Deficiency in Chromosome Congression by the Inhibition of Plk1 Polo Box Domain-dependent Recognition

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Polo-like kinase 1 (Plk1) is one of the key regulators of mitotic cell division. In addition to an N-terminal protein kinase catalytic domain, Plk1 possesses a phosphopeptide binding domain named polo box domain (PBD) at its C terminus. PBD is postulated to be essential for Plk1 localization and substrate targeting. Here, we developed a high-throughput screening system to identify inhibitors of PBD-dependent binding and screened a chemical library. We isolated a benzotropolone-containing natural compound derived from nutgalls (purpurogallin (PPG)) that inhibited PBD-dependent binding in vitro and in vivo. PPG not only delayed the onset of mitosis but also prolonged the progression of mitosis in HeLa cells. Although apparently normal bipolar spindles were formed even in the presence of PPG, the perturbation of chromosome alignment at metaphase plates activated the spindle assembly checkpoint pathway. These results demonstrate the predominant role of PBD-dependent binding on smooth chromosome congression at metaphase.

In eukaryotes, Plk1 (or its orthologs) possesses a wide variety of essential functions during mitosis (1, 2). Although levels of Plk1 increase in late G2 and rapidly decrease by proteolysis at the end of M phase, Plk1 localization changes dramatically when cells transit through the various mitotic stages. During late G2 and prophase, Plk1 localizes primarily at the centrosomes, where it is involved in centrosome maturation, separation, and microtubule nucleation (3–5). At this stage, Plk1 also regulates mitotic entry through the phosphorylation and activation of Cdc25 (6, 7). In parallel, the phosphorylation of Wee1 by Plk1, which is primed by Cdk1, promotes the destruction of Wee1, the inhibitory kinase of mitotic entry (8, 9). By metaphase, a fraction of Plk1 relocalizes to the kinetochores, where it is involved in the regulation of the spindle assembly checkpoint pathway and proper chromosome segregation. Plk1 sensitizes the tension between sister chromatids and regulates the stability of kinetochore-microtubule interactions by phosphorylating kinetochore proteins such as BubR1 and proteins that harbor S3F/32 epitopes (4, 10–14). Although Plk1 localization at chromosome arms is required for loss of arm cohesion during M-phase (15, 16), localized Plk1 activity at the kinetochore is important for the proper accumulation of kinetochore proteins such as BubR1, Mad1, Mad2, Cdc20, and centromere/kinetochore-associated protein-E (CENP-E) (4, 10–12, 17, 18). During anaphase, Plk1 is concentrated in the spindle midzone, where it may facilitate microtubule sliding. After chromosomal segregation, Plk1 remains in the central spindle and midbody, where it is involved in formation of the cleavage furrow (19–21).

These various localizations of Plk1 are mediated by its non-catalytic C-terminal half, the polo box domain (PBD), which comprises two polo box motifs (22, 23). Recently, Yaffe and co-workers (24, 25) discovered that the PBD constitutes a phosphopeptide binding domain which binds with maximal affinity to phosphopeptides containing the sequence S(pS/pT)(P/X). Crystallographic studies have confirmed the specific binding of phosphopeptides to an interface formed by the two polo-box motifs (25). In this structure two residues of human Plk1, His-538 and Lys-540, are shown to be essential for this binding, interacting directly with the phosphate group of the phosphopeptide. This PBD-dependent binding not only is important for the subcellular localization but is also necessary for the targeting of Plk1 to specific substrates (23, 25); Plk1 also binds to other proteins such as HSP90 via PBD-independent mechanisms as well (26).

Although the roles of Plk1 in mitotic progression as described above have been elucidated mainly by depletion of Plk1 using small interfering RNA or specific antibodies, the role of PBD-dependent binding has been examined through overexpression of the PBD (23, 27). Overexpressed PBD perturbed the subcellular localization of endogenous Plk1, implicating the requirement of the PBD for proper localization of Plk1 (23, 27). There are many defects induced by Plk1 depletion (e.g. defects in γ-tubulin and aurora-A recruitment to the centrosome, centrosome separation, spindle formation, chromosome congression, and loss of arm cohesion); however, only a few processes, such as chromosome congression, mitotic spindle stabilization, and cytokinesis, are impaired by PBD overexpression (23, 27).
These results indicate that only Plk1 activity itself, but not PBD-dependent localization, is required for some processes, such as bipolar spindle formation (23, 27).

