Phosphoprotein profiling by Kinetworks™ analysis of M-phase-arrested HeLa cells by nucodazole treatment revealed that a novel mitosis-specific phosphorylation event occurred in the nucleolar protein B23/nucleophosmin at a conserved Ser-4 residue. Consistent with the resemblance of the Ser-4 phosphorylation site to the Polo-like kinase 1 (Plk1) consensus recognition sequence, inhibition of Plk1 by a kinase-defective mutation (K82M) abrogated B23 Ser-4 phosphorylation, whereas activation of Plk1 by a constitutively active mutation (T210D) enhanced its phosphorylation following in vivo transfection and in vitro phosphorylation assays. Depletion of endogenous Plk1 by RNA interference abolished B23 Ser-4 phosphorylation. The physical interaction of Plk1 and B23 was further demonstrated by their co-immunoprecipitation and glutathione S-transferase fusion protein pull-down assays. Interference of Ser-4 phosphorylation of B23 induced multiple mitotic defects in HeLa cells, including aberrant numbers of centrosomes, elongation and fragmentation of nuclei, and incomplete cytokinesis. The phenotypes of B23 mutants are reminiscent of a subset of those described previously in Plk1 mutants. Our findings provide insights into the biochemical mechanism underlying the role of Plk1 in mitosis regulation through the identification of Ser-4 in B23 as a major physiological substrate of Plk1.

The onset of mitosis is executed through a series of extensive morphological reorganizations of the cellular architecture, including nuclear envelope breakdown, bipolar mitotic spindle formation, chromosome condensation, and segregation, to ensure the faithful partitioning of genetic and cytoplasmic components. It is known that these changes are carried out through a number of reversible phosphorylation events on relevant substrate proteins, catalyzed by their respective upstream protein kinases. Whereas the roles of cyclin-dependent kinases (CDKs), the key regulators of eukaryotic cell cycle, are well established and widely appreciated, there is emerging evidence for the involvement of yet other protein kinases in the established and widely appreciated (1), there is emerging evidence for the involvement of yet other protein kinases in the regulation of cell cycle progression (2).

Polo-like kinases (Plks) are a conserved family of serine/threonine kinases that have emerged as pivotal components of the cell cycle regulation machinery over the past few years (3, 4). They are structural and functional homologues of POLO from Drosophila melanogaster, characterized by the conservation of the polo box domains in their C termini, which are required to localize the kinases to subcellular structures such as mitotic spindle, centrosomes, and the midbody in dividing cells (5, 6). So far, four Plks (Plk1, Plk2, Plk3, and Sak) have been identified in mammalian cells (7). Among them, Plk1 is most extensively studied and has been implicated in the regulation of multiple cell cycle events. Consistent with its functional homology with POLO from fruit flies, depletion of Plk1 by either antibody microinjection or antisense oligonucleotides or siRNAs results in defects in spindle formation and chromosome segregation, indicative of its vital roles in regulating mitotic spindle function (8–11). Despite a growing list of its physiological substrates such as Cdc25, Myt1, cyclin B, NudC, cohesin, and TCTP (translationally controlled tumor protein) that has been uncovered in recent studies, the signaling pathway through which Plk1 regulates microtubule dynamics such as bipolar mitotic spindle formation remains to be elucidated (12–17).

Accurate segregation of chromosomes after DNA replication into daughter cells requires correct formation and proper position of a bipolar mitotic spindle, which in turn depends on timely duplication and segregation of centrosomes. Dysregulation of the centrosome cycle has been implicated in the formation of aneuploid cells that are commonly found in human tumors as a result of chromosome missegregation (18, 19). As one of the major components in the pericentriolar material, B23/nucleophosmin (also called NPM, numatrin, or NO38) has been identified recently (20) as a substrate of CDK2/cyclin E. Phosphorylation of Thr-199 on B23 by CDK2/cyclin E at late G1 phase is a prerequisite for centrosome duplication (21). Moreover, a number of additional putative recognition sites for various protein kinases can also be found in B23. However, whether these phosphorylation events actually occur in vivo, how each phosphorylation affects the physiological roles of B23, and the identities of respective upstream kinases still remain to be answered.

