Polo-like Kinase 1-mediated Phosphorylation Stabilizes Pin1 by Inhibiting Its Ubiquitination in Human Cells*

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The Polo-like kinase 1 (Plk1) is a key regulator of mitosis. It is reported that the human peptidyl-prolyl cis/trans-isomerase Pin1 binds to Plk1 from mitotic cell extracts in vitro. Here we demonstrate that Ser-65 in Pin1 is the major site for Plk1-specific phosphorylation, and the polo-box domain of Plk1 is required for this phosphorylation. Interestingly, the phosphorylation of Pin1 by Plk1 does not affect its isomerase activity but rather is linked to its protein stability. Pin1 is ubiquitinated in HeLa S3 cells, and substitution of Glu for Ser-65 reduces the ubiquitination of Pin1. Furthermore, inhibition of Plk1 activity by expression of a dominant negative form of Plk1 or by transfection of small interfering RNA targeted to Plk1 enhances the ubiquitination of Pin1 and subsequently reduces the amount of Pin1 in human cancer cells. Since previous reports suggested that Plk1 is a substrate of Pin1, our work adds a new dimension to this interaction of two important mitotic regulators.

The peptidyl-prolyl cis/trans-isomerase (PPlase)4 Pin1 targets a subset of mitotic phosphoproteins, which contain phosphorylated serine or threonine residues preceding a proline (Ser(P)/Thr(P)-Pro) (1, 2). Prolyl bond isomerization by Pin1 facilitates subsequent enzymatic reactions like cis/trans-isomerization by trans-aryl bond-specific phosphatases like protein phosphatase 2A as demonstrated for Cdc25C, tau (3), and c-Myc (4). Overexpression of Pin1 leads to cell cycle arrest at G2 phase (5). Knockout of the ess1 gene, the Pin1 orthologue in Saccharomyces cerevisiae, or antisense depletion of human Pin1 from HeLa cells induces mitotic arrest (5–7). These data imply that Pin1 is a negative regulator for entry into mitosis and is required for progression through mitosis. Eukaryotic Pin1 homologues consist of two domains: a short N-terminal WW domain and a C-terminal catalytic domain, the PPlase-domain. The WW domain recognizes phosphorylated Pro-rich sequences and might direct substrates to arrange correctly in the Pin1 active site. Phosphorylation of Pin1 by the cAMP-dependent protein kinase A on Ser-16 impairs the nuclear localization and inhibits its binding to substrates (8). Although Pin1 interacts with different mitotic kinases like NIMA (never in mitosis A) (5), Wee1, Myt1 (9), Cdc2-phosphorylated CK2 (10), and Plk1 (9, 11), cAMP-dependent protein kinase A is the only kinase known to date to regulate Pin1 function (8).

Human Polo-like kinase 1 (Plk1), a serine/threonine kinase, which was originally identified and cloned by our group, is highly conserved from yeast to humans (12–14). It represents a key regulator of mitotic progression (15, 16) and is overexpressed in many human tumors (17, 18). Knockdown of Pin1 by antisense oligonucleotides or siRNAs results in G2/M arrest and apoptosis in different cell lines, suggesting essential roles of Plk1 in human cancer (19–21). This is in line with the observation that ectopic expression of kinase active Plk1 in fibroblasts confers a transformed phenotype as shown by the ability of these cells to form tumors in nude mice (22). At the G2/M transition, Plk1 contributes to the control of centrosome maturation (23–26), bipolar spindle formation (27), mitotic checkpoints (28, 29), and activation of the maturation-promoting factor by phosphorylation of Cdc25C (30–32) and cyclin B1 (33–35). In later stages of mitosis, Plks are involved in the activation of the anaphase-promoting complex for mitotic exit (36) and in cytokinesis (37, 38). Moreover, cells treated with siRNAs directed against Plk1 exhibited elevated levels of maturation-promoting factor activity and failed to complete anaphase or cytokinesis, revealing an important role of Plk1 during late stages of mitosis (39).

It has been reported that Pin1 binds to Plk1 from mitotic cells in vitro (9, 11). Still, the function of this interaction has not been analyzed in detail yet. Here we show the phosphorylation of human Pin1 within its catalytic domain by Plk1. This phosphorylation of Pin1 does not alter its enzymatic activity but rather is linked to its protein stability. Pin1 is ubiquitinated in HeLa S3 cells, and substitution of Glu for Ser-65 reduces the ubiquitination of Pin1. Furthermore, inhibition of Plk1 activity by expression of a dominant negative form of Plk1 or by transfection of small interfering RNA targeted to Plk1 enhances the ubiquitination of Pin1 and subsequently reduces the amount of Pin1 in human cancer cells. Since previous reports suggested that Plk1 is a substrate of Pin1, our work adds a new dimension to this interaction of two important mitotic regulators.

EXPERIMENTAL PROCEDURES

Cell Culture, Synchronization, Transfection, and Preparation of Extracts—HeLa S3, NIH-3T3, and 293T cells were grown according to the supplier’s suggestions. Cells were synchronized as described (34). Transfection was performed using FuGENE6 transfection reagent according to the manufacturer’s instructions (Roche Applied Science). Cell lysis was performed in radioimmune precipitation buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM Na2VO4, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 1 mM NaF, and protease inhibitor mixture complete (Roche Applied Science)).

