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Phosphorylation of Threonine 210 and the Role of Serine 137 in the Regulation of Mammalian Polo-like Kinase*

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The mammalian polo-like kinase (Plk) plays a critical role in M-phase progression. Plk is phosphorylated and activated by an upstream kinase(s), which has not yet been identified in mammalian cells. Phosphopeptide mapping and phosphoamino acid analyses of Plk labeled *in vivo* and phosphorylated *in vitro* by *Xenopus* polo-like kinase kinase-1 (xPlkk1) or by lymphocyte-oriented kinase, its most closely related mammalian enzyme, indicate that Thr-210 is a major phosphorylation site in activated Plk from mitotic HeLa cells. Although the amino acid sequence surrounding Ser-137 is similar to that at Thr-210 and is conserved in Plk family members, Ser-137 is not detectably phosphorylated in mitotic mammalian cells or by xPlkk1 *in vitro*. Nevertheless, the substitution of either Thr-210 or Ser-137 with Asp (T210D or S137D) elevates the kinase activity of Plk. The kinase activity of the double mutant S137D/T210D is not significantly different from that of T210D or S137D, demonstrating that substitution of both residues does not have an additive effect on Plk activity. Expression of the S137D mutant construct arrested HeLa cells in early S-phase with slightly separated centrosomes, whereas cells expressing wild type and T210D were arrested or delayed in M-phase. These data indicate that the Ser-137 may have an unexpected and novel role in the function of Plk.

Progression through the mammalian cell cycle depends on the periodic control of various cyclin-dependent kinases (for reviews, see Refs. 1 and 2). In addition, members of the polo-like kinase (Plk)¹ family influence multiple events during cell division (3, 4). Mammalian Plk is involved in bipolar spindle formation, chromosome separation (5–7), centrosome maturation (8), and regulation of the anaphase-promoting complex (9, 10). In *Xenopus*, Plk may play an important role at the onset of mitosis; *Xenopus* Plk (Plx1) phosphorylates and activates the phosphatase Cdc25C, a positive regulator of Cdc2-cyclin B (11).

In addition to proteolytic regulation, post-translational mod-

ifications such as phosphorylation and dephosphorylation are also a key regulatory mechanism of protein kinases in the cell cycle. Throughout late S and early G₂ phases, Cdc2 is kept inactive as the result of phosphorylation of Thr-14 and Tyr-15 in the ATP-binding site. Phosphorylation at these sites is catalyzed by the protein kinases Wee1 and Myt1 (12, 13). Activation of Cdc2-cyclin B at the G₂/M transition requires dephosphorylation of both residues by Cdc25C (14–16). Plk is also regulated by phosphorylation. During mitosis, the phosphorylated enzyme is detected as a more slowly migrating form. Phosphatase treatment increases the mobility and reduces the activity of Plk (17–19). The original studies on Thr-210 in Plk demonstrated that substitution of aspartate at this site significantly elevates protein kinase activity (20). This residue is in the activation loop between protein kinase subdomains VII and VIII, and phosphorylation within this loop results in the activation of several kinases including Cdc2 and Mek1 (1, 21). These data suggest that aspartate mimics the phosphorylation of serine or threonine in the activation loop and, moreover, raise the possibility that Thr-210 is a physiologically important phosphorylation site. xPlkk1 was identified in *Xenopus* egg extracts and was cloned as a major upstream kinase of Plx1 (22). Microinjection of xPlkk1 into *Xenopus* oocytes accelerates the time of Plx1 activation as well as the transition from G₂ to M phase of the cell cycle (23). The upstream activating kinase of Plk in mammalian cells still remains to be identified, although there are enzymes closely related to xPlkk1, such as LOK (24) and STE20-like kinase (SLK) (25), in mammalian cells. We have shown² that LOK functions *in vitro* in a fashion similar to xPlkk1; however, as yet, there are no data that indicate it is the relevant upstream activator of Plk *in vivo*. However, in previous studies, the site(s) of activating phosphorylation in Plk were not identified.

In this study, we provide direct evidence that Thr-210 is a major phosphorylation site in mitotic Plk and that this site is important for Plk activation. In contrast, although substitution of Ser-137, which is embedded in a similar sequence as Thr-210, with aspartate also activates Plk, this residue is not phosphorylated *in vitro* by xPlkk1 or *in vivo* in mitotic cells and does not appear to be involved in activation of Plk during mitosis. Nevertheless, experiments with Ser-137 mutants suggest that this site has the potential to have a biologically significant role in regulating Plk activity during other stages of the cell cycle.

EXPERIMENTAL PROCEDURES

Cell Culture, Synchronization, Metabolic Labeling, and Transfection—HeLa cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and penicillin/streptomycin (Invitrogen). To synchronize cells at the G₁/S transition, HeLa cells were treated with a double thymidine block (2.5 mM). To obtain mitotically synchronized cells, G₁ cells, which were synchronized by treat-

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¹ The abbreviations used are: Plk, polo-like kinase; Plx1, *Xenopus* Plk; xPlkk1, *Xenopus* Plk kinase-1; LOK, lymphocyte-oriented kinase; HA, hemagglutinin; GFP, green fluorescent protein; EGFP, enhanced GFP; GST, glutathione S-transferase; PBS, phosphate-buffered saline; WT, wild type; HPLC, high pressure liquid chromatography.

