Isolation and characterization of the Pin1/Ess1p homologue in Schizosaccharomyces pombe

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SUMMARY

Pin1/Ess1p is a highly conserved WW domain-containing peptidyl-prolyl isomerase (PPIase); its WW domain binds specifically to phospho-Ser/Thr-Pro sequences and its catalytic domain isomerizes phospho-Ser/Thr-Pro bonds. Pin1 PPIase activity can alter protein conformation in a phosphorylation-dependent manner and/or promote protein dephosphorylation. Human Pin1 interacts with mitotic phosphoproteins, such as NIMA, Cdc25 and Wee1, and inhibits G2/M progression in Xenopus extracts. Depletion of Pin1 in HeLa cells and deletion of Ess1p in S. cerevisiae result in mitotic arrest. In addition, Pin1/Ess1p play roles in transcription in S. cerevisiae and in mammalian somatic cells. The S. pombe genome sequence has an open reading frame (ORF) that has 47% identity with Pin1. Expression of this ORF rescued the growth defect caused by ess1 deletion in S. cerevisiae, indicating that S. pombe Pin1p is a functional Pin1 homologue. Overexpression of pin1+ in S. pombe caused slow growth and a G1 delay. Deletion of pin1+ (pin1Δ) did not affect cell cycle progression or cell growth, but increased sensitivity to the cyclophilin inhibitor, cyclosporin A, suggesting that cyclophilin family PPIases have overlapping functions with the Pin1p PPIase. Deletion of pin1Δ did not affect the DNA replication checkpoint, but conferred a modest increase in UV sensitivity. Furthermore, the pin1Δ allele caused a synthetic growth defect when combined with either cdc25-22 or wee1-50 but not the cdc24-1 temperature-sensitive mutant. The pin1Δ strain showed increased sensitivity to the PPI1/P2A family phosphatase inhibitor, okadaic acid, suggesting that Pin1p plays a role in protein dephosphorylation as a result of its ability to increase the population of phospho-Ser/Thr-Pro peptide bonds in the trans conformation that is required for P2A-mediated dephosphorylation. Our genetic data also suggest that Pin1p might function as a positive regulator of Cdc25p and Wee1p.

Key words: pin1+, Synthetic growth defect, S. pombe, ESS1, Cyclosporin A, Okadaic acid

INTRODUCTION

Eukaryotic mitotic entry is controlled by Cdc2, a cyclin-dependent kinase (CDK), and cyclin B (Nurse, 1994). Briefly, Cdc2 binds to cyclin B during G2 but remains inactive due to inhibitory phosphorylation of Thr14 and Tyr15 by the Myt1 and Mik1/Wee1 protein kinases. Mitotic entry requires dephosphorylation of these two residues by protein phosphatase, Cdc25. The activity and subcellular localization of Cdc25 are also controlled by phosphorylation. In interphase Cdc25 is sequestered in the cytoplasm in a hypophosphorylated, low activity form that binds 14-3-3 proteins, but becomes hyperphosphorylated and activated, and moves into the nucleus during M phase. In addition to Cdc2/cyclin B, another Ser/Thr protein kinase, NIMA, is involved in controlling the G2/M transition in A. nidulans (Fry and Nigg, 1995).

The human protein Pin1 was identified through a two-hybrid screen as a protein that interacts with A. nidulans NIMA. Pin1 has 45% identity with the S. cerevisiae homologue, Ess1p (Hanes et al., 1989; Lu et al., 1996) and functionally substitutes for Ess1p (Lu et al., 1996), indicating that the Pin1/Ess1p protein and its functions are highly conserved in eukaryotes. Based on sequence conservation, Pin1 contains two recognizable protein domains, a WW domain at the N-terminus and a peptidyl-prolyl cis-trans isomerase (PPIase) domain at the C-terminus. The WW domain is a small 40 residue protein interaction domain (Sudol, 1996). There are four classes of WW domain; three recognize short proline-rich motifs, and the fourth recognizes phosphoserine (pSer) or phosphothreonine (pThr)-proline motifs (Sudol and Hunter, 2000). The Pin1 WW domain and Nedd4 WW2 are members of the fourth group and interact with phospho-Ser/Thr-Pro motifs in several proteins in a phosphorylation-dependent manner (Lu et al., 1999b; Verdence et al., 2000). There are three families of PPIases, the cyclophilins, the FKBP family and the parvulin family, which includes Pin1/Ess1p. PPIase activity can accelerate protein folding and/or transport (Schmid, 1995). Complexes between cyclophilins or FKBP and immunosuppressant drugs, such as cyclosporin A (CsA), sequester the calcium-activated protein phosphatase calcineurin and block antigen-induced proliferation of T cells (Liu et al., 1991).