Through the recent, remarkable progress in chemical biological analysis, several specific inhibitors of Plk1 activity have been identified (28–32) with which the roles of Plk1 on mitotic progression have been confirmed. In the present study we have developed a high-throughput screening system to identify small molecule inhibitors of PBD-dependent binding and implemented this assay in a screen of compounds that were stored in RIKEN NPDepo (Natural Products Depository) (33). We found several hit compounds, and one of them, purpuragallin (PPG), inhibited PBD-dependent binding in vitro and in vivo. Using PPG, we have shown that PBD-dependent binding is predominantly required for stabilization of the mitotic spindle and smooth mitotic chromosome congression.

**EXPERIMENTAL PROCEDURES**

**Cell Cultures**—Cell culture conditions and synchronization were performed as described (8, 34). PPG (Chromodex, CA) was dissolved in DMSO at a concentration of 10 mM and added to the culture media. In the control population, DMSO was added instead. HeLa cells expressing EYFP-tagged α-tubulin were established from cells transfected with an expression vector (35) encoding the α-tubulin-EYFP fusion protein (obtained from Dr. Wei Jiang, Burnham Institute, San Diego, CA) after G418 selection.

**Screening System for Inhibitors of PBD-dependent Binding**—DNA fragments encoding monomeric Venus (mVenues; A206K-mutated Venus (36); provided from Dr. Atsushi Miyawaki, BSI, RIKEN, JAPAN) were amplified by PCR and inserted into the EcoRI site between GST and PBD of the GST-PBD from Plk1 expression vector (wild-type or H538A/K540M mutant (25); provided from Dr. Mike Yaffe, MIT). PBD of Plk2 (amino acids 355–685) and Plk3 (amino acids 334–646) were also expressed as mVenues fusion proteins in pRSET-B expression plasmids (Invitrogen). Phosphopeptides with target sequences (derived from human Wee1A; C-EEGFGSSp-SPVKSPAAP) for PBD-dependent binding or its derivatives (without phosphorylation or serine-to-alanine mutation at the underlined position) were chemically synthesized and purified. Phosphopeptides with an optimal binding sequence for PBD-dependent binding were selected from the screening performed as described (9). PPG was added to bacterial lysates before mixing and incubated for 16 h in growth medium in the presence of 50 μM PPG or DMSO before fixation. For staining with the anti-γ-tubulin antibody, cells were first fixed with 3.7% formalin in phosphate-buffered saline for 1 min then further fixed and extracted with methanol at −20 °C for 5 min. For staining CENP-E, cells were pre-extracted for 1 min with 0.1% Triton X-100 in PHEM buffer (100 mM PIPES, 20 mM HEPES, pH 6.9, 5 mM EGTA, 2 mM MgCl₂) followed by a 5-min fixation with 3.7% formalin in PHEM. For analysis of cold-stable microtubules, cells were incubated for 10 min on ice in growth medium supplemented with 20 mM HEPES at pH 7.3, then fixed for 10 min at room temperature with 3.7% formalin in PHEM plus 0.2% Triton X-100. For other analyses, cells were fixed for 5 min with 3.7% formalin in PHEM plus 0.5% Triton X-100, blocked in blocking solution (0.5% bovine serum albumin and 50 mM glycin in phosphate-buffered saline), and then incubated with antibodies diluted in blocking solution. Primary antibodies used were mouse ab17057 anti-Plk1 monoclonal antibody (mAb; 1:200, Abcam), rabbit polyclonal anti-γ-tubulin antibody (1:1,000, Sigma), mouse anti-α-tubulin DM1α mAb (1:10,000, Sigma), mouse anti-BubR1 8G1 mAb (1:500, MBL), human CREST (1:1,200, Cortex Biochem), and mouse anti-CENP-E mAb (37) (1:500, obtained from Dr. Tim Yen, Fox Chase Cancer Center, Philadelphia, PA). Antibodies conjugated with Alexa fluorophores (Invitrogen) were used at 1:400 dilutions as secondary antibodies, and DNA was stained with 4′,6-diamidino-2-phenylindole (in Vectashield mounting medium, Vector Laboratories, Burlingame, CA).

