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Review

Phosphorylation-dependent prolyl isomerization: a novel signaling regulatory mechanism

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Abstract. Protein phosphorylation on serine or threonine residues preceding proline (Ser/Thr-Pro) plays an essential role for regulating various cellular processes, including cell cycle progression. Although phosphorylation has been proposed to regulate the function of a protein by inducing conformational changes, much less is known about what phosphate additions actually do and how the functions of phosphoproteins are coordinated. Proline is important for determining protein structure because it exists in cis or trans conformation and can put kinks into a polypeptide chain. We have shown that phosphorylation on Ser/Thr-Pro motifs reduces the cis/trans isomerization rate of Ser/Thr-Pro bonds. At the same time, proteins containing phosphorylated Ser/Thr-Pro motifs are substrates for the prolyl isomerase Pin1. The WW domain of Pin1 acts as a phosphoserine/threonine-binding module binding a defined subset of mitosis-specific phosphoproteins, such as Cdc25 and tau. These interactions target the enzymatic activity of Pin1 close to its substrates. In contrast to other prolyl isomerases (peptidyl-prolyl isomerases, PPIases), Pin1 has an extremely high degree of substrate specificity, specifically isomerizing phosphorylated Ser/Thr-Pro bonds. Therefore, Pin1 binds and regulates the function of a defined subset of phosphoproteins. Furthermore, inhibiting Pin1 function is lethal for dividing cells. Interestingly, Pin1, which can restore the biological function of phosphorylated tau, is sequestered in the neurofibrillary tangles in Alzheimer's brains. Thus, we have proposed a novel signaling regulatory mechanism, where protein phosphorylation creates binding sites for Pin1, which can then latch on to and isomerize the phosphorylated Ser/Thr-Pro peptide bond. In turn, this may change the shape of the protein, regulating its activity, dephosphorylation, degradation or location in the cell. This new post-phosphorylation regulatory mechanism appears to play an important role in normal cell function, such as mitotic progression, and in the pathogenesis of some human pathologies, such as Alzheimer's disease.

Key words. Alzheimer's disease; mitotic regulation; Pin1; peptidyl-prolyl isomerase; prolyl isomerization; protein phosphorylation.

Introduction

To reproduce and multiply, every cell must execute an orderly series of events, called the cell cycle, which usually contains four phases, G1 (gap 1), S (DNA synthesis), G2 (gap 2), and M (mitosis). The events during mitosis are some of the most dramatic in biology. The chromosomes condense, the nuclear membrane disappears, the mitotic spindle assembles, and eventually chromosomes are pulled apart to the oppo-

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site poles of the dividing cell. Many of these events are regulated by phosphorylation of proteins on serine or threonine residues immediately preceding proline (Ser/Thr-Pro) due to activation of cyclin-dependent protein kinase (CdK) Cdc2 at the entry into mitosis. Cdc2 has been conserved during evolution and considerable progress has been made in understanding its upstream regulators [1–4]. Activation and inactivation of the cyclin B/CDC2 complex, frequently referred to as the mitosis-promoting factor, are critical for entry into and exit from mitosis, respectively, in all eukaryotic cells so far examined [1, 3]. At the G2/M transition, activation of Cdc2 requires multiple events, including the synthesis and binding of cyclin B, constitutive phosphorylation of Cdc2 on an activating site by CAK and, finally, Cdc25-dependent dephosphorylation of inactivating sites that have been phosphorylated by Wee1 and Myt1 [1, 3, 5]. Irreversible inactivation of Cdc2 at the metaphase/anaphase boundary requires the ubiquitin-dependent proteolysis of its cyclin subunit [3, 6, 7]. However, the mechanism by which Cdc2 activation elicits a series of mitotic events is less well understood.

Like all other Cdks and MAP kinases, Cdc2 belongs to a subfamily of proline-directed protein kinases that phosphorylate proteins on Ser/Thr-Pro sites [8]. At the G2/M transition, abrupt activation of Cdc2 leads to the phosphorylation of a large number of proteins. These phosphoproteins are localized in various mitotic structures [8] and most are recognized by the MPM-2 monoclonal antibody, which was originally generated using total mitotic extracts from HeLa cells as antigens [9]. MPM-2 appears to recognize conserved phosphoepitopes that contain the minimal phosphorylated Ser/Thr-Pro motif on about 50 mitosis-specific phosphoproteins [9–12]. This remarkable and puzzling specificity of MPM-2 for a defined subset of mitotic phosphoproteins has been seen in all eukaryotic organisms so far examined [9, 13–16]. The MPM-2 antigens include many proteins that play a crucial role during mitosis, such as never-in-mitosis mutant A (NIMA), Myt1, Wee1, Cdc25, topoisomerase IIa, tau, Map 4, INCENP, and Cdc27 [17–24]. Interestingly, MPM-2 does not recognize many proteins that are also phosphorylated on Ser/Thr-Pro sequences during interphase. These results suggest common phosphorylated epitopes present in mitotic phosphoproteins. However, it has remained unclear what role these phosphoepitopes play during mitotic progression, why such epitopes are highly conserved during evolution, and what their endogenous ligand(s) is.

Serine/threonine phosphorylation has been thought to regulate the function of proteins through conformation changes, thereby triggering an organized and programmed set of structural modifications that occur during mitosis. In fact, phosphorylation of certain proteins has actually been shown to regulate specific mitotic events. For example, phosphorylation of nuclear lamin A, small GTP-binding proteins Rab1A and Rab4B, and the kinesin-related motor Eg5 by Cdc2 plays an essential role in regulating nuclear lamina disassembly, intracellular membrane transport, and bipolar spindle formation during mitosis, respectively [25–27]. However, it is not clear what the phosphorylation on the Ser/Thr-Pro motif actually does and how these abrupt changes in the phosphorylation state at the G2/M transition lead to an organized and programmed set of mitotic events.

By searching for proteins that physically interact and functionally suppress the kinase NIMA, one of such MPM-2 antigens, we have recently isolated a novel protein, Pin1 [28]. Pin1 is highly conserved and essential specifically for mitotic progression. It contains a protein-interacting module, called the WW domain, and a catalytically active peptidyl-prolyl isomerase. Pin1 is structurally and functionally distinct from members of two other well-characterized families of prolyl isomerases, the cyclophilins and the FK506-binding proteins (FKBPs) [28]. Prolyl isomerases are ubiquitous enzymes that catalyze the typically slow prolyl isomerization of proteins, allowing relaxation of local energetically unfavorable conformational states [29, 30]. Interestingly, phosphorylation on Ser/Thr residues immediately preceding Pro not only alters the prolyl isomerization rate [31], but also creates a binding site for the WW domain of Pin1 [32, 33]. The WW domain acts as a novel phosphoserine-binding module targeting Pin1 to a highly conserved subset of mitosis-specific phosphoproteins [34]. Furthermore, Pin1 displays a unique phosphorylation-dependent prolyl isomerase activity that specifically isomerizes phosphorylated Ser/Thr-Pro bonds and regulates the function of mitotic phosphoproteins [32, 33]. These results suggest a novel signaling regulatory mechanism, where the isomerase Pin1 binds proteins that have been phosphorylated by Pro-directed kinases, and induces a conformational change to regulate their function. Pin1 could provide a new post-translational level of control to allow the general increase in protein phosphorylation resulting in the organized and programmed set of mitotic events. Interestingly, Pin1 is the first molecule that is able to restore the biological function of phosphorylated tau without dephosphorylation. Significantly, in brains of patients with Alzheimer's disease, Pin1 binds phosphorylated tau and is sequestered into the neurofibrillary tangles, resulting in depletion of soluble Pin1. Since depletion of Pin1 induces mitotic arrest and apoptosis, Pin1 may play a critical role in the pathogenesis of Alzheimer's disease.

Pin1 is a conserved prolyl isomerase essential for cell division

The NIMA kinase is essential for mitosis in the filamentous fungus *Aspergillus nidulans* [35–37]. Furthermore, we have previously demonstrated that an NIMA-like mitotic pathway is also required for entry into mitosis in other eukaryotic cells, including human cells [38]. Consistent with this idea, an NIMA homologue has been recently isolated from fission yeast and shown to induce chromosome condensation, as is the case for NIMA [39, 40]. These results indicate that the NIMA-like mitotic pathway is likely to be important for mitosis in most eukaryotic cells. To search for potential protein components of the putative mammalian NIMA pathway, we took advantage of the fact that expression of NIMA in yeast is lethal, likely due to NIMA-induced mitotic arrest, as shown in all other cells examined [4]. We modified a yeast two-hybrid screen and identified three human proteins, Pin1–3, which physically interact with the *Aspergillus* NIMA and functionally suppress the lethal NIMA phenotype in yeast [28]. We now know that all three Pin proteins are involved in mitotic regulation [28, 32–34, 41–43]. This review will focus on the structure and function of Pin1.