² Y.-J. Jang, Y. Terada, and R. L. Erikson, unpublished data.

ment with mimosine (300 μ M) for 16 h, were washed with fresh medium and were released into medium containing 200 ng/ml nocodazole for 18 h. To obtain metabolically labeled endogenous Plk, actively growing HeLa cells were arrested with 200 ng/ml nocodazole for 12 h. The mitotic cells were collected by shake-off and washed with labeling medium (phosphate-free Dulbecco's modified Eagle's medium plus 10% dialyzed serum, 10% normal Dulbecco's modified Eagle's medium, and 200 ng/ml nocodazole). Cells were labeled with [32 P]orthophosphate (ICN) at a final concentration of 0.7 mCi/ml for 4 h before washing and lysis. Transfection was performed by the standard calcium chloride method (26) except that HEPES-buffered saline was used.

Generation and Expression of Plk Mutants and Constructs in Sf9 or Mammalian Cells—Mutations at the indicated sites in the Plk construct were generated by PCR. All the PCR products were cloned into pGEX vector (Promega) and sequenced. The mutant proteins were expressed in *Escherichia coli* by isopropyl-1-thio- β -D-galactopyranoside induction and confirmed by Western blot with anti-Plk antibody. To generate recombinant baculoviruses, the pGEX constructs were cloned into pAcGHLT vector (Pharmingen) and were transfected into Sf9 cells with BaculoGoldTM (Pharmingen). For expression in mammalian cells, all constructs were cloned into pCMV-Tag2 vector (Stratagene), which encodes eight amino acids of FLAG (DYKDDDDK) epitope at the N terminus. Recombinant baculoviruses for expression of His-xPlkk1 were a kind gift of James Maller. Recombinant LOK or xPlkk1 proteins were prepared from Hi5 cells untreated or treated with 0.1 μ M okadaic acid for 3 h with the use of TALON affinity resin and were further purified as described previously (22). Fractions containing xPlkk1 proteins were stored in small portions at -80°C .

Preparation of Cell Extracts, Immunoprecipitation, and Kinase Assay—Cells were lysed with Nonidet P-40 buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM EGTA, 25 mM β -glycerophosphate, 25 mM NaF, 0.5 mM Na_3VO_4 , 10 mM *p*-nitro phenylphosphate, and 1% Nonidet P-40) supplemented with protease inhibitors and centrifuged at $15,000 \times g$ for 20 min. The supernatant was incubated with antibody, anti-Plk monoclonal antibody (Zymed Laboratories Inc.) for metabolically labeled endogenous Plk or anti-FLAG monoclonal antibody (Sigma) for transfected Plk. Protein A/G- or protein G-PLUS Agarose (Zymed Laboratories Inc.) was added to isolate the immunocomplexes. Kinase reactions were carried out as described (20).

To examine the effect of wild type or T210D Plk on the activation of Cdc2-cyclin B, HeLa cells were transfected with plasmids encoding HA-Plk or HA-PlkT210D. About 60% of the total cell population expressed Plk1 under these conditions. At 12 h, mimosine was added to a final concentration of 0.3 mM, and cells were incubated for an additional 20 h. Cells were released from the mimosine block by washing with serum-free medium and incubated in complete medium before collection to measure Cdc2-cyclin B histone kinase activity in immunocomplex assays. Cell extracts were incubated with polyclonal anti-Cdc2 antibody, and immunocomplex assays were performed as described previously (20). Histone H1 phosphorylation was measured with a Fujix BAS 2000 phosphorimaging device and MacBAS v2.5 software.

Chymotryptic Peptide Mapping and Phosphoamino Acid Analysis—The radiolabeled proteins were electrophoresed on a 8% polyacrylamide gel (1:120 acrylamide:bisacrylamide) and transferred to a polyvinylidene difluoride membrane (Millipore). For phosphopeptide mapping, the pertinent region of the membrane was blocked with 1 ml of blocking solution (0.5% polyvinylpyrrolidone in 100 mM acetic acid) for 30 min at 37°C . The membrane pieces were washed with HPLC-grade water and with freshly made 50 mM ammonium bicarbonate and incubated in 200–300 μ l of 50 mM ammonium bicarbonate containing 1 mM dithiothreitol and 20 μ g of α -chymotrypsin (Sigma) at 37°C overnight. The supernatant was dried, dissolved in 100 μ l of HPLC-grade water, and dried again. This washing step was repeated three times. The final dried samples were spotted onto a TLC plate (5716-7, EM Science). Electrophoresis in the first dimension was performed at 1000 V for 30 min in 1% ammonium carbonate containing 0.5 mM EDTA with the HTLE-7000 system (C.B.S. Scientific). The second dimension was developed for 10–12 h with *n*-butanol:pyridine:acetic acid:water (375:250:75:300). For phosphoamino acid analysis, the phosphopeptides hydrolyzed in 6 N HCl were spotted and analyzed by electrophoresis at pH 1.9 in the first dimension (1.5 V for 20 min) and pH 3.5 in the second dimension (1.3 V for 16 min) with the HTLE-7000 system (27).