Although both Plk1 and Plk3 have been implicated in regulating centrosomal functions (22), the underlying mechanisms are still poorly understood. In the present study, we have identified Ser-4 as a novel mitotic phosphorylation site on B23 by characterizing a cross-reacting signal with an antibody raised against a phosphorylation site epitope in Mek1/2, the upstream kinases of Erk1/2 identified through a Kinetworks™ phosphoprotein analysis. Detailed characterization of this phosphorylation event through employment of Plk1 mutants and RNA interference revealed that Plk1 is the major kinase directly responsible for B23 Ser-4 phosphorylation during mitosis. Interfering with Ser-4 phosphorylation of B23 in HeLa cells (23)
cells by overexpression of its mutants carrying point mutations at this phosphorylation site leads to abnormalities in centri- some duplication, chromosome segregation, and cytokinesis. Our results indicate that Plk1 exerts its roles in M-phase event regulation in part through its phosphorylation of B23 Ser-4.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Culture Conditions**—HeLa (human cervical carcinoma), HEK 293 (human embryonic kidney cells), MCF-7 (human breast carcinoma), and A549 (human lung carcinoma) were maintained in either Dulbecco’s modified Eagle medium or minimum essential medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) at 37°C in a humidified atmosphere containing 5% CO₂. The culture dishes used for HEK 293 were coated with 2% poly-L-lysine (Sigma) for 20 min prior to each use.

**Antibodies and Chemicals**—Anti-phospho-Mek1/2 (Ser-217/Ser-221), phospho-CDK1 (Thr-161), phospho-Plk3 (Thr-210), and anti-heat shock protein 90 were used from Zymed Laboratories Inc. (South San Francisco, CA) and Abgent (San Diego, CA), respectively. Anti-FLAG mouse monoclonal antibody and anti-γ-tubulin rabbit polyclonal antibody were from Sigma. Protein kinase inhibitors including olomoucine, SB203580, PD98059, DRB, and LC94002 and calf intestine alkaline phosphatase were from Calbiochem. Active CDK1/cyclin B1 was expressed from baculovirus (Upstate Biotechnology, Inc., Lake Placid, NY). Dephosphorylated form of α-casen and other chemicals were purchased from Sigma, unless otherwise stated.

**Plasmid Constructs**—PcDNA3-Plk1 wild-type (WT) was kindly provided by Dr. Kenji Fukasawa (University of Cincinnati). B23 gene was amplified by PCR, and a FLAG tag was incorporated into the C-terminal coding sequence. The resulting product was then subcloned into pcDNA3.1 (Invitrogen). pcDNA3-B23-S4A-FLAG and -B23-S4E-FLAG were re-inserted into pcDNA3.1 (Invitrogen). pcDNA3-B23-S4A-FLAG and -B23-S4E-FLAG were re-inserted into pcDNA3.1-H1 vector from Ambion (Austin, TX) was constructed according to the manufacturer’s protocol. Plk1 siRNA targeting human Plk1 sequence, GGGCGGCTTTGCCAAGTGCTT, corresponding to nucleotides 183–203 relative to the start codon, was synthesized. A scrambled sequence supplied by the manufacturer was used as a negative control. The resulting siRNA vectors were transfected into HEK 293 cells. Forty eight hours post-transfection, lysates were prepared, and the levels of both Plk1 and B23 Ser-4 phosphorylation were examined by immunoblotting.

**Small Interference RNA**—For Plk1 depletion, plasmid psiS-3.1-H1 vector from Ambion (Austin, TX) was constructed according to the manufacturer’s protocol. Plk1 siRNA targeting human Plk1 sequence, GGGCGGCGTCAGAGTCAGCTT, corresponding to nucleotides 183–203 relative to the start codon, was synthesized. A scrambled sequence supplied by the manufacturer was used as a negative control. The resulting siRNA vectors were transfected into HEK 293 cells. Forty eight hours post-transfection, lysates were prepared, and the levels of both Plk1 and B23 Ser-4 phosphorylation were examined by immunoblotting.

**Immuno precipitation, Kinase Assays, and GST-B23 Pull-down**—For kinase assays or GST pull-down proteomine, protein A-agarose beads (Amersham Biosciences) were pre-incubated with 5 µg of antibodies for 4 h, followed by incubation with total cell lysates as indicated overnight at 4°C.

**For CDK1 activity assay**, 5 µg of active CDK1/cyclin B1 were incubated with 5 µg of histone H1 or GST-B23 (WT) in 50 µl of CDK1 assay buffer (25 mM β-glycerophosphate, 20 mM MOPS, 2 mM MgCl₂, 0.25 mM dithiothreitol, and 5 µM β-mercaptoethanol, 10 mM 3-(N-morpholino)propanesulfonic acid, pH 7.2) at 30°C for 90 min after addition of the sample buffer to stop the reaction.

**For Plk1 activity assay**, Plk1 was either immunoprecipitated with anti-Plk1 antibody from 500 µg of untransfected cell lysates or anti-HA antibody from 500 µg of Plk1-transfected cell lysates. Immunocomplexes were subjected to immunoblotting using Plk1 antibody.

