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The mitotic peptidyl-prolyl isomerase, Pin1, interacts with Cdc25 and Plx1

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The *cis/trans* peptidyl-prolyl isomerase, Pin1, is a regulator of mitosis that is well conserved from yeast to man. Here we demonstrate that depletion of Pin1-binding proteins from *Xenopus* egg extracts results in hyperphosphorylation and inactivation of the key mitotic regulator, Cdc2/cyclin B. We show biochemically that this phenotype is a consequence of Pin1 interaction with critical upstream regulators of Cdc2/cyclin B, including the Cdc2-directed phosphatase, Cdc25, and its known regulator, Plx1. Although Pin1 could interact with Plx1 during interphase and mitosis, only the phosphorylated, mitotically active form of Cdc25 was able to bind Pin1, an event we have recapitulated using *in vitro* phosphorylated Cdc25. Taken together, these data suggest that Pin1 may modulate cell cycle control through interaction with Cdc25 and its activator, Plx1.

Keywords: Cdc25/mitosis/peptidyl-prolyl isomerase/Pin1/Plx1

Introduction

Entry into mitosis is regulated by the Ser/Thr kinase Cdc2/cyclin B in all eukaryotic cells studied to date (reviewed in Nurse, 1990). Phosphorylation of critical substrates by this kinase complex is believed to underlie many of the structural rearrangements which occur as cells enter mitosis. To avoid execution of mitosis at inappropriate points in the cell cycle, the activity of Cdc2/cyclin B is tightly regulated. As cyclin B is synthesized, it binds to Cdc2 which is inactive in its monomeric form. Although the Cdc2/cyclin B complex is potentially active at the time of formation, it is held in check during interphase by phosphorylation at two sites on Cdc2, Tyr 15 and Thr 14 (Norbury *et al.*, 1991; Krek *et al.*, 1992; Solomon *et al.*, 1992). Wee1, a nuclear enzyme, and Myt1, a membrane-bound enzyme, can both phosphorylate Tyr 15, but only Myt1 is able to catalyze phosphorylation of Thr 14 (Atherton-Fessler *et al.*, 1994; Kornbluth *et al.*, 1994; Watanabe *et al.*, 1994; McGowan and Russell, 1995; Mueller *et al.*, 1995a,b; Liu *et al.*, 1997). At G_2/M, Tyr 15 and Thr 14 are rapidly dephosphorylated by the dual-specificity Cdc25 phosphatase (Dunphy and Kumagai, 1991; Gautier *et al.*, 1991; Strausfeld *et al.*, 1991; Millar and Russell, 1992). Phosphorylation at Thr 161 of Cdc2 is also required for Cdc2/cyclin B activity, but there is no evidence to suggest that this phosphorylation event is cell-cycle regulated (Solomon, 1993).

Although Cdc2/cyclin B is a key regulator of mitosis, an additional Ser/Thr kinase, NIMA, is also required for mitotic entry in the filamentous fungus, *Aspergillus nidulans* (Osmani *et al.*, 1987). NIMA activity, like that of Cdc2/cyclin B, peaks at the G_2/M transition (Osmani *et al.*, 1991a). In *A.nidulans* cells lacking functional NIMA, the cell cycle arrests in G_2 (Morris, 1976; Bergen *et al.*, 1984), while deregulated expression of NIMA induces premature mitosis (Osmani *et al.*, 1988). Physiologically relevant substrates of NIMA kinase have yet to be identified and the relationship between NIMA-dependent and Cdc2/cyclin B-dependent pathways of entry into mitosis has not been completely clarified. It remains an open question whether NIMA acts in series or in parallel with Cdc2/cyclin B to promote mitosis (Osmani *et al.*, 1991b; Ye *et al.*, 1995).

Various cloning strategies have been unsuccessful in identifying functional NIMA homologs in higher eukaryotes. However, a yeast 2-hybrid screen using an *A.nidulans* NIMA as a 'bait' to screen a human cDNA library uncovered a novel mitotic regulator, Pin1 (Lu *et al.*, 1996). Overexpression of Pin1 in HeLa cells caused a G_2 cell cycle arrest, implicating Pin1 as a negative regulator of mitosis (Lu *et al.*, 1996). Moreover, knock-out of a gene encoding a Pin1 homolog in *Saccharomyces cerevisiae* (*ESS1*), or anti-sense depletion of Pin1 from HeLa cells, led to mitotic arrest suggesting that Pin1 might also be required for exit from mitosis (Hanes *et al.*, 1989; Lu *et al.*, 1996). Pin1 is a member of the prokaryotic parvulin family of peptidyl-prolyl isomerases (PPIases) and is unrelated in sequence to the well-characterized cyclophilin A and FK506-binding PPIases (Rahfeld *et al.*, 1994; Schmid, 1995; Lu *et al.*, 1996). PPIases, in general, enhance the rate of *cis* to *trans* isomerization around peptide bonds immediately preceding proline residues and have been implicated in intracellular protein folding and trafficking (Schmid, 1995). Eukaryotic parvulin-type PPIases, including *S.cerevisiae* Ess1 (Hanes *et al.*, 1989), *Drosophila* dodo (Maleszka *et al.*, 1996) and human Pin1, possess an N-terminal protein sequence motif, the WW domain, believed to mediate protein–protein interactions (Sudol, 1996).

The isolation of Pin1 in a screen for interactors of a known mitotic regulator, NIMA, suggested that Pin1 might exert its cell cycle-regulatory effects through modulation of a NIMA-like pathway. However, we present data here to suggest that Pin1 may impinge upon the Cdc2/cyclin B-dependent pathway of mitotic regulation. Specifically, we show that Pin1 can physically interact with the Cdc2/

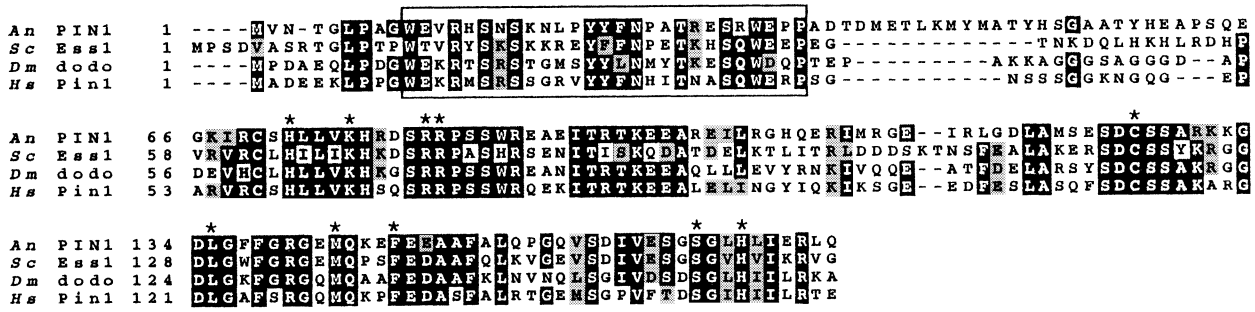

Fig. 1. Alignment of the deduced amino acid sequence of *Aspergillus* Pin1 (*An* PIN1) to yeast (*Sc* Ess1), *Drosophila* (*Dm* dodo) and human (*Hs* Pin1) Pin1 homologs. Residues that are identical in at least three of the four proteins are shaded black, conservative replacements are shaded grey and dashes denote gaps in the alignments. The core of the N-terminal WW domain of the proteins is boxed, and the conserved residues shown by the crystal structure of human Pin1 to be within the active site of the PPIase moiety are indicated by asterisks. The sequences were aligned by the ClustalW 1.6 program, and shaded using BOXSHADE 3.21.

cyclin B regulator, Cdc25, and with the Cdc25-directed Ser/Thr kinase, Plx1. Moreover, we have found that Pin1 selectively binds the phosphorylated form of Cdc25 and that Cdc2/cyclin B activity can convert unphosphorylated Cdc25 into a Pin1 interactor. This suggests that Pin1 may impact the cell cycle through interaction with regulators of the Cdc2/cyclin B kinase.

Results

In the yeast two-hybrid screen originally used to isolate human Pin1, expression of active NIMA kinase was lethal to the *S.cerevisiae* host (Lu *et al.*, 1996). Therefore, the screen selected not only for expression of NIMA interactors but also for proteins that would restore yeast viability. To avoid biasing the search for NIMA-binding proteins in favor of negative regulators of NIMA, we repeated the yeast two-hybrid screen using as the bait an enzymatically inactive variant of NIMA [NIMAK40M, Lys to Met at residue 40 (Lu *et al.*, 1993)], which was not, in our system, detrimental to yeast growth. In addition, we used an *A.nidulans* cDNA library as the source of 'prey-'encoding cDNAs, to search for NIMA interactors in a system where the *nimA* gene was of demonstrated importance. Surprisingly, in a screen of 7.9 million colonies, we isolated *A.nidulans* Pin1 19 times, suggesting that Pin1 is likely to be either an abundant or particularly avid NIMA interactor which does not require NIMA enzymatic activity for efficient binding. As shown in Figure 1, the predicted *A.nidulans* Pin1 protein is 49.4% identical to human Pin1 (Lu *et al.*, 1996), 47.2% identical to *S.cerevisiae* Ess1 (Hanes *et al.*, 1989) and 51.5% identical to *Drosophila* dodo (Maleszka *et al.*, 1996), demonstrating that this mitotic regulator is well-conserved from yeast to man. This sequence has been deposited in DDBJ/EMBL/GenBank (AF035768) and further characterization of this clone will be described elsewhere.

