of wortmannin that were originally assumed to reflect the inactivation of PIKKs may be due to inhibition of Plks.

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