Direct quantification of polo-like kinase 1 activity in cells and tissues using a highly sensitive and specific ELISA assay

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Communicated by R. L. Erikson, Harvard University, Cambridge, MA, December 20, 2008 (received for review September 30, 2008)

Polo-like kinase 1 (Plk1) plays a pivotal role in the regulation of cellular proliferation. Plk1 is overexpressed in ≈80% of human tumors of diverse origins, and overexpression of Plk1 promotes neoplastic transformation of human cells. A growing body of evidence suggests that deregulation of Plk1 closely correlates with prognosis of various cancers in humans. Thus, accurate assessment of Plk1 deregulation would provide clear clinical advantages. However, because of the limited amount of cancer tissues available, quantification of the Plk1 activity has not been feasible. Here, we report the development of a rapid, highly sensitive, and specific ELISA-based Plk1 assay that can quantify the level of Plk1 activity with a small amount (2–20 μg) of total cellular proteins. Unlike the conventional immunocomplex kinase assay, this assay directly utilizes total cellular lysates and does not require a Plk1 enrichment step such as immunoprecipitation or affinity purification. Using this assay, we demonstrated that Plk1 activity is elevated in tumors but not in the surrounding normal tissues and that the level of Plk1 activity significantly diminishes after an antiproliferative chemotherapy. The method described here may provide an innovative tool for assessing the predisposition for cancer development, monitoring early tumor response after therapy, and estimating the prognosis of patients with cancers from multiple organ sites.

p-T78 peptide | PBIP1 | polo kinase | polo-box domain | PBIPtide

Protein kinases belong to a large family of enzymes, many of which play critical roles in a wide range of cellular processes including cell division, proliferation, apoptosis, and differentiation. Deregulation of various kinases has been linked to numerous diseases including cancers. Found in a wide array of organisms from yeast to mammals, polo-like kinases (collectively, Plks) constitute a conserved subfamily of Ser/Thr protein kinases that play pivotal roles in cellular proliferation. Plks are characterized by the presence of a highly conserved polo-box domain (PBD) in the C-terminal noncatalytic region that appears to be critical for targeting the catalytic activity to its specific binding partners and physiological substrates (for reviews, see refs. 1 and 2). In mammals, 3 closely related (Plk1, Plk2/Snk, and Plk3/Prk) and 1 distantly related (Plk4/Sak) kinases have been identified, and they exhibit apparently distinct expression patterns and physiological functions (3). Among them, Plk1 has been the focus of extensive studies because of its strong association with neoplastic transformation of human cells. Plk1 mRNA is overexpressed in a broad spectrum of human cancers including breast, ovarian, non-small-cell lung, head/neck, colon, endometrial, and esophageal carcinomas and leukemias, and its overexpression appears to be sufficient to override cellular checkpoints and induce genetic instability, thus promoting tumorigenesis (4–6). In agreement with these observations, several studies show that the level of Plk1 transcript positively correlates with aggressiveness of tumor progression and that patients with high Plk1 expression in their tumors exhibit a significantly poorer rate of survival than those with low Plk1 expression (7–10). These data suggest that the level of Plk1 expression serves as a prognostic marker for many types of malignancies. Thus, a reliable and convenient assay for measuring Plk1 protein level or kinase activity would greatly facilitate both diagnosis and prognosis of various cancers in humans.

The currently available assays for measuring Plk1 kinase activity typically rely on immunocomplex kinase assays using a generic kinase substrate, casein. They are not only laborious and time-consuming but also insensitive and use a large amount of cellular lysates. The accuracy of these assays is also limited because of nonspecific phosphorylation of the generic substrate casein by other contaminating kinases. Here, we describe development of an ELISA-based Plk1 assay that employs a principle to rapidly and accurately quantify the Plk1 activity with high sensitivity and specificity. Because Plk1 expression is tightly correlated with cell proliferation activity and most, if not all, chemotherapeutic agents aim at inhibiting hyperproliferative cancer cells, this assay could offer wide applications in monitoring the effect of anti-Plk1 or other antiproliferative therapeutic agents.

Results

Plk1 Kinase Assay Using GST-PBIPtide as a Plk1 Affinity Ligand and in Vitro Substrate. Previous results showed that Plk1 efficiently phosphorylates a centromeric protein PBIP1 at T78, and this phosphorylation event generates a docking site for a high-affinity interaction between the PBD of Plk1 and *p*-T78 PBIP1 (11). By taking advantage of the specific Plk1-dependent PBIP1 phosphorylation and subsequent interaction, we examined whether a GST-fused PBIP1 peptide bearing the T78 motif (hereon referred to as GST-PBIPtide) could precipitate Plk1 through the Plk1-generated *p*-T78 epitope and whether the precipitated Plk1 could further phosphorylate as yet unphosphorylated GST-PBIPtide. To test this possibility, we first generated GST-PBIPtide$_4$ (PBIPtide containing 4 repeats of the T78 motif to enhance the phosphorylation level and binding affinity) and expressed it in *Escherichia coli*. The resulting bead-associated GST-PBIPtide$_4$ was incubated with mitotic HeLa lysates in a conventional immunoprecipitation buffer, precipitated, and then reacted in a kinase reaction mixture (KC buffer; see *Materials and Methods*) in the presence of [γ-^{32}P]ATP. The GST-PBIPtide coprecipitated Plk1, which, in turn, phosphorylated and

Author contributions: J.-E.P., L.L., and K.S.L. designed research; J.-E.P. and L.L. performed research; J.P., R.K., and K.S. contributed new reagents/analytic tools; J.-E.P., L.L., R.K., K.S., S.H.Y., and K.S.L. analyzed data; and J.-E.P., L.L., S.H.Y., and K.S.L. wrote the paper.

