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A multicopy suppressor of a cell cycle defect in *S.pombe* encodes a heat shock-inducible 40 kDa cyclophilin-like protein

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Cyclophilins are peptidyl-prolyl *cis–trans* isomerases (PPIases) which have been implicated in intracellular protein folding, transport and assembly. Cyclophilins are also known as the intracellular receptors for the immunosuppressive drug cyclosporin A (CsA). The most common type of cyclophilins are the 18 kDa cytosolic proteins containing only the highly conserved core domain for PPIase and CsA binding activities. The *wis2*$^+$ gene of the fission yeast *Schizosaccharomyces pombe* was isolated as a multicopy suppressor of *wee1-50 cdc25-22 win1-1*, a triple mutant strain which exhibits a cell cycle defect phenotype. Sequence analysis of *wis2*$^+$ reveals that it encodes a 40 kDa cyclophilin-like protein, homologous to the mammalian cyclophilin 40. The 18 kDa cyclophilin domain (CyP-18) of wis2 is followed by a C-terminal region of 188 amino acids. The C-terminal region of wis2 is essential for suppression of the triple mutant defect. Furthermore, this region of the protein is able to confer suppression activity on the 18 kDa *S.pombe* cyclophilin, cyp1, since a hybrid protein consisting of an 18 kDa *S.pombe* cyclophilin (cyp1) fused to the C-terminus of wis2 shows suppression activity. We also demonstrate that the level of *wis2*$^+$ mRNA increases 10- to 20-fold upon heat shock of *S.pombe* cells, suggesting a role for *wis2*$^+$ in the heat-shock response.

Keywords: cell cycle/cyclophilin/fission yeast/heat shock/*S.pombe*

Introduction

Cyclophilins form a ubiquitous and highly conserved group of proteins, found in all prokaryotic and eukaryotic cells investigated. The first cyclophilin discovered was a cytosolic 18 kDa bovine protein which displayed strong binding affinity to the immunosuppressive drug cyclosporin A (CsA; Handschumacher *et al.*, 1984). This protein was later shown to be identical to a previously identified peptidyl-prolyl *cis–trans* isomerase (PPIase; Fischer *et al.*, 1989; Takahashi *et al.*, 1989).

In vitro, cyclophilins accelerate rate-limiting *cis–trans* peptidyl-prolyl isomerization steps in the folding pathway of peptide and protein substrates (Lang *et al.*, 1987; Takahashi *et al.*, 1989). It has also been suggested that, in addition to the isomerase activity, cyclophilins affect the yield and kinetics of protein folding *in vitro* by preventing the aggregation of early folding intermediates of protein substrates (Freskgard *et al.*, 1992). The data on cyclophilin function *in vivo* is fragmentary, but several studies have implicated cyclophilins in protein folding, transport and assembly (reviewed in Stamnes *et al.*, 1992; Galat, 1993). The best example of this is the photoreceptor-specific *Drosophila melanogaster* cyclophilin, ninaA, which is essential for the proper folding and intracellular transport of the photopigment rhodopsin, Rh1 (Ondek *et al.*, 1992). In support of a chaperone function for ninaA is the recent finding that it forms a stable complex with Rh1, and that maturation of Rh1 is dependent on the levels of ninaA (Baker *et al.*, 1994). Another line of investigation which lends support to the function of cyclophilins as molecular chaperones is the finding that the levels of mRNA of several members of this family are increased upon heat shock (Sykes *et al.*, 1993; Luan *et al.*, 1994). Disruption of either of the *Saccharomyces cerevisiae* cyclophilin genes *CYP1* or *CYP2* reduces cell survival following exposure to high temperature, providing further evidence for a functional role of cyclophilins during heat shock (Sykes *et al.*, 1993).

Cyclophilins share the property of PPIase activity with another, structurally unrelated family of proteins, the FKBPs, which have strong binding affinity to the immunosuppressive agent FK506 (reviewed in Galat, 1993). Collectively, cyclophilins and FKBPs are known as immunophilins. When bound to its cognate drug, the PPIase activity of the immunophilin is abolished, but loss of PPIase activity does not appear to be responsible for the immunosuppression effect of the drugs. Rather, the mechanism of action of CsA and FK506 involves the formation of CsA–cyclophilin and FKBP–FK506 complexes, which inhibit the activity of the Ca^{2+}/calmodulin-dependent phosphatase calcineurin. The inhibition of calcineurin activity is thought to disrupt the T-cell receptor signal transduction pathway, which normally leads to cytokine gene transcription (Friedman and Weissman, 1991; Liu *et al.*, 1991a; Clipstone and Crabtree, 1992). The immunophilin–drug complexes also block Ca^{2+}-dependent signal transduction pathways in yeast (Foor *et al.*, 1992) and plant cells (Luan *et al.*, 1993). First it was thought that neither cyclophilins nor FKBPs bind calcineurin in the absence of the cognate drug (Liu *et al.*, 1991a). However, more recent research (Cardenas *et al.*, 1994) has demonstrated physical interaction between the *S.cerevisiae* immunophilin FKBP12 and calcineurin, and between cyclophilin A and calcineurin, in the absence of FK506 and CsA respectively. Moreover, genetic analysis in *S.cerevisiae* demonstrated related phenotypes of calcineurin,

FKBP12 and cyclophilin A. For example, calcineurin mutations prevented recovery from pheromone arrest and conferred hypersensitivity to LiCl, whereas FKBP12 mutants increased recovery from pheromone arrest and enhanced LiCl resistance, suggesting that FKBP12 normally acts to inhibit calcineurin (Cardenas *et al.*, 1994).

The family of cyclophilins has been expanding rapidly, revealing a high degree of sequence conservation across the 18 kDa domain containing the PPIase and CsA binding activity (CyP-18 domain), compared with the diversity of flanking amino acid sequences (Stamnes *et al.*, 1992). Short amino acid extensions at the N- and C-termini of the CyP-18 domain have been shown to play a role in targeting cyclophilins to their specific subcellular localization, such as the endoplasmic reticulum or the mitochondria (reviewed in Galat, 1993). Several proteins have also been identified that contain a CyP-18 domain in addition to other functional regions (Anderson *et al.*, 1993; Galat, 1993).

Here we describe a tentative link between cyclophilin function and the cell cycle. We have been characterizing the *Schizosaccharomyces pombe* $wis1^+$–$wis5^+$ genes which were isolated as multicopy suppressors of the cell cycle defect of the strain *wee1-50 cdc25-22 win1-1* (Warbrick and Fantes, 1992). The wee1 and cdc25 proteins are negative and positive regulators, respectively, of the cdc2 protein kinase, the universal key regulator of entry into mitosis (reviewed in Nurse, 1990). The activity of cdc2 at the G_2–mitosis transition is regulated by binding to a regulatory mitotic cyclin (encoded in *S.pombe* by the $cdc13^+$ gene) and by reversible phosphorylation (Gould and Nurse, 1989; reviewed in Nurse, 1990). The $cdc25^+$ gene product of *S.pombe* is a protein tyrosine phosphatase which dephosphorylates cdc2 on Tyr15, thereby activating the cdc2 kinase activity (Moreno *et al.*, 1989; Millar *et al.*, 1991). The $wee1^+$ gene product is a protein kinase which phosphorylates cdc2 on Tyr15, thereby rendering cdc2 inactive (Featherstone and Russell, 1990; Parker *et al.*, 1991). The $win1^+$ gene has been implicated in regulating entry into mitosis on the basis of genetic interactions (Ogden and Fantes, 1986).

We report here the sequence analysis and preliminary characterization of the $wis2^+$ gene and its protein product. Sequence analysis of $wis2^+$ predicts a 40 kDa protein product with significant sequence similarity to the cyclophilins. The 18 kDa cyclophilin domain lies at the N-terminus and is followed by a C-terminal region of 188 amino acids. We report a structure–function analysis of $wis2^+$, employing its suppression of the cell cycle defect of *wee1-50 cdc25-22 win1-1* as the *in vivo* test. We also demonstrate that the $wis2^+$ mRNA level is increased upon heat shock, suggesting a role for wis2 in conditions of elevated temperature.