Expression of Recombinant Protein, Purification, and Pull-down Assays—Chimeric His or GST-Pin1 constructs were expressed in Escherichia coli (BL21, DE3, Codon Plus) as described (34). After cell lysis, proteins were purified using Ni2+-nitrilotriacetic acid-agarose beads (Qiagen, Hilden) or glutathione-Sepharose 4B beads (Amersham Biosciences) according to the manufacturer’s recommendations. For kinase assays, proteins were eluted with imidazole or glutathione. Proteins were digested with Factor Xa (Amersham Biosciences) or tobacco...
etch virus (TEV) protease (Invitrogen) followed by dialysis in kinase buffer to remove different tags. For pull-down assays, bead-coupled proteins were washed three times with lysis buffer (250 mM NaCl, 50 mM Tris-Cl, pH 8.0, 1% Triton X-100, 10% glycerol), followed by washing with TIF buffer twice (150 mM NaCl, 20 mM Tris-Cl, pH 8.0, 1 mM MgCl₂, 0.1% Nonidet P-40, 10% glycerol). Subsequently bead-coupled proteins were incubated with extracts from nocodazole-synchronized HeLa S3 cells overnight. After extensive washing, proteins were loaded on SDS-PAGE followed by Western blot analysis.

**Kinase Assays and Pin1 Isomerization Assays**—If it is not described otherwise, 0.2 µg of GST-Plk1 or its mutants purified from S9 insect cells (40) and 2 µg of substrate were used for kinase assays as described previously (34). Synthetic peptides representing the polo-box 1 of Plk1 were subjected to kinase assays with Plk1 as described previously (41). PPlase activities of Pin1 and Pin1 variants were determined by protease-coupled and protease-free assays as described (3). For the stoichiometric analysis of Plk1 using Pin1-Cat as substrate, 4.6 pmol of GST-Plk1 and 74 pmol of Pin1-Cat were used in kinase assays in the presence of 2 µCi of [γ-³²P]ATP and 100 µM ATP at 37 °C for 30 min based on our previous dose and time kinetics with Plk1 and Pin1-Cat. An Eastman Kodak Co. Geldoc system and phosphor imager were used to quantify the amount of incorporated radioactive phosphate in Pin1-Cat.

**Antibodies, siRNAs, Western Blots, and Immunoprecipitations**—Monoclonal Plk1 antibodies used for immunoprecipitations were purchased from Zymed Laboratories Inc. (San Francisco, CA). Rabbit serum IgG, β-catenin antibodies, and polyclonal Plk1 antibodies for Western blot analyses were from Santa Cruz (Heidelberg, Germany). β-actin antibodies were from Sigma. Polyclonal Pin1 antibodies were kind gifts from Dr. Vincent (Quebec, Canada). Polyclonal HA antibodies were from Clontech (Heidelberg, Germany), and monoclonal FLAG-antibodies (M2) were purchased from Sigma. Application of siRNA targeting Plk1 (accession number X75932) corresponding to positions 1416–1438 (sPlk) and a scrambled version as control (sPlks) was performed as described (19). Western blot analyses and immunoprecipitations were performed as described (42).

**Plasmids and Site-directed Mutagenesis**—pGEX 4T-1-Pin1 was kindly provided by Dr. Schnapp (Roche Applied Science). Pin1 constructs were generated by PCR using the following primers containing EcoRI and XhoI sites: Pin-E-5 (GTTGAATTCATGGCGGACGAG-1438 (siPlk) and a scrambled version as control (siPlks) was performed as described (43). pcDNA3.1/FLAG-ubiquitin was used as described (44).

**Generation of Stable Cell Clones for the Expression of Short Hairpin RNA (shRNA)-Plk1—T-Rex HeLa cells stably expressing bacterial TetR were obtained from Invitrogen GmbH (Karlsruhe, Germany) and cultured in Eagle’s minimal essential medium with Earle’s balanced salt solution and Glutamax (Sigma) containing 10% Tet system-approved fetal calf serum (PAA Laboratories GmbH, Pasching, Austria), penicillin/streptomycin (Invitrogen), and 5 µg/ml blasticidin (Invitrogen). T-Rex HeLa cells were transfected with different types of designed expression plasmids, pUS, pDS, and pUS/DS (45). FuGENE 6 (Roche Applied Science) was used as transfection reagent according to the manufacturer’s instructions. Selection of transfected cells was accomplished by using culture medium containing 1.5 mg/ml Genetricin (G418; Invitrogen) and started 24 h after transfection. Altogether, selection of resistant cells lasted for a period of 25 days, whereas culture medium with selective antibiotic was changed every 3 days. After 25 days, Genetricin-resistant cells were trypsinized, centrifuged, and resuspended in culture medium. The densities of different cell suspensions were measured by using a hemacytometer. Suspensions were diluted to a final volume containing one cell/300 µl and plated onto 96-well plates for subsequent expansion.