Recent studies have shown the PPIase activity of Pin1/Ess1p...
is required for cell cycle progression (Lu et al., 1996; Rippmann et al., 2000; Wu et al., 2000). Depletion of Pin1 activity in human tumor cells and deletion of ESS1 in S. cerevisiae result in mitotic arrest (Lu et al., 1996; Rippmann et al., 2000). A Pin1 mutant lacking PPIase activity fails to complement the loss of ESS1 in yeast. Overexpression of Pin1 causes G2 arrest in HeLa cells (Lu et al., 1996), suggesting that Pin1/Ess1p is involved in mitosis. It has also been reported that Pin1 is involved in DNA replication checkpoint control in Xenopus embryos (Winkler et al., 2000). The X-ray crystal structure of Pin1 (Ranganathan et al., 1997) and subsequent biochemical studies have shown that, through the WW domain, Pin1 interacts with many of the MPM-2 antigens in Xenopus mitotic lysates, including Cdc25C (Crenshaw et al., 1998; Shen et al., 1998), Wee1 (Shen et al., 1998) and Myt1 (Wells et al., 1999). Pin1 can also catalyze a conformational change in phosphorylated Cdc25 (Stukenberg and Kirschner, 2001) and stimulate dephosphorylation of Cdc25 by PP2A (Zhou et al., 2000). Pin1 can either inhibit or stimulate the activity of phosphorylated Cdc25C depending on where sites are phosphorylated (Shen et al., 1998; Stukenberg and Kirschner, 2001). An Aro.F/Laro.pS.P.R consensus for phosphopeptide binding to the Pin1 WW domain has been developed using degenerate pSer-containing peptide libraries. This binding consensus is similar to that for MPM-2 binding to phosphoproteins (Yaffe et al., 1997), and also overlaps the target sequence [S/T.P.(X).R/K] for several proline-directed kinases, including the CDKs (Nigg, 1995). The X-ray crystal structure of the Pin1 WW domain bound to a phosphopeptide derived from the RNA polymerase II large subunit C-terminal domain (CTD) has recently revealed the mechanistic basis for WW domain binding to the phospho-Ser/Thr-Pro motif (Verdecia et al., 2000).

In addition to functions in cell cycle control, Pin1 is involved in other biological processes. Pin1 interacts with phosphorylated tau protein in the brain, and can restore its activity to promote microtubule assembly in vitro (Lu et al., 1999a). Pin1 binds the phosphorylated CTD of the largest subunit of RNA polymerase II (Albert et al., 1999; Verdecia et al., 2000) and activates basal reporter gene transcription (Komuro et al., 1999). Ess1p also interacts with the phosphorylated CTD (Morris et al., 1999), and the ptf1 temperature-sensitive ESSI allele shows a defect in pre-mRNA 3′ end processing (Hani et al., 1999), suggesting a general role in transcription. Multicopy suppressor analysis of temperature-sensitive ess1 mutants has revealed that Ess1p plays a role in general transcription/chromatin remodeling (Wu et al., 2000) and in gene silencing via the Sin3p-Rpd3p histone deacetylase (Arevalo-Rodriguez et al., 2000). Pin1 also plays a role in inhibition of endocytic membrane transport during mitosis (Gerez et al., 2000). Pin1-dependent prolyl isomerization is required for dephosphorylation of certain piecepThr.Pro motifs by the PP2A protein phosphatase, including Cdc25C (Zhou et al., 2000). The D. melanogaster homologue of Pin1, dodo, is required for proper dorsalventral patterning of the follicular epithelium during oogenesis, through its ability to bind to and promote ubiquitin-mediated degradation of the CF2 transcription factor following its phosphorylation by ERK MAP kinase (Hsu et al., 2001). Pin1 also physically interacts with phosphorylated NFAT and prevents dephosphorylation of the NFAT transcription factor, which is required for T-cell activation (Liu et al., 2001). Recently, Pin1 has been shown to be overexpressed in breast cancer, and to increase expression of cyclin D1 as a result of its ability to bind to c-Jun phosphorylated at the Ser63/73.Pro motifs in its transactivation domain, and thereby stimulate cyclin D1 promoter activity (Wulf et al., 2001). These observations suggest that Pin1/Ess1p might play an indirect role in cell cycle control, either through effects on the transcription of genes or the activity of proteins required in a general housekeeping sense for growth and cell cycle progression.

Even though Ess1p has been reported to be essential for mitotic progression in S. cerevisiae and in certain human tumor cells (Lu et al., 1996; Rippmann et al., 2000), deletion of the D. melanogaster Pin1/Ess1p homologue, dodo (Maleszka et al., 1996), A. nidulans Pin1 homologue (A. R. Means, personal communication), and the mouse homologue, Pin1 (Fujimori et al., 1999), results in viable organisms, albeit with detectable phenotypes. This suggests that these organisms either have additional Pin1 family genes, or else that they have PPIases that have overlapping functions with Pin1/Ess1p. One goal of our study was to see whether another simple eukaryote, S. pombe, had a homologue of Pin1/Ess1p and, if so, whether this has an essential function.

The S. pombe genome sequencing project has identified an open reading frame (ORF) on chromosome III, which encodes a protein containing both a WW domain and a PPIase domain and which has ~47% identity with human Pin1. Sequence alignment reveals that the conserved and positively charged amino acid residues, which are important for the substrate specificity of Pin1/Ess1p (Ranganathan et al., 1997), are also conserved in the newly identified ORF, indicating that this S. pombe gene, pin1+, is a functional homologue. In light of the remarkable similarity in mitotic regulatory mechanisms between S. pombe and mammalian cells, we investigated the role of pin1+ in cell cycle progression. In this paper, we describe isolation and characterization of pin1+. We have obtained both pin1+ genomic DNA and cDNA clones. Overexpression of pin1+ caused a severe growth defect. Deletion of pin1+ did not cause any significant defects in cell cycle progression but conferred increased sensitivity to the cyclophilin inhibitor, CsA, and protein phosphatase inhibitor, okadaic acid (OA). Furthermore, deletion of pin1+ showed a synthetic growth defect with both cdc25-22 and wee1-50 temperature-sensitive (ts) mutants but not cdc24-1 or pin1Δ, the NIMA homologue. Our data suggest that pin1+ might function as a positive regulator of cdc25+ and wee1+ function and play a role in protein dephosphorylation.