Flow Cytometry and Fluorescence Microscopy—Cells co-transfected with Plk constructs and GFP vector (pEGFP-F, Clontech) were detached from the plates by trypsinization. After fixation in 80% ice-cold ethanol at 4°C for 16 h, cells were stained in PBS supplemented with 15 μ g/ml propidium iodide and 100 μ g/ml RNase A for 30 min at room temperature. Bivariate measurements of green (GFP) and red (propid-

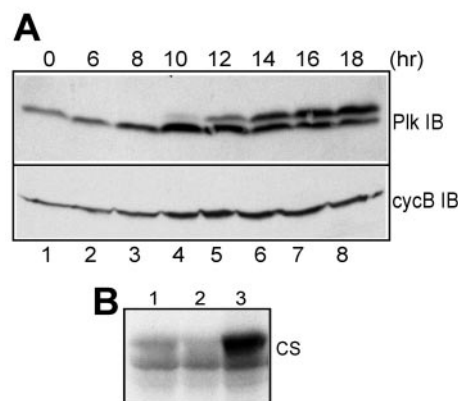


FIG. 1. Plk is phosphorylated and activated in mitotic HeLa cells. **A**, changes in the phosphorylation of Plk protein. After mimosine treatment for 16 h, HeLa cells were released to medium containing nocodazole, and cells were collected at the indicated times. Extracts of HeLa cells collected before release (lane 1) and 6 h (lane 2), 8 h (lane 3), 10 h (lane 4), 12 h (lane 5), 14 h (lane 6), 16 h (lane 7), or 18 h (lane 8) after release were resolved by SDS-PAGE. Proteins were transferred to polyvinylidene difluoride membrane and probed with anti-Plk (upper panel) or anti-cyclin B1 (cycB) monoclonal antibody (lower panel). **IB**, immunoblotting. **B**, catalytic activity of immunocomplexes of Plk from asynchronously growing HeLa cells (lane 1), from cells arrested in G_1 by treatment with mimosine for 16 h (lane 2), and from cells arrested in prometaphase by release into nocodazole-containing medium for 18 h (lane 3). The similar recoveries of Plk in the immunocomplexes were confirmed by Western blotting (data not shown), and the kinase activity was measured with casein (CS) as a substrate as described under "Experimental Procedures."

ium iodide-DNA) fluorescence were made with FACSscanTM (BD Biosciences). A gate was set to select GFP-positive cells with a green fluorescent signal at least 40 times stronger than negative cells. Data were collected for more than 30,000 events with the FL1 signal (GFP) and analyzed using Cell QuestTM (BD Biosciences) and ModFitTM 2.0 (Verity Software House) (28, 29).

For fluorescence microscopy, HeLa cells were seeded onto coverslips and were transfected as described previously. The cells were fixed in 4% paraformaldehyde-PBS followed by treatment with ice-cold methanol. After washing with PBS, the cells were incubated for 2 h in PBS supplemented with propidium iodide (10 μ g/ml). The mounted coverslips were analyzed by fluorescence microscopy, and the visualized cells were captured on the LSM510 imaging system (Zeiss).

RESULTS

Mammalian Plk Is Phosphorylated and Activated during Mitosis—To analyze the modification and activation of Plk during the cell cycle, HeLa cells were synchronized in G_1/S phase with mimosine and transferred into fresh medium with nocodazole to allow entry into M phase. Cells were harvested at various times and analyzed by immunoblotting. Plk from cells arrested in G_1/S phase migrated as a single band (Fig. 1A, lane 1). The slower migrating phosphorylated form of Plk increased as cells proceeded into M phase and was maximal at prometaphase (lane 8). To compare the kinase activity of Plk at various stages, endogenous Plk was immunoprecipitated with anti-Plk antibody from lysates of G_1/S phase and prometaphase cells, and the phosphorylation of casein was assessed. The results shown in Fig. 1B confirm previous studies that Plk activity from mitotic cells is severalfold greater than that from G_1/S cells (17, 19).

xPlkk1 Phosphorylates and Activates Mammalian Plk—Further assessment of Plk phosphorylation and kinase activity was performed in *in vitro* kinase assays. His₆-xPlkk1, which has been characterized as a Plx1-activating kinase in *Xenopus* (22), was purified from insect cells and used to phosphorylate mammalian Plk. GST-tagged kinase-defective Plk (K82M), which lacks autophosphorylation activity (Fig. 2A, lane 1), was phosphorylated by xPlkk1 (lane 3).

