Pyp3 PTPase acts as a mitotic inducer in fission yeast

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Communicated by D.M.Glover

The p34^{cdc2} M-phase kinase is regulated by inhibitory phosphorylation of Tyr15, largely through the actions of the p107^{wee1} tyrosine kinase and p80^{cdc25} protein tyrosine phosphatase (PTPase). In this study we demonstrate that a second PTPase, encoded by *pyp3*, also contributes to tyrosyl dephosphorylation of p34^{cdc2}. *Pyp3* was identified as a high copy suppressor of a *cdc25*[−] mutation. The *pyp3* gene encodes a 33 kDa PTPase that is more closely related to human PTP1B and fission yeast *pyp1* and *pyp2* PTPases than to *cdc25*. *Pyp3* does not share an essential overlapping function with *pyp1* or *pyp2*. We demonstrate that disruption of *pyp3* causes a mitotic delay that is greatly exacerbated in cells that are partially defective for *cdc25* function and that *pyp3* function is essential in *cdc25*-disruption *wee1*[−] strains. *Pyp3* PTPase effectively dephosphorylates and activates the p34^{cdc2} kinase *in vitro*. We conclude that the *pyp3* PTPase acts co-operatively with p80^{cdc25} to dephosphorylate Tyr15 of p34^{cdc2}.

Key words: cell cycle/mitosis/phosphorylation/PTPase/*Schizosaccharomyces pombe*

Introduction

In recent years there has been a rapid advance in the understanding of mechanisms governing the initiation of mitosis. This has come primarily from biochemical studies of the rapid cellular embryonic divisions of frog and starfish oocytes and genetic studies performed in fission yeast (reviewed by Nurse, 1990). These studies have implicated activation of an evolutionarily conserved complex (known as MPF) containing a 34 kDa serine/threonine kinase catalytic core, p34^{cdc2}, and a larger regulatory subunit known as cyclin B, as the critical switch that triggers mitosis. Elucidation of the mechanisms regulating the inhibition and activation of the p34^{cdc2}/cyclin complex has therefore been thought to hold the key to understanding the controls governing the initiation of mitosis. Studies by Gould and Nurse (1989) demonstrated that activation of the p34^{cdc2}/cyclin kinase in fission yeast is immediately preceded by dephosphorylation of the 15th amino acid of the p34^{cdc2} subunit, a tyrosine residue. Observations that alteration of this residue to non-phosphorylatable phenylalanine (*cdc2*-F15) causes mitosis to initiate early and that p34^{cdc2}/cyclin immunoprecipitated from G₂ phase cells is activatable by the human T-cell protein tyrosine phosphatase (PTPase), established that the M-phase function of the p34^{cdc2} kinase is negatively regulated by

phosphorylation on Tyr15 (Gould and Nurse, 1989; Gould *et al.*, 1990).

The activity of MPF in fission yeast is controlled by a hierarchical gene network that impinges on the phosphorylation state on Tyr15 of the p34^{cdc2} molecule and includes the *cdc25*, *wee1*, *nim1* and *mik1* genes (Russell and Nurse, 1986, 1987a,b; Lundgren *et al.*, 1991). Recent biochemical evidence has conclusively demonstrated that dephosphorylation of Tyr15 at the onset of mitosis is directly catalyzed by the *cdc25* gene product, which encodes a novel class of tyrosine phosphatase (Dunphy and Kumagai, 1991; Gautier *et al.*, 1991; Millar *et al.*, 1991b; Strausfeld *et al.*, 1991; Lee *et al.*, 1992; Millar and Russell, 1992). Inactivation of *cdc25* in fission yeast results in a late-G₂ cell cycle arrest in which p34^{cdc2} is maximally phosphorylated on Tyr15 (Gould and Nurse, 1989). The action of the *cdc25* phosphatase is directly counteracted by the *wee1* tyrosine kinase, p107^{wee1}, which acts as a dose-dependent inhibitor of mitosis and has recently been shown to phosphorylate p34^{cdc2}/cyclin B directly on the inhibitory residues *in vitro* (Featherstone and Russell, 1991; Parker *et al.*, 1991, 1992; McGowan and Russell, 1993). Curiously, however, not only is cell viability unaffected when the *wee1* gene is inactivated but the steady state level of p34^{cdc2} tyrosine phosphorylation appears to be unchanged. This puzzle was solved by the discovery of the *mik1* kinase, whose function is essential for viability in the absence of *wee1*, and lead to the hypothesis that *wee1* and *mik1* genes act co-operatively to negatively regulate mitosis. Although disruption of *mik1* alone has no effect on cell cycle progression, simultaneous inactivation of the *wee1* gene leads to what has frequently been referred to as mitotic catastrophe, in which mitosis is initiated prior to the complete and faithful duplication of the genome (Lundgren *et al.*, 1991).

From these lines of experimentation it has become apparent that in fission yeast p34^{cdc2} must be maintained in a tyrosine phosphorylated state during interphase and only dephosphorylated at the onset of mitosis. Dephosphorylation of this residue at inappropriate times in the cell cycle or an inability to dephosphorylate p34^{cdc2} prior to mitosis leads to loss of cell viability. Despite this it has been known for some time that loss of *cdc25* phosphatase activity is not lethal when *wee1* is inactivated (Fantès, 1979; Russell and Nurse, 1986). One would presume that, under these circumstances, p34^{cdc2} is redundantly phosphorylated by the *mik1* kinase. The question then is, does p34^{cdc2} require tyrosine dephosphorylation at mitotic onset in the absence of *cdc25* and if so, what is the nature of this mitotic inducer? Alternatively, is MPF under the influence of an additional operational control yet to be determined? In answering this question, we have uncovered evidence for an additional non-transmembrane phosphotyrosine phosphatase, unrelated to *cdc25*, that regulates the timing of mitotic initiation in fission yeast by direct interaction with the p34^{cdc2}/cyclin kinase.

Fig. 1. *Cdc25*-independent tyrosine dephosphorylation of $p34^{cdc2}$. (A) A *wee1-50 cdc25::LEU2* culture grown in YES medium at 35.5°C to early log phase was incubated at 25°C for 4.5 h. The temperature of the culture was rapidly raised back to 35.5°C and appearance of septated cells determined at 10 min intervals. The first time point (0 min) is the time when the culture was shifted back to 35.5°C. (B) Cells from same culture were harvested, lysed and $p34^{cdc2}$ was affinity precipitated using $p13^{suc1}$ -sepharose. These precipitates were then probed for the presence of either phosphotyrosine (α -pTyr) or $p34^{cdc2}$ polypeptide (α -cdc2).