**Immunofluorescence Microscopy**—B23-S4A- and -S4E-transfected HeLa cells grown on coverslips for 48 h after transfection were fixed in 4% paraformaldehyde in PBS for 15 min, permeabilized with 0.5% Triton X-100 in PBS, and stained with cell lysates incubated with 1:250 anti-Plk1 antibody (1:250) and anti-γ-tubulin rabbit polyclonal antibody. The resulting siRNA vectors were transfected into HEK 293 cells. Forty eight hours post-transfection, lysates were prepared, and the levels of both Plk1 and B23 Ser-4 phosphorylation were examined by immunoblotting.

**RESULTS**

A Phospho-Mek1/2 (Ser-217/Ser-221) Antibody Recognizes a Mitotic-specific Phosphorylated Form of B23—To better understand phosphorylation events occurring during M-phase in mammalian cells, we employed the Kinetworks™ phosphoprotein analysis. This screen utilizes 31 phosphorylation site-specific antibodies to track changes systematically in the phosphorylation states of 33 known signaling proteins in mitotic HeLa cells induced by exposing exponentially growing cells to 100 ng/ml nocodazole for 20 h. Although a number of phosphorylation changes such as increased phosphorylation of retinoblastoma protein Ser-780, Ser-807/Ser-811, and c-Jun Ser-73 were observed as expected, an ~40-kDa phosphoprotein (pp40) was consistently detected by an antibody that was supposed to 

Plk1 Phosphorylates B23 Ser-4

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**Plk1 Phosphorylates B23 Ser-4**

**Phospho-B23 (Ser-4) Purification and LC-MS and MS/MS Analyses**—One hundred twenty milligrams of total protein from HeLa cells treated with 100 ng/ml nocodazole for 20 h were used as starting material for purification. The purification scheme included DEAE-Sepharose, phenyl-Sepharose, Mono Q, and Mono S. At each step, the protein signal was monitored by Western blotting analysis using anti-phospho-Mek1/2 (Ser-217/Ser-221) antibody. The Mono S fractions enriched for the 40-kDa immunoreactive protein were pooled and concentrated by ultrafiltration. The sample was then subjected to SDS-PAGE, followed by Comassie Blue staining of the gel. The 40-kDa immunoreactive protein band was excised and digested in-gel with trypsin (Promega, Madison, WI). A mass spectrometry sample was prepared according to the protocol described by Kinter and Sherman (26). LC-MS, MS/MS, and data search were performed by the Protein Chemistry Center of the University of Victoria (Victoria, British Columbia, Canada).

Small Interference RNA—For Plk1 depletion, plasmid psiS-3.1-H1 vector from Ambion (Austin, TX) was constructed according to the manufacturer’s protocol. Plk1 siRNA targeting human Plk1 sequence, GGGCGGCGTCAGAGTCAGCTT, corresponding to nucleotides 183–203 relative to the start codon, was synthesized. A scrambled sequence supplied by the manufacturer was used as a negative control. The resulting siRNA vectors were transfected into HEK 293 cells. Forty eight hours post-transfection, lysates were prepared, and the levels of both Plk1 and B23 Ser-4 phosphorylation were examined by immunoblotting.
The pp40 protein that cross-reacted with anti-phospho-Mek1/2 antibody is highlighted with a box. It should be noted that the anti-phospho-Mek1/2 antibody has been shown to recognize its cognate targets in epidermal growth factor-stimulated HeLa and A431 lysates (data not shown). Among them, only the known phospho-proteins that are present in the HeLa cells are identified as numbered bands as indicated in the list. The changes in the intensity of phosphorylation signals are evident in nocodazole-treated cells (B) as compared with Me2SO control cells (A). The three times higher intensity in nocodazole-treated cells compared with Me2SO control cells (Fig. 1). The small size of pp40 precluded itself from being one of the original targets of the antibody, Mek1 and Mek2, which are ~45-kDa proteins.

To determine whether pp40 was a nocodazole-specific or a mitosis-associated protein, we monitored the level of pp40 in synchronized HeLa cells by releasing serum-starved cells from G0/G1 block with serum addition. A maximum level of pp40 was seen at 24 h after serum addition, coinciding with the time when cyclin B1 was maximally expressed and CDK1 was maximally phosphorylated at its Thr-161 activation site, followed by a gradual decrease (Fig. 2). Similarly, pp40 was also detected in Hek 293, A549, and MCF-7 cells by the same anti-antibody with higher abundance in mitotic cells than interphase cells (data not shown), indicating that the increase of pp40 was generally associated with mitosis and was not a stress response to nocodazole treatment.

