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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-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.

**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 mitotic cells or 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.

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ment with mimosine (300 μM) 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 mg/ml nocodazole). Cells were labeled with [32P]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 HEPS-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. After expression in mammalian cells, all constructs 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 pFastBacHTL vector (Pharmingen) and were transfected into SF9 cells with BaculoGold™ (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 μM okadaic acid for 2 h with the use of TALON affinity and 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 EDTA, 25 mM β-glycerophosphate, 25 mM NaF, 0.5 mM Na3VO4, 10 mM p-nitro phenylphosphate, and 1% Nonidet P-40) supplemented with protease inhibitors and centrifuged at 15,000 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– 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 Plkk1 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:biacrylamide) 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 μl of 50 mM ammonium bicarbonate containing 1 mM dithiothreitol and 20 μg of α-chymotrypsin (Sigma) at 37 °C overnight. The supernatant was dried, dissolved in 100 μl of HPLC-grade water, and dried again. This washing step was repeated three times. The final dried spots 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 with n-butanol:pyridine:acetic acid:water (375:250:25:100) at 20 °C for 16 h, then dried. The radiolabeled proteins were electrophoresed at pH 1.9 and 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 HA-Plk or HA-PlkT210D and GFP vector 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 μg/ml propidium iodide and 100 μg/ml RNase A for 30 min at room temperature. Bivariate measurements of green (GFP) and red (propidium iodide-DNA) fluorescence were made with FACScan™ (BD Biosciences) as a substrate as described under Experimental Procedures.)

![Flow Cytometry and Fluorescence Microscopy](image)

**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 G1/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 G1/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 G1/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 G1/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. His-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).
Phosphorylation by xPlkk1 and Autophosphorylation Occur on Different Residues in Plk

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 Plk. Panel a, GST-Plk K82M phosphorylated by xPlkk1; panel b, autophosphorylated 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.

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-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).

Thr210 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-210 (corresponding to Thr-210 in mammalian Plk) is re-

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).
required for activation of Plk1 (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 K82M 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

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.” C, DNA profiles. HeLa cells were co-transfected with pEGFP-F and 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.
should be noted that about 60% of the cells is transfected in this
/H9262
blocked at G1/S 12 h post-transfection with mimosine. After
T210D constructs for protein expression and subsequently
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 6). 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.

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 6). 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.

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 G1/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 G2/M 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 G2/M 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
FLAG-S137D fusion construct. Bar, 15 μm. Upper panel, red, DNA; green, GFP; lower panel, red, tubulin; green, GFP.

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
typic 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 G1/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 G2/M 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 G2/M 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
FLAG-S137D fusion construct. Bar, 15 μm. Upper panel, red, DNA; green, GFP; lower panel, red, tubulin; green, GFP.

FIG. 7. The effect of wild type or T210D Plk on the activation of
Cdc2-cyclin B. HeLa cells transfected with vector control (○), HA-Plk (●), or HA-Plk T210D (□) 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.

tyte 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 Plk1 or endogenous enzyme (30). The Xenopus extracts are very homogeneous 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 vivo 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 (134RRRSRL), similar to the Thr-210 site (207RKKTL). Conversion of the homologous residue to aspartate in Plk1 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 Plk1, 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 vitro.

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 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 S137D is not phosphorylated in M phase. We have also shown that the 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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