Results

Tyrosyl dephosphorylation of $p34^{cdc2}$ in a *cdc25* deletion strain

The $p80^{cdc25}$ phosphatase and $p107^{wee1}$ kinase act antagonistically to control the tyrosyl phosphorylation of $p34^{cdc2}$, and thereby regulate the timing of mitosis. Although *cdc25* is normally essential for division, cells lacking *cdc25* are viable if they have a compensating *wee1* mutation (Fantès, 1979; Russell and Nurse, 1986). Tyr15 of $p34^{cdc2}$ is still phosphorylated by a mechanism requiring *mik1* kinase in cells lacking both *wee1* and *cdc25* (Lundgren *et al.*, 1991). This raised the following important question. In cells lacking *wee1* and *cdc25*, is the tyrosyl dephosphorylation of $p34^{cdc2}$ still an obligatory step in the initiation of mitosis? We undertook the following experiment to address this question. *Cdc25::LEU2 wee1-50* cells were grown for 16 h at 35.5°C, a temperature at which *wee1-50* product is inactive. This culture was then incubated at 25°C for 4.5 h, during which the cells arrested in G_2 phase and became highly elongated. The culture was then shifted back to 35.5°C and samples taken at 10 min intervals. Monitoring of septated cells revealed that this culture resumed proliferation soon after the shift back to 35.5°C (Figure 1A).

Fig. 2. Plasmid $p3w$ rescues *cdc25*⁻ mutants. (A) *cdc25-22 leu1-32* or *wee1-50 cdc25::ura4 leu1-32* cells transformed with either control plasmid (pBH2.7), $p3w$, or $pcdc25$ were patched on to selective medium at the permissive temperature (25°C and 36°C, respectively). After 2 days growth the patch was streaked out and the culture incubated at the restrictive temperature (36°C and 25°C, respectively). (B) Restriction map of the *HindIII* insert of $p3w$. The positions of two exons (black) and intervening intron (hatched) are shown. The arrow indicates the direction of the ORF, with the arrowhead at the 3' end of the gene. 200 bp intervals are marked under the map.

The septation index began to rise within 40 min and peaked at 70 min. Cell lysates were generated from duplicate samples and $p34^{cdc2}$ /cyclin was precipitated using $p13^{suc1}$ -sepharose. These precipitates were probed with α -cdc2 antibodies or α -phosphotyrosine antibodies by immunoblotting. As the results in Figure 1B show, most of the $p34^{cdc2}$ underwent tyrosyl dephosphorylation between 20 and 30 min after the release, whereas the level of the $p34^{cdc2}$ polypeptide remained invariant. This strongly suggests that mitotic initiation is still dependent on the tyrosyl dephosphorylation of $p34^{cdc2}$ in *wee1*⁻ *cdc25*-disruption cells. It also clearly demonstrates that a PTPase other than $p80^{cdc25}$ is capable of dephosphorylating $p34^{cdc2}$ to cause the onset of mitosis.

Isolation of a new gene that when overexpressed suppresses *cdc25* deletion

Since $p34^{cdc2}$ undergoes tyrosyl dephosphorylation in the absence of *cdc25*, we considered it plausible that a second *cdc25*-like gene exists in fission yeast. Indeed recent studies have demonstrated the existence of *cdc25* multi-gene families in *Drosophila*, mice and humans (Galaktionov and Beach, 1991; Millar *et al.*, 1991a; Alpey *et al.*, 1992). We reasoned that a single genomic copy of this gene must be unable to counteract the combined activities of *wee1* and *mik1* kinases. However, when cloned on a multi-copy number plasmid this gene could be expected to rescue *cdc25*⁻ mutations. We used such a strategy to clone this gene. A *cdc25-22* strain was transformed with an *Schizosaccharomyces pombe* gene bank and incubated at 36°C. Complementing plasmids were isolated from colonies growing at the restrictive temperature. Most were found to contain either the *cdc25* or *nim1* gene, the latter encoding a mitotic inducer that is believed to be a negative regulator of the *wee1* kinase (Russell and Nurse, 1987b). In addition,

-197 TACAAC TAGAACTAGCGCTATGTGGGGCCGTCACAATGATGATTATTAACAATATATCTCTC
 -131 TTTTCTACTGACTCTGCTCATGCTTTTAGGAATCATTAAAGTCGTTTAGTAGAAATATCTCTTC
 -65 TTTTGTAGACACAAAAAATCTCTGGTGAAGAATTAAAGCGAATAAACGCAAAAAATCAAT

