Polo Box Domain of Plk3 Functions as a Centrosome Localization Signal, Overexpression of Which Causes Mitotic Arrest, Cytokinesis Defects, and Apoptosis*

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Polo-like kinase 3 (Plk3), an immediate early response gene product, plays an important role in the regulation of mitosis, DNA damage checkpoint activation, and Golgi dynamics. Similar to other members of the Plk family, Plk3 has a conserved kinase domain at the N terminus and a Polo box domain consisting of two Polo boxes at the C terminus. In this study, we demonstrate that the Polo box domain of Plk3 is sufficient for subcellular localization of this kinase to the centrosomes, the spindle poles, and the midbody when ectopically expressed in HeLa and U2OS cells. Both Polo boxes are required for the subcellular localization. Overexpression of the Polo box domain, not the kinase domain, of Plk3 causes significant cell cycle arrest and cytokinesis defects, eventually leading to mitotic catastrophe/apoptosis. Interestingly, the Polo box domain of Plk3 is more potent in inhibiting cell proliferation and inducing apoptosis than that of Plk1, suggesting that this domain can provide an additional structural basis for discovery of new anticancer drugs given the current emphasis on Plk1 as a therapeutic target.

Reversible phosphorylation and protein degradation are two major molecular mechanisms that control progression of the cell cycle. Among known gene products that regulate the cell cycle, Plks are a group of protein serine/threonine kinases important for monitoring various transitions during the cell cycle (1, 2). Extensive research in the past demonstrates that Plks have multiple functions in mitosis including modulating the activity of Cdk1/cyclinB (3), mediating fragmentation of the Golgi complex (4–6), and regulating sister chromatid segregation by phosphorylation of cohesin and components of the anaphase-promoting complex (7, 8).

Plks are highly conserved in terms of their structures and functions (1, 2, 9). In mammals, the Plk family consists of four members, namely, Plk1, Plk2, Plk3, and Plk4 (9). In addition to the kinase domain at the N terminus, Plks share one or two stretches of conserved amino acid sequences termed Polo box (9). Without affecting the kinase activity, mutations in the Polo boxes of Cdc5, a Plk ortholog in the budding yeast, result in its inability to complement temperature-sensitive phenotypes (10). Further studies showed that Polo box domains (PBDs)2 of yeast Cdc5 and mammalian Plk1 play an essential role in subcellular localization (11, 12). Through an elegant peptide library screening approach, Yaffe and co-workers (13, 14) demonstrate that PBDs from yeast, Xenopus, and human Plks recognize similar phosphoserine/threonine-containing motifs, functioning as an intact domain that bind to phosphoserine or phosphothreonine. Specific phosphopeptides fit into a conserved and positively charged pocket formed at the edge of the Polo box interface; mutations that interfere or disrupt the phosphodependent interaction impair cell cycle-dependent localization, thus strongly suggesting that the binding of phospholigands to the PBD is essential for mitotic progression (13). Given that overexpression of mammalian Plks is closely associated with cell proliferation and oncogenesis (15, 16) and that the PBD is present in all kinases of the Plk family, the phosphopeptide recognition function of the PBD provides the structural basis for designing anticancer drugs specifically targeting the unique domain.

Mammalian Plk3 was originally identified as an immediate early response gene product (17, 18). Subsequent studies show 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 behaves differently from that of Plk1 in human cells in terms of its regulation during the cell cycle and response to stimulation by growth factors and stresses (17, 19, 20). More prominently, ectopic expression of Plk3 or its mutants perturbs microtubule integrity, resulting dramatic morphological changes, G2/M arrest, and apoptosis (22, 23). Plk3 appears to be also involved in the regulation of Golgi dynamics during the cell cycle perhaps through interaction with MEK1, functioning as a downstream mediator of the kinase in the Golgi fragmentation signaling pathway (6).

In this study, we report the biological function of Plk3 PBD by making a series of Plk3 mutants. We find that the C terminus of Plk3, which contains two Polo boxes, is sufficient for subcellular localization to the centrosomes, the spindle poles, and the midbody. Mutations in the PBD cause abnormal subcellular localization, which affects cell division and viability. Moreover, the Plk3 PBD is more potent in the induction of apoptosis than that of Plk1. Given that many new anticancer compounds that target Plk1 are being developed in pharmaceutical companies, our studies suggest that Plk3 PBD would provide additional structural basis for discovery of more effective compounds targeting cell cycle kinases for cancer intervention.