Pin1 encodes 163 amino acid residues that are arranged in two identifiable domains, an NH_2-terminal WW domain and a COOH-terminal prolyl isomerase domain (fig. 1) [28]. WW domains typically contain 38–40 amino acid residues with two invariant Trp residues and other conserved residues [44, 45]. These small protein modules are found in a variety of proteins involved in cell signaling or regulation, and have been shown to mediate protein-protein interactions [45, 46]. The catalytically active prolyl isomerase domain shares sequence characteristics of the newly discovered family of prolyl isomerases, but displays little similarity with the cyclophilins or FKBPs [28, 47, 48]. Pin1 is 45% identical to Ess1p/Ptf1p, an essential protein in budding yeast, and functionally complemented the *ess1*$^-$ null mutation [28]. These results have demonstrated that Pin1 is structurally and functionally homologous to Ess1p/Ptf1p [49, 50]. Subsequently, Pin1-homologous genes have been identified in all eukaryotic cells so far examined, including mammals, *Xenopus*, *Drosophila*, *Dictylostelium*, budding and fission yeast, as well as *A. nidulans* (fig. 1) [28, 33, 49, 51–53; sequences deposited in GenBank]. In addition, the *Drosophila* Pin1 homologue Dodo also functionally rescues the Ess1/Ptf1 deletion mutation in yeast [51]. These results indicate that Pin1 has been highly conserved during evolution.

To determine the function of Pin1 and its homologues during the cell cycle, we have constructed a haploid yeast strain that has the endogenous Ess1/Ptf1 deleted, but expresses human Pin1 under control of the inducible GAL1 promoter. By manipulating expression of Pin1, we have demonstrated that depletion of Pin1 from yeast induced mitotic arrest and subsequent nuclear fragmentation, without affecting DNA synthesis [28]. Significantly, a similar phenotype was also observed in HeLa cells upon expression of an antisense *PIN1* construct [28]. Conversely, overexpression of Pin1 prevented entry into mitosis in HeLa cells and *Xenopus* extracts [28, 33, 52]. These results have demonstrated that Pin1 is an essential prolyl isomerase that is specifically required for proper progression of mitosis. In addition, some Pin1-like genes (Pin1-Ls) have been isolated [54], [K. P. Lu and X. Z. Zhou, unpublished data], suggesting that multiple Pin1-L genes are likely to be present in mammalian cells. This may explain why Dodo is not essential in *Drosophila* [51].

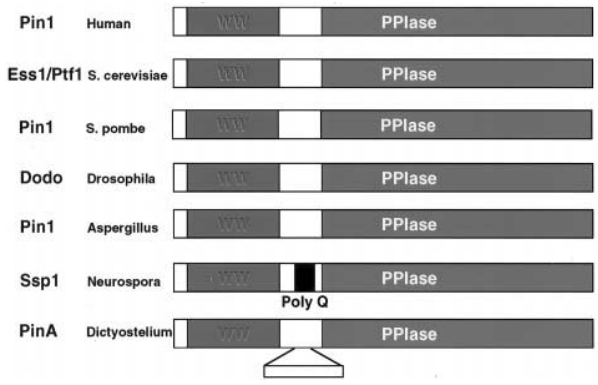
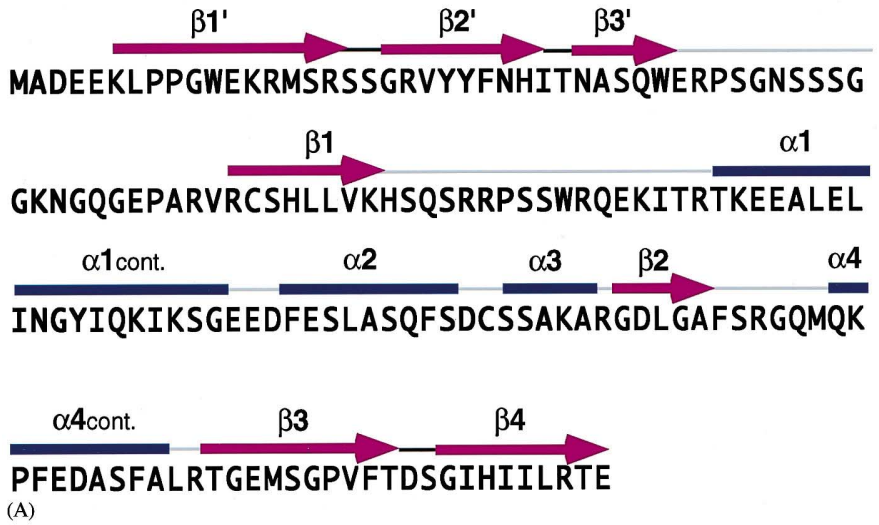


Figure 1. Conservation of the Pin1 structure during evolution. Schematic presentation of the primary domain structure of selected Pin1s isolated from different species.

The X-ray structure of Pin1

To understand the function and regulation of Pin1, we have recently determined its three-dimensional structure using X-ray crystallography [55]. In the Pin1 crystal, there are two easily identifiable structural domains, as predicted from the primary sequence (fig. 2). The NH_2-terminal WW domain, residues 1–39, consists of a triple stranded anti-parallel β-sheet. The overall fold of the WW domain in the Pin1 whole protein is almost identical to the nuclear magnetic resonance solution structure of the isolated YAP WW domain [56], indicating that WW domains likely act as independent protein modules. Interestingly, there is a hydrophobic patch on the surface of the WW domain, which sequesters a polyethylene glycol (PEG) molecule. The PEG molecule makes contacts with several residues, including Tyr23,



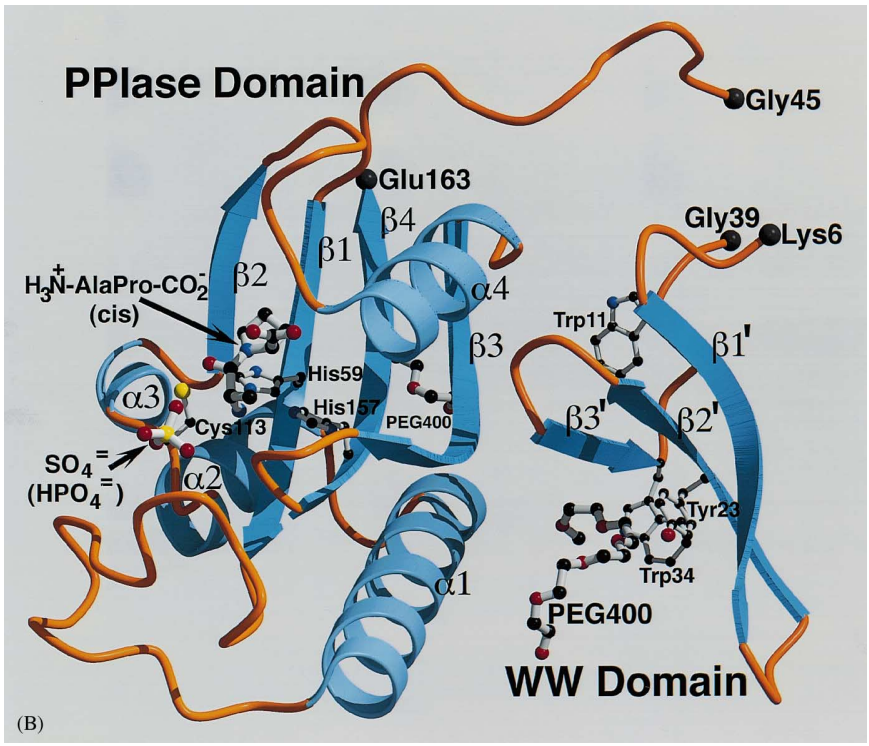


Figure 2. The peptide sequence and X-ray structure of human Pin1. (*A*) The amino acid sequence of the Pin1 protein, with the structural elements found in the crystal structure highlighted. (*B*) X-ray structure of human Pin1. Ribbon representation of the three-dimensional structure of the human Pin1 in a complex with an Ala-Pro dipeptide. While present in the crystallized protein, residues 1–6 and 40–44 are not visible in electron density maps and are considered to be disordered [55]. The NH_2-terminal WW domain is connected to the COOH-terminal catalytic domain by a linker. A sulfate ion is sequestered by a conserved basic cluster in close proximity to the β methyl group of the Ala residue in the bound Ala-Pro dipeptide. Two PEG400 (polyethylene glycol 400) molecules are sequestered from the buffer into the Pin1 structure. Color codes for atoms: red, oxygen; blue, nitrogen; black, carbon; yellow, sulfur (reproduced with permission from Cell Press, Cambridge, MA).

Trp34 and Ser16 (fig. 2). Given that solvent-exposed hydrophobic patches are generally energetically disfavored in proteins, and are often maintained due to functional necessity, this hydrophobic cluster on the surface of the WW domain may be important for Pin1 function.

