The essential mitotic peptidyl–prolyl isomerase Pin1 binds and regulates mitosis-specific phosphoproteins

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Phosphorylation of mitotic proteins on the Ser/Thr-Pro motifs has been shown to play an important role in regulating mitotic progression. Pin1 is a novel essential peptidyl–prolyl isomerase (PPIase) that inhibits entry into mitosis and is also required for proper progression through mitosis, but its substrate(s) and function(s) remain to be determined. Here we report that in both human cells and *Xenopus* extracts, Pin1 interacts directly with a subset of mitotic phosphoproteins on phosphorylated Ser/Thr-Pro motifs in a phosphorylation-dependent and mitosis-specific manner. Many of these Pin1-binding proteins are also recognized by the monoclonal antibody MPM-2, and they include the important mitotic regulators Cdc25, Myt1, Wee1, Plk1, and Cdc27. The importance of this Pin1 interaction was tested by constructing two Pin1 active site point mutants that fail to bind a phosphorylated Ser/Thr-Pro motif in mitotic phosphoproteins. Wild-type, but not mutant, Pin1 inhibits both mitotic division in *Xenopus* embryos and entry into mitosis in *Xenopus* extracts. We have examined the interaction between Pin1 and Cdc25 in detail. Pin1 not only binds the mitotic form of Cdc25 on the phosphorylation sites important for its activity in vitro and in vivo, but it also inhibits its activity, offering one explanation for the ability of Pin1 to inhibit mitotic entry. In a separate paper, we have shown that Pin1 is a phosphorylation-dependent PPIase that can recognize specifically the phosphorylated Ser/Thr-Pro bonds present in mitotic phosphoproteins. Thus, Pin1 likely acts as a general regulator of mitotic proteins that have been phosphorylated by Cdc2 and other mitotic kinases.

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Events of the eukaryotic cell cycle are regulated by an evolutionarily conserved set of protein kinases. The cyclin-dependent kinases (Cdks) are important for driving cells through different phases of the cell cycle and their sequential activation and inactivation are tightly regulated (for review, see Coleman and Dunphy 1994; King et al. 1994; Nurse 1994). At the G₂/M transition, activation of the mitotic Cdk Cdc2 requires multiple events; these include the synthesis and binding of cyclin B, phosphorylation on Cdc2 at a site by CDK-activating kinase (CAK) and, finally, Cdc25 activating sites that have been phosphorylated by Wee1 and Myt1 (for review, see Coleman and Dunphy 1994; King et al. 1994; Nurse 1994).

How activation of a Cdk elicits the downstream events of cell cycle progression is less well understood. Activation of cyclin B/Cdc2 leads to the phosphorylation of a large number of proteins, mainly on sites containing a Ser/Thr-Pro motif (for review, see Nigg 1995).

Protein phosphorylation is believed to alter the functions of proteins to trigger the events of mitosis. In a few cases, mitotic phosphorylation has been shown to regulate mitotic events (Heald and McKeon 1990; Bailly et al. 1991; Blangy et al. 1995); however, it is not understood how the rapid changes in mitotic phosphorylation are converted to the sequential events of mitosis.

An important experimental tool that has uncovered the general role of phosphorylation in mitotic regulation is the MPM-2 (mitotic phosphoprotein monoclonal-2) antibody (Davis et al. 1983). MPM-2 recognizes a phosphorylated Ser/Thr-Pro epitope on ~50 proteins, which are localized to various mitotic structures (Vandre et al. 1986; Westendorf et al. 1994; Matsumoto-Taniura et al. 1996). Several important mitotic regulators are recognized by this antibody, including Cdc25, Wee1, topoisomerase IIa, Cdc27, Map 4, inner centromere proteins (INCENP), and NIMA (never in mitosis A) (Vandre et al. 1991; Taagepera et al. 1993; Coleman et al. 1993; Kuang et al. 1994; King et al. 1995; Mueller et al. 1995; Ye et al. 1995; Stukenberg et al. 1997). Currently six kinases have been shown to phosphorylate proteins in vitro to pro-

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duce the MPM-2 epitope: Cdc2, Polo-like kinase (Plk1), NIMA, MAP kinase, a MAP kinase kinase (MEK), and an unidentified activity ME-H (Kuang and Ashorn 1993; Taagepera et al. 1994; Kumagai and Dunphy 1996; Renzi et al. 1997). However, these kinases also phosphorylate substrates that do not generate the MPM-2 epitope especially in cell cycle stages other than mitosis. This suggests that additional features are required for the recognition by MPM-2. Determination of the optimal MPM-2-binding sequence have confirmed the importance of amino acid residues flanking the phosphorylated Ser/Thr-Pro motif for the MPM-2 recognition (Westendorf et al. 1994; Yaffe et al. 1997).

The identification of the novel human mitotic regulator Pin1 suggests a new regulatory mechanism for mitotic regulation (Lu et al. 1996). Pin1 was identified originally in a yeast two-hybrid screen as a protein that interacts with the essential mitotic kinase NIMA and suppresses its mitosis-promoting activity (Lu et al. 1996). Although a NIMA-like pathway is also required for the G₂/M transition in vertebrate cells (Lu and Hunter 1995), it has been difficult to identify metazoan NIMA functional homologs. In contrast, Pin1 has been identified in all eukaryotic organisms where examined, including plants, yeast, *Aspergillus*, and mammals (Hanes et al. 1989; Lu et al. 1996; Maleszka et al. 1996; sequences have been deposited in GenBank under accession nos. 1688322 and 2739197). Pin1 is an essential peptidyl-prolyl *cis-trans* isomerase (PPIase). It is distinct from two other well-characterized PPIase families: the cyclophilins and the FK506-binding proteins (FKBPs), which are targets for the immunosuppressive drugs cyclosporin A and FK506, respectively (for review, see Schreiber 1991; Fischer 1994; Schmid 1995). PPIases are ubiquitous enzymes that catalyze rotation about the peptide bond preceding a Pro residue, and may accelerate the folding and trafficking of some proteins (for review, see Schmid 1995). Interestingly, inhibition of PPIase activity is not required for the immunosuppressive property of cyclosporin A and FK506. Furthermore, neither the cyclophilins nor the FKBPs are essential for normal cell growth (Schreiber 1991; Fischer 1994; Schmid 1995). Thus, evidence for the biological importance of PPIase enzymatic activity has been limited.

In contrast, Pin1 contains a PPIase domain that is essential for cell cycle progression and its subcellular localization is tightly regulated at the G₂/M transition (Lu et al. 1996). Pin1 is localized in a defined nuclear substructure in interphase, but is concentrated to the condensed chromatin, with some staining in other structures during mitosis. Furthermore, depletion of Pin1 protein in HeLa cells or Pin1/Ess1p in yeast results in mitotic arrest, whereas overexpression of Pin1 induces a G₂ arrest (Lu et al. 1996). These results suggest that Pin1 is an essential mitotic regulator that both regulates negatively entry into mitosis and is required for progression through mitosis.

The crystal structure of human Pin1 complexed with an Ala-Pro dipeptide suggests that the isomerization mechanism of Pin1 includes general acid-base and cova-

lent catalysis during peptide bond isomerization (Ranganathan et al. 1997). More interesting, Pin1 displays a unique substrate specificity. It prefers an acidic residue amino-terminal to the isomerized Pro bond attributable to interaction of the acidic side chain with a basic cluster in Pin1. This basic cluster consists of the highly conserved residues Lys-63, Arg-68, and Arg-69 at the entrance to the active site. In the crystal structure, this conserved triad sequestered a sulfate ion in close proximity to the β -methyl group of the Ala residue in the bound Ala-Pro dipeptide. Because a logical candidate for this negatively charged residue would be a phosphorylated Ser/Thr, we have hypothesized previously that Pin1 may recognize its substrates in a phosphorylation-dependent manner (Ranganathan et al. 1997). Recently, we have further shown that Pin1 is a sequence-specific and phosphorylation-dependent PPIase that can recognize the phosphorylated Ser/Thr-Pro bonds specifically present in mitotic phosphoproteins (Yaffe et al. 1997). However, little is known about the identity of Pin1 target proteins and the role, if any, of Pin1 in regulating these proteins.

