The Identification of Wos2, a p23 Homologue That Interacts With Wee1 and Cdc2 in the Mitotic Control of Fission Yeasts

Manuel J. Muñoz,[1] Eduardo R. Bejarano, Rafael R. Daga and Juan Jimenez

Departamento de Genética, Facultad de Ciencias, Universidad de Málaga, 29071 Málaga, Spain

Manuscript received March 1, 1999
Accepted for publication August 11, 1999

ABSTRACT

The Wee1 kinase inhibits entry into mitosis by phosphorylation of the Cdc2 kinase. Searching for multicopy suppressors that abolish this inhibition in the fission yeast, we have identified a novel gene, here named *wos2*, encoding a protein with significant homology to human p23, an Hsp90-associated cochaperone. The deletion mutant has a modest phenotype, being heat-shock sensitive. Using antibodies raised against bacterially produced protein, we determined that Wos2 is very abundant, ubiquitously distributed in the yeast cell, and its expression dropped drastically as cells entered into early stationary phase, indicating that its function is associated with cell proliferation. In proliferating cells, the amount of Wos2 protein was not subjected to cell cycle regulation. However, *in vitro* assays demonstrated that this Hsp90 cochaperone is potentially regulated by phosphorylation. In addition to suppressing Wee1 activity, overproduction of Wos2 displayed synthetic lethality with Cdc2 mutant proteins, indicating that this Hsp90 cochaperone functionally interacts with Cdc2. The level of Cdc2 protein and its associated H1 kinase activity under synthetic lethal conditions suggested a regulatory role for this Wos2-Cdc2 interaction. Hsp90 complexes are required for CDK regulation; the synergy found between the excess of Wos2 and a deficiency in Hsp90 activity suggests that Wos2 could specifically interfere with the Hsp90-dependent regulation of Cdc2. *In vitro* analysis indicated that the above genetic interactions could take place by physical association of Wos2 with the single CDK complex of the fission yeast. Expression of the budding yeast p23 protein (encoded by the *SBA1* gene) in the fission yeast indicated that Wos2 and Sba1 are functionally exchangeable and therefore that properties described here for Wos2 could be of wide significance in understanding the biological function of cochaperone p23 in eukaryotic cells.

THE protein kinase encoded by *cdc2* plays a key role in the mitotic control of all eukaryotic cells (Nurse 1990). In the fission yeast *Schizosaccharomyces pombe*, the activity of Cdc2 requires binding of the *cdc13*-encoded B-type cyclin, and the Cdc2-cyclin B complex is maintained in a repressed state during interphase by phosphorylation of the Cdc2 subunit on Tyr15 (Gould and Nurse 1989). The major Tyr15 phosphorylation activity is provided by the Wee1 protein kinase (Russell and Nurse 1987a; McGowan and Russell 1993) whereas Cdc25 provides the predominant Tyr15 dephosphorylation activity (Russell and Nurse 1986; Gautier *et al.* 1991), required to activate the Cdc2-cyclin B complex and subsequently to trigger mitosis.

The search for new elements involved in the regulation of Cdc2 by Wee1 in fission yeasts led to the identification of *swo1*, a gene encoding an Hsp90-like protein required for Wee1 activity (Aligue *et al.* 1994; Muñoz and Jimenez 1999). Genetic analysis has shown that the Hsp90/Swo1 chaperone is also required for Cdc2 function (Muñoz and Jimenez 1999), pointing toward Hsp90 as an important element in the regulation of these key cell cycle kinases.

Hsp90 is an abundant molecular chaperone essential to the establishment of many cellular regulation and signal transduction systems. In addition to playing a vital role in thermotolerance and stress responses, the cytoplasmic Hsp90s are essential for establishing the function of steroid hormone receptors, transcription factors, tyrosine and threonine/serine kinases, and tumor suppressors (Pratt 1997). *In vitro*, purified Hsp90 binds to denatured protein and displays antiaggregant properties. However, *in vivo*, Hsp90 activity involves a cohort of associated partners or cochaperones that participate in multiprotein complexes with Hsp90. Several discrete subcomplexes are associated with particular subclasses of target proteins, so that one of these subcomplexes contains Hsp90/Hsp70/Hip and p60/Sti1 (Smith *et al.* 1993), while another contains one of several immunophilins/cyclophilins and the acidic protein p23 (Johnson and Toft 1994).

The p23 protein is one of the most abundant Hsp90-associated proteins whose function in living cells remains uncertain. This cochaperone was first identified in association with steroid receptors (Smith *et al.* 1990) and was later shown to be a major component of Hsp90

Corresponding author: Juan Jimenez, Departamento de Genética, Facultad de Ciencias, Universidad de Málaga, Campus Universitario de Teatinos, 29071 Málaga, Spain. E-mail: jimmar@uma.es

[1] *Present address:* 310 Turker Hall, University of Missouri, Columbia, MO 65211-7400.

complexes (Johnson and Toft 1994). It is thought that p23 interacts with target polypeptides that are indirectly associated *via* Hsp90. However, *in vitro* folding assays have shown that p23 itself can function as a molecular chaperone that binds independently to proteins and prevents them from aggregating (Bose *et al.* 1996; Freeman *et al.* 1996). In this report, we present a genetic and biochemical characterization of a p23 homologue from the fission yeast *S. pombe*. We show that the identified p23 cochaperone is required for heat-shock tolerance and plays a role in the activity of Wee1 and the regulation of Cdc2.