Images were acquired as 0.2-μm distanced optical sections in the z axis using a Delta Vision RT microscope system (Applied Precision) on an Olympus IX-71 base that was equipped with a PlanApo 60×/1.40 oil immersion objective (Olympus). Images at single focal planes were processed with a deconvolution algorithm by Softworx software (Applied Precision). Settings were conservative, with noise filtering set to a medium and eight deconvolution cycles. Maximal intensity projections were shown in all figures except for the right part of Fig. 6C (section) in which single optical sections were shown to visualize the individual kinetochores more clearly. Images shown in the same panel have been scaled identically.

**Live-cell Imaging**—HeLa cells stably expressing histone H2B-EFGP (38) (obtained from Dr. Hiroshi Kimura, Kyoto University, Japan) or α-tubulin-EYFP were seeded on glass-bottom dishes (MatTek Corp., Ashland, MA) and synchronized at the G1/S boundary by double thymidine block as described above.
One hour after the release, 50 \mu M PPG was added, and another 7 h later the culture dish was placed onto a heated sample stage (37 °C) within a heat chamber filled with 5% CO₂ for imaging. Images were acquired at 3-min intervals for 5 h from 9 h after the release using the same microscope system described above using an UplanApo 20×/0.70 objective (Olympus) and analyzed with Softworx software (Applied Precision).

RESULTS

Development of the Screening System for PBD-dependent Binding Inhibitors—We have established a screening system to isolate small molecule inhibitors of PBD-dependent binding in a high-throughput manner (Fig. 1A). To quantitate PBD, the open reading frame of a fluorescent protein (monomeric Venus (36) was inserted between GST and PBD in a GST-PBD expression vector (25), and the GST-mVenus-PBD construct was expressed in bacteria. Phosphopeptides of the PBD binding sequence derived from human Wee1A (C-EFGFGSSpSPVKSPAAP) (9) were chemically synthesized and covalently bound to maleimide-activated 96-well plates. The bacterial lysates expressing GST-mVenus-PBD were mixed with the test compounds and put into each well; unbound PBD was washed away, and bound PBD was quantitated by spectrofluorometry. When small molecules that compete with the phosphopeptides for PBD binding are present in the assay, PBD will fluoresce less.

The level of bound PBD was increased when greater amounts of phosphopeptides were bound on the plates (Fig. 1B). When mutant PBD, in which two amino acids (His-538 and Lys-540) that are known to interact directly with the phosphate group of the target phosphopeptides (25) were mutated was used, no significant binding was detected (Fig. 1B). It is also known that binding of the PBD is strictly dependent on the phosphoserine (or phosphothreonine) and the serine preceding it (24, 25) (SpS; underlined in the peptide sequence above). When phosphopeptides in which the serine preceding the phosphorylation site was substituted to alanine (ApS) or when unphosphorylated peptides (SS) were used, the binding was also diminished (Fig. 1B). These results indicate that the specificity of PBD-dependent binding is properly reproduced in our system.

The phosphopeptides named poloboxtides are experimentally determined to be the optimal sequence for PBD-dependent binding (25). When bacterial lysates containing PBD were mixed with poloboxtides before putting into the wells, the binding was inhibited in a dose-dependent manner (Fig. 1C). Similar but less inhibition was observed when lysates were mixed with the phosphopeptides with a less optimal binding sequence (SpS, Fig. 1B). But mutant phosphopeptides (ApS, Fig. 1B) that do not bind to PBD could not inhibit the binding. These results indicate that small molecule inhibitors of PBD-dependent binding, which have similar effects like poloboxtides, can be screened out using this assay system.