The authors declare no conflict of interest.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0812135106/DCSupplemental.

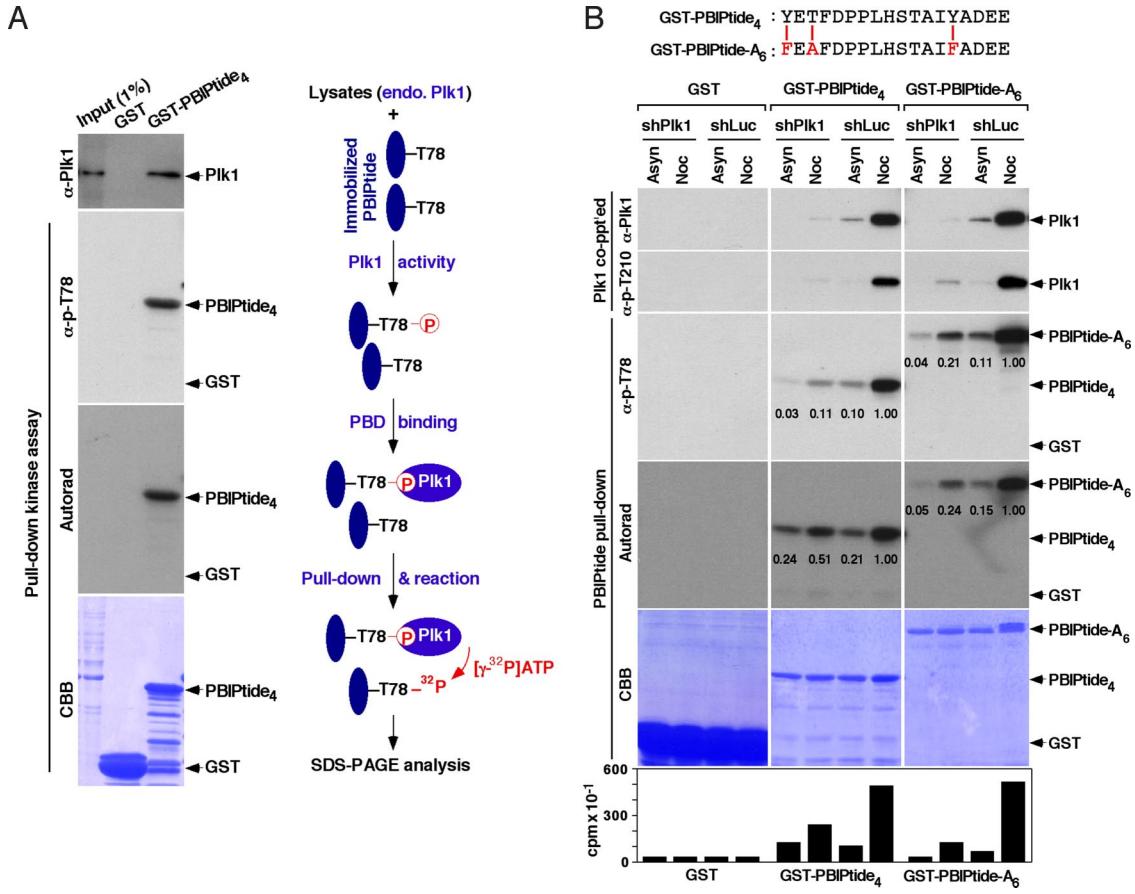

Fig. 1. Development of a Plk1 kinase assay using GST-PBIPtide as a Plk1 affinity ligand and in vitro substrate. (*A*) GST-PBIPtide not only precipitates Plk1 but also serves as Plk1 substrate in vitro. (*Left*) Mitotic HeLa lysates were incubated with either bead-immobilized control GST or GST-PBIPtide$_4$ in TBSN buffer containing phosphatase inhibitors. Beads were precipitated and washed and then subjected to in vitro kinase assays in the presence of [γ-^{32}P]-ATP. Samples were separated by SDS/PAGE, transferred to PVDF, and then exposed (Autorad). The membrane was immunoblotted with the indicated antibodies and then stained with Coomassie (CBB). Note that the anti-*p*-T78 signal (α-*p*-T78 panel) is relatively weak because the incubation was carried out in TBSN at 4 °C. (*Right*) Schematic diagram illustrating the experimental procedures described in *Left*. The blue ellipsoids depict bead-associated GST-PBIPtide containing the T78 motif. (*B*) A direct Plk1 kinase assay with total cellular lysates using GST-PBIPtides as substrate. Asynchronously growing HeLa cells (Asyn) or cells expressing shRNA directed against control luciferase (shLuc) or Plk1 (shPlk1) were harvested. Where indicated, cells were treated with nocodazole (Noc) for 16 h to arrest the cells in prometaphase. Total HeLa lysates (100 μg) were prepared in KC-plus buffer and incubated with the control GST or the indicated GST-PBIPtide in the presence of [γ-^{32}P]-ATP at 30 °C for 30 min. The bead-associated GST-PBIPtides were precipitated and washed with KC-plus buffer before boiling with SDS/PAGE sample buffer. The samples were separated as above, transferred, and then exposed (Autorad). After immunoblotting, the membrane was stained with Coomassie (CBB). The GST-PBIPtide bands were excised, and incorporated ^{32}P was quantified. The signals in the anti-Plk1 and anti-*p*-T210 immunoblots indicate the amount of Plk1 coprecipitated (Plk1 co-ppt'ed) with the GST-PBIPtide and the level of the *p*-T210 epitope among the Plk1 precipitates, respectively. Numbers indicate the levels of the *p*-T78 epitope (α-*p*-T78 panel) or ^{32}P incorporation (Autorad) relative to those in the nocodazole (Noc)-treated, control luciferase RNAi (shLuc) cells.