Pin1 negatively regulates entry into mitosis

In order to understand the molecular basis for Pin1's effects on mitotic regulation, and given the high degree of Pin1 sequence conservation across species, we turned to the *in vitro* cell cycle reconstitution system provided by *Xenopus* egg extracts. Upon addition of sperm chromatin to 'cycling extracts' of *Xenopus* eggs, nuclei form *in vitro* around the added chromatin templates and then undergo repeated rounds of S and M phase (Murray and Kirschner, 1989; Murray *et al.*, 1989; Dasso and Newport, 1990). To determine whether Pin1 could affect these S/M oscillations, we produced recombinant *A.nidulans* Pin1 in bacteria and added it to cycling extracts at the beginning of S phase. Nuclear formation occurred on schedule and DNA replication was not perturbed by Pin1 addition (data not shown). However, we found that Pin1 addition prevented entry into mitosis: nuclear envelope breakdown and chromosome condensation were completely inhibited (Figure 2B and E), as was activation of Cdc2/cyclin B indicated by the relatively insignificant level of histone H1 kinase activity (Figure 2F, lower panel) compared with that observed in control extract (Figure 2F, upper panel). Although activation of the histone H1-directed kinase was suppressed by Pin1 addition, cyclin B synthesis was not impaired, as shown by immunoblotting extract aliquots with antisera directed against cyclin B2 (Figure 2G). Addition of GST protein or the isolated WW or PPIase domains of Pin1, prepared in a manner identical to that used for full length Pin1, did not impede progression of these extracts into mitosis (Figure 2A, C and D, respectively). The cell cycle arrest promoted by full length Pin1 could be overriden by addition of high levels of recombinant glutathione-*S*-transferase (GST)-cyclin B (data not shown; previously shown to escape inhibition by regulatory enzymes in the extract) (Smythe and Newport, 1992), further demonstrating that Pin1 had not disabled the extract's ability to respond to Cdc2 activation. These results revealed that Pin1 could act as a negative regulator of mitosis in the *Xenopus* extract system and implied that proteins interacting with Pin1 were important for mitotic regulation *in vitro*.

Pin1-binding proteins are required for activation and maintenance of Cdc2/cyclin B activity

Since addition of Pin1 to cycling extracts inhibited entry into mitosis, we hypothesized that Pin1 might interact with key mitotic regulators in the extract. To address this possibility, we investigated whether depletion of Pin1-interacting proteins from egg extracts would prevent mitotic entry *in vitro*. Since extracts statically arrested in one phase of the cell cycle are more amenable to biochemical analysis than cycling extracts, we prepared extracts arrested in either S phase (by addition of cycloheximide to prevent cyclin B synthesis) or M phase (by

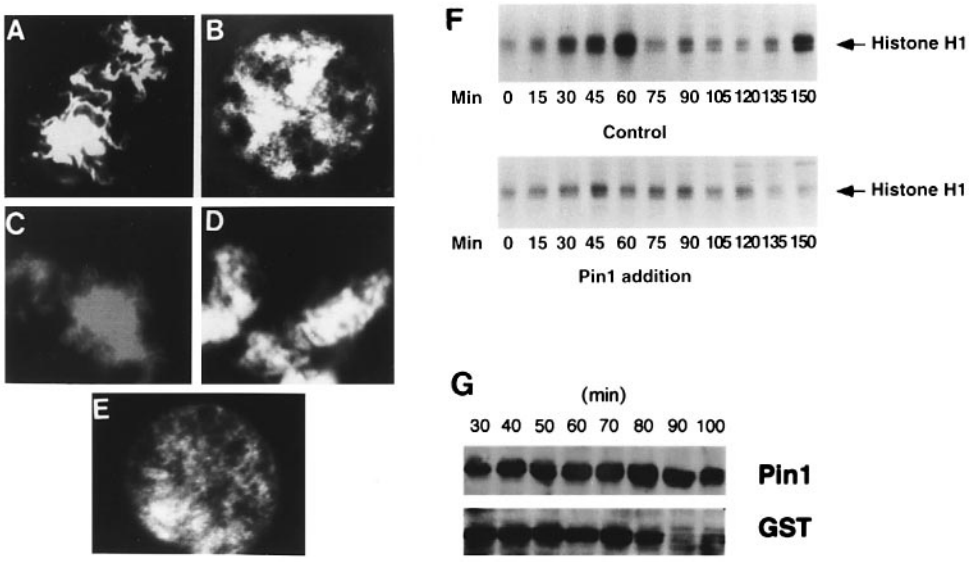

Fig. 2. Exogenously added Pin1 prevents entry into mitosis in *Xenopus* egg extracts. Bacterially produced fusion proteins were added to cycling extracts containing sperm chromatin and incubated at room temperature. (**A**) GST protein. (**B**) Full-length *A.nidulans* Pin1. From a separate extract: (**C**) WW domain (aa 1–41 of Pin1); (**D**) PPIase domain (aa 44–176 of Pin1); (**E**) full-length Pin1. Samples were monitored for nuclear envelope breakdown (NEBD) and chromatin condensation at 15 min intervals by withdrawing samples, staining with Hoechst 33258 and observing nuclei by fluorescence microscopy. Between 60 and 75 min after the start of the room temperature incubation (in several different extracts), extracts containing GST, the WW domain or the PPIase domain (A, C, D) entered mitosis as indicated by nuclear envelope breakdown and DNA condensation, while extracts containing recombinant full-length Pin1 (B) and (E) remained arrested in interphase with decondensed DNA and an intact nuclear envelope throughout the course of the 3 h experiment. (**F**) At the indicated times after the start of the room temperature incubation, aliquots of samples prepared as for Figure 2A and B were assayed for their ability to phosphorylate exogenously added histone H1 in the presence of [γ-^{32}P]ATP. Extracts supplemented with GST (upper panel); extracts supplemented with Pin1 (lower panel). Phosphorylated histone was resolved by SDS–PAGE and subjected to autoradiography. (**G**) At the indicated times after the start of the room temperature incubation, aliquots of samples prepared as for Figure 2A and B were removed, resolved by SDS–PAGE, and processed for immunoblotting with a monoclonal antibody directed against cyclin B2. Note that cyclin is degraded at 90 min in the GST-containing sample, indicative of entry into mitosis, while cyclin accumulates and is stable in the Pin1-containing extract.

addition of EGTA to prevent cyclin B proteolysis). Pin1 was synthesized as a fusion protein with GST to facilitate coupling to Sepharose through the GST moiety and these 'Pin1 beads' (or GST beads alone) were incubated in either S or M phase arrested extracts. The beads were pelleted from the extract by gentle centrifugation and the supernatants ('depleted extracts') were supplemented with sperm chromatin and an ATP-regenerating cocktail. When we attempted to drive the depleted S phase extracts into mitosis by addition of recombinant cyclin B (which can bind to and activate endogenous monomeric Cdc2), extracts depleted using GST beads alone rapidly entered mitosis (within 30 min at concentrations of cyclin B from 0.5 to 10 μM), as determined by observation of chromosome condensation and nuclear envelope breakdown, while extracts depleted using Pin1 beads remained arrested in interphase at cyclin B concentrations ⩽2 μM (Figure 3A and Table I). Moreover, assay of histone H1-directed kinase activity in the depleted extracts revealed that depletion of Pin1-binding partners prevented the appropriate activation of Cdc2 in response to cyclin B addition (Figure 3B). These results substantiate data from the cycling extracts suggesting that Pin1-interacting proteins are required for entry into mitosis and argue that Pin1 binding partners are required for proper activation of Cdc2/cyclin B complexes.

Given the effects of Pin1 on activation of Cdc2/cyclin B, we evaluated whether Cdc2/cyclin B activity could be maintained in the absence of Pin1-binding proteins. Surprisingly, when we assayed the histone H1 kinase activity in depleted mitotic egg extracts, we found that depletion of Pin1-binding partners reduced Cdc2/cyclin B kinase activity to levels normally found in interphase (Figure 3C). Depletion of extracts with GST beads alone had no effect. These results demonstrate that Pin1 interactors are required not only for activation of Cdc2/cyclin B but also for maintenance of its activity.

Cdc2/cyclin B complexes do not associate with Pin1

One possible explanation for the failure of Pin1 bead-depleted extracts to enter mitosis or to maintain mitotic levels of Cdc2/cyclin B activity was that Cdc2/cyclin B itself was removed from the extract through interaction with Pin1. To determine if this was the case, we incubated GST–Pin1 beads in M phase arrested extracts, pelleted and washed the beads, and resolved the bead-bound material by SDS–PAGE. Examination of the GST–Pin1 binding proteins with anti-Cdc2 sera revealed only trace levels of this protein associated with Pin1 beads (Figure 3D, lane 7). This particular SDS–polyacrylamide gel was not of sufficient resolution to distinguish the different phosphorylation states of Cdc2, an indicator of the enzyme's activation state (but see Figure 5A). However, we were unable to find any cyclin B1 (not shown), cyclin B2 (Figure 3E), or histone H1 kinase activity (Figure 3C) associated with the Pin1 beads after incubation in mitotic extracts. This result indicates that the small amount of Cdc2 found in association with Pin1 beads does not represent the active, cyclin B-complexed sub-population

of Cdc2. Finally, soluble Pin1 protein could not suppress active histone H1 kinase activity when added directly to a mitotic extract (Figure 3F), nor could it inhibit the activity of purified Cdc2/cyclin B when added directly to an active preparation of the kinase (Figure 3G), suggesting that Pin1 is probably not a direct Cdc2 inhibitor akin to the Cdk2 inhibitor, $p21^{cip/waf1}$. Collectively, these results suggest that some regulator(s) of Cdc2, rather than Cdc2 itself, might be the target of Pin1.

To detect candidate Pin1-interacting proteins associated with GST–Pin1 beads, we incubated these beads, or GST beads alone, with either mitotic or interphase extract, pelleted and washed the beads, and resolved the bead-bound material by SDS–PAGE. Silver staining of the gel revealed ~12 proteins from mitotic extracts associated with Pin1 but not GST beads, and six proteins specifically associated with Pin1 during interphase (Figure 4). While this gel provides only a rough estimate of the number of Pin1-interacting proteins in the extract, we can conclude that: (i) under the conditions used, GST itself binds many proteins but specific Pin1-interactors are detected; and (ii) Pin1 beads appear to interact specifically with a relatively small number of proteins in the extract, at least at a level detectable by silver staining.