Purpurogallin Inhibits PBD-dependent Binding in Vitro and in Vivo—We implemented this screening system in a screen of compounds that were stored in RIKEN NPDepo (Natural Products Depository) (33). Among about 2500 compounds screened, a benzotropolone-containing natural compound derived from nutgalls PPG (Fig. 2A) inhibited PBD-dependent binding most strongly (Fig. 1, C and D). Interestingly, however, compounds similar to PPG (2, 3, 4, and 5; Fig. 2A) that lacked the 2-hydroxyl group did not have the inhibitory activity, indicating that the 2-hydroxyl group of PPG is essential for this activity.

Mammalian cells contain two other protein kinases, Plk2 and Plk3, which contain PBD consisting of two polo boxes. These PBDs also bind to the phosphopeptides with the target sequence for PBD of Plk1 (25). Thus, we examined the effect of PPG on the binding of these PBDs using mVenus fused PBD of Plk2, Plk1, and Plk3. As shown in Fig. 2B, PPG most efficiently inhibited the PBD of Plk1-dependent binding. PPG also inhibited PBD of Plk2-dependent binding, but it did not inhibit PBD of Plk3-dependent one (Fig. 2B). Because Plk2 is expressed only in the G₁ phase of the cell cycle (39), the effects of PPG on the progression of M-phase as demonstrated below are considered to be caused by the inhibition of Plk1 PBD-dependent binding.

The inhibitory activity of PPG was confirmed in two other systems. Previously, we demonstrated the specific recognition of Wee1A by PBD in a GST pulldown assay (9). When PPG was added in this assay, PPG actually inhibited the binding at an IC₅₀ value of ~0.3 µM (Fig. 2C). Next, we examined the effect of PPG in vivo using cultured HeLa cells. PBD-dependent binding is required for the proper subcellular localization of Plk1 (23,
In prometaphase and metaphase cells, endogenous Plk1 was localized to centrosomes and kinetochores, which were identified by the staining of anti-γ-tubulin and CREST antibodies, respectively (Fig. 2D, control panels). When cells were treated with PPG, the centrosomal and kinetochore localization of endogenous Plk1 nearly disappeared (Fig. 2D, green signals in PPG panels). These results indicate that PPG inhibits the PBD-dependent binding not only in vitro but also in vivo.

Effects of Inhibition of PBD-dependent Binding on Mitotic Progression—We examined the role of PBD-dependent binding on the progression of mitosis using PPG. PPG was added to synchronized HeLa cells at early S-phase (1 h after the release from the G1/S boundary), and cell cycle progression was monitored by fluorescence-activated cell sorter analysis (Fig. 3A). In both control and PPG-treated cells, DNA content reached 4C (content at G2/M phase) by 8 h after the release, indicating that PBD-dependent binding does not have a significant role in S-phase progression. At 10 h after the release, control cells started to divide into 2 daughter cells, and most cells entered the G2 stage with 2C DNA content at 12 h. In contrast, almost all the PPG-treated cells remained with 4C DNA content by 13 h after release. The degradation of cyclin B1 and Plk1 that occurred at the exit of mitosis was not detected at 13 h after release in PPG-treated cells. These results indicate that inhibition of PBD-dependent binding by PPG slightly delayed the onset of M phase, and the predominant phenotype of the inhibition is retardation of the mitotic exit.

When PPG was added into a non-synchronized cell population, the increase in the number of cells with 4C DNA was also observed at 12 h after PPG addition (Fig. 3D). By 1 day after the addition, many cells with sub-G1 DNA content (2C) were observed in this population, indicating that PPG induced apoptotic cell death after prolonged M phase. The accumulation of rounded-up cells and apoptotic cells was also observed not only in PPG-treated HeLa cells but also in normal rat kidney cells and NIH/3T3 cells after the treatment of PPG for 18 h (Fig. 3E). Interestingly, however, compounds similar to PPG but with no PBD binding inhibitory activity (compounds 2–5 in Fig. 2A) did not have such an effect, strongly suggesting that the PPG-induced mitotic delay is caused by the specific inhibition of PBD-dependent binding in these mammalian cells (Fig. 3F).