Results

Nucleotide sequence of $wis2^+$

The plasmid pwis2-3 was isolated from a gene library as a multicopy suppressor of the conditional lethal phenotype of *wee1-50 cdc25-22 win1-1* (Warbrick and Fantes, 1992). *wee1-50 cdc25-22 win1-1* exhibits a conditional lethal phenotype when grown on minimal medium at the restrictive temperature of 35°C. Under such conditions, the majority of the triple mutant cells are highly elongated, a phenotype characteristic of cell cycle mutants, and show abnormal morphologies of branched and bent cells. In contrast, *wee1-50 cdc25-22 win1-1* cells transformed with pwis2-3 undergo continued division, albeit at a cell size slighter greater (~1.5-fold) than that of wild-type cells, and are of normal shape.

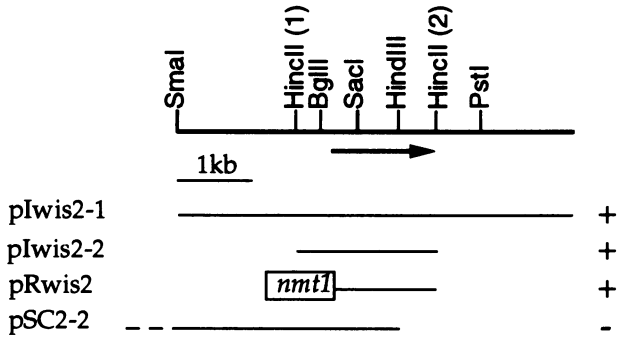

Fig. 1. Restriction map and activity of subclones. Restriction sites within the insert of pIwis2-1 are indicated on the top bar. The predicted open reading frame is indicated by an arrow. pIwis2-1 and pIwis2-2 are clones in pIRT2, pRwis2 is a clone in pREP1. pSC2-2 is a clone in pDB248 (Warbrick and Fantes, 1992). +/– indicates suppression of the lethal phenotype of *cdc25-22 wee1-50 win1-1* on minimal medium at 35°C. The suppression activity of pRwis2 was tested under derepressing conditions for the regulated *nmt1* promoter.

Previous subcloning and transposon mutagenesis analysis predicted that the $wis2^+$ gene would lie within pIwis2-1, a subclone containing a 5 kb fragment of pwis2-3, to the right of the *Bgl*II restriction site indicated in Figure 1 (Warbrick and Fantes, 1992). The subclone pSC2-2 was found to be non-functional (Warbrick and Fantes, 1992), suggesting that sequences which lie to the right of the *Hin*dIII site indicated in Figure 1 are important for $wis2^+$ suppression activity. The DNA sequence was determined for a 1960 nt region extending from 135 nt to the left of *Hin*cII (1) indicated in Figure 1, to 80 nt to the right of the *Pst*I site indicated. This nucleotide sequence contained an uninterrupted open reading frame (ORF) of 1068 nucleotides (Figure 2). Consistent with the predicted ORF of 1068 nt, a transcript of ~1500 nt was detected by Northern hybridization (see Figure 8). This transcript was shown to be present at a constant level throughout the cell cycle (P.Kersey, personal communication).

To confirm that this ORF was indeed responsible for wis2 activity, a multicopy plasmid, containing the 1.6 kb *Hin*cII fragment ligated into the vector pIRT2 (pIwis2-2), was introduced into *wee1-50 cdc25-22 win1-1* and shown to suppress its conditional lethal phenotype. As no other ORFs longer than 200 nt are present in either orientation within the *Hin*cII fragment, it is most likely that the 1068 nt ORF is responsible for the suppression activity. To test this, the predicted $wis2^+$ ORF was placed under the control of the *S.pombe* regulatable *nmt1* promoter, and its ability to suppress *wee1-50 cdc25-22 win1-1* was examined. The cloning of the $wis2^+$ ORF involved the introduction of an *Nde*I restriction site at the putative initiation codon of $wis2^+$, and the resulting *Nde*I–*Hin*cII fragment was cloned downstream of the *nmt1* promoter of pREP1 (Maundrell, 1990). The resulting plasmid, pRwis2, was able to suppress the triple mutant phenotype in the absence of thiamine (de-repressing conditions for the *nmt1* promoter) but not in the presence of thiamine

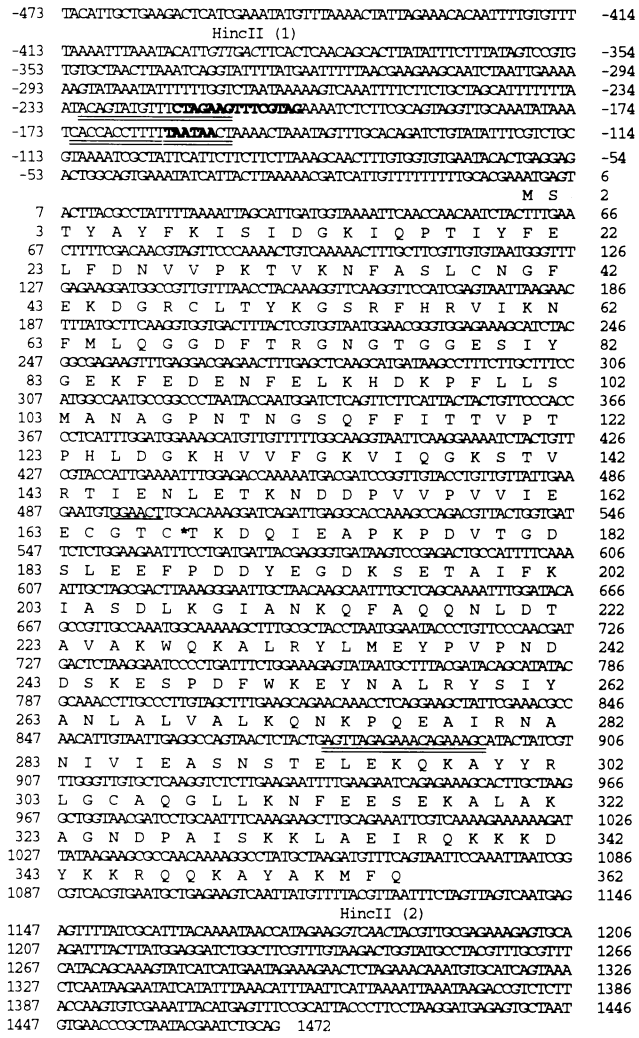

Fig. 2. Nucleotide and predicted amino acid sequence. Nucleotide and amino acid sequences are numbered relative to the predicted start codon. In bold are the two consecutive stop codons in-frame with the ORF (position −161 to −155) and the predicted HSE (heat-shock element; position −219 to −204). Double underlined sequences indicate the positions of synthetic oligonucleotide primers used in PCR (see text for details). A star between the amino acids Cys167 and Thr168 indicates the border between the CyP-18 domain and the C-terminal region of wis2. The underlined sequence at position 493–497 indicates the site of introduction of the *Kpn*I restriction site for the construction of the cyp1–wis2 fusion protein.

(repressing conditions for the *nmt1* promoter), providing further evidence that the predicted ORF is responsible for wis2 activity.

We also analysed *wis2* cDNA sequences in order to confirm that the predicted ATG start codon of *wis2*$^+$ is the actual initiating ATG. The predicted ATG start codon is the first ATG downstream of two consecutive in-frame stop codons (see Figure 2). Therefore, unless these stop codons are part of an intron, the predicted start ATG codon is likely to serve as the initiation codon for translation. Two oligonucleotide primers were designed to anneal to sequences upstream of the predicted *wis2*$^+$ ORF, and were used in conjunction with a primer within the ORF at positions 878–896 (Figure 2) in PCR, using as a template an *S.pombe* cDNA library. When the downstream of the 5′ primers was used, an amplified product of the size of ~1 kb was obtained, consistent with the expected size of 1.09 kb (data not shown). No amplified product was obtained when the 5′ primer further upstream of *wis2*$^+$ ORF at positions −233 to −215 was used. The ability of the downstream 5′ primer (which contains the two in-frame stop codons) to bind specifically to sequences in the cDNA library strongly suggests that the regions covered by the primer are not part of an intron and, therefore, the first ATG codon downstream of the two in-frame stop codons is likely to function as the initiating ATG codon.