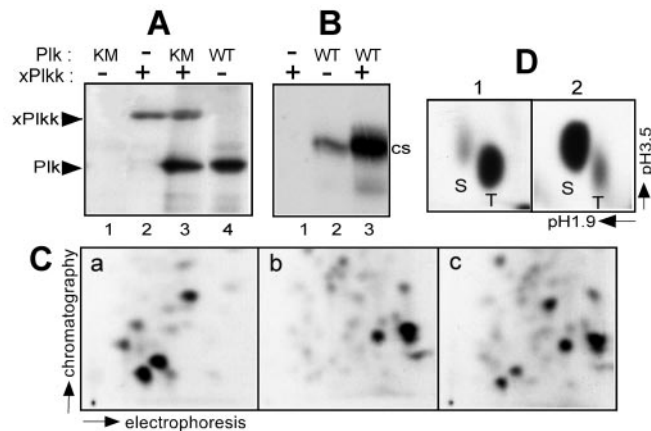


FIG. 2. Mammalian Plk is phosphorylated on threonine and is activated by xPlkk1 *in vitro*. A, phosphorylation of GST-Plk K82M by xPlkk1. Recombinant GST-Plk proteins were phosphorylated by recombinant His₆-xPlkk1 purified from Hi5 cells. Kinase reactions were carried out as described under "Experimental Procedures." Proteins were subjected to SDS-PAGE and visualized by autoradiography. His₆-xPlkk1 and GST-Plk WT were autophosphorylated (lanes 2 and 4) but not GST-Plk K82M (lane 1). GST-Plk K82M was phosphorylated upon incubation with xPlkk1 (lane 3). As shown in B, His₆-xPlkk1 did not phosphorylate casein (lane 1). Casein phosphorylation by GST-Plk WT alone (lane 2) and after incubation with xPlkk1 (lane 3) is shown. C, two-dimensional analyses of chymotryptic phosphopeptides of recombinant Plk. Panel a, GST-Plk K82M phosphorylated by xPlkk1; panel b, auto-phosphorylated GST-Plk WT; panel c, mixture of panels a and b. D, phosphoamino acid analysis of Plk K82M phosphorylated by xPlkk1 (panel 1) and autophosphorylated Plk WT (panel 2).

When GST-Plk wild type (WT) was incubated and phosphorylated by purified xPlkk1 or LOK, catalytic activity was elevated severalfold (Fig. 2B, lane 3). xPlkk1 did not show detectable phosphorylation of casein. These results demonstrate that xPlkk1 can phosphorylate and activate mammalian Plk *in vitro*. Similar results have also been obtained using purified LOK, a mammalian enzyme closely related in sequence to xPlkk1 (data not shown).

Phosphorylation by xPlkk1 and Autophosphorylation Occur on Different Residues in Plk—As shown for xPlkk1, purified wild type Plk has significant autophosphorylation activity (Fig. 2A, lane 4). To investigate whether the phosphorylation by an upstream kinase and autophosphorylation occur on a common residue, phosphopeptide maps of both GST-Plk K82M phosphorylated by xPlkk1 and autophosphorylated GST-Plk WT were compared. Although the chymotryptic digest of phosphorylated K82M yielded 5–6 phosphopeptides (Fig. 2C, panel a), that of autophosphorylated Plk yielded several phosphopeptides that did not migrate with those of phosphorylated K82M (panels b and c). Phosphoamino acid analysis revealed that the site(s) phosphorylated by xPlkk1 is mainly threonine (Fig. 2D, panel 1), whereas the autophosphorylated site(s) occur primarily on serine (panel 2). These data indicate that phosphorylation by an upstream kinase and autophosphorylation in Plk occur on different residues and that phosphorylation on threonine is likely to be important for Plk activation.

Phosphorylation *In Vivo* and *In Vitro* Occurs on the Same Threonine Residue—To characterize Plk phosphorylation during mitosis, HeLa cells were metabolically labeled with [³²P]orthophosphate during treatment with nocodazole as described under "Experimental Procedures." Endogenous Plk was immunoprecipitated from cell lysates, subjected to SDS-PAGE, and transferred to polyvinylidene difluoride membrane, and radiolabel incorporated into Plk was detected by autoradiography (Fig. 3A, lane 1). In parallel, the proteins from the immunocomplex and the cell lysate were monitored by Western blot with anti-Plk antibody (lanes 2 and 3). Approximately one-