Perturbation of Chromosome Congression Activated the Spindle Checkpoint Pathway in PPG-treated Cells—Previous studies have shown that inhibition of Plk1 activity induces promet-
In these arrested cells, abnormal spindles, typically monopolar ones, were frequently observed (3–5, 29–32). In PPG-treated cell populations, however, we could not find any mitotic cells with monopolar spindles. Mitotic spindles in PPG-treated cells were apparently normal, but careful examination after staining with anti-α- and γ-tubulin antibodies revealed that centrosomes at the spindle poles in PPG-treated cells were frequently unfocused and more distanced than in control cells (mean ± S.D.; 9.21 ± 1.21 versus 7.83 ± 0.64 μm; n = 20 each; significantly different in t test, p < 0.01), indicating a weaker tension between the centrosomes (Fig. 4, see below).

In this observation we also found abnormal chromosome congression in most PPG-treated mitotic cells (Fig. 4, PPG).

**FIGURE 3.** PPG induces retardation of mitotic progression and apoptosis. A, HeLa cells were synchronized at the G1/S boundary and released, and PPG was added 1 h after the release. Cell cycle progression was monitored by DNA content determination by fluorescence-activated cell sorter analysis. B, cells in A were examined under phase microscopy. Rounded-up but undivided cells accumulated in the PPG-treated populations at 10.5 and 11.5 h after release, when most of the cells were divided into two daughter cells in the control population. C, cell cycle progression was monitored biochemically. Levels of serine 10-phosphorylated histone H3, cyclin B1, and Plk1 were examined by immunoblotting of total cell lysates from each time point. D, HeLa cells were treated with PPG (50 μM) for 12 and 24 h, and the cell cycle was monitored by fluorescence-activated cell sorter analysis. DNA contents of 2C (left) and 4C (right) are indicated by triangles in each panel. E, HeLa cells, normal rat kidney (NRK) cells, and NIH/3T3 cells were synchronized at S-phase and then cultured in the presence or absence of PPG for 18 h. F, HeLa cells were treated with 50 μM PPG or its related compounds (Fig. 2A) for 18 h and examined under phase microscopy. Accumulation of rounded-up cells was observed only in PPG-treated cell population.

**FIGURE 4.** Representative phenotype of PPG-treated mitotic cells. HeLa cells treated with or without PPG (50 μM; 16 h) were fixed and processed for immunofluorescence microscopy to analyze the status of mitotic spindles (α-tubulin; red), centrosomes (γ-tubulin; green), and chromosomes (DNA; blue).
Microtubule Interactions Are Destabilized in PPG-treated Cells—Recently, several groups reported that Plk1 interacts with BubR1 at the kinetochores of unaligned chromosomes (11, 13, 14, 17, 40). This interaction was shown to be primed by cyclin-dependent kinase 1 phosphorylation of BubR1 in a PBD binding-dependent manner. Plk1 senses the loss of this tension and phosphorylates kinetochore proteins, including BubR1. The phosphorylation of BubR1 by Plk1 does not appear to be necessary for spindle checkpoint function but, instead, is important for the stability of kinetochore-microtubule interactions. Because PPG reduced the kinetochore localization of Plk1 and BubR1, it is possible that kinetochore-microtubule interactions were not stabilized by PPG treatment, although mitotic spindles of PPG-treated cells were apparently normal. Thus, we examined the stability of kinetochore-microtubule interactions. Previously, it was shown that kinetochore microtubules were preferentially stabilized at cold temperatures, but spindles without kinetochore attachment were unstable and disappeared by the cold treatment (41, 42). When cells were fixed 10 min after cold treatment, the mitotic spindles of control cells did not change significantly, and the kinetochore-microtubule interactions remained; however, the spindles of PPG-treated cells almost disappeared (Fig. 6C). In these cells, kinetochores of sister chromosomes were not separated anymore, indicating that the tension of microtubules disappeared after cold treatment (Fig. 6C, insets). Thus, the spindles of PPG-treated cells seem to be apparently normal, but their kinetochore-microtubule interactions are destabilized. This unstable kinetochore-microtubule interaction seems to be the reason for the distanced centrosomes of PPG-treated cells and may be responsible for the deficiency in smooth chromosome congression.