MATERIALS AND METHODS

Yeast strains, media, and general methods: The standard media and genetic procedures used in this work have been previously described (Moreno *et al.* 1991). The fission yeast *S. pombe* 972 h^- and 975 h^+ haploid wild-type strains were used. The thermosensitive (ts) strains *cdc2-33*, *cdc2-M26*, *cdc2-56*, *cdc2-L7*, and *cdc25-22* and the strains *adh:wee1-50* (a strain overproducing the thermosensitive Wee1-50 protein from the *adh* promoter), *CDC2Dm* (in which the endogenous *cdc2* is replaced by its Drosophila homologue), and *swo1-w1* (partially defective in Hsp90 function) have been previously described (Nurse *et al.* 1976; Nasmyth and Nurse 1981; Russell and Nurse 1987a,b; Bejarano *et al.* 1995; Muñoz and Jimenez 1999). Double mutants were constructed by tetrad dissection. The strain *nmt:wos2* (the *wos2* gene is expressed from the *nmt* thiamine-repressible promoter; Maundrell 1993) and *wos2::ura4* (in which the *wos2* gene is replaced by the *ura4* gene) are described in this study. Yeast transformation was achieved using the electroporation procedure (Prentice 1992). Flow cytometry analysis was performed as described in Sazer and Sherwood (1990). Cells were fixed with ethanol, stained with propidium iodine, and a Becton Dickinson FACScan system was used. Fixed cells were also stained with 4′, 6-diamidino-2-phenylindole (DAPI) and nuclei visualized in a Nikon microscope equipped with epifluorescence and a CCD camera. Calcofluor staining was used to visualize the cell wall and septum material. Double staining with DAPI and calcofluor was used to visualize nuclei and septum in the same cells. Under the conditions used, brightness of calcofluor is more intense than that of DAPI but the former photobleaches more rapidly. Thus, capturing fluorescent images early or late after excitation and using different exposure times allows us to visualize calcofluor and DAPI independently. Anti-α-tubulin (a generous gift from Dr. K. Gull) was used for microtubule localization (1/25 dilution) and anti-Wos2 polyclonal rabbit serum (1/400 dilution) for the localization of Wos2, following described procedures (Alfa *et al.* 1993). Bound antibodies were detected using fluorescein-conjugated secondary antibodies (Sigma, St. Louis; 1/40 dilution). The synchronous culture was prepared by means of the *cdc25-22 ts* mutation. A culture of a *cdc25-22* strain was synchronized by restricting growth at 36° for 4 hr. The cells were then released into fresh medium at the permissive temperature of 25°, and aliquots were removed at 20-min intervals over 8 hr. The septation index was monitored in a Nikon microscope using dark-field microscopy. Protein samples were prepared for Western blot analysis of Wos2. For stationary culture experiments, cells were grown at 32° in minimal liquid medium until glucose (the limiting nutrient in the medium used) became exhausted. Samples of total RNA and protein were also used to visualize the *wos2* mRNA and Wos2 protein, by standard Northern and Western blot analysis, respectively. Heat shock was achieved by transferring cultures exponentially growing at 25° in liquid media to 48° in a water bath. Aliquots were then taken at time intervals and placed in ice. Drops from these aliquots with equal number of cells were then placed in a YE plate to monitor growth after incubation for 2 days at 25°, or viable cells were counted by counting colonies formed from diluted samples plated in YE, incubated for 5 days at 25°.

Cloning of *wos2*: An *adh:wee1-50 leu1-32* strain was transformed with a *S. pombe* genomic library constructed in the pDB262 plasmid (Wright *et al.* 1986). Prototrophic transformed cells were selected at 35° in minimal medium agar plates, and the colonies formed were replica plated into YE agar plates and incubated at 25°. Colonies growing under these conditions were microscopically screened, and one of them was selected because of the mitotic instability of the *adh:wee1-50* suppressing activity, indicating that it is located in a plasmid. The plasmid was recovered from one of these colonies, and the suppressing activity localized to a 3.9-kb *S. pombe* genomic DNA fragment. This fragment was entirely sequenced in both strands and a single gene found (*wos2*) responsible for the suppression activity (GenBank accession no. L41166). This gene contained a single intron with conventional features found in *S. pombe* introns (Prahala *et al.* 1992). Using this 3.9-kb DNA fragment as a probe, *wos2* was physically mapped to a single locus in chromosome I linked to *mst1*, between *rad16* and *nuc2*.

Deletion of *wos2*: To construct a *wos2*-deleted strain (*wos2::ura4*) an *Xba*I-*Ava*I fragment in *wos2* was replaced with the *ura4* gene in the 3.9-kb *wos2* genomic clone. This construct was used to delete the *wos2* gene in a diploid strain h^+/h^- *ade6-M210*/*ade6-M216 ura4-d18*/*ura4-d18*. Tetrad dissection indicated that these diploids produced four viable meiotic spores. Stable ura^+ diploids were selected, and Southern blot analysis indicated that the *wos2* gene was deleted in these ura^+ haploid spores. Southern blot analysis was carried out by digesting 1–2 μg DNA of the desired strain, separating the digested DNA on 0.9% agarose gels and blotting onto Hybond-N^+ (Amersham, Piscataway, NJ) membranes according to the manufacturer's instructions. These membranes were hybridized in Church buffer at 65° (Church and Gilbert 1984) and probed with *wos2* DNA labeled by the random priming method. The filters were washed in 0.1× standard saline citrate (SSC)/0.1% SDS at 65° and autoradiographed or analyzed using a PhosphorImager system (Fuji).