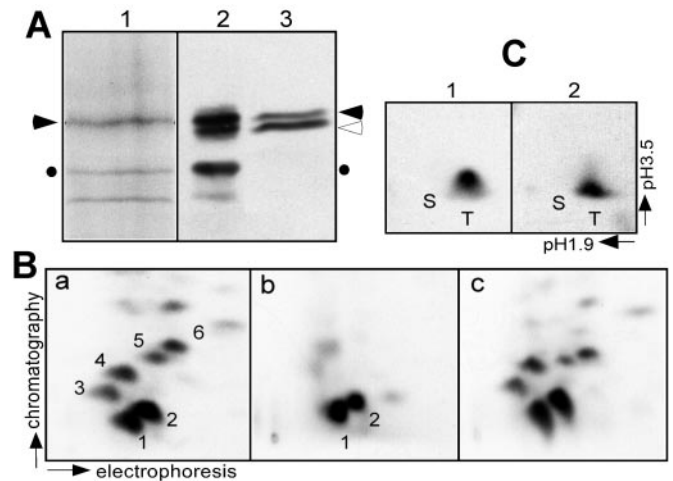


FIG. 3. A common threonine residue is phosphorylated *in vivo* and *in vitro*. A, detection of Plk radiolabeled *in vivo*. HeLa cells were synchronized in M phase by nocodazole treatment for 12 h. After being shaken off, the mitotic HeLa cells were labeled metabolically with [³²P]orthophosphate as described under "Experimental Procedures." The endogenous Plk was immunoprecipitated with anti-Plk antibody and analyzed by SDS-PAGE. The labeled Plk was detected by autoradiography (lane 1) or by Western blot analysis with the same antibody (lane 2, from the immunoprecipitate; lane 3, from the cell lysate). The band with reduced electrophoretic mobility is the mitotic-specific phosphorylated form of Plk (filled arrowhead), and the lower band (open arrowhead) represents unphosphorylated Plk. The filled circle indicates the IgG heavy chain. B, two-dimensional analyses of chymotryptic phosphopeptides of recombinant K82M phosphorylated by xPlkk1 (panel a), of metabolically labeled endogenous Plk (panel b), and of the mixture of panels a and b (panel c). Numbers represent the chymotryptic phosphopeptides. C, phosphoamino acid analyses of *in vitro* phosphorylated Plk (panel 1) and *in vivo* phosphorylated endogenous Plk (panel 2). The major phosphopeptides (spots 1 and 2) were scraped from the cellulose plates and analyzed.

third of endogenous Plk from nocodazole-treated cells displayed retarded electrophoretic mobility. Radiolabel was detected solely in this slower migrating form (filled arrowhead), indicating that this form corresponds to Plk phosphorylated during mitosis in HeLa cells.

To investigate whether *in vivo* and *in vitro* phosphorylation occurs on the same residue, the phosphopeptide map of Plk labeled *in vivo* was compared with that of kinase-defective Plk phosphorylated *in vitro*. The labeled protein from the membranes (Fig. 2A, lane 3 for *in vitro* labeled Plk and Fig. 3A, lane 1 for *in vivo* labeled Plk) was excised and subjected to phosphopeptide mapping. From Plk labeled *in vivo*, two major phosphopeptides were detected; these were in exactly the same positions as those on the phosphopeptide map of Plk phosphorylated *in vitro* (Fig. 3B, panels a–c).

Phosphoamino acid analyses on individual spots (panel a, spots 1–6) showed that the major phosphopeptides (spots 1 and 2) contain phosphothreonine (Fig. 3C, panel 1). The two spots (Fig. 3B, panel b), which are coincident with the major phosphopeptides in panel a, also yielded phosphothreonine (Fig. 3C, panel 2), indicating that same threonine residue(s) may be phosphorylated *in vitro* and *in vivo*. Two other phosphopeptides (spots 4 and 6) also yielded phosphothreonine, whereas the remaining peptides (spots 3 and 5) yielded phosphoserine (data not shown), which is consistent with the phosphoamino acid analysis (Fig. 2D).

Threonine 210 of Plk Is Major Phosphorylation Site—There are several conserved serine and threonine residues in the N-terminal catalytic domain of Plks (20, 30). The replacement of Thr-210 with Asp elevates the kinase activity of Plk produced in Sf9 cells and in budding yeast (20). In *Xenopus*, Thr-201 (corresponding to Thr-210 in mammalian Plk) is re-

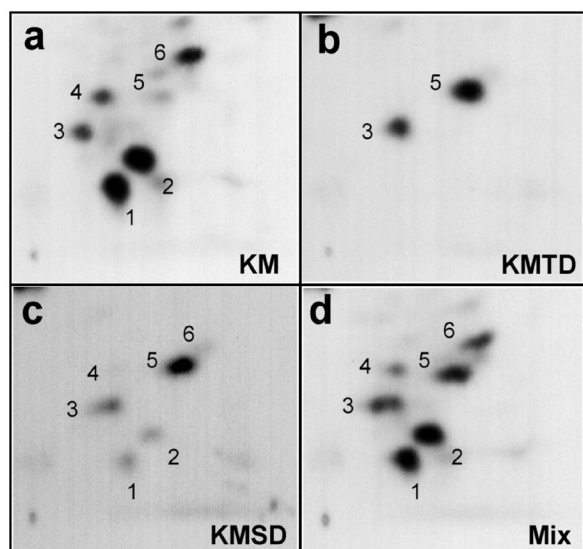


FIG. 4. **Threonine 210 is the major phosphorylation site for the activation of Plk in M phase.** Two-dimensional analyses of chymotryptic phosphopeptides of recombinant Plk phosphorylated by xPlkk1. Panel a, GST-K82M (K_M); panel b, GST-K82M/T210D (KMTD); panel c, GST-K82M/S137D (KMSD); panel d, a mixture of panels a–c. In panel b, spots 1, 2, 4, and 6 were completely absent. These are diminished on the map of KMSD (panel c).

quired for activation of Plx1 (30). These results do not, however, provide direct evidence that Thr-210 is a phosphorylation site in mammalian Plk.

To investigate whether the phosphothreonine residue in Plk labeled *in vitro* and *in vivo* is indeed Thr-210, we generated a mutant in which Thr-210 is substituted with Asp in the kinase-defective background (KMTD). After phosphorylation *in vitro* with xPlkk1, the phosphopeptide map of KMTD was compared with that of the K82M mutant. All threonine-containing peptides, which were detected in the map of K82M mutant (Fig. 4a, spots 1, 2, 4, and 6), were undetectable in the map of the KMTD mutant (Fig. 4b), indicating that xPlkk1 phosphorylates Thr-210. We propose that the four threonine-containing peptides result from partial digestion, which is likely as the recovery of the minor phosphopeptides is not as reproducible as spots 1 and 2.