The predicted wis2$^+$ *gene product is a 40 kDa cyclophilin-like protein*

Translation of the *wis2*$^+$ ORF predicts a polypeptide of 40.1 kDa containing 356 amino acids (Figure 2). The predicted molecular weight of wis2 is consistent with the detection of a ~40 kDa protein in strains overexpressing *wis2*$^+$(see below). A search of the GenEMBL and SwissProt databases revealed that an 18 kDa cyclophilin domain (CyP-18) lies at the N-terminus of wis2 (first 168 amino acids). The wis2 CyP-18 domain is most similar to cyclophilins from *Toxoplasma gondii* (63% identity; High *et al.*, 1994), *Candida albicans* (62% identity; Koser *et al.*, 1990) and the 18 kDa type cyclophilin of *S.pombe* (62% identity; de Martin and Philipson, 1990). A striking difference between the wis2 CyP-18 domain and other eukaryotic cyclophilins is the replacement of the sole and highly conserved tryptophan residue with a histidine residue (position 124 in the wis2 sequence). This tryptophan residue is important for high affinity binding to CsA, but seems to have little effect on PPIase activity (Liu *et al.*, 1991b). Over its entire length, wis2 was found to be most similar to bovine and human cyclophilin 40 (CyP-40; Kieffer *et al.*, 1993) and human ERBC (Ratajczak *et al.*, 1993), a protein almost identical to human CyP-40 which was isolated as a component of the inactive estrogen receptor complex (see also Discussion below). The homology between wis2 and Cyp-40/ERBC extends throughout the amino acid sequence (44% identical in a 360 amino acid overlap, and 31% identity over the non-CyP-18 domain). Interestingly, the CyP-18 domains of bovine CyP-40, human CyP-40 and ERBC (61% identical to the CyP-18 domain of wis2) also contain a histidine residue at the position corresponding to Trp121 of human CyP-18 (see Figure 3).

The non-CyP-18 regions of wis2 and CyP-40 also show a significant similarity (27 and 31%, respectively) with mammalian FKBP-59, an immunophilin of the FKBP family (Lebeau *et al.*, 1992; Tai *et al.*, 1992), which previously has been identified both as a heat-shock protein (hsp56; Sanchez, 1990) and as a component of a variety of inactive steroid receptor complexes (Tai *et al.*, 1986). FKBP-59 is a 59 kDa protein containing two domains which are structurally related to the cytoplasmic binding protein for FK506 (FKBP-12), a third domain which is distantly related to the first two, and a C-terminal tail (Callebaut *et al.*, 1992). It is the third domain and the C-terminal tail which are structurally related to the non-CyP-18 domain of CyP-40 and wis2 (see Figure 4).

A putative calmodulin binding site has been identified in the C-terminal tail of FKBP-59 (Lebeau *et al.*, 1992), and is conserved in the predicted sequences of CyP-40 and wis2 (Kieffer *et al.*, 1993; and see Figure 4). In

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  Sp-wis2  ..........  ..........  ......MSTY AYFKISIDGK IQPTIYFELF 24
  Sp-cyp1  ..........  ..........  .......MSN CFFDVIANGQ PLGRIVFKLF 23
Tg-CyP-20  KHPTRLRPGS LPCVAFCLYS SRLSTMPNPR VFFDISIDKK PAGRIEFELF
Hu-CyP-18  ..........  ..........  ......VNPT VFFDIAVDGE PLGRVSFELF 25
Hu-CyP-40  ..........  ...MSHPSPQ AKPSNPSNPR VFFDVDIGGE RVGRIVLELF 37
consensus  ..........  ..........  .......npr vfFdisidg. plgrivfeLF

  Sp-wis2  DNVVPKTVKN FASLCNGFEK D.....GRCL TYKGSRFHRV IKNFMLQGGD 69
  Sp-cyp1  DDVVPKTAAN FRALCTG.EK GYG.......  .YAGSTFHRV IPQFMLQGGD 64
Tg-CyP-20  ADVVPKTAEN FRALCTG.EK GTGR.SGKPL YYKGCPFHRI IPQFMCQGGD
Hu-CyP-18  ADKVPKTAEN FRALSTG.EK GFG.......  .YKGSCFHRI IPGFMCQGGD 66
Hu-CyP-40  ADIVPKTAEN FRALCTG.EK GIGHTTGKPL HFKGCPFHRI IKKFMIQGGD 87
consensus  advVPKTaeN FraLctG.EK g.g...g..l  .ykGspFHRi IpqFM.QGGD

  Sp-wis2  FTRGNGTGGE SIYGEKFEDE NFELKHDKPF LLSMANAGPN TNGSQFFITT 119
  Sp-cyp1  FTRGNGTGGK SIYGEKFPDE NFALKHNKPG LLSMANAGPN TNGSQFFITT 114
Tg-CyP-20  FTRMNGTGGE SIYGEKFADE NFSYKHSEPF LLSMANAGPN TNGSQFFITT
Hu-CyP-18  FTRHNGTGGK SIYGEKFEDE NFILKHTGPG ILSMANAGPN TNGSQFFICT 116
Hu-CyP-40  FSNQNGTGGE SIYGEKFEDE NFHYKHDREG LLSMANAGRN TNGSQFFITT 137
consensus  FtrgNGTGGe SIYGEKFeDE NF.lKHdkpg lLSMANAGpN TNGSQFFItT
                    *
  Sp-wis2  VPTPWLDGKH VVFGKVIQGK STVRTIENLE TKNDDPVVPV VIEECGTCTK 169
  Sp-cyp1  VVTPWLDGKH VVFGEVTEGM DVVKKVESLG SNSGATRARI VIDKCGTV.. 162
Tg-CyP-20  VPCPWLDGKH VVFGKVVAGQ EVVKMMEAEG RSNGQPKCAV EISSCGQLS*
Hu-CyP-18  AKTEWLDGKH VVFGKVKEGM NIVEAMERFG SRNGKTSKKI TIADCGQLE. 165
Hu-CyP-40  VPTPWLDGKH VVFGQVIKGI GVARILENVE VKGEKPAKLC VIAECGELKE 187
consensus  vptpwLDGKH VVFGkVieGm .vv..mEnlg skngkp.k.. vIaeCG.l..
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Fig. 3. Alignment of the CyP-18 domain of wis2 with cyclophilins. The Cyp-18 domain of wis2 is aligned with cyp1, the 18 kDa type cyclophilin of *S.pombe* (de Martin and Philipson, 1990), CyP-20 of *Toxoplasma gondii* (High *et al.*, 1994), human CyP-18 (Harding *et al.*, 1986) and human CyP-40 (Kieffer *et al.*, 1993). On the bottom line, upper case letters indicate identical (5/5) amino acids. Lower case letters indicate identity between at least three amino acids. An asterisk indicates the position of the conserved tryptophan residue of the CyP-18 domain, which is replaced by a His residue in wis2 and CyP-40. All sequences are numbered according to the amino acid position relative to the predicted initiating methionine, except for CyP-20 of *T.gondii*, of which the position of the initiating methionine is unknown (High *et al.*, 1994).

addition, a region consisting of a three unit TPR-like domain was identified in both FKBP-59 and human ERBC (Ratajczak *et al.*, 1993). TPR domains contain a 34 amino acid motif that are presumed to form jointed helical structures each with a 'knob' and 'hole', and it has been suggested that these structures play a role in protein–protein interactions (Hirano *et al.*, 1990; Sikorski *et al.*, 1990; Lamb *et al.*, 1994). TPR domains have been found in a diverse group of proteins functioning, for example, in mitosis, transcription, splicing, import and neurogenesis (reviewed in Goebl and Yanagida, 1991). Two out of the three TPR units identified in ERBC are conserved in wis2, while the third unit in wis2 only remotely resembles the first two. It should also be noted that the TPR units identified in ERBC are imperfect when compared with the consensus sequence of TPR units suggested by Hirano *et al.* (1990) and Sikorski *et al.* (1990). The repeats of wis2, ERBC or FKBP-59 are more similar to those identified in STI1, a stress-inducible protein from yeast, IEF, a heat shock- and transformation-sensitive human protein and MAS70, a yeast outer mitochondrial membrane protein (Ratajczak *et al.*, 1993).