generated the *p*-T78 epitope on neighboring GST-PBIPtide molecules (Fig. 1*A*). Under the same conditions, the control GST failed to precipitate Plk1 and generate the *p*-T78 epitope (Fig. 1*A*). As expected if the Plk1 activity in the total lysates was responsible for generating the *p*-T78 epitope, Plk1 immunoprecipitated from cultured lysates efficiently phosphorylated GST-PBIPtide and generated the *p*-T78 epitope in a kinase activity-dependent manner [supporting information (SI) Fig. S1].

We then examined whether the Plk1 activity present in total cellular lysates could be directly measured by incubating the lysates with GST-PBIPtide. To this end, total cellular lysates were prepared from either control luciferase RNAi (shLuc) or Plk1 RNAi (shPlk1)-treated HeLa cells (Fig. S2) in a KC-plus buffer (kinase reaction mixture supplemented with 100 mM NaCl, 0.5% Nonidet P-40, and protease inhibitors), mixed with GST-PBIPtide$_4$, and then reacted in the presence of 15 μCi of [γ-^{32}P]ATP. Consistent with the levels of Plk1 expression (Fig. 1*B*, α-Plk1) and activational Plk1 phosphorylation at T210 (Fig. 1*B*, α-*p*-T210), the control shLuc cells arrested with nocodazole (M phase) exhibited a high level of T78 phosphorylation onto PBIPtide$_4$ (Fig. 1*B*; α-*p*-T78 in the center column). By contrast, asynchronously growing shLuc cells (Asyn) displayed a significantly diminished level of the *p*-T78 epitope. As expected, cells silenced for Plk1 (shPlk1) showed a very low level of T78 phosphorylation. However, the same sample exhibited a much higher level of ^{32}P incorporation onto PBIPtide$_4$ (Fig. 1*B*; compare the autorad with the α-*p*-T78 blot in the center column), suggesting that the Thr and Tyr residues other than the T78 residue within PBIPtide$_4$ are phosphorylated during the incubation with total lysates. To eliminate these potential phosphorylation sites, we mutated Y68, T70, and Y81 within the GST-PBIPtide (thus leaving the T78 residue as the only phosphorylation site) and then generated a GST-PBIPtide variant bearing 6 repeats of the PBIPtide(Y68F T70A Y81F) triple mutant (herein referred to as GST-PBIPtide-A$_6$). Examination of Plk1-dependent GST-PBIPtide-A$_6$ phosphorylation showed that the level of the *p*-T210 epitope (Plk1 activity) was closely correlated with that of the *p*-T78 epitope on PBIPtide-A$_6$ (Fig. 1*B*; compare the 2nd and 3rd panels in the right column). Additionally, PBIPtide-A$_6$ displayed a mildly,