The central scaffolding of the COOH-terminal catalytic domain consists of a four-stranded anti-parallel β-sheet and four α-helices surrounding the flattened half β-barrel [55]. The core β-sheet and the α4-helix of the Pin1 prolyl isomerase domain fold in a similar fashion to FKBP12, despite the lack of primary sequence similarity. A set of absolutely conserved catalytic residues in the Pin1 subfamily of prolyl isomerases project outward from the barrel structure and define the binding pocket for the proline (Leu122, Met130, Phe134) and the peptide bond that undergoes cis to trans isomerization [47, 48, 55]. Similarly, a hydrophobic pocket containing aromatic and aliphatic residues sequesters the aliphatic Pro side chain in FKBPs [55, 57–59]. However, a striking difference is observed at the entrance to the Pro-binding pocket, which determines the substrate specificity of prolyl isomerases at the i-1 position relative to Pro. In FKBP, hydrophobic residues (Ile90, Ile91, Phe36) at this pocket can explain its preference for hydrophobic residues N-terminal to proline in peptide substrates. Interestingly, a cluster of basic residues is formed at the spatially analogous positions by the side chains of conserved residues Arg68 and Arg69, which are characteristics of the Pin1 subfamily of prolyl isomerases [43]. Although Arg68 and Arg69 in Pin1 and Ile90 and Ile91 in FKBP arise from different primary sequence positions, they are superimposed in the three-dimensional structure [55]. This positively charged cluster sequestered a crystallographically well-ordered sulfate ion in close proximity to the β methyl group of the Ala residue in the bound Ala-Pro dipeptide [55]. The spatial proximity of this tripartite basic cluster in the active site to the bound dipeptide suggests that Pin1 possesses a strong preference for negatively charged chains in the residue NH_2-terminal to Pro in substrates. Indeed, a Glu, a phosphorylated Ser or a phosphorylated Thr side chain modeled on the Ala of the Ala-Pro dipeptide superimposed their respective anionic groups, carboxylate or phosphate, on the bound sulfate ion in the complex crystal structure [55]. Furthermore, Pin1 prolyl isomerase activity increases 100-fold when a negatively charged Glu residue is placed immediately upstream of Pro [55]. These structural analyses suggest that Pin1 might prefer a negatively charged residue immediately preceding Pro residue in substrates.

Pin1 is a phosphorylation-specific prolyl isomerase

Proline residues provide a potential backbone switch in the polypeptide chain controlled by the cis/trans isomerization about the peptidyl-prolyl bond catalyzed by prolyl isomerases [29, 30, 60–62]. There are two families of conventional prolyl isomerases, cyclophilins (Cyps), FKBPs, and a newly identified third family of prolyl isomerases, which includes Pin1. These three prolyl isomerase families are unrelated in their primary sequences and three-dimensional structures, yet they all catalyze a similar reaction. There are eight cyclophilin family genes, four FKBP genes, and one Pin1 homologue in budding yeast, and there are at least five cyclophilins, six FKBPs, and at least two characterized members in the third family in mammals, Pin1 and Par14 [62, 63] with some more to come. It is worth pointing out that in contrast to Pin1, Par14 does not catalyze the isomerization of phosphorylated Ser/Thr-Pro bonds [63].

Although cis/trans isomerization about the prolyl bond occurs spontaneously, acceleration of this process by prolyl isomerases could play a role in protein folding or refolding by catalyzing a rate-limiting step [29, 30, 61, 62]. The original finding that the in vitro folding of ribonuclease A involves a mixture of slow- and fast-folding species differing in the isomeric state of prolyl peptide bonds prompted the hypothesis that catalysis of this isomerization would represent a general mechanism for accelerating protein folding in vivo. Although direct evidence for prolyl-isomerase-mediated cellular protein folding has been limited, indirect evidence indicates that the formation of the collagen triple helix is limited by prolyl isomerization, and facilitated by a Cyp family prolyl isomerase [29]. For example, the mitochondrial Cyps from *Saccharomyces cerevisiae* and *Neurospora crassa* catalyze the refolding of proteins following mitochondrial import. Similarly, the folding of procollagen I in suspended chick embryo tendon fibroblasts is accelerated by cellular Cyp activity, which renders procollagen overhydroxylated due to a prolonged residence time of the non-triple helical state in the endoplasmic reticulum [64]. Furthermore, prolyl isomerase may also be involved in assembly/disassembly of protein complexes, protein trafficking, and direct regulation of protein activity [30, 62]. For example, the photoreceptor Cyp, NinaA, is essential for rhodopsin maturation and forms a complex with substrate rhodopsins in the visual system of *Drosophila* [65]. Cyp40 is part of the steriod receptor complex [66, 67], and also binds and inhibits the DNA-binding activity of the c-Myb transcription factor [68]. Furthermore, FKBP12 interacts with the cardiac ryanodine receptor and modulates its calcium release activity, with knockout of FKBP12 resulting in severe dilated cardiomyopathy and ventricular septal defects in mice [69]. These diverse effects suggest that PPIases play an important role in regulating a wide range of cellular processes.

The most well-characterized function of the Cyps and FKBPs is their role in the immune system, because of their importance as cellular receptors for clinically relevant immunosuppressive drugs [29, 30, 60–62]. When the Cyps and FKBPs bind the immunosuppressive drugs cyclosporin A and FK506, respectively, there are

two common outcomes: inhibition of prolyl isomerase activity and inhibition of the common target, calcineurin. Interestingly, it is inhibition of the calcineurin phosphatase activity that prevents lymphocytes from responding to antigen-induced mitogenic signals, resulting in the immunosuppression. Surprisingly, inhibition of the prolyl isomerase activity is apparently unrelated to the immunosuppressive property of the drug/isomerase complexes [29, 30, 60, 61]. In addition, genetic analysis of prolyl isomerase function in budding yeast has yielded unexpected results. All the known members of the Cyp and FKBP family genes can be disrupted without any significant phenotype [70]. Therefore, evidence for the biological importance of prolyl isomerase activity has been elusive.

Prolyl isomerization of Ser/Thr-Pro motifs is particularly relevant for cell cycle control, since the known specificity of the Cdks, including Cdc2, is for Ser/Thr-Pro in a variety of Cdk targets [8, 71, 72]. Although phosphorylation on Ser/Thr-Pro has been proposed to alter the conformation of a protein, little is known about the actual conformational changes and whether phosphorylation regulates the conformation of the Ser/Thr-Pro bonds. As an initial step to address this question, we synthesized a series of peptides that contain the phosphorylated or non-phosphorylated Ser/Thr-Pro motif and characterized their conformations [31]. Phosphorylation on Ser/Thr-Pro significantly altered the content of the cis isoform and reduced the rate of the cis to trans isomerization, when compared with the non-phosphorylated analogues. In contrast, little effect was observed with phosphorylation on the Tyr-Pro bond. This may be because the side chain of Tyr is longer than that of Ser or Thr. Furthermore, studies of the pH dependence of the isomerization of the phosphopeptides have revealed that the cis to trans isomerization rate was most affected when the phosphate of pThr was in the dianionic state [31]. These effects of phosphorylation on isomerization were specific for phosphorylated Ser/Thr since neither phosphorylated Tyr nor glutamic acid affected the prolyl isomerization [31]. Thus, these results have demonstrated that protein phosphorylation specifically restrains the isomerization rate of the Ser/Thr-Pro peptide bonds.

Given that phosphorylation on the Ser/Thr-Pro motif alters the cis/trans isomerization rate, we hypothesized that Pin1 is a prolyl isomerase that would isomerize the pSer-Pro peptide bonds [32]. We therefore measured Pin1 isomerase activity against a variety of chromogenic oligopeptide substrates, with members of the Cyps and FKBPs as controls [32]. We found that neither Cyp18 nor FKBP12 was able to effectively catalyze isomerization of peptides with pSer/Thr-Pro moieties, compared to peptides lacking phosphate. These results have indicated that phosphorylation on the Ser/Thr-Pro motif renders the prolyl-peptidyl bond resistant to the catalytic action of conventional prolyl isomerases, and also suggested the need for a different enzyme to catalyze this reaction. In contrast, Pin1 displays the unique phosphorylation-specific substrate specificity [32]. Pin1 displayed little activity towards the Ala-Pro peptide bond. Incorporating negatively charged side chains of Glu and Asp immediately preceding the Pro residue, which could mimic the pSer, resulted in significant increases in isomerization activity. The most striking feature of Pin1 is that its isomerase activity is highly specific for peptides with pSer/Thr-Pro bonds. Pin1 displayed very low levels of isomerase activity for substrates containing Ser-Pro or Thr-Pro bonds. However, phosphorylation of these peptides on Ser or Thr residues dramatically increased the k_{cat}/K_m values about 300-fold [32]. The specificity constant of Pin1 prolyl isomerase activity was increased up to 1300-fold with the best available substrate, compared to its non-phosphorylated counterpart [32]. Similar phosphorylation-specific prolyl isomerase activity was later also observed in Ess1/Ptf1 and Ssp1, the Pin1 homologues in budding yeast and *N. crassa*, respectively [53]. These findings have demonstrated the dramatic differences in substrate specificity between the Pin1 subfamily of prolyl isomerase and the other prolyl isomerases.

These striking differences in substrate specificity result from a different organization of the Xaa-Pro binding pocket in the enzymes (fig. 3). Since a hydrophobic pocket containing aromatic and aliphatic residues sequesters the aliphatic Pro side chain in all three families of prolyl isomerases [55, 57–59], the residues responsible for determining substrate preference must reside at the entrance to the Pro-binding pocket. In Pin1 and all its homologues identified so far, the side chains of conserved residues Arg68 and Arg69 form a basic patch of residues at the entrance to the Pro-binding pocket (fig. 3B). Furthermore, substitution of both Arg68 and Arg69 by Ala residues reduced the k_{cat}/K_m over 500-fold compared to wild-type Pin1 for the phosphorylated substrate, to values similar to those obtained with the non-phosphorylated peptide [32]. Moreover, when the non-phosphorylated peptide was used as a substrate, the catalytic activity of this double-Ala Pin1 mutant was the same as wild-type Pin1 [32]. These data have provided strong evidence that this cluster of basic residues is responsible for the phosphorylation-specific substrate specificity of the Pin1 subfamily of enzymes. Conspicuously, bacterial and human parvulins, which are related to the catalytic domain of Pin1, were unable to catalyze the cis/trans isomerization of phosphorylated Ser/Thr-Pro bonds and, instead, preferred the Arg-Pro bond [32, 47, 63, 73]. This preference can be easily explained by the fact that the two positively charged residues, Arg68 and Arg69, in Pin1s are re-

placed by two Glu residues in parvulin (fig. 3A) [47, 63]. These studies have demonstrated that the unique structural features in the active site of Pin1 determine its phosphorylation-specific substrate specificity.

