Plk1 Activation by Ste20-like Kinase (Slk) Phosphorylation and Polo-Box Phosphopeptide Binding Assayed with the Substrate Translationally Controlled Tumor Protein (TCTP)[†]

Tim M. Johnson,[‡] Robin Antrobus,[§] and Louise N. Johnson*[,‡]

Laboratory of Molecular Biophysics and Glycobiology Institute, Biochemistry Department, University of Oxford, Parks Road, Oxford OX1 3QU, United Kingdom

Received October 23, 2007; Revised Manuscript Received January 9, 2008

ABSTRACT: The mitotic protein kinase Plk1 catalyzes events associated with centrosome maturation, kinetocore function, spindle formation, and cytokinesis and is a target for anticancer drug design. It is composed of a N-terminal kinase domain and a C-terminal polo-box domain (PBD). The PBD domain serves to localize the kinase on cognate phosphorylated substrates, and this binding relieves the inhibition of the kinase by the PBD. Similar to many protein kinases, Plk1 is activated by phosphorylation on a threonine residue, Thr210, in the activation segment. In this work, we describe expression in *Escherichia coli* cells and purification of full-length Plk1 in quantities suitable for structural studies and use this material for quantitative characterization of the activation events with the substrate translationally controlled tumour protein (TCTP). The presence of the PBD-binding phosphopeptide enhances phosphorylation by the activating Ste20-like kinase (Slk). Native Plk1 exhibits a basal catalytic efficiency k_{cat}/K_M of 9.9×10^{-5} s^{-1} μM^{-1}. Association with a polo-box-binding phosphopeptide increased the catalytic efficiency by 11× largely through an increase in k_{cat} with no change in K_M. Phosphorylation by Slk increases catalytic efficiency by 202× with a 2.3-fold reduction in K_M and 88-fold increase in k_{cat}. Phosphorylation and the presence of the PBD-binding phosphopeptide result in an increase in catalytic efficiency of 1515× with a 2.3-fold decrease in K_M and a 705-fold increase in k_{cat} over the unmodified Plk1. Knowledge of kinase regulatory mechanisms and the structures of the Plk1 individual domains has allowed for a model to be proposed for these activatory events.

Plk1 is a major regulator of mitotic progression; it plays a role in centrosome maturation, kinetochore function, spindle formation, chromosome segregation, and cytokinesis (*1–4*). RNAi experiments have suggested that Plk1 is essential for proper spindle assembly but may not be essential for mitotic entry and anaphase promoting complex activation (*5*). Plk1 is a candidate for therapeutic intervention in the treatment of cancer (*6, 7*). The enzyme is highly expressed in proliferating cells and overexpressed in many cancers. Downregulation of Plk1 expression causes reduced proliferation in tumor cell lines but does not affect untransformed cells. A number of promising inhibitors have been reported (*8–13*), and the structure of the Plk1 kinase domain with a pyrrolo-pyrazole inhibitor has been solved (*14*).

Plk1 (MW 68 254, 603 amino acids) and its isoforms Plk2 and Plk3 consist of a kinase domain followed by a C-terminal polo-box domain (PBD).[1] The PBD is critical to Plk1 localization and function and negatively regulates the kinase activity of the catalytic domain (*15, 16*). In a seminal study, Elia et al. (*17*) established that the PBD binds preferentially to a sequence motif Ser-(pSer/pThr)-Pro. This motif, whose phospho form could be generated by the action of other kinases, such as CDK1, is present in several Plk1 substrates, leading to the notion that, during cell-cycle progression, Plk1 uses its PBD to locate either to its appropriate substrate as a priming site for further phosphorylation or to dock Plk1 to an appropriate scaffold, such as the centrosomes or kinetochores, for phosphorylation of targets within these assemblies. It also appears that phosphopeptide binding to the PBD stimulates Plk1 activity (*18*), suggesting a conformational switch in Plk1 regulation and a dual function for the PBD. For other substrates, such as the microtubule-associated protein involved in cytokinesis PCR1 and the mitotic kinesin MKlp2, which are important for localization of Plk1 to the central spindle in anaphase, Plk1 is its own priming kinase, allowing further mechanisms for regulation (*19*). The structure determination of the PBD, both free and complexed to an optimal peptide sequence MQS(pT)PL (using the single-letter amino acid code), explained the origin of the specificity for the phosphopeptide and showed that there are no significant conformational changes in the PBD itself upon binding the phosphopeptide (*18, 20*). More recent structural studies with a Cdc25C PBD binding peptide and

[†] This work has been funded by the Medical Research Council Grant G0400957.

* To whom correspondence should be addressed. Telephone: 44-1865-275365. Fax: 44-1865-285353. E-mail: louise.johnson@bioop.ox.ac.uk.

[‡] Laboratory of Molecular Biophysics.

[§] Glycobiology Institute.

[1] Abbreviations: DFP, diisopropyl fluorophosphate; DLS, dynamic light scattering; FA, formic acid, GEF, guanine exchange factor; MBS, MOPS-buffered saline; PBD, polo-box domain of Plk1; Slk, Ste20-like kinase; TCTP, translationally controlled tumor protein.

10.1021/bi702134c CCC: $40.75
Published on Web 02/26/2008

in studies on the localization of Plk1 to the centrosome have shown that the requirement for a phospho threonine or serine may be dispensed (*21*).

Plk1, similar to many other protein kinases, contains a threonine residue (Thr210) in the activation segment. In CDK2/cyclin A, phosphorylation of the equivalent residue (Thr160) is essential for full activity. Phosphorylation of Thr160 leads to a change in conformation of the activation segment that results in the correct conformation for substrate recognition (*22, 23*). Phosphopeptide mapping showed that phosphorylation of Plk1 by *Xenopus* polo-like kinase kinase (xPlkk1) gave Thr210 as the major phosphorylation site (*24*). Thr210 is also an *in vivo* phosphorylation site, and the phosphomimetic mutation Thr210Asp is activatory, while Thr210Val is inhibitory (*24*). Slk, the human homologue of xPlkk1, has been shown to phosphorylate and activate Plk1 *in vitro* and stimulates Plk1 activity *in vivo* (*25, 26*), but it has not been definitively established that this kinase is the *in vivo* upstream activator of Plk1.

Plk1 has been shown to phosphorylate a number of targets that include BRAC2, Cdc25C, claspin, cyclin B, Chk2, Emi1, Grasp65, MKLp1/2, Myt1, Nlp, NudC, RhoA, ROCK2, TCTP, and Wee1 (*2, 15, 27*). The translationally controlled tumour protein (TCTP) is a microtubule-associated protein implicated in cell growth, cell-cycle progression, and malignant transformation (*28*). Plk1 phosphorylation of TCTP leads to a decrease in the microtubule-stabilizing activity of TCTP and promotes an increase in microtubule dynamics that occurs after metaphase (*29*). Mutation of TCTP phosphorylation sites gives an increase in multinucleate cells and cell death. A central role of TCTP in cell proliferation and survival in a tissue-specific manner in mammals has been demonstrated in a knockout mouse model (*30*). The protein also plays a role in antiapoptosis through interaction with Bcl-xL and Mcl-1 (*31, 32*). The structure determination of *Schizosaccharomyces pombe* TCTP by nuclear magnetic resonance (NMR) revealed an unexpected relationship with guanine nucleotide free chaperones, a family of proteins that bind the GDP/GTP free form of the Ras superfamily (*33*). More recently, a link between TCTP and guanine exchange factors (GEFs) and Plk1 and GEFs has been indicated (*34, 35*).

In this study, we have used TCTP as a model substrate for phosphorylation studies by Plk1 to explore Plk1 regulation by binding of a PBD phosphopeptide and phosphorylation by Slk. We show that Plk1 phosphorylates TCTP at a single site, Ser46. Phosphopeptide binding to the PBD promotes efficient phosphorylation of Plk1 by the Slk kinase domain. Four levels of Plk1 activation are demonstrated, progressing from the basal level with the nonphosphorylated/unbound PBD, nonphosphorylated/bound PBD, phosphorylated/unbound PBD, to the fully active, phosphorylated and PBD-domain-bound form. We outline a procedure for the production of milligram quantities of pure Plk1 expressed in *Escherichia coli* cells.