Overexpression of *wos2*: To isolate cDNAs coding for *wos2*, *Escherichia coli* colonies transformed with a cDNA library of *S. pombe* (Moreno and Nurse 1994) were probed with the 3.9-kb genomic fragment of *wos2*, and a variety of cDNAs were selected because of their different sizes. The different cDNAs were sequenced, and the largest one was subcloned into the pREP3X *S. pombe* expression vector (in this construct, the expression of *wos2* is driven by the *nmt1* thiamine repressible promoter) (Maundrell 1993). To construct a stable strain overproducing *wos2* (the *nmt:wos2* strain), the promoter-cDNA-transcriptional stop construct was inserted into the pJK148 integrative plasmid. An h^- *leu1-32* strain was transformed with this pJK-*wos2* construct linearized within the *leu1* gene, and a stable leu^+ transformant was selected. Southern blot analysis indicated that a single copy of the construct was integrated at the *leu1-32* locus. Similarly, the *nmt:wos2* construction was subcloned into an *ura4* integrative plasmid. An h^- *ura4-D18* strain was transformed with this construction linearized within the *wos2* gene, and stable ura^+ transformants were selected. Southern blot analysis indicated that a single copy of the construction was integrated at the *wos2* loci. These strains overexpressed *wos2* when grown in minimal medium without thiamine. Samples were routinely taken 19 hr after

induction. A total of 5 μg/ml thiamine was added to turn the *nmt1* promoter off. The ura$^+$ and leu$^+$ markers were used to construct strains overexpressing *wos2* in different genetic backgrounds or to select a strain overexpressing *wos2* from two copies of the *nmt:wos2* construction (2× *nmt:wos2*).

Expression of the *S. cerevisiae* p23 homologue in the fission yeast: A budding yeast homologue to *wos2* was first identified as sequence *YKL518*, derived from the *S. cerevisiae* genome project (Jacquier *et al.* 1992). More recently, this gene has been characterized as a p23 homologue named *SBA1* (Fang *et al.* 1998). Oligonucleotides 5′-CGATTCATAATAGTCAT GTCCG-3′ and 5′-GCACTCCAGGTTGATTTGCTCC-3′ were used to clone by PCR amplification the YKL518 open reading frame (ORF) of *S. cerevisiae*, and the resulting DNA fragment was subcloned into the pREP3x plasmid. This construct allows the *nmt*-driven expression of the amplified DNA in *S. pombe*.

Northern and Western blot analyses: In Northern blot analysis, total RNA was prepared as described (Moreno *et al.* 1991) and probed with the 3.9-kb purified fragment containing the *wos2* gene. Filters were analyzed using a PhosphorImager system (Fuji). Western blot procedures were performed by loading equivalent amounts of protein in SDS-polyacrylamide gels, and the blot was carried out using GeneScreen Plus membranes according to the manufacturer's (New England Nuclear, Boston) instructions. To obtain polyclonal antibodies against Wos2, the large cDNA encoding *wos2* was subcloned in an *E. coli* expression vector (Sambrook *et al.* 1989); the *E. coli*-overproduced Wos2 protein was extracted from a polyacrylamide gel, and preparation of protein conjugates for rabbit immunization was as described (Sambrook *et al.* 1989). Western blots were made using serum (1/1000 dilution) against this gel-purified Wos2 protein overproduced in *E. coli*, anti-α-tubulin (Sigma; 1/10,000 dilution), or monoclonal anti-Cdc2 (1/1000 dilution) provided by Paul Nurse (ICRF, London). The blots were developed with an enhanced chemiluminescence method (ECL, Amersham).

Immunoprecipitation and H1 kinase assay: Conventional experiments of immunoprecipitation in *S. pombe* extracts were carried out following described methods (Moreno *et al.* 1991). The immunoprecipitation of cell extracts obtained with 2 μl of anti-Wos2 or with 2 μl of anti-Cdc2 was used for histone H1 kinase assays as described in Moreno *et al.* (1991). This procedure was also performed to assay Wos2 phosphorylation. An aliquot of each sample was used for Western blot analysis and Coomassie blue staining as controls.

[^{35}S]Methionine labeling and *in vitro* association assays: For *in vitro* experiments, [^{35}S]methionine-labeled Wos2, Cdc2, and Cdc13 proteins were synthesized using the TnT Coupled Reticulocyte Lysate system kit (Promega, Madison, WI), according to the manufacturer's instructions. Aliquots of 2 μl of reticulocyte lysates containing the synthesized proteins were mixed, and immunoprecipitation with 1 μl of anti-Wos2, 1 μl of anti-Cdc2, or 1 μl of anti-Cdc13 antibodies was carried out as described in Moreno *et al.* (1991). The labeled proteins were resolved by electrophoresis in polyacrylamide gels and visualized by autoradiography.

RESULTS

Isolation of *wos2*: Wee1 encodes a tyrosine kinase that inhibits entry into mitosis by phosphorylation of the Cdc2 kinase. Overproduction of Wee1 causes a lethal G2 arrest. Since Wee1 is not essential for yeast growth or viability (Russell and Nurse 1987a), searching for multicopy suppressors of this lethal effect is a powerful selective procedure to isolate genes encoding negative regulators of Wee1. The *adh:wee1-50* strain was used to this end. This strain overproduces a Wee1-50 thermosensitive protein (Russell and Nurse 1987a), which results in viable "wee" cells at 35°, where Wee1-50 is inactive, but yields a lethal cell cycle block upon incubation at 25°, where the protein becomes active. Using this strain, we isolated a 3.9-kb *S. pombe* DNA fragment from a genomic library, which, in a multicopy plasmid, rescued the lethality caused by the overproduction of Wee1-50. Subcloning and DNA sequencing indicated that a single gene (here named *wos2*, for ***w**ee1-50 **o**verexpression **s**uppressor*) was responsible for the *wee1-50* overexpression suppressor phenotype (Figure 1A). This gene physically mapped in chromosome I, between *rad16* and *nuc2*. We also analyzed the suppression activity of *wos2* using a different conditional system to overexpress *wee1*. Strains harboring a *wee1* wild-type allele or a *wee1-50 ts* allele, subjected to the expression of the thiamine repressible promoter *nmt* (Maundrell 1993), grow on thiamine-containing media but produce elongated arrested cells under derepressed conditions.

