Negative regulation of mitosis by two functionally overlapping PTPases in fission yeast

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We have identified a third protein tyrosine phosphatase (PTPase) gene in fission yeast, *pyp2*, encoding an 85 kDa protein. Disruption of *pyp2* has no impact on cell viability, but *pyp2* is essential in strains lacking the 60 kDa *pyp1* PTPase. The two *pyp* PTPases are ~42% identical in their C-terminal catalytic domains and share weak homology in their N-terminal regions. Both genes play a role in inhibiting the onset of mitosis. Disruption of either gene rescues the G₂ arrest caused by mutation of the *cdc25* mitotic inducer, though the effect of *pyp1*-disruption is more pronounced. Disruption of *pyp1* advances mitosis, suppresses overexpression of the tyrosine kinase encoded by the *wee1* mitotic inhibitor, and causes lethal mitotic catastrophe in *cdc25* overproducer cells. Cells bearing inactive *wee1* are unresponsive to disruption of *pyp1*. Overexpression of *pyp1* or *pyp2* delays the onset of mitosis by a *wee1*-dependent mechanism. These data reveal an unexpected second role for protein tyrosine phosphorylation in the mitotic control that acts by promoting the inhibitory *wee1* pathway.

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

Introduction

The cell cycle of the fission yeast *Schizosaccharomyces pombe* is controlled at two major transitions, one in late G₁, analogous to *START* in *Saccharomyces cerevisiae*, and the other in late G₂ that regulates entry into mitosis. During exponential growth the G₂/M transition is rate-limiting and ensures that mitosis and cell division are only permitted when the cells have attained a critical mass. Since *S.pombe* cells grow exclusively by length extension, changes in the control coordinating cellular growth with the G₂/M transition are reflected by modification of cell length at division. The molecular events that bring about the onset of mitosis are generally believed to be well conserved over a wide range of species and are centred around the activation of an evolutionarily conserved protein kinase complex, sometimes called M-phase promoting factor or MPF (reviewed by Nurse, 1990). MPF consists of a 34 kDa catalytic core and a larger regulatory subunit known as cyclin B. In *S.pombe* these components are encoded by the *cdc2* and *cdc13* genes, respectively. Activation of the p34^{cdc2}/cyclin kinase in

fission yeast is achieved by dephosphorylation of the 15th amino acid of the p34^{cdc2} subunit, a tyrosine residue (Gould and Nurse, 1989).

Genetic analyses of fission yeast have been exploited to identify the proteins regulating phosphorylation of p34^{cdc2}. Of particular interest are genes involved in regulating cell size at division and, by inference, the timing of p34^{cdc2}/cyclin activation. These include the *cdc25* gene product, p80^{cdc25}, a mitotic inducer which oscillates in level during the cell cycle (Russell and Nurse, 1986; Moreno *et al.*, 1990). *Cdc25* encodes a novel class of tyrosine phosphatase that dephosphorylates Tyr15 of p34^{cdc2} (Dunphy and Kumagai, 1991; Gautier *et al.*, 1991; Millar *et al.*, 1991; Strausfeld *et al.*, 1991; Lee *et al.*, 1992; Millar and Russell, 1992). The *cdc25* gene is counteracted at the genetic level by the *wee1* and *mik1* gene functions that operate in a co-ordinate fashion to negatively regulate mitotic entry (Russell and Nurse, 1987a; Lundgren *et al.*, 1991). The *wee1* gene encodes a tyrosine kinase, p107^{wee1}, that directly phosphorylates p34^{cdc2}/cyclin B on Tyr15 (Featherstone and Russell, 1991; Parker *et al.*, 1991, 1992; McGowan and Russell, 1993). Although deletion of *mik1* has no obvious effect on cell cycle control, *mik1* is essential when the *wee1* kinase is inactivated and, by this criteria, is also assumed to phosphorylate p34^{cdc2} directly. Inactivation of both kinases leads to dephosphorylation and activation of p34^{cdc2} that results in the initiation of a premature and inappropriate mitosis, termed mitotic catastrophe, that culminates in a dramatic loss of cell viability (Lundgren *et al.*, 1991).

The mechanism of mitotic entry in fission yeast is also of interest in being the only documented case of protein regulation by tyrosine phosphorylation in this unicellular organism. This contrasts with the pivotal role of tyrosyl phosphorylation in the transduction of extracellular signals leading to mitogenesis and cell proliferation of animal cells, and its deregulation in the neoplastic transformation of many malignant cancer cells (Hunter, 1989; Ullrich and Schlessinger, 1990). Indeed a great deal of attention has been focused on a number of receptor and non-receptor tyrosine kinases identified as the transforming proteins of the acutely oncogenic retroviruses (Hanks *et al.*, 1988; Cantley *et al.*, 1991). Molecular studies of their counterparts, the protein tyrosine phosphatases (PTPases), were initiated after the purification and microsequencing of a 35 kDa soluble PTPase from human placenta, PTP1B, which revealed a striking homology to the cytoplasmic domain of the leukocyte cell surface protein, CD45 (Tonks *et al.*, 1988; Charbonneau *et al.*, 1989). Realization that the PTPase family are closely structurally related in the catalytic domain sparked the rapid identification of a host of PTPases from a variety of species by homology screening, including one from fission yeast, *pyp1*, and two from budding yeast (Guan *et al.*, 1991, 1992; Ottilie *et al.*, 1991; Ota and Varshavsky, 1992). Although

to date no specific function has been ascribed to the yeast PTPases other than p80^{cdc25}, the discovery of yeast PTPases that more closely resemble PTP1B has raised the intriguing possibility that protein modification by tyrosine phosphorylation may play a more general role in the mechanisms of signal transduction and cell cycle progression in these organisms.

The PTPases have been broadly classified in two groups, those that possess a transmembrane spanning region and appear to be exclusively membrane-associated and those that are predominantly cytoplasmic in origin (Fischer *et al.*, 1991). Analogous to receptor-linked tyrosine kinases, the PTPases bearing extracellular domains are thought to transmit as-yet-unidentified signals. Less is known of the function or regulation of the non-transmembrane PTPases of which fission yeast *pyp1* is a member. Here we report the discovery of a second member of the non-transmembrane PTPase family in fission yeast, *pyp2*, that shares an essential overlapping role with *pyp1*. In addition, we have uncovered a surprising role for both PTPases in the control process regulating the onset of mitosis.