MATERIALS AND METHODS

The PBD-binding phosphopeptide MQS(pT)PL was synthesized by Dr. G. Bloomberg (University of Bristol).

Expression and Purification of Full-Length Plk1. Plk1 cDNA (a gift from Professor Erich Nigg, Max-Planck-Institute of Biochemistry, Martinsried, Germany) was cloned into pGEX 6p1 expression vector. *E. coli* (BL21 DE3) cells were transformed and grown to an OD_{600} of 0.6 at 37 °C in the presence of 100 μg/mL ampicillin. The cells were cooled to 20 °C, and expression was induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 12 h. Cells were harvested and resuspended in MOPS-buffered saline (MBS) [200 mM NaCl, 40 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS) at pH 7.4, 0.01% monothioglycerol, 1 mM ethylenediaminetetraacetic acid (EDTA), and 0.01% sodium azide]. Plk1 was found to be stable and monodisperse [as indicated by dynamic light scattering (DLS) in MOPS buffer, but in other buffers, such as *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) at pH 7.5, phosphate at pH 7.0, and 2-(*N*-morpholino)ethanesulfonic acid (MES) at pH 6.2, peak broadening was observed, indicative of oligomerization or aggregation]. Resuspended cell pellets were flash-frozen and stored at −80 °C prior to use. Cells pellets were lysed by homogenization in the presence of 100 μM DFP, 5 mM benzamidine, and one protease inhibitor cocktail tablet (Sigma) per 1 L of cell culture equivalent. The whole cell lysate was clarified by centrifugation at 100000*g* for 1 h. A glutathione-sepharose column was pre-equilibrated in MBS, and the supernatant from the centrifugation of solubilized cell pellets was loaded. The column was washed with 500 mL of MBS containing 1.0 M NaCl in the presence of 100 μM DFP and 5 mM benzamidine to remove any contaminants and degradation products. The GST−Plk1 fusion protein was then eluted by application of 5 column volumes of MBS containing 20 mM glutathione. GST was cleaved from Plk1 using precision protease in a molar ratio of 1:50 at 4 °C overnight. The cleaved protein was applied to a preparative S200 gel-filtration column pre-equilibrated in MBS supplemented with 5 mM benzamidine and 100 μM DFP. A total of 1 column volume of MBS with inhibitors 100 μM DFP and 5 mM benzamidine was run, and the elution peak was collected. Full-length Plk1 elutes prior to the GST dimer, but there is a substantial overlap of the peaks. GST was removed with an additional glutathione-sepharose column, and Plk1 was further purified on a S200 gel-filtration column. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) analysis of the 4 mL fractions showed a single band of 70 kDa, corresponding to full-length Plk1. Protein-degradation products, which have proven problematic in the past, appeared to have been largely eliminated by the use of DFP, which is a more potent protease inhibitor than PMSF. Typically, 8–10 mg of pure protein were obtained from 4 L of cell culture. The full-length Plk1 contains eight additional residues at the N terminus (GPLGSPEF) from the 3C protease cleavage of the pGEX vector product and nine additional residues (LLEKPPHRD) at the C terminus as a result of cloning. In addition, sequencing showed that there was an insert of a glycine residue after Arg356. Because this occurs in the linker region between the kinase and PBD domains, it is not expected to influence function.

DLS measurements were made with a Viscotek 802 DLS instrument at 20 °C taking the average measurement of 10 consecutive 10 s scattering experiments.

Expression and Purification of TCTP and Slk. Human TCTP was expressed as a GST fusion protein from the pGEX 6p2-TCTP plasmid (a gift from Christophe Cans, Molecular Engines Ltd., France). A single colony was used to inoculate

4 L of Luria Broth (LB) and grown to an OD_{600} of 0.6 in the presence of 100 μg/mL ampicillin. The cells were pelleted by centrifugation and resuspended in MBS containing 1 protease inhibitor cocktail tablet per 1 L of culture. Cells were flash-frozen and lysed when required by homogenization. The lysate was clarified by centrifugation at 100000*g* for 1 h, and the lysate was applied to a 4 mL glutathione sepharose column pre-equilibrated in MBS and 5 mM benzamidine. After washing with 5 column volumes of MBS, the protein was eluted with 20 mM glutathione in MBS. The GST tag was cleaved by incubation with a 1:50 molar ratio of precision protease (3C protease) to GST−TCTP overnight at 4 °C, and the products were further purified by gel filtration.

Slk domain (residues 19–320) was expressed from the vector pNIC28-Bsa4 (a gift from Stefan Knapp, Structural Genomics Consortium, Oxford, U.K.) as a histidine-tagged protein in BL21 Rosetta cells. A culture of 4 L was grown at 37 °C to an OD_{600} of 0.6. The cells were cooled to 20 °C, and expression was induced with 1 mM IPTG for 4 h. Cells were pelleted by centrifugation and resuspended in binding buffer (30 mM MOPS at pH 7.4, 300 mM NaCl, 20 mM imidazole, and 0.01% MTG). The cells were lysed by homogenization, and the resulting lysate was clarified by centrifugation at 100000*g*. The lysate was applied to a Ni−NTA column, and the column was washed with 50 mL of wash buffer (30 mM MOPS at pH 7.4, 1 M NaCl, 20 mM imidazole, and 0.1% MTG). The column was then washed with 50 mL of binding buffer (defined above), and the protein was eluted with 50 mL of elution buffer (30 mM MOPS at pH 7.4, 300 mM NaCl, and 150 mM imidazole). The eluted protein was concentrated to 10 mL and applied to a S200 gel-filtration column pre-equilibrated with 20 mM MOPS at pH 7.4, 250 mM NaCl, and 0.01% MTG. The resulting elution peak was pooled and concentrated as required for phosphorylation of Plk1.

Slk-Mediated Activation of Plk1. Assays were carried out using p81 paper chromatography to detect phosphorylated proteins (*36*). Full-length Plk1 (3 μM) was prephosphorylated with Slk1 (1 μM) in the presence of 0.5 mM unlabeled ATP and 10 mM $MgCl_2$. The reaction was started by the addition of ATP, and aliquots were taken over a time course from 5 to 60 min at 30 °C. These aliquots were then added to reaction tubes containing 0.5 mM γ^{32}P-labeled ATP (specific activity of 50 cpm/pmol), 10 mM $MgCl_2$, and 100 μM TCTP with or without 5 mM PBD-binding phosphopeptide. After 10 min, aliquots were spotted onto p81 paper squares and the reaction was stopped by the addition of 0.1% phosphoric acid. The papers were washed 5 times in 0.1% phosphoric acid to remove free ATP and then dried for Cerenkov counting. The amount of phosphate incorporated into the substrate was determined from the counts obtained with reference to the specific radioactivity of the ATP.

To determine if the presence of the PBD-binding phosphopeptide had an effect on the ability of Slk to phosphorylate Plk1, phosphorylation time-course reactions were carried out containing 2.3 μM Plk1 and either 4.3 or 8.6 μM Slk, 0.5 mM γ^{32}P-labeled ATP (specific activity of 100 cpm/pmol), and 10 mM MgCl, in the presence or absence of 5 mM PBD-binding phosphopeptide at 30 °C. Control reactions were also carried out with only Plk1 or only Slk, where Slk or Plk1 were replaced with MBS, respectively. After starting the reaction by the addition of the Plk1/Slk mixture, aliquots were taken at 5, 15, 30, and 45 min and run by SDS−PAGE. The resulting gels were dried and subjected to overnight autoradiography for visualization of the levels of phosphorylation.