1 ATGCTTTTAAAGAAATATCTACAGAAATGGTGTCTTAACACCCCTTGATCACAATAAAAGAAAA
 1 M S F K E V S T E N G V L T P L I T I K E K

67 GCGTACATGATTATTTAGGAGCTCAATGAGGAAGAGATAGAGCTGTTAAATCTAGGTGCGCAAG
 23 A Y M I I E G L N E E E L R L K C D I V K I P K

133 CTAAGTAAGAAAGCGCTGGCTAGAAACGATATCCACATAGTCCCTTATGAAATCTGCTGT
 45 L S K K A L A R N A Y S N I V P Y E N T R V

199 CGTTTAGATCCAAATGCGGAAGAGCATGTGATTACATTAATGCTTCCATAGTAAAAATCCCTCT
 67 R L D P M W K E A C D Y I N A S I V K I P S

265 GGGAAACATTTATCGCTACTCAGGTAGGAATAAAAGAAATGCGGGATTGTAACGGCTTTGG
 89 G K T F I A T Q

331 CCTTTTTCTTTTCACTATTTTTTGTAAACATAATTAACCTAGTCTCTTTATTTATATAGGGT
 G

397 CCAACGTCGAACAGTATTGATGTTTTTGGAAAGATGGTCTGGCAATCTGTGCCAAAATCAGGCATC
 98 P T S N S I D V F W K M V W Q S V P K S G I

463 ATCGTTATGCTTACCAATTTAGGGAACGCCATCGCTGAAATGTGATATCTATTGCGCCATGGA
 120 I V M L T K R E R H L K C D I V K I P K

529 CTGTTTGAGACACTTAATATCGGTGATTAAAGCGTTATTTGGTAAAGTTTATCTTTGACATCA
 142 L F E T L N I G D L S V I L V K V Y T L T S

595 TTAATGAAGTTCAAGTCTCGTATTTGAGCTGAACAGGACGGGTCAAGAAAAAATTTGCAT
 164 L N E V Q V R E F E L N K D G V K K K I L H

661 TTTTACTATAACGGTGGCGTACTTTGGTGCACACATACCTCTCACTACTATCACTGACTCGC
 186 F Y Y N G W P D F G A P H T F S L L S L T R

727 TATATAAAAGTCTCTCATATCTCCGACCTTGAACAGCACCATTATAGTCCATTTGTCAGCG
 208 Y I K S L S Y S P D F E T A P I I V H C S A

793 GGTGTGCGCAGAACGGGAACCTTCATGGCGTTATTTGAGATTTATCTCAACAGATGACTCTACT
 230 G C G R T G T F M A L F E I L S Q T D D S T

859 TCACTTCGAAATTTGAGGTGATAATATCGCAATATAGTTCCAGTCTTCGGTCACAAAGATG
 252 S T S K F E V D N I A N I V S S L R S Q R H

925 CAATCTGTTCATCGGTCGATCAGCTGTTTTCTTTATACAGTATCTCAAGAGCTGCTTCAAGGG
 274 Q S V Q S V D Q L V F L Y T V S Q E L L Q G

991 AAGGAATTTCTTCTTCCTCAGTATAGCCTTTGGTTAGAGTTTGCTGATAGTTCGATGATGTTA
 296 K E F L L P Q L *

1057 AAAAATCGTCTCATGAATTTTTCGAATTAACATATATTTTATAAGAGGATCTCTTTTTT
 1123 TATTCAAAATTTATTTTATTGCTAGCAGCTTTTATTTCTTGATATCCAGTTTGGACCGCTA
 1189 ATGATTAATAATGATTTCATTTACTTTATATGGCACTATTTCTTAGAGACCTATATAATGATCAA

Fig. 3. *Pyp3* DNA and predicted protein sequence. The complete genomic sequence of the 1028 nucleotide open reading frame, interrupted by a 109 bp intron and some adjoining sequence is shown. A 303 amino acid ORF is translated using the single letter amino acid code. The sequences underlined represent the amino acids believed to form the catalytic active site of the PTPase family.

several extragenic suppressor plasmids were found to contain a novel 3.1 kb *HindIII* insert whose restriction enzyme map bore no relation to the maps of *cdc25* or *nim1* (Figure 2). One of these plasmids was designated p3w. As compared with plasmids carrying *cdc25* or *nim1*, the plasmid p3w was rather ineffective at rescuing *cdc25-22*. At 36°C p3w transformants were about two to three times larger than wild type and formed slower growing colonies (Figure 2).

We wanted to eliminate the possibility that p3w harbored an activity that could stimulate residual activity that the p80^{*cdc25-22*} protein might retain at 36°C. Therefore we asked whether the p3w plasmid could rescue the cell cycle arrest arising from a *cdc25*-disruption. *Cdc25::ura4 weel-50* cells bearing either plasmid borne copies of *cdc25* or the p3w plasmid were able to form colonies at 25°C, whereas cells transformed with a control plasmid did not (Figure 2A). This formally demonstrates that p3w bypasses the requirement for *cdc25*. In principle this would most likely occur either by independently promoting the tyrosyl dephosphorylation of p34^{*cdc2*} or by negatively regulating the *weel* kinase.

The p3w high copy suppressor gene product is homologous to protein tyrosine phosphatases

Subcloning and insertion-disruption analysis of the 3.1 kb *HindIII* insert in p3w indicated that sequences required for rescue of *cdc25-22* included those between two *EcoRV* sites

located near the center of the DNA fragment (Figure 2B). The entire 3.1 kb insert was sequenced and revealed two contiguous open reading frames of 288 bp and 621 bp separated by 105 bp of predicted intron sequence (Figure 3). Most of the 621 bp ORF was located between the two *EcoRV* sites, indicating that it was part of the high copy suppressor gene. No other extended open reading frames were identified. To confirm the presence of an intron directly, the gene was isolated from an *S.pombe* cDNA library by PCR using oligonucleotides located shortly upstream and downstream of the ORF (see Materials and methods). A 909 bp fragment was identified, which upon sequencing was found to correspond directly to the complete ORF having the intron removed.

The predicted sequence of the 303 amino acid protein was used to screen protein sequence databases for primary structure similarity. The sequence was found to be similar to the catalytic domain of the previously identified family of protein tyrosine phosphatases typified by human PTP1B (Figure 4). A region encompassing the PTPase catalytic domain of this newly identified gene product was ~30% identical to human PTP1B and the two recently identified fission yeast PTPases encoded by *pyp1* and *pyp2* (Charbonneau *et al.*, 1989; Otilie *et al.*, 1991; Millar *et al.*, 1992). Significantly, the PTPase encoded on p3w had only very weak similarity to *cdc25*, at a level equivalent to the similarity between *cdc25* and PTP1B, and therefore it cannot be classified as a member of the *cdc25* family. On this basis the newly identified fission yeast PTPase gene was named *pyp3*. Like *pyp1* and *pyp2* proteins, the catalytic domain of *pyp3* PTPase is located in the C-terminal portion of the protein, but *pyp3* does not contain a long N-terminal extension that is present in both *pyp1* and *pyp2* proteins.