Results

Isolation of the *pyp2* gene

Disruption of the fission yeast *pyp1* gene does not alter cell viability, nor has any obvious cell cycle phenotype been ascribed to it (Ottillie *et al.*, 1991). Reasoning that *pyp1* might share an overlapping function with another PTPase gene, we continued our experiments aimed at isolating fission yeast PTPase genes using the polymerase chain reaction (PCR). Degenerate oligonucleotides were designed based on the conserved amino acid sequence found amongst members of the mammalian PTPase family. Among the 16 PCR clones sequenced, four clones represented an identical 0.4 kb sequence that bore high sequence homology to the PTPase family but was not identical to *pyp1*, and was thus named *pyp2*. The complete *pyp2* gene was isolated by screening a λ gt11 *S.pombe* genomic library. The nucleotide sequence and deduced amino acid sequence of *pyp2* are shown in Figure 1. The *pyp2* gene encodes a polypeptide having 711 amino acid residues with a predicted molecular mass of 85 kDa. This is somewhat larger than the *pyp1* gene which

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1  CTATCTTTTTGCACTTTTATATCCAGCGCACTTCGCGACTTCTATTTATTTGACAAAAGGAGCTTTCTCTGTTCAACATCAATAGGCAATACAAAGGAAAAATCGAGTTCAGTTT
121 TTCTTTTTCATCACAAAAAATAACCACTCATCCAAGAATACAAAATTTAATTTTGTGTTATTTCTGTTTATTAATAATTTATTTATTTGCTGTTGATTTCGTCTTTTCAAT
241 CAACAAGTTTCTCATTCCTGTTGTCACGTGCGCCAGGAGTTCTTTGCTTATTTTGTGCTGTTGATTGAACGCAAAAAGTCAATAATGTTATTTTATACAGTTTCTTCT
361 CTCTTACGTTTCCATTTTGTGTTATATCTTTTTTCCGAAAGCACACTGTAATAACTCAAAAATTTCTCTTAACCGGCACACCTTTCACACTCGCTGCTATCTTTTTTTCGTTTT
481 AATTGTGTGCCATTTAAAAGTTTCTGTATATATATTTAGCTTGGCCAACTGTGTCTGTGCTCTTACTTAGGATATAACATCTTCAAGGTGTTTTCAAAAATTTGTGCTACT

601 ATGCTCCATCTTCTGTCTAAGACGAATTTAACTCTACTCTTAAGTCTTTTGAAGACAAACCGAGAGCGTTTCTTGGATTATTGATTGCGCCTGCACCTCAAAATATGCTGTTAGCCAT
1  M L H L L S K D E F N S T L K S F E E Q T E S V S W I I D L R L H S K Y A V S H
721 ATAAAAATGCCATCAACGTTTCTTCCAACCTGCGTTGTTACGTGCGTCTTTTGACATTGGAAAGGTTTTCGCTGTATAAAATGCAACGTAAGTCTCGTTGGATGAATTAAT
41 I K N A I N V S L P T A L L R R P S F D I G K V F A C I K C N V K V S L D E I N
841 GCCATATCTATACGACTCTAGCATGGCTGGCATGAACGATTTATGATTGGTACAGAAAGTTTCGACGTGGTGGATATTCAAAAAATTTATTTATTAAGCAATGGATTGAAGCC
81 A I F L Y D S S M A G M N R I Y D L V Q K F R R G G Y S K K I Y L L S N G F E A
961 TTTGCTTCTCTCATCCGAGCGCATTTGTCTCTACCGAAATGGTCAAGGAGTCGGTCCCATACAAAATGACATCAATGAGAATTGCAAGCTGGATATCTTCATTTATCCGATCCATCT
121 F A S S H P G F S I V S T E M V K E S V P Y K I D I N E N C K L D I L H L S D P S
1081 GCGGTTTCTACCCCTATTTACCAGATTATGCTTTCCATTGAGAGTTCCTATTAACATCCCACCCTTTATGCACACCTTCGGTAGTCTCCGATACCTTTAGTGAGTTCGCGAGTCAT
161 A V S T P I S P D Y S F P L R V P I N I P P P L C T P S V V S D T F S E F A S H
1201 GCGGAATACCTGGATTTTCAGGTTTAAACCCGTTTCGATTCACTCTCTACTGCTTCTTCTGTTGCTGCTGCAATCTATATATGGTTACCCCTCTCTCCCCAAATTCAGCTTTT
201 A E Y P G F S G L T P F S I H S P T A S S V R S C Q S I Y G S K S P P N S A F
1321 CAAGCTGAAATGCCATATTTCCAATCTCTCCGCAATTTCTTGGCATCTTCTGTCTAGTAGCCTGATGAACAGAAAACTTTTTTATCGTAGGCAATGCCCTCAGCAAACTCCT
241 Q A E M P Y F P I S P A I S C A S S C P S T P D E Q K N F F I V G N A P Q Q T P
1441 GCCAGGCCATCTACGATCGGTGCTTCTATCCCTCCTCGAATAATCAGAGCGGCTTCTGCTCTCGGCTCGTAGCTTTAGCAACTATGTTAAATCCAGCAACGTCGTCATCCCA
281 A R P S L R V P S Y P S S N N Q R P S A S R V R S F S N Y K S S N V N P S
1561 AGTTGTCTCAAGCTTCTTGGAAATTTCCACGGAAGTCAATGAAACGTGATAGCAATGCACAGAATGATGGTACTAGTACGATGACAAGCAAACTTAACCACTCTGTGGTTTATCA
321 S L S Q A S L E I I P R K S M K R D S N A Q N D G T S T M T S K L K P S V G L S
1681 AACACACGAGATGCTCCAAACCGCGGTCTAAGAAGAGCTAACAAACCGTCTTTAATAAGAGACCAAGGAAGCAATTTCTCAAGGAAAAACAAAGGACCCCTTACTTGTATCC
361 N T R D A P K P G G L R R A N K P C F K E T K G S I F S K E N K G P F T C N P
1801 TGGGTGCCAAAAAGGTTTCTCTCTCTCTGTGAGGTGCTTGGGATTTAAATCTGCTTCTATTTTATAAGTTTAAAAGACTTGAGGAATGGAATGACTAGATCCCTAGCGTTT
401 W G A K K V S P P P C E V L A D L N T A S I F Y K F K R L E E M E M T R S L A F
1921 AATGACAGTAAATCTGATTGGTGTGTTAGCTTCCAGCCGCTCCACTTCCATTTCAAGAAAAATCGTTACACAGATATCGTCCCTACGATAAAACAGAGTCCGCTAGCCGTTC
441 N D S K S D W C C L A S S R S T S I S R K N R Y T D I V P Y D K T R V R L A V P
2041 AAAGGATGTTCTGATTATATTAATGCTTACATATAGCGTTGGAATAAAAAATATATGCTGCGCAGGCCCTAAGCCGGGAACCTTTTAGACTTTTGGGAAATGGTTTGGCATAAC
481 K G C S D Y I N A S H I D V G N K K Y I A C Q A P K P G T L L D F W E M V W H N
2161 TCAGGAACCAATGGTGTATCGTATGCTACCAAACTCTGATGAGGCGGAAGTGAAGATGTTCTCAATATTGGCCAGATAACAAAGATCAGCATTATGCTTGAAGGCGGATTACGC
521 S G T N G V I V M L T N L Y E A G S E K S Q Y W P D N K D H A L C L E G L R
2281 ATATCTGTTCAAAAATATGAAACCTTTGAAGATTGAAGGTCAACACTCATTTGTTTTCGATTGGATAAACCTAATGGTCTCCAAAGTATATACATCACTTTTGGGTGCACAGTGGTTT
561 I S V Q K Y E T F E D L K V N T H L F R L D K P N G P P K Y I H H F W V H T W F
2401 GACAAAACCATCCAGATATTGAAAGCATCACGGGAATCATAGTGTATTGATAAGTGTCCCAATGATGGCAATGTTCTGTTCACTGTTGACGAGCGTAGGACGCACTGGTACTTTT
601 D K T H P D I E S I T G I I R C I D K V P N D G P M F V H C S A G V G R T F
2521 ATTGCTGTAGACCAATACCTCAGGTACCAAAAAACATTTTACCAGACGACCAATTTGGAAGATTCAAAAGATTTCATATTCAATTGTGTTAACTCGTTGAGATCACACGGATGAAA
641 I A V D Q I L Q V P K N I L P K T T N L E D S K D F I F N C V N S L R S Q R M K
2641 ATGGTTCAAAACCTTTGAGCAATTCAAATTTCTACGACGCTGTGATTATTTAAATAGCGGCTTCAAGCCCTTGATGACTTAACGAAACGACTGTTCTTTAATTTTC
681 M V Q N F E Q F K F L Y D V V D Y L N S G V N Q A S K P L M T U
2761 TGTGTTGTACACACTATGTTCTATTTATGTGAGATTGTGAATTCCTCATTTTACATATTCTGACGCGAAGGTTATGTTAAAAACTTAACCACTCTCCGGAATCATGTGTA
2881 TTCTCTCTTATTGTTGTTAAAAATGATTTTATCTCATGCGTGAATGAATGTTATATAGTTATTAATGAATCTGTTTATGTTGGAATGGAATAATGTTAAAGACTAAATAGT