Pin1-binding partners include key regulators of Cdc2/cyclin B

Since the activity of the Cdc2/cyclin B complex is known to be tightly regulated by phosphorylation (Dunphy, 1994), we predicted that the state of phosphorylation of Cdc2/cyclin B in extracts depleted of Pin1-binding proteins would be altered. To address this, mitotic extracts depleted of Pin1 or GST-binding proteins were incubated at room temperature, boiled in sample buffer, resolved by SDS–PAGE and processed for immunoblotting with anti-Cdc2 sera. As described previously, the mobility of Cdc2 on

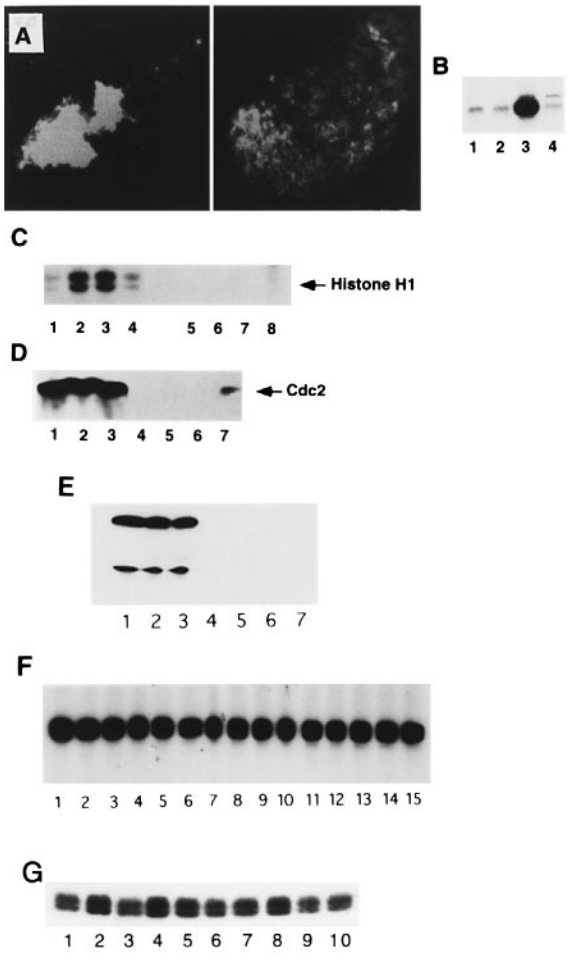

Fig. 3. Pin1-binding proteins are required for activation and maintenance of Cdc2/cyclin B activity. (**A**) Crude interphase extracts were depleted with 1/10 volume of glutathione–Sepharose beads linked to equivalent amounts of either GST or GST–Pin1 ('pin 1 beads'). After 1 h rotation at 4°C, beads were removed by gentle centrifugation, then sperm chromatin (500/μl), an ATP-regenerating cocktail, and GST–cyclin B were added to the depleted extracts. Nuclear morphology was monitored at 10 min intervals by Hoechst staining and fluorescence microscopy. Thirty min after cyclin B addition, extracts depleted with GST beads entered mitosis (left), while extracts depleted with Pin1 beads remained in interphase throughout the course of the 3 h experiment (right). Nuclei were photographed 40 min after GST–cyclin B addition. Note that the GST–cyclin B added was a non-degradable variant. (**B**) Samples treated as above were also assayed for histone H1-directed kinase activity before and after GST–cyclin B addition. Lane 1, interphase extract depleted with GST beads; lane 2, interphase extract depleted with Pin1 beads; lane 3, interphase extract depleted with GST beads and supplemented with GST–cyclin; lane 4, interphase extract depleted with Pin1 beads and then supplemented with GST–cyclin. (**C**) Mitotic extracts (100 μl) were depleted of GST or GST–Pin1-binding proteins as was done for interphase extracts in (A). After depletion, extracts were incubated for 20 min at room temperature. Aliquots (2 μl) from undepleted interphase extract (lane 1); undepleted mitotic extract (lane 2); mitotic extract depleted with GST beads (lane 3); mitotic extract depleted with Pin1 beads (lane 4) were measured for Cdc2/cyclin B-associated histone H1 kinase activity. We also assayed the material associated with the GST beads after incubation in mitotic extract (lane 7) and the material associated with the Pin1 beads after incubation in mitotic extract (lane 8). GST beads (lane 5) and Pin1 beads (lane 6) which had not been exposed to extract were also assayed. The reactions were subject to SDS–PAGE and autoradiography. After depletion of Pin1-binding proteins from mitotic extracts, the Cdc2-associated histone H1 kinase activity dropped to basal levels seen in interphase extracts. (**D**) Mitotic extracts (2.5 μl) depleted of either GST-binding or GST–Pin1-binding proteins were resolved by SDS–PAGE and processed for Western blotting with anti-Cdc2 sera. In addition, the beads pelleted from the extract were washed and processed for SDS–PAGE and Western blotting. Virtually all of the Cdc2 remained in the extract after depletion, with trace amounts of Cdc2 associated with the GST–Pin1 beads. Lane 1, undepleted mitotic extract; lane 2, mitotic extract depleted with GST beads; lane 3, mitotic extract depleted with Pin1 beads; lane 4, GST beads prior to extract exposure; lane 5, Pin1 beads prior to extract exposure; lane 6, material bound to GST after incubation with mitotic extract; lane 7, material bound to Pin1 beads after incubation with mitotic extract. (**E**) Samples treated identically to those loaded in lanes 1–7 for part (D) were processed for immunoblotting with a monoclonal antibody directed against cyclin B2. The upper band is cyclin B2, the lower band is a protein which cross-reacted with the anti-cyclin antibody. (**F**) Buffer alone (lanes 1, 6 and 11) or the following proteins: GST (lanes 2, 7 and 12), full length Pin1 (lanes 3, 8 and 13), the isolated PPIase domain (lanes 4, 9 and 14), or the WW domain (lanes 5, 10 and 15) was added to mitotic extract at a final concentration of 1 mg/ml (lanes 2–5), 2 mg/ml (lanes 7–10) or 4 mg/ml (lanes 12–15). After 40 min incubation, samples were withdrawn and assayed for histone H1-directed kinase activity. None of these treatments inhibited active Cdc2/cyclin from a mitotic extract. (**G**) Pin1 does not suppress histone H1 kinase activity. Active Cdc2/GST–cyclin B complexes were purified from interphase cytosol in the presence of an ATP-regenerating system. The histone H1 phosphorylation activities of the complexes were assayed in 20 μl reactions following pre-incubation in the presence of Pin1 (60, 12, 2.4 or 0.48 μg in lanes 3, 4, 5 and 6, respectively), GST (60, 12, 2.4 or 0.48 μg in lanes 7, 8, 9 and 10, respectively), or Pin1 dilution (XB) buffer (lanes 1 and 2). Baculovirus-expressed Cdc25 (400 ng) was added to the reaction shown in lane 2.

Table I. Effect of depletion of Pin1 binding proteins on cyclin B-induced mitosis

Concentration of cyclin B added (μM)	Time of entry into mitosis after cyclin B addition (min)	
	Extracts depleted with GST beads	Extracts depleted with Pin1 beads
0.25	70	n.o.[a]
0.5	30	n.o.
1	30	n.o.
2	30	n.o.
5	30	40
10	30	30

[a]n.o., not observed in >180 min.

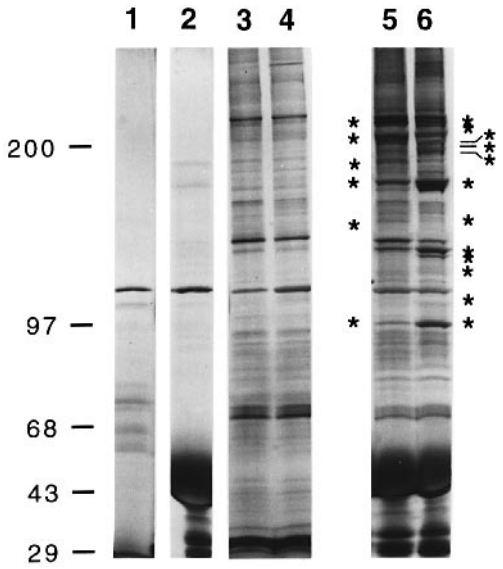

Fig. 4. Pin 1 interacts with specific proteins in *Xenopus* egg extracts. GST (lanes 3 and 4) or GST–Pin1 (lanes 5 and 6) beads were incubated in either interphase (lanes 3 and 5) or mitotic (lanes 4 and 6) extracts. Untreated GST and GST–Pin1 beads were loaded in lanes 1 and 2, respectively. After pelleting and washing, the bead-bound material was resolved by SDS–PAGE and developed with a silver staining kit (Bio-Rad). For each lane, 50 μl of extract (at 40 mg/ml total protein) was incubated with 10 μl of protein-linked glutathione–Sepharose beads. Asterisks indicate bands that associate specifically with GST–Pin1.

SDS–PAGE reflects its state of phosphorylation at the negative regulatory sites, Thr 14 and Tyr 15 (in our gel system Cdc2 from egg extracts does not shift in mobility in response to changes in phosphorylation at the activating site, Thr 161). Specifically, three forms of Cdc2 are detectable: the slowest mobility form corresponds to Cdc2 phosphorylated at both Thr 14 and Tyr 15; the fastest mobility form represents Cdc2 which is unphosphorylated at these sites; and the intermediate mobility form contains a mixture of singly phosphorylated forms (at Thr 14 or Tyr 15) (Kornbluth *et al.*, 1994). Under normal circumstances, the only form of Cdc2 detected in a mitotic extract is the unphosphorylated form because Wee1 and Myt1, the enzymes which phosphorylate Thr 14 and Tyr 15, are both relatively inactive, while Cdc25 phosphatase is active (Mueller *et al.*, 1995a,b). Figure 5A shows that depletion with Pin1 beads promoted the appearance of the Thr 14, Tyr 15-doubly phosphorylated form of Cdc2 in

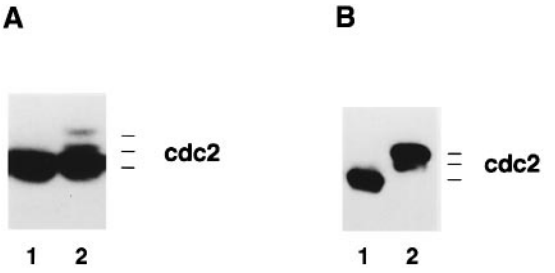

Fig. 5. Cdc2/cyclin B is rephosphorylated after depletion of Pin1-binding proteins. (**A**) After depletion of mitotic extracts with GST–Pin1 beads as was done in Figure 3C, 2 μl aliquots of the depleted extract were processed for SDS–PAGE on a high resolution gel and Western blotting with anti-Cdc2 sera in order to visualize the differently phosphorylated forms of Cdc2. Lane 1, mitotic extract after depletion with GST beads; lane 2, mitotic extract after depletion with Pin1 beads. In lane 2, the doubly phosphorylated, most slowly migrating, form of Cdc2 appears. (**B**) GST–cyclin B was added to interphase extracts depleted with either GST beads or Pin1 beads. GST–cyclin B-bound Cdc2 was retrieved on glutathione–Sepharose beads from the depleted samples. These protein complexes were processed for SDS–PAGE and Western blotting with anti-Cdc2 sera to visualize the different phosphorylated forms of Cdc2. Lane 1, interphase extract depleted with GST beads and supplemented with cyclin B; lane 2, interphase extract depleted with Pin1 beads and supplemented with cyclin B.

