A Spindle Checkpoint Arrest and a Cytokinesis Failure by the Dominant-negative Polo-box Domain of Plk1 in U-2 OS Cells*

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Polo kinases play critical roles for proper M-phase progression. They are characterized by the presence of two regions of homology in the C-terminal non-catalytic domain, termed polo-box 1 (PB1) and polo-box 2 (PB2). Here we demonstrate that both PB1 and PB2 are required for targeting the catalytic activity of Plk1 to centrosomes, midbody, and kinetochores. Expression of either kinase-inactive PLK1/K82M or the C-terminal plk1ΔN induced a pre-anaphase arrest with elevated Cdc2 and Plk1 activity. Prophase-arrested cells exhibited randomly oriented spindle structures, whereas metaphase cells exhibited aberrant bipolar spindles with Mad2 localization at kinetochores of misaligned chromosomes. Microtubule nucleation activity of centrosomes was not compromised. In vivo time-lapse studies revealed that expression of plk1ΔN resulted in repeated cycles of bipolar spindle formation and disruption, suggestive of a defect in spindle stability. A prolonged arrest frequently led to the generation of micronucleated cells in the absence of sisterchromatid separation and centrosome duplication, indicating that microtubule nucleation is not a result of accumulated cytokinesis failures. Interestingly, bypass of the mitotic arrest by dominant-negative polo-box checkpoint components led to a failure in completion of cytokinesis. We propose that, in mammalian cells, the polo-box-dependent Plk1 activity is required for proper metaphase/anaphase transition and for cytokinesis.

The polo-like protein kinases (Plks) are a conserved subfamily of Serine/Threonine protein kinases that play a variety of roles during M-phase progression. Characterization of mutations in the prototype of these members, Drosophila polo, revealed that polo is important for the function of spindle poles and bipolar spindle formation at several developmental stages (1, 2). Microinjection of anti-Plks antibodies into either Xenopus embryos or cultured mammalian cells resulted in the formation of monopolar spindles (3, 4) with centrosome maturation defect, as evidenced by a decreased amount of γ-tubulin and MPM-2 antigens recruited to the centrosomes (4). Overexpression of a kinase-inactive form of mammalian polo-like kinase Plk1 in most tumor cell lines was shown to induce centrosome maturation defect in combination with mitotic catastrophe and apoptosis (5). These observations raised the possibility that, in Plk1-deficient cells, a defect in centrosome maturation is the primary cause of a defect in bipolar spindle formation, which ultimately leads to cell death.

A growing body of evidence suggests that Plks also play important roles in mitotic entry, mitotic exit, and cytokinesis. Xenopus polo-like kinase Plxl phosphorylates and activates Cdc25, which in turn dephosphorylates and activates Cdc2 (6), suggesting a direct role of Plx1 in regulating mitotic entry. In addition, studies in various organisms have demonstrated that Plks are important in mitotic exit. Plk1 has been suggested to directly phosphorylate components of anaphase-promoting complex (APC) including Cdc27 and activate the latter (7). Activated APC subsequently ubiquitinates mitotic cyclins, leading to their degradation and the inactivation of Cdc2 (for review, see Refs. 8 and 9). Plks also appear to contribute to the onset of cytokinesis in various low eucaryotic organisms (for review, see Ref. 10). In addition, recent studies show that Drosophila polo9 and polo10 mutants are defective in the metaphase/anaphase transition due to the activation of a tension-dependent spindle checkpoint (11), suggesting that polo activity is required to promote the onset of anaphase.

Microtubule organization is facilitated by both centrosomes and kinetochores of sisterchromatids. γ-Tubulin, a highly conserved centrosomal protein, plays a critical role in microtubule nucleation (12–14). The plus end of growing microtubules attaches to the kinetochores before sisterchromatids. Genetic analyses in budding yeast have identified Bub1, Bub3 (15), Mad1, Mad2, Mad3 (16), and Mps1 (17) as components regulating this pathway. In metazoan cells, homologues of these checkpoint proteins were shown to localize to unattached kinetochores to inactivate Cdc20, a protein that directs the APC to a specific set of substrates (for review, see Refs. 18 and 19). Recent studies in mammalian cells show that Mad2 detects spindle attachment to the kinetochores, whereas Bub1 and BubR1 sense kinetochore tension (20, 21). Although distinct Mad2/Cdc20 and BubR1/Cdc20 complexes have been suggested (22), they may function in a concerted...
manner since loss of either one abrogates spindle checkpoint arrest in vivo (23, 24).