Kinetochoore Localization of CENP-E Perturbed by PPG Treatment—CENP-E is a mitotic kinesin-like motor protein and is required not only for stable kinetochore-microtubule interactions but also for chromosome alignment in prometaphase (10, 43). The proper localization of CENP-E at the kinetochore is dependent on localized Plk1 at the kinetochore (10, 12). Because functional disruption of CENP-E by various methods has consistently resulted in the appearance of unaligned chromosomes at prometaphase (43–45), which is very similar to that observed in PPG-treated cells, we predicted that the mis-localization of Plk1 at the kinetochore by PPG would result in mislocalization of CENP-E at the kinetochore in prometaphase cells. The kinetochore localization of CENP-E was examined using a CENP-E-specific monoclonal antibody (37). In control prometaphase cells, CENP-E was detected at the kinetochore; however, the kinetochore localization of CENP-E was totally perturbed in the PPG-treated cells (Fig. 6D). In these cells, however, the total CENP-E level was not significantly affected (Fig. 6E). These results suggest that PBD-dependent localization of Plk1 at the kinetochore is required for smooth chromosome congression through proper kinetochore localization of CENP-E.

DISCUSSION

We have established a screening system to identify small molecule inhibitors of PBD-dependent binding. Phosphopep-
tides with target sequences for PBD-dependent binding were covalently bound to wells of 96-well plates, and the binding of fluorescent protein-fused PBD to the phosphopeptides was quantified by spectrofluorometry. When small molecules are present in the assay that compete with phosphopeptides to bind PBD, PBD will fluoresce less. We have confirmed that a phosphopeptide with the optimal binding sequence for the PBD (poloboxtide) (24) actually inhibits the binding of PBD (Fig. 1C). However, it should be noted that a relatively high concentration of poloboxtides (5 μM for 50% of inhibition) is required for the inhibition (Fig. 1C), when we consider that poloboxtides are known to bind PBD with a $K_d$ of 280 nM (25). The reason for this inefficient inhibition is not clear, but only compounds with strong inhibitory effects can be screened out in this assay.

It is known that the recognition of target peptides by the PBD is strictly dependent on a phosphoserine (or phosphothreonine) and a serine preceding the phosphorylation site (i.e. Ser-Ser(P) or Ser-Thr(P)) (24, 25). We confirmed that this binding specificity was reproduced in our system. When peptides without the essential serine or without phosphorylation were used, no significant binding was detected (Fig. 1B).

Among the about 2500 compounds screened, the compound with the highest inhibitory effect was PPG. PPG is a natural benzotropolone compound extracted from Quercus sp. nutgall. PPG has also been shown to inhibit tyrosine-specific protein kinases (46), human immunodeficiency virus 1 integrase (47), DNA synthesis of tumor cells (48), prolyl endopeptidases, (49) and the interaction between Bcl-XL and BH3 peptides (50) at IC$_{50}$ values of 27.5, 2, 200–500, 16, and 2.2 μM, respectively. In contrast, PPG inhibited the PBD-dependent binding at IC$_{50}$ of 0.3 μM, indicating that the inhibitory effect on PBD-dependent binding is significantly more specific than that for other targets reported (Fig. 2C).
Inhibitor of Polo Box Domain-dependent Recognition

We found that the 2-hydroxyl group of PPG is essential for the inhibition of PBD-dependent recognition, because compounds that lack this group did not have the activity (Figs. 2A and 3F). In contrast, two compounds without the 2-hydroxyl group (3 and 4 in Fig. 2A) inhibited the interaction between Bcl-xL and BH3 peptides, although compound 5 in Fig. 2A did not (50). Therefore, the mode of action of PPG may be different between these two systems.