***Pyp3* PTPase dephosphorylates and activates p34^{*cdc2*} in vitro**

It was previously shown that ectopic expression of human T cell PTPase in fission yeast caused premature onset of mitosis and rescued *cdc25-22*, presumably by directly dephosphorylating p34^{*cdc2*} (Gould *et al.*, 1990). Thus the cloning of a *S.pombe* PTPase-like gene as a high copy suppressor of *cdc25-22* was not completely unexpected. By analogy it suggested that the *pyp3* PTPase might bypass the requirement for *cdc25* by directly dephosphorylating p34^{*cdc2*}. A key prediction following from this proposal was that *pyp3* PTPase should dephosphorylate and activate p34^{*cdc2*} in an *in vitro* assay using purified components. To test this prediction the complete open reading frame of *pyp3* was cloned into a bacterial expression vector to generate a fusion gene encoding glutathione-S transferase followed by *pyp3*. Soluble GST-pyp3 protein was readily produced at high levels in bacteria. The fusion protein was easily purified to near homogeneity by passage over a glutathione sepharose (GSH-sepharose) column (Figure 5A). GST-pyp3 was found to efficiently dephosphorylate *para*-nitrophenyl-phosphate (pNPP), an organic compound that acts as a substrate for all classes of phosphatases (Figure 5B). The reaction was linear both during the time course of the assay and over a range of GST-pyp3 concentrations. Kinetic analysis revealed that GST-pyp3 dephosphorylates pNPP with a specific activity of ~17.6 μmol phosphate released per min per mg, a rate similar to previously described PTPases. This activity is sensitive to micromolar

Fig. 4. Homology between *pyp3* protein and PTPases. Homologies of the catalytic domains of fission yeast *pyp1*, *pyp2*, *pyp3* and human PTP1B. Boxes indicate identities amongst all four proteins; shading indicates identities and conservative substitutions amongst all four proteins; consensus indicates that at least three of four proteins have identical amino acids. Homologies generated using GeneWorks 2.1 (Intelligenetics, Inc.).

We next determined whether GST - pyp3 directly activates p34^{cdc2}/cyclin. Antibodies raised against a synthetic peptide corresponding to the C-terminus of p34^{cdc2} were used to immunoprecipitate p34^{cdc2} from fission yeast cells arrested in late G₂. This complex was treated with either GST, GST - pyp3 or GST - cdc25 proteins. As compared with GST - cdc25, the GST - pyp3 protein was found to activate fully the histone H1 kinase activity of the immunoprecipitated p34^{cdc2}/cyclin (Figure 5C). In a separate experiment in which p34^{cdc2}/cyclin was isolated by p13^{suc1}-sepharose affinity chromatography it was shown that GST - pyp3 caused nearly complete tyrosyl dephosphorylation of p34^{cdc2} (Figure 5D). These data strongly indicate that pyp3 acts as a high copy suppressor of *cdc25*⁻ mutations by directly carrying out the function of p80^{cdc25}, namely to dephosphorylate Tyr15 of p34^{cdc2}.

If *pyp3* acts to catalyze the tyrosyl dephosphorylation of *p34^{cdc2}* directly *in vivo*, we would then predict that high overexpression of *pyp3* should advance the timing of mitosis and cause a *wee* phenotype in the same manner that *cdc25* overexpression does. To test this prediction, the *pyp3* ORF was cloned behind the thiamine-repressible *nmt1* promoter (Maundrell, 1990) on a multi-copy episomal plasmid and introduced into various strains. *Pyp3* overexpression in fact did advance mitosis, causing cells to divide at half the size of wild type (Table I). The phenotype was identical to that caused by overexpression of *cdc25* (Russell and Nurse, 1986). *Pyp3* overexpression also rescued *cdc25-22*; in fact, the *wee* phenotype caused by *pyp3* overexpression was fully epistatic to the *cdc25-22* cell cycle arrest phenotype (Figure 6 and Table I). Furthermore, overexpression of *pyp3* induced a lethal mitotic catastrophe in *cdc2-3w* and *wee1-50* cells

Fig. 5. GST–pyp3 dephosphorylates and activates p34^{cdc2}. **(A)** GST–pyp3 made in bacteria was purified by glutathione affinity chromatography, eluted with excess glutathione, and then subjected to SDS–PAGE and stained with Coomassie blue. As is typical of proteins expressed using pGEX vectors, several species differing slightly in molecular weight were detected. **(B)** GST–pyp3 (1 μ g) was incubated in phosphatase buffer in the presence of 50 mM pNPP for various lengths of time as indicated and release of free phosphate measured by increase in absorbance at 410 nm as described in Materials and methods. **(C)** p34^{cdc2}/cyclin complex was immunoprecipitated from extracts of late G₂ arrested *cdc25-22* cells using an antibody directed against the C-terminus of p34^{cdc2}. Washed immunoprecipitates were then incubated in the presence of GST (open bar), GST–pyp3 (closed bar) or GST–cdc25 (hatched bar) at 50 μ g/ml for 30 min at 30°C. Histone H1 phosphorylation assays were then performed. **(D)** p34^{cdc2} was affinity precipitated from extracts of late G₂ arrested *cdc25-22* cells using p13^{suc1}-sepharose and incubated in the presence of either GST or GST–pyp3 proteins at 50 μ g/ml for 30 min at 30°C. Precipitates were resolved by SDS–PAGE and probed after blotting for phosphotyrosine-containing p34^{cdc2} (α -pTyr) or p34^{cdc2} polypeptide (α -cdc2).

Table I. Overexpression of *pyp3* advances mitosis

	T (°C)	pREP1	pREP1- <i>pyp3</i>
Wild type	32	wt	wee
<i>cdc25-22</i>	35.5	<i>cdc</i> ⁻	wee
<i>wee1-50</i>	35.5	wee	mc
<i>cdc2-3w</i>	32	wee	mc
<i>cdc2-33</i>	35.5	<i>cdc</i> ⁻	<i>cdc</i> ⁻

Phenotypes of transformants grown in EMM medium lacking thiamine were scored. They fell into the following classes: wt, wild type, dividing at 14–15 μ m; wee, dividing at 7–8.5 μ m; *cdc*⁻, arresting cell division as extremely long cells; mc, mitotic catastrophe, premature lethal mitosis as previously described (Russell and Nurse, 1986). Phenotypes of pREP1-*pyp3* and pREP1 transformants grown in media supplemented with thiamine were similar to those of pREP1 transformants grown in media lacking thiamine.