In addition to the kinase domain, the polo subfamily members are characterized by the presence of conserved regions of homology in the C-terminal non-catalytic domain. Alignment of the C-terminal regions of known polo kinases revealed that they are composed of two domains with significant homology (polo-box 1 (PB1), from amino acids 405 to 494, and polo-box 2 (PB2), from amino acids 505 to 598 in Plk1) (25). In budding yeast, a highly conserved motif in PB1 of the budding yeast polo kinase Cdc5 is critical for its subcellular localization and mitotic functions. In mammalian cells, however, it is not yet known whether the PB1 and PB2 are important for subcellular localization or for a specific function of Plk1 during M-phase progression. To address these issues, we expressed various forms of Plk1 in U-2 OS cells using an adeno virus expression system. We show that both PB1 and PB2 are required for efficient subcellular localization of Plk1. Expression of plk1ΔN, which contains both PB1 and PB2, induces a pronounced defect in establishing normal bipolar spindles and leads to the activation of a Mad2-dependent spindle checkpoint pathway. These arrested cells exhibited apparently normal centrosome maturation and microtubule nucleation activity, suggesting that expression of plk1ΔN induced a defect in forming normal bipolar spindles without influencing centrosome function. In addition, bypass of the spindle checkpoint arrest led to a failure in completing cytokinesis. Our data suggest that the polo-box-dependent Plk1 function is required for bipolar spindle formation and cytokinesis.

EXPERIMENTAL PROCEDURES

Cell Culture and Synchronization—U-2 OS osteosarcoma cell line (American Type Culture Collection) was maintained in McCoy's 5A medium supplemented with 10% fetal bovine serum (HyClone Laboratories Inc.) and 100 units/ml penicillin G plus 100 μg/ml streptomycin (Invitrogen). For the double thymidine block and release experiment, U-2 OS cells were arrested for 14 h with 2.5 mM thymidine (Sigma) with a 7-h release interval. Arrested cells were either released into fresh medium to follow cell cycle progression or into the medium containing 400 μg/ml nocodazole (Sigma) to induce mitotic arrest. To examine the effect of various forms of Plk1 expression on cell cycle progression, cells were infected with various Plk1 viruses at a multiplicity of infection (m.o.i.) of 10, 4 h before release from the double thymidine block. To carry out microtubule re-growth assay, U-2 OS cells were treated with 200 ng/ml nocodazole for 16 h, washed, and released into prewarmed medium at 37 °C. Cells were allowed to re-colonize for 8 h. These cells were fixed and stained with DAPI to immunostain with an anti-α-tubulin antibody (see below).

Plasmid Construction and Generation of Plk1 Adenoviruses—Recombinant adenoviruses were made according to the procedure described previously (26). Briefly, a 1.8-kilobase SalI-NotI (end-filled) fragment containing the full-length murine Plk1 cDNA-N-terminally tagged with hemagglutinin (HA) epitope was first cloned into pShuttle-CMV vector digested with SalI and EcoRV. A copy of EGFP (CLONTECH) was inserted into the XhoI site of the N-terminal Plk1 sequence to generate pShuttle-CMV-HA-EGFP-P1k1. The C-terminal form of Plk1 (plk1ΔN) lacking amino acids 51–356 was generated by digesting the pShuttle-CMV-HA-EGFP-P1k1 with Nael and Smal and re-ligating the resulting fragment. The triple plk1ΔN/W414F/Y415A/L457A (plk1ΔNFAA) mutant was generated by using the Sculptor in vitro mutagenesis system (Amersham Biosciences). PB1 (amino acids 357–502) and PB2 (amino acids 503–603) constructs were generated by replacing the full-length Plk1 with respective PCR fragments. All the viruses were generated in HEK293 (ATCC) cells and purified two times by CsCl ultracentrifugation. To determine viral titers, viruses were serially diluted and infected into U-2 OS cell in 24-well plates, and then the proportion of GFP-positive cells was enumerated.

Flow Cytometry Analysis and Mitotic Indices—To analyze cell cycle progression, flow cytometry analyses were carried out using FACSCalibur (BD PharMingen), and data were analyzed by Cellquest software (BD Pharmingen). To determine the percentage of mitotic cells, cells were harvested, fixed in 4% paraformaldehyde, and then stained with 0.1 μg/ml 4′,6-diamidino-2-phenylindole (DAPI). The percentage of mitotic figures was determined by counting more than 200 cells/time point using fluorescence microscopy.

Transfections—U-2 OS cells were transiently transfected with 2 μg of plasmid DNA using FuGENE 6 transfection reagent (Roche Molecular Biochemicals) according to the manufacturer's protocol. To determine the effect of double microtubule checkpoint on plk1ΔN-induced mitotic arrest, various constructs were co-transfected with pDsRed2-N1 (CLONTECH)-plk1ΔN at a 5:1 ratio. Mitotic indices of co-transfected cells were determined by counting only the red fluorescent cells.

Immunoblot Analyses—An affinity-purified C-terminal anti-Plk1 antibody (Zymed Laboratories Inc.), a monoclonal anti-HA antibody (Berkeley Antibody Co.), an anti-Cdc2 antibody (Upstate Biotechnology Inc.), an anti-Cdc27 antibody (Santa Cruz Biotechnologies), and an anti-phospho-histone H3 antibody (Upstate Biotechnology Inc.) were used at 0.5 μg/ml. Immunoblotting was carried out as described previously (27).

Immunoprecipitation and Kinase Assays—Anti-Plk1 immunocomplex kinase assays were carried out as described previously (28). Briefly, supernatants (S15) obtained from centrifugation at 15,000 × g for 20 min were incubated with an anti-Plk1 antibody (Zymed Laboratories Inc.) in the presence or absence of its epitope peptide for 2 h, then protein A-Sepharose 4B (Zymed Laboratories Inc.) was added to precipitate the antibodies. To determine Cdc2 kinase activity, the S15 was incubated with 10 μl of yeast plk1ΔN anti-cortisone conjugate (Calbiochem), then the precipitates were subjected to kinase reactions using 3 μg of histone H1 (Calbiochem) as a substrate.