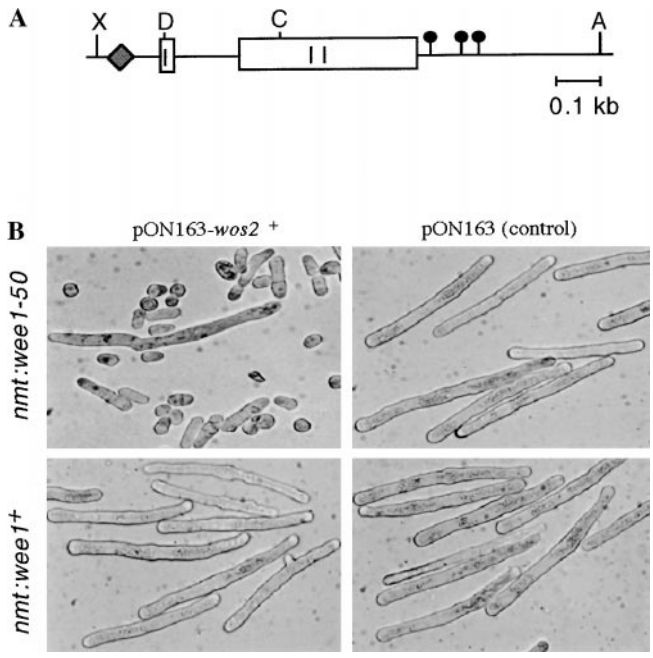

Figure 1.—Identification of *wos2*. (A) Schematic representation of the genomic fragment responsible for the Wee1-50 suppression activity. The only gene in this fragment (*wos2*) contains a small intron (exons I and II are in boxes). The TATA box (◆), transcriptional ends (●), and restriction sites used in this work (*Xba*I-X-, *Dra*I-D-, *Cla*I-C-, and *Ava*I-A-) are indicated. (B) Suppression activity of the cloned *S. pombe wos2* gene. The DNA fragment containing *wos2* was subcloned into the pON163 multicopy plasmid (pON163-*wos2*), and its suppression activity assayed in *S. pombe* cells overexpressing either the *wee1-50* allele or the wild-type *wee1* allele from the *nmt* promoter. Plasmid pON160 was used as a control. Suppression efficiently occurred in *nmt:wee1-50* cells, yielding "wee" cells (on plasmid loss cells become elongated) as happens in the *adh:wee1-50* strain, but cells overexpressing the wild-type *wee1* gene were not suppressed by multicopy expression of *wos2*.

When using these strains, we found that multicopy expression of *wos2* suppressed the lethal arrest of the *nmt:wee1-50* strain, too, indicating that the suppression activity of *wos2* relied on the encoded Wee1-50 protein and not on the promoter used for its overexpression (Figure 1B).

However, the multicopy expression of *wos2* did not suppress the arrest caused by the wild-type protein, indicating that the Wee1-50 mutant version is more sensitive to the action of Wos2 (Figure 1B). Such interaction also occurred in a *nim1*-deleted background, the only upstream inhibitor of *wee1* described in fission yeasts (Russell and Nurse 1987b), and consequently *wos2* interacts with Wee1 in a *nim1*-independent manner.

When searching for homologous sequences in DNA and protein databases, we found that Wos2 shares 30% identity to the human p23, and it is also homologous to the *S. cerevisiae* protein encoded by DNA sequence *YKL518* (Figure 2A). This sequence was initially described from the genome sequencing project of this microorganism (Jacquier *et al.* 1992), but very recently it has been identified as the budding yeast homologue to vertebrate p23 proteins (encoded by the *SBA1* gene; Fang *et al.* 1998). Thus, Wos2 could identify a p23-like cochaperone of fission yeast. A detailed sequence analysis revealed that it contains a glycine-rich sequence common to Hsp70 and many other molecular chaperones (Thode *et al.* 1996).

Proteins involved in chaperone functions and the stress response are often required for heat-shock tolerance. To examine a possible role of *wos2* in this response, we constructed a deletion mutant strain lacking Wos2 protein (Figure 2B). The *wos2* deletion had no effect on cell growth or viability, but *S. pombe* cells deleted for *wos2* were heat-shock sensitive (Figure 2C), indicating that Wos2 is actually a stress-related protein. Therefore, from the above combined results we conclude that Wos2 is likely a p23 protein in fission yeasts.

The putative protein sequence encoded by *wos2* shows some other striking properties in common with *YKL518*/*SBA1* and p23 homologues. The carboxyl-terminal half of these proteins has very few basic amino acids and, by contrast, contains clumps of acidic residues (Figure 2D). This poly-acidic sequence is also found in many other proteins, including cell cycle regulatory elements such as the *S. cerevisiae* Sic1 protein (Schwob *et al.* 1994) and a large number of cyclins, although the biological significance of this similarity is unknown.

Expression of *wos2*: To further elucidate the biological function of *wos2* and its possible regulation, we studied the expression of this gene in different phases of the *S. pombe* cell cycle and under different physiological conditions. No significant cell-cycle-dependent changes were observed in Wos2 during two rounds of synchronous cell divisions (Figure 3A), indicating that the level of this protein was not subject to oscillations during the cell cycle. The *wos2* messenger RNA was extremely abundant in proliferating cells, being roughly >20-fold the level of *cdc2* mRNA (assessed by PhosphorImager analysis of Northern blots). However, its expression dropped drastically as cells entered into early stationary phase (while the amount of *cdc2* transcripts stayed constant; Figure 3B). The amount of Wos2 protein also decreased in starved cells (Figure 3B), indicating that its function is probably associated with cell proliferation. Immunofluorescence analysis revealed punctate staining of Wos2 in exponentially growing cells, which suggests that Wos2 is not a freely diffusible molecule but is restricted to dispersed macromolecular aggregates found in the nucleus and the cytoplasm (Figure 3C). As described for the expression of protein p23 in avian cells (Johnson *et al.* 1994), expression of Wos2 was not significantly induced by temperature, either by growth at 35° or by heat shock for 10–20 min at 48° (data not shown).