Fig. 6. Overexpression of *pyp3* rescues *cdc25-22* and causes mitotic catastrophe in *cdc2-3w* cells. Left panel: overexpression of *pyp3* from the plasmid pREP1-*pyp3* (*nmt:pyp3*) allows *cdc25-22* cells to form colonies at 35.5°C. *Cdc25-22* arrest is not rescued by pREP1 (Control) or pREP1-*pyp1* (*nmt:pyp1*). Right panel: overexpression of *pyp3* from the plasmid pREP1-*pyp3* (*nmt:pyp3*) is lethal in a *cdc2-3w* strain. *Cdc2-3w* cells bearing pREP1 (Control) or pREP1-*pyp1* (*nmt:pyp1*) are able to form colonies at 32°C.

at the restrictive temperature (Figure 6 and Table I), exactly as has previously been observed for overexpression of *cdc25* (Russell and Nurse, 1986). Overexpression of *pyp3* did not rescue *cdc2-33*, consistent with the model that *pyp3* advances mitosis by causing the premature activation of p34^{cdc2} M-phase kinase (Table I). To demonstrate that these effects were specific to the *pyp3* PTPase and not a general property of all PTPases, a similar set of experiments was performed in which the fission yeast *pyp1* PTPase was overexpressed in parallel cultures and the phenotypes compared. As the results in Figure 6 demonstrate, overexpression of *pyp1* was completely unable to rescue *cdc25-22* or to cause a mitotic catastrophe in a *cdc2-3w* strain. As described in the accompanying paper, *pyp1* and *pyp2* overexpression actually has the opposite effect, in that it delays the onset of mitosis (Millar *et al.*, 1992). Taken together these data strongly suggest that the *pyp3* PTPase is capable of dephosphorylating p34^{cdc2} *in vivo*.

Pyp3 contributes to the activation of p34^{cdc2}/cyclin *in vivo*

Having acquired convincing evidence that *pyp3* acts as a high copy suppressor of *cdc25-22* by directly dephosphorylating Tyr15 of p34^{cdc2}, we then turned our attention to the *in vivo* requirement for *pyp3* activity. Specifically, we asked three

Fig. 7. *Pyp3* does not share an overlapping function with *pyp1* or *pyp2*. (A) Map of *pyp3::ura4* construction. (B) Phase contrast photographs of wild type, *pyp3::ura4* alone and in combination with *pyp1* and *pyp2* disruptions.

Table II. Loss of *pyp3* delays mitosis

	T (°C)	<i>pyp3</i> ⁺ (μ m)	<i>pyp3::ura4</i> (μ m)
Wild type	32	14.1 $\pm$ 0.2	15.0 $\pm$ 0.4
<i>cdc25-22</i>	26	19.7 $\pm$ 0.5	30.3 $\pm$ 1.7
<i>nim1::LEU2</i>	32	17.3 $\pm$ 0.2	19.2 $\pm$ 0.5
<i>adh:wee1-50</i>	25	<i>cdc</i> ⁻	<i>cdc</i> ⁻
	27	34.2 $\pm$ 3.1	<i>cdc</i> ⁻
	29	18.3 $\pm$ 0.3	27.2 $\pm$ 0.7

Cell length at division was measured.

questions: (i) is *pyp3* essential for viability, (ii) does *pyp3* contribute to the dephosphorylation of p34^{cdc2} in a wild type background, and (iii) is *pyp3* essential for the dephosphorylation and activation of p34^{cdc2} in *wee1*⁻ *cdc25*⁻ strains? To answer these questions we endeavoured to create a genomic disruption of *pyp3*. The genomic *pyp3* sequence in p3w was disrupted by removing sequences corresponding to the entire catalytic domain of the *pyp3* PTPase and replacing them with *ura4*⁺ (Figure 7A). This construct was used to disrupt one genomic copy of *pyp3* in a *ura4-D18/ura4-D18* diploid strain. A heterozygous *pyp3*⁺/*pyp3::ura4* genotype was confirmed by Southern hybridization analysis (data not shown). Random spore

analysis produced a 1:1 ratio of uracil prototrophs and auxotrophs, indicating that haploid *pyp3::ura4* cells were viable. Southern hybridization and out-crossing confirmed the *pyp3::ura4* genotype in several haploid strains (data not shown). The *pyp3::ura4* disruptants were slightly longer than wild type length at cell division (Table II), but had a similar doubling time to that of wild type cells. These data demonstrate that *pyp3* is not normally essential for viability, although loss of *pyp3* results in a small increase in cell size at division.

The *pyp1* or *pyp2* genes in themselves are also not essential, but spores lacking both PTPase genes are dead (Millar *et al.*, 1992). Therefore we analyzed whether *pyp3* might share an essential overlapping function with either the *pyp1* or *pyp2* PTPases. *Pyp3::ura4* disruptants were crossed to cells bearing a disruption of either the *pyp1* or *pyp2* genes and tetrads dissected. Of eight asci dissected from a *pyp1::ura4* × *pyp3::ura4* cross, six gave rise to tetrads with four uracil prototrophs and were therefore assumed to be parental ditypes (PD), one tetrad gave rise to three uracil prototrophs and one auxotroph and was assigned as a tetratype (TT), and the remaining ascus gave rise to two prototrophs and two auxotrophs and thus was assigned as a non-parental ditype (NPD). The *pyp1::ura4 pyp3::ura4* double mutants arising from the TT and NPD asci were fully viable (Figure 7B), exhibiting the semi-wee phenotype typical of *pyp1::ura4* cells (Millar *et al.*, 1992). *Pyp2::ura4* cells were crossed to *pyp3::ura4* in a similar manner and 13 tetrads dissected. Again all the asci from this cross gave rise to four viable spores. On the basis of uracil prototrophy these could be identified as nine PD, one NPD and three TT. The *pyp2::ura4 pyp3::ura4* double mutants had a normal growth rate and morphology (Figure 7B). Thus we conclude that *pyp3* does not share an important overlapping function with either *pyp1* or *pyp2*. Interestingly, the *pyp1* and *pyp2* genes are linked, separated by a genetic distance of ~20 cM (Millar *et al.*, 1992). Remarkably, the observed ratio of PD:NPD:TT for crosses described above suggests that all three PTPase genes are genetically linked, although this remains to be proven.

The small cell size increase arising from *pyp3*-disruption suggested that the *pyp3* PTPase might normally share with *p80^{cdc25}* the job of inducing mitosis by dephosphorylating *p34^{cdc2}*. If this were so, we could expect that the cell size increase caused by *pyp3*-disruption would be exaggerated in cells that were delayed for the initiation of mitosis because of partial loss of *cdc25*. To test this, we crossed *pyp3::ura4* into a *cdc25-22* background. *Cdc25-22* gene product function is partially compromised at 26°C, such that *cdc25-22* cells divide at a cell length of ~20 µm, versus wild type cells which undergo mitosis at ~14 µm (Table II). *Pyp3::ura4 cdc25-22* cells divided at an extremely large size (30.3 ± 1.7 µm) when grown at 26°C. Similar results were obtained using another temperature-sensitive *cdc25* strain, *cdc25-M51* (data not shown). These data show that *pyp3* plays a very important role as a mitotic inducer in cells that are partially compromised for *cdc25* function.