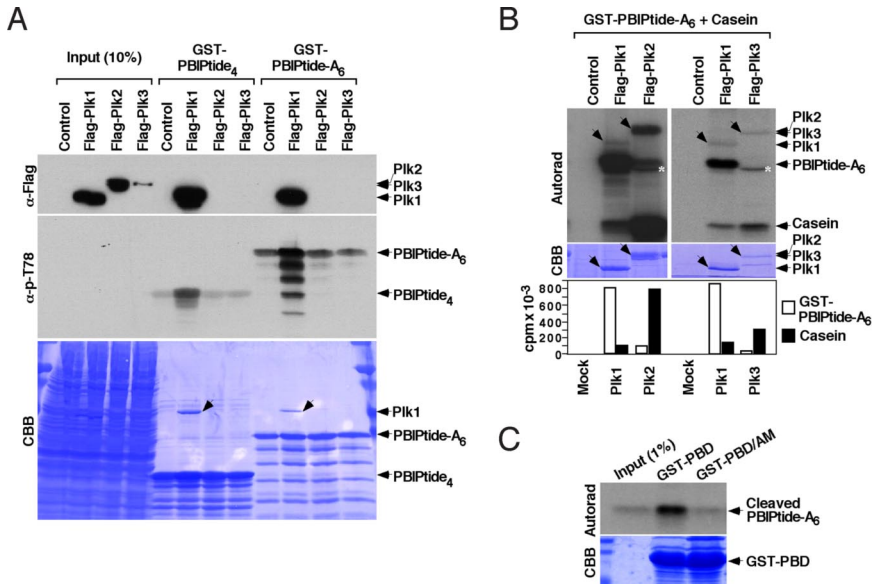

Fig. 2. Plk1, but not Plk2 or Plk3, phosphorylates and binds to the T78 motif of GST-PBIPtides. (*A*) 293T lysates expressing control vector, Flag-Plk1, Flag-Plk2, or Flag-Plk3 were prepared in KC-plus buffer and incubated with the indicated GST-PBIPtides immobilized to the GSH-agarose beads. The GST-PBIPtide precipitates were separated by SDS/PAGE, transferred, and then immunoblotted with anti-Plk1 and anti-*p*-T78 antibodies. Afterward, the membrane was stained with Coomassie (CBB). The level of Plk3 expression is low because of its cytotoxicity. The low levels of the *p*-T78 signal detected in the control vector-, Plk2-, and Plk3-expressing cells are likely due to the endogenous Plk1 activity. The multiple tiers of the *p*-T78 signals are due to GST-PBIPtide degradation. Arrowheads indicate Plk1 coprecipitated with GST-PBIPtides. (*B* and *C*) Plk1-dependent phosphorylation onto the T78 motif of GST-PBIPtide-A_6 is sufficient for the PBD binding. (*B*) (*Top* and *Middle*) Flag-Plk1, Flag-Plk2, and Flag-Plk3 immunoprecipitates prepared from transfected 293T cells were subjected to in vitro kinase assays using both GST-PBIPtide-A_6 and casein as substrates in the same reaction. Samples were separated by SDS/PAGE for autoradiography (Autorad). Arrowheads indicate autophosphorylated signals corresponding to Plk1, 2, or 3 in the gels stained with Coomassie (CBB), whereas asterisks denote nonspecific signals. Incorporated ^{32}P into GST-PBIPtide-A_6 and casein were quantified (*Bottom*). (*C*) The Plk1-phosphorylated, bead-bound, GST-PBIPtide-A_6 was digested with thrombin, and the resulting soluble ^{32}P-PBIPtide-A_6 was incubated with either bead-bound GST-PBD or GST-PBD(H538A K540M) in TBSN buffer. Precipitates were washed and then analyzed as in *B*.

but reproducibly, lower level of ^{32}P incorporation after Plk1 depletion than the original PBIPtide$_4$ (Fig. 1*B*; autorad in the right column), suggesting an improved sensitivity to Plk1 activity. In a separate experiment, either mitotic HeLa lysates or *Xenopus* cytostatic factor (CSF)-arrested extracts efficiently generated the T78 epitope in both PBIPtide$_4$ and PBIPtide-A_6, and the resulting *p*-T78-containing PBIPtides interacted with its respective endogenous Plk1 or Plx1 (Fig. S3), demonstrating that the FAF mutations in PBIPtide-A_6 do not alter the efficiency of T78 phosphorylation and subsequent binding to the PBD.

Plk1, but Not Plk2 or Plk3, Phosphorylates and Binds to the T78 Motif of GST-PBIPtides. Next, we examined whether Plk2 or Plk3 contribute to the generation of the *p*-T78 epitope (because of a dissimilar PBD-binding module, the distantly related Plk4 has not been tested). To this end, total lysates prepared from cells transfected with Flag-Plk1, Flag-Plk2, or Flag-Plk3, were incubated with the indicated GST-PBIPtides in the KC-plus buffer and then precipitated. Remarkably, lysates expressing Plk1 efficiently generated the *p*-T78 epitope on GST-PBIPtides, yielding a Coomassie-stainable level of Plk1 binding to the *p*-T78 GST-PBIPtides (Fig. 2*A*). In stark contrast, the lysates expressing Plk2 or Plk3 did not significantly generate the *p*-T78 epitope or bind to the GST-PBIPtides (Fig. 2*A*). The low levels of the *p*-T78 epitope in the vector-, Plk2-, and Plk3-expressing lysates are likely due to the endogenous Plk1 activity.

We next tested whether Plk1 directly phosphorylates and generates the *p*-T78 epitope on GST-PBIPtide-A_6 and whether this event is sufficient for PBD binding. Similar to the previous observation with GST-PBIPtide$_4$ (12), Plk1 preferentially phosphorylated GST-PBIPtide-A_6 in a kinase reaction that contained both PBIPtide-A_6 and a generic substrate, casein. In contrast, both Plk2 and Plk3 exhibited a strong preference for casein over PBIPtide-A_6 (Fig. 2*B*). Furthermore, GST-PBD, but not the respective PBD(H538A K540M) phosphate-pincer mutant (13), efficiently coprecipitated the phospho-PBIPtide-A_6 (Fig. 2*C*). Thus, Plk1 specifically phosphorylates GST-PBIPtide-A_6 and generates the *p*-T78 epitope and this event is sufficient for the subsequent interaction with its own PBD.

Measurement of the Kinase Activity of Plk1 by a T78-Based ELISA. By exploiting the high specificity in Plk1-dependent PBIPtide phosphorylation and the ensuing PBD-*p*-T78 interaction, we then generated a PBIPtide-based ELISA designed to measure the Plk1 activity by either of 2 approaches: (*i*) quantifying the level of the *p*-T78 epitope (α-*p*-T78 antibody) or (*ii*) measuring the level of Plk1 binding (α-Plk1 antibody) (Fig. 3*A*). To perform these assays, a 96-well plate was coated with various amounts of GST-PBIPtide containing the T78 motif and then applied with a range of total cellular lysates prepared in the KC-plus buffer to allow efficient phosphorylation onto PBIPtide during incubation (≈2–10 μg of total HeLa lysates were sufficient for reactions with ≈0.5–1 μg of GST-PBIPtide$_4$ or GST-PBIPtide-A_6 per well; see Fig. S4). Attesting to the specificity of Plk1-dependent PBIPtide phosphorylation, depletion of Plk1 greatly diminished the levels of both the *p*-T78 epitope and bound Plk1 on GST-PBIPtides (Fig. 3*B*). Consistent with the results shown in Fig. 1*B*, PBIPtide-A_6 exhibited a mildly, but reproducibly, increased sensitivity to Plk1 activity compared with PBIPtide$_4$. Notably, the level of the Plk1 binding (α-Plk1 signal) was significantly lower than the level of the *p*-T78 generation (α-*p*-T78 signal) (Fig. 3*B*), in part because Plk1 binds to a fraction of the total *p*-T78 epitope generated. Because of the apparently elevated sensitivity, we chose GST-PBIPtide-A_6 for further analysis.