To address the above questions, we identified Pin1-binding proteins in human cells and *Xenopus* extracts. Our results indicate that although Pin1 levels are constant throughout the cell cycle, the interaction of Pin1 and its targets is cell cycle regulated and depends on mitotic phosphorylation of target proteins. Pin1 interacts directly with a large subset of mitosis-specific phosphoproteins, which includes Cdc25, Wee1, Myt1, Plk1, Cdc27, and E-MAP115, as well as some others recently identified by a screen for mitotic phosphoproteins (Stukenberg et al. 1997). Many of these Pin1-interacting proteins are also recognized by the MPM-2 antibody. In functional assays, microinjection of Pin1 inhibits mitotic division in *Xenopus* embryos and entry into mitosis in *Xenopus* extracts, as is the case in HeLa and yeast cells. Furthermore, Pin1 binds the mitotically phosphorylated form of Cdc25 in vitro and in vivo, and it binds Cdc25 on the important phosphorylation sites and inhibits its activity. This characterization of the Pin1-Cdc25 interaction can explain at least partially the ability of Pin1 to inhibit the G₂/M transition. All of these activities of Pin1 are dependent on the ability of Pin1 to mitotic phosphoproteins, as the activities are disrupted by point mutations that inhibit the ability of Pin1 to recognize this unique class of phosphoproteins. These results suggest that Pin1 acts as a general regulator of mitosis-specific phosphoproteins, presumably by catalyzing phosphorylation-dependent Pro isomerization.

Results

Pin1 levels are constant through the cell cycle

Whereas overexpression of Pin1 results in G₂ arrest, depletion of Pin1 induces mitotic arrest without affecting DNA synthesis (Lu et al. 1996). To determine the basis for this cell cycle specificity, we first asked

whether Pin1 protein level fluctuated during the cell cycle. To address this question, HeLa cells were synchronized at the G_1/S boundary. At different times after the release from the block, cells were harvested and analyzed by flow cytometry or lysed and analyzed for protein expression by immunoblotting. Analysis of DNA content and cyclin B1 levels indicated that the HeLa cells progressed synchronously through different phases of the cell cycle (Fig. 1A and B), as shown previously (Heintz et al. 1983). Total Pin1 levels did not change significantly during the cell cycle (Fig. 1B). The total cellular Pin1 concentration in HeLa cells was estimated to be $\sim 0.5 \mu\text{M}$, based on immunoblotting analysis using anti-Pin1 antibodies with recombinant Pin1 protein as a standard (data not shown).

Figure 1. Pin1 levels are constant during the cell cycle. HeLa cells were synchronized at the G_1/S boundary by double thymidine and aphidicolin block and released to enter the cell cycle. To block cells at mitosis, nocodazole (Noc, 50 ng/ml) was added to cells at 8 hr after the release and incubated for another 4 hr (12 Noc) or 6 hr (14 Noc). To obtain pure G_1 cells, mitosis-arrested cells (14 Noc) were plated in a nocodazole-free media for another 4 hr (18 Noc⁻). Cells were harvested at the indicated times and aliquots were subjected to flow cytometric analysis to determine the cell cycle status; the remaining cells were lysed in RIPA buffer. The same amount of total proteins were separated on a SDS-containing gel, transferred to a membrane that was cut into three pieces and probed with anti-cyclin B, anti-Cdc2, and anti-Pin1 antibodies, respectively. Cdc2 levels were similar in all lanes (data not shown). (A) Flow cytometric analysis of the DNA content; (B) immunoblot analysis of cyclin band Pin1 protein levels.

Pin1 directly binds a subset of conserved mitotic phosphoproteins

Because the levels of Pin1 do not fluctuate during the cell cycle, its mitosis-specific function is likely conferred by some other mechanisms. Therefore, we tested for a cell cycle-dependent interaction between Pin1 and its binding proteins. A glutathione *S*-transferase (GST) fusion protein containing full-length Pin1 was bacterially expressed, purified, and then used to probe for interacting proteins in S-phase, mitosis, or G_1 -phase by Far-western analysis. As shown in Figure 2A, the ability of Pin1 to interact with cellular proteins remained relatively low during S-phase, increased when cells progressed through G_2/M (10-hr point), and was almost completely lost when cells moved to the next G_1 (14-hr point). However, if cells were not allowed to progress into the next cell cycle but, rather, were blocked at mitosis by adding nocodazole (14+N), Pin1-binding activity increased even further (Fig. 2A). Because the binding activity was detected using denatured proteins, the protein-protein interaction between Pin1 and these proteins must be direct. To examine whether this Pin1 interaction with its target proteins occur under nondenaturing conditions and to estimate the number of Pin1-interacting proteins, glutathione beads containing GST and GST-Pin1 were incubated with interphase and mitotic extracts, and beads were extensively washed and proteins bound to beads were separated on SDS-containing gels and stained with Coomassie blue. Whereas no detectable proteins were precipitated by GST beads from either interphase or mitotic extracts (data not shown), GST-Pin1 beads specifically precipitated ~ 30 clearly Coomassie-stainable bands from mitotic extracts, but only four to seven minor bands from interphase extracts (Fig. 2B). These two results together indicate that Pin1 interacts mainly with a subset of proteins in a mitosis-specific manner.

Recently we have shown that both Pin1 and MPM-2 bind similar peptides containing phosphorylated Ser-Pro flanked by hydrophobic residues or Arg, and that many, but not all, of the mitotic proteins precipitated by GST-Pin1 are also recognized by the MPM-2 antibody (Yaffe et al. 1997) (Figs. 2B and 3C), suggesting a possible interaction between Pin1 and MPM-2 antigens. To further validate this interaction, we first determined whether GST-Pin1 can deplete MPM-2 antigens from cell lysates. Mitotic extracts from HeLa cells were incubated with different amounts of GST-Pin1, followed by analyzing MPM-2 antigens remaining in the depleted supernatants (Fig. 2C). At a concentration (8 μM) that was ~ 15 -fold higher than the endogenous level, Pin1 depleted the majority of MPM-2 antigens (Fig. 2C), indicating that Pin1 strongly interacts with most MPM-2 antigens. We then determined whether endogenous Pin1 interacts with MPM-2 antigens in vivo, Pin1 was immunoprecipitated from either interphase or mitotic HeLa extracts using anti-Pin1 antibodies in the presence or absence of various phosphatase inhibitors. The resulting Pin1 immunoprecipitates were probed with MPM-2. Several MPM-2

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Figure 2. Pin1 interacts directly with a subset of conserved mitosis-specific phosphoproteins both in vitro and in vivo. (A) Cell cycle regulation of Pin1-binding proteins. HeLa cells were harvested from different phases of the cell cycle as described in Fig. 1 and subjected to flow cytometric analysis (*bottom*) and Far Western analysis using GST-Pin1 as a probe. Equal loading of proteins was shown in each lane by protein staining and anti-Cdc2 immunoblotting analysis (data not shown). Arrows points to Pin1-binding proteins increased at mitosis. (B) Detection of Pin1-binding proteins. GST and GST-Pin1 were incubated with interphase (I) and mitotic (M) HeLa extracts at a final concentration of 20 μ M and glutathione beads were added, followed by extensive wash. GST-Pin1 precipitated proteins were separated on SDS-containing gel and stained by Coomassie blue R250. GST did not precipitate any specific Coomassie stainable proteins from either I or M extracts (data not shown). (C) GST-Pin1 can deplete MPM-2 antigens. I and M HeLa cell extracts were either not depleted (Control), or incubated with GST or GST-Pin1 beads. After removing the beads, the supernatants were subjected to immunoblotting analysis using MPM-2. To determine the concentration of Pin1 required to deplete MPM-2 antigens, mitotic extracts were incubated with increasing concentrations of GST-Pin1 and then GST-agarose beads were added to precipitate GST-Pin1-binding proteins. Proteins present in the depleted supernatants, together with control total interphase and mitotic extracts, were subjected to immunoblotting analysis using MPM-2. (D) Coimmunoprecipitation of