To further analyze the phenotype caused by the overexpression of *wos2*, we isolated cDNAs for this gene. Three different polyadenylated cDNAs were isolated, corresponding to the three transcripts observed in Northern blot analysis (Figure 3B). The largest *wos2* cDNA was expressed under the control of the thiamine repressible *nmt* promoter, and the construct integrated at the *leu1* locus in the *S. pombe* genome in a single copy. The genetic interactions previously described by using a multicopy plasmid system to overexpress the genomic *wos2* gene took place as well upon *wos2* overexpression from the *nmt* promoter in this strain. This *nmt:wos2* strain was further used for genetic analysis.

Genetic interactions of *wos2* and *cdc2*: Interestingly, overproduction of Wos2 showed allelic interactions identical to those caused by a partial Hsp90 inactivation (Muñoz and Jimenez 1999). In addition to suppressing Wee1-50 activity, the overexpression of *wos2* produced a large proportion of elongated cells (~20%) in combination with the *cdc2-33 ts* allele at 25°, permissive temperature. These elongated cells were multinucleate and had multiple septa. The phenotype was exacerbated at 29°, while cells retained a wild-type phenotype under *nmt:wos2* repression under this condition (Figure 4). At 29° in the absence of thiamine 100% of the cells were arrested and, as determined by flow cytometry analysis (Figure 4), ~70% of them were 4C or 8C (DNA content per cell) in agreement with the proportion of binucleate and tetranucleate cells observed under the microscope under these conditions.

A more drastic effect was observed when the overexpression of *wos2* took place in a *cdc2-M26 ts* mutant, where >50% of the cells had condensed chromatin at 25° and many exhibited a novel septation defect. In these cells, the septum was initiated but exit of septum formation did not occur, and therefore the septum was continuously produced, sometimes along the cell (Figure 4). In some other cells, the septation pattern mimics that of *S. pombe cdc16*$^{-}$ mutants (Chang and Nurse