Immunofluorescence Microscopy—U-2 OS cells were grown on collagen (Vitrogen 100; Celsitix)-coated glass coverslips and extracted for 5 min with a microtubule-stabilizing (PMEG) buffer (100 mM Pipes, 5 mM MgSO4, 5 mM EDTA, 0.5 mM EGTA, 0.9 mM glycerol, pH 6.9) plus 0.25% Triton X-100 for staining kinetochores or PMEG without detergent for staining centrosomes and microtubules. Cells were then fixed with 4% paraformaldehyde for 10 min and washed 4 times with PBST (PBS plus 0.1% Triton X-100). The coverslips were then incubated for 2 h in PBS plus 3% bovine serum albumin containing a rabbit anti-Plk1 antibody (Zymed Laboratories Inc.), a rabbit anti-Cep135 antibody (A5-CEP) (29), a rat anti-α-tubulin antibody (Accurate), a scleroderma CREST autoimmune antisemum (Cortex Biochem), a mouse anti-γ-tubulin (Sigma), or a rabbit anti-Mad2 antibody (a gift of Rey-Hui Chen, Cornell University). After washing 5 times with PBST for 5 min each, coverslips were further incubated for 1 h with a fluorescein isothiocyanate-labeled goat anti-rabbit IgG for Plk1 staining, a Texas Red-labeled goat anti-rabbit IgG for Mad2 and Cep135 staining, a Texas Red-labeled goat anti-mouse IgG for γ-tubulin staining, or a Texas Red-labeled goat anti-human IgG for CREST staining. All the secondary antibodies were purchased from Jackson ImmunoResearch Laboratories and were used at a 1:200 dilution. To stain chromosomes, cells were treated with PBST containing 0.1 μg/ml of DAPI (Sigma). Confocal fluorescent images were collected with a Leica TCS spectrophotometer confocal microscope or with a Texas Red-Axiovert 100iM confocal microscope.

In Vivo Time-lapse Microscopy—Generation of U-2 OS cell lines expressing EGFP-tubulin (human α-tubulin; CLONTECH), DsRed-centrin (30), GFP-histone H2B will be described elsewhere. Cells were cultured on a 35-mm dish on the stage of the Axiovert S-100 inverted microscope equipped with an environmental chamber (Zeiss). Time-lapse images were captured every 3 min by a SenSys digital camera (Photometrics) and analyzed by Openlab software (Improvision). Videos of cells depicted in Fig 4, A–C, and Fig 5D are available as on-line supplemental material.

RESULTS

Both PB1 and PB2 Are Required for Efficient Subcellular Localization of Plk1 in U-2 OS Cells—Studies in budding yeast show that the highly conserved motif (amino acids 410–439 in Plk1) present in PB1 of both mammalian Plk1 and budding yeast Cdc5 is critical for the subcellular localizations and mitotic functions of these kinases (27, 31). To determine the role of the PB1 and PB2 domains of Plk1 in its own organism, we expressed various forms of PLK1 in U-2 OS cells using an adenoviral expression system (Fig. 1A). These proteins were

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N-terminally fused with both HA and EGFP to facilitate protein detection and subcellular localization. Asynchronously growing cells were infected with various viruses at a m.o.i. of 10 to examine the expression level of these constructs. Total cellular proteins were prepared for immunoblotting with an anti-HA antibody 24 h post-infection. As shown in Fig. 1B, each of the HA-EGFP-fused proteins were expressed at similar levels, although the expression level of control HA-EGFP was severalfold higher than those of Plk1 fusions (Fig. 1B). Previous studies show that Plk1 localizes to centrosomes, midbody, and kinetochores (28, 32, 33). Thus, we examined the ability of these mutant proteins to localize to these sites. Expression of either EGFP-PLK1/K82M or EGFP-plk1ΔN, which contain the entire C-terminal domain of Plk1, yielded two distinct fluorescent dot signals and a single strong band at an apparent midbody (Fig. 1C). Punctate signals were also apparent in nucleus (Fig. 1C and see also 1E). Immunostaining with an antibody specific to Cep135 pericentriolar protein revealed that the two strong green fluorescent dots localized at the centrosomes (Fig. 1C). In addition, an anti-α-tubulin staining revealed that both EGFP-Plk1K82M and EGFP-plk1ΔN clearly localized to the centrosome, which was flanked by the remnants of spindle microtubules (Fig. 1D and data not shown). In contrast, both EGFP-PB1 and EGFP-PB2 constructs largely yielded diffused signals in cytoplasm (Fig. 1, C and D; see Table I for quantification), suggesting that PB1 or PB2 alone is not sufficient to localize at distinct subcellular structures. In addition, introduction of the triple W414F/V415A/L427A mutations into the PB1 of plk1ΔN severely impaired the ability of this protein to localize properly (Table I).