Because the phospho-Mek1/2 antibody was initially raised against a phosphopeptide corresponding to the sequences surrounding Ser-217/Ser-221 of Mek1, it seemed likely that pp40 shares a similar epitope with Mek1/2 and potentially represented a new class of Mek-like kinases, as suggested by Okano and Rustgi (28). To reveal the molecular identity of pp40, we partially purified it from the total lysate of nocodazole-treated HeLa cells by sequential liquid chromatography over DEAE-Sephacel, phenyl-Sepharse, Mono Q and Mono S. These columns were developed with linear or step NaCl gradients. The elution profile of pp40 was monitored by immunoblotting with the phospho-Mek1/2 antibody at each step. Following Mono S chromatography, the fractions enriched for pp40 were pooled and separated on a SDS-PAGE gel. The band corresponding to pp40 was excised after Coomassie Blue staining and digested in-gel with trypsin. The sample was then subjected to LC-mass chromatography, the fractions enriched for pp40 were pooled and separated on a SDS-PAGE gel. The band corresponding to pp40 was excised after Coomassie Blue staining and digested in-gel with trypsin. The sample was then subjected to LC-mass chromatography. Five tryptic peptides from LC-MS were further characterized with tandem MS (MS/MS). A Mascot data base search revealed that pp40 was human B23/nucleophosmin (Fig. 3A).

The molecular identity of pp40 was further supported by reciprocal co-immunoprecipitation data. B23 was detected in phospho-Mek1/2 antibody immunoprecipitates by immunoblotting with a B23 antibody. Conversely, pp40 was found in anti-B23 immunoprecipitates (Fig. 3B). Immunofluorescence microscopy with the phospho-Mek1/2 antibody revealed a signal exclusively localized within the nucleolus in interphase and translocated into cytoplasm during M-phase, consistent with the behavior of B23 (data not shown).

B23 Is Phosphorylated at Ser-4—Because the phospho-Mek1/2 antibody only reacts with the Ser-217/Ser-221-phosphorylated forms of Mek1 and Mek2, it was likely that pp40 might represent a phosphorylated form of B23. This was further supported by the fact that the increase of pp40 signal in response to nocodazole treatment was not due to the change in B23 protein level, as indicated by immunoblotting of nocodazole-treated and untreated HeLa lysates with a B23 antibody (Fig. 4, A and B). Treatment of lysates from nocodazole-treated HeLa, MCF-7, and A549 cells with 35 units of alkaline phosphatase for 4 h at 37 °C resulted in the disappearance of pp40, whereas the B23 protein level remained relatively constant, indicating the phospho-Mek1/2 antibody recognized a phospho-epitope on B23 (Fig. 4, C and D).

To identify the phospho-epitope on B23 recognized by the phospho-Mek1/2 antibody, we aligned the human B23 full-length sequence with the antigenic phosphopeptide of the antibody using ClustalW algorithm (29), and we found that only...
Fig. 3. pp40 corresponds to the nucleolar protein B23/nucleophosmin. Purified pp40 from nocodazole-treated HeLa lysate was digested in-gel with trypsin, and the eluted fragments were subjected to LC-mass spectrometry followed by tandem MS of five tryptic peptides. Mascot database search indicated that pp40 is human B23/nucleophosmin (A). Reciprocal immunoprecipitation (IP) with anti-B23 or phospho-Mek1/2 antibody from nocodazole-treated HeLa lysate further supported assignment of pp40 as B23 (B). WB, Western blot.

The flanking sequences of Ser-4 on B23 shared limited similarity with the antigenic peptide of the antibody (Fig. 4E). Three consecutive residues including Ser-4 (B23/Ser-217 (Mek1) and two adjacent residues (Asp at -1 and Met at +1 positions) were identical between these two sequences.

To test whether Ser-4 of B23 is the phosphorylation site recognized by the antibody, we constructed a nonphosphorylatable mutant of B23 (B23-S4A) by replacing Ser-4 with Ala and expressed B23 wild-type (B23-WT) and B23-S4A in Escherichia coli as GST fusion proteins. The ability of nocodazole-treated HeLa cell lysates to phosphorylate GST-tagged B23-WT and B23-S4A was tested through in vitro phosphorylation assays. As shown in Fig. 4, F–H, whereas B23-WT could be phosphorylated by HeLa lysate in response to nocodazole treatment, as determined by immunoblotting with anti-phospho-Mek1/2 antibody, the Ser-to-Ala substitution in B23 completely eliminated this phosphorylation event, indicating that Ser-4 phosphorylation might be targeted by the phospho-Mek1/2 antibody. The failure of B23-S4A phosphorylation by nocodazole-treated cell lysate could not be attributed to potentially incorrect folding of the bacterially expressed protein caused by Ser-4 mutation, as both B23-WT and -S4A could be readily phosphorylated by cell lysate at Thr199 (Fig. 4G), a CDK2/cyclin E phosphorylation site (21), as detected by immunoblotting with an antibody against phospho-B23 (Thr-199).