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Fig. 1. Sequence of *pyp2*. Nucleotide sequence and predicted amino acid sequence of *pyp2*. The deduced amino acid sequence (single letter code) is shown underneath the nucleotide sequence.

encodes a protein of predicted molecular mass of 60 kDa. Neither *pyp2* nor *pyp1* contain sequences that code for potential transmembrane spanning domains, and are thus unlikely to be integral membrane proteins. Northern hybridization of total mRNA from a wild type cell with a *pyp2*-specific probe indicates that the *pyp2* mRNA is ~2.6 kb, consistent with the size of the *pyp2* open reading frame (Figure 2).

Alignment of *pyp2* protein sequence with other members of the PTPase family reveals that the *pyp2* catalytic domain is located in the C-terminal half of the protein. The *pyp2*

Fig. 2. Northern blot of *pyp2* mRNA. Total RNA of *S.pombe* was hybridized with a *pyp2*-specific probe. Positions of small and large ribosomal RNAs are indicated.

Fig. 3. *pyp2* is homologous to the family of protein tyrosine phosphatases. The amino acid sequences of *pyp1* (middle) (Ottile *et al.*, 1991) and PTP1B from rat brain (bottom) are aligned with *pyp2* (top) (Guan *et al.*, 1990) using the PILEUP program of Wisconsin GCG. The conserved amino acid residues are shown in black. Gaps (blank space) were introduced to maximize the alignment. Sequence alignment starts at position 5 for *pyp2* and position 9 for *pyp1*.

sequence can best be aligned with the *pyp1* sequence. In the catalytic domain the *pyp2* protein shares ~42% sequence identity to *pyp1* and ~29% to PTP1 from rat brain (Figure 3). In addition, the *pyp2* gene product shares significant sequence homology to *pyp1* in the N-terminal half of the protein, but not to any other members of the PTPase family (Figure 3). Thus sequence comparison suggests that *pyp1* and *pyp2* PTPases are functionally related.

PTPase activity of *pyp2* protein

To demonstrate that the *pyp2* protein possesses PTPase activity, we endeavoured to express full length *pyp2* in *Escherichia coli* as a GST fusion protein. Unfortunately the fusion protein was insoluble. We have previously observed that the phosphatase domains of rat brain PTP1 and *cdc25* PTPase were more soluble than the full length protein when expressed in *E.coli* (Guan and Dixon, 1991b; Millar *et al.*, 1991). Thus a truncated version of *pyp2* containing the C-terminal catalytic domain was produced as a GST fusion protein. This fusion protein was soluble and was purified by glutathione affinity chromatography (Figure 4A). The upper band corresponds to GST-*pyp2* while the lower band corresponds to glutathione S-transferase, which was confirmed by immunoblotting using a GST-specific polyclonal antibody (data not shown). The fusion protein was active towards *p*-nitrophenylphosphate and tyrosine phosphorylated Raytide (Figure 4B and C), but was ineffective against two peptides H2 and H6 phosphorylated on serine and threonine residues respectively (data not shown). Kinetic analysis revealed that the GST-*pyp2* dephosphorylated pNPP with a V_{max} of 0.4 μ M/mg/min and a K_m of 15.8 mM. In addition the phosphatase activity of GST-*pyp2* was effectively inhibited by sodium orthovanadate (IC_{50} = 90 nM) and by $ZnCl_2$ (IC_{50} = 30 μ M), but not by high concentrations of NaF (50 mM), thus demonstrating that *pyp2* phosphatase is also sensitive to the same inhibitors as many previously described PTPases (Tonks *et al.*, 1988). On this basis we conclude that *pyp2* can be classified as an authentic member of the PTPase family.

Pyp1 and *pyp2* PTPases perform an essential function

To study the physiological function of *pyp2* protein, a gene disruption experiment was performed. A large fragment of the internal coding sequence of *pyp2* was replaced by the *ura4⁺* selectable marker in plasmid pGEM-*pyp2::ura4* (see Materials and methods). This construct deleted 71% of the coding sequence of *pyp2* and removed the entire catalytic domain. This construct was used to create a genomic disruption of *pyp2* in a diploid strain. Disruption of the endogenous *pyp2* gene was confirmed by Southern blot analysis (data not shown). Sporulation of this diploid and subsequent tetrad analysis showed that the *pyp2* gene is non-essential for vegetative growth. The *pyp2*-disruptant strains had a normal growth rate and by microscopic observation appeared identical to wild type.