Deletion and overexpression of wis2$^+$ in wild-type and cell cycle mutant cells

The *wis2*$^+$ gene was deleted from the chromosome by one-step gene replacement (Rothstein, 1983). The deletion was performed using the 1.8 kb *S.pombe ura4*$^+$ gene, which was used to replace all coding sequences of *wis2*$^+$ excluding 12 bp at the 3′ end (see Figure 5). A linearized DNA fragment containing the disrupted *wis2* gene was integrated into the chromosome of a diploid strain by homologous recombination. Six ura$^+$ diploid strains were subjected to Southern blot analysis, and preliminary analysis of *Eco*RI hybridization fragments suggested that

A

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Hu-FKBP59  PYGLERAIQR MEKGEHSIVY LKPSYAFGSV GKEKFQIPPN AELKYELHLK 250
Hu-CyP40   ECG......E LKEGDDGGIF PK.....DGS GDSHPDFPED A....DIDLK 214
wis2       ..........  ..TKDQIEAP KP.....DVT GDSLEEFPDD Y....EGD.K 195
consensus  ..........  ...gd.s.i. .k.....dgs Gds...fP.d a....evdlK

Hu-FKBP59  SFEKAKESWE MNSEEKLEQS TIVKERGTVY FKEGKYKQAL LQYKKIVSWL 300
Hu-CyP40   DVDKI.....  ......LLIT EDLKNIGNTF FKSQNWEMAI KKYAEVLRYV 253
wis2       SETAI.....  ......FKIA SDLKGIANKQ FAQQNLDTAV AKWQKALRYL 234
consensus  s..ki.....  ......l.is edlK.ign.. Fk.qn...A. .kykk.lryl

Hu-FKBP59  EYESSFSNEE AQKA......  .QALRLASHL NLAMCHLKLQ AFSAAIESCN 343
Hu-CyP40   D.SSKAVIET ADRA.....K LQPIALSCVL NIGACKLKMS NWQGAIDSCL 297
wis2       M.EYPVPNDD SKESPDFWKE YNALRYSIYA NLALVALKQN KPQEAIRNAN 283
consensus  e.ess.sne. a.ka......  .qalrls..l Nla.c.LK.. a.q.Ai.scn

Hu-FKBP59  KALELDSNNE ....KGLFRR GEAHLAVNDF ELARADFQKV LQLYPNNKAA 389
Hu-CyP40   EALELDPSNT ....KALYRR AQGWQGLKEY DQALADLKKA QGIAPEDKAI 343
wis2       IVIEASNSTE LEKQKAYYRL GCAQGLLKNF EES....EKA LAKAGNDPAI 329
consensus  .alEld.sne ....KalyRr g.a...lk.f d.a.ad..Ka lq.ap.dkAi

Hu-FKBP59  KTQLAVCQQR IRRQLAREKK LYANMFERLA EEENKAKAEA SSGDHPTDTE 416
Hu-CyP40   QAELLKVKQK IKAQKDKEKA VYAKMFA*.. .......... .......... 370
wis2       SKKLAEIRQK KKDYKKRQQK AYAKMFQ... .......... .......... 356
consensus  .a.La...Qk ikaqk.rekk .YAkMF.... .......... ..........
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B

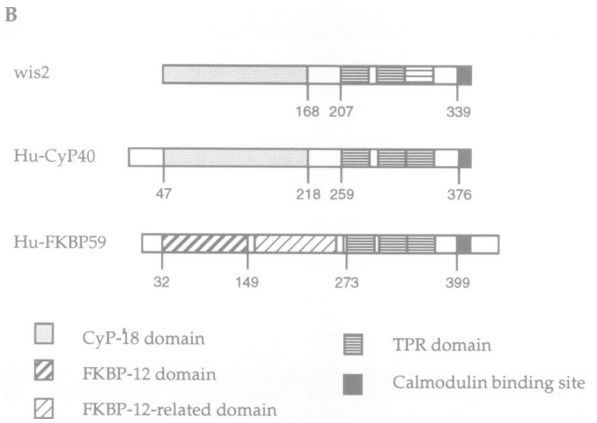

Fig. 4. Comparison between wis2, CyP-40 and FKBP-59. (**A**) The C-terminal region of wis2 is aligned with human CyP-40 and human FKBP-59. The consensus sequence derived from comparisons of the above protein sequences and that of rabbit FKBP-59 and bovine CyP-40 is shown at the bottom line. A putative calmodulin binding site is boxed (Lebeau *et al.*, 1992). (**B**) Schematic representation of wis2, CyP-40 and FKBP-59 proteins.

in all diploids strains the same single integration event had occurred (data not shown). One of these ura$^+$ diploid strains was analysed further by Southern blotting, and *Pst*I bands with the expected sizes of 3.75, 2.2 and 2.1 kb were obtained (Figure 5A). Tetrad analysis showed that all four haploid spores of the heterozygous diploid produced colonies at 28°C, indicating that the *wis2*$^+$ gene is non-essential for viability under normal growth conditions. Southern hybridization of restriction-digested DNA from the haploid spore clones with radiolabelled *Pst*I fragment of *wis2*$^+$ showed the expected pattern of hybridizing bands (2.2 and 2.1 kb for ura$^+$ cells and 3.75 kb for ura$^-$ cells). The lack of any hybridization signal in ura$^+$ haploid cells when probed with the *Bgl*II–*Hin*dIII fragment of *wis2*$^+$, which contains those sequences removed in the DNA construct used for *wis2* disruption, confirmed that ura$^+$ haploid cells represent a deletion of the *wis2*$^+$ gene (Figure 5B).

Microscopic examination failed to detect any difference in cell size or morphology between *wis2*$^+$-deleted cells (*wis2*Δ) and wild-type cells, over a range of temperatures from 20 to 36°C. Possible interactions of *wis2*Δ with cell

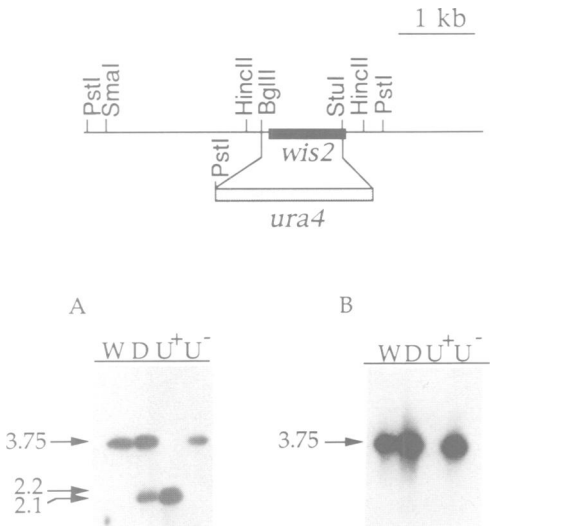

Fig. 5. Deletion of *wis2* gene. Upper panel. Schematic representation of deletion by the one-step gene replacement method (Rothstein, 1983). The *S.pombe ura4*$^+$ gene was used to replace the *Bgl*II–*Hin*cII fragment of *wis2*$^+$ (for details see Materials and methods). Lower panels. Southern blot analysis of *wis2*-deleted strains. *S.pombe* genomic DNA prepared from a disruptant diploid (D), its haploid ura$^+$ (U$^+$; *wis2*Δ) and ura$^-$ (U$^-$) progeny cells, and haploid wild-type cells (W). Genomic DNA samples were digested with *Pst*I, separated by agarose gel electrophoresis and Southern blotted. (**A**) Probed with a *Pst*I fragment from pIwis2-1. (**B**) Probed with a *Bgl*II–*Hin*dIII fragment from pIwis2-1.