To verify the above results in vivo, we transfected HEK 293 cells with either a FLAG-tagged B23-S4A or B23-WT construct. After 20 h of nocodazole treatment, the overexpressed FLAG-tagged B23-WT or -S4A proteins were immunoprecipitated with anti-FLAG antibody, and the immunoprecipitates were immunoblotted with anti-FLAG or anti-phospho-Mek1/2 antibody. As can be seen in Fig. 4, I and J, although the expression levels of B23-WT and B23-S4A were similar, the phospho-Mek1/2 antibody recognized two bands in B23-WT-transfected cell lysates, but only the lower band was detected in B23-S4A-transfected cell lysates. The differential immunoreactivities of the two bands with anti-FLAG and B23 antibodies indicated that the upper band corresponded to the overexpressed FLAG-tagged B23 proteins, whereas the lower band corresponded to endogenous B23. The disappearance of the upper band upon replacing Ser-4 with Ala confirmed that Ser-4 was the phosphorylation site recognized by the phospho-Mek1/2 antibody. The presence of the endogenous phospho-Ser-4 B23 in anti-FLAG immunoprecipitates might be due to the formation of oligomers between overexpressed B23 and endogenous B23 proteins. This is supported by the previous finding that the oligomeric forms are the major functional entities of B23 in cells (30). Its appearance in both B23-WT and B23-S4A-transfected cells indicated that the Ser-4 phosphorylation was at least not required for all the B23 molecules involved in oligomerization and that eliminating the Ser-4 phosphorylation...
site did not disrupt B23 oligomeric activity, despite the fact that Ser-4 is localized at its N-terminal domain which is essential for oligomerization (31).

Furthermore, the human B23 Ser-4 site along with its flanking sequences is highly conserved among its homologues from rat, mouse, and frog (data not shown). More interesting, a protein of similar size recognized by the phospho-Mek1/2 antibody was induced during starfish oocyte maturation process.2 The presence of Plk1 or B23 was found in the immunoprecipitates of B23 or Plk1, respectively (A and D). A higher level of Plk1 was detected in the GST-tagged B23-WT pull-down from nocodazole-treated HeLa lysate than from MeSO control (E). The amount of GST-B23 protein used in the pull-down assays is shown in F.

To confirm the physical interaction between Plk1 and B23, a reciprocal immunoprecipitation experiment with both anti-Plk1 and anti-B23 antibodies was performed. Immunoblotting using their respective antibodies showed the presence of B23 in anti-Plk1 immunoprecipitate and Plk1 in anti-B23 immunoprecipitate (Fig. 6, A–D). In agreement with immunoprecipitation data, bacterially expressed GST-B23-WT protein was able to pull down Plk1 from HeLa cell lysate (Fig. 6, E and F), indicating that Plk1 interacts with B23. Moreover, a higher level of B23 and Plk1 interaction was observed in nocodazole-treated lysates compared with MeSO control (E). This result is consistent with the notion that Plk1 interacts with B23 in a nocodazole/M-phase-dependent manner.

To further explore if Plk1 is the kinase responsible for B23 Ser-4 phosphorylation, we transfected HeLa cells with plasmids carrying HA-tagged Plk1 kinase-defective mutant (Plk1-K82M) and constitutively active mutant (Plk1-T210D), respectively, and we examined the level of B23 Ser-4 phosphorylation 48 h after transfection by using phospho-Mek1/2 antibody (32). Empty vector-transfected cells were used as a negative control. Whereas both types of Plk1 proteins were expressed at comparable levels after transfection, as visualized by immunoblotting with anti-HA antibody (Fig. 7C), overexpression of Plk1-T210D enhanced B23 Ser-4 phosphorylation, and overexpression of Plk1-K82M attenuated its phosphorylation compared with vector-transfected cells (Fig. 7A). Because comparable levels of B23 were detected in all transfected cells (Fig. 7B), the changes
of B23 Ser-4 phosphorylation could not be attributed to the changes at B23 protein level.

To rule out that the effect on B23 Ser-4 phosphorylation seen above was an artifact of a result of Plk1 overexpression in vitro, we examined the level of B23 Ser-4 phosphorylation in the cells in which Plk1 was depleted by small interfering RNA (siRNA). HEK 293 cells were transfected with pSilencer™-1-H1 vectors carrying Plk1-specific siRNA targeting the human Plk1 nucleotides 183–203 relative to the first nucleotide of the start codon as a nonspecific control. At 48 h post-transfection, the levels of Plk1 as well as B23 Ser-4 phosphorylation were monitored by immunoblotting. Treatment of Plk1 siRNA resulted in the depletion of most endogenous Plk1 (Fig. 7, A). Correlating with the loss of Plk1, B23 Ser-4 phosphorylation decreased dramatically compared with control siRNA-treated cells (Fig. 7E).