Test of the Ability of Slk to Phosphorylate TCTP. Because Slk is present in the Plk1 TCTP phosphorylation reactions above as well as the kinetic experiments outlined below, it was necessary to determine if it was able to phosphorylate TCTP. Prephosphorylation of Plk1 (7.5 μM) with Slk (60 μM) was carried out in the presence of 0.5 mM ATP, 10 mM $MgCl_2$, and 4 mM PBD-binding peptide. Aliquots were taken over a time course of 5–60 min and assayed against 100 μM TCTP in the presence of 0.5 mM γ^{32}P-labeled ATP (specific activity of 50 cpm/pmol) and 10 mM $MgCl_2$. The same procedure was carried out in a control reaction, in which Plk1 was substituted by MBS. These reactions were then run on a 12.5% SDS−PAGE gel, which was then dried and subjected to autoradiography.

Kinetic Analysis of Plk1. Plk1 (4.2 μM) was activated by phosphorylation with the Slk kinase domain (40 μM) both in the absence and presence of 4 mM PBD-binding phosphopeptide at 30 °C for 30 min in 50 mM MOPS at pH 7.4, 200 mM NaCl, 0.01% monothioglycerol, 0.5 mM ATP, and 10 mM $MgCl_2$. Slk was added in a high molar excess to ensure rapid, complete phosphorylation. The phosphorylated Plk1 was diluted to 10 nM and assayed against substrate TCTP (concentrations from 6.25 to 400 μM) in the presence of 0.5 mM γ^{32}P-labeled ATP (specific radioactivity of 100 CPM/pmol). The final concentration of Slk in the Plk1 kinase reactions against TCTP was 95 nM. Slk-mediated phosphorylation of Plk1 appears to be stopped at the diluted concentration of enzyme used in the assay as indicated by the lack of incorporation of radioactively labeled phosphate into Plk1 during the assay. The reactions were started by the addition of the diluted enzyme to the ATP/Mg/TCTP substrate mix and allowed to proceed for 5 min at 30 °C.

In the kinase assays, the PBD-binding phosphopeptide was diluted 420-fold from the prephosphorylation reaction to the final kinase reaction, giving a final concentration of 6.0 μM. At least 0.5–1.0 mM of phosphopeptide is required to facilitate accelerated phosphorylation of Plk1 by Slk. To determine if there is an additive effect on the activity of phosphorylated Plk1 by the presence of high phosphopeptide concentrations in the assay, similar reactions were carried out but with phosphopeptide added to the final reaction tubes at higher concentrations (2.5 mM). Similar experiments were carried out with nonphosphorylated Plk1 in the absence and presence of high peptide concentrations. Concentrations of enzyme used in the kinase reactions were 500, 250, 10, and 10 nM in the nonphosphorylated/6 μM phosphopeptide, the nonphosphorylated/2.5 mM phosphopeptide, the phosphorylated/6 μM phosphopeptide, and the phosphorylated/2.5 mM phosphopeptide reactions, respectively. The incubation time for the nonphosphorylated/zero phosphopeptide assay was increased from 5.0 to 7.0 min.

Phosphorylated TCTP substrate was purified from free ATP by p81 paper chromatography (*12*). Briefly, the papers were washed 5 times in 1% phosphoric acid and air-dried, and the incorporated radioactivity was measured by Cerenkov counting. Kinetic constants were calculated by nonlinear regression analysis.

Determination of the Optimal PBD-Binding Phosphopeptide Concentration for Slk-Mediated Phosphorylation of Plk1. Plk1 (10 μM) was incubated with Slk (80 μM) in the presence of 0.5 mM γ^{32}P ATP (100 cpm/pmol), 10 mM $MgCl_2$, and PBD-binding phosphopeptide (concentrations of 0, 0.1, 0.5, 1.0, 2.0, and 4.0 mM). A total of 5 μL samples were taken from 20 μL total volumes at each peptide concentration at 0, 20, 40, and 60 min, and the reaction was stopped by adding 2× SDS sample buffer. Samples were then run by SDS−PAGE, and the gel was dried and subjected to autoradiography for visualization of phosphorylated bands.

Mass Spectrometry of Phosphorylated TCTP and Plk1. Plk1 (5 μM) in MBS was phosphorylated by Slk (70 μM) also in MBS, in the presence of 1.0 mM ATP, 10 mM $MgCl_2$, and 4 mM PBD-binding peptide at 30 °C for 30 min. The reaction mix was diluted $^1/_{20}$, added to the TCTP reaction mix (21.0 mM ATP, 10.0 mM $MgCl_2$, and 100 μM TCTP) with a final Plk1 concentration of 62.5 nM, and incubated at 30 °C for 45 min. The reaction was put on ice, and the buffer was exchanged into low salt buffer (20 mM NaCl and 10 mM MOPS at pH 7.4).

TCTP was dephosphorylated using λ-protein phosphatase (New England Biolabs) at 30 °C o/n. Phosphorylated and dephosphorylated TCTP were each dialysed into 0.1% formic acid (FA) and diluted in 50:50 MeCN/0.1% FA to a concentration of 10 pM/μL for MS data acquisition. Intact mass spectra were obtained using a QTof1 (Micromass, Manchester, U.K.), fitted with a nanoESI source and operated using MassLynx version 4.0. Capillary voltage was maintained at 3300 V, and cone voltage was maintained at 40 V. Raw spectra were combined and processed using MaxEnt1.

To identify phosphorylation sites, TCTP was dialysed into either 25 mM NH_4HCO_3 for tryptic digestion or 50 mM Tris-HCl at pH 4.0 for Glu-C digestion. Samples were diluted to 10 pM/μL and digested overnight at 37 °C. A total of 5 μL of each were dried down and resuspended in 6.5 μL of 0.1% FA for LC−MSMS acquisition using a Q-TOF 1 coupled to a CapLC (Waters, Milford, MA). Peptides were concentrated and desalted on a 300 μm i.d./5 mm C18 precolumn and resolved on a 75 μm i.d./25 cm C18 PepMap analytical column (LC packings, SanFrancisco, CA) using a 45 min 5–95% MeCN gradient containing 0.1% FA at a flow rate of 200 nL/min. Spectra were acquired in positive mode, and MS to MS/MS switching was controlled in an automatic data-dependent fashion with a 1 s survey scan followed by three 1 s MS/MS scans of the most intense ions. Precursor ions selected for MS/MS were excluded from further fragmentation for 2 min. Spectra were processed using ProteinLynx Global server 2.1.5 and searched against the SWISS-PROT database (release 53.2) using the MASCOT search engine (Matrix science, London, U.K.). Searches were restricted to the human taxonomy allowing carbamidomethyl cysteine as a fixed modification, with oxidized methionine and phosphoSTY as potential variable modifications. Data were searched allowing 0.5 Da error on all spectra and up to three missed cleavages to accommodate calibration drift and incomplete digestion. All data were checked for consistent error distribution.

To identify phosphorylation sites in Plk1 phosphorylated by Slk, GST−Plk1 fusion (5 μM) in MBS was phosphorylated by Slk (70 μM) also in MBS, in the presence of 1.0 mM ATP, 10 mM $MgCl_2$, and 4 mM PBD-binding peptide at 30 °C for 30 min. The mixture was applied to a gluthathione-sepharose column. The column was washed in MBS to remove Slk, and the phosphorylated-Plk1 fusion was eluted with 20 mM glutathione and applied to a gel-filtration S200 column in MBS, 20 mM MOPS at pH 7.5, and 100 mM NaCl. The Plk1 fusion was cleaved with GST−3C protease and reapplied to the glutathione-sepharose column to remove 3C protease and GST. Reduced and alkylated Plk1 was dialysed into either 25 mM NH_4HCO_3 for tryptic, Arg-C, and Glu-C digestion or 50 mM Tris-HCl at pH 4.0 for Glu-C digestion. Samples were diluted to 10 pM/μL and digested for 3 h or overnight at 37 °C. A total of 5 μL of each were dried down and resuspended in 6.5 μL of 0.1% FA for LC−MSMS acquisition using a Q-TOF 1 coupled to a CapLC (Waters, Milford, MA) as detailed above. Because of increased sample complexity, all digests were analyzed using a 45 and 300 min 5–95% MeCN gradient. Spectra were processed using ProteinLynx Global server 2.1.5 and searched against the SWISS-PROT database (release 54.6x) using the MASCOT search engine (Matrix Science, London, U.K.). Searches were restricted and evaluated as described above.