MATERIALS AND METHODS

Yeast strains and genetic methods

All S. pombe strains in this study (Table 1) are derived from the wild-type strain 972 (h+) and 975 (h-). The wild-type S. cerevisiae strain, HKH1, is a derivative of yeast strains SEY6210 (Robinson et al., 1988). Standard genetic techniques and media used for both fission and budding yeast have been described (Alfa et al., 1993; Guthrie and Fink, 1991; Moreno et al., 1991).

The S. pombe pin1+–GFP fusion construct was generated by standard PCR procedures (Sambrook et al., 1989). An XhoI restriction site was created immediately 5′ to the AUG start codon of the pin1+ gene and a SacI site was introduced immediately 5′ to the stop codon.
This PCR fragment was subcloned into the XhoI and SacI restriction sites of the plasmid pSG572, which contains GFP and the nmt transcription terminator, to generate plasmid p97, which encodes a fusion protein with GFP at the C-terminus of Pin1p. The XhoI-SacI DNA fragment from plasmid p97, which contains pin1p-GFP-nmt terminator, was subcloned into the XhoI and SacI sites of the integration vector, pJK210, to generate plasmid p107. For construction of the pin1p-GFP expression allele, plasmid p107 was digested with Nhel and transformed into wild-type strain (Sp1). Transformsants with a Ura+ phenotype, indicative of pin1p-GFP integration, were streaked out for single colonies to generate the pin1p-GFP expression strain, Sp35.

The pin1p overexpression construct was generated by standard PCR procedures (Sambrook et al., 1989). An XhoI restriction site was created immediately 5′ to the AUG start codon of the pin1p gene and an Xmal site was introduced immediately 3′ to the stop codon. This PCR fragment was subcloned into the XhoI and Xmal restriction sites of the overexpression vector pREP3X, which contains the nmt transcription promoter and terminator, to generate plasmid p95. Plasmids pREP3X and p95, respectively, were transformed into wild-type strain (Sp1) to generate the control (Sp13) and overexpression strain (Sp15).

The pin1 deletion construct was generated by standard PCR procedures (Sambrook et al., 1989). In the first PCR reaction, an KpnI restriction site was introduced approximately 1 kb upstream of the AUG start codon of the pin1p gene and an XhoI site was introduced immediately 3′ to the AUG start codon. This PCR fragment was subcloned into the KpnI and SacI restriction sites of the plasmid pAF1 (Ohi et al., 1996), which contains the his3+ gene, to generate plasmid p85. For the second PCR reaction, the forward primer introduced an Xmal site preceding the stop codon of pin1p and the reverse primer created a SacII site 1 kb from the end of the pin1p codon region. The second PCR fragment was inserted into the Xmal and SacII sites of p85 to generate plasmid p87. Thus, the entire coding region of pin1p was replaced by the wild-type his3+ gene. For construction of the pin1 deletion allele, wild-type S. pombe strains with complementary mating types, Sp1 and Sp3, were crossed followed by replica-plating to select for Ade+ cells, indicative of diploid cells. The KpnI-SacI DNA fragment from p87 containing deletion construct was purified from agarose gel and transformed into diploid cells. His+ cells were selected and subjected to tetrad analysis. Spores with a His+ phenotype (Sp23 and Sp25), indicative of pin1 deletion, were streaked out to obtain single colonies and stored in 50% glycerol solution.

For generation of the pin1Δcdc25-22 allele, the pin1 deletion strain Sp25 was crossed to cdc25-22 (Sp62) followed by random spore analysis (RSA). Spores were germinated on YES plates at 25°C and then replica-plated to YES plates and synthetic-histidine plates, which were incubated at 36°C and 25°C, respectively. The spores with a His+ phenotype, indicative of pin1 deletion, that were temperature sensitive, indicative of cdc25-22, were selected to generate a double mutant strain Sp84 (pin1Δcdc25-22). To generate the pin1Δ wee1-50 double mutant Sp88, the pin1 deletion strain Sp25 was crossed to wee1-50 (Sp66) followed by RSA. Spores were germinated on YES plates at 25°C and then replica-plated to YES-phloxin B plates and synthetic-histidine plates, which were incubated at 36°C and 25°C, respectively. The spores with a His+ phenotype, indicative of pin1 deletion, that had a dark pink color, indicative of wee1-50, were selected. To generate the pin1Δ fin1Δ double deletion strain, Sp191, the pin1 deletion strain Sp25 was crossed to fin1Δ (Sp145) followed by RSA. Spores were germinated on YES plates at 25°C and then replica-plated to synthetic-ura and synthetic-histidine plates, which were incubated at 25°C. The spores that had a His+ phenotype, indicative of pin1 deletion, and Ura+, indicative of fin1Δ, were selected.

The S. cerevisiae ESS1 gene was cloned by PCR using genomic

Table 1. Yeast strains

<table>
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<th>S. pombe</th>
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<td>Sp1</td>
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</tr>
<tr>
<td>Sp3</td>
<td>h+ ura4-D18 leu1-32 his3-D1 ade6-M210</td>
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<tr>
<td>Sp13</td>
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</tr>
<tr>
<td>Sp15</td>
<td>h+ ura4-D18 leu1-32 his3-D1 ade6-M216 pREP3X-pin1p</td>
</tr>
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<td>h+ ura4-D18 leu1-32 his3-D1 ade6-M210 pin1Δ::his3+</td>
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<tr>
<td>Sp25</td>
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</tr>
<tr>
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</tr>
<tr>
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<tr>
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<th>S. cerevisiae HKH1</th>
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DNA as templates. A Bg/II restriction site and a Sac/II site were introduced approximately 1 kb upstream and downstream, respectively, of the coding region of the ESS1 gene. The wild-type ESS1 DNA fragment was subcloned into the Bg/II and Sac/II sites of plasmid pEGFP-N1 (Clontech). To generate the ess1 deletion construct, the Ban/I DNA fragment (147 bp) and EcoRV DNA fragment (212 bp) in the ESS1 coding region were deleted. Thus, the coding sequences of the WW domain and most of the PPIase domain were deleted. Then, the Sp3 DNA fragment from pRS303 (Sikorski and Hieter, 1989), which contains wild-type HIS3, was subcloned into the EcoRV site to generate plasmid p45. To generate the ess1 deletion allele, plasmid p45 was digested with restriction enzymes Bg/II and Sac/II and transformed into wild-type diploid strain HKH1. Transformants with a His+ phenotype, indicative of ESS1 deletion, were selected and subjected to tetrad analysis.