In support of this, after Plk1 was immunodepleted from nocodazole-treated HeLa cell lysate by Plk1 antibody, we found that the resulting supernatant lost its B23 Ser-4 phosphorylation activity toward bacterially expressed GST-B23 (data not shown). Meanwhile, anti-Plk1 immunoprecipitates exhibited a strong phosphorylation activity in vitro toward B23-WT, but not B23-S4A, and the resulting phosphorylation could be detected either by autoradiography as the incorporation of [32P]ATP (Fig. 7G) or by immunoblotting with phospho-Mek1/2 antibody (Fig. 7H).

To exclude the possibility that other kinases that could potentially associate with Plk1 in anti-Plk1 immunoprecipitate might contribute to B23 Ser-4 phosphorylating activity, we immunoprecipitated HA-tagged Plk1-T210D or Plk1-K82M proteins from transfected HeLa cells by using anti-HA antibody, and we examined their respective abilities to phosphorylate GST-B23 at Ser-4 in vitro. We found that only Plk1-T210D, not Plk1 K82M, could phosphorylate Ser-4 of B23 (Fig. 7, K–M), indicating that it was Plk1 activity, not an associated kinase, that was responsible for B23 Ser-4 phosphorylation. Taken together, our results demonstrated that Plk1 is a bona fide B23 Ser-4 kinase.

**Phosphorylation of B23 Ser-4 Is Specific for Plk1**—Because Plk3, another member of the mammalian Polo-like kinase family, has been implicated in stress responses including mitotic spindle disruption induced by nocodazole (33, 34), we next explored whether Plk3 could also contribute to B23 Ser-4 phosphorylation.

To address this question, we applied insect expressed His₆-Plk3 in an in vitro phosphorylation assay using bacterially expressed B23-WT or B23-S4A as substrate, and we monitored the incorporation of [32P]ATP into B23 by autoradiography. As shown in Fig. 8A, whereas active Plk3 exhibited a strong phosphorylation activity toward c-casein, a putative Plk substrate for in vitro kinase assays, it was able to phosphorylate B23-WT but not B23-S4A, indicating that Plk3 could also phosphorylate B23 at Ser-4 in vitro.

To further clarify this point, we monitored both Plk1 and Plk3 activities over the course of the cell cycle as well as...
response to treatment by nocodazole with and without olomoucine. Treatment by nocodazole induced a significant activation of Plk1, whereas only a moderate increase was observed for Plk3 (Fig. 8, B and C). Furthermore, only Plk1 activation by nocodazole could be inhibited dramatically by olomoucine. In fact, a slight induction of Plk3 activity was seen after olomoucine treatment, probably due to the accumulation of cells arrested before G2/M transition when a higher Plk3 activity was detected (Fig. 8, B and C). The change of B23 Ser-4 phosphorylation was in parallel with that of Plk1 in response to drugs (Fig. 8D). Anti-Plk1 immunoprecipitate exhibited its peak α-casein phosphorylation activity 23 h after serum re-addition, coinciding with the time when B23 Ser-4 was maximally phosphorylated (Fig. 8, E and G). In contrast, anti-Plk3 immunoprecipitate reached its highest activity level at ~7 h (Fig. 8F). The correlation of Plk1 activity profiles with B23 Ser-4 phosphorylation during the cell cycle and following drug treatment indicated that Plk1, not Plk3, is the physiological upstream kinase of B23 Ser-4.

**DISCUSSION**

Accumulating evidence from recent studies (35) has indicated that mammalian Plk1 is implicated in a variety of mitotic processes, such as onset of mitosis, centrosome duplication and maturation, chromosome segregation, cytokinesis, and DNA damage checkpoint control, which is echoed by its dynamic pattern of subcellular localization to centrosomes, kinetochores, and the midbody. However, complete understanding of pleiotropic activities of Plk1 still awaits the identification of its physiological substrates in each process. In this study, we identified a novel mitotic-specific phosphorylation event occurring at Ser-4 of B23 through characterizing a cross-reacting antibody. Further character-
ization of this phosphorylation event revealed that Plk1 is the upstream kinase directly responsible for B23 Ser-4 phosphorylation. Examination of B23 Ser-4 mutant phenotypes using indirect immunofluorescence microscopy provided the evidence for the involvement of B23 Ser-4 phosphorylation in mediating the roles of Plk1 in a series of cell cycle events including centrosome duplication, sister chromatid segregation, and cytokinesis.