The sequence similarity in the N-terminal halves of *pyp1* and *pyp2* proteins suggested that the two PTPases might function redundantly. To determine whether *pyp2* shares an overlapping function with *pyp1*, we attempted to create a strain having both PTPase genes disrupted. A *pyp1::ura4* strain was crossed to *pyp2::ura4* and 23 tetrads were dissected. Fourteen tetrads gave rise to four viable colonies that were all uracil prototrophs and thus could be assigned

Fig. 4. Tyrosine phosphatase activity of GST-pyp2. (A) SDS-PAGE analysis of purified GST-pyp2 fusion protein. The C-terminal phosphatase domain (residues 408–711) was expressed in fusion with glutathione S-transferase. C, crude extract of overexpressing *E. coli*; P, purified fusion protein; M, mol. wt markers (96, 66, 45, 31, 22 kDa from top to bottom). The GST-pyp2 fusion protein is indicated by an arrow. (B) Hydrolysis of pNPP. The hydrolysis of pNPP was monitored by increase in absorbance at 410 nm. The amount of fusion protein used in each assay is indicated. (C) Dephosphorylation of Raytide. Raytide was phosphorylated exclusively on tyrosine by p43^{v-abl} kinase. Phosphorylated Raytide was incubated with various concentrations of GST-pyp2 (as indicated) and dephosphorylation was measured by the release of ³²P.

as parental ditypes. The other nine tetrads produced three viable colonies and one single cell that could not be distinguished from an ungerminated spore. Of the three viable colonies in these nine tetrads, two were uracil prototrophs and the other a uracil auxotroph. This indicated

that the nine 3:1 tetrads were tetratypes in which the *pyp1::ura4 pyp2::ura4* spore failed to germinate. To confirm this, three tetrads bearing four *ura*⁺ colonies and six tetrads bearing two *ura*⁺ and one *ura*⁻ colonies were analysed by Southern blot hybridization using probes to the *pyp1* and *pyp2* genes. An illustrative Southern blot is shown in Figure 5. By this means tetrads with four viable colonies were confirmed as parental ditypes and tetrads with three viable colonies as tetratypes in which the spore lacking both PTPases was inviable. These results prove that *pyp1* and *pyp2* proteins perform at least one essential overlapping function. It should also be noted that the high frequency of parental ditypes and absence of non-parental ditypes in the *pyp1::ura4* × *pyp2::ura4* cross indicated that the two genes were genetically linked. We calculate that the two PTPase genes are separated by a genetic distance of ~20 cM.

Loss of *pyp1* advances the timing of mitosis

In the course of our experiments we noticed that cells lacking *pyp1* divided at a considerably smaller size ($10.4 \pm 0.2 \mu\text{m}$) than wild type ($14.1 \pm 0.2 \mu\text{m}$) (Table I). We term this phenotype semi-wee. The *pyp1::ura4* cells grew and divided as rapidly as *pyp1*⁺ cells, indicating that the semi-wee phenotype was specifically due to advancement of mitosis, as opposed to a growth defect. This possibility was tested by evaluating the interaction of *pyp1*-disruption with other mutations altering the mitotic control. For example, a key attribute of *wee1*⁻ mutations is their ability to rescue the cell cycle arrest phenotype arising from mutations that impair the function of the *cdc25* mitotic inducer (Fantès, 1981). To analyse whether this was true for *pyp1*, disruptants of *pyp1* were crossed to a *cdc25-22* strain. *Cdc25-22* is a temperature-sensitive mutation, such that at the permissive temperature of 26°C *cdc25-22* cells divide at a cell size (~20 μm) that is somewhat longer than wild type, while at 35.5°C these cells undergo cell cycle arrest in late G₂. At 26°C, *pyp1::ura4 cdc25-22* cells underwent division at a substantially smaller cell size (~12 μm) than did the *pyp1*⁺ *cdc25-22* control cells (Figure 6A and Table I). Moreover, growth of the double mutants at 35.5°C revealed that disruption of *pyp1* effectively suppressed the *cdc25-22* cell cycle arrest phenotype (Figure 6B and Table I). *Pyp1*-disruption did not have an epistatic interaction with *cdc25-22*, in that the double mutant cells divided at ~25 μm when grown at 35.5°C. This is consistent with the fact that *pyp1*-disruption does not cause a full *wee* phenotype. Overexpression of the mitotic inhibitor tyrosine kinase, p107^{wee1}, causes cells to cell cycle arrest as highly elongated cells. At the permissive temperature of 25°C, *pyp1*⁺ *adh:wee1-50* cells are unable to form colonies, whereas *pyp1::ura4 adh:wee1-50* cells undergo cell division at ~28 μm , approximately twice the size of wild type (Table I). These data show that *pyp1*-disruption causes an advancement of mitosis that operationally is similar to inactivation of the *wee1* mitotic inhibitor.

Pyp1 functions as a mitotic inhibitor in the *wee1* pathway

Mutations that advance mitosis, such as *pyp1*-disruption, are most likely to have their effect by causing premature activation of the p34^{cdc2}/cyclin B kinase. The activation is brought about by dephosphorylation of Tyr15 of the *cdc2* subunit. Since Tyr15 of p34^{cdc2} is the only site of tyrosine

Fig. 5. Disruption of both *pyp1* and *pyp2* is lethal. Genomic Southern to confirm *pyp1* and *pyp2* gene disruption. Results of two representative tetrads are shown. Tetrad A gave three viable spores (lanes 1, 2 and 3) while tetrad B gave four viable spores (lanes 4, 5, 6 and 7). Genomic DNA isolated from the three colonies of tetrad A and the four colonies of tetrad B were digested with *EcoRI* and then resolved on an agarose gel. The left half was hybridized with a *pyp2*-specific probe. The right half was hybridized with a *pyp1*-specific probe. *pyp2* and *pyp2::ura* represent the expected bands for *pyp2*⁺ and *pyp2*-disruptant, respectively. Similarly, *pyp1* and *pyp1::ura* represent the expected bands for *pyp1*⁺ and *pyp1*-disruptant.

phosphorylation in the complex, we discarded the possibility that p34^{cdc2}/cyclin B complex was a substrate of *pyp1* phosphatase. This left open two likely possibilities for how *pyp1* functions as a mitotic inhibitor. In one model *pyp1* PTPase negatively regulates the ability of p80^{cdc25} to dephosphorylate Tyr15 of p34^{cdc2}. Alternatively, *pyp1* PTPase might stimulate p107^{wee1} to phosphorylate Tyr15. These models were easily tested by determining whether the semi-wee phenotype caused by *pyp1*-disruption was additive with the wee phenotypes caused by overexpression of *cdc25* or inactivation of *wee1*. We first tested the latter possibility using the temperature-sensitive *wee1-50* mutation, which causes no phenotype at 25°C, whilst at 35.5°C it imparts a full wee phenotype with cells dividing at $7.6 \pm 0.4 \mu\text{m}$. *Pyp1::ura4 wee1-50* cells were semi-wee at 25°C, typical of *pyp1*-disruption in a *wee1*⁺ background. At 35.5°C, *pyp1::ura4 wee1-50* cells exhibited a classical wee phenotype, dividing at $7.8 \pm 0.1 \mu\text{m}$ (Table I). In all terms, including growth rate and cell viability, these cells were indistinguishable from *pyp1*⁺ *wee1-50* controls. An identical result is observed in cells that are wee because they overexpress the *nim1* kinase, which has been proposed to negatively regulate *wee1* function (Russell and Nurse, 1987b). *Pyp1::ura4 adh:nim1* and *pyp1*⁺ *adh:nim1* cells exhibited identical wee phenotypes (data not shown). These results show that cells lacking active *wee1* are unresponsive to *pyp1*-disruption, and thus support the conclusion that *pyp1* PTPase acts as a mitotic inhibitor by a mechanism that depends on *wee1*. Moreover, the data indicate that *pyp1*-disruption does not enhance the rate of tyrosyl dephosphorylation of p34^{cdc2}, since mitotic advancement by this mechanism would be expected to be further additive with inactivation of *wee1*. An ancillary conclusion is that loss of active *pyp1* does not have a significant negative impact on *mik1* kinase, since this mechanism would also be expected to have an additive interaction with *wee1-50*.