Pin1 and MPM-2 antigens. Using anti-Pin1 antibodies, Pin1 was immunoprecipitated from I and M lysates in the presence or absence of various phosphatase inhibitors, followed by immunoblotting with MPM-2. Arrows point to the MPM-2 antigens that are immunoprecipitated by Pin1 antibodies. (E) Precipitation of *Xenopus* MPM-2 antigens by human Pin1. *Xenopus* interphase (I) extracts were driven into mitosis (M) by addition of nondegradable cyclin B. Both I and M extracts were incubated with agarose beads containing GST or GST-Pin1, followed by immunoblotting analysis with MPM-2 antibody. Arrows point to proteins that are specifically precipitated by GST-Pin1.

antigens were coimmunoprecipitated with anti-Pin1 antibodies (Fig. 2D, right). However, the coimmunoprecipitation between Pin1 and the MPM-2 antigens was not detected in the absence of phosphatase inhibitors (Fig. 2D, left). These results indicate that stable complexes between Pin1 and MPM-2 antigens exist in vivo, likely in a phosphorylation-dependent manner, and that Pin1 does not form complexes with all Pin1-binding proteins at the same time in vivo. Taken together, these results demonstrated that Pin1 interacts directly both in vitro and in vivo with a subset of mitotic phosphoproteins that overlaps with the set of proteins known as MPM-2 antigens.

Because both Pin1 and MPM-2 antigens are highly conserved, it is possible that Pin1-binding proteins are also conserved. To examine this possibility, we exam-

ined the interaction between human Pin1 and mitotic phosphoproteins in *Xenopus* extracts. When GST-Pin1 was incubated with interphase or mitotic egg extracts, Pin1 precipitated specifically a subset of MPM-2 antigens from mitotic extracts, with molecular masses similar, although not identical, to those present in human cells (Fig. 2C,E). Again, this interaction between Pin1 and *Xenopus* MPM-2 antigens was specific as it was not detected whether the precipitation was performed with control GST glutathione beads (Fig. 2E). Thus, the interaction between Pin1 and mitosis-specific protein is evolutionarily conserved from amphibians to mammals.

Mutations in the binding pocket abolish the ability of Pin1 to interact with most mitotic phosphoproteins

The above results demonstrate that Pin1 binds directly

Figure 3. Generation of Pin1 mutants unable to bind phosphoproteins. (A) Model for a phosphorylated Ser-Pro dipeptide bound to the active site of Pin1 in the Syn 90 conformation. A basic cluster consisting of conserved Lys-63, Arg-68, and Arg-69 sequesters a sulfate ion in close proximity to the β -methyl group of the Ala residue in the bound Ala-Pro dipeptide. The Phospho-Ser has been modeled on the original Ala in an extended low energy conformation and in the maximal overlap of the extended Phospho-Ser side chain with the sulfate ion. Steric clashes of the Phospho-Ser side chain with the Pin1 active site in the *cis* or *trans* conformations would necessitate an active site rearrangement, or a transition of the Phospho-Ser side chain to a higher energy conformation. Atoms in the active site have been color coded for clarity. Oxygen is red, nitrogen is blue, carbon is black, and sulfur is yellow. Syn 90 indicates the conformation of the Phospho-Ser-Pro peptide bond being between *cis* (0) and *trans* (180). [Reproduced, with permission, from Ranganathan et al. (1997)] (B) PPIase activity of mutant proteins. Two Pin1 mutants were generated that contain either single Ala substitution at His-59 (Pin1^{H59A}) or double Ala substitutions at Arg-68 and Arg-69 (Pin1^{R68,69A}), which are the amino acids implicated in binding the Pro residue or putative phosphate in the substrate, respectively, as shown in A. Both Pin1 and mutants were expressed and purified as GST fusion proteins. Purified Pin1 and the mutants were subjected to PPIase assay using two different substrates; one substrate had an Ala as the amino acid amino-terminal to the Pro (AAPF, open bars), and the second substrate has a Glu amino-terminal to the Pro (AEPF, solid bars). (C) Phosphoprotein-binding activity of mutant proteins. After incubated with I or M extracts, proteins associated with GST-Pin1, GST-Pin1^{R68,69A} or GST-Pin1^{H59A} beads were probed with MPM-2. M1 and M2 represent mitotic extracts prepared from two independent experiments. Although the exact intensity of MPM-2 staining in each precipitated protein varies as a result of changes in the phosphorylation state, overall patterns are quite similar in all different experiments.

numerous conserved mitotic phosphoproteins in a mitosis-dependent manner. To insure that this interaction is highly specific for Pin1, site-specific mutations were introduced into Pin1. A high resolution X-ray structural and preliminary functional analysis of Pin1 (Ranganathan et al. 1997) suggest that a basic cluster consisting of Lys-63, Arg-68, and Arg-69 could coordinate a putative phosphate group in the substrate (Fig. 3A). Ala substitutions at these residues (Pin1^{R68,69A}) should cause a reduction in the ability to bind phosphorylated residues amino-terminal to the target Pro residue in the substrate. In addition, His-59 has been shown to have an intimate contact with the cyclic side chain of the catalyzing Pro residue (Fig. 3A). An Ala substitution at His-59 of Pin1 (Pin1^{H59A}) should therefore disrupt the interaction between Pin1 and the substrate Pro residue.

The mutant proteins were expressed and purified as GST fusion proteins, and both their PPIase activity and their ability to bind mitotic phosphoproteins were determined. PPIase activity was assayed with two peptide substrates: AEPF, which has an acidic residue at the position amino-terminal to the catalytic Pro residue, and AAPF, which does not. As shown previously (Ranganathan et al. 1997), Pin1 had a strong preference for the AEPF substrate (Fig. 3B). The PPIase activity of Pin1^{R68,69A} was reduced >90% against AEPF, whereas the reduction was very small against AAPF. Moreover, Pin1^{R68,69A} had little preference for either substrate (Fig. 3B). These results confirm that residues Arg-68 and Arg-69 are critical for promoting strong selection for a negatively charged residue at the position amino-terminal to

the substrate Pro residue. The PPIase activity of Pin1^{H59A} was barely detectable against either peptide substrate, confirming the importance of His-59 in Pin1 substrate binding or catalysis (Fig. 3B).

To determine whether the Pin1 mutants interact with mitotic phosphoproteins, GST-Pin1, GST-Pin1^{R68,69A}, and GST-Pin1^{H59A} fusion proteins were incubated with interphase or mitotic HeLa cell extracts and associated proteins subjected to MPM-2 immunoblotting analysis. As shown in Figure 3C, Pin1 interacted specifically with MPM-2 antigens in two independently prepared mitotic extracts, but the binding activity of both Pin1^{R68,69A} and Pin1^{H59A} was significantly reduced compared to the wild-type protein. A few proteins including the most strongly reacting p55 band could still be recognized. The two Pin1 mutants also failed to bind most mitotic phosphoproteins from *Xenopus* extracts (data not shown). Thus mutating the residues that are implicated in binding either the substrate's putative phosphate group or the substrate's Pro residue abolish the ability of Pin1 to bind MPM-2 antigens. This suggests that the active site residues in Pin1 contact both the phosphorylated Ser/Thr and the Pro residues on MPM-2 antigens.