Stably transfected HeLa cells were synchronized to the G1/S boundary by double-thymidine block. Briefly, cells were treated with 2 mM thymidine for 16 h, released with washing from fresh medium, and then induced with 5 µg of doxycycline (Sigma). Cells were grown for 8 h and subjected again to thymidine for a further 16 h. Finally, cells were released to the nocodazole-containing medium and harvested at the indicated time points.

**RESULTS**

**Plk1 and Pin1 Interact in Vitro**—Two studies investigating the role of Pin1 during mitosis describe the physical interaction of Pin1 with the mammalian Ser/Thr-kinase Plk1. Whereas Plk1 from mitotic cell extracts was efficiently bound to bacterially expressed Pin1, Plk1 from interphase extracts failed to associate with Pin1, indicating that this interaction is restricted to mitosis (9, 11). To study this interaction in more detail, we investigated the association of Pin1 deletion proteins with Plk1 from mitotic HeLa S3 cells. In pull-down analyses, full-length human Pin1 was able to bind endogenous Plk1 from HeLa S3 cells (Fig. 1A). A more detailed investigation on the binding properties of different Pin1 domains revealed that the catalytic subunit of Pin1 was not capable of binding to Plk1. In contrast, a region of Pin1 containing the WW domain was required for the association with Plk1.
FIGURE 1. Plk1 interacts with and phosphorylates Pin1. A, interaction of Plk1 and Pin1 in vitro. Top, GST and GST-Pin1 proteins were incubated with extracts from nocodazole synchronized HeLa S3 cells followed by Western blot with Plk1-specific antibodies. 20% of the cell lysate was loaded as input control (bottom). B, phosphorylation of Pin1 by Plk1. GST and GST-Pin1 were subjected to kinase assays with Plk1 expressed in Sf9 cells (top). Input of GST proteins was visualized by Coomassie staining (bottom). C, Plk1 phosphorylates Pin1 in its PPIase domain. Each of the C-terminal truncated Pin1 proteins was incubated with Plk1 in the kinase assay (top). Coomassie staining of Pin1 proteins in the same membrane served as input control (bottom). D, a schematic representation of C-terminal truncated Pin1 proteins and their phosphorylation intensity. E, the phosphorylation in Pin1-(1–70) by Plk1 is specific. GST-Pin1-(1–70) was subjected to kinase assays with Plk1WT or the hyperactive Plk1T210D or kinase-dead Plk1K82M (top). Coomassie staining of GST-Pin1-(1–70) proteins in the same membrane served as input control (bottom).
Ser-65 within the PPlase Domain of Pin1 Is the Major Phosphorylation Site for Plk1—To analyze whether Pin1 serves as substrate for Plk1, we performed in vitro kinase assays. Plk1 from SF9 cells phosphorylated GST-Pin1 (Fig. 1B). To narrow down the phosphorylation site in Pin1, we subjected different truncated Pin1 proteins to kinase assays (Fig. 1C). Whereas Pin1-(1–62) did not serve as substrate, Plk1 phosphorylated Pin1-(1–70) (Fig. 1, C and D). The enhanced phosphorylation of Pin1-(1–70) and Pin1-(1–87) compared with full-length Pin1 could be due to steric reasons.

We further analyzed the specificity of the Plk1-mediated phosphorylation by subjecting truncated Pin1 proteins to kinase tests with hyperactive Plk1\textsuperscript{T14KD} or kinase-deficient Pin1\textsuperscript{K82M} (46). Whereas Pin1-(1–70) was more efficiently phosphorylated by Plk1\textsuperscript{T14KD} compared with wild-type Plk1, phosphorylation of Pin1-(1–70) by Plk1\textsuperscript{K82M} was not detected (Fig. 1E). Taken together, phosphorylation of Pin1 correlates specifically with the kinase activity of Plk1.

Since the association of Plk1 with Pin1 requires an N-terminal region of Pin1 that includes the WW domain, we analyzed the role of the WW domain for the phosphorylation of Pin1 by Plk1 in more detail. Furthermore, considering that GST-Pin1-(1–70) is a better substrate for Plk1 compared with full-length Pin1 fused to GST (Fig. 1, C and D), we asked whether steric aspects of Pin1 and its GST moiety could be responsible for the reduced phosphorylation in full-length GST-Pin1. To address these questions, we expressed different Pin1 proteins (full-length Pin1 GST-Pin1-(1–70) and the His-tagged catalytic domain of Pin1 (His-Pin1-Cat) in E. coli and removed the GST tag from full-length Pin1 by cleavage with TEV protease. After purification under native conditions, proteins were subjected to kinase assays in increasing concentrations of the substrate (Fig. 2A). The WW domain was not phosphorylated by Plk1. In contrast, subjecting increasing amounts of the full-length protein or the catalytic domain to kinase tests resulted in linear increasing phosphorylation signals. As shown in Fig. 1, C and D, GST-Pin1 exhibited a reduced phosphorylation signal compared with its deleted versions. Removal of the N-terminal GST tag indeed improved phosphorylation of full-length Pin1 by Plk1 (Fig. 2A), suggesting that the GST moiety contributed to steric hindrance. In summary, the WW domain is dispensable for the phosphorylation of the catalytic domain of Pin1 by Plk1.