To analyze whether this effect was restricted to cells that are partially compromised for *cdc25* activity, we next tested the effect of *pyp3*-disruption in cells that lack the mitotic inducer *nim1*. Cells having a *nim1*-disruption underwent cell division at 17.3 ± 0.2 µm, whereas *pyp3::ura4 nim1::LEU2* were 19.2 ± 0.5 µm at division (Table II). These results

Table III. *Pyp3* is essential in a *wee1⁻ cdc25*-disruption background

	T (°C)	<i>pyp3⁺</i> (µm)	<i>pyp3::ura4</i> (µm)
<i>wee1-50</i>	35.5	7.6 ± 0.4	8.0 ± 0.3
<i>wee1-50 cdc25::LEU2</i>	35.5	18.5 ± 1.2	<i>cdc⁻</i>
<i>cdc2-3w</i>	32	9.0 ± 0.3	10.0 ± 0.5
<i>cdc2-3w cdc25::ura4</i>	32	17.2 ± 0.2	23.3 ± 0.5

Cell length at division was measured.

indicate that the mitotic delay arising from *pyp3*-disruption is exaggerated under conditions in which Tyr15 of *p34^{cdc2}* is more effectively phosphorylated. To confirm this we tested the effect of *pyp3*-disruption in cells that overexpress *p107^{wee1}*. We used a strain that has a copy of the temperature-sensitive allele *wee1-50* under the control of the strong *adh* promoter. A *pyp3⁺ adh::wee1-50* strain underwent cell cycle arrest at 25°C, it divided at a very large size at 27°C (~34 µm), and was somewhat elongated at 29°C (~18 µm) (Table II). In contrast, a *pyp3::ura4 adh::wee1-50* strain underwent cell cycle arrest at both 25°C and 27°C, and divided at a highly exaggerated cell size (~27 µm) at 29°C (Table II). These data show that loss of *pyp3* can have quite a major effect on the mitotic control even in cells that have a wild type copy of *cdc25*.

***Pyp3* is essential for *p34^{cdc2}* tyrosyl dephosphorylation in a *wee1⁻ cdc25⁻* background**

Having shown that the PTPase encoded by *pyp3* is capable of dephosphorylating *p34^{cdc2}* *in vitro* and acts as mitotic inducer *in vivo*, we then returned to the original question that prompted these experiments. Namely, is *pyp3* PTPase responsible for dephosphorylating *p34^{cdc2}* in cells lacking *cdc25* and *wee1*? To test this possibility, *wee1-50 pyp3::ura4* cells were mated to *wee1-50 cdc25::LEU2*. The growth of spores of the genotype *wee1-50 pyp3::ura4 cdc25::LEU2* at 35.5°C was selected on medium lacking both uracil and leucine. Random spore analysis revealed no haploid colonies from over 2000 spores plated. On microscopic inspection ~25% of the spores were found to have germinated but were arrested as single highly elongated cells, suggestive of a pre-mitotic arrest. Haploid colonies arose at a high frequency when spores were plated on media supplemented with uracil or leucine. These observations indicated that *wee1-50 pyp3::ura4 cdc25::LEU2* spores germinated at 35.5°C but were unable to undergo division. To confirm this, asci from the same cross were subjected to tetrad dissection. Three NPD tetrads had two spores that grew to give two viable colonies that behaved as *wee1-50 pyp3⁺ cdc25⁺*, and two spores that germinated but underwent *cdc* arrest at 35.5°C (Table III). The inviable spores were classified with the genotype *wee1-50 pyp3::ura4 cdc25::LEU2*. From this we conclude that *pyp3* function is essential for the viability of *cdc25*-disruption *wee1⁻* cells.

The *cdc2-3w* allele bypasses the requirement for both *cdc25* and *pyp3*

Complete loss of the *cdc25* phosphatase is suppressed by certain semi-dominant activating mutations of *cdc2*, notably *cdc2-3w* (Russell and Nurse, 1986). *Cdc2-3w* mutants divide

at $\sim 8\text{--}9\ \mu\text{m}$, whereas *cdc2-3w cdc25::ura4* cells divide at $\sim 17\ \mu\text{m}$ (Table III). These data show that *cdc2-3w* mutants no longer require the *cdc25* phosphatase, although the onset of mitosis is significantly delayed in the absence of *cdc25*. The mechanism by which *cdc2-3w* bypasses the requirement for *cdc25* is unknown, as the *cdc2-3w* mutation is removed from the site of tyrosine phosphorylation and *cdc2-3w* protein is tyrosyl phosphorylated at normal levels in asynchronous cells (Carr *et al.*, 1989; Gould *et al.*, 1990). However, the fact that the onset of mitosis is delayed in *cdc2-3w* cells as a consequence of *cdc25*-disruption suggests that activation of *cdc2-3w* protein is dependent on tyrosine dephosphorylation. To investigate this we asked whether *pyp3* was essential in a *cdc2-3w cdc25::ura4* background. Surprisingly, we found that the *cdc2-3w cdc25::ura4 pyp3::ura4* triple mutant was viable (Table III), dividing at a somewhat longer cell size ($\sim 23\ \mu\text{m}$) than the *cdc2-3w cdc25::ura4* control ($\sim 17\ \mu\text{m}$). Although we cannot rule out the possibility that the $\text{p34}^{\text{cdc}2-3w}$ protein induces mitotic initiation without tyrosyl dephosphorylation, we think it most likely that there is another fission yeast PTPase, as yet undiscovered, that also contributes to the dephosphorylation of $\text{p34}^{\text{cdc}2}$ *in vivo*.