Identification of several mitotic regulators as Pin1 targets

Several known mitotic regulators such as cyclin B, Cdc25, Myt1, Plk1, and Cdc27 are phosphorylated at mitosis (Taagepera et al. 1993; Kuang et al. 1994; King et al. 1995; Mueller et al. 1995). To identify at least a few of

the many Pin1-binding proteins, the proteins that bound GST-Pin1 beads were isolated from HeLa cells or *Xenopus* extracts and probed with antibodies specific for known mitotic phosphoproteins. As shown previously (Strausfeld et al. 1994; Kuang et al. 1994; Golsteyn et al. 1995; Hamanaka et al. 1995), levels of Plk1 and cyclin B1 increased at mitosis, whereas similar amounts of Cdc25C were present in interphase and mitotic HeLa cells. Moreover, a significant fraction of Cdc25C, Plk1, Myt1, Cdc27, and PTP-1B became hyperphosphorylated during mitosis and exhibited a shift in electrophoretic mobility by SDS-PAGE (Fig. 4A,C; data not shown). Although cyclin B1 and PTP-1B were not precipitated by Pin1 in either interphase or mitotic extracts (Table 1), Pin1 bound selectively only to the mitotically hyperphosphorylated form of Cdc25C, Plk1, Myt1, and Cdc27 (Fig. 4A). Furthermore, neither mutant Pin1^{R68,69A} nor Pin1^{H59A} interacted with Cdc25 or Cdc27 (Fig. 4B), indicating that the residues that are implicated in binding either the substrate's putative phosphate group or the substrate's Pro residue are necessary for Pin1 to bind Cdc25 and Cdc27. Similarly, only the mitotic, but not the interphase form of *Xenopus* Cdc27 was precipitated by Pin1 (Fig. 4C). Moreover, pretreatment of the mitotic extract with calf intestine phosphatase (CIP) dephos-

phorylated completely Cdc27 and abolished the interaction between Pin1 and Cdc27 (Fig. 4C), demonstrating a phosphorylation-dependent interaction. These results indicate that the interaction between Pin1 and Cdc25 or Cdc27 is likely to be mediated by a phosphorylated Ser/Thr-Pro motif.

To gain a sense of the generality of the interaction between Pin1 and mitotic phosphoproteins and to confirm the Pin1 interaction with target proteins is indeed mediated by phosphorylation, we examined the ability of Pin1 to bind other known mitotic phosphoproteins and a set of mitotic phosphoproteins identified by a systematic phosphoprotein screen (Stukenberg et al. 1997). Proteins synthesized *in vitro* were phosphorylated in a cell cycle-specific manner by incubating them in either *Xenopus* interphase or mitotic extracts. These labeled proteins were incubated subsequently with GST-Pin1 beads, extensively washed and the bound proteins analyzed by SDS-PAGE (Fig. 4D). To validate this method, Cdc25 was first tested. Again, the mitotically phosphorylated form of *in vitro* translated Cdc25 could be precipitated by GST-Pin1 beads. However, Cdc25 was not recognized by Pin1 if it was incubated in interphase extracts. Moreover, Pin1 did not interact with Cdc25 if the mitotically phosphorylated Cdc25 was treated with phosphatase be-

Figure 4. Pin1 can interact with important mitotic regulators in mitosis-specific and phosphorylation-dependent manner. (A) Binding of Pin1 to human Cdc25C, Plk1, and Myt1. Similar amounts of proteins from I and M HeLa cells were either immunoprecipitated (IP) and immunoblotted using the same anti-Cdc25C or anti-Plk1 antibodies, or subjected to GST bead pulldown assay (Pin1-PP, GST, GST-Pin1), followed by immunoblotting analysis using anti-Cdc25C, anti-Plk1, or anti-Myt1 antibodies. (B) Failure of the Pin1 mutants to bind Cdc25C and Cdc27. GST-glutathione beads containing wild-type and mutant Pin1 proteins were incubated with mitotic extracts and proteins associated with the beads were subjected to immunoblotting analysis using anti-Cdc25C and anti-Cdc27 antibodies. (C) Interaction between Pin1 and *Xenopus* Cdc27. *Xenopus* interphase (I) extracts were driven into mitosis (M) and half of the reaction was treated subsequently with calf intestine phosphatase (M + CIP). These three different extracts were incubated with agarose beads containing GST or GST-Pin1, followed by immunoblotting analysis with anti-Cdc27 antibodies. (D) Interaction between Pin1 and other selected mitotic phosphoproteins. The indicated proteins were synthesized by *in vitro* transcription and translation in the presence of [³⁵S]-methionine and incubated with *Xenopus* I, M, or M + CIP. These proteins were separated on SDS-containing gels either directly (input) or first precipitated by GST-Pin1 beads (GST-Pin1).

Table 1. Interaction between Pin1 and selected mitotic phosphoproteins

Phosphoproteins ^a	Interphase ^b	Mitotic ^b
Cdc25*	–	+++
Plk1*	–	+++
Plx1	–	+++
Wee1	+	++
Myt1*	–	+++
Mos	+	++
Cdc27*	–	+++
NIMA	–	+++
Sox3	–	+++
Xbr-1b	–	+++
MP75 (E-MAP-115)	–	+++
MP110 (Cdc5)	–	+++
MP68	–	+++
MP30	–	++
MP105	+	+
MP48	–	–
Cyclin B*	–	–
PTP-1B*	–	–

The binding between Pin1 and all selected mitotic phosphoproteins was assayed by incubating synthesized proteins with interphase and mitotic *Xenopus* extracts, followed by precipitation with GST–Pin1 beads.

^aThe Pin1 interactions with those proteins indicated with an asterisk (*) were also confirmed by GST–Pin1 pulldown assay from endogenous interphase and mitotic HeLa cell extracts.

^b(+) A weak, but above background, interaction; (++) readily detectable interaction; (+++) strong interaction.

fore the GST–Pin1 incubation (Fig. 4D, GST–Pin1). These results demonstrate that this method can be used to detect mitosis-specific and phosphorylation-dependent interactions between Pin1 and phosphoproteins. Of the 13 mitotic phosphoproteins examined, Pin1 bound 10 in a mitosis and phosphorylation-dependent manner (summarized in Table 1), including Wee1, MP75, and MP110, as shown in Figure 4D. MP75 and MP110 are *Xenopus* proteins related to the microtubule-associated protein E-MAP-115 and the fission yeast G₂ transcription factor Cdc5, respectively. These results indicate that Pin1 may target many but not all mitosis-specific phosphoproteins.

Pin1, but not the mutants, blocks cell cycle progression in Xenopus embryos and entry into mitosis in Xenopus extracts

The above results establish a specific interaction between Pin1 and mitosis-specific phosphoproteins. To demonstrate that this binding is biologically important for Pin1 function, we turned to the experimentally amenable *Xenopus* system. Because Pin1 is conserved from yeast to humans, it is likely that Pin1 exists in *Xenopus*. To confirm this, *Xenopus* egg extracts were immunoblotted with two separate anti-human Pin1 antisera. Both antibodies, but not their respective preimmune

sera, specifically recognized a band that comigrated with human Pin1 at 18 kD (data not shown), indicating that Pin1 is present in *Xenopus*.

Overexpression of Pin1 has been shown to inhibit cell cycle progression in both yeast and HeLa cells (Hanes et al. 1989; Lu et al. 1996). To examine whether increasing the concentration of Pin1 has similar biological effects in *Xenopus*, we injected Pin1 or Pin1 mutants into one cell of two-cell stage embryos and allowed the embryos to develop for 3 hr (about five divisions). Wild-type Pin1-injected cells failed to cleave or cleaved slowly when compared to the cells in the uninjected side (Fig. 5A). A similar concentration (4 μM final) of either Pin1 mutant had little effect on the cell cycle (Fig. 5A). In a separate experiment Pin1 blocked cleavage of the injected cells in a concentration-dependent manner, and at a concentration ~20-fold above the estimated endogenous levels (10 μM), completely inhibited the cell cycle (Fig. 5B). In contrast, higher concentrations of the mutant proteins were needed to block the cell cycle (Fig. 5B). Injection of control BSA had no obvious effect on cell cycle progression (data not shown). These results suggest that Pin1 must bind mitotic phosphoproteins to block cell cycle progression.

To determine the nature of the cleavage block in *Xeno-*

Figure 5. Microinjection of Pin1 protein, but not its mutants, inhibits mitotic division in *Xenopus* embryos. (A) GST fusion proteins containing wild-type Pin1 or its two mutants were microinjected into one blastomere of *Xenopus* embryos at the two-cell stage. The injected embryos were allowed to develop at 18°C for 3 hr (about five cycles) followed by photography of typical embryos. Embryos with a cell cycle arrest had fewer and larger blastomeres on one side. The arrows point to the injected blastomere. (B) GST–Pin1 and the mutants were titrated into the assay described in A except that the embryos were injected at the four-cell stage. The percentage of embryos with a cell cycle block phenotype at each Pin1 concentration was determined and presented graphically.