In HeLa cells a higher concentration of PPG (50 μM) was required to obtain a clear phenotype in the treated cell population. At lower concentrations (15 and 5 μM), we could see the characteristic phenotype in the metaphase chromosome only in a part of the cell population (data not shown). We suspect that PPG penetrates into cells inefficiently because of its acidic hydroxyl groups. Alternatively, it is possible that PPG might be easily inactivated by oxidation in the culture media. Despite this relatively high concentration, the characteristic effect of PPG on chromosome congression strongly indicates that PPG specifically inhibits PBD-dependent recognition. Actually, PPG did not inhibit DNA synthesis at this concentration (Fig. 3A), consistent with a previous report (48).

Previously, the role of PBD-dependent binding was examined by the overexpression of PBD (23, 27). In PBD-overexpressed cells, the localization of endogenous Plk1 to centrosomes and kinetochores was severely perturbed, indicating that PBD-dependent binding is important for the intracellular localization of PBD. It was also found that the phenotypes of PBD overexpression were strikingly different from Plk1 depletion. PBD overexpression did not significantly impair centrosome maturation and separation, loss of sister chromatid arm cohesion, or bipolar spindle formation, all of which are perturbed in Plk1-depleted cells. Instead, PBD overexpression interfered with chromosome congression (23, 27). A defect in mitotic spindle stability was also observed in PBD-overexpressed cells (23). These phenotypes of PBD overexpression were also confirmed by the overexpression of the Plk1 catalytic domain in a Plk1-depleted background (27). Thus, although delocalized Plk1 activity seems to be sufficient for some Plk1 functions, it cannot provide all of the functions required for mitotic progression.

We have identified PPG as an inhibitor of PBD-dependent binding. PPG perturbed the intracellular localization of endogenous Plk1 and reproduced all the phenotypes of PBD overexpression described above. The mislocalization of Plk1 to kinetochores caused by PPG seems to be responsible for these phenotypes. Proteins such as INCENP (51), PBIP1 (18), and BUB1 (17) are postulated to be PBD-dependent binding partners and to be responsible for the localization of Plk1 to kinetochores, but the functional relationship between these proteins remains to be elucidated.

![FIGURE 6. PPG prevents kinetochore localization of BubR1 and CENP-E and establishment of stable microtubule-kinetochore attachment.](image)

A. HeLa cells treated with or without PPG (50 μM; 16 h) were fixed and processed for immunofluorescence microscopy to analyze BubR1 (green) distribution across kinetochores (CREST; red). The status of chromosomes (DNA; blue) is also shown. Representative prometaphase (upper) and metaphase (lower) cells are shown. B, levels of BubR1 at S, G2, and M phase of control or PPG (50 μM)-treated HeLa cells were examined by immunoblotting of total cell lysates. C, HeLa cells treated with or without PPG (50 μM; 16 h) were put on ice for 10 min, fixed, and processed for immunofluorescence microscopy to analyze mitotic spindles (α-tubulin; green) and kinetochores (CREST; red). Maximum-intensity projections of deconvolved stacks (projection) or one 200-nm section (section) are shown. The boxed area in the section panels is magnified for views of kinetochore attachments and is shown in the insets. D, HeLa cells treated with or without PPG (50 μM; 16 h) were fixed and processed for immunofluorescence microscopy to analyze CENP-E (green) distribution across kinetochores (CREST; red) of prometaphase cells. E, levels of CENP-E at S or M phase of control or PPG (50 μM)-treated HeLa cells were examined by immunoprecipitation and immunoblotting analysis using CENP-E antibodies.