To verify the Plk1-dependent PBIPtide phosphorylation and

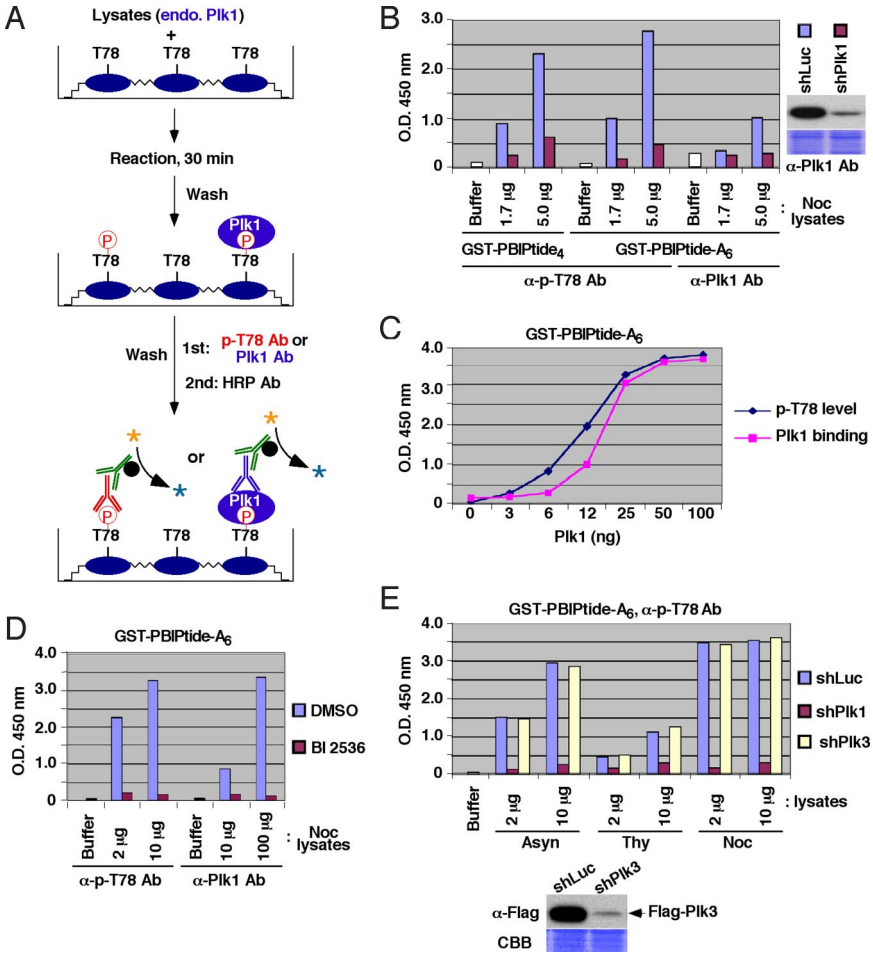

Fig. 3. Development of a Plk1-specific ELISA. (*A*) Scheme illustrating the Plk1 ELISA. The ELISA wells were coated with soluble GST-PBIPtide containing the T78 motif (dark blue) and then reacted with total cellular lysates. The Plk1 activity in the total lysates generates the *p*-T78 epitope to which Plk1 itself binds. After reaction, Plk1 activity is quantified by incubating the ELISA wells with either anti-*p*-T78 antibody (red) (to detect the *p*-T78 epitope generated) or anti-Plk1 antibody (blue) (to detect Plk1 bound to the *p*-T78 epitope), followed by HRP-conjugated secondary antibody (the green antibody with a black dot). The yellow and light blue asterisks indicate 3.3′, 5.5′-tetramethylbenzidine (TMB) substrate and its reaction product, respectively, generated by HRP. (*B*) (*Left*) HeLa cells were silenced for control luciferase (shLuc) or Plk1 (shPlk1) and then treated with nocodazole for 16 h to arrest the cells in prometaphase (a condition that maximizes Plk1 activity). The lysates were then applied onto the ELISA wells coated with GST-PBIPtide$_4$ or GST-PBIPtide-A$_6$, and ELISAs were carried out as described in *Materials and Methods*. Buffer indicates no-lysate control. The same lysates were subjected to immunoblotting analysis to determine the level of Plk1 in the lysates (*Right*). (*C*) Plk1 generates and binds to the *p*-T78 epitope in a concentration-dependent manner. GST-PBIPtide-A$_6$-coated ELISA wells were incubated with the indicated amount of recombinant Plk1 purified from Sf9 cells. The level of the *p*-T78 epitope generated and the amount of Plk1 bound to the *p*-T78 GST-PBIPtide-A$_6$ were quantified by using anti-*p*-T78 and anti-Plk1 antibodies, respectively. (*D*) HeLa cells arrested with nocodazole for 16 h were additionally treated with either control DMSO or a Plk1 inhibitor, BI 2536, for 30 min before harvest. Total lysates were prepared from these cells and applied to the GST-PBIPtide-A$_6$-coated wells. Buffer indicates no-lysate control. (*E*) HeLa cells silenced for control luciferase (shLuc), Plk1 (shPlk1), or Plk3 (shPlk3) were treated with either thymidine (Thy) or nocodazole (Noc) or left untreated for 16 h before harvest. Total cellular lysates prepared from these cells were subjected to ELISAs as in *D*. Buffer indicates no-lysate control. Because detection of endogenous Plk3 with currently available antibodies was not reliable, efficiency of Plk3 depletion by shPlk3 was determined by using cells transfected with Flag-Plk3. The target sequence for shPlk3 was described previously (17).

subsequent PBD binding, we then carried out the above assay using purified recombinant proteins. Results showed that the level of the *p*-T78 epitope generated was proportional to the amount of Plk1 provided. Although the Plk1 amounts that can be quantified in a linear range could be relatively narrow because of the nature of an ELISA-based assay, the increase in the level of the *p*-T78 epitope also closely paralleled the increase in the amount of Plk1 bound to PBIPtide (Fig. 3*C*). In a second experiment, we also found that treatment of cells with a Plk1 inhibitor, BI 2536 (14), for 30 min was sufficient to acutely inhibit the Plk1-dependent *p*-T78 generation and Plk1 binding (Fig. 3*D*), further confirming that Plk1 activity is responsible for these events.