In contrast to Pin1-(1–62), Pin1-(1–70) served as substrate; therefore, we reasoned that phosphorylation of Pin1 occurs between aa 62 and 70. This short stretch encompasses two serine residues at positions 65 and 67. Whereas Pin1-Cat\textsuperscript{WT} and Pin1-Cat\textsuperscript{S65A} were good substrates for Plk1, substitution of Ala for Ser at position 65 in Pin1-Cat nearly abolished the Plk1-mediated phosphorylation (Fig. 2B). Furthermore, we used mutated full-length Pin1 for kinase assays with Plk1. The phosphorylation of GST-Pin1\textsuperscript{S65A} compared with GST-Pin1\textsuperscript{WT} or GST-Pin1\textsuperscript{S67A} was not completely abolished but was clearly reduced (Fig. 2C). Our data suggest that Ser-65 within the PPlase domain is the major phosphorylation site in Pin1 for Plk1.

The Polo-box 1 of Plk1 Is Required for the Phosphorylation of Pin1—Recently, it was reported that the polo-box domain (PBD), which consists of two polo-boxes (PB1 and PB2) is required for substrate binding (47, 48). We demonstrated previously that membrane-permeable peptides containing the highly conserved portion of the polo-box 1 (PB1) of human Plk1 (aa 410–429) inhibit multiple functions of Plk1 (41). This inhibitory effect was hardly observed when we used a mutated derivative of this peptide in which the crucial residues were changed (W414F, V415A, L427A). To explore the impact of the PBD on Pin1 phosphorylation by Plk1, we utilized these synthetic peptides in a concentration range from 1.56 to 50 \textmu M for in vitro kinase assays (Fig. 2D). The wild-type polo-box peptide (number 1) and its mutated form (number 2) at high concentrations of 25–50 \textmu M strongly reduced the phosphorylation signal of full-length Pin1 by Plk1. In contrast, the carrier peptide (number 3) by itself as control exhibited only a weak inhibitory impact on Plk1 kinase activity. In a concentration range of 6.25–12.5 \textmu M, the inhibitory effect of the wild-type polo-box peptide differed from that of the mutated form; the wild-type polo-box peptide induced at least a 2-fold inhibition compared with its mutated form. In addition, the carrier peptide (number 3) showed no effect at all at a concentration of 6.25 \textmu M (Fig. 2D). The results suggest that the wild-type polo-box peptide inhibits specifically the phosphorylation of Pin1 by Plk1. Thus, the PBD of Plk1 is required for the phosphorylation of Pin1.

Phosphorylation on Ser-65 Does Not Alter the Isomerase Activity of Pin1—It has been reported that mimicking phosphorylation on Ser-67 by substitution to Glu abolishes the catalytic activity of Pin1 (3). Since Plk1 phosphorylates Pin1 on Ser-65, which is in the immediate vicinity of Ser-67, we wondered whether a similar inhibitory effect on the catalytic activity of Pin1 could be induced by Plk1. To address this question, we measured at first the stoichiometry of the kinase reaction to ensure efficient phosphorylation of Pin1 by Plk1. Our analysis revealed an overall incorporation of 1.1 mol of phosphate/mol of Pin1, suggesting one major site of phosphate incorporation. Subsequently, we investigated the isomerase activity of Plk1-phosphorylated Pin1. When Pin1-Cat was incubated with Plk1 in the presence or absence of ATP, its isomerase activity remained unchanged (Fig. 3A). It is noted that Plk1 and ATP had no contaminating isomerase activity (Fig. 3A). In addition, the isomerase activities of Pin1\textsuperscript{WT} and Pin1\textsuperscript{S65E} differed only slightly (Fig. 3B). In summary, neither phosphorylation of the catalytic domain of Pin1 by Plk1 nor mimicking phosphorylation on Ser-65 in full-length Pin1 alters the PPlase activity of Pin1 significantly.

Mimicking Phosphorylation on Ser-65 Results in Stabilization of Pin1 in Human Cells—Multiple expression analyses using different point mutations of Pin1 revealed elevated levels of Pin1\textsuperscript{S65E} compared with Pin1\textsuperscript{WT} or Pin1\textsuperscript{S65A} in HeLa S3 cells (data not shown). To test the hypothesis that substitution of Glu for Ser-65 enhances the translation efficacy of full-length Pin1 proteins, we utilized reticulocyte lysates for the expression of different mutant Pin1 proteins. Pin1\textsuperscript{S65A} and Pin1\textsuperscript{S65E} were translated to a similar level in this system (data not shown), indicating that the enhanced level of Pin1\textsuperscript{S65E} in mammalian cells might be due to other cellular mechanisms. Next, we considered protein stability as a determinant for the different protein levels observed. We treated cells 24 h posttransfection with the proteasome inhibitor MG132 (10 \textmu M, 16 h) and analyzed Pin1 levels. The efficiency of MG132 to inhibit proteasomal activity was confirmed by incubating the membrane with antibodies against \beta-catenin (Fig. 4A, top). Compared with Pin1\textsuperscript{WT}, the level of Pin1\textsuperscript{S65E} was remarkably elevated in HeLa S3 cells (Fig. 4A, bottom, lanes 3 and 5). Moreover, the proteasome inhibitor MG132 was able to stabilize Pin1 proteins (Fig. 4A, bottom, lanes 4 and 6).