The chymotryptic map of KMSD, in which Ser-137 was substituted with Asp, yielded the two phosphoserine-containing peptides present in the map of the K_m mutant (Fig. 4c, spots 3 and 5). This indicates that, although the sequences surrounding Ser-137 and Thr-210 are similar, this residue is apparently not phosphorylated *in vivo* or *in vitro* by xPlkk1. The phosphopeptides that contain Thr-210 (spots 1 and 2) were present on the map of the KMSD mutant (Fig. 4, a and c) but at a lower level.

Ectopic Plk Mutants Block Cell Cycle Progression in Mitosis—To investigate whether mutation of Thr-210 or Ser-137 affects the catalytic activity of Plk, we created FLAG-tagged mutants, and the kinase activities of these mutants, expressed in and immunoprecipitated from HeLa cells, were determined. When Thr-210 or Ser-137 was substituted with Asp (TD or SD), Plk displayed increased activity (Fig. 5, A and B, lanes 4 and 6). In contrast, mutation of Thr-210 to Val (TV) abolished its activity (lane 5). Mutation of Ser-137 to Ala (SA) did not change its activity significantly from that of wild type Plk (lane 3). Plk, in which both Ser-137 and Thr-210 were substituted with Asp (SDTD), was not more active than Plk with a TD mutation (lane 8), indicating that substitution of both Ser-137 and Thr-210 with Asp does not have an additive effect on the activity of Plk. This result is in contrast with that found with Plx1, in

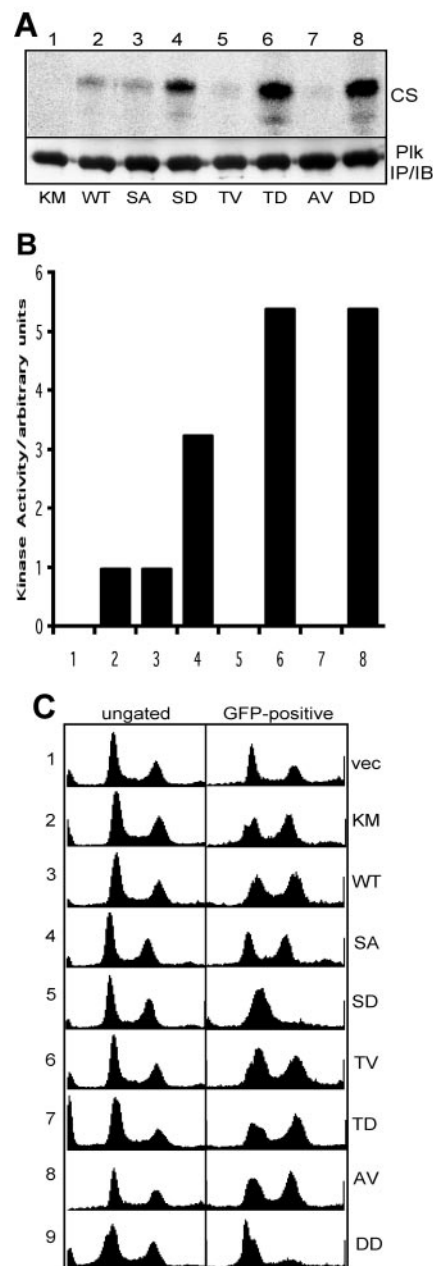


FIG. 5. **Protein kinase activity of the various mutant Plk constructs.** As shown in A, immunoprecipitates from HeLa cells transfected with the various Plk constructs for 40 h were used in *in vitro* kinase assays. Plk activity was measured by phosphorylation of casein (upper panel, CS). The amounts of the immunoprecipitated FLAG-Plk proteins were assessed with anti-FLAG antibody (lower panel, α -FLAG IP, IB). Lane 1, K82M; lane 2, WT; lane 3, S137A; lane 4, S137D; lane 5, T210V; lane 6, T210D; lane 7, S137A/T210V; lane 8, S137D/T210D. B, quantification of casein phosphorylation. Casein phosphorylation was measured as described under "Experimental Procedures." C, DNA profiles. HeLa cells were co-transfected with pEGFP-F and the various Plk constructs and subjected to FACS analysis as described under "Experimental Procedures." Left panels (ungated) and right panels (GFP-positive) represent the DNA profiles from total cells and GFP-positive transfected cells, respectively. Profile 1, DNA profile from HeLa cells with vector (vec); profile 2, with K82M; profile 3, with WT; profile 4, with S137A; profile 5, with S137D; profile 6, with T210V; profile 7, with T210D; profile 8, with S137A/T210V; profile 9, with S137D/T210D.

which the homologous mutations are additive (30).