RESULTS

Full-Length Plk1 Expression in E. coli Cells and Purification. Full-length Plk1 expressed from *E. coli* cells and purified was homogeneous and free of major degradation products and contaminants (Figure 1A). Plk1 gave a symmetrical peak on gel filtration (Figure 1B) and was monodisperse at low concentrations (1 mg/mL) with an approximate hydrodynamic radius of 4.1 nm in DLS experiments (Figure 1C). This radius indicated a molecular mass of 100 kDa for a spherical particle. This is a reasonable approximation for Plk1 (MW 70 114, including the extra eight N-terminal and nine C-terminal residues). Plk1 is a two-domain protein, and its shape is likely to deviate from the spherical model used in the DLS calculation. The choice of buffer and the use of 200 mM salt concentration contributed to maintaining a monodisperse sample. Peak broadening at concentrations above 2–3 mg/mL indicated the possibility of some oligomerization or self-association.

Activation of Plk1 by Slk Is Enhanced by PBD-Binding Phosphopeptide. Slk has been shown to phosphorylate and activate Plk1 (*25, 26*) but the effects of the PBD binding to a phosphopeptide on Slk activation have not been reported. We found that in the absence of PBD-binding phosphopeptide there was an increase in the activity of Plk1 against its substrate TCTP with time of incubation with Slk (lower curve in Figure 2A). In the presence of 5 mM PBD-binding phosphopeptide, there was a significant further increase in activity (upper curve in Figure 2A). After 60 min of incubation, the activity was approximately 6-fold higher in the presence of PBD-binding phosphopeptide than that in the absence of phosphopeptide. We conclude that binding of the PBD-binding phosphopeptide enhances the effects of Slk on Plk1.

Phosphorylation time courses of Plk1 (2.3 μM) in the presence and absence of 5 mM PBD-binding phosphopeptide at two Slk concentrations (4.3 and 8.6 μM) are shown in Figure 2B. The presence of PBD-binding phosphopeptide increased the phosphorylation of Plk1 by Slk (two left panels

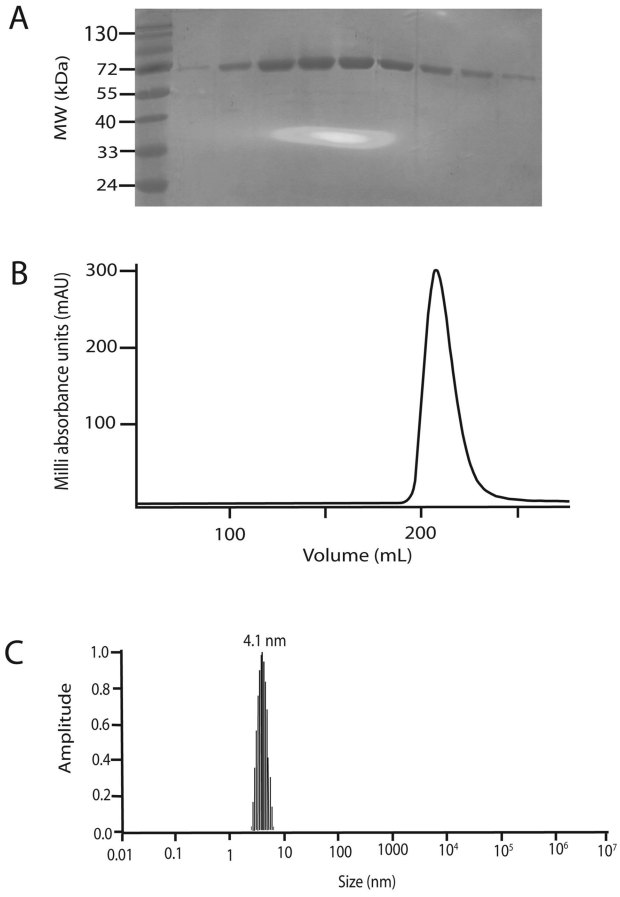

FIGURE 1: Purification and characterization of full-length Plk1. (A) SDS−PAGE analysis after the final S200 gel-filtration column. (B) Peak from preparative S200 gel-filtration column. (C) Results from DLS showing a monodisperse peak with hydrodynamic radius of 4.1 nm.

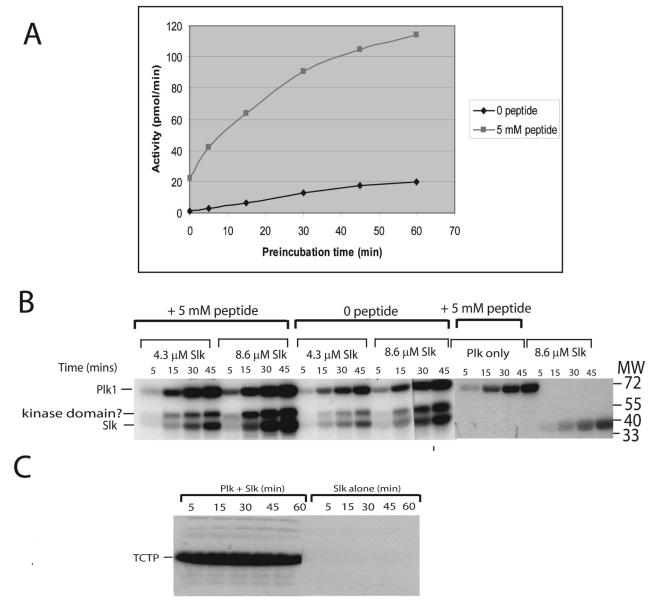

FIGURE 2: Phosphorylation activities of Plk1. (A) Activity of Plk1 against substrate TCTP (100 μM) in the presence (upper curve) and absence (lower curve) of 5 mM PBD-binding phosphopeptide as a function of time of Slk (1 μM) phosphorylation of Plk1 (3 μM). (B) Time course SDS−PAGE autoradiographs showing phosphorylation of Plk1 (2.3 μM) by Slk (4.3 or 8.6 μM) in the presence (left panel) and absence (center panel) of 5 mM PBD-binding phosphopeptide. The right panel shows phosphorylation in the absence of TCTP by Plk1 only and Slk only. (C) SLK does not phosphorylate TCTP. The left panel shows phosphorylation of TCTP by Plk1 activated by Slk. The right panel shows TCTP incubated with Slk alone (see the Materials and Methods).

compared to the two center panels in Figure 2B). The maximal efficiency of PBD-binding phosphopeptide on Slk-mediated phosphorylation of Plk1 occurred at peptide concentrations of 0.5−1.0 mM peptide (results not shown).

In addition to the Plk1 band at 71 kDa, there was a third strongly phosphorylated band at about 40 kDa. This band is likely to correspond to a small amount of degradation product of Plk1 and runs at a similar MW to the kinase domain of Plk1. We judged that the concentration of the 40 kDa protein was significantly lower than that of full-length Plk1, because the band is not visible on a Coomassie-stained SDS gel (Figure 1A). Slk was also phosphorylated under these conditions with increased phosphorylation in the presence of PBD phosphopeptide (Figure 2B).

Plk1 incubated on its own in the presence PBD-binding phosphopeptide was able to autophosphorylate (right panel in Figure 2B). The absolute requirement for the presence of PBD-binding phosphopeptide for this to occur (results not shown) supports the notion that occupation of the PBD-binding site by phosphopeptide results in a more active enzyme that is accessible to substrate, in this case itself. In the absence of Slk, there was no apparent phosphorylation of the additional 40 kDa band. This may indicate that autophosphorylation occurred at a different site to the site of phosphorylation by Slk or was too low to detect. Slk was also able to autophosphorylate (far right panel in Figure 2B). The intensities of the Slk radioactive bands (far right panel in Figure 2B) were slightly less than those seen in the presence of Plk1 (second panel from left in Figure 2B), suggesting that Plk1 was able to phosphorylate Slk.