M phase extracts (lane 2), whereas Cdc2 in extracts depleted with GST beads was maintained in a non-phosphorylated form (lane 1). These results indicate that the inactivation of Cdc2/cyclin B following depletion of Pin1-binding proteins resulted from re-phosphorylation of Cdc2 at its negative regulatory sites, implying that a regulator of Cdc2 phosphorylation was removed from the extract by association with Pin1. It should be noted that the relative proportions of phosphorylated and unphosphorylated Cdc2 found in the Pin1-depleted mitotic extracts were similar to those we have observed during interphase in cycling extracts, and that unphosphorylated Cdc2 remaining in the mitotic extracts after Pin1 depletion is likely to represent inactive Cdc2 that is uncomplexed to cyclin B.

We hypothesized that perturbations of the Cdc2 phosphorylation state might also underlie the inability of Cdc2/cyclin B to trigger entry into mitosis in interphase extracts depleted of Pin1-binding proteins. To test this idea, we added GST–cyclin B to Pin1 bead-depleted, interphase extracts and then retrieved the cyclin-bound Cdc2 on glutathione–Sepharose beads. As shown in Figure 5B, Cdc2 retrieved with GST–cyclin B from control interphase extracts was dephosphorylated, indicative of its active state (lane 1). However, Cdc2 recovered on exogenously-added GST–cyclin B from extracts depleted of Pin1-binding proteins bound to exogenously-added recombinant cyclin B remained phosphorylated at Tyr 15 and/or Thr 14 (lane 2), suggesting that critical regulators of Cdc2 phosphorylation had been removed or altered.

Cdc25 interacts with Pin1

Since removal of Pin1-binding partners from both interphase and mitotic extracts altered the phosphorylation state of Cdc2/cyclin B, we postulated that a critical regulator(s) of Thr 14/Tyr 15 phosphorylation on Cdc2/cyclin B would associate with Pin1. An increase in Cdc2 phosphorylation after depletion of Pin1-binding proteins

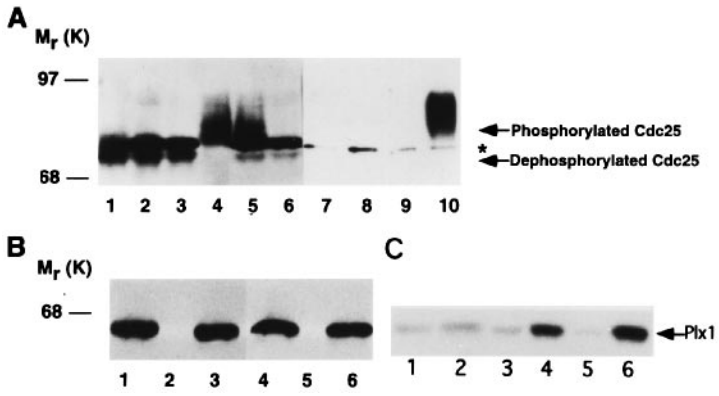

Fig. 6. Pin1 interacts with Cdc25 and with Plx1 in *Xenopus* egg extracts. (**A**) Interphase or mitotic extracts were depleted of either GST or GST–Pin1 binding proteins by incubation with the appropriate resin (GST or Pin1 beads), followed by gentle pelleting of the beads. Extract samples (5 µl) or pelleted beads were resolved by SDS–PAGE and processed for Western blotting with anti-Cdc25 sera. Lane 1, interphase extract; lane 2, interphase extract depleted with GST beads; lane 3, interphase extract depleted with GST–Pin1 beads; lane 4, mitotic extract; lane 5, mitotic extract depleted with GST beads; lane 6, mitotic extract depleted with GST–Pin1 beads; lane 7, GST beads after incubation in interphase extract; lane 8, Pin1 beads after incubation in interphase extract; lane 9, GST beads after incubation in mitotic extract; lane 10, Pin1 beads after incubation in mitotic extract. The phosphorylated form of Cdc25 is, as indicated, shifted in mobility. The Pin1 bead-bound Cdc25 is hypershifted, perhaps because Cdc25-directed phosphatases are separated from the Cdc25 during purification on the Pin1 beads. The asterisk (*) indicates a contaminating bacterial protein, present in the recombinant protein preparations, which cross-reacts with the anti-Cdc25 sera. This band is present in the protein preparations before exposure to extract. (**B**) Either GST or GST–Pin1 beads were incubated with interphase or mitotic extract at 4°C for 60 min. The beads were then pelleted, washed thoroughly, and processed for SDS–PAGE and Western blotting with a monoclonal antibody directed against human Plk1, which recognizes *Xenopus* Plx1. Lane 1, interphase extract; lane 2, GST beads after incubation with interphase extract; lane 3, Pin1 beads after incubation with interphase extract; lane 4, mitotic extract; lane 5, GST beads after incubation with mitotic extract; lane 6, Pin1 beads after incubation with mitotic extract. (**C**) *In vitro* translated, ^{35}S-labeled Plx1 was incubated with GST beads (lane 1); Pin1 beads (lane 2); interphase extract and then GST beads (lane 3); interphase extract and then Pin1 beads (lane 4); mitotic extract and then GST beads (lane 5); mitotic extract and then Pin1 beads (lane 6).

could result from removal of Cdc25 (or a positive activator of Cdc25) or from removal of negative regulators of the kinases, Myt1 and/or Wee1 (see Discussion).

To determine whether Cdc25 was removed from extracts in association with Pin1, we incubated mitotic extracts with Pin1 or GST beads, pelleted the beads and processed the depleted supernatants for immunoblotting with anti-Cdc25 sera. Pin1 beads removed all of the Cdc25 from mitotic extracts, while GST beads alone did not bind detectable levels of the protein (Figure 6A).

Since a positive regulator of Cdc25, Plx1 kinase (Kumagai and Dunphy, 1996), was purified from *Xenopus* egg extracts by virtue of its stable association with Cdc25, we suspected that Plx1 might also be present on the Pin1 beads after incubation in extracts. To determine if this was the case, we incubated Pin1 beads in mitotic extracts and then immunoblotted the Pin1 bead-bound material with antibody directed against a human Plx1 homolog, Plk1 (Lane and Nigg, 1996). This antibody, which recognized baculovirus-produced *Xenopus* Plx1, also recognized a protein of the predicted molecular weight of Plx1 (67 kDa) in egg extracts. Examination of material bound to Pin1 beads after incubation in mitotic extracts revealed that Plx1, like Cdc25, could bind to Pin1 beads (Figure 6B).

When we performed parallel experiments to examine Cdc25 and Plx1 binding to Pin1 beads in interphase, rather than mitotic, extracts of *Xenopus* eggs, we were surprised to find that Plx1 associated with the Pin1 beads, but that Cdc25 did not. Moreover, the concentration of Cdc25 protein in interphase extracts was unaltered by depletion of Pin1-binding proteins (Figure 6A and B). Therefore, Pin1 can bind to Cdc25 from mitotic but not interphase extracts and Plx1 binding to Pin1 can occur independently of Cdc25 association. However, Plx1 translated *in vitro* in reticulocyte lysates could not bind to Pin1 without prior incubation in interphase or mitotic extracts (Figure 6C), suggesting that either post-translational modifications or an additional binding partner is required for the Plx1–Pin1 interaction.

Depletion of Cdc25 can inactivate Cdc2/cyclin B

Given the fact that Pin1 bound both Plx1 and Cdc25, we wished to determine whether the phenotypic consequences of depleting Pin1-binding partners could be attributed to removal of these proteins from the extract. Following depletion of Pin1-binding proteins from mitotic extracts, as described above, Cdc2/cyclin B is inactivated as a histone H1 kinase. We reasoned that re-addition of Cdc25 or Plx1 to the depleted extracts might be expected to restore H1 kinase activity if removal of either of these was responsible for the inactivation of Cdc2. We also wished to assess whether recombinant *A.nidulans* NIMA, a known binding partner of Pin1, might complement the depleted extracts, which would suggest that removal of a NIMA-like *Xenopus* protein was responsible for the inactivation of Cdc2/cyclin B. As shown in Figure 7A, re-addition of recombinant *Xenopus* Cdc25 restored H1 kinase activity to mitotic extracts depleted of Pin1-binding proteins. However, neither active recombinant Plx1 kinase, which was able to phosphorylate Cdc25 *in vitro* (not shown), nor bacterially produced kinase active NIMA (data not shown) restored mitotic H1 kinase activity.

These data suggested that removal of Cdc25 from mitotic extracts resulted in inactivation of active Cdc2/cyclin B. To confirm this conclusion, Cdc25 was immunodepleted from mitotic extracts using antisera directed against *Xenopus* Cdc25 and H1 kinase activity remaining in the depleted extract was measured. As shown in Figure 7B, immunodepletion of a mitotic extract resulted in complete removal of Cdc25 from the extract. The removal

of Cdc25 resulted in the inactivation of Cdc2/cyclin B, providing independent evidence that Cdc25 activity is required not only for the initial activation of Cdc2/cyclin B, but for maintenance of its activity (Figure 7C).

Although Cdc25 depletion by Pin1 beads could account for the inability of depleted mitotic extracts to maintain Cdc2/cyclin B activity, Cdc25 was not removed from interphase extracts by Pin1 beads and thus its removal did not explain the failure of exogenous cyclin B to activate Cdc2/cyclin B in extracts depleted of Pin1-binding proteins. Since Plx1 bound to Pin1 beads during interphase, we tested whether active, baculovirus-produced Plx1 could complement extracts depleted of Pin1-binding partners, restoring the ability to respond to cyclin B. Although the Plx1 we added could phosphorylate Cdc25 *in vitro* (data not shown), its addition to Pin1 bead-depleted interphase extracts did not restore the extract's ability to respond to cyclin, as assessed by observation of nuclear morphology (Figure 7D and E) or measurement of histone H1 kinase activity (data not shown). These data make it unlikely that Plx1 is the Pin1-binding partner whose removal makes interphase extracts refractory to cyclin B addition. However, it is formally possible that a particular subpopulation of Plx1, which cannot be replaced by recombinant Plx1, is specifically depleted by the Pin1 beads.

