Binding of Phosphatase Inhibitor-2 to Prolyl Isomerase Pin1 Modifies Specificity for Mitotic Phosphoproteins†

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ABSTRACT: Inhibitor-2 (I-2) is the most ancient protein that selectively recognizes type-1 protein phosphatase and is phosphorylated by CDK1-cyclinB during mitosis at Thr72 in a conserved PXTP site. Pin1 is a peptide prolyl cis/trans isomerase conserved among eukaryotes that specifically reacts with proteins phosphorylated at Ser/Thr-Pro sites. We tested phospho-T72-I-2 as a substrate for Pin1 and discovered that unphosphorylated I-2 bound Pin1 with micromolar affinity and phosphorylation of the PXTP site or truncation of I-2 reduced binding 10-fold. Ectopic Pin1 coprecipitated endogenous I-2 and ectopic I-2 coprecipitated endogenous Pin1, but only in the absence of detergents, which may account for the interaction not being detected previously. Endogenous I-2 and Pin1 colocalized in HeLa cells and showed nuclear−cytoplasmic redistribution in response to cell density, suggestive of their association in living cells. Recombinant Pin1 binding to different phosphoproteins in mitotic cell extracts was modulated by I-2, and binding to individual mitotic phosphoproteins was increased, decreased or unaffected by I-2, showing that I-2 allosterically modifies Pin1 specificity. This was confirmed by mutation of Ser16 to Ala in the Pin1 WW domain that eliminated I-2 binding and abrogated I-2 effects on Pin1 binding to different phosphoproteins. A S16E mutation to mimic Pin1 phosphorylation restored binding to both I-2 and phospho-T72-I-2, indicating that phosphorylation of both proteins governs their interaction. The results reveal a novel function for I-2, and suggest phosphorylation-dependent regulation of Pin1 specificity during entry and exit of mitosis, in other phases of the cell cycle, and in multiple cell signaling processes.

Phosphorylation of Ser/Thr-Pro sites in proteins has important functions in cell signaling. Cyclin-dependent kinases (CDKs), mitogen-activated protein kinases (MAPKs), and glycogen synthase kinase 3 (GSK-3) are referred to as proline-directed kinases, because, distinct from other kinases, they depend on the Pro residue immediately following Ser/Thr for substrate recognition (*1*). In peptides Pro residues have the unique property of existing in two distinct isomers, cis and trans, therefore they can provide a potential backbone switch in the polypeptide chain that is controlled by isomerization around the peptidyl−prolyl bond. This intrinsically slow conversion is catalyzed by multiple families of cis/trans peptidyl-prolyl isomerases (PPIases) that participate in protein folding and refolding (*2*, *3*), with 13 genes in yeast (*4*). Isomerization of protein backbone cis/trans conformation at Pro residues is crucial for all eukaryotes, but phosphorylation by the Pro-directed kinases reduces the rate for interconverting Thr-Pro dipeptides and renders these sites resistant to conventional FKBP and cyclophilin families of PPIases.

The enzyme Pin1, related to the essential yeast gene ESS1 (*5*), is an unconventional PPIase discovered about 10 years ago (*6*) as a 2-hybrid prey for the centrosomal NIMA kinase (Nek2A). Pin1 has the special property of recognizing (Ser/Thr)-Pro sites only after they are phosphorylated, primarily by CDKs (*7*−*12*). Many of these sites are recognized by the MPM-2 monoclonal antibody and are phosphorylated at the onset of mitosis (*13*−*15*). More recent results suggest there may be a wider repertoire of Pin1 substrates, including cyclinD1, p65/RelA subunit of NF-kB, the steroid receptor coactivator SRC-3, Bruton Tyr kinase, and the neuronal microtubule-binding protein tau that is abnormally phosphorylated in Alzheimer's disease (*16*−*21*). Deletion of the single Pin1-related gene in *Drosophila* or yeast is lethal (*5*, *22*), and antisense knockdown of Pin1 imposes arrest in mitosis (*23*), consistent with a Pin1 function in mitotic chromosome condensation involving binding to topoisomerase (*24*). Pin1 knockout mice are viable without general defects in development or cell cycle control (*25*, *26*). Some unknown compensatory mechanisms, perhaps involving other PPIases, such as the previously unrecognized Pin1-like gene that encodes a protein with phosphosite prolyl isomerase activity (*27*), may account for the viability. Pin1 overexpression is seen in multiple human cancers, including prostate, breast, cervical, colorectal, hepatic, salivary adenoid, esophageal squamous cell, and thyroid carcinomas (*28*−*31*). Overexpression of Pin1 blocks cells at G2 in the cell cycle and produces centrosome amplification and chromosome

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instability (*23*, *32*). How Pin1 produces these effects or contributes to cancer is not understood, and the critical substrates are yet to be identified.

Pin1 is a relatively small protein (163 residues) composed of two domains, an N terminal WW domain (residues 1−39) and a C terminal PPIase catalytic domain (residues 45−163). Both domains can recognize phosphorylated Ser/Thr residues. Therefore, it has been proposed that proteins need to be phosphorylated at two sites: one to dock at the WW site and one to be isomerized at the active site of the PPIase (*12*, *33*). A pair of highly conserved Arg residues in the Pin1 catalytic site is responsible for phosphorylation specificity; these residues are not found in the corresponding location in other known PPIases (*8*). Phosphorylation of Ser16 in the WW domain was reported to prevent phosphosubstrate binding to Pin1, and this site becomes dephosphorylated during mitosis, as a possible mechanism for mitotic activation of Pin1 (*33*). But this scheme predicts that pSer16-Pin1 would be unable to bind substrates during interphase, so there must be other mechanisms to control Pin1 activity. Pin1 has been selected as a target for drug development (*34*), its 3D structure determined by crystallography (*7*) and by NMR (*35*), and Pin1 protein has been used to screen combinatorial chemical libraries for specific inhibitors (*36*). Despite this progress, important questions remain as to whether Pin1 can be used as a diagnostic marker or therapeutic target for human cancer (*37*).

Inhibitor-2 is a PP1-specific[1] inhibitor protein of 204 residues with a Pro-X-Thr-Pro sequence motif that is conserved from yeast to human (*38*, *39*). The PXTP site is phosphorylated in biochemical assays by GSK3, MAPK and CDK kinases (*40*−*43*). The I-2 heterodimer with PP1 has been studied as an "MgATP-dependent phosphatase", wherein the dephosphorylation of Thr72 in I-2 causes conformational activation of the bound PP1. The structure of the heterodimer was recently determined (*44*), but the I-2 phosphosite was not located near the PP1 active site, making it difficult to envision the mechanism for PP1 activation by dephosphorylation of I-2. We found phosphorylation of the PXTP site in I-2 during mitosis and showed that this was catalyzed by CDK1:cyclinB1 (*45*, *46*). Therefore we tested whether Pin1 might react with this mitotic phosphorylation site and instead found that I-2 acts an allosteric regulator that modifies Pin1 binding to its phosphoprotein substrates. Mechanisms for regulation of Pin1 activity are needed to understand how this protein fulfills so many reported functions in cells that are related to human disease.