The size of the fragment (approximately 40 kDa) that was strongly phosphorylated in Figure 2B is likely to correspond to the Plk1 kinase domain produced by trace amounts of protease activity during purification. The high activity of Slk for this fragment suggests that removal of the PBD from the kinase domain renders the Plk1 kinase domain a much better substrate for Slk than the full-length enzyme. This would be consistent with the notion that binding of the PBD-binding phosphopeptide to the PBD causes a conformational change that creates access to the site of phosphorylation by Slk and thereby an increase in the activity of Slk on Plk1.

Slk Does Not Phosphorylate TCTP. Because low concentrations of Slk (95 nM) were present in the Plk1 assay against TCTP, it was important to establish if Slk phosphorylated TCTP. As shown in Figure 2C in assays in which Plk1 was replaced with MBS buffer, there was no phosphorylation of TCTP by Slk. In the presence of Plk1, there is marked phosphorylation of the TCTP over the time course of activation of Plk1 by Slk.

Determination of the Site of Phosphorylation of TCTP by Mass Spectrometry. Mass spectrometry analysis of the Plk1-phosphorylated TCTP gave a molecular mass of 20 460.6. Treatment of the phosphorylated TCTP with phosphatase resulted in a protein with a molecular mass of 20 380.5. The calculated molecular mass of TCTP from the amino acid sequence deduced from the DNA sequence is 20 380.2. The phosphatase-sensitive mass shift (−80 Da) indicates that TCTP contains a single site of phosphorylation. LC−MSMS

Table 1: Kinetic Constants for Plk1 Activity against the Substrate TCTP

	K_M (μM)	k_{cat} (s^{-1})	relative turnover	catalytic efficiency k_{cat}/K_M (s^{-1} μM^{-1})	relative catalytic efficiency
Plk1	172 ± 9	0.017 ± 0.001	1	9.9×10^{-5}	1
Plk1 plus phosphopeptide	182 ± 18	0.23 ± 0.02	14	1.1×10^{-3}	11
phosphorylated Plk1	76 ± 10	1.5 ± 0.3	88	0.02	202
phosphorylated Plk1 plus phosphopeptide	79 ± 9	12 ± 1	706	0.15	1515

and database searching identified TCTP from both tryptic (MOWSE score of 449, sequence coverage of 67%) and Glu-C (MOWSE score of 269, sequence coverage of 51%) digested samples. Glu-C digestion of TCTP generated three peptides (residues 33–60, 41–60, and 41–63), with masses consistent with single-site phosphorylation and MSMS consistent with phosphorylation at Ser46 in all three peptides. Tryptic digestion generated one peptide (residues 39–85), with a mass consistent with monophosphorylation and the MSMS supporting phosphorylation at Ser46. Because all MSMS data identify Ser46, which has a sequence consistent with the epitope for Plk1 phosphorylation (NIDDS[46]LI), we conclude that the site of TCTP phosphorylation by Plk1 is Ser46. Knowledge that phosphorylation under our conditions took place at a single site simplified the kinetic analysis.

Plk1 Sites Phosphorylated by Slk. Mass spectrometry of all digests of Slk-phosphorylated Plk1 identified the major protein component as Plk1 (Plk1_human). Combining the peptides from the tryptic, Arg-C, and Glu-C digests generated total sequence coverage of 86%. All identified sites of phosphorylation were evaluated for MSMS ion intensity, signal/noise, and frequency of occurrence. A total of eight potential sites of phosphorylation were identified within Plk1, fulfilling these criteria. While care must be taken inferring the relative abundance of phosphorylation sites from ion intensities, it was clear from the LC−MSMS data that the phosphorylation site most abundantly represented across the spectrum of digests was Thr210. Phosphorylated residues between S330 and S335 were also well-represented, with ions containing phosphorylated Ser330 more common than either Ser331 or Ser335. Less well-represented but still of significance were phosphorylated residues Thr259, Thr317, Thr320, and Thr341.

Kinetic Analysis of Plk1. The results described above show that both phosphorylation by Slk and binding of PBD-binding phosphopeptide enhance the activity of Plk1. They also show that the activatory phosphorylation event is made more efficient by having the Plk1 PBD engaged with its optimal PBD-binding phosphopeptide. To quantify these effects, we carried out a kinetic comparison between the various activation states of Plk1 (i.e., the nonphosphorylated/nonbound PBD, the nonphosphorylated/bound PBD, the phosphorylated/nonbound PBD, and the phosphorylated/bound PBD). As shown in Table 1, the nonphosphorylated/non-PBD-bound full-length Plk1 gave a relatively low basal activity with k_{cat} of 0.017 s^{-1} and a high K_M of 172 μM. The addition of 2.5 mM PBD-binding phosphopeptide to the reaction resulted in a similar K_M of 182 μM, but k_{cat} was increased 14-fold to 0.23 s^{-1} over the basal activity level. Phosphorylated Plk1 resulted in approximately a 2.4-fold drop in K_M to 76 μM and a further substantial increase in k_{cat} to 1.5 s^{-1}, compared to the nonphosphorylated form in the presence of high peptide (k_{cat} of 0.23 s^{-1}). Finally, phosphorylated Plk1 in the presence of 2.5 mM PBD-binding phosphopeptide throughout the preactivation reaction and kinase assay resulted in a similar K_M to the phosphorylated Plk1 of 79 μM but a marked increase in k_{cat} to 12 s^{-1}. The turnover rate as indicated by k_{cat} increased 700-fold, and catalytic efficiency (k_{cat}/K_M) increased 1500-fold upon going from the nonphosphorylated/non-PBD-bound form to the phosphorylated/PBD-bound form. These results indicate that PBD-binding peptide at high concentration increases the enzymatic activity, possibly through the relief of steric hindrance to the catalytic site by the PBD. However, there is no change in K_M between the non-PBD bound or PBD-bound forms in either the non-phospho or phospho states of Plk1 (Table 1), indicating that binding of the PBD-binding phosphopeptide does not substantially affect the substrate-recognition site. The decrease in K_M upon phosphorylation indicates a likely increase in affinity for the substrate that could be achieved by reconfiguration of the activation segment.

DISCUSSION

The first step for structural studies by X-ray crystallography requires the production of milligram quantities of pure, homogeneous material. Previously, in our hands, this has proven problematic for PLk1, because expression levels were low in insect cells and the protein was prone to degradation. In this work, we have expressed and purified homogeneous Plk1 in milligram quantities from *E. coli* cells. Key steps involved the maintenance of protease inhibitors, especially DFP, during purification, and attention to buffer solutions (preferred buffer: 40 mM MOPS and 200 mM NaCl at pH 7.4) to achieve a monodisperse protein as assessed by DLS. Attempts to crystallize using a number of protein crystallization screens with crystallization robots have not been successful. More work is required that may use different deletion constructs and/or the introduction of surface mutations to reduce surface residue charge and/or entropy. Previous work has shown that Slk can activate Plk1 (*25, 26*) and that phosphorylation of Thr210 is activatory (*24*). In this work, we confirm by mass spectrometry that Thr210 is the major site of phosphorylation by Slk and that Ser330 is also phosphorylated, together with other residues (Thr259, Thr317, Thr320, Ser331, Ser335, and Thr341) at lower frequency. Thr210 is in the activation segment at a classical position for activation as determined from other protein kinase structures. The crystal structure of the Plk1 kinase domain defined the fold up to residue 328 (*14*). There is no structural information for the linker region (residues 329–370) between the kinase domain and the PBD. Ser330 lies just at the start of the linker where the polypeptide chain is directed into the solvent. Residues Thr317 and Thr320 lie in a helical turn on this external C-terminal region of the kinase domain. None of these sites are close to the catalytic site, and their phosphorylation seems unlikely to affect the conformation

of the kinase domain or its activity. Thr259, which is phosphorylated at low frequency, is in a partly buried water-filled pocket but also distant from the catalytic site.