We then examined whether punctate nuclear signals observed with EGFP-Plk1K82M or EGFP-plk1ΔN are indicative of Plk1 localization at kinetochores. In prophase, endogenous Plk1 was detected as two strong dots (most likely centrosomes...
as observed above) and punctate dotted signals (Fig. 1E, fluorescein isothiocyanate (FITC) panel). The dotted signals were co-localized with CREST antigens at kinetochores (Fig. 1E, merged panel). In late stages of mitosis, strong Plk1 localization was also detected at midzone and midbody structures (Fig. 1E). EGFP-plk1ΔN efficiently localized at kinetochores, whereas EGFP-PB1 and EGFP-PB2 did not (Fig. 1E and data not shown). These observations indicate that, as with centrosome and midbody localization, the C-terminal domain of Plk1, but not the PB1 or PB2 alone, is sufficient to localize to kinetochores of mitotic chromosomes.

**Expression of PLK1/K82M or plk1ΔN Leads to a Pre-anaphase Arrest**—The ability of both EGFP-Plk1/K82M and EGFP-plk1ΔN to properly localize to specific subcellular structures led us to investigate whether overexpression of these proteins alters cell cycle progression. To this end, U-2 OS cells were doubly blocked with thymidine, infected with various viruses, and then synchronously released from the G1/S block. Flow cytometry analyses indicate expression of either EGFP-PLK1/K82M or EGFP-plk1ΔN induced a cell cycle arrest after achieving DNA replication (Fig. 2A). Cells with greater than a 4N DNA content were also apparent after a prolonged arrest.
TABLE I
Both polo-box 1 and polo-box 2 are required for proper subcellular localization of Plk1 in U-2 OS cells

| % Localization | Centrosome | Midbody |
|----------------|------------|--------|
| EGFP           | 0 (−)      | 0 (−)  |
| EGFP-plk1K82M  | 100 (+++++) | 100 (+++++) |
| EGFP-plk1ΔN (PB1 + PB2) | 100 (+++++) | 100 (+++++) |
| EGFP-plk1ΔN/FAA | 37 (++)   | 33 (++) |
| EGFP-PB1       | 8 (±)      | 11 (±) |
| EGFP-PB2       | 28 (±)     | 22 (±) |

* Relative intensities of fluorescent signals at specific locations are indicated in parenthesis, +++, strong; −, not detectable. More than 150 cells were counted for each sample.

In contrast, expression of PB1, PB2, or the plk1ΔN bearing the triple FAA mutations (plk1ΔN/FAA) failed to induce these phenotypes (Fig. 2E). These observations indicate that induction of both binucleated and micronucleated morphologies are likely the consequence of the polo-box-dependent mitotic arrest.

To investigate whether the expression of PLK1/K82M or plk1ΔN alters the endogenous Plk1 activity, U-2 OS cells infected with various Plk1 viruses were harvested 9 h after G1 release, a period when Plk1 becomes active. In vitro immunocomplex kinase assays revealed that expression of either PLK1/K82M or plk1ΔN did not inhibit the Plk1 kinase activity (Fig. 2F).

Because expression of PLK1/K82M or plk1ΔN alters the cell cycle progression, we then closely examined whether the expression of these proteins influences the timing of Plk1 activation in cells synchronously releasing from the G1/S block. In control cells, Plk1 activity increased at 9- and 12-h time points, then decreased as the cell cycle progressed. In both PLK1/K82M- and plk1ΔN-expressing cells, Plk1 activity appeared to increase at a similar rate as in control cells and remained active up to 18 h after release (Fig. 2F), suggesting that the Plk1 activation step is not impeded in these cells. These observations are consistent with the steadily increased Cdc27 phosphorylation during the mitotic arrest (Fig. 2B). As with Plk1 activation, Cdc2 activation appeared to occur normally (Fig. 2F).

Expression of PLK1/K82M or plk1ΔN Leads to a Defect in Proper Bipolar Spindle Formation—Anti-Plk1 microinjection (4) or overexpression of a kinase-inactive Plk1 studies (5) suggests that Plk1-deficient cells are defective in centrosome maturation, which leads to monopolar spindle formation and mitotic catastrophe. To examine whether the apparent pre-anaphase arrest induced by PLK1/K82M or plk1ΔN expression is the result of a defect in centrosomes or other subcellular structures, cells released from the G1/S block for 15 h were fixed and subjected to immunostaining with anti-α-tubulin, anti-γ-tubulin, or anti-Cep135, a pericentriolar matrix component. Under various conditions, the amount of γ-tubulin and the localization of Cep135 appeared to be normal in comparison to control cells (Fig. 3B and data not shown), suggesting that centrosome maturation occurred properly in these cells. In contrast, anti-α-tubulin staining revealed that most prophase cells exhibited defects in spindle structures in varying degrees. The most prominent defect associated with PLK1/K82M or plk1ΔN expression was the presence of randomly oriented, disorganized spindles (Fig. 3A, first panel in EGFP-plk1ΔN). Approximately 55% of the prophase cells (~27% of total mitotic cells) exhibited this phenotype (Table II). This observation was in sharp contrast to control cells, which have already established strong bipolar spindles in prophase (Fig. 3A, first panel in EGFP control). In addition, a small fraction of monopolar or multipolar spindle structures (Fig. 3A, second and third panels in EGFP-plk1ΔN, and Table II) were also observed. However, structures resembling bipolar spindles were observed in most metaphase-like cells (Fig. 3A, fourth panel in EGFP-plk1ΔN, and Table II), although they often looked abnormal, with uneven bipolarity. Close examination of these cells revealed that the majority of them (62% for Plk1/K82M and 60% for plk1ΔN; see Table III) exhibited either misaligned or non-congregated chromosome morphologies (Fig. 3A, arrow in the fourth DAPI panel in EGFP-plk1ΔN), suggesting that these cells have a defect in establishing normal bipolar spindles.