In order to determine if the ability of Pin1 to interact with Cdc25 and Plx1 was evolutionarily conserved, we incubated Pin1 beads with extracts from HeLa cells arrested in mitosis with nocodazole or in S-phase with aphidicolin. As seen with *Xenopus* egg extracts, Pin1 specifically associated with mitotic, but not interphase Cdc25c, although Cdc25c could be immunoprecipitated from both extracts (Figure 7G). Unlike our observations in *Xenopus* egg extracts, we found that while Pin1 beads incubated in extract from 4×10^6 mitotic HeLa cells recovered Plk1, Plk1 was not recovered on Pin1 beads incubated in extract from five times as many S phase-arrested cells (Figure 7H). While in S phase Plk1 is relatively less abundant than in M phase, its enzymatic

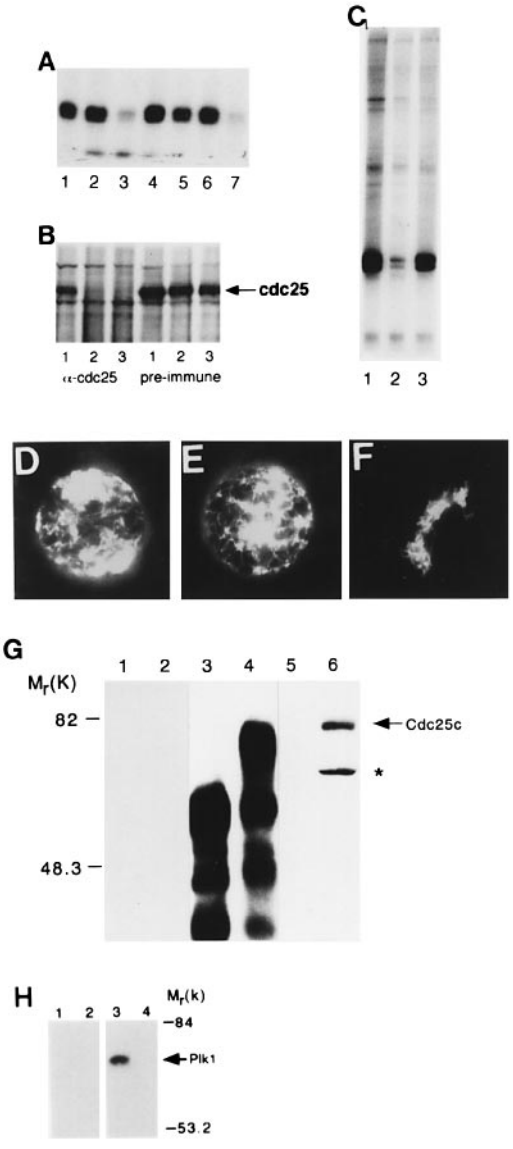

Fig. 7. Depletion of Cdc25 from mitotic extracts can account for the ability of Pin1 beads to deplete Cdc2/cyclin B kinase activity. (**A**) Extracts were depleted of GST or Pin1 binding proteins and assayed for histone H1 kinase activity. Lane 1, mitotic extract; lane 2, mitotic extracts depleted with GST beads; lane 3, mitotic extracts depleted with Pin1 beads; lane 4, mitotic extracts depleted with GST beads and supplemented with recombinant Cdc25 (37 ng/μl); lane 5, mitotic extracts depleted with Pin1 beads and supplemented with recombinant Cdc25; lane 6, mitotic extracts depleted with GST beads and supplemented with recombinant Plx1 (30 ng/μl); lane 7, mitotic extracts depleted with Pin1 beads and supplemented with recombinant Plx1. (**B**) Aliquots of mitotic extract were immunodepleted using protein A–Sepharose linked to either anti-Cdc25 sera or preimmune sera. Aliquots of depleted extract were resolved by SDS–PAGE and immunoblotted with a different anti-Cdc25 sera. It is evident that, after one round of immunodepletion, a small amount of Cdc25 remained in the extract (lane 1). However, multiple depletions (lanes 2 and 3, left side of panel), resulted in complete removal of Cdc25 by anti-Cdc25, but preimmune sera did not (lanes 1–3, right hand side of panel). (**C**) Histone H1 kinase activity was assayed in mitotic extracts (lane 1), or in mitotic extracts immunodepleted of Cdc25 (lane 2). Extracts depleted three times were used. Depletion with preimmune sera did not markedly affect histone H1 kinase activity (lane 3). (**D**) Nuclei were formed in interphase extracts depleted of Pin1-binding proteins. Addition of recombinant GST–cyclin B did not induce mitosis. (**E**) Extracts treated as in (D) were supplemented with recombinant Plx1 protein, which did not restore the ability of GST–cyclin B to induce mitosis. (**F**) Nuclei formed in extracts depleted of GST-binding proteins entered mitosis within 30 min of GST–cyclin B addition. (**G**) Phosphorylated human Cdc25c associates with Pin1 beads. GST or Pin1 beads were added to extracts from nocodazole or aphidicolin-treated HeLa cells. After 1 h incubation at 4°C, the beads were washed extensively, bound proteins were denatured in SDS sample buffer and were subjected to SDS–PAGE. To establish that it was present in both extracts, Cdc25c was immunoprecipitated with a rabbit polyclonal antibody specific for Cdc25c. After transfer to Immobilon P membrane, the Western blot was probed with rabbit anti-Cdc25c and horseradish peroxidase-conjugated protein A. Lanes 1, 2 and 3 contain proteins from 2×10^7 aphidicolin-treated cells which associated with GST beads or Pin1 beads, or were immunoprecipitated with anti-Cdc25c, respectively. Lanes 4, 5 and 6 contain proteins from 2×10^7 nocodazole-treated cells which were immunoprecipitated with anti-Cdc25 sera or were associated with GST beads or Pin1 beads, respectively. The asterisk (*) indicates a protein that is recovered specifically on Pin1 beads and may represent a proteolytic fragment of phosphorylated Cdc25c or Cdc25c phosphorylated at a single or limited number of sites which may be relevant for Pin1 binding, and is not hyperphosphorylated to the level that can occur in the presence of microcystin. (**H**) Human Plk1 binds to Pin1 in mitotic cell extracts. GST (lanes 2 and 4) or Pin1 beads (lanes 1 and 3) (20 μl) were incubated in extracts from 2×10^7 S phase cells (arrested with aphidicolin) (lanes 1 and 2) or 4×10^6 mitotic cells (arrested with nocodazole) (lanes 3 and 4). Proteins retained on the beads were separated by SDS–PAGE and transferred to Immobilon P membrane. The blot was probed with a monoclonal antibody against human Plk1.

activity increases disproportionately relative to the increase in protein as cells enter mitosis (Golsteyn *et al.*, 1995; Hamanaka *et al.*, 1995; Lee *et al.*, 1995). The relative increase in Plk1 activity appears to coincide with phosphorylation. It is possible that post-translational modification of Plk1 may influence its interaction with Pin1, and this regulation may differ between somatic cells and eggs. However, it is clear that Pin1 can interact with the same mitotic regulators in human cells that we identified as Pin1-interactors in *Xenopus* egg extracts.

Pin1 interacts selectively with the phosphorylated form of Cdc25

In mitotic egg extracts, virtually all of the Cdc25 is present in a hyperphosphorylated, activated form, a state that is visualized as a decrease in mobility on SDS–polyacrylamide gels (the apparent M_r shifts from 70 kDa in interphase to >90 kDa in mitosis) (Kumagai and Dunphy, 1992). Given the lack of Cdc25 binding in an interphase extract, it was possible that either Pin1 could not bind to the less active, interphase form of Cdc25 or that factors present in the mitotic extract, but absent from the interphase extract, mediated the Pin1–Cdc25 interaction. In order to determine whether all of the conditions required for association of Cdc25 and Pin1 were present in interphase extracts, we exploited the previously published observation that treatment of interphase extracts, which are completely lacking in cyclin B, with phosphatase inhibitors (e.g. okadaic acid or microcystin) results in the hyperphosphorylation and activation of Cdc25 phosphatase (Kumagai and Dunphy, 1992; Izumi and Maller, 1995). Microcystin-treated interphase extracts were incubated with either GST beads or Pin1 beads, the beads were washed, pelleted and the bound proteins were resolved by SDS–PAGE. Immunoblotting with anti-Cdc25 sera revealed that Pin1 could deplete 'artificially' active Cdc25 from an interphase extract, revealing that Pin1 preferentially interacts with the hyperphosphorylated form of the Cdc25 phosphatase (Figure 8A). In support of this, it appears that Pin1 associates preferentially with the phosphorylated form of human Cdc25c (Figure 7G).

Phosphorylation converts Cdc25 into a Pin1 interactor

To determine if Cdc25 could bind to Pin1 in the absence of other components from the egg extract, we attempted to convert *in vitro* translated, unphosphorylated *Xenopus* Cdc25 into a Pin1 binding partner by phosphorylating the