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pus, GST-Pin1 was added to *Xenopus* egg extracts that had been arrested in second meiotic metaphase because of the activity of the cyostatic factor. These extracts are arrested in mitosis (meiosis II) and reenter the cell cycle in response to the addition of Ca^{2+} . Extracts containing demembrated sperm to monitor nuclear morphology and rhodamine-tubulin to monitor microtubule spindle assembly, were activated with Ca^{2+} . Pin1 was added after the extracts had entered interphase (15 min after the addition of Ca^{2+}), and the subsequent entry of the extracts into mitosis was followed by nuclear morphology and Cdc2 kinase activity using histone H1 as a substrate. Addition of either 10 or 40 μM Pin1, ~20- or 80-fold higher than endogenous levels, completely blocked entry into mitosis as detected by the persistence of interphase nuclei and low Cdc2 kinase activity (Fig. 6A,B). In contrast, the same extracts containing 40 μM of either BSA or the mutant Pin1 proteins entered mitosis by 70–80 min as detected by nuclear envelope breakdown, spindle formation and high histone H1 kinase activity (Fig. 6A,B). Thus, as shown previously in HeLa cells (Lu et al. 1996), increasing the Pin1 concentration causes a cell cycle block in G_2 . More important, Pin1 must bind mitotic phosphoproteins to elicit this phenotype.

Pin1 binds and inhibits mitotically phosphorylated Cdc25

The above results indicate that overexpression of Pin1 inhibits mitotic entry in *Xenopus*, as is the case in HeLa cells and yeast (Lu et al. 1996). Entry into mitosis is regulated by dephosphorylation of Cdc2 by the phosphatase Cdc25, and Cdc25 is activated by mitosis-specific phosphorylation at the MPM-2 epitope at the G_2/M transition (Russell and Nurse 1986; Kuang et al. 1994; Strausfeld et al. 1994). Earlier results indicated that it is the mitotically phosphorylated form of Cdc25 that interacts with Pin1 in vitro (see Fig. 4). Therefore, it is conceivable that the inhibitory effects of Pin1 on entry into mitosis could at least partially be explained through inhibition of Cdc25 activity.

To test this possibility, we first examined whether Pin1 interacts with Cdc25 in vivo and if so, whether this interaction is cell cycle regulated. *Xenopus* eggs were collected at various times after fertilization and subjected to immunoprecipitation using anti-*Xenopus* Cdc25 antibodies as well as histone H1 kinase assay to monitor cell cycle progression. When the resulting Cdc25 immunoprecipitates were immunoblotted with anti-Pin1 antibodies, we found that endogenous Pin1 was precipitated by anti-Cdc25 antibodies (Fig. 7A). Furthermore, this interaction between Pin1 and Cdc25 was cell cycle regulated, significantly increased just before mitosis (Fig. 7A). Similar results were also obtained using synchronized HeLa cells using anti-human Cdc25C (data not shown). Unfortunately, we were not able to detect Cdc25 in anti-Pin1 immunoprecipitates, probably because the amount of Cdc25 precipitated is below the detection of the Cdc25 antibodies. It is worth pointing out that the percentage of coimmunoprecipitable Pin1

Figure 6. Pin1, but not the mutants, blocks mitotic entry in *Xenopus* extracts. (A) *Xenopus* cyostatic factor (CSF)-arrested extracts containing demembrated sperm and rhodamine-tubulin were activated with 0.4 mM Ca^{2+} at time zero, and 15 min later, Pin1 or the mutants was added to 40 μM and at 110 min the nuclear morphology was examined microscopically. (B) The activity of Cdc2 was followed using histone H1 as a substrate in the CSF extract experiment described in A; 40 μM GST-Pin1 (black squares), 40 μM GST-Pin1^{R68,69A} (dark blue triangles), and 40 μM GST-Pin1^{H59A} (brown crossed circles). Also shown are reactions in the same extracts that contained 10 μM GST-Pin1 (green circles) and 40 μM BSA (light blue crossed squares).

and phosphorylated Cdc25 is not high. This might be expected because the complex might not be stable to the stringent immunoprecipitation conditions, the amount of Cdc25 phosphorylated on Pin1-binding sites might be low at this point, or the complex might have a high off rate as the phosphorylated Cdc25 is a substrate of Pin1. Nevertheless, these results suggest that Pin1 is associated with Cdc25 at a time when Cdc25 is partially phosphorylated and yet its activity is low (Izumi et al. 1992; Lee et al. 1994).

Because the interaction between Pin1 and Cdc25 is

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Figure 7. Cell cycle-regulated in vivo association of Pin1 and Cdc25 and the effect of mutating Cdc25 phosphorylation sites on the interaction. (A) Cell cycle-dependent interaction between Pin1 and Cdc25. *Xenopus* eggs were fertilized and assayed for both H1 kinase activity and the Pin1 Cdc25 complex at the indicated times after fertilization. After crushing into a buffer containing 1 μ M okadaic acid, aliquots of soluble extracts were used to assay histone H1 kinase activity to monitor cell cycle progression ($\circ$), and the remaining extracts were immunoprecipitated with anti-Cdc25 antibodies. The resulting immunoprecipitates were subjected to immunoblotting with anti-Pin1 antibodies ($\blacktriangle$). (Left) Pin1 coimmunoprecipitated by anti-Cdc25 antibodies at the two time points (30 and 60 min); (right) relative H1 kinase activity and relative amount of Pin1 that was immunoprecipitated by anti-Cdc25 during the first embryonic cell cycle, with either the maximal amount of Pin1 precipitated or H1 kinase activity being defined as 100%. (B) Failure of Cdc25 mutants to bind Pin1. Wild-type *Xenopus* Cdc25 (WT), T3 mutant Cdc25 (T48A, T67A, and T138A), and T3S2 mutant Cdc25 (T48A, T67A, T138A, S205A, and S285A) were synthesized by in vitro transcription and translation in the presence of [35 S]-methionine and incubated with *Xenopus* I and M extracts. These proteins were separated on SDS-containing gels either directly (input) or first precipitated by GST-Pin1 beads (GST-Pin1).

mediated by phosphorylation of Cdc25 (Fig. 4D), we then examined whether Pin1 interacts with Cdc25 on important phosphorylation sites. At entry into mitosis, Cdc25 is phosphorylated at multiple Thr/Ser-Pro sites (Izumi and Maller 1993; Ogg 1994; Kumagai and Dunphy 1996). Izumi and Maller (1993) have shown that the triple mutation of conserved Thr-48, Thr-67, and Thr-138 (T3 Cdc25), and the quintuple mutation of the three Thr residues plus Ser-205 and Ser-285 (T3S2 Cdc25) prevent most of the shift in electrophoretic mobility of Cdc25 after incubation with mitotic extracts. When they mea-

sured the ability of the Cdc25 mutants to activate Cdc2 in the Cdc25-depleted oocyte extracts and to initiate mitotic entry in oocyte extracts, the activities of T3 and T3S2 mutants were reduced ~70% and 90%, respectively (Izumi and Maller 1993). These results indicate that these Thr/Ser residues are essential for the Cdc25 function. We examined the ability of Pin1 to bind the T3 and T3S2 Cdc25 mutants. As shown previously (Izumi and Maller 1993), the T3 and T3S2 Cdc25 mutants failed to undergo the mobility shift after incubation with mitotic extracts (Fig. 7B, right). Although Pin1 strongly bound the mitotically phosphorylated form of Cdc25, Pin1 almost (T3) or completely (T3S2) failed to bind the Cdc25 mutants, which were incubated with either interphase or mitotic extracts (Fig. 7B, left). Although further experiments are required to pinpoint which phosphorylation sites play the major role in mediating the Pin1 and Cdc25 interaction, these results show that Pin1 interacts with the phosphorylation sites on Cdc25 that are essential for its mitotic activation.

