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TITLE: Elucidating cdc25's Oncogenic Mechanism in Breast Cancer Using Pin1, a Negative Mitotic Regulator

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The current study demonstrates that Pin1 functions in the control of the G2/M transition in *Xenopus laevis* egg extracts. We show that extracts depleted of Pin1 protein enter mitosis prematurely, a phenotype which is dramatically potentiated under circumstances in which the replication checkpoint has been triggered. The replication checkpoint functions as a failsafe mechanism which enables the postponement of the entry into mitosis in response to unreplicated DNA; in this system the extent of the delay correlates with the amount of chromatin added to the extract. Extracts from which Pin1 has been quantitatively depleted undergo the G2/M transition with rapid kinetics regardless of DNA concentration, while control extracts display a robust checkpoint response triggered by the presence of unreplicated DNA.
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Abstract

The current study demonstrates that Pin1 functions in the control of the G2/M transition in *Xenopus laevis* egg extracts. We show that extracts depleted of Pin1 protein enter mitosis prematurely, a phenotype which is dramatically potentiated under circumstances in which the replication checkpoint has been triggered. The replication checkpoint functions as a failsafe mechanism which enables the postponement of the entry into mitosis in response to unreplicated DNA; in this system the extent of the delay correlates with the amount of chromatin added to the extract. Extracts from which Pin1 has been quantitatively depleted undergo the G2/M transition with rapid kinetics regardless of DNA concentration, while control extracts display a robust checkpoint response triggered by the presence of unreplicated DNA.
Introduction

Cancerous growth is fundamentally dependent on the escape from normal cell cycle control. A group of highly conserved proteins cooperate to regulate cell cycle transitions by ensuring high fidelity replication and subsequent compartmentalization of the genome. We are studying the mechanism of action of a novel cell cycle regulator, Pin1, which influences the progression from G2 to M phase. Pin1 consists of an N-terminal WW domain (a protein interaction module with affinity for poly-proline containing sequences) and a C-terminal peptidyl-prolyl isomerase domain. It is found in all eukaryotes, and the budding yeast homolog, ess1, is essential; deletion of the gene results in mitotic arrest. Although it has been reported that Pin1’s WW domain preferentially binds to poly-proline motifs which contain phosphorylated serine or threonine, and that its isomerase activity is similarly directed toward prolines preceded by phospho-serine/threonine, the biological function of Pin1 in vertebrates remains obscure. At the time the project was proposed, the affinity of each of Pin1’s domains for phospho S/T-P sequences had not yet been recognized. We had made the observation that several mitotically phosphorylated proteins from Xenopus extracts bound Pin1. Among these, cdc25 was of particular interest because of its role as a mitotic initiator in activating the mitosis promoting factor (MPF), cdc2/cyclin B, and the correlation of its upregulation in breast cancers with worsened prognoses. Furthermore, the mitotic cell cycle defect which occurs in extracts which have been depleted of Pin1 binding proteins is complemented by the addition of recombinant phosphorylated cdc25. These observations were the premise for my proposed project of investigating cdc25’s oncogenic role in breast cancer using Pin1 as a novel molecular probe.
Initially the investigation of cdc25’s oncogenic role using Pin1 required that I understand to the extent possible the biological nature of Pin1’s own function to allow interpretation and synthesis of any prior and subsequent observations pertaining to cdc25. This was the goal of my first technical objective, and the results are summarized below.

In order to use *Xenopus* egg extracts as a biochemically system amenable to studying cell cycle transitions, I first cloned the frog Pin1 homologue (xPin1). Three clones were isolated by low stringency screening of a *Xenopus* embryonic cDNA library using the human PinI cDNA sequence as a probe. Each insert contained a single identical open reading frame. The predicted polypeptide sequence shares 89% identity with human Pin1 and greater than 45% identity with each of the other family members over its full length of 159 residues (Fig. 1A). Typical of this recently identified eukaryotic class of parvulin prolyl isomerases, the *Xenopus* Pin1 (xPin1) has both an N-terminal WW domain and a C-terminal prolyl isomerase (PPIase) catalytic domain.