Because the phoso-Mek1/2 polyclonal antibody used in this study was originally raised against a phosphopeptide corresponding to the amino acid sequences flanking the two phosphorylation sites, Ser-217 and Ser-221, in the mitogen-activated protein kinase kinase Mek2, and its related isofrom Mek2, it was quite tempting to speculate that the cross-reacting protein with the antibody might correspond to a novel Mek1/2-like protein kinase. This is further corroborated by a study of Cha et al. (36) that was accepted for publication during the preparation of this manuscript, in which they characterized presumably the same cross-reacting protein from the same antibody and identified it as B23. However, instead of Thr-234/Thr-237 as suggested by their work, Ser-4 was identified as the phospo-epitope recognized by the phoso-Mek1/2 antibody in our study. Given the similarity in profiles of B23 phosphorylation during the cell cycle as well as its induction in response to nocodazole treatment observed in both studies, it seems very difficult to reconcile the discrepancy in results from them. However, the inability of purified, active CDK1/cyclin B to phosphorylate B23 directly in vitro at the site recognized by the antibody indicated that the inhibition of B23 phosphorylation at that particular site by the CDK inhibitor olomoucine is mediated through an intermediate kinase activated by CDK1. Thus, CDK1 cannot be the direct upstream kinase of B23 for that site, and Thr-234 and Thr-237, the pre-characterized CDK1 sites, are therefore unlikely to be the epitope recognized by the phoso-Mek1/2 antibody (21). In addition, little similarity was found between the flanking sequences around Thr-234/Thr-237 in B23 and those of Ser-217/Ser-221 in Mek1, whereas three consecutively identical residues are shared by two proteins around Ser-4 of B23, indicating that Ser-4 of B23 is more likely to react with the antibody than Thr-234/Thr-237. Nevertheless, the limited sequence similarity between the antigenic peptide of the antibody and the flanking sequences of B23 Ser-4 has not only demonstrated the need of being cautious in interpreting cross-reacting proteins in proteomic studies, but also validated the phospo-specific antibody-driven proteomics approach for identifying novel phosphorylation events.

By testing the efficiency of systematic mutated peptides derived from Cdc25C as Plk1 substrates, Nakajima et al. (13) identified a consensus phosphorylation motif for Plk1, (D/E)X(S/T)XX(D/E), in which a hydrophobic amino acid (X) at position +1 and an acidic amino acid at position −2 are critical for optimal phosphorylation by Plk1 (where Xaa indicates any amino acid), which is in agreement with several Plk1 substrates characterized previously (37). Consistent with the consensus phosphorylation motif, B23 Ser-4 was found to be the substrate of Plk1 in our study, supported by evidence from both in vitro phosphorylation of recombinant B23 proteins and in vivo transfection experiments with Plk1 mutants. This conclusion is further strengthened by the data of Plk1 depletion through RNA interference approach. The presence of a physical interaction between Plk1 and B23, as evidenced by GST fusion protein pull-down and co-immunoprecipitation data, and the close correlation between B23 Ser-4 phosphorylation and Plk1 activity both during the cell cycle and in their responses to nocodazole and olomoucine treatment further corroborated the conclusion that Plk1 is the physiological upstream kinase of B23. This is the first time that experimental evidence has been presented for the functional relationship between Plk1 and B23, even though the link between two proteins has long been speculated, due to the similarities both in function and in subcellular localization (38, 39).

Plk1 was initially proposed as a trigger kinase of CDK1/cyclin B via activation of Cdc25C (40, 41). However, more recent studies (42, 43) have shown that Plk1 is implicated in a positive feedback loop that consists of both Plk1 and CDK1, and it does not function as the initial activator, rather its activation depends upon CDK1 activity. Our study seems to shed new light on this controversial issue, as the inhibitory effect of olomoucine on Plk1-mediated B23 Ser-4 phosphorylation that we observed here strongly implies that the B23 phosphorylation activity of Plk1 during M-phase is dependent upon CDK1 activation. The conclusion is further corroborated by the recent observation that silencing Plk1 via siRNA resulted in a metaphase arrest but exhibited no effect on mitotic entry when activation of CDK1 is required.4

Despite opposing activities of Plk1 and Plk3 in cell proliferation controls, Plk3 has also been found to associate with centrosomes, mitotic spindle poles, and the midbody and to be involved in regulating mitotic spindles as well as cytokinesis (33, 34, 44). Considering the apparent overlaps between Plk1 and Plk3 in functionality, we speculated that Plk3 might also function as a B23 Ser-4 kinase. Even though active Plk3 was capable of phosphorylating B23 at Ser-4 in vitro, its timing of activity peak during the cell cycle and its distinct response to the treatment of nocodazole and olomoucine indicate that phosphorylation of B23 Ser-4 by Plk3 observed in this study may be due to similarity between two Plks in substrate recognition, and it may not be physiologically relevant. The profile of Plk3 activity during the cell cycle is in agreement with that reported previously (27).