Discussion

Cells lacking both *wee1* and *cdc25* are viable even though $\text{p34}^{\text{cdc}2}$ is still tyrosyl phosphorylated and inactivated by a mechanism requiring the *mik1* kinase (Lundgren *et al.*, 1991). The studies described in this report were undertaken to solve how *wee1*⁻ *cdc25*-disruption cells undergo mitosis without the *cdc25* phosphatase. One could imagine that tyrosyl dephosphorylation of $\text{p34}^{\text{cdc}2}$ might not be necessary for viability in a *wee1*⁻ background. This could occur by a mechanism in which the rate of production of $\text{p34}^{\text{cdc}2}$ /cyclin complex outpaces the rate of tyrosyl phosphorylation of $\text{p34}^{\text{cdc}2}$ by the *mik1* kinase. An alternative hypothesis could be that there exists a second PTPase that shares with $\text{p80}^{\text{cdc}25}$ the responsibility for dephosphorylating Tyr15 of $\text{p34}^{\text{cdc}2}$. In our initial experiment we proved that the second hypothesis is valid. In cells of the genotype *wee1*^{ts} *cdc25*-disruption, $\text{p34}^{\text{cdc}2}$ underwent rapid tyrosyl dephosphorylation following the reversible inactivation of *wee1* gene product. From this result we could confidently predict that a second PTPase exists that dephosphorylates $\text{p34}^{\text{cdc}2}$ and that it could be identified as a high copy suppressor of *cdc25-22*. Using this well-proven approach we cloned a candidate high copy suppressor gene that encoded a predicted PTPase and thus we named it *pyp3*. Surprisingly, this PTPase bore little similarity to the *cdc25* class of PTPases, but instead was found to be most similar to the class typified by human PTP1B. Nevertheless, we were able to prove that *pyp3* PTPase does contribute to the dephosphorylation of $\text{p34}^{\text{cdc}2}$ *in vivo*. This conclusion is based on the following lines of evidence. First, overexpression of *pyp3* causes the cells to initiate mitosis early such that they divide at a smaller size and undergo a lethal premature mitosis in cells lacking the *wee1* kinase, an effect that is dependent on an active $\text{p34}^{\text{cdc}2}$ kinase. Secondly, *pyp3* protein efficiently tyrosine dephosphorylates and activates $\text{p34}^{\text{cdc}2}$ *in vitro*. Third and most importantly, loss of *pyp3* causes a delay in the initiation of mitosis that is lethal in a *wee1*⁻ *cdc25*-disruption background. Taken

together these data indicate that *pyp3* acts in conjunction with *cdc25* to dephosphorylate $\text{p34}^{\text{cdc}2}$ and bring about the onset of mitosis.

Loss of *pyp3* function in an otherwise wild type background has only a small effect on cell size at cell division. In this respect, a striking parallelism exists between the *wee1* and *mik1* kinases on one side and the *cdc25* and *pyp3* phosphatases on the other. The *wee1* and *cdc25* gene products are clearly the dominant components of the control. Although *mik1* and *pyp3* are dispensable for cell cycle progression under normal circumstances, their more subtle roles might still have a significant impact on the long-term efficiency of cell proliferation. In addition there may be circumstances where *mik1* and *pyp3* have more important roles, for example by modulating the mitotic control in response to alterations in nutritional conditions or a checkpoint control, or perhaps in meiosis. Interestingly, Ogden and Fantes (1986) have identified a mutation, *win1-1*, that reverses the ability of *wee1-50* to suppress *cdc25-22*. Although this effect is heavily dependent on the prevailing nutritional conditions, many of the genetic effects observed for *win1-1* appear to be similar to those observed in this study for *pyp3*. Genetic studies, however, have shown that *win1* and *pyp3* are different genes (Peter Fantes, personal communication). However, the possibility remains that *win1* encodes a PTPase of the *pyp3* type. It is conceivable that the *win1* protein is the PTPase that dephosphorylates $\text{p34}^{\text{cdc}2}$ in a *cdc2-3w cdc25::LEU2 pyp3::ura4* strain.

Surprisingly little is known about the physiological roles of PTPases, their substrate specificity or how they are regulated. They have been broadly classified into two main groups; those that span the plasmamembrane and those that are generally believed to be cytosolic in origin (Fischer *et al.*, 1991). Although fission yeast *pyp1*, *pyp2* and *pyp3* PTPases clearly belong to the latter class, genetic evidence suggests that *pyp3* does not share an overlapping function with *pyp1* or *pyp2*, but in fact has an opposing effect on the timing of mitotic initiation. *Pyp3* acts as a mitotic inducer by direct $\text{p34}^{\text{cdc}2}$ interaction with $\text{p34}^{\text{cdc}2}$, whereas the *pyp1* and *pyp2* PTPases negatively regulate mitotic entry, probably by modulating the activity of the *wee1* kinase (Millar *et al.*, 1992).

A total of four tyrosine phosphatases have now been identified in fission yeast, including *cdc25* itself. These findings indicate a far more important and widespread role for regulation of protein function by tyrosine phosphorylation in mitotic control than has previously been suspected. We suspect that *pyp1*, *pyp2* and *pyp3* PTPases may also have other important cellular regulatory functions which remain to be elucidated. The genetic tractability of fission yeast provides an ideal opportunity by which the cellular function and regulation of the PTPases may be examined with the goal that such studies are likely to shed light on the role of their human counterparts.

Materials and methods

Media and general techniques

Media, genetic and molecular methods have been described elsewhere (Mitchison, 1970; Gutz *et al.*, 1974; Russell and Nurse, 1986; Sambrook *et al.*, 1989; Moreno *et al.*, 1991).

Cloning, mapping and DNA sequence analysis

Clones were isolated from a partial *Hind*III digest library constructed in the LEU2/ μm vector pDB262. Selection of *cdc25-22* complementing clones

and integrative genetic mapping were done as previously described (Russell and Nurse, 1986). DNA sequence analysis was performed using subclones in the vector pTZ18 (Pharmacia). Unidirectional deletions were made using exonuclease III and S1 nuclease (Henikoff, 1984). The DNA sequence was determined by the dideoxynucleotide method (Sanger *et al.*, 1977) using the T7 promoter primer (Promega Biotec) and custom-synthesized oligonucleotide primers.