The above results indicate that Pin1 interacts with Cdc25 both in vitro and in vivo. Therefore, we tested whether Pin1 could affect the physiological activity of Cdc25, which is to dephosphorylate and activate the cyclin B/Cdc2 complex. To generate the mitotically phosphorylated form of Cdc25, GST-Cdc25 was incubated in *Xenopus* mitotic extracts, affinity purified on glutathione-agarose beads and eluted. This mitotic Cdc25 was at least ninefold more active than GST-Cdc25 purified in parallel from interphase extracts (data not shown). This mitotic GST-Cdc25 activated the cyclin B/Cdc2 complex, which was kept inactive as a result of inhibitory phosphorylations on Tyr-15 and Thr-14 (Fig. 8A). However, if Pin1 was included in the assay, mitotic Cdc25 failed to activate the Cdc2 complex (Fig. 8). In contrast, neither the mutant Pin1^{R68,69A} at the same concentration, nor BSA at a 25-fold higher concentration had a significant inhibitory effect on Cdc25 activity (Fig. 8A). Fivefold higher concentrations of Pin1^{R68,69A} could inhibit mitotic Cdc25 activity partially (Fig. 8B), a result that is consistent with the requirement for higher concentrations of this mutant protein to arrest the *Xenopus* cell cycle (see Fig. 5 and 6). To rule out the possibility that Pin1 could directly inhibit the cyclin B/Cdc2 complex itself, we examined the effect of Pin1 and its mutants on the activity of dephosphorylated-active cyclin B/Cdc2 under same conditions. Neither Pin1 nor the Pin1 mutant had any effect on Cdc2 activity (Fig. 8C). Taken together, these results indicate that Pin1 could inhibit premature mitotic activation of Cdc25 by interacting with the phosphorylation sites on Cdc25 that are essential for its activation. This offers one explanation for the ability of Pin1 to inhibit mitotic entry.

Discussion

We have demonstrated and characterized a cell cycle-specific and phosphorylation-dependent interaction between the PPLase Pin1 and phosphoproteins. Pin1 binds to a subset of mitosis-specific phosphoproteins, includ-

Figure 8. Pin1, but not the mutant, directly inhibits the ability of Cdc25 to activate cyclin B/Cdc2. (A) GST-Cdc25 (1 μM), which had been incubated in *Xenopus* mitotic extracts and purified on glutathione-agarose, was incubated with 0.62 μM of BSA, GST-Pin1, or GST-Pin1^{R68,69A}. The resulting reaction was incubated for 10 min with beads containing GST-cyclin B/Cdc2 phosphorylated on the inhibitory residues of Cdc2 (Thr-14, Tyr-15). The beads were washed and the activity of cyclin B/Cdc2 was assayed using histone H1 as a substrate. (B) Titration of GST-Pin1 and GST-Pin1^{R68,69A} into the Cdc25 assay, described in A and in Materials and Methods. (C) Effect of Pin1 on Cdc2 kinase activity. Different concentrations of Pin1 and its mutant protein were added to active cyclin B/Cdc2 complex, followed by assaying H1 kinase activity using the same conditions as described in A and B.

ing many MPM-2 antigens, in both human cells and in *Xenopus* extracts; this interaction is direct and depends on the phosphorylation of the Pin1-binding proteins. A limited number of these Pin1-binding proteins have been identified and include Cdc25, Plk1, Wee1, Myt1, Mos, Cdc27, and proteins with sequence similarity to E-MAP-115 and the fission yeast Cdc5. In *Xenopus*, introduction of Pin1 inhibits mitotic division in embryos, and pre-

vents activation of Cdc25 and Cdc2 and entry into mitosis in extracts. Furthermore, just before mitosis, Pin1 not only binds directly the mitotically active form of Cdc25 through the activating phosphorylation sites, but also inhibits its activity. Significantly, these phenotypic and biochemical changes induced by Pin1 are abolished by point mutations in the active site residues of Pin1 that are implicated in binding either the substrate Pro residue or its preceding phosphorylated Ser/Thr phosphate in the substrate. This demonstrates that the ability to bind mitotic phosphoproteins is required for its biological activity. Together with other findings that Pin1 is a phosphorylation-dependent PPLase that is specific for phosphorylated Ser/Thr-Pro peptide bond in mitotic phosphoproteins (Yaffe et al. 1997), Pin1 adds potentially an additional level of mitotic regulation using a novel mechanism involving sequence-specific and phosphorylation-dependent proline isomerization.

The relationship between Pin1 and MPM-2 antigens

In 1983, Davis et al. used total mitotic extracts from HeLa cells as antigens and generated a monoclonal antibody, MPM-2, that specifically recognized phosphoproteins from mitotic cells, but not from interphase cells. This puzzling and remarkable specificity has been seen in all eukaryotic organisms so far examined (Davis et al. 1983; Vandre et al. 1984; Hecht et al. 1987; Keryer et al. 1987; Wordeman et al. 1989). MPM-2 appears to recognize a conserved phosphopeptide that contains the motif phosphorylated Ser/Thr-Pro, on at least 50 mitotic phosphoproteins (Davis et al. 1983; Vandre et al. 1986; Westendorf et al. 1994; Matsumoto-Taniura et al. 1996). However, the importance of the MPM-2 epitope on mitotic phosphoproteins has remained mysterious.

Whereas MPM-2 is a monoclonal antibody, Pin1 is a highly conserved endogenous enzyme. Yet, 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 they both localize to the nuclear speckle during interphase and to mitotic chromosomes at mitosis (Davis et al. 1983; Vandre et al. 1986; Hirano and Mitchison 1991; Kuang et al. 1994; Westendorf et al. 1994; Lu et al. 1996; Matsumoto-Taniura et al. 1996; this study). Second, the interaction either between Pin1 and its interacting proteins or MPM-2 and its antigens is dependent on mitosis-specific phosphorylation of the target proteins (Davis et al. 1983; Westendorf et al. 1994; this study). Third, both inhibit cell cycle progression in *Xenopus* embryos and entry into mitosis in *Xenopus* extracts (this study, Davis et al. 1989; Kuang et al. 1989). Fourth, Pin1 is highly conserved in all eukaryotic cells so far examined, including plants, yeast, *Aspergillus*, and vertebrates, as is the case for the MPM-2 epitope (Davis et al. 1983; Vandre et al. 1986; Hirano and Mitchison 1991; Kuang et al. 1994; Westendorf et al. 1994; Ye et al. 1995; Matsumoto-Taniura et al. 1996). 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 (Yaffe et al. 1997). The observations that the antibody and Pin1 recognize the same proteins and almost the same primary sequences, as well as have similar phenotypes, indicate that the conservation of the MPM-2 epitope can be explained by the recognition of this epitope by a highly conserved mitotic regulator, Pin1.

Pin1 regulation of mitotic entry

Although the G_2 arrest phenotype is induced by overexpression of Pin1, there is enough genetic and biochemical evidence to suggest that Pin1 is working as an inhibitor of premature entry into mitosis. First, Pin1 was isolated originally as a protein that suppresses premature mitotic entry induced by the NIMA kinase in a genetic screen, indicating that Pin1 has the ability to prevent premature entry into mitosis (Lu et al. 1996). Second, depletion of Pin1 from HeLa cells or Pin1 homolog Ess1 from yeast induces mitotic arrest and nuclear fragmentation. Phenotypic analysis reveals that these mitotically arrested HeLa cells are very similar to premature mitotic entry induced by overexpression of NIMA or activated Cdc2 mutant (Krek and Nigg 1991; Lu and Hunter 1995; Lu et al. 1996). These results suggest that depletion of Pin1 may actually induce premature entry into mitosis and mitotic catastrophe. Third, overexpression of Pin1 inhibits entry into mitosis in two completely different genetic systems, *Xenopus*, and humans (this study; Lu et al. 1996). Fourth, we have shown here that Pin1 interacts specifically with many important regulators of Cdc2, whose activation is shown to trigger entry into mitosis. Finally, we have also shown that Pin1 binds a key mitotic inducer Cdc25. This interaction occurs highest just before mitosis, is mediated by the phosphorylation sites on Cdc25 that are essential for mitotic activation, and results in an inhibition of Cdc25 activity to interphase levels. These results consistently suggest that Pin1 acts as an inhibitor of the mitotic activity in G_2 , preventing lethal premature entry into mitosis (Johnson and Rao 1970).