Pin1 directly binds to a defined subset of mitotic phosphoproteins

The above results indicate that Pin1 is a unique prolyl isomerase specific for the pSer/Thr-Pro bonds in vitro and is specifically required for proper progression during mitosis in vivo. What is the basis for the cell cycle specificity of the Pin1 function? To answer this question, we determined Pin1 levels and Pin1-binding activity at different phases of the cell cycle [33]. Although Pin1 levels were constant through the cell-cycle, Pin1 directly bound a number of proteins in a cell cycle-regulated manner, as shown by Farwestern analysis using Pin1 as a probe [33]. Pin1-binding activity was low during G1 and S, increased in G2/M, and was highest when cells were arrested in M. Since the patterns of these Pin1-binding proteins are similar to those of MPM-2 antigens, we asked if Pin1 interacted with MPM-2 antigens [33]. Pin1 beads were added to interphase or mitotic extracts, followed by analysis of MPM-2 antigens present in the beads. Pin1 bound and depleted almost all of the MPM-2 antigens in a HeLa mitotic extract. Furthermore, MPM-2 antigens were co-immunoprecipitated with anti-Pin1 antibodies in a mitosis-specific and phosphorylation-dependent manner [33], indicating a stable complex between MPM-2 antigens and Pin1 in the cell. In addition, Crenshaw et al. [52] have shown that *Aspergillus* Pin1 proteins bind Cdc25 and Plk1, two known MPM-2 antigens. These results have indicated that Pin1 directly binds MPM-2 antigens in a phosphorylation-dependent manner.

The Ser/Thr-Pro motif is the target for phosphorylation by a range of protein kinases including Cdc2 and MAP kinases, and is present in a large number of kinase substrates [8, 71, 72]. However, since Pin1 only binds a defined subset of mitotic phosphoproteins, additional factors must determine whether or not a phosphorylated protein is a target for Pin1 regulation. As an initial attempt to identify the Pin1-binding specificity, we used an oriented degenerate peptide library to examine the optimal Pin1-binding sequences [32]. Although no specific binding was detected when Pin1 was incubated

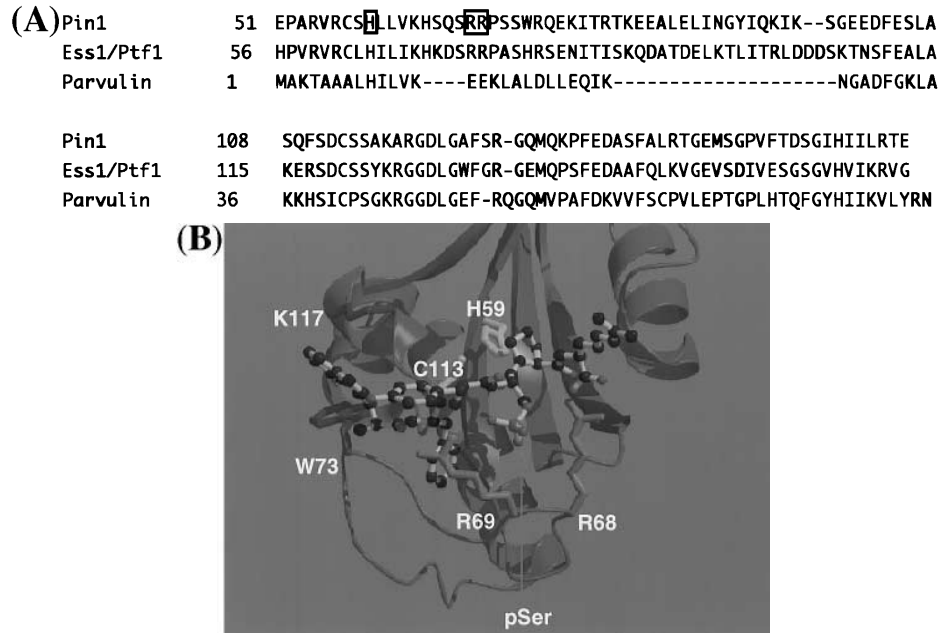


Figure 3. The structural basis of the Pin1-unique substrate specificity. (*A*) Sequence alignment of the PPIase domain of selected Pin1s. Numbers to the left correspond to the first residue on each line. Boxes indicate the amino acid residues that are important for catalysis or substrate specificity, as determined by mutagenesis study described in Yaffe et al. [32]. (*B*) Molecular modeling of the optimal Pin1 peptide substrate bound to the Pin1 active site. The best available substrate Trp-Phe-Tyr-pSer-Pro-Arg is modeled into the active site cleft. The COOH-terminal Arg residue has the aliphatic portion of its side chain against the hydrophobic surface exiting the Pin1 active site. The aliphatic side chain of the Pro ring is shielded from solvent within a hydrophobic cluster of Leu122, Met130, and Phe134, and the Ser phosphate is complexed within a positively charged pocket composed of Arg68 and Arg69, adapted from Yaffe et al. [32].

with non-phosphorylated peptide libraries, strong and specific binding was observed with a pSer-containing degenerate peptide library. Furthermore, Pin1 displayed a marked preference for Pro immediately C-terminal to pSer [32]. These results suggest that Pin1 prefers binding to a pSer-Pro motif. To refine the sequence specificity of Pin1 for its substrates, we used a degenerate peptide library containing a fixed pSer-Pro sequence flanked by three degenerate positions on each side and confirmed that the binding specificity of Pin1 resides in the sequence of amino acids flanking the pSer/Thr-Pro sequence [32].

In light of the similarity of Pin1-binding partners and MPM-2 antigens, we have investigated the sequence specificity for MPM-2 recognition using the same technique. The optimal MPM-2 binding motifs were similar to those identified using different approaches [11] and, more interestingly, were extremely similar to the sequence motifs selected by Pin1 [32]. These results explain the observation that Pin1 specifically interacts with MPM-2 antigens [33]. They also explain the puzzling and remarkable conservation of the MPM-2 epitopes in all eukaryotic cells [9, 13–16]. Whereas MPM-2 is a monoclonal antibody, Pin1 is a highly conserved endogenous enzyme. However, there are strong similarities between the interaction of Pin1 and MPM-2 with their respective targets. First, they both recognize an overlapping subset of conserved mitosis-specific phosphoproteins and localize to the nuclear speckle during interphase and to mitotic chromosomes at mitosis [9–12, 20, 28, 33, 74]. Second, the interaction either between Pin1 and its interacting proteins or MPM-2 and its antigens is dependent upon mitosis-specific phosphorylation of the target proteins [9, 10, 33]. Third, both inhibit cell cycle progression in *Xenopus* embryos and entry into mitosis in *Xenopus* extracts [33, 75, 76]. Fourth, Pin1 is highly conserved in all eukaryotic cells so far examined, including mammals, *Xenopus*, budding and fission yeast, and *Aspergillus*, as is the case for the MPM-2 epitopes [9–12, 20, 23, 28, 33, 74]. Finally, peptide-binding specificities of Pin1 and MPM-2 are almost identical, and both strongly bind peptides containing phosphorylated Ser-Pro flanked by hydrophobic residues or Arg, as shown by screening oriented degenerate peptide libraries [32]. These results indicate that the conservation of the MPM-2 epitopes can be explained by the recognition of these epitopes by the highly conserved mitotic regulator Pin1.

Based on the amino acid preferences deduced for the six positions surrounding the pSer-Pro motif for optimal Pin1 binding, we undertook a weighed screening of the SWISS-PROT protein sequence database. The scan revealed within the top 5% of highest scores a variety of potentially important mitotic phosphoprotein targets for Pin1. Many of these proteins are involved in regulation of the cell cycle, cytoskeletal or spindle structure, DNA replication, transcription, and RNA processing [32]. Among them are those shown to bind Pin1 following mitosis-specific phosphorylation such as Cdc25, Plk1, and NIMA [33, 52]. Other proteins identified in this search, however, had not been previously suspected of interacting with Pin1. To examine whether they can bind Pin1, we have tested two examples, Rab4 and p70 S6 kinases, and found that they both interacted with Pin1 in a mitosis-specific and phosphorylation-dependent manner in vitro [32]. Recent results have further confirmed that Pin1 indeed formed a stable complex with Rab4 during mitosis in vivo, when Rab4 is phosphorylated [L. Gerez, K. Mohrmann, M. van Raak, X. Z. Zhou, K. P. Lu and P. van der Sluys, unpublished data].