FACS analysis and microscopy
Flow cytometry was performed as previously described (Sazer and Scherwood, 1990), except that cells were stained with 1 µM Sytox Green (Molecular Probes). The Becton Dickinson FACScan sorter and CellQuest software were employed to analyze our data.

For observation of Pin1p-GFP and DAPI staining, the Pin1p-GFP expressing strain (Sp35) was cultured in selective medium until cell density reached late log phase (OD655=0.8-1.0). Cells were fixed in 3.7% formaldehyde for 30 minutes at room temperature. Fixed cells were washed three times with PBS, stained with DAPI solution (1 µg/ml) for 10 minutes in the dark and washed with PBS again. Cells were viewed using a fluorescence microscope (Leitz Laborlux) and images were acquired by Adobe PhotoShop software (Adobe Systems Inc.) using the SPOT-2 CCD digital camera (Diagnostic Instruments Inc.).

UV sensitivity assay
Approximately 400 cells from an overnight culture were plated on YES plates followed by UV radiation (UV Stratalinker 2400, Stratagene). Plates were covered with foil and incubated at 32°C for 24 hours. The foil was removed and plates were further incubated at 32°C for 3 days. Colony numbers were counted and survival rates were calculated relative to the control plates that were not UV irradiated.

Cyclosporin A and okadaic acid plate assays
Wild-type (Sp1 and Sp3) and pin1Δ (Sp23 and Sp25) strains were grown on YES plates at 32°C for 24 hours. Cells were streaked on another YES plate or YES plates containing 10 and 60 µg/ml of CsA (Fluka) or 5 µM of okadaic acid (Calbiochem) followed by three days of incubation at 32°C.

RESULTS
Isolation of pin1+ and protein sequence alignment
The S. pombe genome sequencing project has identified an open reading frame (ORF) on the right arm of chromosome III, which has ~47% identity with human Pin1 (Lu et al., 1996) and 46% identity with budding yeast Ess1p (Hanes et al., 1989). This uncharacterized ORF encodes a protein containing both a WW domain and a PPIase domain. Sequence alignment (Fig. 1) reveals that several amino acids that play key roles in binding and enzymatic activities of Pin1 are also conserved in this ORF. These include residues equivalent to Ser16, Arg17 and Tyr23, which form a phosphate-binding module in the WW domain required for it to bind to phospho-Ser/Thr-Pro site that confers phospho-Ser/Thr specificity; and His59, Cys113 and His157, which are essential for catalytic activity (Ranganathan et al., 1997). Furthermore, we found that overexpression of this ORF (cDNA) under control of the GAL1 promoter rescued the severe temperature-sensitive phenotype of S. cerevisiae cells carrying an ess1 deletion (data not shown), indicating that the S. pombe pin1+ gene encodes a functional homologue of Pin1/Ess1p. To study the function of the pin1+ gene, which encodes the Pin1p protein, we have isolated both pin1+ genomic DNA and cDNA clones by PCR using genomic DNA and library cDNA, respectively, as DNA templates. The coding region sequence of pin1+ contains 673 base pairs with a single intron spanning nucleotides 403-546.

Cellular localization of Pin1p
To investigate localization of the endogenous Pin1p, we generated an in-frame pin1+GFP fusion chimera and chromosomal integrant of this fusion construct (Fig. 2A). This integration puts expression of Pin1p-GFP fusion proteins under control of the pin1+ native promoter and the nmt transcription terminator. This integration did not significantly affect cell growth or morphology (data not shown). The localization of the Pin1p-GFP fusion protein was determined by fluorescence microscopy after formaldehyde fixation. The localization of nuclei was determined by DAPI staining. The Pin1p-GFP fusion protein was found localized predominantly in nuclei during both interphase and mitosis (Fig. 2B), indicating that Pin1p is a nuclear protein. This is consistent with the observed localization of Pin1 to nuclear spliceosome speckles when Pin1
is expressed transiently in HeLa cells (Lu et al., 1996). By contrast, overexpressed Pin1p-GFP fusion protein (under the nmt1 promoter) was present in both nuclei and cytoplasm (data not shown).

**Overexpression of pin1** causes a severe growth defect and a G1 delay during cell cycle progression

To investigate the consequences of pin1 overexpression, we placed pin1 cDNA under control of the derepressible nmt1 promoter, which is repressed in the presence of thiamine. The wild-type strain (Sp1) was transformed with either an empty vector or a vector encoding nmt1-pin1 to generate control (Sp13) and overexpression (Sp15) strains, respectively. To induce overexpression of Pin1p, both control and overexpression strains were cultured in repressive media and then shifted to non-repressive media. As shown in Fig. 3A, the control strain grew normally and reached stationary phase (OD595=7.0) after two days culture in the non-repressive medium, the control strain showed a typical S. pombe cell cycle profile in which a majority of cells have 2N DNA content (Fig. 3B, top panel). However, the overexpression strain showed a significant increase in 1N DNA content and a modest increase in DNA content between 1N and 2N, indicative of G1 and S phases, respectively. This observation suggests that overexpression of pin1 causes a delay in G1 and S phases during cell cycle progression, but not a specific cell cycle arrest.