MATERIALS AND METHODS

Materials. The following antibodies were purchased from the indicated suppliers: anti-GST(26H1) monoclonal antibody (Cell Signaling Technology), anti-phospho-Ser/Thr-pro MPM-2 antibody (mouse monoclonal IgG1) (Millipore-Upstate), S-protein HRP conjugate (Novagen), the secondary antibodies for immunofluorescence- Rhodamine Red-X-conjugated AffiniPure Goat Anti-Mouse IgG (H+L) (Jackson ImmunoResearch Laboratories, INC), Oregon green 488 donkey anti-sheep IgG (H+L) (Molecular Probes). Anti-HA (12CA5) monoclonal antibody was obtained from antibody core facility at the University of Virginia and affinity-purified sheep anti-I-2 antibody was described previously (*47*). Phosphosite-specific (PXTP) I-2(Thr72) antibody was produced by BioSource International using a peptide DYTSpTP for immunization and affinity purification. S-protein agarose was purchased from Novagen and glutathione Sepharose beads from Amersham Biosciences. TNT Quick Coupled Transcription/Translation System was purchased from Promega. The rabbit anti-human Pin1 antibody and plasmid DNAs for in vitro transcription and translation were described previously (*48*). Lipofectamine was purchased from Invitrogen. ExGen 500 was purchased from Fermentas. Recombinant I-2 was prepared and phosphorylated at Thr72 by overnight reaction with GSK-3β (New England BioLabs) as described previously (*39*).

Pull-Down and Coprecipitation Binding Assays. The direct binding assay of I-2 with Pin1 was performed in vitro by two different methods, with identical results. In solution different recombinant His_6-S-tagged recombinant I-2 (WT, T72A, P-T72, 14−197) were mixed with GST-Pin1 proteins (WT, S16A, S16E) in buffer containing 50 mM HEPES (pH 7.4), 100 mM NaCl, 10 mM $MgCl_2$, 0.5 mM $MnCl_2$, 5 mM EGTA, 1 mg/mL BSA, 0.5 mM DTT, 1 mM Pefabloc protease inhibitor. After incubation His_6-S-I-2 proteins or GST-Pin1 proteins were pulled down by either S-Sepharose beads or glutathione beads. Alternatively, as described in figure legends, one of the proteins was first bound to beads, the beads pelleted and rinsed, then incubated with the second soluble protein. In both methods after the binding step the beads were pelleted by centrifugation, the supernatant was removed, and the beads washed three times with 1 mL of the same buffer without BSA. Bound proteins were eluted with 2X SDS sample buffer and subjected to 12% SDS−PAGE and Western blotting as previously described (*46*).

The binding of Pin1 with I-2 in cells was tested by coexpression of pTriex-Pin1(WT) with pK-HA_3-I-2 in 293T cells using Lipofectamine 2000 or by individual expression of pTriex-Pin1(WT) or pTriex-I-2(WT) in HeLa cells using ExGen 500 for transfection. After 48 h the cells were scraped off plates in the HeLa cell lysis buffer described above without Triton-X-100. The cell suspension was sonicated and centrifuged at 13000*g* for 10 min. The S-Pin1 or the S-I-2 in the supernatants was pulled down by S-Sepharose beads. The beads were washed 3 times with 1 mL of buffer containing 150 mM NaCl, or other buffers, as indicated. The proteins were eluted from beads with 2X SDS sample buffer and immunoblotted with the indicated antibodies.

For the assay of I-2 effects on the binding of Pin1 with Pin1 substrates glutathione Sepharose beads were prepared by binding 5 μg of GST-Pin(WT) protein. The beads were preincubated with BSA as control or with recombinant I-2(WT) at final concentration of 10 μM in the same buffer used for preparing the cell extracts. After 1 h incubation, the beads were pelleted and the supernatant was removed. The beads were incubated with 1 mg protein of mitotic lysate for 1 h at 4 °C, then pelleted. The supernatant was removed and the beads were washed three times with 1 mL of HeLa cell lysis buffer, and then the protein eluted with 10 mL of

[1] Abbreviations: BSA, bovine serum albumin; GST, glutathione S-transferase; PBS, phosphate-buffered saline; β-ME, β-mercaptoethanol; DTT, 1,4-dithiothreitol; PP1, protein phosphatase 1; I-2, phosphatase inhibitor-2; DAPI, 4′-6-diamidino-2-phenylindole; I, interphase cell lysates; M, mitotic cell lysates; anti-MPM-2 antibody, anti-mitotic protein monoclonal-2 antibody.

2X SDS sample buffer. The samples were immunoblotted by anti-MPM-2 antibody.

Preparation of Mitotic HeLa Cell Extracts. HeLa cells were synchronized by incubating with 200 ng/mL nocodazole for 16 h. Cells were rinsed once with PBS, and the mitotic cells were shaken off and suspended in the buffer containing 50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 50 mM NaF, 20 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 mM DTT, 0.1% 2-mercaptoethanol, 1 mM Pefabloc and 2 μM microcystin LR. After shaking, the cells remaining on the plate were scraped off and used as interphase cells. Collected cells were disrupted by sonication on ice, and cell debris was pelleted by centrifugation (13000*g* for 10 min). The supernatants were stored frozen at −80 °C.

Preparation of Phosphorylated Pin1 Substrate Proteins. Individual Pin1 substrate proteins were prepared using the TNT Coupled Reticulocyte Lysate System from Promega, according to the manufacturer's instructions, except the reaction volume was scaled down to 25 μL and 1 μg of plasmid DNA was used per reaction. For making phosphorylated Pin1 substrates, [^{35}S] methionine-labeled protein pools (12 μL) were mixed with 25 μL of mitotic cell extract in a total 50 μL kinase reaction containing 50 mM Tris (pH 8.0), 10 mM $MgCl_2$, 1 mM DTT, 1 mM ATP. The kinase reaction was performed at room temperature for 1 h. Instead of mitotic cell lysate, interphase lysate from the same culture of HeLa cells was used as control. Proteins were stored at −80 °C.

Pull-Down of In Vitro Translated Proteins by GST-Pin1. Glutathione Sepharose beads with GST-Pin1(WT) or (S16A) mutant were preincubated with BSA or I-2(T72A) as described above. The beads were further incubated with the phosphorylated Pin1 individual substrate proteins for another 1 h at 4 °C. After incubation, the beads were washed three times with 1 mL of HeLa cell lysis buffer and proteins eluted with 10 mL of 2X SDS sample buffer. The samples were resolved by SDS−PAGE and gels dried and analyzed by autoradiography with Kodak X-ray film.

Immunofluorescence Microscopy. HeLa cells were cultured to confluence and then replated at different cell densities onto 22 mm cover slips, grown overnight, then rinsed with PBS and fixed with 2% paraformaldehyde in PBS at 37 °C for 20 min. Cells were permeabilized with 0.1% Triton X-100 in PBS for 5 min, washed three times with PBS and blocked with 3% bovine serum albumin in PBS for 1 h at 37 °C. Primary antibody (rabbit anti-human Pin1 at 1:250 and the sheep anti-human I-2 at 1:500 dilution) was incubated overnight at 4 °C and following three washes with PBS the secondary antibodies plus Hoechst (1:20,000 dilution) were incubated 1 h at 37 °C. The specimens were washed three times with PBS and then mounted with Vectashield (Vector Labs, Burlingham, CA) and microscopic images acquired on a Nikon Microphot SA with a Hamamatsu C4742 digital camera operated by OpenLab software (Improvision) or with a Zeiss confocal microscope and processed in Adobe PhotoShop (*47*).