To determine the identity of other Pin1-binding proteins, we have used three further approaches. The first is to probe Pin1-binding proteins with antibodies that are specific to known mitotic phosphoproteins [33]. The second is to phosphorylate in-vitro-translated mitotic phosphoproteins using *Xenopus* cell-cycle-specific extracts, and then determine if they would bind Pin1 upon mitotic phosphorylation [33, 34]. The third method is to obtain peptide sequences of Pin1-binding proteins using a microsequencing technique [K. P. Lu, X. Z. Zhou and M. Shen, unpublished data]. We have so far identified more than 20 Pin1-binding proteins, some of which are listed in table 1. It should be noted that none of these Pin1-binding proteins contains the optimal binding motifs described above. Therefore, the optimal binding sequence of Pin1 identified using degenerate peptide libraries might not necessarily apply to all Pin1 target proteins. However, all these studies have further confirmed that Pin1 binds a rather specific subset of mitotic phosphoproteins in a phosphorylation-dependent manner.

The WW domain mediates Pin1 substrate recognition by acting as a phosphoserine/threonine-binding module

Protein-interacting modules play an important role in determining the specificity of signal transduction events [77–80]. These modules are typically small conserved domains that bind specific sequences in target proteins, recruiting active enzymes into the signaling network or by placing enzymes close to their substrates. For example, Src homology domain 2 (SH2) and 3 (SH3) domains bind specific phosphotyrosine residues and polyproline motifs on target proteins, respectively, thereby promoting the assembly of signaling molecule complexes around an activated receptor [81, 82]. Following the identification of the Pin1-binding proteins, we wondered how it was possible for Pin1 to interact

Table 1. Pin1 binding to mitotic phosphoproteins involved in various mitotic events.

Proteins	Interphase	Mitotic
Mitotic kinases and phosphatases		
NIMA	–	+++
Cdc25	–	+++
Plk1	–	+++
Wee1	+	+++
Myt1	–	+++
MAPs		
tau	–	+++
MP75 (E-MAP-115)	–	+++
Small G proteins		
Rab4	–	+++
DNA-binding proteins		
CENP-F	–	+++
Incenp	–	+++
Sox3	–	+++
Xbr-1b	–	+++
MP110 (Cdc5)	–	+++
APC		
Cdc27	–	+++
Others		
MP68	–	+++
MP30	–	++
No binding		
MP48	–	–
Cyclin B	–	–
PTP-1B	–	–
Cdc2	–	–

The binding between Pinl and all selected mitotic phosphoproteins was determined as described [33, 34, 43]. –, no detectable interaction; +, a weak but above background interaction; ++, readily detectable interaction; +++, strong interaction.

with so many proteins. Both the primary sequence and crystal structural analyses indicate that Pin1 contains two separate domains, the prolyl isomerase and WW domain [28, 55]. Interestingly, the WW domain contains a hydrophobic cluster, which can be traced to the active site of the isomerase domain by a conserved path of hydrophobicity on the molecular surface [55]. Given that solvent-exposed hydrophobic patches are generally energetically disfavored in proteins, and are often maintained due to functional necessity, clusters of hydrophobic residues are predicted structural features of protein-protein interaction surfaces [83–85]. Therefore, we hypothesized that the WW domain plays an important role in targeting Pin1 to the substrates by interacting with mitotic phosphoproteins via its hydrophobic patch [34]. We have investigated the role of the WW domain in Pin1 substrate recognition by examining the ability of the WW domain to interact with Pin1 target proteins [34]. Pin1 was divided into the WW domain and the isomerase domain which were then incubated with mitotic extracts. Interestingly, only the Pin1 WW domain, but not the isomerase domain, bound almost all of the Pin1-binding proteins [34]. These results have shown that it is the WW domain that is responsible for binding phosphoproteins, and suggested that the WW domain might be a phosphoserine-binding module. This finding was rather unexpected because WW domains were believed to bind proline-rich motifs [45, 46, 86–88] (fig. 4A). Therefore, it was critical to demonstrate that the interaction between the WW domain and phosphorylated Ser/Thr-containing sequences is indeed specific and biologically significant.

If the WW domain is a phosphorylated Ser/Thr-binding module, at least the following four criteria should be met. First, the interactions between the WW domain and phosphoproteins should depend on phosphorylation of target proteins. Second, the WW domain should bind specific pSer- or pThr-containing sequences present in the target proteins. Third, the interactions should be competed by a phosphoserine-containing peptide, but not by its non-phosphorylated counterpart. Finally, the WW domain should display a reasonable affinity for a phosphopeptide.

To examine the first prediction, the interactions between the Pin1 WW domain and specific target proteins were determined [34]. Proteins synthesized in vitro were phosphorylated in a cell-cycle-specific manner by incubating them with either *Xenopus* interphase or mitotic extracts. Like Pin1, the isolated WW domain bound the Cdc25C that was phosphorylated by mitotic extracts, but not by interphase extracts. However, the WW domain failed to interact with Cdc25C if the mitotically phosphorylated Cdc25C was dephosphorylated prior to the binding [34]. These results have demonstrated that the WW domain binding depends on phosphorylation of target proteins.

To determine whether the WW domain of Pin1 binds specific pSer- or pThr-containing sequences in target proteins, we have identified the Pin1- and its WW-domain-binding sequences in the mitotic phosphatase Cdc25 and the microtubule-binding protein tau using peptide-binding assays [34, 42]. Although both proteins have been shown to be phosphorylated on more than a dozen Ser/Thr sites during mitosis, we have demonstrated that via the WW domain, Pin1 bound to very specific phosphorylated Ser/Thr-Pro motifs. Specifically, the WW domain bound only two phosphorylated Thr-Pro motifs, pThr47 and pThr68, in Cdc25, and only one single phosphorylated Thr-Pro motif, pThr231, in tau [36]. Furthermore, point mutations of these specific Thr residues completely abolished the ability of Pin1 to bind either Cdc25 or tau [34, 42]. Therefore, the Pin1 WW domain binds specific phosphoserine residues present in target proteins.

We next asked whether the WW domain binds other phosphoproteins via the same pSer-binding pocket [34]. Pintide (WFYpSPRLKK), which was originally identified to be the optimal Pin1-binding peptide by screening degenerate peptide libraries [32], was used to

compete with phosphoproteins for binding. When Pin1 or its WW domain was incubated with various concentrations of Pintide or the control peptide before incubation with mitotic extracts, the phosphoprotein-binding activity was significantly reduced by Pintide, but not with the non-phosphorylated peptide, in a concentration-dependent manner [34]. Pintide effectively prevented either Pin1 or the WW domain from binding to MPM-2 antigens with a similar potency. These results have demonstrated that a phosphopeptide can completely compete with phosphoproteins in binding to Pin1 or its WW domain in a phosphorylation-dependent manner. Finally, to further confirm that the WW domain is a pSer-binding module, we measured binding constants of the WW domain for phosphopeptides using a quantitative fluorescence polarization assay [34].