Cells expressing EGFP-plk1ΔN were doubly stained with both anti-α- and anti-γ-tubulin antibodies to examine whether the cells with randomly oriented spindle structures possess proper microtubule-organizing centers. Two closely placed...
FIG. 2. A, expression of PLK1/K82M or plk1ΔN induces mitotic arrest. Four hours before release from double thymidine block, U-2 OS cells were infected with indicated viruses at an m.o.i. of 10. Samples were harvested at the indicated time points for analysis. Control, control EGFP virus; PLK1/K82M, EGFP-Plk1/K82M virus; plk1ΔN, EGFP-plk1ΔN virus; 1N, 1N DNA content; 2N, 2N DNA content. B, cells were infected with the indicated viruses then released from G\textsubscript{1}/S block as in A. Total cellular proteins prepared at the indicated time points were subjected to immunoblotting analyses with an anti-Cdc2 antibody (top panel), an anti-Cdc27 antibody (middle panel), or an anti-phospho-histone H3 antibody (bottom panel). control/noc., control EGFP virus-infected cells released into nocodazole-containing medium; P-Cdc2, phosphorylated Cdc2; P-Cdc27, phosphorylated Cdc27; P-H3, phosphorylated histone H3. noc., nocodazole-treated total lysates. Numbers indicate hours after G\textsubscript{1}/S release. C, mitotically arrested cells induced by expression of plk1ΔN. Merged images (EGFP-plk1ΔN (green), tubulin (red), and DAPI (blue)) of a field of mitotically arrested cells are shown. D, quantification of cells at different stages of the cell cycle after release from G\textsubscript{1}/S block. U-2 OS cells were infected and released from the double thymidine block as in Fig. 2A then fixed and stained with DAPI. Data are an average of three independent experiments. More than 200 cells were counted for each time point. E, quantification of cells with binucleated and micronucleated morphologies. Asynchronously growing U-2 OS cells were infected with indicated viruses. Samples were fixed and stained with DAPI at the indicated time points to determine the percentage of cells with bi- and multinucleated chromosome morphologies. plk1ΔN/FAA, EGFP-plk1ΔN/FAA virus; PB1, EGFP-PB1 virus; PB2, EGFP-PB2 virus. F, expression of PLK1/K82M or plk1ΔN does not influence Plk1 and Cdc2 activation. Left, cells infected with indicated viruses and released 9 h from G\textsubscript{1}/S block were harvested and subjected to anti-Plk1 immunocomplex kinase assays. Right, samples prepared as in B were subjected to anti-Plk1 immunocomplex kinase assays. IP, immunoprecipitation. K82M, EGFP-Plk1/K82M virus; ΔN, EGFP-plk1ΔN virus; ΔN+pep, ΔN sample immunoprecipitated with an anti-Plk1 antibody preincubated with its epitope peptide; noc., nocodazole-treated sample; p13 ppt, p13\textsuperscript{caso} precipitation; H1, histone H1.


g-tubulin signals were apparent in all the cells examined (n = 152) (Fig. 3B). In addition, the amount of g-tubulin reactivity, which reflects a functional maturation of centrosomes, appeared to be normal. To determine directly whether the expression of plk1ΔN influences the microtubule nucleation capacities of microtubule-organizing centers, cells expressing either EGFPPlk1ΔN or control EGFPP were treated with nocodazole to depolymerize microtubules. After removing nocodazole, cells were transferred to fresh medium to allow for the re-growth of microtubules. Immunostaining for α-tubulin indicated that nocodazole treatment completely depolymerized the microtubules of both EGFPPlk1ΔN and control EGFPP-expressing cells (Fig. 3C, 0 min). Comparison of the length of re-grown microtubules for 5 or 10 min revealed similar kinetics of microtubule polymerization activities between these two samples (Fig. 3C), indicating that expression of EGFPPlk1ΔN did not influence the microtubule nucleating activity of the centrosomes under these conditions.