RESULTS

Binding of I-2 and Phospho-I-2 to Pin1. Different recombinant His_6-S-peptide-I-2 proteins were incubated with recombinant GST-Pin1. After repeated washing, proteins adsorbed on S-protein beads were eluted and analyzed by Western blotting (Figure 1A). GST-Pin1 alone as a control sample did not bind to the S-protein beads (lane 1). We observed binding of GST-Pin1 to wild type I-2(WT) or the I-2(T72A) phosphosite mutant (lanes 2 and 3), showing that phosphorylation of the PXTP site in I-2 was not required for binding. Here the opposite applies, because phosphorylation of I-2 in the PXTP site by reaction with GSK3β essentially eliminated binding of GST-Pin1 (lane 4). GST-Pin1 also did not bind to truncated I-2 (residues 14−197), a form that does not exhibit high affinity association with PP1 (*39*). This indicates that binding of Pin1 and PP1 requires the same region of I-2 and therefore they are probably mutually exclusive partners. To confirm the results we reversed the binding assay and did a GST pull-down (Figure 1B). GST-Pin1 bound to glutathione beads was mixed with different purified I-2 proteins. We titrated the amount of I-2

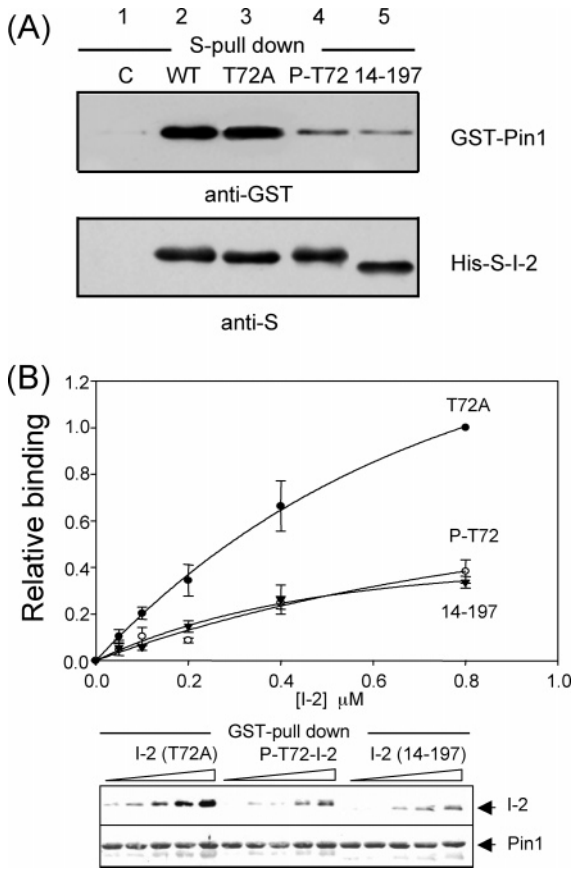

FIGURE 1: Binding of Pin1 with I-2 proteins. (A) Direct binding of GST-Pin1 to immobilized recombinant I-2 proteins. GST-Pin1 was incubated 1 h at 4 °C with S-Sepharose beads loaded with His_6-S-peptide tagged recombinant I-2 (WT), I-2(T72A), P-T72-I-2 or I-2(14−197) in buffer without detergent as described in Materials and Methods. The beads were washed by centrifugation, and the bound proteins immunoblotted with anti-GST (top panel) and anti-S antibodies (bottom panel). (B) Binding of His_6-S-I-2 proteins to immobilized GST-Pin1. Glutathione Sepharose beads with GST-Pin1 bound were incubated with various concentrations of His_6-S-I-2(T72A), P-T72-I-2 or I-2(14−197) as described in Materials and Methods. Proteins were eluted and immunoblotted utilizing pairs of antibodies detected simultaneously with different fluorescently labeled secondary antibodies using dual wavelength scanning (Odyssey, Li-Cor Industries). Sheep anti-I-2 antibody and mouse monoclonal anti-GST antibody were detected using rabbit anti-sheep at 680 nm and goat anti-mouse at 800 nm. The intensity of the bands were quantitated and normalized to GST. The binding curves were made using SigmaPlot 9.0 software.

proteins to demonstrate concentration-dependent binding to Pin1. Analysis of the binding curve for I-2(T72A) gave a $K_{0.5}$ of ~1 μM, well below the estimated 5−30 μM concentration of I-2 in tissue culture cells.[2] The calculated apparent affinity was reduced 10-fold by either phosphorylation of T72 or truncation of I-2. These reciprocal pull-down assays gave the same results, demonstrating differential binding of I-2 and phospho-T72-I-2 to Pin1.

I-2 and Pin1 Associate Together and Colocalize in Cells. We coexpressed HA_3-tagged I-2 and S-tagged Pin1 in HEK 293T cells and recovered complexes by immunoprecipitation from cell extracts (Figure 2A). Association of I-2 with Pin1 was disrupted if detergents were used for cell lysis. We tested Triton-X-100, IGEPAL CA-630 (NP-40), Tween 20 and CHAPS, and all prevented recovery of complexes. Binding between I-2 and Pin1 was stable in salt, and complexes resisted washing with 500 mM NaCl (Figure 2A). Individual expression of either S-tagged I-2 or S-tagged Pin1 in HeLa cells afforded recovery of their endogenous partner, Pin 1 and I-2, respectively (Figure 2B). This showed that ectopic I-2 could associate with Pin1 in cells and ectopic Pin1 could associate with I-2 in cells. Attempts to coprecipitate endogenous I-2 and Pin1 together were not successful using either anti-I-2 or anti-Pin1 antibodies. We attributed this to the relatively small size of both proteins (23 and 16 kDa, respectively), suspecting that the binding of antibodies interfered with protein−protein association. We previously published that I-2 is concentrated in the nucleus of HeLa cells plated at low density, but is excluded from the nucleus of cells at high density (*47*). We found by immunofluorescent staining that endogenous Pin1 exhibited the same pattern of nuclear vs cytoplasmic distribution as endogenous I-2 in low- and high-density cells (Figure 2C). Redistribution and colocalization of Pin1 and I-2 in either the nucleus or cytoplasm depending on cell density was consistent with their association together in living cells.

I-2 Alters Binding of Pin1 with Mitotic Phosphoproteins. Pin1 binds proteins with phosphorylated Ser/Thr-Pro sites, and the MPM-2 antibody recognizes many, but not all, mitotic proteins precipitated by GST-Pin1 (*8*). We tested whether interaction with I-2 affected binding of Pin1 to MPM-2-positive proteins. Extracts from mitotic HeLa cells were immunoblotted directly with MPM-2 to show various proteins present (Figure 3, lane 1). These extracts were incubated with GST-Pin1 on glutathione beads that were preincubated with either I-2 (lane 3), or BSA as a control (lane 2). Proteins bound to GST-Pin1 were analyzed by Western blotting using anti-MPM-2 antibody. Anti-GST staining demonstrated equal loading (Figure 3, lower panel). Binding of Pin1 with some proteins was increased by I-2 relative to BSA (lane 3, arrows), whereas binding of at least one other protein was diminished by I-2 binding to Pin1 (lane 2 vs 3, open arrow). These results indicated that I-2 association with Pin1 altered specificity for different mitotic phosphoproteins.