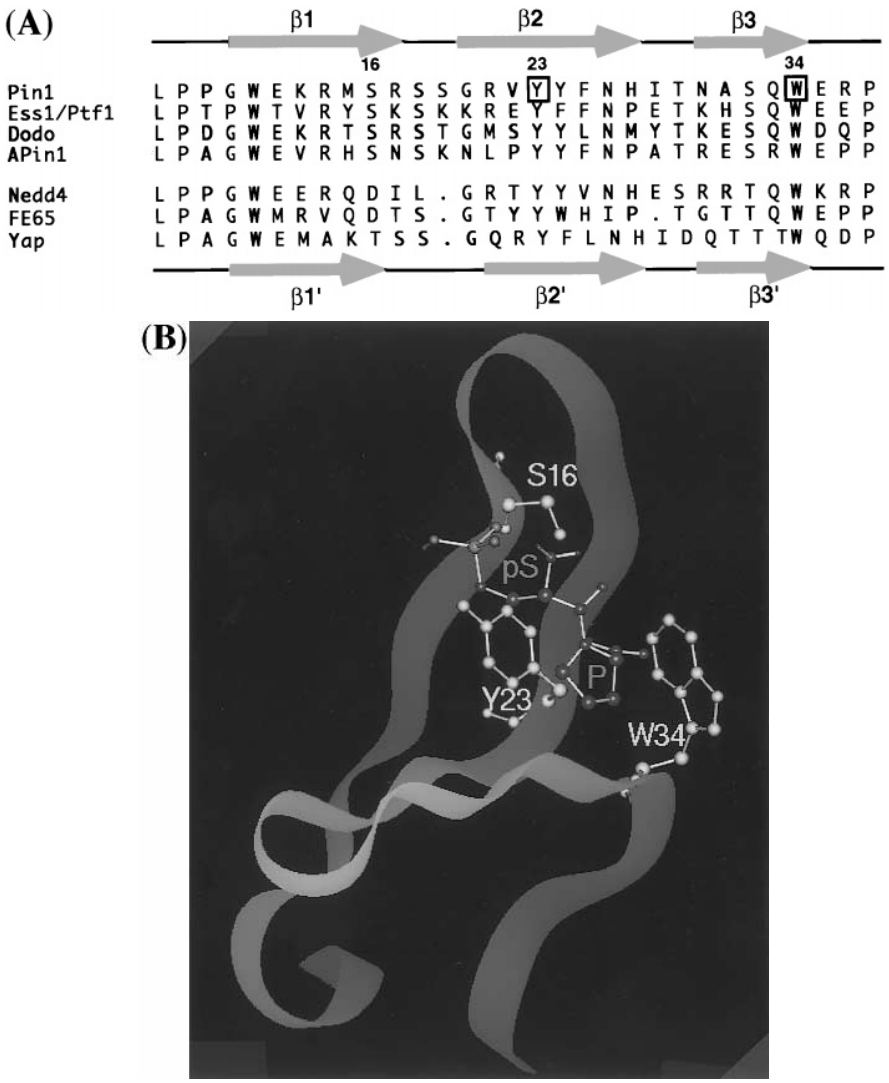


Figure 4. The function of the Pin1 WW domain as a pSer/Thr-Pro-binding module. (*A*) Sequence alignment of selected WW domains. The top and bottom lines illustrate the X-ray structural elements in native Pin1 and the nuclear magnetic resonance structural elements in the isolated YAP WW domain, respectively. The numbers above the sequences refer to the human Pin1. Boxes indicate the amino acid residues that are important for binding phosphoprotein substrates, as determined by mutagenesis analysis [34]. (*B*) Molecular modeling of the WW domain in a complex with the phosphorylated Ser-Pro motif. Starting from the co-ordinates of the WW domain, along with the co-crystallizing PEG in the Pin1 crystal, a phosphorylated Ser-Pro dipeptide is modeled into the hydrophobic cluster of the WW domain. The Pro ring sits in a hydrophobic crevice, stabilized by the hydrophobic interactions with side chains of Tyr23 and Trp34, whereas the pSer fits into a space between Ser16 and Tyr23, stabilized by the hydrogen bonding interactions between the phosphate moiety of phosphorylated Ser and the hydroxyl group of Tyr23.

Although no binding at all was detected between the non-phosphorylated control peptide and Pin1, its WW domain or isomerase domain, binding with the Pintide analogue was readily detected. Pin1 displayed two binding sites for Pintide with different affinities, with a K_d of 1.2 and 11.0 μM, respectively. Interestingly, the isolated WW domain and isomerase domain had only one binding site and their affinities corresponded to those of the high- and low-affinity sites, respectively [34]. These results have demonstrated that both the WW and the isomerase domain can directly bind the phosphopeptide, the affinity of the former being much higher than that of the latter. Collectively, the above results demonstrate that the WW domain directly binds with a high affinity to the phosphopeptide and a defined set of mitotic phosphoproteins and that these interactions are mediated by phosphoserine residues [34]. These properties are reminiscent of those of the SH2 domain and phosphotyrosine interactions [81, 82]. Thus, the Pin1 WW domain is a phosphoserine-binding module [34].

To determine the structural basis and function of the WW domains and pSer interaction, we focused on the Pin1 WW domain [34]. In the Pin1 crystal, the WW domain contains a hydrophobic cluster sequestering a PEG molecule, which forms close contacts with Ser16, Tyr23, and Trp34 located at three different strands of the β-sheet (fig. 4B) [55]. Since a hydrophobic patch at the molecular surface is usually conserved for interaction, and a statistical analysis ranks the propensity of Tyr to bind phosphate next to that of Arg [83, 84], the hydrophobic cluster at WW domains may be a phosphoserine-binding pocket. To examine this possibility, Pin1 proteins containing mutations at the above three residues and at Arg14, a residue close to Tyr23 in the structure [55], were generated and tested for their ability to bind phosphoproteins and peptides. Substitution of Arg14 or Ser16 with Ala did not appear to change binding to phosphopeptide or phosphoproteins significantly [34]. In contrast, a single Ala point mutation of either Tyr23 or Trp34 completely abolished the ability of Pin1 to bind either phosphoproteins or the phosphopeptide with high affinity [34], consistent with disrupting the pSer-binding ability of the WW domain. To examine the importance of the hydroxyl group of Tyr23, it was substituted with Phe. This mutation significantly reduced the ability of Pin1 to bind the phosphoproteins and phosphopeptide, demonstrating the importance of the Tyr hydroxyl group in mediating pSer-binding [34]. Interestingly, a similar Tyr-mediated pSer-binding has also been observed in the complex of phosphorylated CREB and CBP [6]. Therefore, a pSer-Pro dipeptide is modeled into the hydrophobic cluster of the WW domain in the location of the PEG molecule, shown in figure 4B. The Pro ring sits in a hydrophobic crevice stacked between the aromatic rings of Tyr23 and Trp34, whereas the pSer fits into a space between Ser16 and Tyr23, with the phosphate moiety being directed within hydrogen-bonding distance of the Tyr23 hydroxyl proton. Further structural analysis of the Pin1 WW domain and phosphorylated Ser/Thr-Pro-containing peptides will be useful to define the phosphorylated Ser/Thr-Pro-binding pocket and binding specificity of the WW domain.

To address the importance of the WW domain in Pin1 function in vivo, we took advantage of the fact that the *PIN1* yeast homologue, *ESS1/PTF1*, is essential for cell growth and that human Pin1 can carry out this essential function [28]. A temperature-sensitive *ptf1* mutant phenotype is completely rescued by *PTF1* or *PIN1* [34]. To determine whether the WW domain is important for Pin1 to exert its essential function, the WW domain and the isomerase domain of Pin1 were separately expressed in the same vector, but neither domain was able to complement the *ptf1* phenotype [34]. These results indicate that the WW domain is indispensable in vivo. Furthermore, all WW domain mutants that were able to bind phosphoproteins rescued the *ptf1* phenotype. However, all Pin1 mutations, which disrupted the interactions between the Pin1 WW domain and phosphoproteins, abolished the ability of Pin1 to support cell growth [34]. These results demonstrate that phosphoprotein-binding activity of the WW domain is essential for Pin1 to function in vivo.

Pin1 regulates the biological function of mitotic phosphoproteins

Given that Pin1 is a phosphorylation-specific prolyl isomerase that binds a defined subset of mitosis-specific proteins, the obvious question is whether Pin1 affects the function of Pin1-binding proteins. The fact that Pin1 was originally isolated as a protein that physically interacts and functionally suppresses the mitotic function of the mitotic phosphoprotein NIMA suggests that Pin1 regulates the biological activity of its target proteins [28]. To further address this question, we have chosen two other Pin1 target proteins that have well-defined, but completely different, biological activity.

The Pin1 target protein is tau, a microtubule-binding protein that is important for the dynamic change in the microtubule structure in mitotic cells. We have demonstrated that Pin1 not only binds phosphorylated tau, but also functionally restores its biological activity [42], as described below. This is consistent with the finding that depletion of Pin1 induces mitotic arrest with a collapsed microtubule network in HeLa cells, or a short mitotic spindle in budding yeast [28]. The other target protein is Cdc25C, a phosphatase that dephosphorylates and activates Cdc2 at entry into mitosis [20, 89–

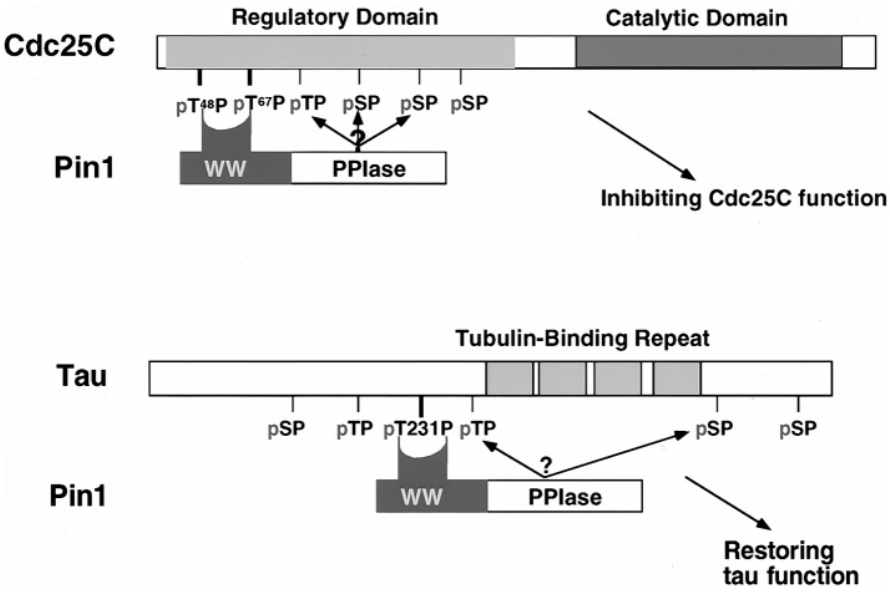


Figure 5. The relationship between Pin1 and its substrates. The WW domain of Pin1 binds the specific phosphorylated Ser/Thr-Pro motif in Pin1 target proteins by acting as a phosphorylated Ser/Thr-binding module and places the phosphorylated Ser/Thr-Pro-specific isomerase domain close to its substrates, such as Cdc25 and tau, thereby regulating their function.