**Deletion of pin1 does not cause significant defects in cell growth, cell cycle progression or checkpoint control**

In light of the important role of ESS1 in S. cerevisiae (Lu et al., 1996), we tested whether pin1 is essential for viability in S. pombe using a one-step disruption method and tetrad dissection. A wild-type diploid strain was deleted for one copy of ESS1 followed by tetrad dissection. The deletion was confirmed by two independent genomic PCR analyses (data not shown). Rather than a phenotypic segregation of two viable spores to two inviable spores, we observed tetrads that showed four viable spores and a phenotypic segregation of two His+ spores to two

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**Fig. 3.** Overexpression of pin1 causes a severe growth defect. (A) Wild-type (Sp13) and overexpression (Sp15) strains were grown on plates containing thiamine for two days and inoculated into non-repressive medium for 20 hours, which is the time required for induction of pin1 overexpression. Cells with overexpressed pin1 were re-inoculated into non-repressive medium to OD595=0.05 and cultured at 32°C. For every 12 hours, aliquots of cells were taken and the cell densities (OD595) were measured.

(B) Same as A except that cell aliquots were fixed in 1 ml of cold 100% ethanol, resuspended in 50 mM sodium citrate and stained with 1 μM Sytox Green. Flow cytometry was performed with a Becton Dickinson FACScan and data were analyzed by CellQuest software.

**Fig. 4.** Survival analysis of ESS1 and pin1. (A) Wild-type diploid S. cerevisiae cells deleted for one copy of ESS1 were subjected to sporulation followed by tetrad dissection. Spores were grown on YES plates at 37°C for 9 days (left panel), or at 30°C for 4 days (middle panel), or at 37°C for 6 days (right panel). (B) A wild-type, two independent S. pombe pin1Δ strains and a rad3Δ strain were grown in YES medium overnight and then plated on YES plates followed by UV irradiation. Plates were cultured at 32°C with a foil cover for 1 day and without a foil cover for the subsequent 3 days. Colony numbers were calculated and survival rates were determined based on control plates, which were not irradiated. The data in this figure represent the average of three independent experiments with a standard deviation of less than 7%.
and tetrad analysis using a wild-type diploid content, indistinguishable from wild-type (data not shown). This observation suggests that deletion of viability in organisms, we re-examined whether Ess1p is essential for homologue, pin1 + these strains were streaked out on a YES plate. The top right panel shows the schematic diagram of how formation. The top right panel shows the schematic diagram of how (bottom right) for 5 days to compare cell growth and colony plates and cultured at 29°C (top left), 32°C (bottom left) and 36°C (bottom right) for 5 days to compare cell growth and colony formation. The top right panel shows the schematic diagram of how these strains were streaked out on a YES plate.

His− spores (data not shown). These viable His+ spores displayed normal morphology and growth rate (see Fig. 5A; and data not shown). These observations taken together suggest that pin1+ is not essential for cell viability.

To investigate whether deletion of pin1+ affects cell cycle control, we performed FACS analysis using asynchronous cultures with cell densities in the early, mid and late exponential phases. A wild-type (Sp1), two independent pin1Δ::his3+ strains (Sp23 and Sp25) and a rad3Δ strain (Sp41), as a second control, showed a major peak of 2N DNA content, indistinguishable from wild-type (data not shown). This observation suggests that deletion of pin1+ does not affect cell cycle control significantly.

Given that deletion of the D. melanogaster Pin1/ESS1 homologue, dodo (Maleszka et al., 1996), and the mouse homologue, Pin1 (Fujimori et al., 1999), results in viable organisms, we re-examined whether Ess1p is essential for viability in S. cerevisiae. We performed one-step disruption and tetrad analysis using a wild-type diploid S. cerevisiae strain (HKH1). We found that most tetrads containing ess1Δ showed a phenotypic segregation of two wild-type to two slow-growth spores at 30°C and 17°C (Fig. 4A, middle and left panels, respectively). Nine of eleven tetrads showed a phenotypic segregation of two viable to two inviable spores at 37°C (Fig. 4A, right panel). Moreover, the slow-growth colonies also had a His+ phenotype (data not shown). These results suggest that ESS1 is not absolutely essential for cell viability, and that deletion of ESS1 causes a severe temperature-sensitive (ts) growth phenotype in S. cerevisiae.

To determine whether pin1+ plays a role in DNA damage check point control, we exposed S. pombe cells to UV light followed by colony formation assay to determine survival rates. As shown in Fig. 4B, a rad3Δ deletion strain (Sp41) showed a UV sensitivity that is comparable to previous reports. In comparison to the wild-type strain (Sp1), two independent pin1 deletion strains (Sp23 and Sp25) only showed a modest increase in UV sensitivity in the presence of increasing dosages of UV radiation. In addition, we challenged the pin1 deletion strain with the DNA damaging reagent, bleomycin sulfate. The pin1 deletion strain exhibited an elongated morphology and a survival rate similar to the WT strain upon bleomycin treatment (data not shown). These results taken together suggest that pin1+ does not play a major role in DNA damage checkpoint control.