Is Pin1 a general regulator of mitosis-specific phosphoproteins?

Phosphorylation is a common mechanism to regulate interactions between proteins. For example, the SH2 domain and the 14-3-3 protein have been shown to mediate protein-protein interactions by recognizing phosphorylated Tyr and phosphorylated Ser/Thr motifs, respectively (Pawson 1995; Muslin et al. 1996). In contrast to other phosphoprotein-binding proteins, Pin1 is the first to bind and regulate mitosis-specific phosphoproteins and is also the first to have an intrinsic prolyl isomerase activity. Interestingly, we have also found that phosphorylation of Ser/Thr-Pro, but not Tyr-Pro sequences, renders peptides resistant to the isomerase action of both cyclophilins and FKBP, suggesting the need for a differ-

ent enzyme to catalyze this reaction (Yaffe et al. 1997). Significantly, the novel PPIase Pin1 preferentially isomerizes Pro residues preceded by phosphorylated Ser or Thr with ~1300-fold selectivity compared to unphosphorylated peptides (Yaffe et al. 1997). Therefore, Pin1 is a phosphorylation-dependent PPIase that is specific for the phosphorylated Ser/Thr-Pro moieties present in mitotic phosphoproteins. These results suggest a new two-step mechanism for mitotic regulation. The first event is phosphorylation at specific Ser-Pro or Thr-Pro sites by the mitosis-specific activation of Ser/Thr kinases, creating binding sites for Pin1. Second, Pin1 binds the phosphorylated Ser/Thr-Pro motifs and induces local conformational changes through prolyl isomerization. These local conformational changes can alter the activity of a phosphoprotein, as shown previously for NIMA (Lu et al. 1996) and here for Cdc25. In addition, Pin1 could also regulate the ability of its target proteins to interact with other proteins or to be degraded.

Why the cell would use an additional regulator for proteins that have been already regulated by phosphorylation? As mentioned above, Pin1 may act as an inhibitor of mitotic entry in interphase to prevent premature mitotic entry. During mitosis, by acting as a general modulator of mitotic phosphoproteins, Pin1 may provide a means for temporally synchronizing or amplifying the activity of mitotic proteins. For example, the first protein to be phosphorylated by mitotic kinases may be kept inactive by direct binding to Pin1. Only after sufficient levels of mitotic phosphoproteins have been accumulated, would Pin1 inhibition be relieved simultaneously on target proteins. This would relieve inhibition of mitotic phosphoproteins and allow an abrupt wave of signaling to proceed in a synchronous manner. Alternatively, Pin1 might sequentially form complexes with different phosphoproteins whose concentration or affinity for Pin1 may change during mitosis. In this manner, Pin1 could regulate the activities of different proteins at distinct times during mitosis. Interestingly, a similar model has been shown to regulate the function of the Cdk inhibitor p27, which forms sequentially a complex with cyclin D/Cdk4 and cyclin A/Cdk2 during progression through G_1 and S (Poon et al. 1995; Reynisdottir and Massague 1997). How Pin1 exerts its functions during mitotic progression are questions for future study.

Materials and methods

Expression, purification, and kinetic analysis of recombinant Pin1 proteins

Pin1 was expressed and purified by Ni^{2+} -NTA agarose column as an amino-terminally His₆-tagged fusion protein, followed by removing the tag using thrombin, as described (Lu et al. 1996; Ranganathan et al. 1997). To generate an amino-terminally GST-Pin1 fusion protein, Pin1 cDNA was subcloned into a pGEX vector and the resulting fusion protein was expressed and purified by glutathione-agarose column, as described (Lu et al. 1993, 1996). GST-Pin1 was stored in the agarose bead at 4°C for 2 weeks or eluted from the beads and concentrated to 20 mg/ml with a Centricon-10 (Amicon), followed by storing at -80°C.

Both preparations were stored in a buffer containing 20 mM HEPES (pH 7.5), 50 mM NaCl, and 1 mM DTT, as described (Ranganathan et al. 1997). All proteins were quantified by the method of Bradford (Bio-Rad) using BSA as a standard.

Site-directed mutations of Pin1 were introduced using PCR-based techniques and verified by DNA sequencing. The corresponding mutant proteins were expressed and purified using the same procedures as those described for wild-type Pin1. PPIase activity was measured, as described previously (Lu et al. 1996), with the exception that the absorbance of *p*-nitroaniline (at 395 nm) was followed every second for 2–10 min. and data were analyzed offline using a kinetic computer program written by G. Tucker-Kellogg (C. Walsh laboratory at Harvard Medical School).

Analysis of Pin1 and its binding proteins during cell cycle

HeLa cells were arrested at the G₁/S boundary using double thymidine and aphidicolin block, and released to enter the cell cycle, as described (Heintz et al. 1983; Lu and Hunter 1995). To accumulate cells at mitosis, nocodazole (50 ng/ml) was added to cells at 8 hr after the release for the time indicated in text. To obtain a large quantity of interphase and mitotic cells, HeLa cells were incubated with double thymidine and aphidicolin or nocodazole for 16 hr, which resulted in >90% of cells being arrested at the G₁/S boundary or mitosis, respectively. Cells were harvested and a aliquot of cells was subjected to flow cytometry analysis, as described (Lu and Hunter 1995). The remaining cells were lysed in RIPA buffer [10 mM sodium phosphate (pH 7.4), 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 50 mM NaF, 1 mM sodium orthovanadate, 10 µg/ml of aprotinin, 50 µg/ml of PMSF, and 1 mM DTT] and the same amount of total proteins was subjected to immunoblotting analysis using various antibodies for Far Western analysis using GST–Pin1 as a probe. For Far Western analysis, after blocking with 5% BSA, membranes were incubated with 2 µg/ml of GST–Pin1 in TBST for 2 hr, followed by incubation with anti-GST monoclonal antibodies (UBI) and the ECL detection procedures.

Microinjection of Xenopus embryos

Unfertilized eggs were incubated with sperm, dejellied, and 4 µM of the indicated protein (~10-fold above the estimated endogenous levels) was injected in one cell of two-cell stage embryos (30 embryos each protein). The injected embryos were allowed to develop at 18°C to stage 8 and pictures were taken of typical embryos. The titration of Pin1 and the mutants was essentially as described above except that the indicated protein was injected into one cell of the four-cell stage (18 embryos each Pin1 concentration) to the indicated final concentration and allowed to develop for 3 hr. The cell cycle blocks by GST–Pin1 were not homogeneous as cells that were injected with greater concentrations of GST–Pin1 were cleaved fewer times indicating a tighter cell cycle block. To be consistent, cell cycle-blocked embryos were scored as those that contained at least one cell on the injected side that was more than five times larger than uninjected cells.

Preparation of Xenopus CSF extracts

Xenopus colony-stimulation factor (CSF) extracts were prepared from unfertilized eggs, as described previously (Murray 1991) and used immediately. To examine the effect of Pin1 on mitotic entry, a fresh CSF extract containing demembranated sperm (150/µl) and rhodamine–tubulin (2 µg/ml) was activated by ad-

dition of 0.4 mM calcium chloride for 15 min, before the indicated concentrations of various Pin1 proteins were added and mitotic entry was followed for 2 hr by nuclear morphology, nuclear envelope breakdown, spindle formation, and Cdc2 kinase activity, as described previously (Murray 1991). The cell cycle state of nuclei within the extracts were >90% synchronous and typical nuclei were photographed.