If the conclusion that disruption of *pyp1* impairs the *wee1*-dependent mechanism of tyrosine phosphorylation of

Table I. Loss of either *pyp1* or *pyp2* advances the timing of mitosis

	T (°C)	<i>pyp1</i> ⁺	<i>pyp1::ura4</i>	<i>pyp2::ura4</i>
<i>wt</i>	32	14.1 ± 0.2	10.4 ± 0.2	13.8 ± 0.3
<i>cdc25-22</i>	26	19.7 ± 0.5	12.2 ± 0.2	17.8 ± 0.5
	30	30.6 ± 1.0	n.d.	26.5 ± 0.8
<i>adh: wee1-50</i>	37	<i>cdc</i>	24.6 ± 2.9	35.9 ± 3.1 ^a
	25	<i>cdc</i>	28.5 ± 1.0	n.d.
<i>wee1-50</i>	25	14.4 ± 0.3	10.0 ± 0.5	12.9 ± 0.4
	35.5	7.6 ± 0.4	7.8 ± 0.1	7.5 ± 0.3
<i>nim1::LEU2</i>	32	17.3 ± 0.2	13.2 ± 0.2	n.d.

^aCell size measurement made in medium supplemented with 0.5 M sorbitol.

Cell size measurements of septated cells grown in liquid YES medium. 'cdc' denotes cell division cycle arrest; 'n.d.' denotes 'not determined'.

p34^{cdc2} was correct, we would then expect the semi-wee phenotype caused by *pyp1*-disruption to be additive with wee phenotype caused by overexpression of *cdc25*. For this experiment we used a strain having a copy of *cdc25* under the control of the thiamine-repressible *nmt1* promoter (*nmt:cdc25*). This strain exhibits a typical wee phenotype in media lacking thiamine. *Pyp1::ura4 nmt:cdc25* and *pyp1*⁺ *nmt:cdc25* cells were grown on thiamine-free medium for 4 days at 32°C. As the results in Figure 7A demonstrate, the growth rate of *pyp1::ura4 nmt:cdc25* cells was highly impaired relative to *pyp1*⁺ *nmt:cdc25* cells. Microscopic examination revealed that many of the *pyp1::ura4 nmt:cdc25* cells underwent lethal premature mitosis, i.e. mitotic catastrophe (Figure 7B). This phenotype was similar to that caused by simultaneous overexpression of *cdc25* and inactivation of *wee1* (Russell and Nurse, 1986), although not quite as severe. This is consistent with the semi-wee phenotype caused by *pyp1*-disruption. Together these results strongly suggest that *pyp1* acts as a mitotic inhibitor via the *wee1* tyrosine kinase pathway.

Fig. 6. *Cdc25-22* cell cycle arrest is rescued by disruption of *pyp1*. (A) Cells of the indicated genotype were cultured to log phase at 26°C. Photomicrographs show that *pyp1::ura4* causes *cdc25-22* cells to divide at a substantially smaller size. (B) Cells of the indicated genotype were streaked on plates and incubated for 3 days at 25°C or 35.5°C. Photographs show that *pyp1::ura4* rescues the cell division defect of *cdc25-22* cells grown at 35.5°C. The unlabelled strain is an additional control (*pyp3::ura4 cdc25-22*), see Millar et al. (1992).

Genetic evidence indicates that *pyp1* PTPase and *nim1* kinase regulate *wee1* kinase by independent mechanisms

Previous experiments have suggested that the *wee1* mitotic inhibition pathway is subject to negative regulation by the *nim1* protein kinase (Russell and Nurse, 1987b). Thus the conclusion that the *wee1* pathway is positively regulated by tyrosine dephosphorylation directed by *pyp1* raises the intriguing possibility that *nim1* and *pyp1* function counteractively to regulate the phosphorylation of the same site or sites. If this were true, and no other kinase phosphorylated these sites, then a prediction that follows is that disruption of *pyp1* should have no consequence in a strain lacking the *nim1* kinase. We tested this prediction by creating a *pyp1::ura4 nim1::LEU2* strain. *Nim1::LEU2* cells exhibit a mitotic delay, undergoing cell division at $17.3 \pm 0.2 \mu\text{m}$. The double mutant strain divided at $13.2 \pm 0.2 \mu\text{m}$, showing that *pyp1* PTPase was still acting to delay mitosis in cells lacking the *nim1* kinase (Table I). These data are most simply interpreted to indicate that *pyp1* and *nim1* proteins regulate the *wee1* kinase by different mechanisms.

Pyp2 also contributes to the inhibition of mitosis

Our observations that *pyp1* and *pyp2* PTPases are not only structurally related but also have an essential overlapping function, prompted us to examine the effect of a loss of *pyp2* on the timing of mitosis. In a wild type background *pyp2*-disruption had a negligible effect on cell size at division (Table I). For a more sensitive assay of the effect of *pyp2*-disruption on the mitotic control we constructed a *cdc25-22 pyp2::ura4* strain. At 30°C, *cdc25-22 pyp2*⁺ cells divided at $30.6 \pm 1.0 \mu\text{m}$, whereas *cdc25-22 pyp2::ura4*

Fig. 7. Overexpression of *cdc25* is additive with loss of *pyp1*. (A) Cells bearing an integrated copy of *nmt:cdc25* gene integrated behind the inducible *nmt1* promoter in either a *pyp1*⁺ background (top and bottom patches) or *pyp1::ura4* (left and right patches) were grown on minimal medium lacking thiamine for 48 h and streaked out on the same plate and allowed to grow for an additional 4 days at 32°C. (B) Microscopic examination of colonies from plate shown in A.

divided at $26.5 \pm 0.8 \mu\text{m}$ (Table I). A similar cell size differential was measured in cells growing at 26°C . The decrease in cell division size observed in the *pyp2*-disruption background indicated that *pyp2* has a weak role as a mitotic inhibitor. This conclusion was supported by the observation that *cdc25-22 pyp2::ura4* cells formed colonies when incubated at 36.5°C on rich medium in the presence of sorbitol (Figure 8), whereas *cdc25-22 pyp2*⁺ cells died or infrequently formed microcolonies under identical conditions. These results taken together suggest that both the *pyp1* and *pyp2* PTPases act to negatively regulate mitotic entry, with *pyp1* having the dominant role. Note that a *pyp2::ura4 weel-50* double mutant was no smaller than *pyp2*⁺ *weel-50* cells when grown at 35.5°C (Table I), consistent with the idea that *pyp2* exerts its effect on mitosis by regulating *weel* kinase. This result also suggests that *pyp2*-disruption does not have a significant effect on *mik1* kinase activity.