Furthermore, our recent characterization of mouse embryonic fibroblast cells derived from Plk3 null mice using Kinetochore phosphoprotein analysis is revealed that the loss of Plk3 exerted no effect on B23 Ser-4 phosphorylation, providing support that it is Plk1 not Plk3 that is responsible for B23 Ser-4 phosphorylation.4 However, we cannot exclude the possibility that Plk3 may phosphorylate B23 at different site(s).

B23 is a multifunctional protein and is implicated in ribosome biogenesis, protein trafficking between the nucleus and the cytoplasm, preserving protein conformation, and activity through its chaperoning activity (38). More recently, it has been shown to be involved in centrosome duplication through phosphorylation of Thr-199 by CDK2/cyclin E at late G1 phase. Whereas initial functional characterization of Plk1 by antibody microinjection indicated that Plk1 was involved in centrosome maturation and not duplication (9), recent work from Liu and Eriksen (45) using RNA interference approach demonstrated that the depletion of Plk1 inhibited centrosome amplification in hydroxyurea-arrested U2OS cells. Consistent with the latter finding, our study demonstrated that alterations of B23 Ser-4 phosphorylation by mutations induced aberrant centrosome numbers in HeLa cells. However, whether the abnormality in centrosome numbers is due to the deregulation of centrosome duplication or interruption of a duplication-related event such as re-licensing of centrosome for duplication is unclear. Given the facts that the B23 Ser-4 phosphorylation does not correlate

4 W. Dai, unpublished data.
temporally with that of centrosome duplication as well as that it does not exert any effects on Thr-199 phosphorylation, it seems unlikely that B23 Ser-4 phosphorylation plays a direct role in centrosome duplication. The exact mechanism underlying the abnormality of centrosomes induced by mutations of Ser-4 of B23 remains to be elucidated.

Consistent with its localization at the midbody in dividing cells, involvement of Plk1 and its orthologues in cytokinesis has been demonstrated in many organisms by a number of studies (6, 46–48). We observed that unschshedlated phosphorylation of B23 Ser-4 led to the appearance of doublets or tetraplets in a fraction of cells overexpressing B23-S4E. Because no B23 has been reported to locate at the midbody so far, we suspect that cytokinesis aberration may be attributed to the possible formation of multipolar spindles directed by multiple centrosomes in B23-S4E cells, which is consistent with the roles of mitotic spindle poles in determining the position and orientation of the cleavage furrows (49, 50). As a result, the daughter cells derived from this defective cytokinesis are likely to suffer from chromosome segregation errors such as loss of chromosomes, leading to genetic instability as well as the formation of aneuploid cells, the hallmarks of carcinogenesis.

We have shown that overexpression of B23-S4A resulted in the formation of elongated and unsevered nuclei of dumbbell-like appearance, reminiscent of the phenotype of Plk1-deficient cells generated by using the RNA interference approach (45). Although it seems likely to be a consequence of deficiency in sister chromatid segregation, as suggested by Liu and Erikson (45), more studies are needed to clarify this point. In support of this notion, B23 has been shown to associate with the kinetochore/centromere region of mitotic chromosomes (51). Recently, Plk1 was found to phosphorylate and dissociate cohesion from chromatids, leading to chromatid segregation at anaphase (16). The question remains as to whether the phosphorylation of B23 Ser-4 is directly involved in controlling sister chromatid segregation or missegregation of chromatids is simply due to the lack of duplicated centrosomes.

Our preliminary fluorescence-activated cell sorter analysis showed that no significant difference was seen in cell cycle profile before and after inhibition of Ser-4 phosphorylation, indicating the phenotype observed above were not general consequences of cell cycle blocks. The subtle effects on the cell cycle progression by Ser-4 mutations may be attributed to the fact that HeLa cells used in this study are an immortalized tumor cell line, and thus certain checkpoint controls that could normally monitor and eliminate any aneuploid cells as a result of chromosome missegregation or aberrant cytokinesis through apoptosis might be defective.

Given its vital roles in mitotic controls, the activity of Plk1 has been increasingly recognized as a prognostic marker for a number of human tumors, including non-small cell lung cancer, squamous cell carcinomas of the head and neck, melanomas, oropharyngeal carcinomas, and colorectal cancers (52). Similarly, an elevated level of expression and/or phosphorylation of B23 has been observed in highly proliferating cells, including cancer cells. Centrosome amplification has been shown to be a major factor causing genomic instability and aneuploidy in tumor cells. With the identification of the functional relationship between Plk1 and B23 and the elucidation of the role of B23 Ser-4 phosphorylation mediated by Plk1 in centrosome-associated cell cycle events, our study has not only furthered our understanding of the mechanism underlying the involvement of Plk1 in tumorigenesis but has also identified the phosphorylation of B23 Ser-4 as a useful indicator for tumor progression and a potential target for both high throughput screenings for anti-cancer drugs and tumor therapeutic interventions.

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