91]. Cdc25C is activated by mitosis-specific phosphorylation on the MPM-2 epitopes at the G2/M transition [92–94]. Since it is the mitotically phosphorylated form of Cdc25C that interacts with Pin1 [33, 52], it is conceivable that the inhibitory effects of Pin1 on entry into mitosis could be at least partially explained through inhibition of Cdc25C activity. To test this possibility, we first examined whether Pin1 interacts with Cdc25C in vivo and, if so, whether this interaction is cell cycle regulated. Both in HeLa cells and *Xenopus* extracts, the interaction between Pin1 and Cdc25C was indeed cell cycle regulated [33, 52]. The interaction significantly increased just prior to mitosis. Further experiments have identified that both Pin1 and its WW domain bound only two conserved pThr-Pro sites (Thr48 and Thr67) (fig. 5) [34]. Significantly, Izumi and Maller [92] have shown that phosphorylation of these two Thr residues is important for activating Cdc2 and for initiating mitotic entry in *Xenopus* extracts. These results have shown that Pin1 interacts with the phosphorylation sites on Cdc25C that are essential for its mitotic activation. Finally, to examine whether Pin1 can regulate activity of Cdc25C, Pin1 was incubated with the mitotically phosphorylated and active Cdc25c. Pin1 reduced Cdc25C activity to a level similar to that of Cdc25C incubated with interphase extracts (fig. 5) [33], indicating that Pin1 prevents the mitotic activation of Cdc25C. This offers one explanation for the ability of Pin1 to inhibit mitotic entry.

Pin1 is an new enzyme involved in Alzheimer's disease

To examine the potential role of the Pin1-mediated post-phosphorylation regulatory mechanism in human diseases, we chose Alzheimer's disease because various studies suggest an important role of aberrant activation of mitotic events in the disease process, as described below. One of the two neuropathological hallmarks in Alzheimer's disease is the neurofibrillary tangle, which consistently correlates with cognitive and memory decline in the patient [98, 99]. These tangles consist of paired helical filaments (PHFs), the main component of which is hyperphosphorylated tau [98–102]. Tau protein normally stabilizes the internal microtubule structure of neurons that functions to transport proteins and other molecules through the cells [98–102]. When phosphorylated, tau loses its ability to bind microtubules and promote their assembly [103–105]. Furthermore, dephosphorylation of tau in PHFs by phosphatases restores the ability of tau to bind microtubules and promote their assembly [105, 106]. These results lead to the hypothesis that phosphorylation of tau abolishes its ability to promote microtubule assembly, leading to destabilization of the neuronal cytoskele-

ton and cellular demise. The importance of tau for neuronal function has been convincingly demonstrated by the recent findings that mutations in tau cause hereditary forms of fronto-temporal dementia (FTDP-17) [107–110]. The signature lesions in FTDP-17 brains are aggregates composed of hyperphosphorylated tau, similar to those in brains of Alzheimer's patients [111]. Interestingly, some FTDP-17 mutations also disrupt the ability of tau to bind microtubules and promote microtubule assembly [112, 113], suggesting that the interaction between tau and microtubules is critical for the normal function of neurons. Furthermore, the absence of senile plaques and Lewy bodies in FTDP-17 [111] suggests that the tau pathology in Alzheimer's disease may not be just a secondary effect of the disease process, but may rather lead directly to neuronal loss. Therefore, restoring the function of phosphorylated tau may prevent or reverse PHF formation in Alzheimer's disease.

There is growing evidence that aberrant activation of mitotic events may play an important role in the development of Alzheimer's disease. First, tau from fetal brain tissue, still undergoing cell division, has an elevated phosphate content and is recognized by diagnostic phosphorylation-dependent Alzheimer's disease antibodies [114]. Second, a series of monoclonal antibodies recognizing independent phosphorylated epitopes in the PHFs show strong immunoreactivity with cells in mitosis, but not in the interphase of the cell cycle, in various cell types [115]. Conversely, the mitosis marker MPM-2 also reacts strongly with neurofibrillary tangles, neurotic processes, senile plagues and neurons in Alzheimer's disease brains, but has no staining in normal human brains [115]. Third, various studies have shown that mitotic events are aberrantly activated in neurons of the Alzheimer's disease brain, including re-expression of Cdc2 kinase and cyclin B [42, 115–117]. Indeed, common Ser/Thr sites of tau are phosphorylated in normal mitotic cells and in the Alzheimer's disease brain [42, 115, 116, 118–121]. Fourth, the appearance of mitotic phosphoepitopes occurs prior to PHF formation in Alzheimer's disease brain [119]. Fifth, phosphorylation of amyloid precursor protein and production of amyloidogenic fragments from amyloid precursor protein that are characteristics of Alzheimer's disease neurons also occur in normal mitotic cells [122, 123]. Finally, there is a tight link between apoptosis and the mitotic mechanism [124], and induction of mitosis in post-mitotic neuronal cells leads inevitably to cell death [125]. Indeed, apoptosis is elevated in hippocampal brain tissue in Alzheimer's disease [126, 127]. All these results support the notion that aberrant activation of mitotic mechanism in neurons leads to phosphorylation of proteins, such as tau, and ultimately drives the cells into apoptosis, as seen in Alzheimer's disease. However, it remains to be determined how mitotic events occur in the neuron and how they lead to the neuropathology of the disease.

Since tau is an MPM-2 antigen phosphorylated on multiple Ser/Thr-Pro motifs during mitosis [121], we examined whether Pin1 binds tau and found that it does so in a mitosis-specific and phosphorylation-dependent manner [42], as shown for other binding proteins [33]. We therefore hypothesized that Pin1 may bind and regulate the function of tau in the Alzheimer's disease brain. To test the hypothesis, we first examined the relationship between Pin1 and tau in extracts of normal and Alzheimer's disease brain. Pin1 bound tau that was present in Alzheimer's disease extracts or in purified PHFs, but not in age-matched normal brain extracts [42]. Furthermore, Pin1 co-purified with PHFs after a series of purification procedures and specifically bound to the neurofibrillary tangles when added to Alzheimer's disease brain sections. Moreover, endogenous Pin1 redistributed from its normal nuclear location to the neurofibrillary tangles in Alzheimer's disease brains. These results indicate that Pin1 binds PHFs in Alzheimer's disease brains [42]. Importantly, this specific binding sequestered Pin1 into the tangles, depleting soluble Pin1 in Alzheimer's disease brains [42]. Since depletion of Pin1 triggers mitotic arrest and apoptosis, sequestration of Pin1 itself may contribute to neuronal loss in Alzheimer's disease [28].

To determine the biological significance of the Pin1 and tau interaction, we mapped the Pin1-binding site in tau. By screening tau phosphopeptides for Pin1 binding, we found that Pin1 specifically bound to only a single tau peptide containing phosphorylated Thr231 via its WW domain, with a K_d of about 40 nM. In contrast, no binding was observed at all between Pin1 and the non-phosphorylated counterpart, demonstrating an absolute requirement of Thr231 phosphorylation for the Pin1 binding [42]. Furthermore, mutagenesis analysis and competition assays using three monoclonal antibodies specific for the phosphorylated Thr231 tau sequence demonstrated that phosphorylation of the Thr231 residue in tau was both necessary and sufficient for mediating the interaction between Pin1 and tau [42]. These results have demonstrated that Pin1 binds tau phosphorylated on Thr231 (fig. 5). Since Thr231 is a major Cdc2 phosphorylation site [115, 116, 128], these results are consistent with the findings that Pin1 binds mitotically phosphorylated tau and is sequestered on to PHFs in Alzheimer's disease brains where Cdc2 is abnormally upregulated [42].

The high-affinity interaction between Pin1 and phosphorylated tau suggests that Pin1 might affect the biological activity of the latter. Upon phosphorylation by many protein kinases, including Cdc2, tau loses its

ability to bind microtubules and promote their assembly [103–105, 129]. To examine whether Pin1 could restore the biological activity of phosphorylated tau, we generated phosphorylated tau in vitro using purified Cdc2, and determined its ability to bind microtubules and promote their assembly in the presence or absence of Pin1 [42]. Phosphorylation of tau by Cdc2 disrupted the ability of tau to bind microtubules and promote their assembly, but both of these activities of phosphorylated tau were fully restored by Pin1. In contrast, a Cyp did not have any detectable effects on phosphorylated tau. Furthermore, point mutation that affected either phosphoprotein binding or phosphorylation-specific isomerase activity of Pin1 completely abolished or significantly reduced the ability of Pin1 to restore the function of phosphorylated tau. In addition, Pin1 was also detected in the fraction of tau-bound microtubules and did not induce dephosphorylation of tau [42]. These results have demonstrated that Pin1 not only binds phosphorylated tau, but also functionally restores its biological activity (fig. 5). This is consistent with the finding that depletion of Pin1 induces mitotic arrest with a collapsed microtubule network in HeLa cells or a short mitotic spindle in budding yeast [28].

The above findings have further supported the mitotic mechanism of Alzheimer's disease, and also suggested the following model for the role of Pin1 in Alzheimer's disease. Pin1 may be needed to prevent abnormal activation of mitotic events in neurons and to control the function of phosphoproteins, such as tau, if they are phosphorylated due to transient and aberrant activation of Pro-directed kinases. However, during development of Alzheimer's disease, a long-term and sustained activation of protein kinases would result in continuous hyperphosphorylated tau, which binds and sequesters Pin1. This leads to at least two potential detrimental consequences [42]. On the one hand, hyperphosphorylation of tau may create more binding sites than the capacity of the available Pin1. Therefore, hyperphosphorylated tau is not able to bind microtubules, affecting the normal function of neurons. On the other hand, since depletion of Pin1 itself can induce mitotic events and trigger apoptosis, sequestration of Pin1 may trigger or accelerate the formation of PHFs, and cause neuronal loss. In short, our findings suggest that the Pin1-mediated post-phosphorylation regulatory mechanism may play a central role during the pathogenesis of Alzheimer's disease. Since the aggregates of hyperphosphorylated tau are also a common neuropathological feature of several other neuronal degenerative diseases, such as FTDP-17 [111], Pin1 might also be involved in these diseases. Future studies on the role of Pin1 in these neuronal degenerative diseases, including the identification of possible Pin1 mutations, would help elucidate the molecular events that lead to the development of these diseases. In addition, overexpression of Pin1 in neurons and/or prevention of Pin1 sequestration into PHFs might have a therapeutic effect on Alzheimer's and related diseases.