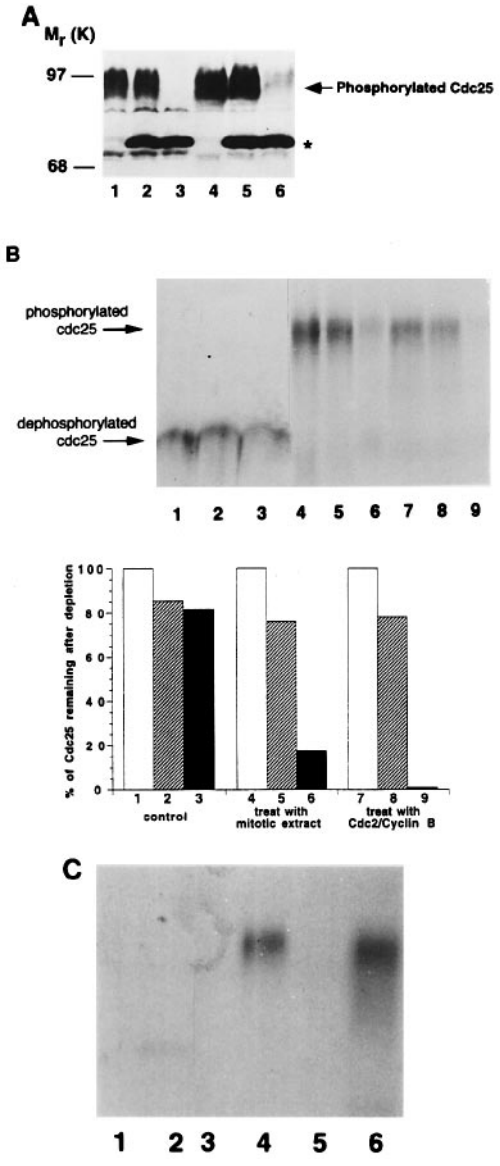

Fig. 8. Pin1 binds to the phosphorylated form of Cdc25. (**A**) Interphase or mitotic extracts treated with 5 μM microcystin were depleted with either GST or GST–Pin1 beads using the same method as in Figure 6A. Extract samples (5 μl) were resolved by SDS–PAGE and processed for Western blotting with anti-Cdc25 sera. Lane 1, interphase extract treated with 5 μM microcystin; lane 2, interphase extract treated with 5 μM microcystin and depleted with GST beads; lane 3, interphase extract treated with 5 μM microcystin and depleted with GST–Pin1 beads; lane 4, mitotic extract treated with 5 μM microcystin; lane 5, mitotic extract treated with 5 μM microcystin and depleted with GST beads; lane 6, mitotic extract treated with 5 μM microcystin and depleted with GST–Pin1 beads. The asterisk (*) indicates a contaminating bacterial protein, present in the recombinant GST and GST–Pin1 protein preparations, which cross-reacts with anti-Cdc25 sera. (**B**) (upper panel): Radiolabeled Cdc25, translated *in vitro*, was incubated in buffer, mitotic extract, or with purified Cdc2/cyclin B complexes at 23°C for 20 min. Either GST or Pin1 beads were added to these reactions and incubated at 4°C for 60 min. Beads were washed thoroughly and processed for SDS–PAGE and autoradiography. Lane 1, input ^{35}S-labeled Cdc25; lane 2, ^{35}S-labeled Cdc25 remaining in buffer after depletion with GST beads; lane 3, ^{35}S-labeled Cdc25 remaining in buffer after incubation with Pin1 beads; lane 4, input ^{35}S-labeled Cdc25 added to mitotic extracts; lane 5, ^{35}S-labeled Cdc25 remaining in mitotic extracts after depletion with GST beads; lane 6, ^{35}S-labeled Cdc25 remaining in mitotic extracts after depletion with Pin1 beads; lane 7, ^{35}S-labeled Cdc25 treated with Cdc2/cyclin B; lane 8, cyclin B/Cdc2-treated ^{35}S-labeled Cdc25 remaining after depletion with GST beads; lane 9, Cdc2/cyclin B-treated ^{35}S-labeled Cdc25 remaining after depletion with Pin1 beads. Non-specific protein interactions and adsorption to glutathione–Sepharose were prevented in samples not containing egg extract by addition of heat-treated fetal bovine serum. (Lower panel): Relative levels of Cdc25 in lanes 1–9 of part (B). Numbering corresponds to lane numbers in (B). White boxes: undepleted; cross-hatched boxes: GST-depleted; black boxes: Pin1-depleted. (**C**) Cdc25 associated with GST or Pin1 beads after incubation in extracts, as described in part (B). Lane 1, ^{35}S-labeled Cdc25 in buffer bound to GST beads; lane 2, ^{35}S-labeled Cdc25 in buffer bound to Pin1 beads; lane 3, ^{35}S-labeled Cdc25 incubated in mitotic extracts and bound to GST beads; lane 4, ^{35}S-labeled Cdc25 incubated in mitotic extracts and bound to Pin1 beads; lane 5, ^{35}S-labeled Cdc25 treated with Cdc2/cyclin B and bound to GST beads; lane 6, ^{35}S-labeled Cdc25 treated with Cdc2/cyclin B and bound to Pin1 beads.

Cdc25 *in vitro*. Conversion of *in vitro* translated Cdc25 (which co-migrates with interphase Cdc25 on SDS–PAGE) into a form which co-migrates with mitotically phosphorylated Cdc25 can be accomplished by incubating the reticulocyte lysate containing unphosphorylated Cdc25 with catalytically active Cdc2/cyclin B complexes purified from *Xenopus* egg extracts (although the direct phosphorylation of Cdc25 may be catalyzed not only by the added Cdc2/cyclin B, but by other kinases present in the reticulocyte lysate). While unphosphorylated *in vitro* translated Cdc25 did not associate with Pin1 beads, incubation with Cdc2/cyclin B conferred upon Cdc25 the ability to interact with Pin1 (Figure 8B and C). These data suggest that one or more phosphorylation sites on Cdc25 may be required for Pin1 binding. In accordance with this, mutation of the known sites of serine phosphorylation on Cdc25 to alanine greatly reduces the ability of Cdc25 to interact with Pin1, even after incubation of mutant Cdc25 in mitotic extracts (Katharine Winkler and A.R.Means, unpublished observations).

Pin1 binding does not affect the in vitro phosphatase activity of Cdc25

The fact that Pin1 interacts specifically with the active form of Cdc25 raises the question of whether Pin1 binding affects the Cdc25 phosphatase activity. We therefore purified the Cdc25 phosphatase using a baculovirus vector for expression of His-tagged Cdc25 in Sf9 cells. Treatment of the Sf9 cells with okadaic acid prior to lysis resulted in the production of hyperphosphorylated Cdc25, presumably mediated by the action of kinases in the insect cells (Figure 9A). Cdc25 produced in this manner and purified on a nickel chelate column was able to bind Pin1 (Figure 9B).

As an indirect assay of Cdc25 activity, we prepared inactive Cdc2/cyclin B complexes by incubating GST–cyclin B in interphase extracts in the presence of sodium orthovanadate (to inhibit endogenous Cdc25). These complexes were retrieved on glutathione–Sepharose, washed extensively to remove the orthovanadate and incubated with purified active Cdc25 in the presence of either soluble Pin1 or GST. As shown in Figure 9C, Pin1 protein did not specifically interfere with the ability of Cdc25 to stimulate the histone H1 kinase activity of Cdc2/cyclin B (there was an equivalent inhibition of Cdc25 activity by both GST and Pin1 protein at high molar excess of Pin1 and GST, e.g. >25:1, Pin1/GST:Cdc25).

To prepare a suitable substrate for a more direct assay of Cdc25 phosphatase activity, ^{32}P-labeled Cdc2/cyclin B complexes phosphorylated at Thr 14/Tyr 15 were prepared as follows. Recombinant GST–cyclin B, which binds to free Cdc2, was incubated in the cytosolic fraction of a mitotic extract in the presence of unlabeled ATP. This allowed the production of active complexes phosphorylated, but not radiolabeled, on Thr 161. These complexes were purified on glutathione–Sepharose and incubated in buffer with the isolated membrane fraction from an interphase extract and [γ-^{32}P]ATP, which results in radioactive labeling of Tyr 15 and Thr 14 by the Myt1 kinase (Kornbluth *et al.*, 1992). The phosphorylated Cdc2/cyclin B complexes were then incubated with active, baculovirus-produced Cdc25 in the absence or presence of soluble Pin1. As indicated in Figure 9D, even at a 100:1 ratio of Pin1:Cdc25, the phosphatase activity of Cdc25 was unaffected by Pin1 in this assay. This strongly suggests that Pin1 does not affect Cdc25 by direct modulation of its enzymatic activity, a finding which is corroborated by the fact that we are able to detect significant Cdc25 activity on Pin1 beads incubated in mitotic extract (Figure 9E).

Discussion

Cell cycle transitions are largely mediated by a complex series of phosphorylation/dephosphorylation reactions occurring in a tightly regulated manner. Here we report that the peptidyl-prolyl isomerase, Pin1, can impinge upon the regulatory networks controlling entry into mitosis in an *in vitro* cell cycle reconstitution system. Specifically, we have shown that Pin1 associates with two key regulators of Cdc2/cyclin B: Cdc25 and its positive regulator Plx1. These data, coupled with earlier demonstrations that Pin1 overexpression or depletion could affect the regulation of mitosis, raise the possibility that protein isomerization may play a role in regulating cellular factors important for mediating cell cycle transitions.

Phosphorylation and the Cdc25–Pin1 interaction

We have demonstrated that Pin1 can interact with the Cdc2-directed phosphatase Cdc25 only when Cdc25 is phosphorylated. Moreover, phosphorylation of an *in vitro* translated form of Cdc25 by Cdc2/cyclin B conferred the ability to bind Pin1, demonstrating both that Cdc25 can interact directly with Pin1 (Cdc2/cyclin B itself does not appreciably associate with Pin1), and that phosphorylation plays a role in the recognition of Cdc25 by Pin1.

While our manuscript was in preparation, the crystal structure of Pin1 complexed to a model dipeptide substrate (Ala–Pro) was solved (Ranganathan *et al.*, 1997). Structural analysis suggested that Pin1 would favor binding of substrates containing acidic side chains in the residue immediately N-terminal to the Pro, which was confirmed using a series of tetrapeptide substrates in *in vitro* assays of Pin1 PPIase activity. The authors proposed that Pin1 might preferentially isomerize substrates with a phosphoSer or phosphoThr residue at position –1 relative to the isomerized Pro, consistent with their findings that inorganic phosphate inhibited the isomerase activity of Pin1, perhaps by occupying the site on Pin1 normally occupied by the relevant phosphoamino acid. The selective binding of phosphorylated Cdc25 by Pin1, coupled with data from the crystal structure, suggest that phosphoSer/phosphoThr residues created by kinases with a preference for Ser–Pro/Thr–Pro sites might contribute to interaction with binding sites on the Pin1 protein. Indeed we and others have found that the monoclonal antibody, MPM-2, which recognizes mitotically phosphorylated proteins (Davis *et al.*, 1983), can detect a number of mitotically phosphorylated proteins, other than Cdc25, which can interact with Pin1 (J.Yang, D.G.Crenshaw, S.Kornbluth and A.R.Means, unpublished observations; Yaffe *et al.*, 1997).