We transfected the various mutant constructs into HeLa cells for 40 h and analyzed both the DNA content by FACS and the morphological changes by fluorescence microscopy as described under "Experimental Procedures" to evaluate the phe-

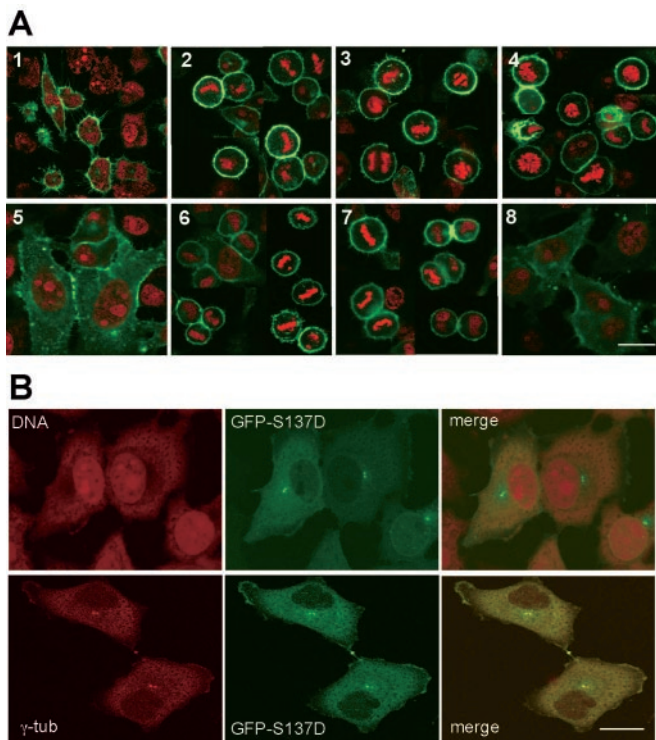


FIG. 6. The effect of Plk mutants on cell cycle progression. HeLa cells were transfected for 40 h as described under "Experimental Procedures." **A**, phenotypes of HeLa transfectants. **Panel 1**, vector alone. HeLa cells transfected with FLAG-K82M, WT, S137A, T210V, or T210D (**panels 2–4, 6, and 7**, respectively). Cells transfected with FLAG-S137D or S137D/T210D arrest in G₁ phase (**panel 5** or **panel 8**). **Bar in 8**: 15 μm. **Red**, DNA. **B**, localization of GFP-S137D and γ -tubulin (γ -tub) in HeLa transfectants. **Bar**, 15 μm. **Upper panel**, red, DNA; green, GFP; **lower panel**, red, tubulin; green, GFP.

notypic changes elicited. The G₂/M population of HeLa cells transfected with wild type and Thr-210 mutant constructs increased slightly (**Fig. 5C, panels 3, 6, and 7**). The cells expressing the kinase-defective mutant showed a similar phenotype as the wild type and Thr-210 mutants, which accumulated in M-phase (**Figs. 5C, panel 2, and 6A, panels 2, 3, 6, and 7**) in agreement with a previous report (19). This suggests that ectopic expression of Plk does not affect the G₂/M transition but does disturb mitotic progression. However, when the S137D mutant, which has catalytic activity similar to that of T210D, was expressed, more than 90% of the transfected cells showed G₁/S DNA content (**Fig. 5, A and B, lanes 4 and 6, and Figs. 5C and 6A, panel 5**). The double mutant, S137D/T210D, resulted in accumulation of a G₁/S population as well (**Figs. 5C, panel 9, and 6A, panel 8**), indicating that the block caused by S137D is not abrogated by T210D. Cells were then transfected with the GFP-S137D fusion construct. GFP-S137D localized to centrosomes (**Fig. 6B**), as shown by staining with anti- γ -tubulin antibody; the GFP and γ -tubulin signals, which colocalize, appeared as two slightly separated dots.

To determine whether Plk T210D accelerates entry into G₂/M, HeLa cells were transfected with vector, wild type, or T210D constructs for protein expression and subsequently blocked at G₁/S 12 h post-transfection with mimosine. After incubation for an additional 20 h, the cells were released from the mimosine block, and the activity of Cdc2-cyclin B was measured in immunocomplexes using histone as a substrate. The timing and degree of Cdc2-cyclin B activity were not changed as the result of Plk expression, and cells expressing each construct appeared to enter G₂/M normally (**Fig. 7**). It should be noted that about 60% of the cells is transfected in this

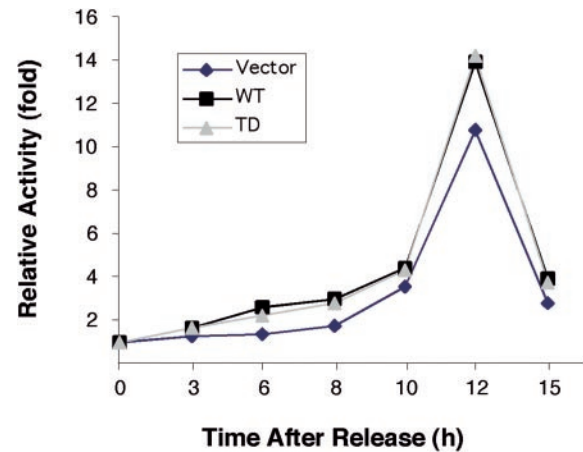


FIG. 7. The effect of wild type or T210D Plk on the activation of Cdc2-cyclin B. HeLa cells transfected with vector control ($\blacklozenge$), HA-Plk ($\blacksquare$), or HA-Plk T210D ($\triangle$) were blocked with mimosine at 12 h post-transfection and further incubated in mimosine to block cells at the G₁/S boundary. After release from the block, cells were collected at the indicated time points, and Cdc2-cyclin B immunocomplexes were assayed for histone H1 phosphorylation.

type of experiment and that Cdc2-cyclin B activity is measured on the entire population. In experiments using *Xenopus* egg extracts, the addition of Plk T201D, which is homologous to Plk T210D, accelerates Cdc2-cyclin B activation by about 30 min as compared with wild type Plx1 or endogenous enzyme (30). The *Xenopus* extracts are very homogenous as compared with transfected cells, and the difficulty in achieving a high degree of synchrony in an animal cell population makes it difficult to detect minor shifts in activation.