Construction of a *pyp3::ura4* allele

The 3.1 kb *Hind*III fragment of p3w was cloned into pT7T3 18U after cleavage with *Hind*III to form pT7T3 18U-pyp3. This plasmid was cleaved with *Eco*RV, which removes all the catalytic domain and the *ura4* gene inserted by blunt end ligation to form pT7T3 18U-pyp3::ura4. The *ura4* fragment was isolated from pIRT2U-ura4 after cleavage with *Hind*III and the overhanging ends were filled in with Klenow DNA polymerase. The *Hind*III fragment containing *pyp3::ura4* sequence was used to disrupt one copy of *pyp3* in a *ade6-210/ade6-M216 leu1-32/leu1-32 ura4-D18/ura4-D18 h⁺/h⁻* diploid. Confirmation of the *pyp3::ura4* disruption was performed by Southern blot hybridization using the *Hind*III fragment of p3w.

Overexpression of *pyp3* in *S. pombe*

The *pyp3* cDNA was amplified by PCR from a *S. pombe* cDNA library. The 5' oligonucleotide CCGGATCCCCATATGTCTTTTAAAGAAGTATC, incorporating *Bam*HI and *Nde*I sites (shown italicized) hybridized to sequences surrounding the ATG initiation codon, whereas the 3' oligonucleotide CCGGATCCGAATTCATACTGAGGAAGAAGAAATTCC incorporating *Bam*HI and *Eco*RI sites (shown italicized) hybridized to sequences surrounding the TGA termination codon. PCR amplification generated a 909 nucleotide fragment that was cleaved with *Nde*I and *Bam*HI and cloned into pREP1 (Maundrell, 1990) to form pREP1-pyp3. pREP1-pyp3 was used to transform strains bearing the *leu1-32* mutation and leucine prototrophs selected. The phenotype of overexpression was determined after at least 48 h growth in the absence of thiamine.

Production and purification of bacterially expressed *S. pombe pyp3*

The full length *pyp3* gene was amplified from a cDNA library as described above and ligated into pGEX-2T (Smith and Johnson, 1988) after cleavage with *Bam*HI and *Eco*RI to form pGEX-2T-pyp3. DH5 *Escherichia coli* were transformed with pGEX-2T-pyp3 and GST-pyp3 protein isolated from overexpressing cells exactly as previously described (Millar *et al.*, 1991b). The eluate was dialyzed against 2 mM EDTA, 2 mM DTT, 25 mM imidazole, pH 7.2 and concentrated to a final protein concentration of between 1 and 5 mg/ml.

Phosphatase assays

pNPP assays were performed as previously described (Millar *et al.*, 1991b). TY peptide, a 19mer corresponding to amino acids 7–26 of fission yeast p34^{cdc2} (a gift of K. Gould and P. Nurse, ICRF, Oxford) was phosphorylated using GST-*wee1* (McGowan and Russell, 1992). 2D-phosphoamino acid analysis confirmed that the peptide was phosphorylated solely on tyrosine. Tyrosine phosphatase assays were performed in 50 mM β -mercaptoethanol, 5 mM EDTA, 50 mM imidazole-HCl, pH 7.2 containing 50 nM phosphorylated peptide (~50 000 c.p.m. per reaction). Free phosphate and phosphorylated peptide were resolved by thin layer chromatography (TLC) using cellulose F sheets (J.T. Baker Inc., NJ) in TLC buffer (isobutyric acid/butanol/acetic acid/water/pyridine; 65:2:5:29:3) and visualized by autoradiography (Boyle *et al.*, 1991).

Phosphotyrosine content and activity of p34^{cdc2}

Immunoprecipitations or affinity precipitations with p13^{suc1}-sepharose beads were performed essentially as previously described (Millar *et al.*, 1991b). *cdc25-22* strains were grown in YES medium at 25°C to early log phase (OD₆₀₀ = 0.2–0.6) and then grown for a further 4.5 h at 35.5°C. p34^{cdc2} was immunoprecipitated using either antiserum 9525 raised against a peptide corresponding to the C-terminus of *S. pombe* p34^{cdc2} (NH₂-KRALQQNYLRDFH-COOH) or p13^{suc1} sepharose, prepared as previously described (Brizuela *et al.*, 1987). Antibodies were precipitated with protein A-sepharose beads. Beads were washed three times with RIPA buffer and precipitated proteins resolved by SDS-PAGE and transferred electrophoretically to nitrocellulose membranes. Membranes were probed with either an antiserum raised against bacterially expressed full length p34^{cdc2} (9808-5) or with a monoclonal antibody to phosphotyrosine (PY20, ICN). Detection was performed using an alkaline peroxidase-conjugated anti-rabbit or anti-mouse IgGs (Promega, USA) and chemiluminescence visualization (ECL, Amersham) according to the manufacturer's instructions.

Dephosphorylation and activation of p34^{cdc2} in vitro

Inactive and tyrosine phosphorylated p34^{cdc2} bound to cyclin B was immunoprecipitated from *cdc25-22* cells arrested in late G₂ using antisera 9525 as above. Treatment with phosphatase *in vitro* and measurement of p34^{cdc2}-associated H1 kinase activity was also performed exactly as previously described (Millar *et al.*, 1991b).

SDS-gel electrophoresis

Slab gel electrophoresis was performed using a 6–15% acrylamide and 0.1% SDS gel. After electrophoresis, the slabs were fixed in 40% methanol, 7.5% glacial acetic acid and dried down on to Whatman 3 mm filter paper for autoradiography with Fuji X-ray film (Fuji Photo Co. Ltd, Japan). For most purposes, dried gels were exposed to films for 1–7 days.

Materials

The GST expression vector pGEX-2T and prepacked glutathione sepharose columns were purchased from Pharmacia LKB Biotechnologies. [γ -³²P]ATP (4500 Ci/mmol) were purchased from ICN Radiochemicals. Histone H1, aprotonin, leupeptin, DTT, PMSF and protein A-sepharose beads were from Boehringer Mannheim. pNPP was bought from Sigma. All other reagents were of the highest grade available.

Acknowledgements

Technical assistance was provided by Autumn Chapman. Krishna Sadhu performed the disruption of *pyp3*. We would like to thank Avelino Bueno and Clare McGowan for helpful discussions. J.B.A.M. is a Lucille P. Markey Visiting Fellow and is supported by the Lucille P. Markey Charitable Trust and by the Science and Engineering Research Council, UK. G.L. was supported by Fondation Pour La Recherche Medicale. This work was funded by an NIH grant awarded to P.R.

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Received on August 7, 1992; revised on September 18, 1992