We presumed that Pin1\textsuperscript{S65E} might be more resistant to proteasome-mediated degradation compared with its wild-type counterpart. To analyze the stability of Pin1\textsuperscript{S65E} in more detail, we measured the half-life of different Pin1 proteins by using the protein synthesis inhibitor cycloheximide (CHX). Plasmids encoding Pin1\textsuperscript{WT}, Pin1\textsuperscript{S65A} and Pin1\textsuperscript{S65E} were transfected into HeLa S3 cells, and 24 h posttransfection cells were treated with CHX (75 \textmu M) for the indicated time. Based on Fig. 4B, Pin1\textsuperscript{WT} and Pin1\textsuperscript{S65A} (top and middle panels) exhibited half-lives of \textasciitilde2.8 and 3.5 h, respectively. Substitution of Glu for Ser-65 extended the half-life of the protein to 7.5 h. The degradation of Pin1 proteins in the presence of CHX could be antagonized by MG132 (Fig. 4B, last lane).
Taken together, phosphorylation of Pin1 on Ser-65 prolongs the half-life of Pin1 by rendering it more resistant to proteasome-mediated proteolysis.

FIGURE 2. Plk1 phosphorylates Ser-65 in the catalytic domain of Pin1. A, Plk1 phosphorylates Pin1 in its catalytic domain in a dose-dependent manner. His-tagged Pin1-WW (aa 1–54), Pin1-Cat (aa 44–163), and GST-tagged full-length Pin1 were expressed in bacteria, followed by cleavage with TEV protease to remove the N-terminal GST tag from full-length Pin1. Increasing amounts of Pin1 (0.5–2.5 μg) were subjected to kinase assays with Plk1 in the presence or absence of ATP, followed immediately by isomerase assays. The isomerase activities are illustrated in a diagram (top), and the comparison is listed in a table (bottom). B, mimicking phosphorylation of Ser-65 in Pin1 exerts no substantial effect on the catalytic activity of Pin1. The isomerase assays were performed with full-length Pin1-WT or Pin1–65E, and the results are presented in B.

FIGURE 3. Phosphorylation of Pin1 by Plk1 or mimicking phosphorylation on Ser-65 does not influence the PPIase activity. A, phosphorylation of Pin1-Cat by Plk1 prior to isomerase assays does not alter the enzymatic activity of Pin1-Cat. Pin1-Cat proteins were subjected to kinase assays with Plk1 in the presence or absence of ATP, followed immediately by isomerase assays. The isomerase activities are illustrated in a diagram (top), and the comparison is listed in a table (bottom). B, mimicking phosphorylation of Ser-65 in Pin1 exerts no substantial effect on the catalytic activity of Pin1. The isomerase assays were performed with full-length Pin1-WT or Pin1–65E, and the results are presented in B.

expressed in bacteria, followed by cleavage with TEV protease to remove the N-terminal GST tag from full-length Pin1. Increasing amounts of Pin1 (0.5–2.5 μg) were subjected to isomerase assays with Plk1 (top). The input was visualized by Coomassie staining (bottom). B, Ser-65 within the catalytic domain of Pin1 is the major phosphorylation site for Plk1. GST-Pin1-Cat proteins were expressed in bacteria, treated with TEV protease to remove the GST moiety, and subsequently subjected to kinase assays with Plk1 (top). Substrates in the same gel were stained with Coomassie as input control (bottom). C, Plk1 targets Ser-65 in full-length Pin1. GST-Pin1 proteins were subjected to kinase assays with Plk1 (top). The subsequent Coomassie staining served as input control (bottom). D, membrane-permeable polo-box peptides inhibit phosphorylation of Pin1 by Plk1. In vitro kinase assays were performed by using full-length Pin1 as substrate with the indicated concentrations of synthetic wild-type peptide of polo-box 1 (aa 410–429, termed number 1 (No. 1)) derived from Plk1 (41), its mutated form (No. 2), and the control antennapedia peptide (No. 3). Phosphorylated products were separated using a 12% SDS-PAGE. Top, autoradiograph of phosphorylated Pin1. Pin1-WT or Pin1–65E, and GST-Pin1 proteins in the same gel were stained with Coomassie as input control (middle). Bottom, intensity of Pin1 phosphorylation was standardized to Pin1 input using a Kodak gel documentation system.
Plk1 Regulates Stability of Pin1