Relapse of Mitotic Progression by Expression of plk1ΔN—To closely investigate the nature of the pre-anaphase arrest associated with plk1ΔN expression, we conducted in vivo time-lapse studies using U-2 OS cells transfected with a plasmid expressing either GFPtubulin, DsRed-centrin, or GFP-H2B. These cell lines were transfected with a plasmid expressing either control DsRed or DsRed N-terminally fused to plk1ΔN. GFPtubulin cells expressing control DsRed appeared to grow normally with a doubling time similar to that of parental cells (data not shown). In contrast, as with EGFPPlk1ΔN expression, a large fraction of cells expressing DsRed-plk1ΔN resulted in mitotic arrest. Twenty-four hours post-transfection, 19 interphase cells, which weakly expressed DsRed-plk1ΔN, were chosen to closely monitor phenotypic changes during the arrest. Among them, 12 cells remained arrested at pre-anaphase for more than 20 h, whereas 7 cells died about 10–15 h after entering mitosis. One of the latter cells is shown in Fig. 4A. Consistent with normal microtubule nucleation activity, these cells all established apparent bipolar spindles 10–15 min after the onset of mitosis (Fig. 4A). However, these cells exhibited repeated cycles of bipolar spindle formation and disruption during the arrest (Fig. 4A), suggesting that these cells were not able to maintain stable bipolar spindle structures to execute metaphase/anaphase transition. One interesting observation was the induction of a cleavage furrow-like structure (arrow in Fig. 4A) after a prolonged arrest. However, this furrow soon regressed, and cell death followed (Fig. 4A), suggesting the occurrence of an aborted cytokinesis.

Because centrosomes play a critical role in bipolar spindle formation, we then investigated whether centrosome separation occurs normally upon plk1ΔN expression. Cells expressing control EGFP steadily increased the distance between two strong centrin signals (presumably two mother centrioles) and executed cytokinesis 35 min after onset of mitosis (Fig. 4B, top panels). In contrast, similar to the release of bipolar spindles, cells expressing EGFPPlk1ΔN (n = 11) frequently retrogressed centrosome separation even after achieving a full separation (Fig. 4B; compare between 0:12 and 2:06 time points). Similar to Fig. 4A, a prolonged mitotic arrest led to an aborted cytokinesis (Fig. 4B, arrow in DsRed-centrin plk1ΔN panel) followed by micronucleation. The number of centrioles did not increase in these cells (Fig. 4B), suggesting that another round of cell cycle did not occur during the arrest.

Normal bipolar spindle formation is critical for proper sister chromatid separation. To determine whether the generation of micronuclei is due to a defect in chromosome separation or due to accumulated cytokinesis failures, the behavior of chromosome structures were closely monitored in cells stably expressing GFP-H2B. Among 15 cells examined, most cells congregated chromosomes at the metaphase plate within 20 min after the onset of mitosis (Fig. 4C). However, all the cells expressing plk1ΔN resulted in repeated cycles of chromosome congression to and relapse from the metaphase plate (Fig. 4C), reminiscent of a defect in bipolar spindle formation. In addition, prolonged arrest at pre-anaphase resulted in an apparent cytokinesis defect (Fig. 4C, top panel, 25:47 time point) or abrupt generation of micronucleated cells in the absence of chromosome separation (Fig. 4C, bottom panels). Mitotic exit is thought to be a prerequisite step for the onset of cytokinesis. Thus, these observations together with the results above suggest that micronucleated morphology is not the result of accumulated cytokinesis failures in the presence of continuous cell cycle progression. Rather, it is more likely the result of fragmentation of unseparated chromosomes after mitotic exit in the absence of proper bipolar spindle formation.

Bypass of plk1ΔN-induced Spindle Checkpoint Arrest Leads to a Failure in Completion of Cytokinesis—To examine whether the apparent spindle defect induced by PLK1K82M or plk1ΔN expression resulted in spindle checkpoint activation, immunostaining with an anti-Mad2 antibody was carried out. Control prophase cells expressing EGFP alone exhibited distinct Mad2 localization. A low number (between 1 and 10) of localized Mad2 signals (Fig. 5A, first panel under control, and B) was still evident in ~45% of control metaphase cells, whereas Mad2 localization was not detectable in cells progressing through anaphase (Fig. 5A, the second panel under control). In contrast, all the PLK1K82M or plk1ΔN-expressing cells (n = greater than 32 for each) possessed clear Mad2 localizations at kinetochores of metaphase-like chromosomes under the same conditions (Fig. 5A and B), indicative of improper spindle attachment to the metaphase kinetochores.

It is possible that the observed pre-anaphase arrest could be due to an unknown defect at a step before the dissociation of Mad2 from metaphase kinetochores. In budding yeast, overexpression of CDC20 allows cells to bypass the spindle damage checkpoint, because elevated Cdc20 activates APC (37, 38). A recent report showed that a C-terminally truncated cdc201–153, which binds constitutively to Mad2 but not to APC, alleviates the spindle checkpoint (39). In addition, a C-terminal deletion bub11–331 mutant (40) or a truncated form of BubR1, bub1d (22), disrupts the mitotic checkpoint. Thus, we examined whether introduction of these components alleviates the mitotic block induced by expression of plk1ΔN. Expression of CDC20 strongly alleviated the mitotic arrest. In addition, expression of either cdc201–153, bub11–331, or bub1d allowed cells to bypass the mitotic block (Fig. 5C), indicating that the