To elucidate the biological function of Pin1 in cell cycle progression, we removed the protein from *Xenopus* extracts by immunodepletion. To do this we purified GST-xPin1 fusion protein from bacteria by affinity chromatography using GSH sepharose, and eluted xPin1 by cleavage of the fusion protein with thrombin (Fig. 1B). This recombinant (and PPIase active) xPin1 was used to generate polyclonal antiserum which recognizes a single polypeptide of 18 kDa in *Xenopus* extract (Fig. 1C). Using recombinant xPin1 as a standard, we estimate that the endogenous Pin1 concentration in extracts is 20 ng/μL, and that it does not vary in quantity during the cell cycle and the protein itself is extraordinarily stable. Even extracts manipulated so as to undergo cell cycle transitions in the presence of the protein synthesis inhibitor, cycloheximide, showed no decrease in protein levels over the course of the assay, and injection of antisense Pin1 into *Xenopus* oocytes had no effect on the protein levels over a course of several days. Furthermore, addition of γ32P-ATP to cycling extracts did not reveal any phosphate incorporation into Pin1, and there was no apparent cell cycle change in the protein’s migration by SDS-PAGE and immunoblot. Therefore we suspected that Pin1 itself is a constitutive regulator which functions in the cell cycle by responding to or enabling functions in a way which is dependent upon the biochemical climate of the cell. Despite Pin1’s abundance and stability, we were able to quantitatively deplete the endogenous protein in either two or three rounds of immunodepletion with Protein A matrix (silica beads) pre-bound to anti-xPin1 antibody.

The mitotic arrest phenotype of the yeast ess1 deletion strain, also reported for 60% of HeLa cells 72 hours after transfection with anti-sense human Pin1, implies a conserved function for Pin1 in mitotic exit. To test this directly, cytostatic factor arrested egg extracts (CSF extracts) were depleted of Pin1 or mock-depleted (Fig. 2A). In response to calcium addition, CSF extracts ordinarily transition rapidly into interphase, characterized by nuclear formation, chromatin de-condensation and initiation of replication. We found that control extracts and those devoid of Pin1 were equally capable of proceeding through this phase of the cell cycle (Fig. 2, panels B and C). Thus, in this system Pin1 is not required for either mitotic exit or DNA replication.
Another explanation for the previously reported phenotypes is that the mitotic arrest occurs because the cells have inappropriately entered mitosis and therefore lack the cues which allow continued cell cycle progression. To test this hypothesis we removed Pin1 from interphase extracts and compared the cell cycle progression of these extracts with that of mock-depleted control extracts. We consistently observed that the Pin1 depleted extracts were accelerated in their M-phase entry, both by cytological examination and in the activation of Histone H1 kinase activity (Fig. 3, panels A and B). This was true not only in interphase extracts which are treated with cycloheximide necessitating the addition of recombinant cyclin B, but also in cycling extracts which synthesize endogenous cyclin B (Fig. 3C, solid lines). The degree of difference in the timing of mitotic entry between control and Pin1-depleted extracts varied from ten minutes to greater than sixty minutes. We noticed a qualitative correlation between the magnitude of the effect and the concentration of sperm chromatin used in the assay (Fig. 3C, solid vs. dashed lines). Furthermore, in the instances in which the acceleration of mitosis was modest, inactivation of MPF and the transition into interphase occurred normally. However, the Pin1 depleted extracts which more dramatically preempted the regulation of mitotic entry often failed to exit mitosis (Fig. 3C).

Increasing the concentration of DNA lengthens interphase in *Xenopus* extracts; this has been attributed to a G2 checkpoint triggered in response to unreplicated DNA. We postulated that removal of Pin1 from extracts results in a failure of this checkpoint. Because of the ambiguity introduced when manipulating the cell cycle length using chromatin to titrate replication factors, we took advantage of the DNA polymerase inhibitor, aphidicolin, which allowed us to keep the concentration of nuclei in the extracts constant and selectively stall the replication process. In mock-depleted extracts, the presence of unreplicated DNA resulted in delay of mitotic entry of 45 minutes (Fig. 4A, dotted vs. solid line). That this delay was a bona fide replication checkpoint was supported by the observation that caffeine, known to disable the checkpoint upstream of chkl/cds1 kinase activation, restored to aphidicolin-treated extracts the timing of mitotic entry observed for controls (Fig. 4A, dashed line). Like caffeine treatment, depletion of Pin1 from extracts prevented engagement of the checkpoint delay of mitotic entry in response to aphidicolin (Fig. 4A, solid line w/squares).