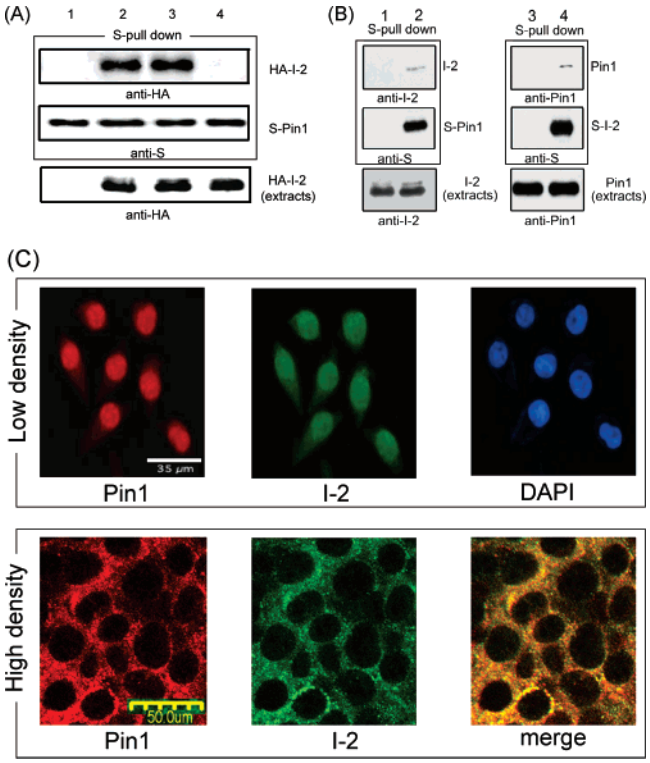

FIGURE 2: Association of Pin1 with I-2 in cells. (A) HEK293T cells were transfected with pTriex-Pin1 and empty vector (lane 1) or cotransfected with pTriex-Pin1 and pKHA-I-2(WT) (lanes 2, 3, 4). After 48 h cell extracts were prepared using buffer without detergent (lane 1, 2, 3) or cell lysis buffer containing 1% Triton-X-100 (lane 4). The extracts were incubated with S-Sepharose beads and the beads were washed with buffer containing 150 mM NaCl without detergent (lanes 1, 2); buffer with 500 mM NaCl without detergent (lane 3); or buffer including 150 mM NaCl and 1% Triton-X-100 (lane 4). The proteins were eluted and Western blotted with anti-HA antibody (top panel) to show coprecipitated I-2 and with anti-S antibody (middle panel) to show pull-down yield of Pin1. The cell extracts were immunoblotted with anti-HA antibody to show the expression levels of ectopic I-2 (bottom panel). (B) HeLa cells were transfected with pTriEx-4 empty vector (lanes 1, 3) or transfected with pTriEx-Pin1 (lane 2) or pTriEx-I-2 (lane 4). After 48 h cell extracts were prepared without detergent and incubated with S-Sepharose beads. The beads were washed with the same lysis buffer containing 150 mM NaCl. The proteins were eluted and Western blotted with anti-sheep I-2 antibody (top panel of lanes 1, 2) or anti-Pin1 antibody (top panel of lanes 3, 4). Anti-S antibody was used to detect the S-tagged Pin1 (middle panel of lanes 1, 2) and S-tagged I-2 (middle panel of lanes 3, 4). The cell extracts (bottom panels) were blotted to show the endogenous I-2 and Pin1. (C) Cellular localization of endogenous Pin1 and I-2 at different cell densities. HeLa cells were replated at either low density (top panel) or high density (bottom panel) on coverslips, fixed and examined by indirect immunofluorescence using rabbit anti-human Pin1 antibody and sheep anti-human I-2 antibody. DAPI staining was used to determine the position of the nuclei in low-density cells using wide-field fluorescence microscopy. Confocal images of the high-density cells were merged to show overlap.

I-2 Alters Pin1 Binding to Individual Substrates. We examined Pin1 binding to several individual mitotic phosphoproteins that had been identified by a systematic phosphoprotein screen (*48*). The proteins were synthesized by in vitro transcription and translation in the presence of [^{35}S]-methionine and were phosphorylated under identical reaction conditions with either mitotic or nonmitotic HeLa extracts as a source of kinases. Many proteins phosphorylated by mitotic extracts exhibited a shift to lower electrophoretic mobility during SDS−PAGE, seen by comparing the lanes

[2] Immunoblotting of a range of 5−50 ng of purified recombinant human I-2 with different volumes of human ARPE-19 cell extracts showed 5 ng of I-2 per 10 μg cell protein, or 22 pmol/mg total protein. Using 10^6 cells per mg protein and per cell volume of 4 pL gives 5.5 μM as the calculated cellular concentration. Compared to these cells HeLa had >6-fold higher relative I-2 levels.

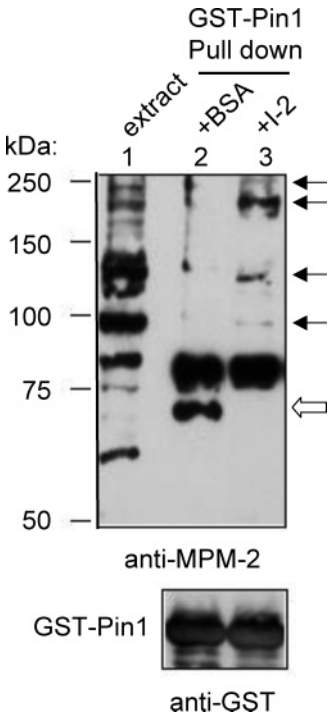

FIGURE 3: Effects of I-2 on binding of Pin1 with phosphoproteins from mitotic HeLa cells. Glutathione beads saturated with GST-Pin1 were preincubated with BSA (lane 2) or I-2(WT) (lane 3), washed by centrifugation and mixed with mitotic HeLa cell extracts. After washing, proteins were eluted from the beads and subjected to immunoblotting with anti-MPM-2 antibody (top panel) or anti-GST antibody as a loading control (bottom panel). Mitotic extracts were blotted with MPM-2 antibody (lane 1) to show all the proteins reactive with MPM-2.

labeled I and M in Figure 4. To assay effects of I-2 on Pin1 binding, these mitotic phosphorylated proteins were incubated with GST-Pin1 beads preincubated with saturating amounts of I-2(T72A) to form Pin1::I-2 complexes, or preincubated with BSA as control. The phosphorylated form of in vitro translated CDC25 bound to GST-Pin1, validating the assay (Figure 4A). About 3-fold more CDC25 bound to Pin1 complexed with I-2(T72A) compared to the control Pin1 incubated with BSA. A total of 9 different mitotic phosphoproteins were tested for binding to Pin1 or to Pin1::I-2 complex. Based on the results we divided these proteins into four groups: (A) proteins whose binding to Pin1 was increased by I-2 (Figure 4A), (B) proteins where binding of the most highly phosphorylated form(s) (lowest mobility) was selectively enhanced by I-2 (Figure 4B); (C) proteins whose binding to Pin1 was decreased by I-2 (Figure 4C); (D) proteins whose binding to Pin1 was not affected by I-2 (Figure 4D). The results demonstrate that I-2 changes the binding of Pin1 with individual substrates. Both increases and decreases suggest that I-2 interaction modulates Pin1 specificity and makes it likely that I-2 itself participates in recognition of the phosphoproteins.