MATERIALS AND METHODS

Cell Culture—HeLa and U2OS cell lines were obtained from the American Type Culture Collection. Cells were cultured in dishes or on Lab-Tek II chamber slides (Fisher Scientific) in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and antibiotics (100 μg of penicillin and 50 μg of streptomycin sulfate/ml) with 5% CO2.

Plasmid Construction—Plk3 and its mutants were expressed as enhanced green fluorescence protein (EGFP) fusion products (all fused at the C terminus). Constructs of pEGFP-Plk3-full-length (pEGFP-Plk3-FL,
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amino acids 1–652), pEGFP-Plk3-Polo box domain (pEGFP-Plk3-PBD, amino acids 312–652), pEGFP-Plk3-kinase domain (pEGFP-Plk3-KD, amino acids 1–334), pEGFP-Plk3-Polo box 1 (pEGFP-Plk3-PB1, amino acids 471–491), pEGFP-Plk1-full-length (pEGFP-Plk1-FL, amino acids 1–603), pEGFP-Plk1-Polo box domain (pEGFP-Plk1-PBD, amino acids 294–603), pEGFP-Plk1-kinase domain (pEGFP-Plk1-KD, amino acids 1–307), were generated by inserting respective PCR fragments into multiple cloning sites of pEGFP-N3 (BD Biosciences Clontech). The full-length of Plk3 cDNA construct was obtained by ligating the synthetic fragment coding for Plk3 N-terminal 1–39 amino acids to the corresponding cutting site of Plk3-A cDNA (22). Various kinase or PBD mutant constructs (e.g. pEGFP-Plk3-WV467FA, pEGFP-Plk3-DHF589AAA, pEGFP-Plk3-T219D, and pEGFP-Plk3-T219E) were made through site-directed mutagenesis using QuikChange™ II site-directed mutagenesis kits from Stratagene. Plk3-Polo box domain and its corresponding mutant were also fused at the C terminus with a FLAG tag (Flag-Plk3-PBD and Flag-Plk3-WV467FA). Plk3 cDNAs of deletion or mutation constructs were thoroughly sequenced to confirm the mutation status as well as the insertion site.

Transfection—HeLa and U2OS cells cultured in dishes or on chamber slides were transfected with plasmid constructs for either 16 or 36 h using the Lipofectamine method. Untransfected control cells or cells transfected with vector alone or with various constructs were processed for analysis by immunofluorescence microscopy. In some experiments, transfected cells were lysed, and equal amounts of proteins were subjected to immunoblotting analysis. Each transfection experiment was repeated at least three times.

Immunoblotting—Cultured cells were lysed as described earlier (22). Equal amounts (50 μg) of protein lysates were analyzed by SDS-PAGE followed by immunoblotting with antibodies to GFP (Invitrogen, 1:1000), procaspase 3 (Cell Signal, 1:100), or cysbin D (DAKO, 1:10,000). Specific signals were detected with horseradish peroxidase-conjugated goat secondary antibodies (Sigma) and enhanced chemiluminescence reagents (Amersham Biosciences).

Fluorescence Microscopy—Subcellular localization of Plk3 was determined by double immunofluorescence analysis. Cells were quickly washed with phosphate-buffered saline (PBS) and fixed with methanol for 7 min at room temperature. Fixed cells were treated with 0.1% Triton X-100 in PBS for 5 min and then washed three times with ice-cold PBS. After blocking with 2% bovine serum albumin in PBS for 15 min on ice, cells were incubated for 1 h at room temperature with the rabbit monoclonal GFP antibody (Invitrogen, 1:5000) and/or mouse anti-γ-tubulin IgG (Sigma, 1:250) in a 2% bovine serum albumin solution, washed with PBS, and then incubated with rhodamine red X-conjugated anti-mouse IgGs and/or fluorescein isothiocyanate-conjugated anti-rabbit IgGs (Jackson ImmunoResearch) at room temperature for 1 h in the dark. Cells were finally stained with 4′,6′-diamidino-2-phenylindole (DAPI) (Fluka, 1 μg/ml) for 2 min. Fluorescence microscopy was performed, and images were captured using a digital camera (Optronics) using Optronics MagFire and Image-Pro Plus softwares. The living cells ectopically expressing EGFP fusion proteins or GFP alone were visualized under a Nikon fluorescence microscope.

Flow Cytometry—HeLa cells transfected with or without Plk3 or its mutants for 36 h were analyzed for the cell cycle and apoptotic statuses. Brieﬂy, cells were ﬁxed with methanol, treated with Triton X-100 (0.25% in PBS) for 5 min, washed with PBS with 1% bovine serum albumin, and incubated for 1 h with a rabbit monoclonal GFP antibody (Invitrogen, 1:5000). Cells were rinsed with PBS and then incubated with a second antibody conjugated with fluorescein isothiocyanate for 1 h, rinsed with PBS containing 1% bovine serum albumin, and stained with DAPI (1 μg/ml).