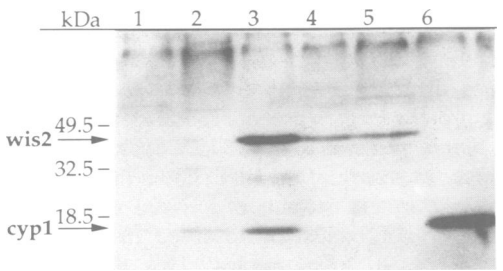

Fig. 6. Detection of the proteins wis2, cyp1 and cyp1–wis2. Total protein extracts were prepared of the following strains: *wis2*Δ (lane 1), wild-type 972 *h*$^-$ (lane 2), and *cdc25-22 wee1-50 win1-1 leu1-32 h*$^-$ transformed with pRwis2 (lane 3), pIwis2-1 (lane 4), pIcyp1-wis2 (lane 5) or pIcyp1 (lane 6). Cells containing the pRwis2 plasmid were grown under derepression conditions for full activity of the *nmt1* promoter in pRwis2. Each lane contains ~40 μg of total protein. Protein extracts were separated on SDS–PAGE gels and blotted under denaturing conditions. The enhanced chemiluminescence (ECL) detection system was employed using polyclonal antibodies raised against human CyP-A. Autoradiography films were exposed for 5–10 s.

cycle mutations were investigated by crossing a *wis2*Δ strain with strains carrying the following mutations: *win1-1*, *cdc25-22*, *wee1-50*, *cdc2-33* and *cdc13-117*. No defects associated with the deletion of *wis2*$^+$ were noticed in any case. Also, no interactions were observed with a deletion of the *wis1*$^+$ gene, a MAPKK homologue isolated in the same screen as *wis2*$^+$ (Warbrick and Fantes, 1991). We have also investigated whether the *wis2* deletion allele has any effect in the double mutant *wee1-50 cdc25-22* and the triple mutant *wee1-50 cdc25-22 win1-1*. However, no effects on cell length at division or any other effects were observed (data not shown). The lack of effect of deletion of *wis2* in *wee1-50 cdc25-22 win1-1*, a genetic background in which overexpression of *wis2*$^+$ shows a strong suppression phenotype, could be explained by the presence of another gene which acts redundantly with *wis2*$^+$.

The effect of overexpression of *wis2*$^+$ was examined in wild-type cells and in a variety of cell cycle mutants, as genetic interactions might give a clue to the mechanism by which *wis2*$^+$ suppresses the cell cycle defect of *wee1-50 cdc25-22 win1-1*. pRwis2 (strongly overexpressing *wis2*$^+$ from the *nmt1* promoter, see above) was used to transform various strains. These include wild-type and the cell cycle mutants described above. Wild-type cells transformed with pRwis2 in the absence of thiamine showed an ~40-fold increase in *wis2*$^+$ mRNA level compared with the wild-type level (assessed by PhosphorImager analysis of Northern blots, data not shown). No phenotypic effect associated with the introduction of pRwis2 was observed in the above genetic backgrounds, except in the background of the triple mutant *wee1-50 cdc25-22 win1-1*, where pRwis2 suppressed the elongated and lethal phenotype of the strain, as expected.

Finally, possible effects of *wis2*$^+$ deletion or overexpression in the presence of CsA were investigated. We observed that the presence of CsA in the growth medium (50–400 μg/ml) resulted in an increased proportion of septated cells, as well as the presence of multiseptated cells, similar to the effect reported by Yoshida *et al.* (1994). As members of the cyclophilin family are known to mediate the effects of CsA in mammalian and *S.cerevisiae* cells, the effects of CsA on a *wis2*Δ strain and on cells overexpressing *wis2*$^+$ were examined. Neither deletion of *wis2*$^+$ nor overexpression affected the phenotype of *S.pombe* cells treated with CsA, suggesting that *wis2*$^+$ does not participate in mediating its effect.

Detection of wis2 protein

Detection of the wis2 protein was performed by immunoblotting with polyclonal antibodies raised against human Cyp-A protein (an 18 kDa cytosolic cyclophilin; a kind gift from F.Etzkorn and C.Walsh). Total *S.pombe* protein extracts were separated on SDS–PAGE gels, blotted and antibody binding proteins were detected by using the ECL immunodetection system (Figure 6). Cells transformed with pIwis2-1 exhibited increased expression of an ~40 kDa protein (lane 4) compared with wild-type (lane 2), or cells transformed with the control plasmid pIRT2 (data not shown). The increased level of a 40 kDa protein species is consistent with the predicted molecular weight of wis2, and indicates that the human CyP-A antibodies cross-react with the overexpressed *S.pombe* wis2 cyclophilin-like protein.

In cells carrying pRwis2, a further increase in the level of the 40 kDa protein was observed under derepressing conditions for the *nmt1* promoter (lane 3). The appearance of ~18 and ~30 kDa protein bands was also observed in cells transformed with pRwis2, and may indicate a proteolytic degradation of the overexpressed wis2 protein. The presence of an elevated level of the 18 kDa species may reflect the presence of a particularly sensitive proteolytic site between the cyclophilin and the C-terminal domains of wis2.

Reaction of the antibodies with protein extracts of wild-type and *wis2Δ* cells (lanes 1 and 2) was poor and, therefore, difficult to interpret. However, a signal with an 18 kDa protein species probably indicates a cross-reaction with *S.pombe* proteins of the 18 kDa cyclophilin subgroup. In a longer exposure of the blot shown in Figure 5A (data not shown), protein extracts of a *wis2Δ* strain showed the same pattern of bands as observed in wild-type cells, including the 40 kDa species. This may indicate the presence of another 40 kDa cyclophilin-like protein, in addition to wis2.

The C-terminal domain of wis2⁺ is essential for its suppression activity and appears to confer specificity on the cyclophilin domain

The unusual structure of wis2 prompted us to carry out a structure–function analysis, using the suppression of the conditional lethal phenotype of a *wee1-50 cdc25-22 win1-1* strain to test for *in vivo* function. This suppression activity is referred to hereafter as wis2 activity. Previous subcloning analysis (Warbrick and Fantes, 1992) suggested that the C-terminal domain is required for wis2 activity: the subclone pSC2-2 lacks most of the C-terminal region (129 amino acids) and is unable to confer wis2 activity (see Figure 1).

The requirement for the C-terminal domain of wis2 for activity was investigated further by examining the ability of the 18 kDa *S.pombe* cyclophilin-like protein, cyp1 (de Martin and Philipson, 1990) to confer wis2 activity. The *Eco*RI fragment of a genomic clone of *cyp1⁺* was cloned into the *S.pombe* plasmid pIRT2. This *Eco*RI fragment contains 500 bp upstream of the predicted initiating ATG codon, the entire predicted *cyp1⁺* ORF and 270 bp of the downstream sequences. The resultant plasmid, pIcyp1, was transformed into the triple mutant strain. The *cyp1⁺* clone was unable to suppress the conditional lethal phenotype: no colony formation occurred at the restrictive temperature on minimal medium, and cells displayed the typical lethal phenotype of highly elongated, bent and branched cells (Figure 7). An increased level of an ~18 kDa protein species was identified with the anti-human Cyp-A antibodies in protein extracts of cells transformed with pIcyp1, confirming overexpression of the *cyp1⁺* gene product (see Figure 6, lane 6).

The observation that *cyp1⁺* could not confer wis2 activity when overexpressed may be explained in at least two ways: the wis2 cyclophilin domain is itself necessary, but not sufficient, for wis2 activity, or an intrinsic substrate specificity is contained within the cyclophilin domain. In order to test these possibilities, the C-terminal region of wis2 was fused in-frame to the N-terminal CyP-18 domain of cyp1, and the ability of the hybrid protein to confer wis2 activity was examined.