Overexpression of *pyp2* delays the onset of mitosis

Overexpression of *cdc25* or human T cell PTPase in *S.pombe* causes mitosis to initiate early, consistent with accelerated tyrosyl dephosphorylation of the p34^{cdc2} kinase (Russell and Nurse, 1986; Gould *et al.*, 1990). Our observations showing that deletion of either *pyp1* or *pyp2* also advances mitotic initiation, prompted us to examine the effect of overexpressing these genes. At present we lack a genomic clone of *pyp1*, therefore we were unable to perform dosage experiments with this gene. Transformation of cells with a multicopy vector (about five copies per cell) having a genomic clone of *pyp2* had no apparent effect on cell size or growth rate, but this was perhaps to be expected given the weak effect of *pyp2*-disruption. To examine the phenotype arising from higher overexpression of *pyp2*, we cloned the *pyp2* open reading frame downstream of the thiamine-repressible *nmt1* promoter. Northern analysis confirmed that *pyp2* mRNA was greatly overexpressed when *nmt:pyp2* cells were grown in media lacking thiamine (data

not shown). Induction of *pyp2* expression appeared to slow cellular growth, such that the doubling rate in medium lacking thiamine was $\sim 25\%$ slower than that seen in cells grown in medium containing thiamine. Control cultures, transformed with a similar vector lacking *pyp2* open reading frame, grew at the faster growth rate in the absence or presence of thiamine. Induction of *pyp2* expression also caused a significant increase in cell size at division (Table II). In media lacking thiamine the *nmt:pyp2* cells divided at $18.4 \pm 1.2 \mu\text{m}$, whereas control cells grown in the same media underwent division at $14.6 \pm 0.6 \mu\text{m}$. Consistent with the proposal that *pyp2* inhibits mitosis in a *weel*-dependent mechanism, we observed that the *pyp2* overexpression had no significant effect on cell division size in a *weel-50* strain grown at 35.5°C (Table II). However, inactivation of *weel* did not suppress the cell growth defect due to *pyp2* overexpression. This suggests that the two phenotypes arising from *pyp2* overexpression, namely the cell division size increase and cell growth defect, occur by different mechanisms.

The cell cycle delay caused by *pyp2* overexpression was greatly accentuated in *cdc25-22* cells grown at the permissive temperature (Table II). Preliminary results have shown that *pyp1* overexpression via the thiamine-repressible *nmt1* promoter causes an identical phenotype (data not shown). In addition, overexpression of *pyp1* driven by the strong *adh* promoter has been found to cause cell cycle arrest in a *cdc25-22* background (S.Ottile, personal communication). These data reinforce the conclusion that *pyp1* and *pyp2* contribute to mitotic inhibition in fission yeast. Moreover, they indicate that the *pyp1* and *pyp2* substrate that is involved in regulating mitosis is not fully dephosphorylated in wild type cells. Experiments are underway to clone a genomic copy of *pyp1*, so that gene dosage experiments can be performed.

Discussion

In this study we have established two important facts. The first is that *pyp1* and the newly identified PTPase gene *pyp2* share at least one essential function in the life cycle of fission yeast. This is the first evidence that PTPases of the PTP1B-like class have a critical function in yeast. The other important fact to emerge from these studies is that *pyp1*, and to a lesser extent *pyp2*, are involved in a control that inhibits the onset of mitosis. Remarkably these PTPases function to counteract the third PTPase known in fission yeast, the *cdc25* mitotic inducer. The fact that all three yeast PTPases operate in cell cycle controls, indicates that tyrosine phosphorylation plays a more general role in the regulation of growth and division in yeast than was previously suspected. Furthermore

Fig. 8. Disruption of *pyp2* rescues *cdc25-22*. *Cdc25-22* cells (top), *cdc25-22 pyp1::ura4* cells (bottom right) or *cdc25-22 pyp2::ura4* cells (bottom left) were patched on to the centre of a plate containing YES medium supplemented with sorbitol at the permissive temperature of 25°C . The patches were then streaked and the plate incubated at 36.5°C for 4 days.

Table II. Overexpression of *pyp2* delays the onset of mitosis

	T ($^\circ\text{C}$)	pREP3	pREP3- <i>pyp2</i>
<i>wt</i>	32	14.6 ± 0.6	18.4 ± 1.2
<i>cdc25-22</i>	26	21.9 ± 1.4	32.4 ± 3.1
<i>weel-50</i>	35.5	7.8 ± 1.2	8.6 ± 1.4
<i>cdc2-33</i>	35.5	<i>cdc</i> ⁻	<i>cdc</i> ⁻

Cell size measurements of septated cells grown in liquid EMM medium supplemented with thiamine as indicated (Maudrell, 1990).

it raises the exciting possibility that some of the elements of tyrosine-mediated signal transduction in more complex organisms may occur in yeast.

The first hint that fission yeast PTPases were involved in delaying the onset of mitosis was the observation that *pyp1*-disruption cells divide at a reduced cell size, intermediate between wild type and *wee* cells. The fact that *pyp1*-disruption effectively rescues the cell cycle arrest phenotype due to *cdc25*⁻ mutants or overexpression of the mitotic inhibitory p107^{wee1} kinase genetically confirmed that *pyp1* PTPase contributed to the inhibition of mitosis. We considered that advancement in the timing of mitosis could, in principle, be attributed to either activation of *cdc25* mitotic induction pathway or partial inhibition of *wee1* mitotic induction pathway. To distinguish between these possibilities the loss of *pyp1* function was analysed in a variety of mutants altered in their ability to undergo mitotic initiation. The following lines of evidence suggest that disruption of the *pyp1* gene causes a partial loss of *wee1* function. First, loss of *pyp1* function has no effect in the absence of *wee1*, suggesting that *pyp1* acts to inhibit mitosis by a *wee1*-dependent mechanism. Secondly, overexpression of the *cdc25* phosphatase causes mitotic catastrophe in *pyp1*-disruption cells in the same fashion that overexpression of *cdc25* and inactivation of *wee1* causes mitotic catastrophe (Russell and Nurse, 1986).

The evidence also demonstrates that *pyp2* contributes to the inhibition of mitosis, although its role is smaller than *pyp1*. *Pyp2*-disruptants did not have a significantly reduced cell size phenotype in a wild type background, but the *pyp2*-disruption does exhibit an ability to weakly suppress *cdc25*-22. This result, coupled with the fact that *pyp2* overexpression delays the onset of mitosis by a *wee1*-dependent mechanism, supports the conclusion that *pyp2* also acts to inhibit the onset of mitosis. Together these data indicate that *pyp1* and *pyp2* perform a phenotypically similar function in negatively regulating mitotic initiation. At this time it is unclear whether this overlapping function is essential, as both PTPases also appear to share a function which is required for spore germination.