Fluorescence of cells processed for flow cytometry was measured by the ELITE-ESP cytometer/cell sorter (Coulter) as described previously (22). Each experiment was repeated at least three times.

RESULTS

Although the PBD of human Plk1 is known to guide its subcellular localization to the centrosomes, the spindle poles, and the midbody (8, 11, 12), no studies have been performed to determine whether PBDs of other mammalian Plk homologs would function in the same manner as that of Plk1. Both Plk1 and Plk3 share a similar structural organization in which the KD is at the N-terminal region and the PBD is located at the C terminus (Fig. 1A). However, they do exhibit some functional differences, especially in DNA damage responses and induction of apoptosis (2), we asked whether Plk3 PBD would behave differently from that of Plk1. We first made a series of plasmid constructs that code for various mutant forms of Plk3 as EGFP fusion proteins (Fig. 1B). To determine whether these constructs could be expressed, HeLa cells transfected with various Plk3 plasmid constructs for 16 h were blotted with an anti-GFP antibody. We observed that Plk3 mutant proteins, as well as the wild-type Plk3, were highly expressed with expected sizes and that the levels of expression among various constructs were comparable except for pEGFP-Plk1-KD (Fig. 1C). The low yield of pEGFP-Plk1-KD on the immunoblot was partly because of its nuclear localization, which somehow made it insoluble during lysis; on the other hand, immunofluorescence microscopy revealed that it was...
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FIGURE 2. Centrosomal localization of Plk3. A, living HeLa cells transfected various constructs as indicated were directly observed under a fluorescence microscope 16 h post-transfection. B, HeLa cells transfected with EGFP-Plk1-PBD plasmid construct for 16 h were fixed and stained with antibodies to GFP (green) and γ-tubulin (red). C, HeLa cells transfected with EGFP-Plk3-PBD construct for 16 h were fixed and stained with antibodies to GFP (green) and γ-tubulin (red).

expressed at a level comparable to other Plk plasmid constructs (data not shown). As our early studies show that endogenous Plk3 is localized at the centrosome/spindle pole regions (22), we attempted to map the region that was responsible for the subcellular localization of Plk3 by expressing various EGFP-Plk3 mutant constructs. The examination of live cells under a fluorescence microscope revealed that whereas EGFP showed no specific subcellular localization when ectopically expressed, a significant amount of EGFP-Plk3-FL formed distinct foci (Fig. 2A), reminiscent of centrosomal localization. Supporting this, ectopically expressed EGFP-Plk1 and EGFP-Plk1-PBD also exhibited a focal pattern similar to that of EGFP-Plk3 (Fig. 2A). Moreover, expression of EGFP-Plk3-PBD and kinase-defective Plk3 (EGFP-Plk3-T219E) also resulted in the formation of foci similar to that of full-length Plk3, suggesting that the PBD but not the kinase domain was sufficient for the subcellular localization. Consistently, the kinase domain (EGFP-Plk3-KD) alone or a PBD mutant (EGFP-Plk3-WV467FA) failed to form the foci in living HeLa cells (Fig. 2A).

Early studies show that GFP-Plk1 localizes to the centrosomes and the spindle poles (8). Double immunofluorescence microscopy showed that ectopically expressed EGFP-Plk1-PBD formed distinct foci co-localizing with γ-tubulin, confirming its role in centrosomal localization (Fig. 2B). Co-staining with γ-tubulin also confirmed that Plk3-PBD (Fig. 2C) and several other Plk3 mutant proteins, such as Plk3-T219D (data not shown), localized to the centrosomes and the spindle poles. Furthermore, similar to EGFP-Plk1-PBD, EGFP-Plk3-PBD also localized at the midbody during cytokinesis (Fig. 3A, arrows). It was interesting to note that γ-tubulin was also detected at the midbody (Fig. 3A), which is consistent with an early report that γ-tubulin takes part in the formation of the midbody in mammalian cells (24). The percentage of cells with centrosome, spindle pole, or the midbody localization when they were transfected with Plk3 and its mutant constructs was summarized in Fig. 3B. Without PBD, the kinase domain alone (EGFP-Plk3-KD) was insufficient for centrosome, spindle pole, and midbody localization; consistently, mutations at either PBs (EGFP-Plk3-WV467FA and EGFP-Plk3-DHF589AAA that disrupt PB1 and PB2, respectively) significantly impaired or abolished its subcellular localization (Fig. 3B). Again, additional mutations in the kinase domain (e.g. EGFP-Plk3-T219E) failed to disrupt its proper subcellular localization, indicating that the kinase domain is not essential for guiding its subcellular localization (Fig. 3B).