Synthesis of mitotic phosphoproteins

The indicated mitotic phosphoproteins were translated in vitro using the TNT-coupled transcription/translation kit (Promega) in a total volume of 10 µl in the presence of 8 µCi of [³⁵S]methionine (1000 Ci/mmol) for 2 hr at 30°C. They were then incubated in *Xenopus* interphase and mitotic extracts as described (Stukenberg et al. 1997). These incubated clones were precipitated by Pin1 beads as described below. The *Xenopus* Mos and Wee1 clones were a kind gift of M. Murakami, G. vande Woude (both at the National Cancer Institute, Frederick, MD) and J. Cooper (Fred Hutchinson Cancer Center, Seattle, WA); the *Xenopus* Cdc25 clone was a generous gift of W. Dunphy (California Institute of Technology, Pasadena), T3 and T3S2 Cdc25 mutants were kindly provided by J. Maller (Izumi and Maller 1993).

Production of antibodies

Because antibodies that we raised previously against carboxy-terminal peptide of Pin1 (Lu et al. 1996) did not have a high sensitivity for detecting Pin1, especially for *Xenopus* Pin1, new Pin1 antibodies were produced, which recognize specifically a single 18-kD Pin1 protein in human cells and *Xenopus* extracts. To raise antibodies against *Xenopus* Cdc25, recombinant GST–Cdc25 (the clone was a kind gift of A. Nebraska and T. Hunt, Imperial Cancer Research Fund, South Mimms, Herts, UK) was affinity purified as described by the manufacturer (Pharmacia). The protein was further purified by SDS-PAGE and a gel slice containing Cdc25 was used to immunize rabbits.

GST pull-down, immunoprecipitation, and immunoblotting analysis

To detect Pin1-binding proteins, either HeLa cells were lysed in or *Xenopus* extracts were diluted in a buffer (buffer A) containing 50 mM Tris-HCl (pH 8.0), 200 mM NaCl, 100 mM NaF, 1 mM sodium orthovanadate, 10% glycerol, 1% Triton X-100, 10 µg/ml of aprotinin, 50 µg/ml of PMSF, and 1 mM DTT. The lysates were preclarified with boiled *Staphylococcus aureus* bacteria (CalBiochem) and then incubated with 10 µl of agarose beads containing various GST–Pin1 proteins or control GST for 2 hr at 4°C. The precipitated proteins were washed five times in the same buffer and subjected to immunoblotting analysis. Immunoprecipitation and immunoblotting analysis using MPM-2 antibody (Davis et al. 1983), which was kindly provided by J. Kuang (M.D. Anderson Cancer Center, Houston, TX), Pin1 antibodies (Lu et al. 1996; kindly provided by M. White (Joslin Diabetes Center, Boston, MA) or newly generated), anti-phosphorylated Tyr antibody (UBI), anti-Cdc25C (Ogg et al. 1994) (from H. Piwnicka-Worms and Santa Cruz Biotechnology), anti-Cdc27 (Yu et al. 1998), anti-Plk1 (Zymed), anti-Cdc2 (Solomon et al. 1990), anti-human Myt1 (Booher et al. 1997), anti-human cyclin B1 (Pines and Hunter 1991), and anti-*Xenopus* cyclin B were performed, as described previously (Lu and Hunter 1995; Lu et al. 1996).

Coimmunoprecipitation of Pin1 and Cdc25

To detect Pin1 and Cdc25 interaction during the *Xenopus* cell cycle, ~500 eggs were fertilized in a minimal volume of MMR [100 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, 0.1 mM EDTA, 5 mM HEPES (pH 7.8)], diluted in 0.1 × MMR for 10 min, dejellied as described (Murray 1991) and incubated in CSF-XB [100 mM KCl, 0.1 mM CaCl₂, 2 mM MgCl₂, 10 mM K-HEPES (pH 7.7), 50 mM sucrose, 5 mM EGTA (pH 7.7)]. At the indicated time after fertilization 15 eggs were crushed into 150 µl of ice cold CSF-XB with 1 µM okadaic acid, microcentrifuged for 20 sec, the layer between the yolk and the pellet was removed to a fresh chilled tube. This solution was mixed well and 5 µl was frozen in liquid nitrogen for future H1 kinase assays, and 30 µl was diluted in 10 µl of either α-Cdc25 or control rabbit sera beads in 100 µl of buffer A (containing 5 mM EDTA and 1 µM microcystin but not vanadate). The immunoprecipitation reactions were rotated for ~40 min at 4°C, washed four times, and subjected to immunoblotting with anti-Pin1 antibodies. The associated Pin1 was quantified as described (Stukenberg et al. 1997). Although the amount of Pin1 bound to Cdc25 was barely above the detectable limit, the experiment was reproduced three times with similar results. In one case, a Pin1/Cdc25 complex remained high throughout mitosis.

Cdc2 and Cdc25 assays

Cdc2 was assayed using histone H1 as a substrate, as previously described (Murray 1991; Lu and Hunter 1995). Cdc25 activity was assayed by using the activation of its endogenous substrate, Cdc2/cyclin B complex phosphorylated on Thr-161, Tyr-15, Thr-14 as an indicator using a variation of an established protocol (Kumagai and Dunphy 1996). When cyclin B is added to a *Xenopus* interphase extract at levels insufficient to activate mitosis (referred to as a “subthreshold cyclin concentration”), the added cyclin B binds Cdc2 and the Cdc2 in the complex is phosphorylated by CAK, Wee1, and Myt 1 to accumulate in an inactive form (Solomon et al. 1990). A subthreshold concentration of GST cyclin B (10 µg) was added to 1 ml of *Xenopus* interphase extract for 30 min at room temperature (Solomon et al. 1990). This was diluted eightfold in XB + 3 mM DTT, rotated for 1 hr with 3 ml of GST agarose beads, washed three times in XBIP (XB + 500 mM NaCl and 1% NP-40 + 2 mM DTT), washed two times (once overnight) in EB (80 mM β-glycerol phosphate, 15 mM EGTA, 15 mM MgCl₂ + 2 mM DTT, 500 mM NaCl, and 1% NP-40, and finally twice with EB + 10 mM DTT). These Cdc25 assay beads were stored at 4°C for up to 1 month.

Mitotic GST-Cdc25 was purified by incubating 22 µg of GST-Cdc25 in a *Xenopus* mitotic extract for 30 min at 23°C; this was diluted eightfold in XB and rotated with 50 µl of glutathione-agarose beads (Sigma) for 1 hr at 4°C. The beads were washed five times in XB-IP, twice in XB + 2 mM DTT, and eluted in 25 µl XB + 2 mM reduced GSH. The final concentration of mitotic GST-Cdc25 was 0.36 mg/ml. A 27-fold dilution of this mitotic GST-Cdc25 could fully activate Cdc2 in the assay below, whereas GST-Cdc25 isolated from interphase extracts in parallel lost activity after a threefold dilution (data not shown). Thus, the mitotic extract stimulated the Cdc25 at least nine times over interphase extracts as previously reported (Solomon et al. 1990).

To assay Cdc25 activity 1 µM mitotic GST Cdc25, and the indicated concentration of either Pin1, Pin1^{R68,69A}, or BSA were incubated in a 20-µl reaction in XB + 1 mM ATP for 10 min at room temperature. These reactions were diluted sequentially (1:1, 1:3, 1:9, 1:27) into XB + 1 mM ATP and 10 µl of each was mixed with 10 µl of Cdc25 assay beads for 10 min at room

temperature with constant shaking. Subsequently, the Cdc25 assay beads were washed three times in XB-IP, twice in EB + 1 mM DTT, and assayed for H1 kinase activity as described (Solomon et al. 1990). PhosphorImager analysis of the H1 kinase assays were quantified by the Molecular Dynamics ImageQuant 3.3 software. Figure 8A shows an assay with 1 µM mitotic GST-Cdc25, 0.67 µM of either Pin1, Pin1^{R68,69A} or 16 µM BSA then diluted 27-fold before being mixed with the Cdc25 assay beads and the amount of H1 kinase activity is relative to the amount of activity of the beads without Cdc25 being zero and the BSA reaction being 100%. The most reproducible way to quantify the Cdc25 activity in this assay was by determining the end-point dilution of Cdc25 that could activate Cdc2. Therefore, the Cdc25 activity in Figure 8B is quantified by the end-point dilution of the mitotic GST-Cdc25 at which Cdc2 on the beads could still be significantly activated.

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