**Figure 4. Mimicking phosphorylation on Ser-65 leads to enhanced stability of Pin1, and Pin1 is ubiquitinated in human cells.** A, HeLa S3 cells were transfected with the indicated HA-tagged Pin1 constructs. 24 h posttransfection, cells were treated with methanol alone (lanes 1, 3, and 5) or MG132 in methanol at 10 μM for 16 h (lanes 2, 4, and 6). Pin1 proteins were detected using polyclonal rabbit Pin1 antibodies (bottom). Subsequently, the membrane was treated with β-actin antibodies to confirm equal loading (middle) and with β-catenin antibodies to verify the effect of MG132 (top). Due to different cloning strategies, Pin1-WT migrated slightly faster compared with its mutated derivative (see "Experimental Procedures"). B, mimicking phosphorylation on Ser-65 in Pin1 leads to prolonged half-life of the protein. The indicated HA-tagged Pin1 constructs were transfected into HeLa S3 cells. 24 h posttransfection, cells were treated with 75 μM CHX for the indicated time. In addition to CHX, cells were treated with MG132 (50 μM) to antagonize the CHX-mediated effect. Polyclonal Pin1-specific antibodies were utilized for Western blot analysis. C, Pin1 is ubiquitinated in human cells. HeLa S3 cells were double transfected with HA-Pin1 and FLAG-ubiquitin. In the case of transfecting FLAG-ubiquitin alone (lane 5) or HA-Pin1 alone (lane 6), amounts of cDNA were adjusted with pEGFP-C1 to warrant equal conditions for transfections. 10% of cell extract was loaded as input control of untransfected (lane 1) or double transfected cells (lane 2). Precipitation of extracts from double-transfected cells with IgG (c) served as negative control (lane 3). Expression of HA-Pin1 was analyzed by subjecting 30 μg of the same lysates to Western blot analysis (bottom). Ub, ubiquitin; IP, immunoprecipitation.

Pin1 Is Ubiquitinated in Human Cells—Ubiquitination, the covalent conjugation of ubiquitin to proteins, is essential for the degradation of proteins by the proteasome (49). Since mimicking phosphorylation on Ser-65 leads to enhanced stability of Pin1 due to improved resistance to proteasomal degradation, we hypothesized that phosphorylation might regulate the ubiquitination of Pin1. We analyzed the ubiquitination of Pin1 in HeLa S3 cells by cotransflecting HA-Pin1 and FLAG-ubiquitin. Pin1 was immunoprecipitated with specific antibodies, and the covalently bound ubiquitin was detected via its FLAG tag. A specific signal of ubiquitinated Pin1 was only detectable when cells were cotransfected with HA-Pin1 and FLAG-ubiquitin (Fig. 4c, lane 4). The upper signal in Fig. 4c (lane 4) was confirmed as Pin1-ubiquitin conjugate by Western blot analysis using Pin1-specific antibodies (Fig. 5a, lower left). In control immunoprecipitations using IgG, no ubiquitin signal was observed (Fig. 4c, lane 3). This was also the case for untransfected cells (Fig. 4c, lane 7) and for cells transfected with FLAG-ubiquitin (Fig. 4c, lane 5) or with HA-Pin1 (Fig. 4c, lane 6) alone. Thus, Pin1 is ubiquitinated in HeLa S3 cells.

**Down-regulation of Plk1 Activity Enhances Ubiquitination of Pin1 in Human Cells**—Next, we applied two techniques to study the impact of Plk1 activity on the ubiquitination of Pin1 in 293T cells. First, we analyzed the ubiquitination of Pin1 after down-regulation of Plk1 by siRNA. Second, we cotransfected cells with dominant negative Plk1K82M or hyperactive Plk1T210D (46, 50). We chose 293T cells for this triple transfection experiment, because better transfection efficiencies can be achieved compared with HeLa S3 cells. Cell lysates were subjected to immunoprecipitation and Western blot analysis (Fig. 5a). We quantified signals of ubiquitinated Pin1 as shown in Fig. 5a. As illustrated in Fig. 5b, down-regulation of Plk1 by using siRNA (siPlk) enhanced the ubiquitination of Pin1 4.5-fold compared with a scrambled version of this Plk1-specific siRNA (siPlks). Furthermore, cotransfection of kinase-dead Plk1K82M also increased the ubiquitination of Pin1 3.7-fold in comparison with Plk1T210D. Thus, down-regulation of Plk1 activity leads to enhanced ubiquitination of Pin1.

Plk1 Depletion Leads to Down-regulation of Pin1 Levels during Mitosis—Our previous experiments demonstrated that Plk1 influences the ubiquitination and thereby the stability of Pin1. Regarding this observation, we subsequently tested whether down-regulation of Plk1 by RNA interference translates to reduced levels of Pin1 protein in cells. For this purpose, we used our recently developed inducible genetic system for shRNA-mediated gene silencing (45). This system uses the Tet repressor and a tetracycline-responsive derivative of the H1 promoter for the conditional expression of shRNA targeted to Plk1 in HeLa cells. Prior to the analysis of Pin1 levels in mitosis, we generated HeLa cells that harbor this inducible RNA interference system as a stably integrated cassette for the expression of Plk1-shRNA. Compared with noninduced cells, the levels of endogenous Pin1 decline to 20% after doxycycline-induced Plk1-shRNA expression (data not shown). Because Plk1 is an important element controlling the G2/M transition, cell populations were synchronized and tested for a correlation of the levels of Pin1 and Plk1. HeLa cells were synchronized at the G2/M transition by using a double thymidine block and released into medium containing nocodazole, and the kinetics of endogenous Pin1 levels was monitored in