We then examined the consequence of expressing Plk3 and its mutants on the cell cycle status, because it is involved in mitotic functions (19). As expected, ectopic expression of Plk3-FL enriched G2/M cell population (Fig. 4A). Interestingly, the expression of Plk3 PBD (Plk3-PBD) alone further enriched G2/M cells, whereas the kinase domain (Plk3-KD) significantly reduced the ability of Plk3 to induce cell cycle arrest, suggesting that it is the C-terminal domain containing Polo box domains that inhibit Plk3 function (Fig. 4B). Supporting this, expressing Plk3-T219D with a defective kinase activity did not reduce its ability to induce G2/M arrest, whereas knocking out the function of the PBD through expressing Plk3-WV467FA almost completely abolished the ability (Fig. 4, A and B).

To determine whether Plk3 PBD was capable of inducing mitotic arrest, we measured cyclin B levels in HeLa cells transfected with various expression constructs (Fig. 4C). Immunoblotting revealed that compared with control cells cyclin B was significantly accumulated in the cells ectopically expressing Plk3-PBD and Plk3-FL (Fig. 4C). Disruption of the PBD (Plk3-WV467FA) failed to enrich cyclin B compared with that of control cells expressing EGFP (Fig. 4C).

Consistent with the elevated cyclin B, we observed that cells expressing Plk3-PBD exhibited defects in cytokinesis including chromosome mis-segregation, cytokinesis defects, and formation of binucleated and
polyploid cells (Fig. 5, A and B). A quantitative analysis revealed that cells expressing Plk3-PBD caused significant failures (up to 24% of cells) in mitosis (Fig. 5, A and B). The mitotic defects were closely associated with the presence of a functional PBD because disruption of the domain or expressing the kinase domain alone failed to cause significant mitotic defects (Fig. 5A).

Deregulated mitotic processes often result in mitotic catastrophe, a special form of apoptosis occurring during mitosis. To examine the consequence of cell cycle arrest and mitotic defects induced by the Plk3 PBD, we first examined cellular and nuclear morphologies of HeLa cells extopically expressing transfected Plk3 and its mutants after a 36-h transfection. We observed that although few morphological alterations in cells expressing EGFP alone or EGFP-Plk1-PBD were noticed, dramatic cellular changes (e.g., cell condensation and fragmentation, or shrunken morphology) occurred in those expressing Plk3-FL and Plk3-PBD (Fig. 6A). The nuclei of these cells as revealed by DAPI staining also became condensed (Fig. 6B), which is suggestive of apoptosis. To further confirm the cell death induced by Plk3 PBD, we analyzed the cleavage of procaspase-3, an event occurring during apoptosis, in cells transfected with Plk3-PBD. Immunoblotting revealed that procaspase-3 levels were significantly decreased in cells transfected with Plk3-PBD compared with that of control cells transfected with EGFP alone or with Plk1-PBD (Fig. 6C), thus confirming that the cell cycle arrest and mitotic defects induced by Plk3 PBD lead to apoptosis. Consistently, a significant fraction of sub-G1 (apoptotic) cells, as revealed by flow cytometry, was present when they expressed Plk3-FL (18%) and Plk3-PBD (24%); in contrast, a much smaller amount sub-G1 cells, if any, was present when the cells were transfected with constructs expressing Plk3-KD, Plk3-WV467FA, Plk1-PBD, or EGFP (Fig. 6D).

To determine whether the Polo box domain of Plk3 also dictates centrosome localization in other human cell types, we examined subcellular localization patterns of ectopically expressed Plk3-PBD and Plk3-WV467FA in osteosarcoma U2OS cells. Consistent with those observed in HeLa cells, concentrated signals of Plk3-PBD, but not the PBD mutant (Plk3-WV467FA) superimposed with αtubulin (Fig. 7A), indicating centrosome localization. We also observed that Plk3-PBD but not its mutant localized to the midbody in U2OS cells (data not shown). Sustained expression of Plk3-PBD but not of Plk3-WV467FA also caused dramatic shrinkage of the cytoplasm as well as the nucleus of U2OS cells (Fig. 7B), indicative of apoptosis.