Notably, Plk1 exhibits only 49–51% sequence identities in the kinase domain with Plk2 and Plk3, suggesting that the substrate recognition modes of these kinases are distinct. Highlighting the functional disparities of these kinases, it has been shown that Plk2 is transiently expressed during the G_0/G_1 transition (15). However, Plk3 is reported to be active in S and G_2 phases of the cell cycle (16, 17), during which Plk1 is also progressively accumulated and activated before mitotic entry. Furthermore, overexpression of Plk3 rescues the growth defect associated with the budding yeast polo kinase *cdc5–1* mutation (16). Thus, we examined whether Plk3 contributes to the generation of the *p*-T78 epitope in vivo. Consistent with Plk1-dependent PBIPtide phosphorylation, the level of *p*-T78 was high in nocodazole-treated (M phase) cells but low in thymidine-arrested (S phase) cells (Fig. 3*E*). Depletion of Plk1 drastically diminished the level of the *p*-T78 epitope, whereas depletion of Plk3 did not significantly alter the level of the *p*-T78 epitope under various conditions examined (Fig. 3*E*). These results suggest that Plk3 does not contribute to the generation of the *p*-T78 epitope.

Direct Measurement of Plk1 Activity in Mouse Tissues. To determine whether the above PBIPtide-based ELISA could be used to measure the level of Plk1 activity in mammalian tissues, we compared both the ELISA and the conventional immunocomplex kinase assay using the same lysates prepared from various mouse tissues. We found that the Plk1 activities from the ELISA showed a tight correlation with those from the immunocomplex kinase assays (Fig. 4*A*; see also immunocomplex kinase assays using GST-PBIPtide-A_6 as substrates in Fig. S5). Remarkably, only 4–20 μg of the same lysates (because of low mitotic indices for tissues, we needed more lysates from tissues than from cultured cells) were used for the ELISA compared with 2 mg of total lysates for the immunoprecipitation kinase assay. Thus, the Plk1 ELISA is not only rapid but also sensitive, allowing accurate quantification of Plk1 activity with 100- to 500-fold smaller amounts of total lysates from cells and tissues.

Taking advantage of this highly sensitive assay, we then investigated whether Plk1 activity alters during tumorigenesis using B16-derived xenografted tumors in nude mice (Fig. 4*B*). Plk1 activity was low immediately after grafting but soon reached a maximum level during early stages of tumorigenesis (Fig. 4*C*). However, the level of Plk1 activity began to diminish as the tumor reached a significant volume (≈10 mm in diameter). The levels of Plk1 activity closely mirrored those of Plk1 expression (Fig. 4*C*) and cell proliferation activity (Fig. S6). Measurement of Plk1 activity of tissues taken from different parts of a single tumor revealed that the levels of Plk1 expression and activity tightly correlated with the level of mitotic Cyclin B1 (Fig. S7). These findings suggest that elevated Plk1 activity is critical during early stages of tumorigenesis and strongly support the view that the level of Plk1 functions as an indicator of cell proliferation.

Discussion

Elevated levels of Plk1 expression correlate with poor prognosis for a wide range of human cancers such as non-small-cell lung cancer, oropharyngeal carcinoma, esophageal carcinoma, melanoma, colorectal cancer, hepatoblastoma, and non-Hodgkin lymphoma (5). These observations have marked Plk1 as a potential therapeutic target for cancer, and a number of small-molecule inhibitors of the kinase are being developed for that purpose. Thus, an accurate measurement of Plk1 activity would be important not only as a potential guide to address prognostic issues in individual patients but also as a biomarker to indicate the effectiveness of Plk1-targeted therapy. However, limited supplies of tissue samples have made it difficult to determine Plk1 activity by using conventional Plk1 kinase assays in both experimental models and human material. By exploiting a unique Plk1-dependent PBIP1 phosphorylation and subsequent binding and by using an ELISA-based technology, we have now developed a rapid, sensitive, and specific assay that measures the Plk1 activity in biological material by using the intracellular ATP present in the lysates. This assay is far more sensitive than conventional immunocomplex kinase assays and thereby enables the detection of an elevated Plk1 activity in small amounts of total lysates obtained from cultured cells or xenografted mouse tumors. In addition, the assay allows for quantification of the Plk1 activity in a given lysate by easily relating to recombinant Plk1 standard or normalizing to a control lysate.