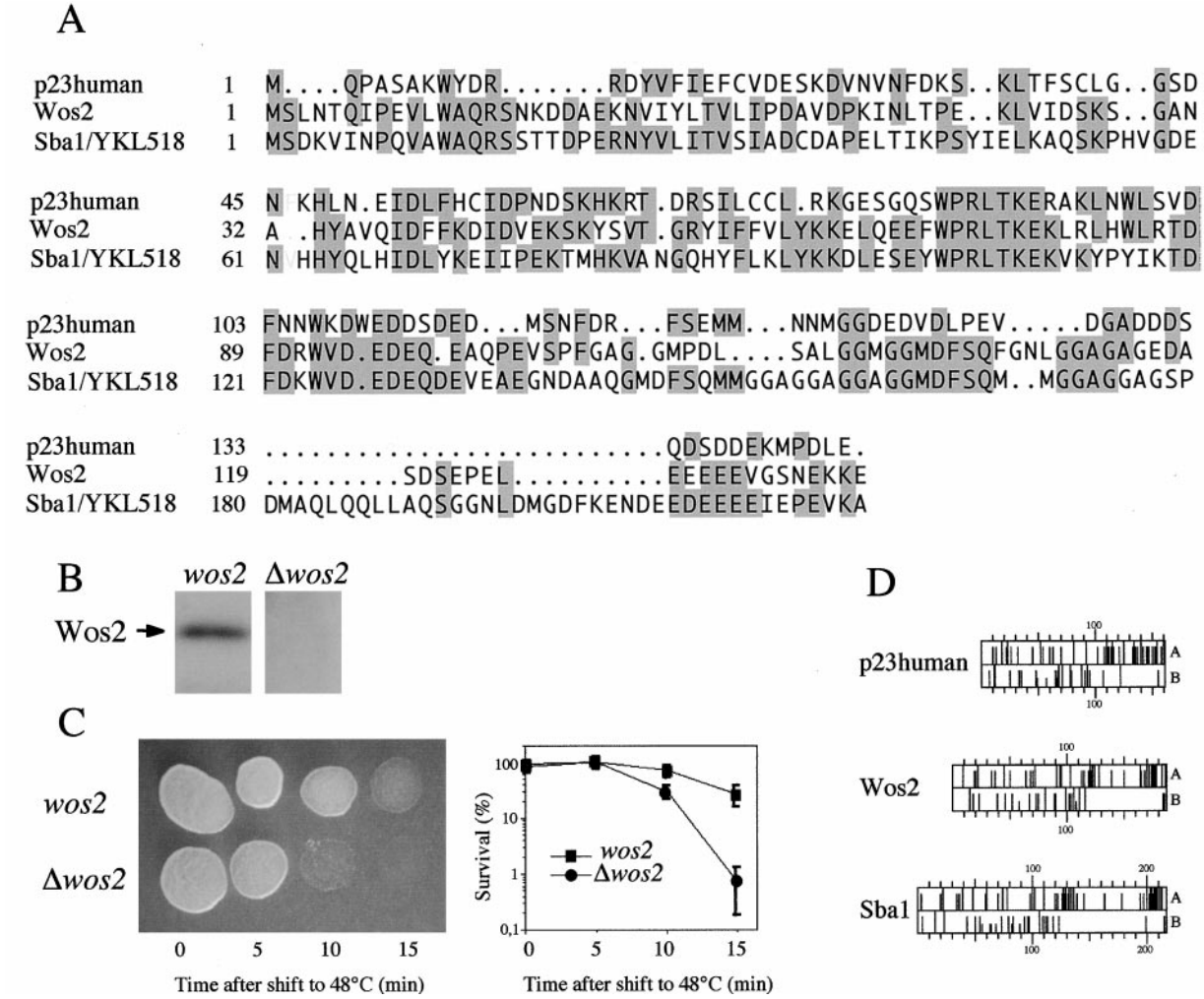

Figure 2.—Sequence and physiological analysis of *wos2* gene function. (A) Identity between the *wos2*-encoded protein and its counterparts from *S. cerevisiae* (Sba1/YKL518) and from the human p23 chaperone (p23 human; identical residues found in at least two sequences are indicated in shadow). (B) Western blot analysis using anti-Wos2 in wild-type cells (*wos2*) and cells deleted for the *wos2* gene (Δ*wos2*). (C) Involvement of *wos2* in the heat-shock tolerance of *S. pombe* cells. Growth temperature of wild-type (*wos2*) or *wos2*-deleted (Δ*wos2*) cells was shifted from 25° to 48°, and time-interval samples were taken to determine cell survival, either by plating a drop with equal number of cells (left) or by counting the number of cells per milliliter that form a colony (average and standard deviation of three independent experiments) (right). (D) Acid-base residue distribution of Wos2, Sba1/YKL518, and human p23 proteins.

1993; Frankhauser and Simanis 1994). The effect of *wos2* over this *cdc2-M26* allele was also enhanced with temperature, yielding 100% arrested cells at 29° and a high proportion of mononucleate cells with 2C DNA content (Figure 4).

A fission yeast strain in which the endogenous *cdc2* gene was replaced by the Drosophila *CDC2Dm* homologue was also used (Jimenez *et al.* 1990). This Drosophila gene is functional in fission yeast, but exhibits a weak interaction with *cdc2*-regulatory proteins such as Wee1 and the B-type cyclin encoded by *cdc13* (Bejarano *et al.* 1995). The overexpression of *wos2* in this *CDC2Dm S. pombe* strain was lethal at any temperature, producing 100% nonseptated Cdc^- cells with a G2 arrest (Figure 4).

The effects on these *cdc2* alleles also occurred in a *wee1*-deficient background, indicating that this interaction is independent of *wee1*. The overexpression of *wos2* in combination with other *cdc2* alleles (such as *cdc2-L7*, *cdc2-56*, or "wee" alleles of *cdc2*), with *ts* alleles in *cdc* genes other than *cdc2* (*cdc10-129*, *cdc17-K42*, *cdc13-117*, and *cdc25-22* were tested), or in a wild-type background had no obvious effects on the cell cycle (see *cdc2-L7* in Figure 4). Therefore, on the basis of this allele-specific interaction, we conclude that Wos2 functionally interacts with the Cdc2 mitotic machinery.

As assessed by Western blotting and histone H1 phosphorylation, the overproduction of Wos2 did not alter the level of Cdc2 protein or its kinase activity (Figure 5). In fact, the level of H1 phosphorylation slightly increased in *cdc2-M26* cells overexpressing *wos2* (Figure 5, lane 2), consistent with the mitotic arrest observed in this strain (Figure 4). These results suggest that Wos2