We were able to attribute the replication checkpoint requirement specifically to Pin1 rather than a co-depleted factor by addition of recombinant xPin1 to depleted extracts. The xPin1 monomer was capable of restoring a functional checkpoint response in Pin1 depleted extracts (Fig. 4B). To achieve the full complementation of the depletion effect observed in this experiment, recombinant xPin1 was added back to roughly five times the endogenous concentration of 20 ng/µL. However, lower concentrations of xPin1 were also effective to lesser degrees in allowing a G2 delay. Because previously we and others had shown that overexpression of Pin1 causes a G2 arrest in various systems, we tested the ability of the xPin1 used in the previous experiment to delay mitosis in an untreated extract. Even at the highest concentrations used in add-back experiments, 100 ng/µL, there was no difference observed between xPin1 and GST treated extracts (Fig. 4C) which precluding the possibility that the re-addition of xPin1 to depleted extracts simply lengthens the cell cycle in a non-specific manner.
Progress through technical objective two is ongoing. Individual phosphorylation site mutations in cdc25 were generated and assessed in their abilities to bind Pin1 before or after incubation with mitotic extract. There was not a single target site which, when removed, resulted in a loss of interaction. In light of the recent recognition that Pin1 binds a number of phospho-peptides, we expect that the cdc25 binding initially observed was dramatic precisely because cdc25 is such a heavily phosphorylated protein in the mitotic extract. Therefore, rather than attempting to further define a Pin1 interaction domain within cdc25, we have mutated the two domains of Pin1 to assess Pin1’s requirements for cdc25 binding. Preliminary indications are that in vitro, a catalytically inactive PPIase mutant retains its ability to bind cdc25, while a mutant in the WW domain loses affinity for the phosphatase. These studies need to be carried out both in extracts and relative to other known Pin1 binding proteins in order to determine whether these are general properties of Pin1’s interactions, or specific to cdc25. We will also attempt to complement the Pin1-depleted extracts with the Pin1 mutants in hope of dissecting the structure-function of Pin1’s checkpoint role.

Because we were unable to show any endogenous association of Pin1 with cdc25, despite many attempts of co-immunoprecipitation, our efforts in this category have been redirected toward elucidating the function of Pin1 itself. In addition to demonstrating a role for Pin1 in the regulation of mitotic initiation, our data establish Pin1 as an essential component of the replication checkpoint signal. When replication is inhibited, the consequences of Pin1 depletion are more likely to be catastrophic to the integrity of the genome. While mock depleted extracts postpone mitotic entry in the presence of unreplicated DNA, Pin1-depleted extracts are incapable of engaging the biochemical brakes and thus lack a functional replication checkpoint. Furthermore, the timing of mitotic entry is similar for Pin1 depleted extracts under all conditions tested and coincides with that of mock depleted extracts treated with caffeine to preempt the checkpoint response to unreplicated DNA, and add-back of xPin1 protein restores a functional checkpoint response to Pin1-depleted extracts. Thus we conclude that Pin1 is required for the appropriate regulation of mitotic entry in a manner responsive to DNA status.

As a novel checkpoint regulator, Pin1 may influence cellular transformation and cancer treatment. Most of the current non-surgical approaches to cancer therapies are rendered less effective (therefore necessitating higher doses) by functional G2 checkpoints such as the replication checkpoint described here or the DNA damage checkpoint. Although we have not yet demonstrated a role for Pin1 in the latter, many of the components of these checkpoints are shared, and it is reasonable to expect that Pin1 might function in both. Thus, if one could pharmaceutically negate Pin1’s function and thereby prevent cells from pausing to preserve genomic integrity, a higher proportion of the cells targeted by the anticancer agents would enter abortive mitoses. Furthermore, disabling the checkpoint in a tissue or cancer-specific manner would specifically sensitize tumor cells to chemotherapeutic agents, allowing lower doses to be used to achieve the same efficacy while sparing other cells of the body which maintain functional checkpoint responses.
Appendix