As an extension of this assay and as a further proof of principle, we measured Plk1 activity in normal and tumor tissues obtained from head and neck cancer patients. Although the level of Plk1 activity varied greatly among tumor samples (likely because of the differences in age, diet, genetic background, tumor site, etc.), elevated Plk1 activity in tumor tissue was manifest in most cases where a difference in matched value was detected (Fig. S8*A*). Furthermore, most tumor samples obtained from patients after antiproliferative chemotherapy exhibited greatly diminished Plk1 activity compared with the tumor samples before therapy (Fig. S8*B*). The number of patients was too small to draw clinical conclusions, but these preliminary results do suggest that the assay could be used to systematically track patients' prognosis and therapy response. Most impressive for these human samples was a good correlation of the ELISA results with the cumbersome immuno-

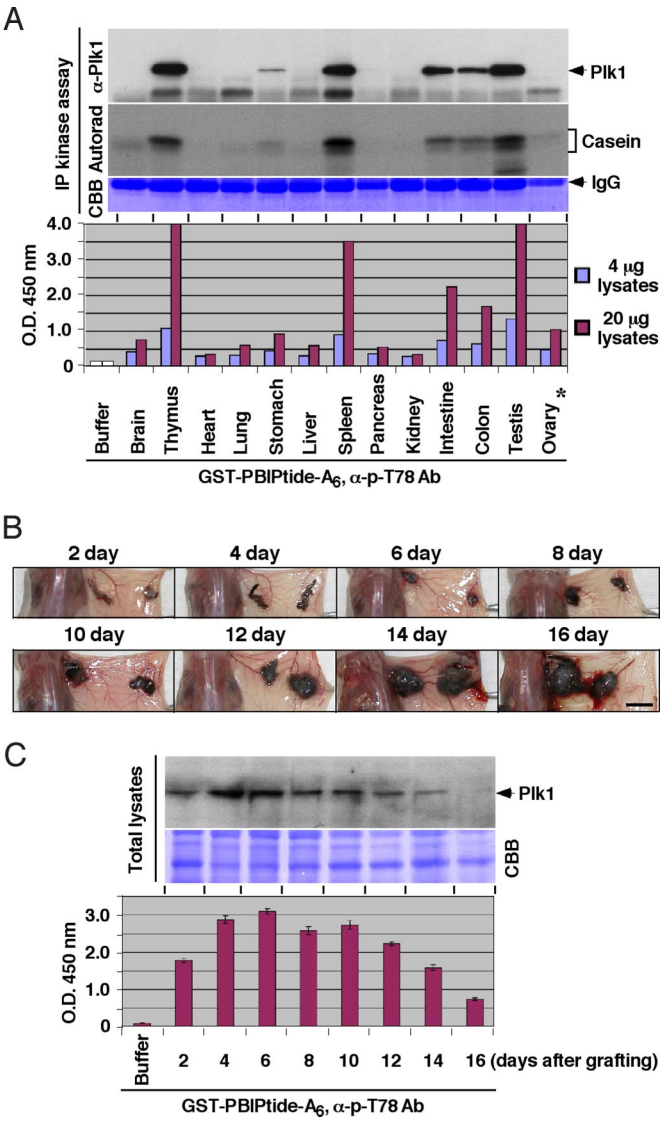

Fig. 4. Direct measurement of in vivo Plk1 kinase activity. (*A*) A tight correlation of Plk1 activities between the conventional immunocomplex kinase assay and the ELISA in various mouse tissues. (*Upper*) Total cellular proteins were prepared from the indicated tissues obtained from 1.5-month-old male and its littermate sister (for ovary only) mice. Anti-Plk1 immunoprecipitation kinase assays were carried out with 2 mg of total lysates and 6 μg of anti-Plk1 antibody for each tissue (except ovary, see below). Samples from in vitro kinase assays were separated by SDS/PAGE, transferred to PVDF membrane for exposure (Autorad), and then immunoblotted with anti-Plk1 antibody. The membrane was then stained with Coomassie (CBB). Asterisk indicates that only half the amount of total lysates (1 mg) and anti-Plk1 antibody (3 μg) was used for immunoprecipitation because of the limited amount of the ovary tissue. (*Lower*) To measure the level of the Plk1 kinase activity in the lysates, Plk1 ELISAs were carried out with either 4 or 20 μg of the same total lysates used in *Upper*. Reactions were terminated at the same time. Buffer indicates no-lysate control. (*B*) Athymic nude mice were s.c. grafted with 4×10^6 cells of B16 mouse tumor line. At the indicated days, mice were killed, and the resulting tumors were surgically removed for subsequent analyses. (Scale bar: 1 cm.) (*C Upper*) Total proteins prepared from the tumors in *B* were separated by SDS/PAGE for anti-Plk1 immunoblotting analyses and then stained with Coomassie (CBB) for loading controls. (*C Lower*) Plk1 ELISAs were carried out with 20 μg of the same total lysates. Bars indicate the standard deviation. See the BrdU and H&E stainings shown in Fig. S6 for comparison.

precipitation kinase assay. Furthermore, compared with the amount of total lysates required for the immunocomplex kinase assay, the ELISA required a 100- to 500-fold smaller amount of the sample lysates, which could be further reduced by using europium-labeled antibodies. This level of specificity and sensitivity provides a particular advantage for analyzing small human tumor samples such as needle biopsies as demonstrated in Fig. S8*B*. Because several Plk1 inhibitors are currently under clinical trials, the ELISA described herein could be of great value in monitoring the early therapy response after the treatment of patients with Plk1 inhibitors.