Plk1 activity after phosphorylation is significantly enhanced when the PBD-binding phosphopeptide is included at high concentrations (Figure 2A), and the presence of the PBD-binding phosphopeptide also increases the rate of Slk phosphorylation of Plk1 (Figure 2B), with maximal efficiency occurring at concentrations of 0.5−1.0 mM PBD-binding phosphopeptide. Slk is also able to autophosphorylate (Figure 2B), and phosphorylation of Slk is observed in the presence of phosphopeptide and Plk1. We cannot exclude the possibility that autophosphorylation and/or phosphorylation of Slk by Plk1 are able to enhance the activity of Slk and lead to the apparent increase in activity of Slk on Plk1 as a function of time. The crystal structures show that Slk expressed in *E. coli* cells is phosphorylated on Thr183 (about 70%) and in some preparations on Ser189 (by about 30%) (PDB codes 2JFL and 2J51). Residues Thr183 and Ser189 are in the activation segment of Slk. It is not known which, if any, of these phosphorylations are activatory for Slk recognition of Plk1, but from sequence alignment, Ser189 is the predicted site for activation. In the crystal structure, Ser189 points toward the solvent. In the crystal dimeric form of Slk, there is a strand exchange of activation segments, so that the activation segment of one Slk kinase enters the catalytic site of the other Slk kinase in the dimer assembly (*37*). Thr183 is involved in a network of interactions that stabilize this structure. Thr183 is close to the catalytic site, so that it could be an autophosphorylation site. The autophosphorylation sites of Slk, seen in the right panel of Figure 2B, are likely to be either or both of these two sites. Their phosphorylation could result in increased activity of Slk observed in Figure 2A in the absence of phosphopeptide, but this was not tested directly. However, the enhancement of activity seen in the presence of the PBD-binding phosphopeptide is most likely to occur by the effects of the phosphopeptide on Plk1, leading to increased accessibility of the Plk1 Thr210 for Slk and subsequent Plk1 activation rather than by effects on Slk itself, because there is no evidence that the kinase domain of Slk binds or is regulated by this specific phosphopeptide. However, these mutual autophosphorylation and phosphorylation activities were not tested experimentally, and the precise activation mechanisms remain to be elucidated. Slk does not phosphorylate TCTP, and hence, the increased activity on TCTP comes solely from Plk1.

Preliminary experiments on optimization of the peptide concentration indicated that a minimal concentration of about 0.5 mM was required but higher concentrations (5 mM) of PBD-binding phosphopeptide were used to ensure that the effects were driven to completion. These concentrations are larger than the K_d for phosphopeptide binding to the free PBD (0.280 μM) obtained by isothermal calorimetry (*18*) or the phosphopeptide binding to free PBD (2.3 μM) obtained by tryptophan fluorescence measurements (*38*), suggesting that the association of the PBD with the kinase domain reduces the affinity of the PBD for the phosphopeptide. The inference that the PBD associates with the kinase domain is supported by the observation that a GST−kinase domain fusion product is able to bring down free PBD (*38*). While the concentrations of the PBD-binding phosphopeptide used in these experiments are likely to be nonphysiological, *in vivo* lower concentrations might be effective if the major role of the PBD binding is to locate the enzyme in the vicinity of the substrate. Our results indicate that the major effects of activation come from phosphorylation of the kinase domain of Plk1 (202-fold increase in k_{cat}/K_M) compared to PBD-binding phosphopeptide (11-fold increase in k_{cat}/K_M).

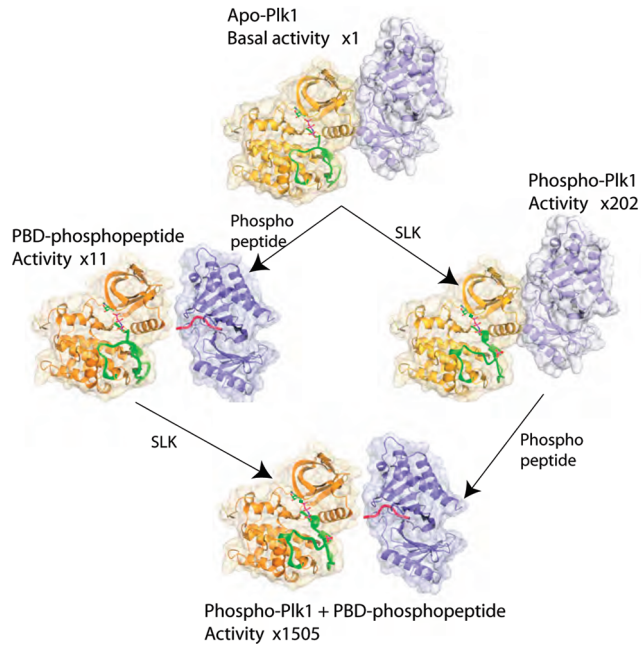

FIGURE 3: Hypothetical model for the activation of Plk1 by Slk phosphorylation and PBD-binding phosphopeptide. The Plk1 kinase and PBD domains are shown as ribbon representation surrounded by van der Waals surface representation. The Plk1 kinase domain is shown in orange with AMPPNP (stick representation) bound at the catalytic site. The activation segment is in green with Thr210 in a stick representation (coordinates from ref *14*; PDB code 2OU7). The PBD is shown in purple (coordinates from ref *20*; PDB code 1Q4K) and has been docked (without energy minimization) against the kinase domain close to the catalytic site. The connecting region from the kinase domain (residue 328) to the start of the PBD (residue 371), for which there is no structural information, is not shown. (Top image) In the Plk1 basal state, it is proposed the PBD blocks access of the substrate to the catalytic site and also may promote the inactive kinase conformation possibly by docking against the C helix of the kinase domain. The activation segment (green) is in the inactive conformation. The relative k_{cat}/K_M is defined as 1. (Middle left image) Upon binding the phosphopeptide (red ribbon) to the PBD, it is proposed that there is a displacement of the PBD relative to the kinase domain allowing for access to the catalytic site. There is no change in the conformation of the PBD upon binding the phosphopeptide. The relative k_{cat}/K_M is increased to 11. (Middle right image) Upon phosphorylation of Plk1 by Slk at Thr210, the activation segment changes conformation so that it can accept the substrate, but the PBD still blocks access (the activation segment was modeled on that for active phosphorylated CDK2). The relative k_{cat}/K_M is 202. (Lower image) Upon both phosphorylation and binding of the PBD, the shift in the PBD allows for access to the substrate and phosphorylation on Thr210 allows for the activation segment to adopt the correct orientation for binding the substrate. The relative k_{cat}/K_M is increased 1505 times that of the unmodified Plk1.

A possible model for the activation of Plk1 is proposed in Figure 3. This model is hypothetical to show possible changes in protein docking, but no attempt has been made to energy refine the model because of insufficient restraints. In the absence of the PBD-binding phosphopeptide, it is assumed that the Plk1 is held in an inactive conformation

by either direct obstruction by the PBD of the catalytic site and access to Thr210 or conformational changes promoted by the association of the PBD with the kinase domain (top image in Figure 3). Upon binding the PBD-binding phosphopeptide, there is a displacement of the PBD or relief of conformational stabilization of the inactive conformation but the affinity for the substrate is low because the activation segment is not in its correct conformation (image at middle left in Figure 3). After observations with other protein kinases, it is assumed that the correct conformation of the activation segment is promoted by phosphorylation of Thr210 by Slk, leading to significant enhancement of activity (image at middle right in Figure 3). In the presence of the PBD-binding phosphopeptide, the Slk phosphorylation reaction is enhanced because binding of the phosphopeptide allows for access of Slk to Thr210 and gives rise to the fully active enzyme (lower image in Figure 3). The notion that the PBD has to be displaced for Slk to access Thr210 is supported by the observation that Slk strongly phosphorylates the free kinase domain (~40 kDa) (Figure 2B). These results are also consistent with the observations that deletion of the PBD results in an increase in activity of the kinase domain (*39, 40*).