We also tested whether pin1+ is involved in DNA replication checkpoint control, as has been reported for Xenopus embryos (Winkler et al., 2000), by challenging the pin1 deletion strain with the DNA synthesis inhibitor, hydroxyurea. We found that both pin1 deletion and wild-type strains showed similar sensitivity to hydroxyurea (data not shown). In addition to hydroxyurea, we also generated a pin1Δcdc19-P1 (the MCM2 homologue) double mutant strain, which had elongated morphology like the cdc19-P1 parental mutant cells at the restrictive temperature (data not shown), suggesting that pin1+ does not play a major role in DNA replication checkpoint control.

Genetic interactions between pin1+ and genes encoding mitotic proteins

It has been reported that Pin1 physically interacts with several mitotic phosphoproteins, such as Cdc25 and Wee1, and negatively regulates the phosphatase activity of Cdc25 (Shen et al., 1998). However, the genetic interactions between Pin1 and its interacting proteins have not been tested. To address this point, we crossed the pin1 deletion strain (Sp25) to temperature-sensitive strains, for either cdc25 (cdc25-22) or wee1 (wee1-50), to generate double mutant strains. Deletion of pin1+ did not change the morphology of cdc25-22 and wee1-50 cells. Double mutant cells were cultured at permissive (29°C), semi-permissive (32°C) and non-permissive (34°C) temperatures to examine whether pin1Δ increased or decreased temperature sensitivity.

As shown in Fig. 5, wild-type (Sp1) and pin1Δ strains (Sp23) grew normally at the three different test temperatures. cdc25-22 ts mutant (Sp62) grew normally and formed colonies at the permissive and semi-permissive temperatures but not the restrictive temperature. By contrast, the pin1Δcdc25-22 double mutant strain (Sp84) did not grow at the semi-permissive temperature (Fig. 5). However, when wild-type pin1+ was integrated into the pin1Δcdc25-22 double mutant strain at the leu1 gene locus (Sp149), the growth defect at 32°C was rescued (Fig. 5). These observations suggest that pin1+ deletion enhances the temperature sensitivity of cdc25-22 and causes a synthetic growth defect with cdc25-22 at the semi-permissive temperature. A similar genetic interaction was observed between wee1+ and pin1+. The wee1-50 mutant cells grew normally and showed a wee phenotype at all tested temperatures, but the pin1Δ wee1-50 double mutant failed to
grow at 34°C. The growth defect at 34°C was rescued when wild-type pin1+ was integrated into the pin1Δ wee-1-50 double mutant at the leu1 gene locus (Sp153) (Fig. 5). These results suggest that pin1 deletion enhances temperature sensitivity of wee1-50 and causes a synthetic growth defect with wee1-50 at the semi-permissive temperature.

To determine whether pin1+ also plays a role in interphase, we generated pin1Δ cdc24-1 and pin1Δ cdc10-M17 double mutant strains. Cdc10p is part of a transcriptional complex required for the G1/S transition, and Cdc24p is a replication factor required for chromosome integrity and DNA replication. Deletion of pin1+ affected the growth of cdc10-M17 cells modestly at the semi-permissive temperature, but did not affect the temperature sensitivity of cdc24-1 cells (data not shown). The synthetic effect of pin1Δ and cdc10-M17 suggests that Pin1p function plays a role in the G1/S transition, as well as a role in mitosis. Because Pin1 was originally isolated as a NIMA-interacting protein (Lu et al., 1996), we also tested the genetic interaction between pin1+ and fin1+, the S. pombe NIMA homologue (Krien et al., 1998). However, we found that the fin1Δ pin1Δ double deletion strains grew normally and formed colonies at 17°C, 32°C and 36°C. There was no appreciable difference in cell morphology between the fin1Δ strain and the fin1Δ pin1Δ double deletion strain. These results taken together suggest that double deletion of pin1 and fin1 does not cause any significant growth defects (data not shown).

**Deletion of pin1+ results in increased sensitivity to CsA and OA**

Given that overexpression of cyclophilin A suppresses the growth defect of an ess1 temperature-sensitive mutant (Wu et al., 2000), it has been suggested that cyclophilin family isomerases have partially overlapping function with Ess1p in *S. cerevisiae*. To test whether cyclophilins complement the loss of pin1+ in *S. pombe*, we streaked both wild-type and pin1Δ cells on plates containing the cyclophilin inhibitor, CsA. As shown in Fig. 6A, after 3 days incubation, two independent wild-type strains grew normally and formed colonies in the presence of two different concentrations of CsA (10 and 60 μg/ml), whereas two independent pin1 deletion strains grew much slower at both concentrations of CsA. Nevertheless, the pin1 deletion strains were able to form colonies after 5 days incubation in the presence of CsA. This observation suggests that cyclophilin family PPIase partly shares an overlapping function with that of Pin1p in *S. pombe* and, consequently, deletion of pin1+ results in increased sensitivity to CsA.

It has been reported that Pin1-dependent prolyl isomerization is required for in vitro dephosphorylation of certain pSer/pThr.Pro motifs by protein phosphatase, PP2A, and that Ess1p and PP2A show reciprocal genetic interactions in budding yeast (Zhou et al., 2000). To test whether pin1+ is involved in protein dephosphorylation in *S. pombe*, we streaked both wild-type and pin1Δ cells on plates containing the PP1/PP2A phosphatase inhibitor, OA. As shown in Fig. 6B, after 3 days incubation, two independent wild-type strains grew and formed colonies in the presence of OA (5 μM), whereas the pin1 deletion strains (Sp23 and Sp25) grew poorly and formed very small colonies. Nevertheless, the pin1 deletion strains were able to form colonies comparable to wild-type cells after 5 days incubation in the presence of OA. This result suggests that pin1+ plays a role in protein dephosphorylation, presumably by increasing the population of phospho-Ser/Thr-Pro peptide bonds in the trans conformation that is required for PP1/PP2A-mediated dephosphorylation.