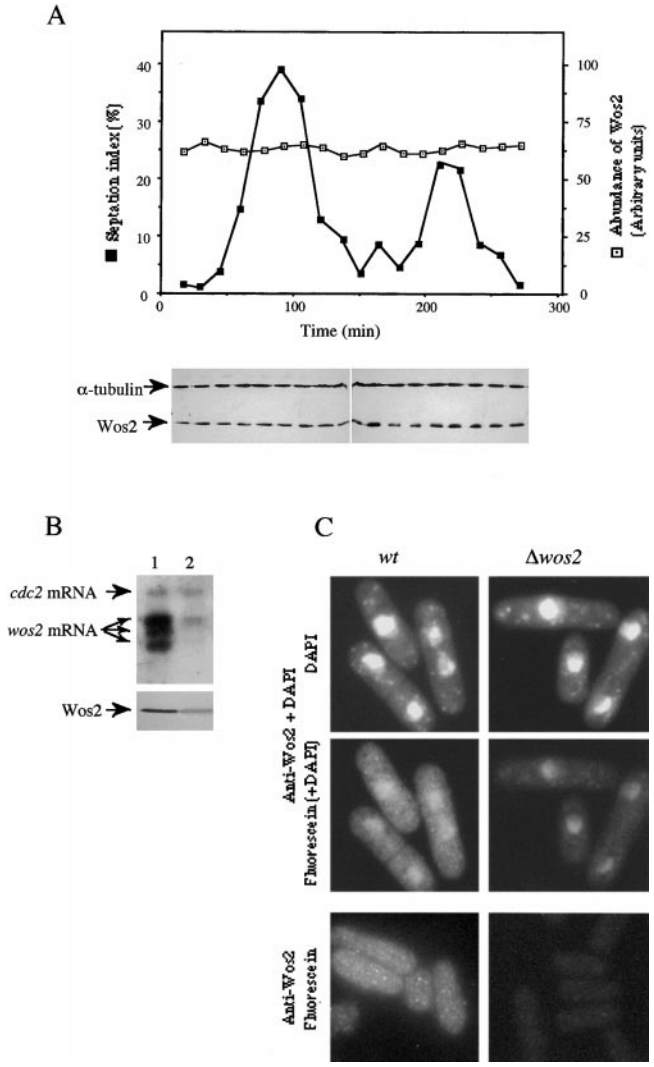

Figure 3.—Expression of *wos2* in *S. pombe* cells. (A) Western blot analysis of Wos2 protein from protein samples of a synchronous culture followed for two generations. α-Tubulin was analyzed as a control. The abundance of Wos2 (densitometric analysis) and the septation index in each sample of the synchronous culture are represented. Cells were synchronized by using a *cdc25-22* mutant strain (see materials and methods). Similar results were obtained in samples from cells synchronized by elutriation (not shown). (B) Northern blot analysis of *wos2* mRNA in total RNA samples from proliferating (lane 1) and stationary starved (lane 2) cells (*cdc2* mRNA was analyzed as a control). The abundance of Wos2 protein was also determined in the same cells by Western blot analysis. (C) Epifluorescence micrographs of wild-type (*wt*) and *wos2*-deleted cells (Δ*wos2*) double stained with DAPI and anti-Wos2 antibody / fluorescein-conjugated secondary antibody (top micrographs). In our filter set the calcofluor signal is still seen in the fluorescein channel, and cells without DAPI staining were also used for the proper immunolocalization of Wos2 (bottom micrographs).

interferes with regulatory functions of Cdc2 rather than its stabilization or kinase activity.

Wos2 interferes with Hsp90 activity: Wee1 and Cdc2 kinases are both highly dependent on Hsp90 activity for function (Aligue *et al.* 1994; Muñoz and Jimenez 1999). Interestingly, all these genetic and biochemical effects caused by overexpression of Wos2 in *cdc2* mutant cells resembled those caused by partial inactivation of the Hsp90 chaperone encoded by *swo1* (Muñoz and Jimenez 1999). This observation strongly suggests that an excess of Wos2 could diminish the activity of Hsp90 on its Hsp90-target kinases. To investigate this hypothesis we analyzed the effect caused by overproducing Wos2 in Hsp90-deficient cells. As shown in Figure 6, cells overproducing Wos2 and those partially defective for Hsp90 (*swo1-w1*) were indistinguishable from wild type at 25°. However, overexpression of *wos2* was lethal in a *swo1-w1* mutant background. This synergy between *nmt:wos2* and *swo1-w1* suggests that overproduction of Wos2 interferes with Hsp90 function *in vivo*. The synthetic cell cycle defect observed by overexpression of *wos2* in *swo1-w1* mutant cells (Figure 6) resembled that produced by overexpression of *wos2* in *cdc2-33* mutant cells (Figure 4). In both cases, identical septation deficiencies and abnormal distributions of DNA content per cell were obtained. This observation suggests that the excess of Wos2 could specifically interfere with the Hsp90-Cdc2 interaction.

In vitro systems have been used to demonstrate that vertebrate p23 physically interacts with target proteins (Bose *et al.* 1996; Freeman *et al.* 1996). To assay for physical interactions, [^{35}S]methionine-labeled Wos2 and Cdc2 were synthesized separately using reticulocyte lysates, and association of these two proteins was assayed by coimmunoprecipitation in mixed lysates. As shown in Figure 7A, anti-Wos2 antibodies coimmunoprecipitated labeled Cdc2 from mixed extracts and, reciprocally, monoclonal anti-Cdc2 copurified Wos2. The anti-Cdc2 antibody was highly specific for the *S. pombe* Cdc2 (Figure 7A); therefore, the existence of Wos2 in anti-Cdc2-purified proteins from Wos2-Cdc2 mixed extracts suggests that Wos2 physically interacts with Cdc2 *in vitro*. The relatively low amount of Wos2 coimmunoprecipitated with anti-Cdc2 indicates that these two proteins interact with low affinity, as has been previously described for other chaperones (Smith 1993; Bose *et al.* 1996; Freeman *et al.* 1996). Polyclonal anti-Wos2 antibodies nonspecifically bind a mild amount of Cdc2 (perhaps through reticulocyte p23 protein), but in equivalent amounts of synthesized Cdc2 protein, the presence of Wos2 significantly increased the level of Cdc2 coimmunoprecipitated with anti-Wos2, in support of a Cdc2-Wos2 association.

The *cdc13*-encoded cyclin binds to Cdc2 with high affinity to form a Cdc2/cyclin B complex. As shown in Figure 7B, Wos2 was not found in anti-Cdc13 immunoprecipitates from mixed extracts containing synthesized Wos2 and Cdc13, but it was copurified when Cdc2 was also added; reciprocally, Cdc13 also coimmunoprecipitated using anti-Wos2 in this extract (Figure 7B). These results confirm that Wos2 may associate with the Cdc2 kinase and with Cdc2/cyclin complexes. Thus, an excess