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the presence or absence of Plk1 knockdown (Fig. 6). Cells treated with doxycycline for the induction of shRNA-Plk1 expression showed effective reduction of Plk1 protein predominantly between 8 and 16 h after release from thymidine. In these cells, Plk1 reduction coincided with a decrease of Pin1 protein as shown 8–16 h after release, indicating that the regulation of Pin1 protein stability in mitotic cells is mediated by Plk1. Furthermore, to investigate the role of Ser-65 of Pin1 in this process, we utilized the inducible shRNA system as described in the legend to Fig. 6A to analyze the impact of Plk1 down-regulation on exogenous Pin1 proteins (Pin1WT, Pin1S65A, and Pin1S65E). Expectedly, as already shown for endogenous Pin1, the amount of exogenous Pin1WT protein reflected the protein levels of down-regulated Plk1 in mitotic cells. By contrast, levels of both Pin1S65A and Pin1S65E remained unchanged independent of the level of endogenous Plk1. Thus, the residue Ser-65 is crucial for the regulation of Pin1 protein levels in mitotic cells by Plk1.

**DISCUSSION**

In the present work, we could demonstrate that Plk1, Ser-65 in the catalytic domain of Pin1 is the major phosphorylation site for Plk1. Mimicking phosphorylation on this residue led to elevated levels of Pin1 in transfected cells. Down-regulation of Plk1 activity enhanced the ubiquitination of Pin1 in human cells. Using RNA interference, we could demonstrate that reduction of Plk1 affected the mitotic level of Pin1. Our data revealed that Plk1 activity positively regulates the stability of Pin1.

Like Plk1, Pin1 is attracting more and more attention as an important regulator of the cell cycle. However, the knowledge of the control of Pin1 is still limited. It could be demonstrated that phosphorylation of the Pin1 WW domain on Ser-16 by cAMP-dependent protein kinase A impairs its ability to function as a Ser(P)/Thr(P) binding module (8). As a consequence, Pin1 function is completely inhibited as demonstrated by overexpression of Pin1S65E. Phosphorylation of the WW domain of Pin1 by cAMP-dependent protein kinase A is the only known posttranslational modification regulating Pin1 function to date. In this report, we identified Plk1 as a kinase responsible for phosphorylating the catalytic domain of Pin1. This reaction is reduced by competitive inhibition using synthetic polo-box peptides, suggesting that the PBD of Plk1 is required for this phosphorylation. It has been demonstrated that the PBD plays multiple roles for binding to substrates, subcellular localization, and regulation of the kinase activity of Plk1 (51–53). Consistent with the function of the PBD, our data imply that the PBD is also involved in the phosphorylation of Pin1 by Plk1.

Furthermore, we identified Ser-65 within the PPIase domain of Pin1 as the major phosphorylation site for Plk1. Surprisingly, neither the phosphorylation of Pin1-Cat by Plk1 nor mimicking phosphorylation on Ser-65 in full-length Pin1 alters the enzymatic activity of Pin1. Ser-65 is in the close vicinity of the two arginine residues (Arg-68 and -69) that are involved in the binding to the targeted Ser(P)/Thr(P) in substrate proteins and are essential for the recognition of the catalytic transition state of the Pin1-substrate complexes. Substitution of leucines or alanines for arginine residues (Arg-68 and -69) reduces the PPIase activity of Pin1 by 0.25 and 0.4%, respectively (3, 54). Moreover, Ser-67, which precedes Ser-65, is also important for Pin1 activity, because mimicking phosphorylation on this residue led to elevated ubiquitination of Pin1 in transfected cells. Down-regulation of Plk1 activity enhanced the ubiquitination of Pin1 in human cells. Using RNA interference, we could demonstrate that reduction of Plk1 affected the mitotic level of Pin1. Our data revealed that Plk1 activity positively regulates the stability of Pin1.

Like Plk1, Pin1 is attracting more and more attention as an important regulator of the cell cycle. However, the knowledge of the control of Pin1 is still limited. It could be demonstrated that phosphorylation of the Pin1 WW domain on Ser-16 by cAMP-dependent protein kinase A impairs its ability to function as a Ser(P)/Thr(P) binding module (8). As a consequence, Pin1 function is completely inhibited as demonstrated by overexpression of Pin1S65E. Phosphorylation of the WW domain of Pin1 by cAMP-dependent protein kinase A is the only known posttranslational modification regulating Pin1 function to date. In this report, we identified Plk1 as a kinase responsible for phosphorylating the catalytic domain of Pin1. This reaction is reduced by competitive inhibition using synthetic polo-box peptides, suggesting that the PBD of Plk1 is required for this phosphorylation. It has been demonstrated that the PBD plays multiple roles for binding to substrates, subcellular localization, and regulation of the kinase activity of Plk1 (51–53). Consistent with the function of the PBD, our data imply that the PBD is also involved in the phosphorylation of Pin1 by Plk1.