Mutation of Ser16 in Pin1 WW Domain Alters I-2 Binding. Pin1 is phosphorylated on Ser16, and this prevents binding to mitotic phosphoproteins (*33*). On the other hand, Pin1(S16A) binds most Pin1 substrates and to synthetic Pintide peptide with undiminished affinity compared to wild type. We assayed the ability of Pin1(S16A) or Pin1(S16E) to bind wild type I-2, I-2(T72A) or P-T72-I-2, whose phosphorylation was confirmed by phosphosite-specific immunoblotting

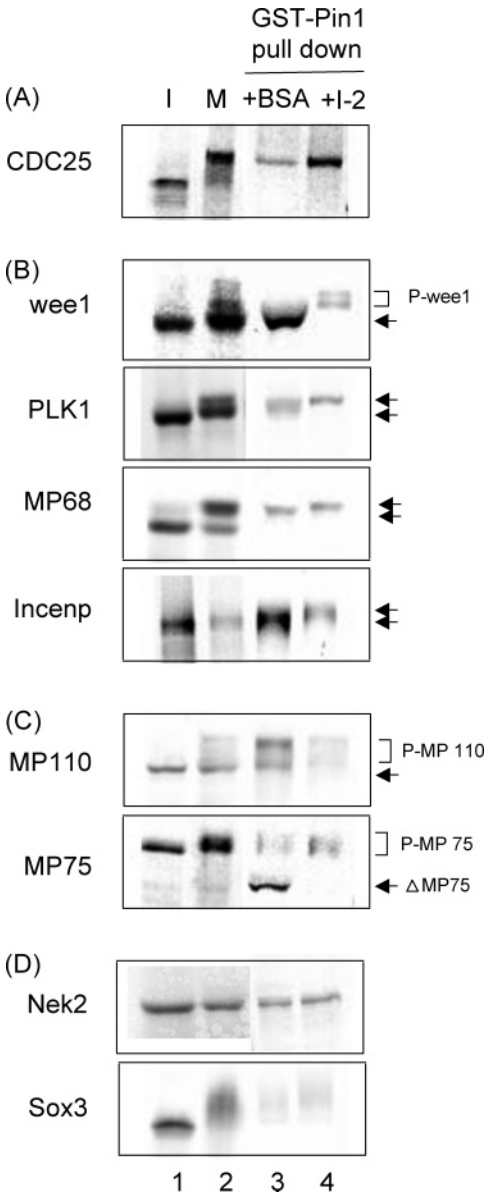

FIGURE 4: Binding of Pin1 with individual phosphoproteins is modulated by I-2. Pin1 substrate proteins were ^{35}S-labeled by in vitro translation and incubated in either interphase (I) or mitotic (M) extracts with Mg-ATP. Aliquots of the samples were treated with SDS sample buffer and directly loaded onto the gel (lanes 1, 2). Another portion of the samples was mixed with GST-Pin1 bound to glutathione Sepharose beads after preincubation with either BSA as a control or I-2(T72A). The beads were washed and proteins eluted and separated by SDS−PAGE (lane 3, 4). The dried gels were analyzed by autoradiography. The results were divided into four groups: (A) I-2 increased binding of substrates to Pin1; (B) I-2 selectively enhances binding Pin1 to hyperphosphorylated form of substrates after incubating; (C) binding decreased by I-2; (D) no change in binding with I-2.

(Figure 5A). The same amounts of recombinant His_6-S-tagged I-2 proteins were bound to S-beads and incubated with either GST-Pin1(S16A) or GST-Pin1(S16E). Bound proteins were eluted and analyzed by Western blotting. Added Pin1(S16A) did not bind to I-2(WT) or I-2(T72A) on beads (lanes 1, 2) and showed low level binding with P-T72-I-2 (lane 3). In contrast, Pin1(S16E) bound to all the I-2 proteins that were tested (lanes 4−6). There was especially robust binding between Pin1(S16E) and P-T72-I-2. These results show that I-2 discriminated between different residues at position 16 in the Pin1 WW domain,

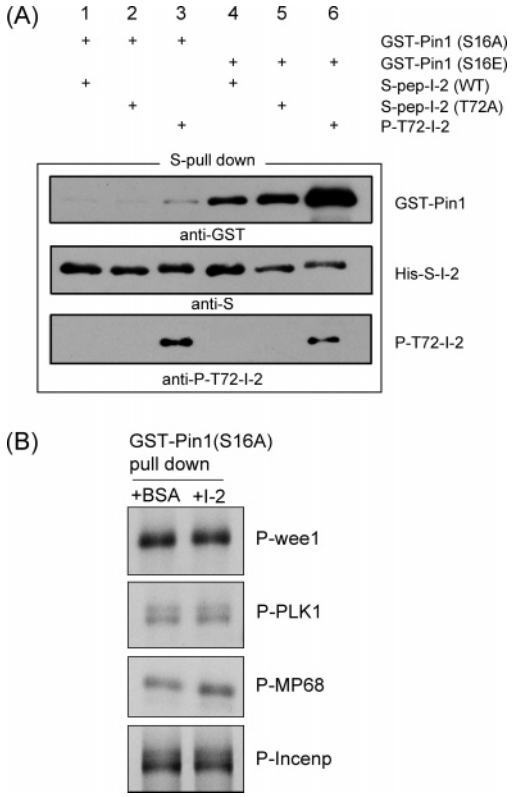

FIGURE 5: Binding of GST-Pin1 (S16A) or GST-Pin1 (S16E) to different I-2 proteins. (A) S-Sepharose beads with bound His-S-I-2(WT), (T72A) or P-T72-I-2 were mixed with recombinant GST-Pin1(S16A) or (S16E) mutant proteins. After incubation the beads were washed and proteins eluted and immunoblotted with anti-GST antibody (top panel), anti-S antibody (middle panel) and phosphosite specific anti-P-T72 antibody to demonstrate I-2 phosphorylation (bottom panel). (B) Binding phosphoprotein substrates by Pin1(S16A) is unaffected by I-2. The phosphorylated proteins including wee1, PLK1, MP68 and Incenp were prepared as described in Materials and Methods and incubated with GST-Pin1-(S16A) on glutathione beads that had been preincubated with either BSA or I-2(T72A). After 1 h incubation, the beads were washed and the proteins eluted and resolved by SDS−PAGE. Dried gels were analyzed by autoradiography.

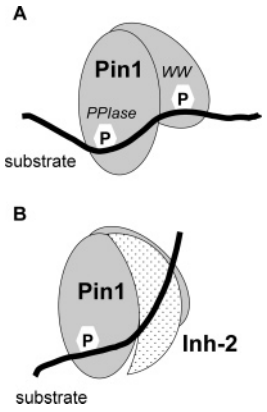

FIGURE 6: Model for association of I-2 with Pin1. Based on the 3D structure Pin1 is depicted as a two lobed protein with a larger PPIase domain and smaller WW domain. A substrate with two separate phosphosites is shown as a thick line with one phosphosite docked in each domain of Pin1. When I-2 binds to the WW domain, involving Ser16 of Pin1, a new composite surface is formed, so a substrate (thick line) interacts with I-2 to position the phospho-Ser/Thr-Pro at the active site in the PPIase domain of Pin1.

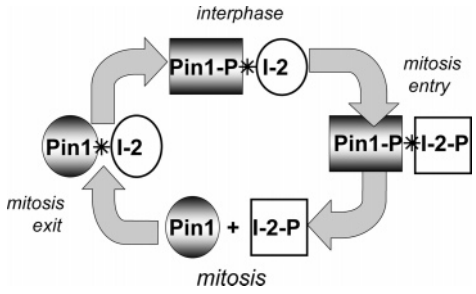

FIGURE 7: Cycle of phosphorylation-dependent binding of I-2 and Pin1. A model to show complexes between Pin1 (shadowed) and I-2 (open) where squares are phosphorylated proteins and circles are unphosphorylated. The asterisks indicate protein−protein binding. Pin1 phosphorylation at Ser16 occurs during interphase and is dephosphorylated in mitosis, conversely I-2 is unphosphorylated during interphase and is phosphorylated at Thr72 in mitosis. The model predicts that different Pin1 complexes are active at the entry and exit of mitosis and during interphase.

and bound with either Ser or Glu, but not Ala. We confirmed the structural integrity and activity of the Pin1(S16A) mutant in a pull-down assay with individual mitotic phosphoproteins (Figure 5B). There was no effect of preincubation with I-2 on the binding of mitotic phosphoproteins by GST-Pin1-(S16A). This contrasts with I-2 alteration of wild type Pin1 binding to each of these same proteins (compare Figures 4B and 5B). The results demonstrate that I-2 changes the binding of Pin1 with phosphoproteins, involving I-2 recognition of Ser16 in the WW domain of Pin1.