A comparison of the kinetic constants of Plk1 in four different activation states, nonphosphorylated with and without 2.5 mM PBD-binding peptide and Slk-phosphorylated Plk1 with and without 2.5 mM PBD-binding peptide (Table 1 and Figure 3), shows that that Slk phosphorylation results in a significant drop in K_M of Plk1 (from 172 to 76 μM), consistent with phosphorylation-mediated reorganization of the catalytic site to a conformation conducive to substrate binding and phosphotransfer. However, it is apparent that, after phosphorylation, binding of the PBD-binding phosphopeptide further enhances activity, as indicated by the significant increase in k_{cat} upon going from the phosphorylated form to the phosphorylated form in the presence of 2.5 mM PBD-binding phosphopeptide (from 1.5 to 12 s^{-1}). By itself, without Slk phosphorylation, the PBD-binding phosphopeptide produces an increase in k_{cat} (from 0.017 to 0.23 s^{-1}) without a change in K_m, consistent with allowing for a better access to the catalytic site but without promoting the correct activation of the activation segment. It is known that there is no significant change in the conformation of the PBD itself upon binding phosphopeptide (*20*). Similarly as discussed above, Plk1 is phosphorylated with a higher efficiency in the presence of a high concentration of PBD-binding phosphopeptide, suggesting a better access to Thr210 in the activation segment.

The work of Yarm (*29*) indicated that Plk1 phosphorylated two sites on TCTP, Ser46 and Ser64. The Ser46 site appeared to be the more important because TCTP mutant Ser46Ala abolished phosphorylation, while the mutant Ser64Ala allowed phosphorylation. We have shown by MS/MS that under our conditions Plk1 phosphorylates only Ser46. The Plk1 consensus phosphorylation site has been identified as Φ/E-E/D-E/D/S-**S/T**-Φ-S/Φ, where Φ is a hydrophobic residue and the site of phosphorylation is in bold, although a number of other nonconsensus sites have also been identified (*41*). TCTP Ser46 is surrounded by a near perfect consensus sequence (IDDS^{46}LI), while that for Ser64 is less than ideal (GTES^{64}TV). This may explain the preference for Ser46 phosphorylation. The structures of TCTP including those of human TCTP (PDB codes 1YZ1 and 2HR9) show that residues 37–66 are disordered and exposed on the surface of the protein. Ser64 is closer to an ordered region, which may make it less accessible to phosphorylation. Ser46 is conserved in higher eukaryotes but not in *Drosophila* or yeast, while Ser64 is not conserved in chicken or lower eukaryotes. In view of the multiple and complex roles in cells for TCTP (as described in the introduction), it is possible that there are other functions for this protein that may or may not involve phosphorylation.

In summary, we describe the expression and purification of fully functional Plk1 from *E. coli* and indicate the requirement for PBD occupancy to facilitate efficient activation by its activating kinase Slk as well as promote full activity once phosphorylated. The results indicate that the structure of the inactive kinase is likely to adopt a conformation in which the PBD either sterically occludes the catalytic site of the kinase and access to Thr210 or promotes a conformational state, in which these sites are not accessible. A variety of mechanisms are known by which a covalently attached domain may regulate protein kinase activity either through direct obstruction of the catalytic site, as in the calcium calmodulin-regulated kinases (reviewed in ref *42*) and in focal adhesion kinase (*43*), or the regulatory domain may act as an allosteric inhibitor through action at a distance that stabilizes an inactive conformation, such as in the Src and Zap70 kinases (*44*). For Plk1, the mechanism used remains to be definitively elucidated by structure.

ACKNOWLEDGMENT

We thank Erich Nigg (Max-Planck-Institute, Martinsried, Germany), Stefan Knapp (Structural Genomics Consortium, Oxford, U.K.), and Christophe Cans (Molecular Engines Ltd., France) for gifts of clones and/or vectors. We also thank Tony Willis for amino acid sequencing, Nick Brown for discussions, and Kin-Yip Cheng and John Sinclair for earlier studies in this laboratory on Plk1.

REFERENCES

1. Barr, F. A., Sillje, H. H., and Nigg, E. A. (2004) Polo-like kinases and the orchestration of cell division. *Nat. Rev. Mol. Cell. Biol. 5*, 429–440.
2. Burkard, M. E., Randall, C. L., Larochelle, S., Zhang, C., Shokat, K. M., Fisher, R. P., and Jallepalli, P. V. (2007) Chemical genetics reveals the requirement for polo-like kinase 1 activity in positioning RhoA and triggering cytokinesis in human cells. *Proc. Natl. Acad. Sci. U.S.A. 104*, 4383–4388.
3. Kang, Y. H., Park, J. E., Yu, L. R., Soung, N. K., Yun, S. M., Bang, J. K., Seong, Y. S., Yu, H., Garfield, S., Veenstra, T. D., and Lee, K. S. (2006) Self-regulated Plk1 recruitment to kinetochores by the Plk1−PBIP1 interaction is critical for proper chromosome segregation. *Mol. Cell 24*, 409–422.
4. Oshimori, N., Ohsugi, M., and Yamamoto, T. (2006) The Plk1 target Kizuna stabilizes mitotic centrosomes to ensure spindle bipolarity. *Nat. Cell Biol. 8*, 1095–1101.
5. Sumara, I., Gimenez-Abian, J. F., Gerlich, D., Hirota, T., Kraft, C., de la Torre, C., Ellenberg, J., and Peters, J. M. (2004) Roles of polo-like kinase 1 in the assembly of functional mitotic spindles. *Curr. Biol. 14*, 1712–1722.
6. Plyte, S., and Musacchio, A. (2007) PLK1 inhibitors: Setting the mitotic death trap. *Curr. Biol. 17*, R280–R283.
7. Strebhardt, K., and Ullrich, A. (2006) Targeting polo-like kinase 1 for cancer therapy. *Nat. Rev. Cancer 6*, 321–330.
8. Lenart, P., Petronczki, M., Steegmaier, M., Di Fiore, B., Lipp, J. J., Hoffmann, M., Rettig, W. J., Kraut, N., and Peters, J. M. (2007) The small-molecule inhibitor BI 2536 reveals novel insights into mitotic roles of polo-like kinase 1. *Curr. Biol. 17*, 304–315.