To exclude the remote possibility that the GFP moiety may modify the function of Plk3-PBD, we made both FLAG-tagged Plk3-PBD and FLAG-tagged Plk3-WV467FA. We observed that ectopically expressed Flag-Plk3-PBD, but not Flag-Plk3-WV467FA, localized to centrosomes (Fig. 7C), indicating that it is the Polo box domain but not a specific tag that functions as a centrosomal localization signal. Consistently, ectopic expression of the functional PBD of Plk3 caused cells to condense and fragment, which are morphological signs of programmed cell death (Fig. 7D).
Together, these lines of experimental evidence indicate that the Polo box domain of Plk3 truly functions as a centrosome localization signal and deregulated expression of this domain induces apoptosis.

**DISCUSSION**

The PBD is very much conserved throughout the evolution, suggestive of critical cellular functions mediated by this unique structure. Extensive studies of mammalian Plk1 and budding yeast homology Cdc5 suggest that the PBD consisting of two identifiable Polo boxes functions as an intact unit (12). Interestingly, Plk4 contains only one Polo box that is also functional and capable of targeting the kinase to proper subcellular localizations during the cell cycle (25). In this study, we have determined the structural basis of Plk3 that guides its subcellular distributions during the cell cycle and biological consequences of expressing Plk3 and various Plk3 mutants. We have shown that Plk3 localizes to the centrosomes during interphase and to the spindle poles during mitosis and that a significant amount of Plk3 is also present at the midbody during cytokinesis. The subcellular distributions of Plk3 are dependent on a functional PBD but not the catalytic domain; both polo boxes are required for the subcellular localizations because disruption of either PB1 (Plk3-WV467FA) or PB2 (Plk3-DHF589AAA) causes mislocalization of Plk3 (Fig. 3B). These observations thus support the notion that the PBD from various Plks may share similar functions in regulating cell division. Structural analyses reveal that two polo boxes of Plk1, exhibiting similar tertiary structures, fold together and form the interface that may provide a site for ligand binding (13). Given the conserved primary amino acid sequences and its distinct function in DNA damage response (2, 17), it is predicted that the Plk3 PBD may have a tertiary structure similar to but not identical to that of Plk1. It is an important task to identify phosphoprotein ligands that specifically bind to Plk3 if the cellular function of this kinase can be fully appreciated.

Overwhelming evidence indicates that the PBD dictates the subcellular localization of Plks to the centrosomes and the spindle poles. It is tempting to speculate that this domain may function as a centrosome-targeting signal because the structural bases that determine centrosomal localization remain unclear. However, our preliminary studies show that core sequences of Polo box one (Plk3-PB1 in Fig. 1A) failed to target...
EGFP to the centrosomes, suggesting that the minimal sequence required for centrosomal localization requires Polo box two as well. Supporting this, Plk3-DHF589AAA, a PB2 mutant, does not localize to the centrosomes and the midbody (Fig. 3B). Recently, Matsumoto et al. (26) have reported that a stretch of 20 amino acids in cyclin E may serve as a centrosomal localization signal. However, the significance of this centrosomal localization signal remains to be determined because this sequence appears to be present in few molecules other than cyclin E, and no known centrosome-specific proteins contain the centrosomal localization signal.

Plks are involved in the regulation of a variety of events during the cell cycle (9). Extensive studies suggest that Plk1 and Plk3 are essential for mitotic progression and cytokinesis. In mammalian cells, depletion of Plk1 by RNA interference largely impairs cytokinesis, resulting in mitotic arrest and formation of dumbbell-like DNA structures (2, 27). Overexpression of the PBD of Plk1 leads to spindle checkpoint failure and defective cytokinesis (12). Our early studies show that overexpression of the PBD of Plk3 functions as a centrosome localization signal but also mitotic events such as cytokinesis. To date, several proteins have been identified that may mediate the function of Plk1 in cytokinesis (28, 29). MKLP1/CHO1, a kinesin-like protein, interacts with and is phosphorylated by Plk1 (30). It is thus interesting to see whether Plk3 also regulates these pro-
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tion of Plk1 in cytokinesis (28, 29). MKLP2, a mitotic kinesin, co-localizes with Plk1 to the midbody, and it can be phosphorylated by Plk1 in vitro (29). In addition, MKLP2, a mitotic kinesin, co-localizes with Plk1 to the spindle poles during late mitosis; the physical interaction between these two proteins and phosphorylation of MKlp2 by Plk1 are necessary for cytokinesis (28). Moreover, NuDC, a dynein-associated nuclear protein, interacts with and is phosphorylated by Plk1 in vitro (30). It is thus interesting to see whether Plk3 also regulates these pro-
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