The question arises as to whether *wee1* kinase is the substrate of *pyp1* and *pyp2* PTPases *in vivo*. Intriguingly, p107^{wee1} autophosphorylates on serine and tyrosine residues *in vitro* (Featherstone and Russell, 1991). However, only phosphoserine has been detected from p107^{wee1} labelled *in vivo* in *S.pombe*. One attractive possibility is that p107^{wee1} autophosphorylates on tyrosine *in vivo*, but undergoes rapid dephosphorylation by the *pyp1* and *pyp2* PTPases, such that the steady state level of phosphotyrosine from p107^{wee1} is very low. Initial phosphoamino acid analysis of p107^{wee1} in strains overexpressing *wee1* and lacking *pyp1* did not reveal any phosphotyrosine (J.B.A. Millar and P. Russell, unpublished results), suggesting that the effect of *pyp1* PTPase on p107^{wee1} may be indirect. Perhaps a less likely explanation is that *pyp2* is sufficient to prevent detection of phosphotyrosine on p107^{wee1}. Construction of conditional mutations of *pyp1* or *pyp2* will allow us to test these possibilities.

The *in vivo* labelling experiments mentioned above need to be cautiously interpreted because they have utilized strains that overexpress *wee1*. These strains have been used because *wee1* is normally poorly expressed and technical limitations have thus far prevented the detection of p107^{wee1} as a phosphoprotein from wild type cells. Therefore it remains

possible that the *wee1* gene product is phosphorylated by a tyrosine kinase whose ability to phosphorylate p107^{wee1} is greatly exceeded in cells overexpressing *wee1*. If this is the case we may be best advised to rely on a genetic approach to identify the kinase that functions in opposition to *pyp1* and *pyp2*. One attractive possibility is that this kinase is encoded by *nim1*, which is believed to function as a mitotic inducer by inhibiting the *wee1* pathway (Russell and Nurse, 1987b). At this time it is not known whether *nim1* kinase directly phosphorylates p107^{wee1}. Regardless of this, the fact that *pyp1*-disruption advances mitosis in a *nim1*-disruption strain argues against a simple model in which *nim1* kinase alone phosphorylates a substrate that is then dephosphorylated by *pyp1* and *pyp2* PTPases.

Another issue which should be considered is the relationship between *mik1* kinase and the PTPases encoded by *pyp1* and *pyp2*. Since the mitotic advancement phenotype caused by *pyp1*-disruption is much more profound than *mik1*-disruption, we can conclude that *mik1* kinase is not the primary target of *pyp1* PTPase. By the same token, since *mik1*-disruption causes a mitotic catastrophe in a *wee1*⁻ background (Lundgren *et al.*, 1991), whereas *pyp1*-disruption has no effect, we can conclude that *pyp1*-disruption does not have a significant negative impact on *mik1* kinase activity. Likewise, *pyp2*-disruption has no apparent additive effect with *wee1*⁻, so it would appear that *pyp2*-disruption also does not result in a significant inhibition of *mik1* activity. These data argue strongly against the notion that *pyp1* and *pyp2* PTPases regulate *mik1* kinase.

Our observations concerning the roles of *pyp1* and *pyp2* have been incorporated into a model of the mitotic control as diagrammed in Figure 9. The biochemical roles of *wee1* kinase and *cdc25* phosphatase in directly controlling the tyrosyl phosphorylation of p34^{cdc2} are documented. The *mik1* kinase very likely also carries out Tyr15 phosphorylation of p34^{cdc2} in a junior capacity to *wee1*

Fig. 9. Mitotic control model. A model incorporating the proposed roles of *pyp1* and *pyp2* PTPases in the control of mitosis. Our genetic data indicate that *pyp1* and *pyp2* gene products contribute to the inhibition of mitosis by enhancing the activity of *wee1* kinase. *Pyp1* has the dominant role. *Wee1* kinase might be the direct substrate of the PTPases, although the available evidence indicates that p107^{wee1} is not phosphorylated on tyrosine *in vivo*. Our data also indicate that the *pyp1* and *pyp2* PTPases do not significantly modulate *mik1* kinase, although they could have minor effects. Our data are most consistent with *pyp1* PTPase and *nim1* kinase regulating the phosphorylation of different sites, although this conclusion may be invalid if *nim1* shares overlapping function with another kinase. See text for elaboration and references.

kinase, although this remains to be proven. Genetic data is strongly in favour of the *nim1* kinase acting as a mitotic inducer by blocking *wee1* kinase function, perhaps by a direct mechanism. The *pyp1* and *pyp2* phosphatases act as mitotic inhibitors by a *wee1*-dependent mechanism, but elucidation of the biochemical process by which this occurs will require further experimentation.

Only a few years ago it was generally believed that one of the primary differences between yeasts and mammalian cells was the importance that tyrosine phosphorylation played in regulating cell proliferation. Indeed prior to the work of Gould and Nurse (1989), reporting inhibitory tyrosine phosphorylation of p34^{cdc2} in *S.pombe*, there was no evidence for the existence of any biologically significant tyrosine phosphorylation in yeast. As documented in this report, the perceived significance of tyrosine phosphorylation in regulating cell cycle control in yeast is growing as the understanding of cell cycle control processes improves. Although recent investigations have revealed some species differences in the details of mitotic control, the overwhelming impression is still of very strong conservation of the basic processes regulating the cell cycle. One of the latest examples of this is the discovery of a human gene encoding a protein kinase with homology to *S.pombe wee1* kinase that not only delays the onset of mitosis when expressed in fission yeast (Igarashi *et al.*, 1991), but also causes cell cycle arrest when overexpressed in human cells and phosphorylates Tyr15 of human p34^{cdc2} *in vitro* (McGowan and Russell, 1993). On the basis of this experience it is likely that human PTPases exist that function as important mitotic inhibitors in a manner similar to *pyp1* and *pyp2* proteins.

Materials and methods

Media and general techniques

Media and genetic methods for studying fission yeast have been reviewed recently (Moreno *et al.*, 1991). General DNA methods were performed using standard techniques (Sambrook *et al.*, 1989). Cell length measurements were made using log-phase cells and an eyepiece drum micrometer at 1200× magnification.

PCR amplification and cloning

Two degenerate oligonucleotides were designed based on the conserved amino acid sequences of YIA^A/TQGP and HCSAGVG. The sequences of the oligonucleotides are 5'-CCGAGGATCCTA^C/TAT^A/TGT^A/GCNC-^A/G^A/GGNCC and 5'-GCACGGATCCCC^A/GACACNGCA^T/C^G/T^A/G^A/GTG. PCR was performed using Gene Amp Kit (Perkin-Elmer Cetus), 1 µg of each primer, 1 µg of *S.pombe* genomic DNA (Clontech) as template. The two initial cycles were 92°C for 2 min; 35°C for 2 min; 72°C for 2 min, followed by 30 cycles in which annealing was at 42°C for 2 min. The PCR products were digested with *Bam*HI and subcloned into M13mp18 for sequencing. Sixteen PCR clones were sequenced and four encoded for a putative new PTPase, *pyp2*.

A λgt11 *S.pombe* genomic library (Clontech) was screened with radiolabelled (Multiprimer DNA labelling kit, Amersham) *pyp2* PCR product (0.4 kb) following standard procedures (Sambrook *et al.*, 1989). Four positive clones (*pyp2*-1 to *pyp2*-4) were isolated. Clone *pyp2*-1 which contains a 5.1 kb *Eco*RI fragment was further characterized and sequenced. The sequence presented in Figure 1 was determined by both orientations using Sequenase (US Biochemicals). Sequence comparison was performed by the Wisconsin GCG software.