The hybrid *cyp1–wis2* gene contains the predicted ORF of *cyp1⁺* (excluding the last codon, encoding valine) followed by *wis2⁺* sequences encoding the C-terminal 188 amino acids (see Materials and methods for details). *cyp1–wis2* was cloned into pIRT2, and the resulting plasmid pIcyp1-wis2 was transformed into the *cdc25-22 wee1-50 win1-1* strain. Western blot analysis indicated that the transformants overexpressed a 40 kDa protein, consistent with the predicted molecular weight of the hybrid protein (Figure 6, lane 5). pIcyp1-wis2 was able

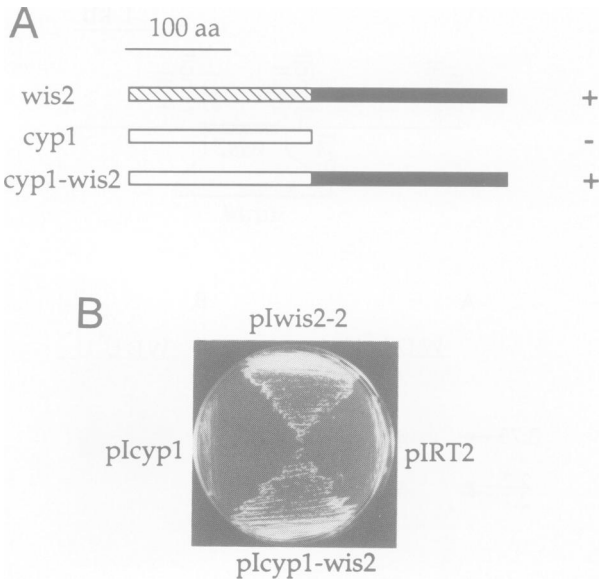

Fig. 7. Suppression activity of *cyp1⁺* and *cyp1–wis2*. (**A**) Schematic diagram representing the *cyp1⁺* and *cyp1–wis2* genes: a striped box represents the CyP-18 domain of wis2, filled boxes represent the C-terminal region of wis2 and open boxes represent cyp1. (**B**) *cdc25-22 wee1-50 win1-1 leu1-32 h⁻* transformed with either pIRT2, pIwis2-2, pIcyp1 or pIcyp1-wis2 were grown on minimal plates at the permissive temperature, then streaked onto fresh minimal plates and incubated at the restrictive temperature (35°C) for 6 days.

to suppress the triple mutant phenotype with comparable efficiency to that of functional subclones of *wis2⁺* in pIRT2 plasmids (Figure 7), consistent with the suggestion that the C-terminal domain of wis2 confers specificity onto the cyp1 CyP-18-like *S.pombe* protein.

wis2⁺ transcript is heat shock induced

The implication of cyclophilins and, in particular, the finding that FKBP-59 is identical to the previously identified heat-shock protein hsp56 (Yem *et al.*, 1992) led us to search for heat-shock elements (HSEs) in the promoter region of *wis2⁺*. HSEs serve as a binding site for the heat shock transcriptional activation factor HSF and consist of inverted repeats of the sequence *n*GAA*n* which is conserved from yeast to mammals (Perisic *et al.* 1989). Indeed, a putative HSE of the sequence CTA**GAAGTTT**-**C**GTAG is present 218 bp upstream of the putative initiating ATG codon (Figure 2). Consequently, we examined the level of *wis2⁺* RNA after heat-shock treatment. A 10- to 15-fold increase of *wis2⁺* mRNA level was observed after a shift of exponentially growing wild-type cells from 25 to 37°C for 30 min. A further 2-fold increase was observed when cells were shifted from 25 to 42°C. A time course experiment in which wild-type cells were shifted from 25 to 42°C showed that *wis2⁺* RNA level increased during the first 30 min, and then began to decline (Figure 8). Although the increased level of *wis2⁺* RNA upon heat shock suggests a role for *wis2⁺* in the heat-shock response, preliminary experiments failed to show a significant difference in the ability of cells deleted for *wis2⁺* to survive heat shock, compared with wild-type cells. This preliminary analysis included time course experiments examining the ability of cells growing at 25°C to survive heat shock at 42 or 49°C during a 45 min period (data not shown).

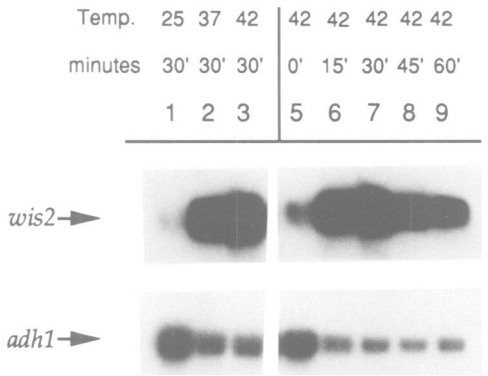

Fig. 8. Increased level of *wis2* transcript upon heat shock. Wild-type cells were grown at 25°C in yeast extract medium and shifted to 37 or 42°C. Samples were taken at the times indicated for preparation of RNA. RNA samples were separated on an agarose formaldehyde gel, Northern blotted and probed sequentially with the *Bgl*II–*Hin*cII fragment of pIwis2-1 and the *Eco*RI fragment internal to the *S.pombe* adh^+ gene as loading control. Relative amounts of RNA were determined by PhosphorImager analysis.

Discussion

Characteristics of the structure and function of wis2

The $wis2^+$ DNA sequence predicts a 40.1 kDa cyclophilin-like gene product. The N-terminus of the gene encodes the 18 kDa domain of cyclophilins and is followed by a C-terminus of 188 amino acids. $wis2^+$ is structurally homologous to bovine and human CyP-40 (isolated as CsA binding proteins; Kieffer *et al.*, 1993) and to human ERBC (isolated as a component of the inactive oestrogen receptor complex; Ratajczak *et al.*, 1993), indicating that the 40 kDa subgroup of cyclophilins is conserved throughout evolution.

Wis2 and CyP-40/ERBC share two distinct features. First, both contain a similar C-terminal region (31% identical), containing a putative calmodulin binding motif and amino acid sequences which resemble TPR domains. Secondly, in both proteins, the highly conserved tryptophan residue of the CyP-18 domain (Trp121 in human CyP-18) is substituted by histidine. Trp121 was suggested, based on mutagenesis analysis, to be important for the interaction with CsA *in vitro* but to have little effect on PPIase activity (Liu *et al.*, 1991b). Also, although human CyP-40 was isolated as a CsA binding protein, its elution from CsA affinity columns prior to CyP-18 suggested a lower affinity for CsA compared with CyP-18 (Kieffer *et al.*, 1992). The importance of the tryptophan residue for high affinity for CsA may partially explain our *in vivo* observation that neither overexpression nor deletion of $wis2^+$ affected the multiseptated phenotype of *S.pombe* cells treated with CsA. Other factors, like subcellular localization or protein–protein interactions, are also likely to determine which members of the cyclophilin family participate in mediating the *in vivo* effects of CsA (see Koser *et al.*, 1991; McLaughlin *et al.*, 1992).

The substitution of tryptophan by histidine both in wis2 and mammalian CyP-40 is striking when compared with the high conservation of Trp121 in other eukaryotic cyclophilins. However, the significance of this substitution for biological activity, if any, is not clear. In particular, the structure–function analysis of wis2, reported here, indicates that replacement of the CyP-18 domain of wis2 with the 18 kDa *S.pombe* cyclophilin cyp1, which contains the conserved tryptophan residue, resulted in a functional hybrid protein with respect to the suppression activity of *wee1-50 cdc25-22 win1-1*. Therefore, the presence or absence of the conserved tryptophan does not appear to affect the *in vivo* function of the suppression activity of wis2.

The C-terminal region of wis2 and CyP-40/ERBC shows structural similarities with FKBP-59, which contains a structurally unrelated PPIase domain. Interestingly, both FKBP-59 and CyP-40/ERBC have been identified in the inactive form of steroid receptors, which also contain hsp90 and hsp70 (Tai *et al.*, 1986, 1992; Ratajczak *et al.*, 1993). It has been suggested that the immunophilins, together with the heat-shock proteins, form hetero-complexes which may facilitate the assembly/disassembly or intracellular transport of steroid receptors (Tai *et al.*, 1992; Pratt, 1993; Ratajczak *et al.*, 1993). The C-terminal non-PPIase domain of either FKBP59 or CyP-40/ERBC is required for association with hsp90 (Pratt, 1993; Radanyi *et al.*, 1994; Hoffmann and Handschumacher, 1995), and mutational analysis indicates that the TPR motifs localized in the C-terminal domain of FKBP-59 have a role for hsp90 binding (Radanyi *et al.*, 1994). The TPR motifs in the C-terminal domain of CyP-40/ERBC are likely to have a similar role. The finding that the *S.cerevisiae* hsp90 homologue, hsp82, associates with a 45 kDa cyclophilin-like protein is the first indication that macromolecular complexes containing hsp90 and immunophilins may be conserved from yeast to human (Chang and Lindquist, 1994). The full sequence of the novel *S.cerevisiae* cyclophilin-like protein is not known yet, but N-terminal amino acid sequence analysis of the 45 kDa protein showed clear homology to cyclophilins; in particular, high homology was found to the human CyP-40 amino acid sequence (Chang and Lindquist, 1994).