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*Fig. 6. Deletion of pin1+ causes increased sensitivity to cyclosporin A (CsA) and okadaic acid (OA).* (A) Sp1 (wild-type), Sp3 (wild-type), Sp23 (pin1Δ) and Sp25 (pin1Δ) cells were streaked out on YES plates containing no CsA (top right), 10 μg/ml CsA (bottom left panel) or 60 μg/ml CsA (bottom right panel) followed by incubation at 32°C for 3 days. The top left panel shows the schematic diagram of how these strains were streaked out on a YES plate. (B) Sp1 (wild-type), Sp3 (wild-type), Sp23 (pin1Δ) and Sp25 (pin1Δ) cells were streaked out on YES plates containing no OA (left panel) or 5 μM OA (right panel) followed by incubation at 32°C for 3 days. The strains were arranged on the plates as in A.


DISCUSSION

It has been reported that human Pin1 is involved in mitosis (Lu et al., 1996) and interacts with several mitotic phosphoproteins (Crenshaw et al., 1998; Shen et al., 1998). Moreover, a lack of Ess1p causes budding yeast cells to arrest in mitosis. Given the great conservation in mitotic regulatory mechanisms between mammalian cells and S. pombe, we have isolated the Pin1 homologue in S. pombe, pin1+, and characterized its potential role in cell cycle control. By expressing a Pin1p-GFP fusion chimera, we showed that endogenous Pin1p is localized exclusively to the nucleus during cell cycle progression. Overexpression of pin1+ caused a severe growth defect and a significant G1 delay during cell cycle progression, which might suggest that S. pombe Pin1p, like the vertebrate Pin1 and S. cerevisiae Ess1p family members, has a role in cell cycle progression. However, overexpression of Pin1p could interfere with cell cycle progression as a result of nonspecific interactions, since the Pin1p-GFP fusion protein was localized to both the nucleus and cytoplasm when overexpressed (data not shown), in contrast to the largely nuclear localization of Pin1-GFP expressed at physiological levels.

Our deletion analyses of pin1+ and ESS1 provide some interesting insights into the nature of Pin1/Ess1p prolyl isomerases. It has previously been reported that ESS1 plays an essential role in S. cerevisiae (Hanes et al., 1989). By contrast, deletion of the D. melanogaster homologue, dodo (Maleszka et al., 1996), and the mouse homologue, Pin1 (Fujimori et al., 1999), does not affect viability. It is plausible that additional Pin1 family genes or genes share an overlapping function with Pin1 exist in higher eukaryotes but not in lower eukaryotes, such as the budding yeast. However, we found that deletion of pin1+ in the fission yeast did not significantly affect viability, cell morphology, cell cycle control or DNA damage checkpoint control. In addition, pin1 deletion did not significantly affect DNA replication checkpoint controls in S. pombe. Consistent with our observations, deletion of the Pin1/Ess1p homologue in A. nidulans does not affect DNA damage or replication checkpoint controls (A. R. Means, personal communication). Given that the genome sequencing project for S. pombe is near completion and pin1+ is the only Pin1/Ess1p homologue found so far, our data suggest that Pin1 function is not essential in S. pombe. Even in S. cerevisiae, the Ess1 gene may not be essential in all cases, since deletion of Ess1 in our strain of budding yeast conferred a severe temperature-sensitive growth phenotype rather than lethality. Our result is consistent with a recent observation that theentralized eggshell phenotype of dodo deletion is greatly enhanced at higher temperatures (30°C) (Hsu et al., 2001). Nevertheless, the discrepancy between our result and previously published data (Hanes et al., 1989) is probably due to intrinsic differences between the wild-type yeast strains that were used, which are known to vary significantly. Our wild-type strain, which has been well characterized and extensively used for other studies (Robinson et al., 1988), may harbour a suppressor of the ess1Δ phenotype. Consistent with previous observations that a temperature-sensitive ess1 mutant is more sensitive to the cyclophilin family PPIase inhibitor CsA (Wu et al., 2000), we found that S. pombe pin1 deletion cells were more sensitive to CsA than wild-type cells, suggesting that PPIases in the cyclophilin family partly share overlapping activities with the activity of Pin1p in S. pombe.

One of the major goals of our study was to investigate Pin1 function using genetic approaches to complement the extensive biochemical studies on Pin1 that have already been reported. For example, despite findings of interactions between Pin1 and proteins involved in mitosis, such as Cdc25, Wee1 and NIMA, the genetic interactions between Pin1 and genes encoding these proteins have not been investigated. Although we did not find a significant effect of Pin1p-deficiency on cell cycle progression, we did observe a synthetic growth defect between pin1 deletion and two temperature-sensitive mitotic mutants, cdc25-22 and wee1-50 at the semi-permissive temperature. We also observed a modest effect of pin1 deletion on the growth of cdc10-M17 cells, suggesting that Pin1p might also play a role in the G1/S transition under some circumstances.

Why does loss of Pin1p enhance the temperature-sensitive phenotype of cdc25 and wee1 ts mutants? Given that Cdc25 phosphatase activity is stimulated by Cdk-mediated phosphorylation, deletion of pin1+ might affect the phosphorylation status and regulation of Cdc25p activity and localization in vivo and thereby confer a synthetic growth defect with the cdc25-22 mutant. Wee1p is also regulated by phosphorylation/dephosphorylation, and lack of Pin1p activity could likewise adversely affect regulation of Wee1p activity. Since Cdk phosphorylation activates Cdc25 and inhibits Wee1p, Pin1p-mediated dephosphorylation of phospho-Ser/Thr-Pro-containing mitotic phosphoproteins, such as Cdc25 phosphorylated by Xenopus Cdc25 phosphorylated by Cdc2, it stimulates the activity of Cdc25 phosphorylated by both Cdc2 and Ptx (Stukenberg and Kirschner, 2001). Thus, it is hard to predict the effect of Pin1 on the function of phosphorylated Cdc25p, and further studies are required to show whether deletion of pin1+ affects the phosphorylation status of Cdc25p and Wee1p and their activities.