In light of the importance of Plk1 activity during oncogenesis and its potential as a therapeutic target, this assay could also be applied in several stages in the overall diagnostic and treatment protocols of an individual patient with cancer. Because levels of Plk1 have prognostic value in a number of cancers (5), determining the level of Plk1 activity at the time of initial tissue diagnosis may provide an important clue for proper therapeutic decisions. Because Plk1 is a marker for cells undergoing mitosis, and the level of Plk1 expression is very low in most normal tissues, this assay could also be used to monitor disease remission, progression, and recurrence. Because of its marked sensitivity, the assay may allow for the detection of circulating tumor cells with high mitotic rate from blood samples or shedding mitotic cells from solid tumors and thus foretell cancer recurrence after a broad spectrum of antiproliferative therapy. In this regard, this assay might be useful to predict the choice of certain chemotherapeutic agents that specifically target mitotic cells and to also assess response to therapy. Given that Plk1 overexpression is tightly associated with tumorigenesis and metastasis, the Plk1 ELISA demonstrated here promises to become a useful method in the diagnosis and treatment of Plk1-associated cancers as well as other pathological conditions associated with a high mitotic rate.

Materials and Methods

Construction of GST-PBIPtides and Cell Culture. Construction of GST-PBIPtides and conditions of cell culture are described in *SI Text*.

GST-PBIPtide Pulldown, Immunoprecipitation, and GST-PBD Pulldown Assays. GST-PBIPtides were expressed and purified from *E. coli* BL21 by using glutathione (GSH)-agarose (Sigma). For GST-PBIPtide pulldown kinase assays, HeLa cells were lysed in TBSN buffer [20 mM Tris-Cl (pH8.0), 150 mM NaCl, 0.5% Nonidet P-40, 5 mM EGTA, 1.5 mM EDTA, 20 mM *p*-nitrophenyl phosphate, and protease inhibitor mixture (Roche]. The resulting lysates were clarified by centrifugation at 15,000 × *g* for 20 min at 4 °C and then incubated with bead-bound GST-PBIPtide to precipitate *p*-T78 PBIPtide-bound Plk1. For efficient Plk1 precipitation with bead-bound GST-PBIPtide in Fig. 2*A*, total cellular lysates were prepared in a KC-plus [kinase reaction mixture (KC; see below) supplemented with 100 mM NaCl, 0.5% Nonidet P-40, and protease inhibitors] buffer that allows more efficient substrate phosphorylation than the TBSN buffer. For ELISA below, GST-PBIPtide bound to the agarose resin was eluted with glutathione (GSH), and then dialyzed before use. Procedures for immunoprecipitation kinase assay and GST-PBD pulldown assay are described in *SI Text*.

Direct Plk1 Kinase Assays with Total Cellular Lysates and GST-PBIPtide. To directly measure the Plk1 kinase activity with total cellular lysates, HeLa cells were lysed in KC-plus buffer and then clarified by centrifugation at 15,000 × *g* for 20 min at 4 °C. The resulting cellular proteins (≈200 μg) were incubated with bead-bound control GST or GST-PBIPtide in the presence of 10 μCi of [γ-^{32}P]ATP (1 Ci = 37GBq) at 30 °C for 30 min. Reactions were terminated by the addition of a large volume of cold KC-plus buffer. Beads were washed with KC-plus buffer and then mixed with SDS/PAGE sample buffer. The resulting samples were separated by 10% SDS/PAGE, transferred to polyvinylidene fluoride (PVDF) (Millipore), and exposed (Autorad). After immunoblotting, the same membranes were stained with Coomassie (CBB). Protein bands were excised and incorporated ^{32}P was measured by liquid scintillation spectrometry.

Antibodies. Primary antibodies used for Plk1 ELISA were anti-PBIP1 *p*-T78 antibody (Rockland Immunologicals, Inc.) and anti-Plk1 antibody (F-8) (Santa Cruz Biotechnology). Other antibodies for immunoblotting analyses are described in *SI Text*.

ELISAs with GST-PBIPtides. To coat a 96-well plate (Beckman–Coulter) with PBIPtide, soluble GST-PBIPtide$_4$ or GST-PBIPtide-A$_6$ was first diluted with 1× coating solution (KPL Inc.) to an optimal concentration (10 μg/ml). The resulting solution was then added into each well (50 μL per well) and incubated for 12–18 h at room temperature. To block the unoccupied sites, wells were washed once with PBS plus 0.05% Tween 20 (PBST), and then incubated with 200 μL of PBS plus 1% BSA for 1 h. The PBIPtide phosphorylation reaction was carried out with 100 μL of total cellular lysates in KC-plus buffer or the indicated amount of recombinant Flag-Plk1 from Sf9 cells for 30 min at 30 °C on an ELISA plate incubator (Boekel Scientific). To terminate the reaction, ELISA plates were washed 4 times with PBST. For detection of the generated *p*-T78 epitope or bound Plk1, plates were incubated for 2 h with 100 μL per well of anti-*p*-T78 or anti-Plk1 antibody at a concentration of 0.5 μg/ml. After washing the plates 5 times, 100 μL per well of HRP-conjugated secondary antibody (diluted 1:1,000 in blocking buffer) was added and the plates incubated for 1 h. Plates were then washed 5 times with PBST and then incubated with 100 μL per well of 3,3′,5,5′-tetramethylbenzidine solution (TMB) (Sigma) as substrate until a desired absorbance was reached. The reactions were stopped by the addition of 0.5 M H_2SO_4. The optical density of the samples was measured at 450 nm by using an ELISA plate reader (Molecular Devices).

ACKNOWLEDGMENTS. We thank Kenneth H. Kraemer, Michael Bustin, and Rachel H. Lee for critical reading of the manuscript; James L. Maller (University of Colorado School of Medicine, Aurora, CO) for providing the anti-Plx antibody; and Ray L. Erikson (Harvard University, Cambridge, MA) and Wei Dai (New York University School of Medicine, New York, NY) for constructs. This work was supported in part by National Cancer Institute intramural grants (to S.H.Y and K.S.L).

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