DISCUSSION

In this study, we have demonstrated that Thr-210 is the major *in vivo* phosphorylation site of activated mammalian Plk during M phase. Plk labeled with ³²P during M phase shows two major phosphopeptides after chymotryptic cleavage (**Fig. 3B, panel b**), both of which contain threonine, and several minor phosphopeptides. The two major phosphopeptides comigrate with phosphopeptides obtained after *in vitro* phosphorylation of kinase-dead (K82M) Plk with xPlkk1. In contrast, these phosphopeptides are absent after *in vitro* phosphorylation of kinase-defective Plk K82M/T210D double mutant (**Fig. 4, panel b**). Several other phosphopeptides are generated by xPlkk1 under these *in vitro* conditions, but they are undetectable in endogenous Plk after *in vivo* metabolic labeling with radioactive phosphate. We conclude, therefore, that they are not essential for activation of Plk *in vivo*. Thr-210 does not appear to be a major *in vitro* autophosphorylation site; in this case, serine phosphopeptides are detected. However, activated M phase Plk that has been metabolically labeled does not contain major phosphoserine-containing peptides, suggesting that autophosphorylation is not a significant event during activation of Plk during M phase.

Ser-137 in mammalian Plk is preceded by three basic amino acids and followed by a hydrophobic residue (¹³⁴RRRSL), similar to the Thr-210 site (²⁰⁷RKKTL). Conversion of the homologous residue to aspartate in Plx1 activates the enzyme (30), suggesting that it may be a physiologically relevant phosphorylation site. Mutation of Ser-137 to Asp-137 in mammalian Plk also results in increased kinase activity (**Fig. 5, A and B**); however, in contrast to Plx1, Plk with both Ser-137 and Thr-210 converted to aspartate is not further activated (30). Moreover, we show here that Ser-137 is not phosphorylated *in vitro* by xPlkk1 (**Fig. 4, panel c**), nor is it apparently phosphorylated in M phase cells. We have not resolved, however, whether or

not Ser-137 is phosphorylated at another point in the cell cycle prior to M phase. We have also used LOK, the mammalian enzyme closely related xPlkk1, to phosphorylate and activate Plk *in vitro* with essentially the same results shown here for xPlkk1. Our studies to date, however, do not implicate LOK as the upstream activating enzyme of Plk *in vivo*.²

To determine whether modification of Ser-137 has the potential to influence the cell cycle, S137D Plk was expressed in HeLa cells, and its influence on the cell cycle was examined. As shown in Fig. 5B, cells expressing S137D had the novel and unexpected phenotype showing a G₁ DNA content, in contrast to all other Plk mutants, in which expression resulted in a high percentage of cells with a G₂ DNA content. Images of cells expressing Plk S137D show that they remain flat and well attached with condensed DNA in the nucleus. Moreover, GFP-S137D localized to centrosomes, which were separated slightly (Fig. 6B) and were similar to those of cells arrested in late G₁ phase by treatment with mimosine (data not shown). These data suggest that Ser-137 modification prior to M phase may be of physiological significance in the cell cycle and that another polo-like kinase kinase may be active at that point. Because of the lack of suitable synchronized cell populations, it has not yet been possible to determine whether Plk is phosphorylated on Ser-137 at other points in the cell cycle. Ectopic expression of all other Plk constructs, including wild type, appear to result in cells blocked in mitosis or cytokinesis. We believe this is likely to be the result of the capacity of the C terminus of Plk to act as a dominant negative, which causes this phenotype after the deletion of the kinase domain (31, 32). However, if HeLa cells are blocked at G₁/S with mimosine shortly after transfection, expression of Plk1 does not appear to alter the timing of Cdc2-cyclin B activation. This result is consistent with the observation that Cdc2-cyclin B is apparently activated normally in cells depleted of Plk with small interference RNA (siRNA) (33).

This report provides direct evidence that Thr-210 is a site for an activating phosphorylation event *in vitro* and the major phosphorylation site *in vivo* as well. In addition, we show that Ser-137 is not phosphorylated in M phase. The discovery that Plk S137D influences cell cycle progression in a unique fashion suggests new approaches for the study of the polo-like kinase family.

While this manuscript was under review, Kelm *et al.* (34) published studies on the phosphorylation of Plx1. They reported that phosphorylation of Thr-201 is an activating event

in vivo, in agreement with the results presented here. They found, however, that although xPlkk1 does phosphorylate Plx1, this phosphorylation does not activate Plx1 and does not occur on Thr-201. The reasons for these discrepancies are unclear.

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Phosphorylation of Threonine 210 and the Role of Serine 137 in the Regulation of Mammalian Polo-like Kinase

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