Further studies revealed that Pin1 proteins could be stabilized in the presence of the proteasome inhibitor MG132, indicating that the degradation of Pin1 is catalyzed by the proteasome. Since the degradation of proteins by the proteasome requires ubiquitination, we analyzed the impact of Plk1 activity on the ubiquitination of Pin1 in human cells. By mutational analysis, we demonstrated that Pin1S65E was less ubiquitinated and exhibited a prolonged half-life compared with wild-type Pin1. Since in vitro phosphorylation of Ser-65 is mediated by Plk1, this result implies a positive effect of Plk1 on the stability of Pin1 by inhibiting its
ubiquitination and subsequently its degradation by the proteasome. Further evidence came from the observation that down-regulation of Plk1 activity by different approaches (siRNA, dominant negative Plk) resulted in an enhanced ubiquitination of Pin1. Moreover, down-regulation of Plk1 in HeLa cells by an RNA interference-based approach revealed the direct correlation between Plk1 down-regulation and reduced levels of Pin1 protein during mitosis. Thus, our analyses demonstrate that the activity of Plk1 is linked to the regulation of the protein stability of Pin1. Moreover, Ser-65 in Pin1 seems to be important for the regulation of Pin1 protein levels by Plk1 in mitotic cells, since Pin1WT but not Pin1S65A or Pin1S65E respond to down-regulation of Plk1. Surprisingly, whereas the substitution of Glu for Ser-65 in Pin1 translated to prolonged half-life of the protein (7.5 versus 2.8 h; see Fig. 4B) and down-regulation of Plk1 led to a reduced stability of endogenous and exogenous Pin1 (Fig. 6), the corresponding substitution to Ala did not result in a reduced half-life of Pin1S65A (Fig. 4B). This observation indicates that Ser-65 plays an important role for the stability of Pin1. i.e., any alteration at this position (phospho-Ser-65, S65A, or S65E) seems to influence the destruction of Pin1, which could be due to altered recognition of this motif by mediators of protein stability or to alterations in the protein conformation of Pin1. Further studies are required to elucidate the mechanism by which mutation of Ser-65 translates to altered stability of Pin1. Nevertheless, our analyses demonstrate that Plk1 is associated with the regulation of Pin1 stability in mitotic cells and that Ser-65 of Pin1 is important for this Plk1-mediated regulation.

Furthermore, we cannot rule out that in addition to Plk1 other kinases contribute to the regulation of Pin1 degradation. As reported by us and others, Plk1 acts synergistically with other kinases to regulate cyclin B1 (34) and more recently Emi2 (55). In the case of cyclin B1, we could demonstrate that priming phosphorylation events by maturation-promoting factor or mitogen-activated protein kinase enhanced phosphorylation of Ser-133 and enabled Plk1 to phosphorylate residue(s) other than Ser-133 that were previously inaccessible for Plk1 (34). Therefore, we assume that priming phosphorylations of serine/threo-

**FIGURE 6.** Plk1 expression is closely correlated with the level of Pin1 in HeLa cells. A, cells, stably transfected with a doxycycline-inducible genetic element for the expression of Plk1-specific shRNA, were synchronized with double thymidine and then released to the nocodazole-containing medium. Cells were induced with doxycycline after the first thymidine treatment. Finally, cells were harvested at the indicated time points, and Western blot analyses were performed by applying Plk1 antibodies (top), Pin1 antibodies (middle) and β-actin antibodies (bottom). The later served as loading control. B, cells harboring an inducible shRNA system targeting Plk1 were additionally transfected with plasmids expressing Pin1WT, Pin1S65A, and Pin1S65E and treated as described in A. At the indicated time points, cellular extracts were prepared for Western blot analyses by using antibodies as in A.
nine residues in the vicinity of Ser-65 could contribute to the regulation of Pin1 stability.

Ubiquitination is essential for the degradation of proteins whose levels are regulated either constitutively or in response to changes in the cellular environment (49). While this article was in preparation, novel reports revealed that Plk1 positively regulates the ubiquitination of two M-phase inhibitors: Wee1 and the early mitotic inhibitor 1 (Emi1) (56, 57). Considering these two publications and our work, Plk1 activity might regulate the ubiquitination of mitotic enzymes in two ways: (i) by promoting the degradation of inhibitors of mitosis (Wee1, Emi1) or (ii) by stabilizing enzymes essential for mitotic progression (Pin1). Furthermore, Lu and colleagues (58, 59) proposed that overexpression of Pin1 promotes the degradation of M-phase inhibitors: Wee1 and the early mitotic inhibitor 1 (Emi1) (56, 57). Considering these two publications and our work, Plk1 activity might regulate the ubiquitination of mitotic enzymes in two ways: (i) by promoting the degradation of inhibitors of mitosis (Wee1, Emi1) or (ii) by stabilizing enzymes essential for mitotic progression (Pin1). Furthermore, Lu and colleagues (58, 59) proposed that overexpression of Pin1 contributes to tumorigenesis. Strikingly, Plk1 activity is highly elevated in tumor cell lines and in primary neoplastic tissues (13, 17, 18, 60). These observations encourage us to suggest that one of the mechanisms contributing to tumorigenesis by Plk1 might be the stabilization of Pin1.

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