Expression of *pyp2* in bacteria

In order to express *pyp2* in *E.coli*, restriction sites *Eco*RI and *Sal*I were introduced into the 5'-end and 3'-end of the *pyp2* gene respectively. This was achieved by PCR amplification using the 5' primer, GCCGAATTCTCCATCTTCTGTCTAA, containing an *Eco*RI site (italicized) and a 3' primer CGCCGTCGACCTTCGGCTCAGATAAT containing a *Sal*I site (italicized) to amplify the entire coding sequence of *pyp2*. The PCR product was digested with *Eco*RI and *Sal*I and subcloned into pGEX-KG (Guan and Dixon, 1991a) to produce pKG-*pyp2*. The plasmid

pKG-*pyp2* was used to express full length *pyp2* protein as a fusion protein with glutathione S-transferase. An internal primer, GCCGAATTCTCC-TCCTTGTGAGG, also bearing an *Eco*RI site (italicized) together with the 3' primer described above were used to amplify the PTPase catalytic domain of *pyp2* by PCR. The resulting construct pKG-*pyp2*ptp can express the truncated form of *pyp2* (including amino acids 408–711) in fusion with glutathione S-transferase. Both the full length and the PTPase domain of *pyp2* were expressed and the fusion protein purified by glutathione agarose affinity chromatography as previously described (Guan and Dixon, 1991a).

Phosphatase assays

Phosphatase assay was performed as previously described (Streuli *et al.*, 1990). Substrates of Raytide (phosphorylated on tyrosine) (Oncogene Science), H2 and H6 peptides (phosphorylated by protein kinase A on serine and threonine, respectively) and *p*-nitrophenyl phosphate were utilized to assay recombinant *pyp2* activity. The amino acid sequence of peptide H2 and H6 are PLSTRTLVSASPGL and PLSTRTLVSSLPLGL, respectively.

Construction of *pyp1::ura4* and *pyp2::ura4* alleles

The *pyp1* gene was cloned by PCR amplification from an *S.pombe* cDNA library. The 5' oligonucleotide CCGCTGCAGGATCCACGATGAATTT-TTCAAACG, incorporating *Pst*I and *Bam*HI sites (shown italicized) hybridized to sequences surrounding the ATG initiation codon, whereas the 3' oligonucleotide GGCCTGCAGTCATGTTAAACCGGGAAAT incorporating a *Pst*I site (shown italicized) hybridized to sequences surrounding the TGA termination codon. PCR amplification generated a 1674 nucleotide fragment that was cleaved with *Pst*I and cloned into pUC12. Plasmid pUC12-*pyp1*-10 had the *Bam*HI sites of the pUC12 and the *pyp1* PCR fragment in close proximity. This plasmid was cleaved with *Bam*HI and religated to form pUC12-*pyp1*-10B. This plasmid was cleaved at the single *Sal*I site located in the *pyp1* open reading frame and a 1.66 kb *Xho*I fragment containing the *S.pombe ura4*⁺ gene was ligated into the *Sal*I site to form pUC12-*pyp1::ura4*. The *ura4* DNA fragment was derived from pTZ19-*ura4-Sac*I/*Xho*I (P.Russell, unpublished data). Plasmid pUC12-*pyp1::ura4* was cleaved with *Sac*I and used to transform a *leu1*-32 *ura4*-D18 h⁺ strain. Stable uracil prototrophs were selected. Confirmation of the *pyp1::ura4* disruption was determined by PCR amplification using the oligonucleotides described above and by Southern blot analysis of genomic DNA.

The 5.1 kb fragment containing the *pyp2* gene was subcloned into the *Eco*RI site of vector pGEM-3Z (Promega) to construct pGEM-*pyp2*. The *ura4* marker was obtained by *Bam*HI digestion of pURA4-*Bam*. Plasmid pGEM-*pyp2* was digested with *Hpa*I and *Nru*I which deletes a 1.5 kb coding sequence of *pyp2*, and blunt ends created with the large fragment of DNA polymerase 1. The 1.8 kb *Bam*HI fragment of *ura4* was subcloned into the *Hpa*I/*Nru*I digested pGEM-*pyp2* to create pGEM-*pyp2::ura4*.

Plasmid pGEM-*pyp2::ura4* was digested with *Eco*RI and transformed into *S.pombe leu1*-32/*leu1*-32 *ade6*-210/*ade6*-216 *ura4*-D18/*ura4*-D18 h⁺/h⁺ by lithium acetate transformation. Uracil prototrophs were selected on medium lacking uracil, and genomic DNA subjected to Southern blot hybridization using a *pyp2*-specific probe. Two colonies bearing a disruption of *pyp2* were plated on to minimal medium containing uracil and leucine at a density of 500 cells per plate. After 3 days a replica plate was made and one set stained with iodine to identify spontaneous conversion of h⁺/h⁺ to h⁺/h⁹⁰ which was able to sporulate. Colonies which gave positive iodine staining were used for tetrad dissection. The linearized plasmid pGEM-*pyp2::ura4* was also transformed into a *S.pombe leu1*-32 *ura4*-D18 *ade6*-216 h⁺ strain. Disruption of *pyp2* in this strain was also confirmed by genomic Southern blot analysis.

To construct *pyp1* and *pyp2* double deletion, *pyp2::ura4 leu1*-32 *ura4*-D18 *ade6*-216 h⁺ was crossed to *pyp1::ura4 leu1*-32 *ura4*-D18 *ade6*-216 h⁺ to create a diploid which was sporulated and the tetrads analysed (see text).

Overexpression of *pyp2*

The *pyp2* coding sequence was amplified by PCR using a 5' primer CTG-CGGATCCACTATGCTCCATCTTCTGTGTC and a 3' primer CTGCGGA-TCCACATTCATTACACGCATGAG both incorporating a *Bam*HI site (shown italicized). PCR amplification generated a 2.3 kb fragment that was digested with *Bam*HI and inserted into the *Bam*HI site of pREP3 (Maundrell, 1990), bearing the selectable *LEU2* marker and the inducible *nmt1* promoter to form pREP3-*pyp2*. Plasmids pREP3 and pREP3-*pyp2* were used to transform strains bearing the *S.pombe leu1*-32 mutation as described and leucine prototrophs selected.

DNA, RNA isolation and hybridization

Schizosaccharomyces pombe cells were cultured in YEA medium (0.5% yeast extract, 3% glucose, 50 mg/l adenine) to stationary phase. Chromosomal DNA isolated from a 10 ml culture was dissolved in 25 µm

of TE, of which one fifth was digested with *EcoRI* and subjected to electrophoresis and Southern blot hybridization. The 5.1 kb *EcoRI* fragment of *pyp2* gene was radioactively labelled and used as a probe. To isolate RNA, *S.pombe* cells were cultured in YEA to exponential growth phase. Approximately 40 µg of total RNA was resolved by formaldehyde agarose gel electrophoresis and transferred to nitrocellulose for hybridization.

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