DISCUSSION

Mitotic phosphorylation sites in many proteins are recognized by the prolyl isomerase Pin1, and the most conserved sequence region in inhibitor-2 (I-2) defines a PXTP site that is phosphorylated during mitosis by CDK:cyclinB (*46*). We tested whether Pin1 would bind to phospho-I-2, but surprisingly found Pin1 bound to unphosphorylated I-2 that was being used as a negative control. Our results demonstrate that I-2 is not a typical substrate for Pin1 isomerization, and instead acts as an allosteric regulator of Pin1 specificity. Pin1 reacts with cell cycle regulatory enzymes including kinases Myt1, Wee1 and Plk1, and Cdc25 phosphatase (*8*, *15*). Phosphorylation of these proteins during mitosis has been thought to be the sole requirement for recognition by Pin1. We demonstrate that I-2 binding to Pin1 introduces an extra layer of complexity to Pin1 substrate specificity and in effect acts as a Pin1 regulatory subunit (Figure 6). The results link together these two highly conserved proteins and suggest a dynamic interplay between them that may have an important role in orchestrating events before and during mitosis (Figure 7), or in other signaling pathways such as beta-catenin degradation and tau hyperphosphorylation in Alzheimer's disease.

Direct protein−protein interaction between I-2 and Pin1 was demonstrated with pure recombinant proteins in reciprocal pull-down assays. Soluble Pin1 bound to immobilized I-2 and soluble I-2 bound to immobilized Pin1 and the soluble proteins bound together in solution and could be pulled down together by different affinity beads. Analysis of the concentration dependence yielded an apparent affinity of about 1 μM, which is well below the estimated micromolar concentrations of I-2 in tissue culture cells. Binding of I-2 to Pin1 was reduced 10-fold by either phosphorylation of I-2 in the PXTP site by GSK3 or deletion of the extreme

ends of I-2, including the ^{9}IKGI^{12} motif critical for high potency inhibition of PP1 (*49*). This implies mutually exclusive binding of I-2 to these partners and highlights the multifunctional nature of I-2, not simply being a PP1 inhibitor. The N terminus of I-2 is separated from the PXTP phosphorylation site by 60 residues, but both regions are implicated in binding to Pin1. I-2 is an inherently unstructured protein that is heat-resistant, with extended and flexible conformation, making it plausible that I-2 can loop out to bring regions well separated in sequence into proximal sites of interaction with a small partner such as Pin1 (M_r 16 kDa). The ability of I-2 to form loops and make multiple contacts with a partner protein is seen in the 3D structure of the I-2 complex with PP1 (*44*).

Binding of I-2 seems to primarily involve the N-terminal WW domain of Pin1 and especially the side chain at residue 16. We saw weak interaction of I-2 with the WW domain of Pin1 in a pull-down assay (not shown). Mutation of Pin1 Ser16 in the WW domain to Ala eliminated binding to I-2 or phospho-I-2, whereas mutation of Ser16 to Glu restored I-2 binding and even supported binding of phospho-I-2. Our suggestion is that an H bond acceptor, namely Ser, P-Ser or Glu, is required in this location to interact with I-2. It is notable that the S16A mutant of Pin1 bound neither I-2 nor phospho-I-2, but still binds phosphoprotein substrates. This suggests that phenotypes ascribed to S16A mutation of Pin1, including mitotic block and apoptosis, may actually be a result of loss of I-2 binding (*33*). Our results suggest a cycle of phosphorylation-dependent regulation for I-2:Pin1 association (see Figure 7): (1) The interphase form of Pin1, phosphorylated at Ser16 (mimicked by S16E), binds unphosphorylated I-2. (2) Early during mitosis I-2 is phosphorylated at Thr72 but remains bound to Pin1, because we show phospho-I-2 binds Pin1(S16E). (3) Later during mitosis Pin1 is dephosphorylated at Ser16 (*33*), causing dissociation of the phospho-I-2. (4) Before entry into the next cell cycle the I-2 is dephosphorylated and rebinds to Pin1 to reform a complex. This provides a plausible scenario for the formation−dissociation−re-formation of distinct Pin1::I-2 complexes during transit through mitosis. We propose that each of the Pin1::I-2 complexes will exhibit different substrate specificity, creating a sequence of Pin1-dependent events for timing of progression through mitosis.

We provide evidence that I-2 and Pin1 associate together in live cells. Tagged proteins were coprecipitated from cell extracts and also individually precipitated their endogenous partner. These complexes resisted salt concentrations up to 0.5 M, but were exceedingly sensitive to ionic and non-ionic detergents. Pull-down assays with recombinant proteins also were sensitive to added detergents, suggesting that I-2 and Pin1 form a complex with a hydrophobic interface readily disrupted by detergents, but stable to salt. The detergent sensitivity probably accounts for I-2:Pin1 complexes not being reported in the literature previously. We observed colocalization of endogenous I-2 and Pin1 within subcompartments of the nucleus (not shown), confirming previous independent reports of Pin1 in nuclear speckles (*33*, *50*). We demonstrate drastic nuclear vs cytoplasmic redistribution of Pin1 and I-2, dependent on cell density. The method reveals intracellular distribution but cannot demonstrate protein−protein binding. We previously reported similar intracellular redistribution of I-2 and PP1δ (*47*). Because the N terminus of I-2 is needed for both Pin1 and PP1 binding, we propose that these I-2 binding partners are mutually exclusive and therefore redistribution probably reflects parallel movement of separate Pin1:I-2 and PP1:I-2 complexes. Consistent with a common site on I-2 for these partners, no coprecipitation of PP1 with Pin1 was observed. Even bound together, I-2 and Pin1 are small enough proteins that they would be expected to freely diffuse through nuclear pores. Therefore the restricted localization depends on interaction with other proteins in the nucleus and cytoplasm. The extensive colocalization supports the idea that I-2 and Pin1 are bound to one another in live cells.

Most important, the binding of I-2 to Pin1 alters the specificity of Pin1 for different phosphoproteins. This was shown first with Pin1 pull-down of proteins from extracts of mitotic HeLa cells, using the MPM2 antibody to detect a variety of mitotic phosphoproteins that are Pin1 binding partners. Pin1 saturated with I-2 showed reduced binding to one prominent protein and enhanced binding to several other proteins reactive with MPM2 antibody. These results suggested that an I-2:Pin1 complex has distinctively different specificity for phosphoproteins compared to Pin1 alone. This idea was confirmed by examination of a panel of nine individual mitotic phosphoproteins known to bind to Pin1 (*48*). The results are compelling. Phosphoproteins Nek2 and Sox3 bind the same to Pin1 alone (with BSA) vs Pin1:I-2 complexes, providing controls. By comparison phosphorylated cdc25 binds more effectively to the Pin1:I-2 complex, whereas binding to MP110 and MP75 is reduced relative to Pin1 without I-2. Other proteins including Wee1, PLK1, MP68 and Incenp show a response where I-2 enhances the binding just to the form of these proteins with the lowest mobility in SDS−PAGE, presumably the most highly phosphorylated form, while suppressing Pin1 binding to the less completely phosphorylated forms. This intriguing result suggests that I-2 restrains Pin1 action until these substrates are fully phosphorylated, thereby producing an abrupt response from a process of gradual or progressive phosphorylation. Such a response could contribute to the G2 to M transition in cells. The assays were repeated using Pin1-(S16A), a mutant protein that does not bind to I-2, and preincubation with I-2 was without effects. We expect that both proteins in a Pin1::I-2 complex contribute to a composite protein surface responsible for substrate specificity and in this complex the phosphosite in the WW domain is occupied by I-2 (see Figure 6). Under these circumstances substrates might not conform to the proposed "double-phosphosite" recognition model that has been widely disseminated, but not rigorously tested.