Apart from $wis2^+$, only one other cyclophilin-like gene has been reported in *S.pombe*. $cyp1^+$ was isolated in a screen of a *S.pombe* genomic library, employing degenerate oligonucleotides containing conserved sequences of cyclophilins (de Martin and Philipson, 1990). We have demonstrated here that $cyp1^+$ is not capable of suppression of the *wee1-50 cdc25-22 win1-1* lethal phenotype. In contrast, a hybrid protein that contains the cyp1 amino acid sequence fused to the C-terminal region of wis2 is an efficient suppressor of *wee1-50 cdc25-22 win1-1*. The C-terminal domain of wis2 is thus able to confer activity on the cytosolic form of the *S.pombe* cyclophilin. These results are consistent with the suggestion that the 18 kDa cyclophilins are involved in housekeeping activities, but become specialized when found as a domain within a complex protein (Stamnes *et al.*, 1992). The C-terminal region of wis2 may confer specificity on the molecule either by targeting the molecule to a specific location or, more likely, by determining protein–protein interactions between wis2 and partner proteins. An intriguing possibility is that the C-terminal region of wis2 and the *S.pombe* hsp90 homologue interact physically, similar to the interactions found between hsp90 and human CyP-40 (Hoffmann and Handschumacher, 1995) or the 45 kDa cyclophilin protein of *S.cerevisiae* (Chang and Lindquist,

1994). Such a hsp90–Cyp-40 interaction may play a role in the suppression activity of wis2 (see also below).

One possibility yet to be examined directly is that the C-terminal domain of wis2 alone is capable of suppressing the lethal phenotype of *wee1-50 cdc25-22 win1-1*. Although this possibility has not been tested experimentally, the high conservation of the CyP-18 domain throughout evolution, and the functional role suggested for the CyP-18 domain in other proteins, strongly suggest that this domain plays a role in the activity of wis2. In a preliminary analysis, we aimed to address the question of the role of the CyP-18 domain of wis2 in the suppression activity by introducing a short deletion (Ser102–Gly112) or any of two point mutations (replacement of His129 by proline or leucine) into the CyP-18 domain of wis2. These mutations are localized to highly conserved amino acids, which have also been mapped, based on the solution for the three-dimensional structure of human CyP-18 (Kallen *et al.*, 1991; Thériault *et al.*, 1993), close to or at the PPIase active site. The resultant mutated genes were no longer able to confer wis2 activity when expressed from a multicopy yeast plasmid (overexpression of wis2 mRNA was confirmed by Northern blot analysis; data not shown). However, Western blot analysis suggested that the mutated proteins were not expressed in *S.pombe* cells to the same level as the wild-type wis2 protein (data not shown). At the moment, it is not clear whether the inability to confer wis2 activity stems from a specific defect within the cyclophilin active site, whether it is a consequence of the instability of the mutated proteins *in vivo* or due to the inability of the anti-human CyP-18 antibodies to recognize the mutated wis2 proteins.

Implication of wis2$^+$ in heat-shock response and cell cycle

The isolation of *wis2$^+$* as a multicopy suppressor of the cell cycle defect of a *wee1-50 cdc25-22 win1-1* strain suggests it has a role in the regulation of mitosis. When overexpressed, the effect of *wis2$^+$* is only apparent in the absence of the major control elements wee1 and cdc25. Overexpression of *wis2$^+$* has no apparent effect on single mutants defective for any of the major regulators of mitosis, cdc2, cdc13 or wee1. The importance of the wee1 and cdc25 defects in the suppression activity of *wis2$^+$* is also indicated by its ability to suppress the defects of other strains carrying *wee1-50* and *cdc25-22*, specifically, in combination with *mcs3*, *mcs4* or *mcs6* mutations (Warbrick and Fantes, 1992). The *mcs* mutations were isolated as suppressors of mitotic catastrophe phenotype in *wee1-50 cdc2-3w* cells (Molz *et al.*, 1989), and this incidentally lends support to the involvement of *wis2$^+$* in regulation of the G_2–mitosis transition.

In cells lacking both cdc25 and wee1, the phosphorylation level of cdc2 Tyr15 appears to be determined by other 'backup' elements such as mik1 and pyp3 (Lundgren *et al.*, 1991; Millar *et al.*, 1992), and regulation under these conditions may be much less stringent than normal. Indeed, *wee1 cdc25* double mutant cells are very heterogeneous in size, suggesting that the control is quite finely balanced. One explanation for the action of wis2 is that it interacts with an element such as mik1 or pyp3, but that this interaction only becomes apparent when mik1 and pyp3 become major regulators of entry into mitosis.

Table I. Strains used in this study

ED812	*972 h$^-$*
ED566[a]	*win1-1 wee1-50 cdc25-22 leu1-32 h$^-$*
ED623[a]	*win1-1 leu1-32 h$^+$*
ED628	*cdc2-33 leu1-32 ura4-D18 h$^-$*
ED678	*win1-1 leu1-32 ura4-D18 ade6-M216 h$^-$*
ED918	*cdc13-117 ura4-294 leu1-32 h$^-$*
ED866	*cdc25-22 leu1-32 ura4-D18 h$^-$*
ED909	*wee1-50 leu1-32 ura4-D18 h$^-$*
ED947[b]	*wis1::his1$^+$ his1-102 ade6-M216 ura4-D18 h$^+$*

[a]Ogden and Fantes, 1986.
[b]S.Stettler, personal communication.

An alternative explanation is that wis2 interacts with an essential component of the mitotic regulatory machinery such as cdc2 or cdc13 but that, again, this is only important in cells when the regulation is rather labile.

Deletion of the gene has not revealed any effects so far, even in genetic backgrounds where we would most expect it: no phenotypic effects were observed when the *wis2*-deleted allele was combined with the combination of *wee1-50*, *cdc25-22* and *win1-1* alleles. The most likely explanation is that another gene exists in *S.pombe* which acts redundantly with *wis2$^+$*. This explanation is plausible, particularly in view of the high redundancy already suggested for the family of cyclophilins (for example, see McLaughlin *et al.*, 1992).

Whatever its molecular targets, the likely function of wis2 as a molecular chaperone suggests possible ways in which it might interact with the mitotic control machinery: for instance, it might serve to stabilize particular regulatory proteins, or mediate their transport between the cytoplasm and the nucleus. As wis2 presumably acts to mediate positively progression through the G_2–mitosis transition, we have examined the level of proteins of two major positive regulators of the G_2–mitosis transition. We have been unable to detect any difference in the levels of cdc13 or cdc25 proteins in a *wis2Δ* strain compared with the level of these proteins in wild-type cells (J.C., unpublished observations). However, other investigations suggest that the heat-shock response and cell cycle control are connected: heat shock specifically delays mitosis in *S.pombe* and other cells (Polanshek, 1977), and a mutation in the HSF of *S.cerevisiae* shows a cell cycle arrest phenotype consistent with a G_2 delay (Smith and Yaffe, 1991). A recent intriguing observation is that a mutation in the *S.pombe* hsp90 homologue, *swo1-26*, suppresses the lethal phenotype of overproduction of wee1, perhaps by direct interaction between the proteins (Aligue *et al.*, 1994). It is possible, particularly in view of the emerging evidence of conserved physical association of cyclophilin-40 and hsp90, that wis2 exerts its effect on mitosis by modulating the activity of an hsp90 homologue.

Materials and methods

Yeast strains and media

Details of the strains used in this study are given in Table I. Media for the propagation of *S.pombe* were as described by Moreno *et al.* (1991). The standard genetic procedures of Gutz *et al.* (1974) were followed. All strains were derived from the two wild-type strains 972 *h$^-$* and 975 *h$^+$*. CsA was a gift of Sandoz Pharma, Basle, Switzerland. For stock solution, 1 mg of CsA was dissolved in 1 ml of absolute ethanol/0.1% Tween 20; filter sterilized and kept in a glass container at

4°C. CsA was added to a final concentration of 50–400 μg/ml in liquid or agar containing media.