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**Table II**

| Abnormality | Control | Plk1K82M | plk1ΔN |
|-------------|---------|----------|--------|
| % Abnormality | %       | %        | %      |
| Prophase    |         |          |        |
| Randomly oriented | 0.3 | 27.0 | 27.1 |
| Monopolar   | 1       | 8.7      | 5.3   |
| Bipolar     | 43.8    | 10.6     | 11.3  |
| Multipolar  | 0.3     | 2.5      | 3.6   |
| Metaphase   |         |          |        |
| Bipolar     | 54.3    | 50.1     | 51.1  |
| Multipolar  | 0.3     | 1.1      | 1.6   |
| Total cell number | n = 424 | n = 531 | n = 506 |

* Four hours before release from double thymidine block, U-2 OS cells were infected with indicated viruses at an m.o.i. of 10. Fifteen hours after release, cells were fixed and subjected to immunostaining with an anti-α-tubulin antibody to determine spindle morphologies.
FIG. 3. A, aberrant spindle morphologies induced by expression of plk1ΔN. U-2 OS cells were infected with indicated viruses as in Fig. 2A. Fifteen hours after release from double thymidine block, cells were fixed with paraformaldehyde and then subjected to immunostaining with an anti-α-tubulin antibody. Cells expressing plk1ΔN exhibited randomly oriented and disorganized (first panel), monopolar (second panel), multipolar (third panel), and apparent bipolar (fourth panel) spindle morphologies. Cells with bipolar spindle structures frequently exhibited misaligned chromosomes (arrows). EGFP, EGFP signals for the indicated constructs; α-tubulin, an anti-α-tubulin staining; DAPI, DAPI staining of chromosomal DNA; Merge, overlapped images.

B, cells with randomly oriented spindles exhibit two microtubule-organizing centers. U-2 OS cells infected with EGFP-plk1ΔN virus were prepared as in A and then subjected to double immunostaining with an anti-α-tubulin (green) and an anti-γ-tubulin (red). EGFP+α-Tub, EGFP-plk1ΔN + α-tubulin signal; γ-Tub, γ-tubulin signal. C, expression of plk1ΔN does not inhibit microtubule nucleation activity. Cells expressing either control EGFP or EGFP-plk1ΔN were treated with nocodazole to depolymerize microtubules. Upon allowing the cells to re-grow microtubules for various length of time, cells were fixed and subjected to an anti-α-tubulin staining.
plk1ΔN-dependent pre-anaphase arrest is due to the activation of a spindle checkpoint pathway. Under these conditions, neither the control vector nor the kinase-inactive BubR1/K795A mutant alleviated the mitotic arrest (Fig. 5C).

To investigate the effect of plk1ΔN expression on M-phase progression after bypassing the mitotic block, EGFP-H2B cells co-transfected with DsRed-plk1ΔN and bubr1Δd were closely monitored by in vivo time-lapse microscopy. Cells with a high level of DsRed-plk1ΔN fluorescent signals became micronucleated or apoptotic (data not shown). However, most cells with moderate DsRed-plk1ΔN signals (5 of 8 cells) exhibited an apparently normal mitotic progression until the formation of condensed, non-refractile, midbody structures with a clear fissure developing between the two dividing cells (Fig. 5D, 0:44 time point). The fissure, however, gradually regressed, resulting in a binucleated cell with two distinct nuclei (Fig. 4D, 4:00 time point). These observations suggest a defect in completion of cytokinesis. The remaining 3 cells exhibited a similar defect.
but with somewhat delayed sisterchromatid separation (data not shown). These observations suggest that the polo-box-dependent Plk1 activity is also required for the completion of cytokinesis.

DISCUSSION

The C-terminal Domain of Plk1 Is Sufficient for Subcellular Localization in U-2 OS Cells—Plk1 has been shown to localize to centrosomes and spindle kinetochores early in mitosis and
also to interzone and midbody structures during late mitosis and cytokinesis. This dynamic subcellular localization presages multiple functions of Plk1 during M-phase progression (28, 33). Our data demonstrated that, as with Plk1K82M, the C-terminal domain of Plk1 (plk1ΔN) efficiently localizes to centrosomes, midbody, and kinetochores, whereas PB1 or PB2 alone does not. In addition, introduction of the triple W414F/V415A/A427A mutations at the highly conserved motif in PB1 severely impaired the capacity of plk1ΔN to localize to these sites. Taken together, these data indicate that both intact PB1 and PB2 are required for efficient subcellular localization of Plk1. Although our data do not completely rule out a differential role of PB1 or PB2 for a specific event, both PB1 and PB2 appear to function together to interact with the binding target(s) at distinct subcellular structures. Understanding how Plk1 localization to these multiple sites is temporally and spatially regulated during mitotic progression and which protein it interacts with at specific locations will require further investigation.