9. McInnes, C., Mazumdar, A., Mezna, M., Meades, C., Midgley, C., Scaerou, F., Carpenter, L., Mackenzie, M., Taylor, P., Walkinshaw, M., Fischer, P. M., and Glover, D. (2006) Inhibitors of polo-like kinase reveal roles in spindle-pole maintenance. *Nat. Chem. Biol. 2*, 608–617.
10. Peters, U., Cherian, J., Kim, J. H., Kwok, B. H., and Kapoor, T. M. (2006) Probing cell-division phenotype space and polo-like kinase function using small molecules. *Nat. Chem. Biol. 2*, 618–626.
11. Steegmaier, M., Hoffmann, M., Baum, A., Lenart, P., Petronczki, M., Krssak, M., Gurtler, U., Garin-Chesa, P., Lieb, S., Quant, J., Grauert, M., Adolf, G. R., Kraut, N., Peters, J. M., and Rettig, W. J. (2007) BI 2536, a potent and selective inhibitor of polo-like kinase 1, inhibits tumor growth in vivo. *Curr. Biol. 17*, 316–322.
12. Lansing, T. J., McConnell, R. T., Duckett, D. R., Spehar, G. M., Knick, V. B., Hassler, D. F., Noro, N., Furuta, M., Emmitte, K. A., Gilmer, T. M., Mook, R. A., Jr., and Cheung, M. (2007) In vitro biological activity of a novel small-molecule inhibitor of polo-like kinase 1. *Mol. Cancer Ther. 6*, 450–459.
13. Steegmaier, M., Baum, A., Solca, F., Peters, J. M., Grauert, M., and Hoffmann, M. (2005) BI 2536, a potent and highly seective inhibitor of polo-like kinase (Plk1) induces mitotic arrest and apoptosis in a broad spectrum of tumour cell lines. *Clin. Cancer Res. 11*, 9147.
14. Kothe, M., Kohls, D., Low, S., Coli, R., Cheng, A. C., Jacques, S. L., Johnson, T. L., Lewis, C., Loh, C., Nonomiya, J., Sheils, A. L., Verdries, K. A., Wynn, T. A., Kuhn, C., and Ding, Y. H. (2007) Structure of the catalytic domain of human polo-like kinase 1. *Biochemistry 46*, 5960–5971.
15. Lowery, D. M., Lim, D., and Yaffe, M. B. (2005) Structure and function of polo-like kinases. *Oncogene 24*, 248–259.
16. van de Weerdt, B. C., and Medema, R. H. (2006) Polo-like kinases: A team in control of the division. *Cell Cycle 5*, 853–864.
17. Elia, A. E., Cantley, L. C., and Yaffe, M. B. (2003) Proteomic screen finds pSer/pThr-binding domain localizing Plk1 to mitotic substrates. *Science 299*, 1228–1231.
18. Elia, A. E., Rellos, P., Haire, L. F., Chao, J. W., Ivins, F. J., Hoepker, K., Mohammad, D., Cantley, L. C., Smerdon, S. J., and Yaffe, M. B. (2003) The molecular basis for phosphodependent substrate targeting and regulation of Plks by the polo-box domain. *Cell 115*, 83–95.
19. Neef, R., Gruneberg, U., Kopajtich, R., Li, X., Nigg, E. A., Sillje, H., and Barr, F. A. (2007) Choice of Plk1 docking partners during mitosis and cytokinesis is controlled by the activation state of Cdk1. *Nat. Cell Biol. 9*, 436–444.
20. Cheng, K. Y., Lowe, E. D., Sinclair, J., Nigg, E. A., and Johnson, L. N. (2003) The crystal structure of the human polo-like kinase-1 polo box domain and its phospho-peptide complex. *EMBO J. 22*, 5757–5768.
21. Garcia-Alvarez, B., de Carcer, G., Ibanez, S., Bragado-Nilsson, E., and Montoya, G. (2007) Molecular and structural basis of polo-like kinase 1 substrate recognition: Implications in centrosomal localization. *Proc. Natl. Acad. Sci. U.S.A. 104*, 3107–3112.
22. Brown, N. R., Noble, M. E., Endicott, J. A., and Johnson, L. N. (1999) The structural basis for specificity of substrate and recruitment peptides for cyclin-dependent kinases. *Nat. Cell Biol. 1*, 438–443.
23. Russo, A. A., Jeffrey, P. D., and Pavletich, N. P. (1996) Structural basis of cyclin-dependent kinase activation by phosphorylation. *Nat. Struct. Biol. 3*, 696–700.
24. Jang, Y. J., Ma, S., Terada, Y., and Erikson, R. L. (2002) Phosphorylation of threonine 210 and the role of serine 137 in the regulation of mammalian polo-like kinase. *J. Biol. Chem. 277*, 44115–44120.
25. Ellinger-Ziegelbauer, H., Karasuyama, H., Yamada, E., Tsujikawa, K., Todokoro, K., and Nishida, E. (2000) Ste20-like kinase (SLK), a regulatory kinase for polo-like kinase (Plk) during the G2/M transition in somatic cells. *Genes Cells 5*, 491–498.
26. Walter, S. A., Cutler, R. E., Jr., Martinez, R., Gishizky, M., and Hill, R. J. (2003) Stk10, a new member of the polo-like kinase kinase family highly expressed in hematopoietic tissue. *J. Biol. Chem. 278*, 18221–18228.
27. Tsvetkov, L. M., Tsekova, R. T., Xu, X., and Stern, D. F. (2005) The Plk1 polo box domain mediates a cell cycle and DNA damage regulated interaction with Chk2. *Cell Cycle 4*, 609–617.
28. Bommer, U. A., and Thiele, B. J. (2004) The translationally controlled tumour protein (TCTP). *Int. J. Biochem. Cell Biol. 36*, 379–385.
29. Yarm, F. R. (2002) Plk phosphorylation regulates the microtubule-stabilizing protein TCTP. *Mol. Cell. Biol. 22*, 6209–6221.
30. Chen, S. H., Wu, P. S., Chou, C. H., Yan, Y. T., Liu, H., Weng, S. Y., and Yang-Yen, H. F. (2007) A knockout mouse approach reveals that TCTP functions as an essential factor for cell proliferation and survival in a tissue- or cell type-specific manner. *Mol. Biol. Cell 18*, 2525–2532.
31. Liu, H., Peng, H. W., Cheng, Y. S., Yuan, H. S., and Yang-Yen, H. F. (2005) Stabilization and enhancement of the antiapoptotic activity of mcl-1 by TCTP. *Mol. Cell. Biol. 25*, 3117–3126.
32. Yang, Y., Yang, F., Xiong, Z., Yan, Y., Wang, X., Nishino, M., Mirkovic, D., Nguyen, J., Wang, H., and Yang, X. F. (2005) An N-terminal region of translationally controlled tumor protein is required for its antiapoptotic activity. *Oncogene 24*, 4778–4788.
33. Thaw, P., Baxter, N. J., Hounslow, A. M., Price, C., Waltho, J. P., and Craven, C. J. (2001) Structure of TCTP reveals unexpected relationship with guanine nucleotide-free chaperones. *Nat. Struct. Biol. 8*, 701–704.
34. Hsu, Y. C., Chern, J. J., Cai, Y., Liu, M., and Choi, K. W. (2007) *Drosophila* TCTP is essential for growth and proliferation through regulation of dRheb GTPase. *Nature 445*, 785–788.
35. Petronczki, M., Glotzer, M., Kraut, N., and Peters, J. M. (2007) Polo-like kinase 1 triggers the initiation of cytokinesis in human cells by promoting recruitment of the RhoGEF Ect2 to the central spindle. *Dev. Cell 12*, 713–725.
36. Hastie, C. J., McLauchlan, H. J., and Cohen, P. (2006) Assay of protein kinases using radiolabeled ATP: A protocol. *Nat. Protoc. 1*, 968–971.
37. Oliver, A. W., Knapp, S., and Pearl, L. H. (2007) Activation segment exchange: A common mechanism of kinase autophosphorylation? *Trends Biochem. Sci. 32*, 351–356.
38. Cheng, K.-Y. (2006) Structural and functional studies on the mitotic polo-like kinase 1, D.Phil. Thesis, University of Oxford, Oxford, U.K.
39. Lee, K. S., and Erikson, R. L. (1997) Plk is a functional homolog of *Saccharomyces cerevisiae* Cdc5, and elevated Plk activity induces multiple septation structures. *Mol. Cell. Biol. 17*, 3408–3417.
40. Mundt, K. E., Golsteyn, R. M., Lane, H. A., and Nigg, E. A. (1997) On the regulation and function of human polo-like kinase 1 (PLK1): Effects of overexpression on cell cycle progression. *Biochem. Biophys. Res. Commun. 239*, 377–385.
41. Johnson, E. F., Stewart, K. D., Woods, K. W., Giranda, V. L., and Luo, Y. (2007) Pharmacological and functional comparison of the polo-like kinase family: Insight into inhibitor and substrate specificity. *Biochemistry 46*, 9551–9563.
42. Pellicena, P., and Kuriyan, J. (2006) Protein−protein interactions in the allosteric regulation of protein kinases. *Curr. Opin. Struct. Biol. 16*, 702–709.
43. Lietha, D., Cai, X., Ceccarelli, D. F., Li, Y., Schaller, M. D., and Eck, M. J. (2007) Structural basis for the autoinhibition of focal adhesion kinase. *Cell 129*, 1177–1187.
44. Deindl, S., Kadlecek, T. A., Brdicka, T., Cao, X., Weiss, A., and Kuriyan, J. (2007) Structural basis for the inhibition of tyrosine kinase activity of ZAP-70. *Cell 129*, 735–746.

BI702134C