Phosphorylation-dependent prolyl isomerization is a novel biochemical regulatory mechanism

Protein phosphorylation on Ser/Thr-Pro motifs is a common mechanism critical for regulating various cellular processes, such as progression through different phases of the cell cycle [1, 3, 5]. Proline residues exist in cis or trans conformation and can put kinks into polypeptide chains. We have shown that phosphorylation on Ser/Thr-Pro motifs not only reduces the cis/trans isomerization rate of Ser/Thr-Pro bonds, but also renders peptides resistant to the isomerase action of the conventional prolyl isomerases, Cyps and FKBPs [31, 32]. At the same time, proteins containing phosphorylated Ser/Thr-Pro motifs are substrates for the prolyl isomerase Pin1 [32, 33]. The WW domain of Pin1 acts as a phosphoserine/threonine-binding module binding a defined subset of mitosis-specific phosphoproteins, including key mitotic regulators [32, 33]. These interactions target the enzymatic activity of Pin1 close to its substrates. In contrast to other prolyl isomerases, Pin1 has an extremely high degree of substrate specificity, specifically isomerizing phosphorylated Ser/Thr-Pro bonds [32]. Therefore, Pin1 binds and regulates the function of mitosis-specific phosphoproteins [28, 33, 42]. Importantly, inhibiting Pin1 function leads to mitotic arrest and apoptosis in yeast and human cells [28]. The results suggest a new two-step mechanism for mitotic regulation (fig. 6). The first event is phosphorylation at specific Ser-Pro or Thr-Pro sites by the mitosis-specific activation of Pro-directed protein kinases. However, the resulting pSer/Thr-Pro moieties are likely to prefer different conformations from the Ser or Thr residues, because relaxation to a new stable conformation is limited by the reduced prolyl isomerization of the pSer/Thr-Pro bond resulting from addition of the phosphate group. Thus, these phosphoproteins are likely to exist in a different conformation until Pin1 binds and relaxes the structures. Pin1 would determine the duration of the certain conformational state of mitotic phosphoproteins. These local conformational changes might regulate the activity of a phosphoprotein, such as Cdc25, and NIMA [28, 33], alter the ability of a phosphoprotein to interact with other proteins, such as tau [42], to be dephosphorylated or to be degraded, or change the subcellular localization of a phosphoprotein. Therefore, in contrast to the other prolyl isomerases, whose primary function is to facilitate post-translational folding events [29, 30, 60, 61], the

Pin1 subfamily of prolyl isomerases is used to regulate the function of proteins after they have been fully folded and phosphorylated (fig. 6).

During the cell cycle, this post-phosphorylation regulatory mechanism would provide a means for temporally and spatially orchestrating the activity of mitotic proteins into an abrupt wave of signaling that proceeds in a synchronous manner. At the G2/M transition, activation of cyclin B/Cdc2 leads to phosphorylation of a large number of proteins, which are responsible for various structural modifications that occur during mitosis. The Pin1-mediated post-phosphorylation regulatory mechanism would allow the general rapid increase in protein phosphorylation to be converted into the organized and programmed set of mitotic events. In addition, this mechanism may help co-ordinate the function of the multiply phosphorylated proteins. A strikingly common feature among most Pin1-binding proteins, such as Cdc25 and tau, is that they are phosphorylated on multiple Ser/Thr residues clustered at their regulatory domain, [10, 11, 93, 94]. Furthermore, it is often necessary to have phosphorylation on multiple sites in order to display the activity, or to mutate multiple phosphorylation sites to disrupt their function [93]. These results suggest that multiple phosphorylation events are required for regulating the function of Pin1 target proteins. However, little is known about the coordination of multiple phosphorylation events into 'all-or-nothing' activity. Interestingly, the pTyr-binding SH2 domains are critical for generating processive phosphorylation by non-receptor tyrosine kinases [95]. The SH2 domain in these kinases prefers to bind phosphotyrosine residues that have been phosphorylated by its own catalytic domain [96, 97]. The resulting high local concentration of the kinases triggers progressive phosphorylation of protein substrates on multiple sites [95]. Therefore, we propose that one biological function of Pin1 is to facilitate the progressive isomerization of proteins that have been phosphorylated by mitotic kinases at multiple sites. The progressive isomerization is triggered by binding of the higher-affinity WW domain of Pin1 to a Ser-phosphorylated site on a substrate protein. Once bound, the high local concentration drives isomerization of all sites that are sterically accessible to the lower-affinity catalytic isomerase domain. This would provide a means to generate coordinate 'all-or-nothing' activity of heavily phosphorylated proteins.

There are at least two possible models that allow Pin1 to catalyze isomerization of its substrate. One is that every phosphorylated Ser/Thr-Pro motif of a Pin1 substrate first binds to the WW domain of Pin1 and is then transferred to and isomerized by the catalytic domain. The other possibility is that the WW domain binds only one or two phosphorylated Ser/Thr-Pro motifs in a Pin1 substrate and the catalytic domain isomerizes the

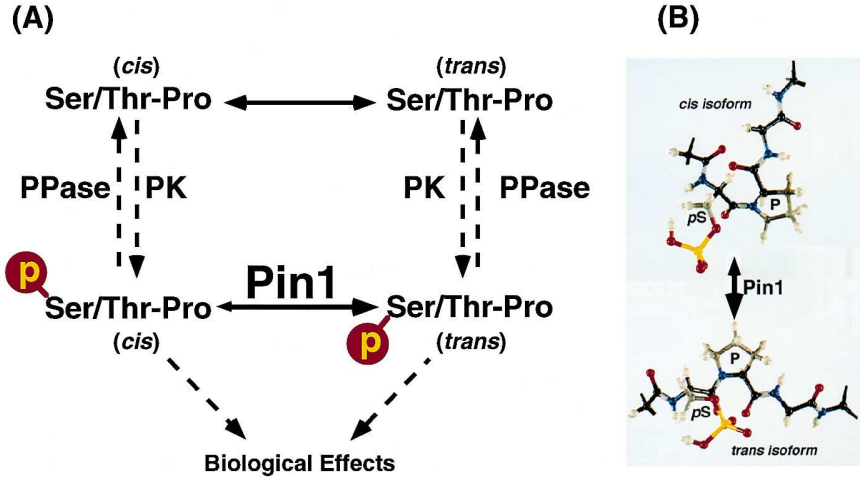


Figure 6. Pin1-mediated post-phosphorylation regulatory mechanism. Phosphorylation on the Ser/Thr-Pro motif by Pro-directed protein kinases restrains the cis/trans prolyl isomerization and, at the same time, becomes the substrate for Pin1. Since the cis and trans isomers of the phosphorylated Ser/Thr-Pro peptide bond exist in the distinct conformations, by catalyzing cis/trans isomerization of the phosphorylated Ser/Thr-Pro bond, Pin1 is likely to regulate the conformation and, subsequently, the function of proteins that have been phosphorylated by Pro-directed protein kinases. Thus, Pin1 would provide a novel post-phosphorylation level of control in the protein phosphorylation/dephosphorylation cascade. It remains to be determined whether cis or trans isomers are the substrates for the kinases (PK) or phosphatases (PPase) or are biologically active (dashed arrows).

rest of the phosphorylated Ser/Thr-Pro bonds in the protein. Since our results have shown that the WW domain only binds one phosphorylated Ser/Thr-Pro motif in tau and two sites in Cdc25C [34, 42], the latter model is more likely. However, further experiments are needed to elucidate the molecular mechanism by which Pin1 regulates the function of its downstream target proteins.

Future directions

In summary, our studies of the novel mitotic regulator Pin1 have shown that phosphorylation-dependent prolyl isomerization is a newly described signaling mechanism that plays an important role in regulating cell cycle progression and in human pathologies, such as Alzheimer's disease. However, these studies have left many interesting and important questions. For example, it remains to be determined whether it is the cis or trans isoform of the Ser/Thr-Pro motif that is a good substrate for Pro-directed kinases and, once phosphorylated, which isoform is biologically active and the preferred substrate for phosphatases. What are the physiological targets for Pin1 during the different phases of mitosis? Are there any differences between phosphorylated Ser-Pro and phosphorylated Thr-Pro motifs regarding their binding to the WW domain and/or isomerization rate? How does Pin1 regulate the function of its target protein? How is Pin1 function regulated during the cell cycle? Given the essential role of various Pro-directed protein kinases in regulating other phases of the cell cycle and other cellular functions, are there Pin1-like prolyl isomerases in the cell that specifically function at these events? What role does Pin1 play in the pathogenesis of Alzheimer's and other diseases? And last but not least, will Pin1 be a good novel drug target? Future studies in these directions should lead to a better understanding of the physiological and pathological roles of this novel post-phosphorylation regulatory mechanism.

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