However, phosphorylation is not required for binding of all proteins to Pin1 since the enzymatically inactive variant of NIMA, NIMAK40M, which was used to isolate Pin1 in our two-hybrid screen, is unphosphorylated when produced in bacteria (Lu *et al.*, 1993), yet can interact

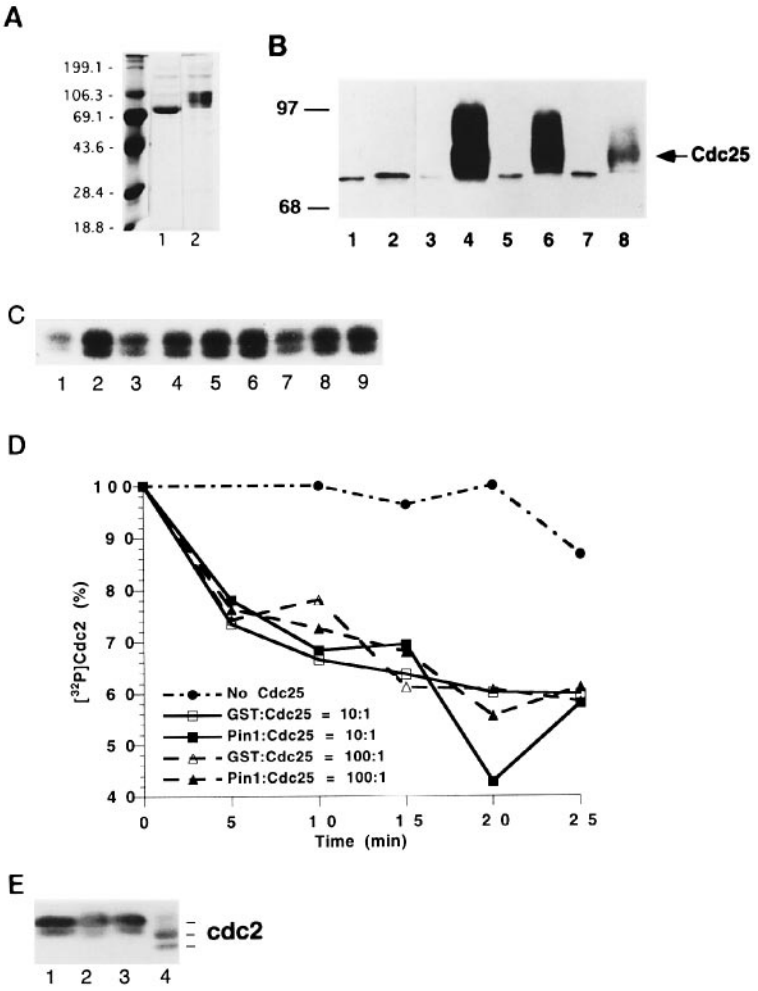

Fig. 9. Pin1 does not suppress the phosphatase activity of Cdc25. (**A**) Cdc25 purified from Sf9 cells infected with baculovirus expressing Cdc25 protein. Lane 1, Cdc25 protein purified from untreated Sf9 cells; lane 2, Cdc25 purified from Sf9 cells treated with okadaic acid prior to harvesting and lysis of the cells. (**B**) An active form of Cdc25 was purified from Sf9 cells by treating cells with okadaic acid to drive them into a state resembling mitosis. Varying concentrations of Cdc25, mixed with 25 μg/μl BSA in mitotic extract buffer containing 1 μM microcystin were incubated with either GST or Pin1 beads for 1 h at 4°C. Then beads were washed and processed for SDS–PAGE and Western-blotted with anti-Cdc25 sera. Lane 1, GST beads; lane 2, Pin1 beads; lane 3, GST beads after incubation with 27 μg/ml Cdc25; lane 4, Pin1 beads after incubation with 27 μg/ml Cdc25; lane 5, GST beads after incubation with 9 μg/ml Cdc25; lane 6, Pin1 beads after incubation with 9 μg/ml Cdc25; lane 7, GST beads after incubation with 3 μg/ml Cdc25; lane 8, Pin1 beads after incubation with 3 μg/ml Cdc25. (**C**) Pin1 does not specifically inhibit Cdc25 activation of Cdc2/cyclin B. Inactive Cdc2/GST–cyclin B complexes were purified from *Xenopus* interphase extracts on glutathione–Sepharose in the presence of sodium orthovanadate. The complexes were assayed for histone H1 kinase activity in the absence (lane 1) or presence (lanes 2–9) of 400 ng of baculovirus-expressed *Xenopus* Cdc25 isolated from okadaic acid-treated Sf9 cells. Cdc25 was preincubated with 60, 12, 2.4 and 0.48 μg of Pin1 (lanes 3, 4, 5 and 6, respectively), 60, 12 and 2.4 μg of GST (lanes 7, 8, and 9, respectively), or Pin1 dilution (XB) buffer (lanes 1 and 2). (**D**) Pin1 does not affect the ability of Cdc25 to dephosphorylate Cdc2 on Thr 14/Tyr 15. Active Cdc25 was purified from Sf9 cells as described above. GST–Cyclin B was incubated in mitotic extracts and the resulting active Cdc2/cyclin B complex was retrieved on glutathione–Sepharose beads (note that this complex should be phosphorylated on Thr 161 with unlabeled phosphate). This Cdc2/cyclin B complex was then labeled with ^{32}P on Thr 14 and Tyr 15 by incubation with interphase membranes in the presence of 5 mM vanadate. The ^{32}P-labeled Cdc2/cyclin B complex was washed extensively to remove vanadate and incubated for the indicated times in buffer control (closed circles), Cdc25 with a 10-fold molar excess of GST protein (open square), Cdc25 with a 10-fold molar excess of Pin1 protein (closed square), Cdc25 with 100-fold molar excess of GST protein (open triangle), Cdc25 with a 100-fold molar excess of Pin1 (closed triangle). The dephosphorylation rate was measured by the decrease of the ^{32}P content of Cdc2 with Phosphorimager after SDS–PAGE. (**E**) Cdc2 phosphorylated at Thr 14 and Tyr 15 was prepared by incubation of GST–cyclin B in interphase extracts containing sodium orthovanadate. The Cdc2/cyclin B complexes were retrieved on glutathione–Sepharose, washed extensively, and eluted with glutathione. The phosphorylated Cdc2/cyclin B complexes were then incubated with GST beads (lane 1), Pin1 beads (lane 2), GST beads pre-incubated with active recombinant Cdc25 and then washed (lane 3), and Pin1 beads pre-incubated with active recombinant Cdc25 and then washed (lane 4).

with Pin1 *in vitro* (data not shown). These data suggest that Pin1 may have at least two distinct modes of protein interaction: one phosphorylation-dependent and one phosphorylation-independent. Since neither the WW domain nor the PPIase domains alone could arrest the cell cycle *in vitro*, it is likely that both phosphorylation-dependent and -independent Pin1 protein interactions mediated by these separate domains are required for intact Pin1 protein to affect the cell cycle.

Pin1 and regulation of cell cycle progression

The ability of Pin1 beads to deplete an activity required for the maintenance of Cdc2/cyclin B activity in mitotic extracts could be accounted for by the quantitative deple-

tion of Cdc25 from these extracts. Indeed, quantitative removal of Cdc25 from mitotic extracts by immunodepletion also resulted in inactivation of Cdc2/cyclin B. These data suggest that the basal level of Myt1 and/or Wee1 in mitotic extracts is sufficient to rephosphorylate Cdc2/cyclin B when Cdc25 is absent. It is interesting to note that depletion of the isolated cytosolic fraction of mitotic extracts with Pin1 beads does not result in suppression of histone H1 kinase activity (data not shown). Rather, re-addition of membrane to the depleted cytosol is required for full suppression of histone kinase activity, suggesting that Myt1 is needed to maintain Cdc2/cyclin B in an inactive state.

Although Cdc25 removal can account for the effect of depleting Pin1-binding partners from mitotic extracts, Cdc25 is not removed from interphase extracts treated in the same manner. Despite the fact that *Xenopus* Plx1 can interact with Pin1 in interphase, it is not quantitively depleted from these extracts and re-addition of recombinant, active Plx1 to Pin1 bead-depleted interphase extracts does not restore the ability of depleted extracts to respond to cyclin B. Like mitotic extracts depleted of Pin1-binding partners, interphase extracts depleted of Pin1 interactors are refractory to cyclin B1 addition only when membrane is present (data not shown). Since Myt1 should be fully active during interphase, this raises the intriguing possibility that a negative regulator of Myt1 is removed in association with Pin1 beads. Definitive evidence of this will require further experimentation.

Pin1 and Cdc25 regulation

Our data suggest that Pin1 does not inhibit entry into mitosis through direct inhibition of Cdc25 phosphatase activity. Although Pin1 could bind to and remove all of the Cdc25 from mitotic extracts, it did not inhibit the Cdc2-directed Cdc25 phosphatase activity, at least as could be measured *in vitro*. The lack of *in vitro* modulation of enzymatic activity cannot be taken as evidence against the hypothesis that Pin1 exerts its effects, at least in part, through modulation of Cdc25 function. This is highlighted by the fact that the active NIMA kinase is toxic to yeast, yet yeast expressing NIMA can be rescued by Pin1 expression, presumably through negative regulation of NIMA. However, Pin1-bound NIMA is not grossly impaired in its *in vitro* catalytic activity (Lu *et al.*, 1996).

Recently, it has been reported that phosphorylation of Cdc25 at Ser 216 creates a binding site for a 14-3-3 protein (Peng *et al.*, 1997). This phosphorylation, catalyzed by the kinase chk1, appears to play a role in the G_2 checkpoints activated in response to DNA damage or incomplete replication, and mutants which prevent phosphorylation at this site cause override of checkpoint-induced G_2 arrest (Furnari *et al.*, 1997; Peng *et al.*, 1997; Sanchez *et al.*, 1997). Like Pin1 binding, phosphorylation of Cdc25 and consequent binding of 14-3-3 protein does not appear to affect the phosphatase activity of Cdc25, yet the cell cycle is profoundly affected by these events. We would propose, as has been proposed for the 14-3-3/Cdc25 interaction, that Pin1 may alter the subcellular localization of its binding partners or may act by sequestering Cdc25 at appropriate times in the cell cycle or in response to activation of particular cell cycle checkpoints. It is also possible that Pin1 binds Cdc25 phosphorylated at a specific site and prevents its further phosphorylation and activation until all requirements for mitotic entry are met or in some other way prevents conversion of Cdc25 from an interphase to mitotic state. Given the fact that Pin1 seems to be a nuclear protein (Lu *et al.*, 1996), it may serve a specific role in modulating Cdc25 dynamics within the nucleus. These possibilities offer many avenues for future investigation.

The original isolation of Pin1 as a NIMA interactor, coupled with its ability to impinge upon mitotic regulation, suggested that NIMA might be the physiological target of Pin1. Although isolation of a *Xenopus* Pin1 homolog and its removal from extracts will be necessary to demonstrate definitively that Pin1 physiologically regulates mitosis in this system, our data reveal that mitotic regulators other than NIMA are also Pin1-binding proteins, and these interactions could well account for the observed effects of Pin1 overexpression or depletion in the human and yeast systems.