Furthermore, because phosphorylation of I-2 at the PXTP site and phosphomimetic mutation of Ser16 in Pin1 alter binding of the proteins to one another, we predict that cell cycle dependent phosphorylation regulates the stability of Pin1:I-2 complexes in cells. Other sites of phosphorylation in Pin1 (e.g., Ser65 (*51*)) and in I-2 may influence complex stability, substrate binding and/or Pin1 PPIase catalytic activity. Our model proposes dynamic alterations in Pin1 complexes in cells before, during and after mitosis (Figure 7), and each Pin1 complex is likely to have distinctive specificity. A remaining challenge is to demonstrate a sequence of Pin1-dependent changes in different proteins at various times, which would permit construction of models

that generate a temporal sequence of events for mitotic progression. Last, the Pin1:I-2 complex beckons analysis of the protein structure and screening for interacting small molecules with potential therapeutic applications.

REFERENCES

1. Zhu, G., Fujii, K., Belkina, N., Liu, Y., James, M., Herrero, J., and Shaw, S. (2005) Exceptional disfavor for proline at the P + 1 position among AGC and CAMK kinases establishes reciprocal specificity between them and the proline-directed kinases, *J. Biol. Chem. 280*, 10743−10748.
2. Harding, M. W. (1991) Structural and functional features of the peptidyl prolyl cis-trans isomerase, cyclophilin, *Pharmacotherapy 11*, 142S−148S.
3. Schmid, F. X. (1993) Prolyl isomerase: enzymatic catalysis of slow protein-folding reactions, *Annu. Rev. Biophys. Biomol. Struct. 22*, 123−142.
4. Arevalo-Rodriguez, M., Wu, X., Hanes, S. D., and Heitman, J. (2004) Prolyl isomerases in yeast, *Front. Biosci. 9*, 2420−2446.
5. Hanes, S. D., Shank, P. R., and Bostian, K. A. (1989) Sequence and mutational analysis of ESS1, a gene essential for growth in Saccharomyces cerevisiae, *Yeast 5*, 55−72.
6. Lu, K. P., Hanes, S. D., and Hunter, T. (1996) A human peptidyl-prolyl isomerase essential for regulation of mitosis, *Nature 380*, 544−547.
7. Ranganathan, R., Lu, K. P., Hunter, T., and Noel, J. P. (1997) Structural and functional analysis of the mitotic rotamase Pin1 suggests substrate recognition is phosphorylation dependent, *Cell 89*, 875−886.
8. Yaffe, M. B., Schutkowski, M., Shen, M., Zhou, X. Z., Stukenberg, P. T., Rahfeld, J. U., Xu, J., Kuang, J., Kirschner, M. W., Fischer, G., Cantley, L. C., and Lu, K. P. (1997) Sequence-specific and phosphorylation-dependent proline isomerization: a potential mitotic regulatory mechanism, *Science 278*, 1957−1960.
9. Lu, P. J., Wulf, G., Zhou, X. Z., Davies, P., and Lu, K. P. (1999) The prolyl isomerase Pin1 restores the function of Alzheimer-associated phosphorylated tau protein, *Nature 399*, 784−788.
10. Joseph, J. D., Yeh, E. S., Swenson, K. I., Means, A. R., and Winkler. (2003) The peptidyl-prolyl isomerase Pin1, *Prog. Cell Cycle Res. 5*, 477−487.
11. Lu, K. P., Finn, G., Lee, T. H., and Nicholson, L. K. (2007) Prolyl cis-trans isomerization as a molecular timer, *Nat. Chem. Biol. 3*, 619−629.
12. Lu, K. P., and Zhou, X. Z. (2007) The prolyl isomerase PIN1: a pivotal new twist in phosphorylation signalling and disease, *Nat. Rev. Mol. Cell Biol.*, submitted.
13. Davis, F. M., Tsao, T. Y., Fowler, S. K., and Rao, P. N. (1983) Monoclonal antibodies to mitotic cells, *Proc. Natl. Acad. Sci. U.S.A. 80*, 2926−2930.
14. Albert, A. L., Lavoie, S. B., and Vincent, M. (2004) Multisite phosphorylation of Pin1-associated mitotic phosphoproteins revealed by monoclonal antibodies MPM-2 and CC-3, *BMC Cell Biol. 5*, 22.
15. Shen, M., Stukenberg, P. T., Kirschner, M. W., and Lu, K. P. (1998) The essential mitotic peptidyl-prolyl isomerase Pin1 binds and regulates mitosis-specific phosphoproteins, *Genes Dev. 12*, 706−720.
16. Ryo, A., Suizu, F., Yoshida, Y., Perrem, K., Liou, Y. C., Wulf, G., Rottapel, R., Yamaoka, S., and Lu, K. P. (2003) Regulation of NF-kappaB signaling by Pin1-dependent prolyl isomerization and ubiquitin-mediated proteolysis of p65/RelA, *Mol. Cell 12*, 1413−1426.
17. Lim, J., and Lu, K. P. (2005) Pinning down phosphorylated tau and tauopathies, *Biochim. Biophys. Acta 1739*, 311−322.
18. Li, H., Wang, S., Zhu, T., Zhou, J., Xu, Q., Lu, Y., and Ma, D. (2006) Pin1 contributes to cervical tumorigenesis by regulating cyclin D1 expression, *Oncol. Rep. 16*, 491−496.
19. Yi, P., Wu, R. C., Sandquist, J., Wong, J., Tsai, S. Y., Tsai, M. J., Means, A. R., and O'Malley, B. W. (2005) Peptidyl-prolyl isomerase 1 (Pin1) serves as a coactivator of steroid receptor by regulating the activity of phosphorylated steroid receptor coactivator 3 (SRC-3/AIB1), *Mol. Cell. Biol. 25*, 9687−9699.
20. Yu, L., Mohamed, A. J., Vargas, L., Berglof, A., Finn, G., Lu, K. P., and Smith, C. I. (2006) Regulation of Bruton tyrosine kinase by the peptidylprolyl isomerase Pin1, *J. Biol. Chem. 281*, 18201−18207.
21. Butterfield, D. A., Abdul, H. M., Opii, W., Newman, S. F., Joshi, G., Ansari, M. A., and Sultana, R. (2006) Pin1 in Alzheimer's disease, *J. Neurochem. 98*, 1697−1706.
22. Maleszka, R., Hanes, S. D., Hackett, R. L., de Couet, H. G., and Miklos, G. L. (1996) The Drosophila melanogaster dodo (dod) gene, conserved in humans, is functionally interchangeable with the ESS1 cell division gene of Saccharomyces cerevisiae, *Proc. Natl. Acad. Sci. U.S.A. 93*, 447−451.
23. Suizu, F., Ryo, A., Wulf, G., Lim, J., and Lu, K. P. (2006) Pin1 regulates centrosome duplication, and its overexpression induces centrosome amplification, chromosome instability, and oncogenesis, *Mol. Cell. Biol. 26*, 1463−1479.
24. Xu, Y. X., and Manley, J. L. (2007) The prolyl isomerase Pin1 functions in mitotic chromosome condensation, *Mol. Cell 26*, 287−300.
25. Fanghanel, J., Akiyama, H., Uchida, C., and Uchida, T. (2006) Comparative analysis of enzyme activities and mRNA levels of peptidyl prolyl cis/trans isomerases in various organs of wild type and Pin1-/- mice, *FEBS Lett. 580*, 3237−3245.
26. Liou, Y. C., Ryo, A., Huang, H. K., Lu, P. J., Bronson, R., Fujimori, F., Uchida, T., Hunter, T., and Lu, K. P. (2002) Loss of Pin1 function in the mouse causes phenotypes resembling cyclin D1-null phenotypes, *Proc. Natl. Acad. Sci. U.S.A. 99*, 1335−1340.
27. Zhu, J. X., Dagostino, E., Rejto, P. A., Mroczkowski, B., and Murray, B. (2007) Identification and characterization of a novel and functional murine Pin1 isoform, *Biochem. Biophys. Res. Commun. 359*, 529−535.
28. Wulf, G., Ryo, A., Liou, Y. C., and Lu, K. P. (2003) The prolyl isomerase Pin1 in breast development and cancer, *Breast Cancer Res. 5*, 76−82.
29. Ayala, G., Wang, D., Wulf, G., Frolov, A., Li, R., Sowadski, J., Wheeler, T. M., Lu, K. P., and Bao, L. (2003) The prolyl isomerase Pin1 is a novel prognostic marker in human prostate cancer, *Cancer Res. 63*, 6244−6251.
30. Wulf, G. M., Ryo, A., Wulf, G. G., Lee, S. W., Niu, T., Petkova, V., and Lu, K. P. (2001) Pin1 is overexpressed in breast cancer and cooperates with Ras signaling in increasing the transcriptional activity of c-Jun towards cyclin D1, *EMBO J. 20*, 3459−3472.
31. Kim, C. J., Cho, Y. G., Park, Y. G., Nam, S. W., Kim, S. Y., Lee, S. H., Yoo, N. J., Lee, J. Y., and Park, W. S. (2005) Pin1 overexpression in colorectal cancer and its correlation with aberrant beta-catenin expression, *World J. Gastroenterol. 11*, 5006−5009.
32. Zhou, X. Z., Kops, O., Werner, A., Lu, P. J., Shen, M., Stoller, G., Kullertz, G., Stark, M., Fischer, G., and Lu, K. P. (2000) Pin1-dependent prolyl isomerization regulates dephosphorylation of Cdc25C and tau proteins, *Mol. Cell 6*, 873−883.
33. Lu, P. J., Zhou, X. Z., Liou, Y. C., Noel, J. P., and Lu, K. P. (2002) Critical role of WW domain phosphorylation in regulating phosphoserine binding activity and Pin1 function, *J. Biol. Chem. 277*, 2381−2384.
34. Lu, K. P. (2003) Prolyl isomerase Pin1 as a molecular target for cancer diagnostics and therapeutics, *Cancer Cell 4*, 175−180.
35. Kowalski, J. A., Liu, K., and Kelly, J. W. (2002) NMR solution structure of the isolated Apo Pin1 WW domain: comparison to the x-ray crystal structures of Pin1, *Biopolymers 63*, 111−121.
36. Wildemann, D., Erdmann, F., Alvarez, B. H., Stoller, G., Zhou, X. Z., Fanghanel, J., Schutkowski, M., Lu, K. P., and Fischer, G. (2006) Nanomolar inhibitors of the peptidyl prolyl cis/trans isomerase Pin1 from combinatorial peptide libraries, *J. Med. Chem. 49*, 2147−2150.
37. Yeh, E. S., and Means, A. R. (2007) PIN1, the cell cycle and cancer, *Nat. Rev. Cancer 7*, 381−388.
38. Tung, H. Y. L., Wang, W., and Chan, C. S. M. (1995) Regulation of Chromosome Segregation by Glc8p, a Structural Homolog of Mammalian Inhibitor 2 that Functions as both an Activator and an Inhibitor of Yeast Protein Phosphatase 1, *Mol. Cell. Biol. 15*, 6064−6074.
39. Li, M., Satinover, D. L., and Brautigan, D. L. (2007) Phosphorylation and functions of inhibitor-2 family of proteins, *Biochemistry 46*, 2380−2389.
40. Hemmings, B. A., Resink, T. J., and Cohen, P. (1982) Reconstitution of a Mg-ATP-dependent protein phosphatase and its activation through a phosphorylation mechanism, *FEBS Lett. 150*, 310−324.
41. Ahmad, Z., Camici, M., DePaoli-Roach, A. A., and Roach, P. J. (1984) Glycogen synthase kinases. Classification of a rabbit liver casein and glycogen synthase kinase (casein kinase-1) as a distinct enzyme, *J. Biol. Chem. 259*, 3420−3428.