![FIGURE 5. Chromosome congression on the metaphase plate was perturbed by PPG.](image)

A. HeLa cells expressing EGFP-tagged histone-H2B were synchronized and treated with or without PPG (50 μM; added 1 h after the release). Live cell images were taken, and one representative cell from the control or PPG-treated population (supplemental Videos 1 or 2, respectively) was selected and is shown. Times (min) after chromosome condensation were shown. B, quantitation of A. C, distribution of prometaphase, prometaphase, and metaphase (from chromosome condensation to separation) are shown in red, and those of anaphase (from chromosome separation to decondensation) are indicated by blue bars. All live cell images of the whole population can be seen in supplemental Videos 3 or 4. D, HeLa cells expressing EYFP-tagged α-tubulin were synchronized and treated with PPG (50 μM; added 1 h after the release). Live cell images were taken, and a representative cell from the population (supplemental Video 5) was selected and is shown. Times (min) after the start of microtubule nucleation are shown.

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The localization of a checkpoint protein, BubR1, to the kinetochore is dependent on Plk1 (11, 17). Their interaction is PBD-dependent, and phosphorylation of BubR1 by Plk1 is essential for the stabilization of kinetochore-microtubule interactions (13, 14, 40). Actually, in PPG-treated cells, kinetochore localization of Plk1 and BubR1 was perturbed, and the kinetochore-microtubule interaction was destabilized, as shown in the cold treatment assay (Fig. 6C). It remains unknown how Plk1-BubR1 interactions stabilize the kinetochore-microtubule interaction, but PPG may become a good tool to elucidate this mechanism.

The most characteristic phenotype of PPG treatment was the abnormal chromosome congression at metaphase. The majority of chromosomes in the treated cells aligned at the metaphase plate, but some chromosomes were unaligned and located close to spindle poles. A similar phenotype was previously observed not only in PBD-overexpressed cells (23, 27) but also in the CENP-E (centromere-associated protein E, a member of the kinesin-7 family) knock-down cells (43–45). At prometaphase, as soon as one sister kinetochore captures a microtubule emanating from one spindle pole, the chromosome is transported toward that pole, becoming mono-oriented. The microtubule-based motor CENP-E is responsible for gliding unattached kinetochores along neighboring K-fibers, helping mono-oriented chromosomes achieve congression before bi-orientation (43). Because the process of spindle attachment is basically a stochastic process, if CENP-E does not exist on the kinetochores, the mono-oriented chromosome will remain unattached for long periods. Because the localization of CENP-E has been shown to be dependent on Plk1 (10), we examined the localization of CENP-E and found that the treatment of PPG completely perturbed the kinetochore localization of CENP-E (Fig. 6D). Because it has been shown that a kinetochore protein, NudC (nuclear distribution protein C), interacts with Plk1 and that this interaction is responsible for CENP-E localization and chromosome congression (12), the interaction between Plk1 and NudC may be PBD-dependent and responsible for the characteristic phenotype of PBD-overexpressed and PPG-treated cells.

Overexpression of Plk1 is frequently observed in human tumors and can have prognostic value. Moreover, down-regulation of Plk1 by antisense oligonucleotides and small interfering RNA is highly effective in inhibiting cancer cell proliferation both in vitro and in vivo. Thus, Plk1 is now one of the most attractive molecular targets for cancer therapy. Actually, several novel Plk1 inhibitors have been reported (28–32), and some of them have proceeded to clinical trials (28, 31). While this manuscript was in preparation, another group independently reported that thymoquinone and its derivative have an inhibitory activity to PBD-dependent recognition (52). Their compounds also induced Plk1 mislocalization, chromosomal congression defect, mitotic arrest, and apoptosis at a similar concentration to that for PPG. Consistent with the present study, the mono-polar spindle could not be observed either. Although they did not show that thymoquinone derivatives also perturb the localization of CENP-E on the kinetochores and induce the chromosome congression defect, our results suggest that it may be so. Because inhibition of PBD-dependent binding can perturb a subset of Plk1 functions, an inhibitor of binding such as thymoquinone or PPG may become another attractive lead compound of Plk1 inhibition and a drug for cancer therapy. The screening system for PBD-dependent binding that we have established here should become a powerful system to identify Plk1-targeting compounds with novel inhibitory mechanisms.

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