It is not yet clear whether a NIMA-like pathway for regulating mitosis exists in vertebrate cells. Conceivably, Pin1 may represent a conduit between Cdc2 and NIMA-like pathways in all cells. Alternatively, NIMA may represent a more recent evolutionary specialization that capitalized on a pre-existing Pin1-dependent pathway to regulate mitosis in *A.nidulans*. Data presented here suggest that Pin1 may modulate mitosis through direct interaction with Cdc2/cyclin B regulators. Like these evolutionarily conserved cell cycle regulators we have identified as Pin1 interactors, Pin1 has been conserved from yeast to man, suggesting that interactions between Pin1- and Cdc2-dependent pathways of mitotic regulation may be part of an evolutionary ancient pathway which is universally important for regulating mitosis.

Materials and methods

Two-hybrid screen

All vectors, control plasmids, the *A.nidulans* two-hybrid library and yeast strain YRG-2 were from Stratagene. The entire open reading frame of kinase inactive (lysine 40 to methionine mutation) *A.nidulans nim*A was inserted into pBD-GAL4 in-frame with the DNA binding domain of Gal4 resulting in the bait plasmid, pBD-NIMAK40M. This plasmid, which carries the *TRP1* gene for selection in yeast, was co-transformed with an *A.nidulans* cDNA two-hybrid library in pAD-GAL4 (which carries *LEU2*) into yeast (strain YRG-2; ***Mat***a *ura3-52 his3-200 ade2-101 lys2-901 leu2-3 112 gal4-542 gal80-538 LYS2::GAL1*$_{\mathrm{UAS}}$-*GAL1*$_{\mathrm{TATA}}$-*HIS3 URA3::GAL4*$_{17\text{-mers}(\times 3)}$-*CYC1*$_{\mathrm{TATA}}$-*lacZ*). The yeast were plated onto selective media lacking tryptophan, leucine and histidine and, after 7–10 days, His^+ colonies were replated onto the same selective plates. Co-transformants were assayed for β-galactosidase expression using a colony lift assay performed according to the Stratagene manual, except that Whatman #5 filters were used. Blue colonies were replated and the β-galactosidase assays were repeated. Library plasmids isolated from β-galactosidase-expressing, His^+ yeast were recovered and used to retransform YRG-2. These isolates were mated with yeast strain Y187 (***Mat***α *ura3-52 his3-200 ade2-101 trp1-901 leu2-3 112 gal4*Δ, *met*$^-$ *gal80*Δ *URA3::GAL1*$_{\mathrm{UAS}}$-*GAL1*$_{\mathrm{TATA}}$-*lacZ*) transformed with the empty pBD-GAL4 vector, pBD-NIMAK40M, or the control plasmids p53 or plaminC. The library clones, from those strains that were viable on minus leucine, tryptophan and histidine plates only in the presence of the library plasmid and pBD-NIMAK40M, were analyzed further.

Preparation of Xenopus egg extracts

Interphase egg extracts, mitotic extracts and demembranated sperm chromatin were prepared as previously described (Smythe and Newport, 1991). Cycling extracts that oscillate between S phase and mitosis were prepared from electrically activated eggs according to the protocol of

Murray and Kirschner (1989) and incubated at room temperature in the presence of sperm chromatin (500/μl extract). For visual monitoring of nuclear envelope breakdown and chromosome condensation, 1.5 μl samples were withdrawn at 15 min intervals and diluted 1:1 with 10 μg of Hoechst 33258 per ml in 37% formaldehyde and examined by fluorescence microscopy.

Production of GST and Pin1 proteins

The bacterial strain BLR1(DE3)pLysS (Novagen) was transformed with the pGEX-2T vector (Pharmacia) or the *A.nidulans* Pin1 cDNA in pGEX-2T. For production of the isolated WW domain, amino acids 1–41 of *A.nidulans* Pin1 were fused in-frame to the C-terminus of GST. For production of the PPIase domain, amino acids 46–176 of Pin1 were fused to the C-terminus of GST. Using PCR, a stop codon was inserted after the final amino acid of the WW domain and clones were inserted into pGEX-2T. The recombinant fusion proteins were expressed and purified as described (Solomon *et al.*, 1990). For cleavage of the resultant GST–Pin1 fusion proteins with thrombin, ~2.5 μg GST–Pin1 protein on the glutathione–Sepharose beads was incubated with 10 U of thrombin (Sigma) at room temperature for 1 h in thrombin cleavage buffer (50 mM Tris pH 8.0, 150 mM NaCl, 2.5 mM $CaCl_2$, and 0.1% β-mercaptoethanol). After cleavage, the supernatant was incubated with aminobenzamidine beads at 4°C for 1 h to allow thrombin removal by binding to the beads. After pelleting of the beads, the supernatant was diluted into XB buffer (100 mM KCl, 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM K-HEPES pH 7.7 and 50 mM sucrose) and concentrated by centrifugation in a Centricon 10 microconcentrator. Proteins were aliquoted and stored at –80°C.

Production of His-tagged Cdc25 protein and sea urchin cyclin B1 GST-fusion protein

Recombinant baculovirus containing histidine-tagged versions of the full-length XCdc25 protein was kindly provided by Dr W.Dunphy. Active XCdc25 proteins were expressed by treating cells with 100 nM okadaic acid for 3 h after 46 h infection and purified as described by Kumagai and Dunphy (1996). A plasmid encoding a nondegradable sea urchin cyclin B1 (missing 13 N-terminal amino acids) fused to GST was kindly provided by Dr C.Smythe and purified according to previously described methods (Solomon *et al.*, 1990).

Histone H1 kinase assays

To assay the histone H1 kinase activity, 2 μl aliquots of extracts were withdrawn at 15 min intervals into 2 μl EB buffer (80 mM β-glycerophosphate pH 7.3, 20 mM EGTA and 15 mM $MgCl_2$), frozen immediately in liquid nitrogen and stored at –80°C. Histone H1 kinase activity was assayed as described previously (Kornbluth *et al.*, 1994).

Depletion experiments

GST or GST–Pin1 fusion proteins were coupled to glutathione–Sepharose beads. Beads were blocked with 10 mg/ml BSA in interphase or mitotic extract buffer, or fetal bovine serum. For extract depletions, beads were diluted 1:10 into interphase or mitotic extracts and rotated at 4°C for 1 h. Beads were pelleted by centrifugation and washed three times with interphase or mitotic extract buffer before Western blotting or kinase assays.

Preparation of differently phosphorylated forms of the Cdc2/cyclin B complex

Cdc2/cyclin B complexes dephosphorylated at Tyr 15 and Thr 14 were prepared by incubating sea urchin GST–cyclin B and an ATP-regenerating cocktail with the cytosolic fraction of interphase extracts at room temperature for 1 h. GST–cyclin B/Cdc2 complexes were retrieved by incubation with glutathione–Sepharose and the beads were washed throughly prior to use. The inactive form of the Cdc2/cyclin B complex was prepared by incubating GST–cyclin B and ATP-regenerating cocktail with both the cytosolic and membrane fractions of interphase extracts in the presence of sodium orthovanadate to inhibit Cdc25. Complexes were then bound to glutathione–Sepharose as above.

In vitro translation of Xenopus Cdc25 protein and binding to Pin1 beads

For *in vitro* transcription and translation, a *Xenopus* Cdc25 cDNA cloned into pBluescript was incubated for 2 h at 30°C in a TNT Coupled Reticulocyte Lysate System (Promega) in the presence of ^{35}S Trans-label (ICN Pharmaceuticals, Covina, CA). Boiled fetal bovine serum was used as a blocking agent to prevent non-specific interactions of the *in vitro* translated Cdc25 with GST or GST–Pin1 Sepharose beads.

HeLa cell extracts

Exponentially growing HeLa cells were treated for 17 h with nocodazole (Sigma, 50 ng/ml final concentration) or aphidicolin (Sigma, 5 μg/ml final concentration) to arrest exponentially growing HeLa cells in prometaphase or S phase of the cell cycle. The cells were shaken or trypsinized off dishes, collected by low speed centrifugation and washed in PBS. Cell lysis was achieved by the addition of 1 ml of modified RIPA buffer (containing 50 mM Tris–Cl pH 8.0, 1% NP-40, 150 mM NaCl, 1 mM DTT, 0.1 μg/ml Pefabloc SC, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 5 μg/ml pepstatin A and 10 μM microcystin) per 2×10^7 cells, incubation on ice for 30 min and brief sonication. The cell lysate was centrifuged at 90 700 *g* for 20 min at 4°C and 1 ml of supernatant was subsequently added to 20 μl glutathione–Sepharose (Pharmacia) coupled to either GST or GST–Pin1. The slurry was rocked for 1 h at 4°C, and the beads were pelleted gently and washed with modified RIPA buffer. Bound proteins were released by boiling in SDS sample buffer, separated on a 10% SDS–polyacrylamide gel and transferred to Immobilon P membrane (Millipore). These Western blots were probed with rabbit polyclonal antibody specific for Cdc25c (Santa Cruz) or monoclonal antibody to human Plk1 (generously provided by Dr Erich Nigg).

Assay for potential inhibition of Cdc25 by Pin1

Baculovirus-expressed, hexahistidine-tagged *Xenopus* Cdc25 (400 ng), purified from okadaic acid-treated Sf9 cells, was preincubated for 12 min at room temperature with Pin1, GST or dilution buffer. Kinase reaction mixture, containing [γ-^{32}P]ATP, was added and this mixture was transferred to 'inactive' Cdc2/GST–cyclin B complexes that were formed in interphase extracts in the presence of sodium orthovanadate, recovered on glutathione–Sepharose (Pharmacia), washed with kinase buffer, and diluted ~16-fold by mixing with fresh glutathione–Sepharose. After a further 15 min at room temperature, 1 μg histone H1 was added to initiate the kinase reaction which proceeded for 10 min at room temperature. The reaction was terminated with an equal volume of 2× SDS sample buffer and boiling, and the reactions were then electrophoresed on a 12% SDS–polyacrylamide gel, stained and destained, dried and exposed to Kodak X-OMAT AR film. To assay for the potential inhibition of Cdc2/cyclin B by Pin1, the assay proceeded as above, except that 'active' Cdc2/GST–cyclin B complexes, isolated in the absence of vanadate, were assayed. Cdc25 was not added, except in a control reaction.

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