42. Wang, M., Guan, K.-L., Roach, P. J., and DePaoli-Roach, A. A. (1995) Phosphorylation and activation of the ATP-Mg-dependent protein phosphatase by the mitogen-activated protein kinase, *J. Biol. Chem. 270*, 18352−18358.
43. Puntoni, F., and Villa-Moruzzi, E. (1995) Phosphorylation of the inhibitor-2 of protein phosphatase-1 by cdc2-cyclin B and GSK3, *Biochem. Biophys. Res. Commun. 207*, 732−739.
44. Hurley, T. D., Yang, J., Zhang, L., Goodwin, K. D., Zou, Q., Cortese, M., Dunker, A. K., and DePaoli-Roach, A. A. (2007) Structural basis for regulation of protein phosphatase 1 by inhibitor-2, *J. Biol. Chem. 282*, 28874−28883.
45. Leach, C., Shenolikar, S., and Brautigan, D. L. (2003) Phosphorylation of phosphatase inhibitor-2 at centrosomes during mitosis, *J. Biol. Chem. 278*, 26015−26020.
46. Li, M., Stefansson, B., Wang, W., Schaefer, E. M., and Brautigan, D. L. (2006) Phosphorylation of the Pro-X-Thr-Pro site in phosphatase inhibitor-2 by cyclin-dependent protein kinase during M-phase of the cell cycle, *Cell Signalling 18*, 1318−1326.
47. Leach, C., Eto, M., and Brautigan, D. L. (2002) Domains of type 1 protein phosphatase inhibitor-2 required for nuclear and cytoplasmic localization in response to cell-cell contact, *J. Cell Sci. 115*, 3739−3745.
48. Stukenberg, P. T., Lustig, K. D., McGarry, T. J., King, R. W., Kuang, J., and Kirschner, M. W. (1997) Systematic identification of mitotic phosphoproteins, *Curr. Biol. 7*, 338−348.
49. Huang, H.-b., Horichi, A., Watanabe, T., Shih, S.-R., Tsay, H.-J., Li, H.-C., Greengard, P., and Nairn, A. C. (1999) Characterization of the inhibition of protein phosphatase-1 by DARPP-32 and inhibitor-2, *J. Biol. Chem. 274*, 7870−7878.
50. Rippmann, J. F., Hobbie, S., Daiber, C., Guilliard, B., Bauer, M., Birk, J., Nar, H., Garin-Chesa, P., Rettig, W. J., and Schnapp, A. (2000) Phosphorylation-dependent proline isomerization catalyzed by Pin1 is essential for tumor cell survival and entry into mitosis, *Cell Growth Differ. 11*, 409−416.
51. Eckerdt, F., Yuan, J., Saxena, K., Martin, B., Kappel, S., Lindenau, C., Kramer, A., Naumann, S., Daum, S., Fischer, G., Dikic, I., Kaufmann, M., and Strebhardt, K. (2005) Polo-like kinase 1-mediated phosphorylation stabilizes Pin1 by inhibiting its ubiquitination in human cells, *J. Biol. Chem. 280*, 36575−36583.

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