Expression of Dominant-negative Forms of Plk1 Leads to Unstable Bipolar Spindles without Interfering with Centrosome Function—Previously, anti-Plk1 microinjection studies (4) showed that Plk1-deficient cells are defective in centrosome maturation and, consequently, in bipolar spindle formation. However, these studies failed to address whether Plk1 activity is required for proper bipolar spindle formation independently of centrosome maturation. To investigate whether expression of PLK1/K82M or plk1ΔN interferes with various cellular events at an early stage of mitotic block, we examined the cells released from G2/M block for 15 h. Under these conditions, centrosome maturation did not appear to be influenced, as evidenced by apparently normal microtubule nucleation activities. Consistent with this notion, in vivo time-lapse studies revealed that, as with control cells, plk1ΔN-expressing cells established apparent bipolar spindles within 10–15 min after the onset of mitosis. In contrast to normal microtubule nucleation, however, expression of either PLK1/K82M or plk1ΔN induced randomly oriented, disorganized spindles in a large fraction of prophase cells. The majority of cells with metaphase-like chromosome structures exhibited largely defective bipolar spindle structures with misaligned chromosomes. In addition, repeated cycles of formation and collapse of bipolar spindles were evident, indicating a clear defect in spindle stability during mitotic arrest. These data suggest that the dominant-negative capacity of Plk1K82M or plk1ΔN might not be sufficient to interfere with centrosome maturation, whereas it could impair spindle function. Thus, we suggest that proper bipolar spindle formation or maintaining stability is likely the most critical step that requires the polo-box-dependent Plk1 activity. Consistent with this notion, a recent report suggested that Xenopus polo kinase, Plx1, promotes microtubule stabilization and spindle assembly by inhibiting the function of a microtubule-destabilizing protein, Op18 (41).

**A Mad2-dependent Spindle Checkpoint Activation by Domin-negative Forms of Plk1**—Plk1 has been reported to phosphorylate a subset of APC components and activate APC (7). This step is critical for inactivation of Cdc2 and mitotic exit. It is generally believed that the checkpoint-dependent inhibitory complex binds to Cdc20 and inhibits APC-mediated ubiquitination until all the kinetochores of sisterchromatids are properly attached with spindles under tension. Cells expressing PLK1/K82M or plk1ΔN exhibited a typical phenotype of cells unable to pass beyond the metaphase/anaphase checkpoint, as they have high levels of Cdc2 activity with Mad2 localized at the kinetochores of metaphase chromosomes. In addition, Plk1 is activated and Cdc27 is phosphorylated normally in these cells, suggesting that spindle checkpoint activation is likely to be the cause of the pre-anaphase arrest. Consistent with this notion, introduction of either cdc20+/−, bub1+/−, or bubr1d alleviated this mitotic arrest. Whether spindle checkpoint activation directly resulted from a primary failure to correctly assemble and establish the bipolar spindle structures is not yet clear. Endogenous Plk1 localized to centrosomes and kinetochores throughout M phase, suggesting that it may have a role in spindle attachment at these structures. However, expression of PLK1/K82M or plk1ΔN did not appear to disrupt localization of endogenous Plk1 (data not shown). Neither did the expression of these constructs appear to interfere with endogenous Plk1 activation (Fig. 2F). Thus, we speculate that Plk1K82M or plk1ΔN may bind to and titrate out physiological substrates or binding targets of Plk1 at these sites essential for bipolar spindle formation or stability.

Recent studies with Drosophila polo(9) and polo(10) mutants revealed that these mutations led to a mitotic arrest as a consequence of tension-dependent spindle checkpoint activation (11). However, the arresting phenotype of polo(9) or polo(10) mutants differs from the Plk1 dominant-negative phenotype in that these mutants exhibit apparently normal bipolar spindles with partially separated sisterchromatids. Whether these apparent differences in arresting phenotypes reflect the different regulatory hierarchies of spindle checkpoint pathway in these evolutionarily distant organisms are not clear at present. In both systems, however, polo activity appears to be important for proper metaphase/anaphase transition.

**Generation of Micronucleated Cells as a Consequence of Mitotic Exit**—Previous transient transfection experiments in HeLa cells (42) revealed that expression of either wild-type or a kinase-inactive form of Plk1 induces abnormal interphase cells with micronucleated morphology. How these cells are generated and whether these morphologies are indicative of a failure in cytokinesis have not been clear. Our in vivo time-lapse studies revealed that plk1ΔN-expressing cells often attempted an aborted cytokinesis before generating micronucleated morphology. Because it is thought that mitotic exit is a prerequisite step before cytokinesis, a prolonged mitotic arrest may have led to an adaptive mitotic exit. Interestingly, centrosome duplication did not occur during the arrest, indicating that cells did not proceed through another cell cycle. Taken together, our data suggest that the previously described Plk1-dependent micronucleated morphology (42) is not the result of accumulated cytokinesis failures with continuous cell cycle progression. Rather, it is likely to be the result of an abrupt exit from mitosis in the absence of proper bipolar spindle formation and chromosome separation. These uncoordinated events may ultimately lead to cell death.

A requirement for the Plks to promote the onset of cytokinesis has been suggested in various organisms (for review, see Ref. 10). However, it has not been clear whether Plk1 directly contributes to cytokinesis in mammalian cells. The plk1ΔN-
expressing cells exhibited aborted cytokinesis with partial contraction, although it could be an indirect effect of a prolonged mitotic arrest. Interestingly, when mitotic block was bypassed, these cells exhibited a failure in completion of cytokinesis. These data suggest that the polo-box-dependent Plk1 activity is likely to be required in this process. A deeper understanding of the mechanisms as to how Plk1 contributes to cytokinesis will require the identification of polo-box-interacting proteins and additional Plk substrates important for this event.

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