As an alternative to enhancing dephosphorylation of Cdc25p and Wee1p, Pin1p, through its PPIase activity, might conceivably act as a phosphorylation-specific chaperone for many phospho-Ser/Thr-Pro-containing mitotic phosphoproteins, and play a more general role in mitotic progression. The ability of Pin1 to alter the conformation of phosphorylated Cdc25 would be one example (Stukenberg and Kirschner, 2001). This idea would be consistent with our finding that pin1Δ cells are more sensitive than wild-type cells to CsA, which inhibits cyclophilin PPIase activity. Thus, the ability of Pin1p PPIase to alter conformation in a phosphorylation-dependent manner may protect the mutant Cdc25p and Wee1p proteins from irreversible unfolding at the semi-permissive temperature and, therefore, Pin1p would be required to maintain their activity in vivo. In this manner, deletion of pin1+ might enhance the temperature sensitivities of the cdc25 and wee1 ts mutants.
Our genetic results taken together with the increased OA sensitivity of the pin1Δ strain are consistent with the recent observations that Pin1-dependent prolyl isomerization enhances in vitro dephosphorylation of Cdc25 and other proteins by PP2A (Zhou et al., 2000), and suggest a conserved role of Pin1/Ess1p in protein dephosphorylation. In S. cerevisiae, Ess1 and PP2A display reciprocal genetic interactions; overexpression of a Pho22 PP2A catalytic subunit suppresses the growth defect of an ess1 temperature-sensitive mutant strain, and Pin1 overexpression partially rescues the growth defect in pho22–172 mutant (Zhou et al., 2000). Therefore, we tested for genetic interactions between pin1Δ and PP2A by generating double deletion strains. ppa2Δ encodes the major PP2A activity in S. pombe and deletion of ppa2Δ has been shown to enhance the effects of the wee1-50 mutation and partially suppress the cdc25-22 mutant phenotype (Kinoshita et al., 1990; Kinoshita et al., 1993). ppa2Δ cells grow somewhat slower than wild-type cells, and are about 20% smaller. The pin1Δ ppa2Δ double deletion strain, however, did not show any additional growth defect or decrease in size, nor did the cells exhibit increased OA sensitivity compared with pin1Δ (data not shown). Presumably, this is due to compensation by the ppa1+ gene product, which is a second PP2A isoform that shares partly overlapping functions with that encoded by ppa2Δ (Kinoshita et al., 1990; Kinoshita et al., 1993). We also did not observe any synthetic effects of combining pin1Δ with ppa1Δ, or with dis2Δ or sds21Δ, which lack one or other PP1 isoform. Not only are there many mitotic targets for proline-directed kinases, but there are also many interphase targets for proline-directed kinases that create phospho-Ser/Thr-Pro motifs, including RNA polymerase II. Therefore a lack of Pin1p could decrease dephosphorylation of proteins involved in many processes, in addition to mitosis.

Pin1p probably has functions in several processes in addition to cell cycle progression, but does not have an essential role in any of them. For instance, Pin1/Ess1p can function in transcription through interaction with the phosphorylated CTD of RNA polymerase II or transcription factors phosphorylated at Ser/Thr-Pro motifs (Albert et al., 1999; Arevalo-Rodriguez et al., 2000; Hani et al., 1999; Komuro et al., 1999; Morris et al., 1999; Verdecia et al., 2000; Wu et al., 2000; Wulf et al., 2001) or endocytosis (Gerez et al., 2000). In support of a role for Ess1p in transcription, overexpression of genes involved in transcription and chromatin remodeling, such as CTD phosphatase (FCP1) and HDAC complex (SAP30), can suppress the growth defect of an ess1 temperature-sensitive mutant (Wu et al., 2000). Furthermore, Ess1p is required for 3′-end formation of pre-mRNA/transcription termination (Hani et al., 1999). Therefore, Pin1p might also have a role in transcription, and deletion of pin1Δ could thereby indirectly increase the temperature sensitivities of cdc25-22 and wee1-50 cells by affecting the expression of other proteins required for Cdc25p and Wee1p function.

In summary, the functions of Pin1/Ess1p family proteins are complex. They appear to serve rather general functions through their ability to promote phosphorylation-dependent isomerization of phospho-Ser/Thr-Pro motifs, which can result in an alteration in protein activity via a conformational change, as well as by priming proteins for dephosphorylation at these sites. Nevertheless, specific phosphoproteins and individual phospho-Ser-Thr-Pro motifs are likely to be preferred targets for Pin1/Ess1p. Consequently, depending on which Pin1/Ess1p targets are present in the organism and/or cell type, Pin1/Ess1p function will be more important for some processes than others, and this will lead to distinct loss-of-function phenotypes and dependencies. The issue of function is complicated by the fact that there are clearly redundant activities partly shared with Pin1/Ess1p in most cells, with cyclophilin family members and PP2A being the most obvious. Thus, although these proteins are structurally and functionally highly conserved, it is hard to pinpoint specific functions. Further genetic analysis of the S. pombe pin1Δ gene may provide additional insights into the function of this highly conserved family of proteins.

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