Plasmids

A subclone of *wis2*$^+$ in the Pharmacia vector pTZ18R (pSC3-16; Warbrick and Fantes, 1992) was used as the parental plasmid for *wis2* subclones. This plasmid is referred to here as pTZwis2-1. The genomic insert of pTZwis2-1 was released by double restriction digest with *Sma*I and *Sal*I, and was cloned between the *Sma*I and *Sal*I polylinker sites of the yeast multicopy vector pIRT2 (Booher and Beach, 1986). The resultant plasmid was named pIwis2-1. Subsequently, the 1.6 kb *Hin*cII fragment of pIwis2-1 was cloned into the *Sma*I site of the vector pIRT2 to give pIwis2-2. For stronger overexpression of *wis2*$^+$, the gene was cloned into the pREP1 plasmid under the control of the *nmt1* promoter (Maundrell, 1990). For this cloning, an *Nde*I restriction site was introduced into pTZwis2-1 at the putative initiation codon of *wis2*$^+$ by oligonucleotide-directed *in vitro* mutagenesis (Mutagene Phagemid Kit, Bio-Rad), using the oligonucleotide 5′-CATTGTTTTTTTTTGCAC*CAT*-ATGACTTAC-3′. The resulting *Nde*I–*Hin*cII fragment of the mutated pTZwis2-1 plasmid was cloned downstream of the *nmt1* promoter of pREP1, to give pRwis2.

An *Xba*I genomic fragment of *cyp1*$^+$ cloned into the pBS$^+$ plasmid (Stratagene) was a kind gift of R.de Martin (pBScyp1; de Martin and Philipson, 1990; the name of the gene was agreed upon personal communication). The *cyp1*$^+$ gene was released from this plasmid by *Eco*RI digestion, the cohesive ends were filled-in, and the fragment was ligated with pIRT2 plasmid digested with *Sma*I. The resultant plasmid from this ligation was named pIcyp1.

DNA sequence analysis

The sequence of *wis2*$^+$ was determined by a combination of the phagemid system devised by Vieira and Messing (1987) and the chain termination sequencing method of Sanger *et al.* (1977). Nested deletions were prepared from pTZwis2-1, using the Pharmacia Nested Deletion Kit. Dideoxy sequencing reactions using T7 DNA polymerase were performed with the Pharmacia T7 Sequencing Kit. The sequence of 1965 bp was determined on both strands. Analysis and homology searches were performed using the University of Wisconsin GCG package. The DNA sequence reported here has been deposited with the EMBL sequence database under the accession number X91981.

Sequence analysis was also performed to confirm all of the mutations introduced either into the *wis2*$^+$ or *cyp1*$^+$ genes.

DNA and RNA manipulation

Yeast RNA was prepared as described by Kaufer *et al.* (1985) and DNA as described by Beach *et al.* (1982). Southern and Northern blot analysis was carried out using Gene Screen Plus (NEN) membranes following the preferred protocol suggested by the manufacturer. DNA probes were labelled with [^{32}P]dCTP using the random oligonucleotide labelling procedure of Feinberg and Vogelstein (1983).

Protein manipulations

Yeast total protein was prepared following the method described by Moreno *et al.* (1991). Protein concentration was estimated using the Pierce BCA Protein Assay kit. The Mini Trans blot cell (Bio-Rad) was used for blotting proteins from SDS–polyacrylamide gels by electric current. The membrane used for the blotting was 0.2 μm PVDF (polyvinylidene difluoride) membrane (Bio-Rad). Transfer was allowed to proceed for 1–1.5 h at 100–150 V. The Amersham ECL (enhanced chemiluminescence) system was used to detect the immobilized proteins. Using this system, wis2, cyp1 and the cyp1–wis2 fusion protein can be detected using polyclonal antibodies raised against human CyP-A, a kind gift of F.Etzkorn and C.Walsh, Harvard Medical School, Boston, MA.

Polymerase chain reaction analysis of S.pombe cDNA library

A *S.pombe* cDNA library made in the yeast vector pREP3X was a gift from C.Norbury, IMM, ICRF, Oxford. The PCRs were performed using the GenAmp DNA reagent kit (Perkin Elmer Cetus). DNA template (0.1 μg) was used for the amplification reactions. Amplification was performed over 30 cycles as follows: 94°C for 2 min (denaturing), 55°C for 2 min (annealing), 72°C for 4 min (elongation). These cycles were completed by 72°C for 5 min, and then 25°C for 30 min.

Construction of the wis2 deletion

A *Hin*dIII genomic fragment containing the *ura4*$^+$ gene was obtained from the plasmid pUC8/ura4 (E.Warbrick, personal communication). The *ura4*$^+$ gene was released by *Hin*dIII digest and was cloned into pBluescript KS$^+$ (Stratagene) at the *Hin*dIII restriction site, to give pBCura4. The *ura4*$^+$ gene was then released from pBCura4 by double restriction digest with *Bam*HI and *Hin*cII. The released fragment was ligated with pTZwis2-1 double digested with *Bgl*II and *Stu*I restriction enzymes, resulting in the plasmid pTZwis2::ura4. The replaced *wis2* sequence consists of 135 bp of 5′-non-coding sequences and 1056 bp of ORF sequences. The 4.2 kb *Kpn*I–*Sph*I fragment of pTZwis2::ura4 was used for yeast transformation by electroporation. This fragment contained the *ura4*$^+$ sequences flanked by ~1.8 and ~2.4 kb of sequences adjacent to *wis2*$^+$. Approximately 2 μg of gel-purified *wis2* disrupted fragment were used to transform a diploid of the genotype *ade6-M210/ade6-M216 ura4-D18/ura4-D18 leu1-32/leu1-32 h*$^+$*/h*$^-$, and ura$^+$ transformants were selected.

Construction of cyp1–wis2 hybrid

Construction of the *cyp1–wis2* hybrid was carried out to replace *wis2*$^+$ sequences encoding the CyP-18 domain with the *cyp1*$^+$ ORF. The *cyp1*$^+$ ORF contains two *Kpn*I restriction sites. The *Kpn*I site at position 208–214 in pBScyp1 (see Plasmids, above) was removed by oligonucleotide-directed *in vitro* mutagenesis (Mutagene Phagemid Kit, Bio-Rad), so that the *Kpn*I site at position 477–483 could be used for subsequent ligations. The removal of the *Kpn*I site using the oligonucleotide 5′-CTTTCCACCGGTTCCGTTACCGCG-3′ resulted in a silent mutation at the predicted Gly60 residue of the cyp1 protein, and gave the plasmid pBScyp1-K. In addition, a *Kpn*I site was introduced by oligonucleotide-directed *in vitro* mutagenesis into pTZwis2-1 at the border between the cyclophilin and the C-terminal region of *wis2*$^+$ (see Figure 2). Introduction of this *Kpn*I site with the oligonucleotide 5′-GAAGAATGTGG*TAC*CTGCACAAAGGATC-3′ resulted in a silent mutation of the predicted Gly164 and Thr165 residues of *wis2*$^+$. Subsequently, the *Kpn*I fragment of pBScyp1-K, containing the *cyp1* ORF excluding the last 3 bp, was ligated with pTZwis2-1-K digested with *Kpn*I, such that it replaced the CyP-18 domain of *wis2*$^+$. The resultant plasmid was named pTZcyp1-wis2. The hybrid gene *cyp1–wis2* was released from pTZcyp1-wis2 by *Sma*I and *Sal*I double restriction digest and was cloned between the *Sma*I and *Sal*I polylinker sites of the yeast multicopy vector pIRT2, to give pIcyp1-wis2. Note that *cyp1–wis2* is predicted to be transcribed from the promoter of the *cyp1*$^+$ gene.

Construction of wis2 mutated genes at the CyP-18 domain

Mutations at the CyP-18 domain of *wis2*$^+$ were introduced into pTZwis2-1 by oligonucleotide-directed *in vitro* mutagenesis (Mutagene Phagemid Kit, Bio-Rad). The oligonucleotides used were: 5′-GGATGG-AAAGC(C/T)TGTTGTTTTTGGC-3′ for the introduction of the point mutations which replaced His129 with leucine or proline; and 5′-CATGATAAGCCTTTCTTGCTTTCTCAGTTCTTCATTACTAC-3′ for the deletion of the 11 amino acids Ser102–Gly112. The mutated genes were released from the plasmid pTZwis2-1 by double restriction digest with *Sma*I and *Sal*I and were cloned between the *Sma*I and *Sal*I polylinker sites of the yeast multicopy vector pIRT2.

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