Key Research Accomplishments

- Cloning of *Xenopus* Pin1
- Purification of recombinant xPin1 protein
- Generation of polyclonal anti-xPin1 antibody
- Demonstration that Pin1 is not required for mitotic exit or DNA replication
- Demonstration that Pin1 does regulate the timing of mitotic entry
- Demonstration that Pin1 is required for the replication checkpoint
**Figure 1.** Identification of the *Xenopus laevis* Pin1 homologue. (A) Sequence alignment of *Xenopus* Pin1 (xPin1) with homologues from human, *Drosophila* (dodo), *Aspergillus nidulans* (PinA), and budding yeast (Essl). Shading indicates identical or similar residues among at least three family members. (B) Purified recombinant xPin1 was purified from bacteria as a GST fusion protein and cleaved away from the GST with thrombin to elute from GSH-sepharose. 1 µg of indicated proteins separated by SDS-PAGE and stained with Coomassie blue. (C) Cleaved xPin1 was used to generate polyclonal antibodies in rabbits. Following precipitation with 50% (NH₄)₂SO₄, the serum was diluted 1:5000 for immunoblots. Here recombinant xPin1 (left lanes) was used as a standard for quantification of the endogenous Pin1 concentration (lane X contains 0.5 µL *Xenopus* extract).
Figure 2. Pin1 is not required for the exit from mitosis in *Xenopus* extracts. (A) Immunoblot of Pin1 remaining in CSF extracts following each stage of immunodepletion. Anti-xPin1 serum or serum from non-immunized rabbits was pre-incubated with Affi-prep Protein A (Biorad) and PBS + 2 mg/mL BSA at 1:4:25 with protein A measured by volume of slurry as purchased. Supernatant was removed and extract exposed to matrix, transferring to a fresh batch after 40 minutes rotating at 4°C. Complete depletion required two or three rounds (1°, 2°, 3°) depending on extract variety. (B) 3° depleted extracts were supplemented with sperm chromatin and ATP regenerating mix and released from CSF arrest with 400 μM CaCl₂. DNA replication was detected by incubating 10μL aliquots of the extract in the presence of α³²P-dCTP for 30 minute intervals. Reactions were then stopped, subjected to proteinase K digestion, separated by agarose gel electrophoresis and visualized by autoradiography. (C) Separate aliquots were removed from the same extracts at various times, fixed and stained with Hoechst, and observed by fluorescence microscopy.
Figure 3. Pin1 functions in the regulation of the G2/M transition. (A) Interphase extracts were depleted of Pin1 (B, inset) and progression of 3° extracts through the cell cycle was monitored (following supplementation with 100 nuclei/µL, ATP regenerating mix, and addition of His6-human cyclin B1) by freezing aliquots at 10 minute intervals and subsequently assessing the histone H1 kinase activity present in each sample. Autoradiography was done to detect labeled substrate. (B) Radioactivity was quantified using a Molecular Dynamics Phosphorimager.
Fig. 3 cont’d. (C) Cycling extracts were depleted of Pin1 (inset), and 2° extracts were supplemented with ATP regenerating mix and demembranated sperm nuclei to 100/μL (solid lines) or 500/μL (dashed lines). Mitotic index was monitored by fluorescence microscopy.
Figure 4. Pin1 is required for the checkpoint arrest in response to unreplicated DNA. (A) Interphase extracts depleted of Pin1 (inset, 2° depletions shown) were supplemented with sperm chromatin (200 nuclei/µL), ATP regenerating mix, and 50 µg/µL aphidicolin (except as indicated, dotted line), in the presence (dashed line) or absence (solid lines) of 5 mM caffeine. Cytology was monitored over time by microscopy following the addition of His6-humanΔcyclinB1 (non-degradable).
Fig 4 cont’d. (B) Recombinant xPin1 restores the checkpoint function. Extracts were depleted of Pin1 (inset, 2° depletions shown) and treated as for A. In one case 100 ng/μL xPin1 was added back to the Pin1-depleted extract (dashed line).
Fig 4 cont’d. (C) Titration of xPin1 into undepleted extract. The timing of mitotic entry of interphase extracts was compared in the presence of increasing concentrations of xPin1 (solid lines) or GST control (dashed line).
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