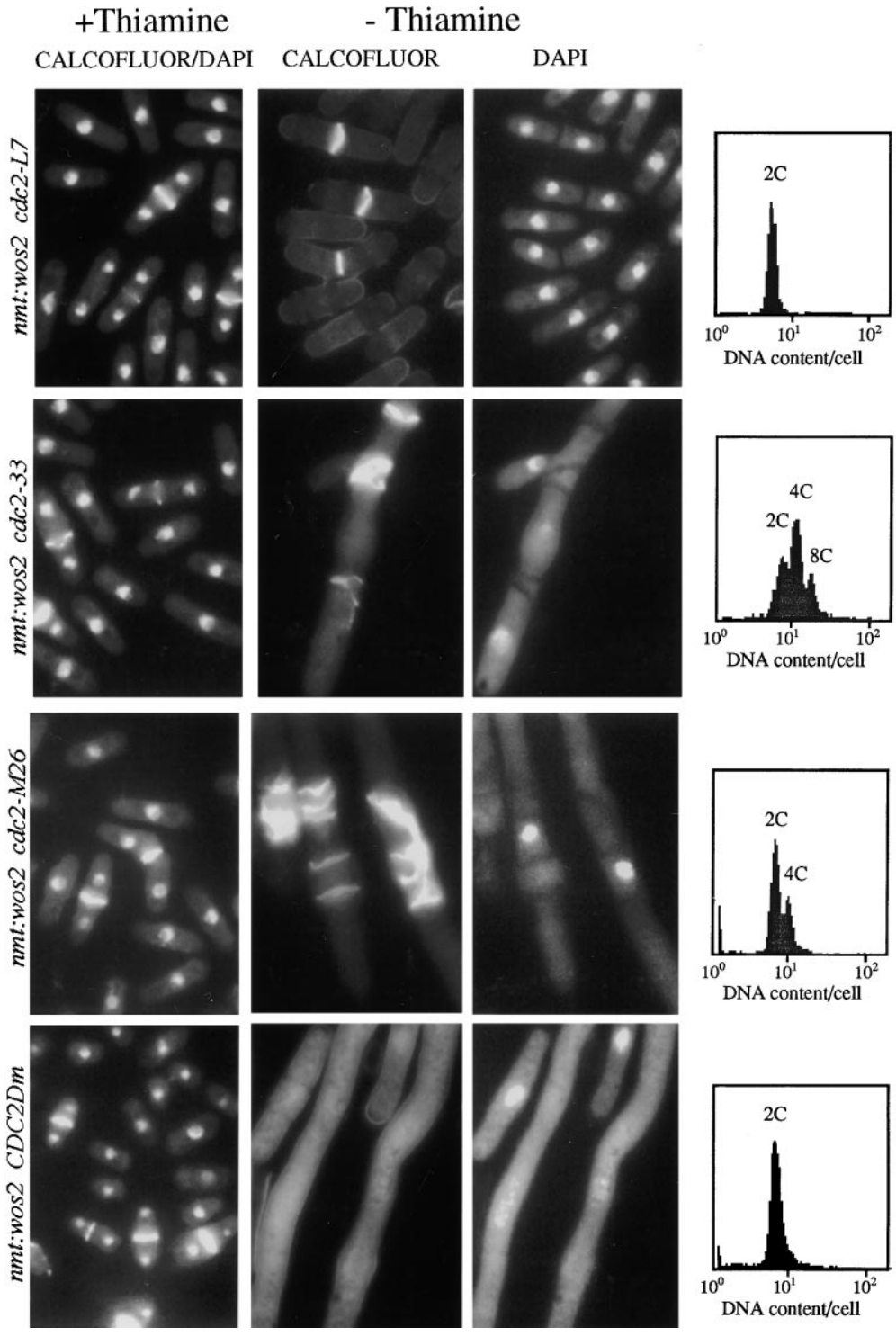

Figure 4.—Genetic interactions between *wos2* and *cdc2*. Calcofluor and DAPI staining of cells harboring the *nmt:wos2* construction in combination with different *cdc2* alleles (*cdc2-L7*, *cdc2-33* *cdc2-M26*, and *CDC2Dm*) incubated in media with thiamine (control) and without this vitamin (overexpressing *wos2*) at 29°. Calcofluor and DAPI staining are shown simultaneously in cells growing in the presence of thiamine. Flow cytometry analyses (DNA content per cell) of these strains incubated at 29° in media without thiamine are also shown. Populations containing 2C, 4C, and 8C represent mononucleate, binucleate, and tetranucleate cells, respectively.

of Wos2 could reduce Hsp90 function *in vivo* by competitive binding to common target kinases.

We failed to demonstrate such physical interaction by measuring Cdc2 kinase activity (H1 phosphorylation) in immunoprecipitates of Wos2 obtained from yeast crude extracts, but surprisingly, the immunoprecipitated Wos2 protein was efficiently phosphorylated in this assay (Figure 8). This phosphorylation took place in the absence of Cdc2 and Wee1 kinase activity (assayed at 37° in *cdc2-33* and *wee1-50* genetic backgrounds) and in cells lacking the Spc1 MAP kinase (deleted for the *spc1* gene; Shiozaki and Russell 1995). The nature of this kinase activity is now being investigated, but despite its source, it demonstrates that Wos2 may be phosphorylated *in vitro*, according to the large number of putative phosphorylation sites that can be found on its protein sequence; phosphorylation sites were also described earlier for the avian p23 (Johnson *et al.* 1994).

The physiological expression of *wos2* is extremely high in proliferating cells (Figure 5). Thus, the addi-

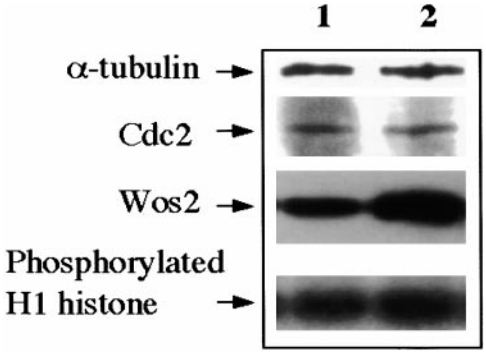

Figure 5.—Effects of the overproduction of Wos2 in the abundance of Cdc2 and the level of Cdc2 kinase activity. Cellular proteins from *wos2* (lane 1) and *wos2*-overexpressing cells (lane 2) in a *cdc2-M26* background incubated at 29° (100% arrested cells) were separated on SDS-polyacrylamide gel, transferred to a membrane, and the abundance of Cdc2 and Wos2 in the membrane visualized by immunostaining (anti-α-tubulin was used as a loading control). Crude extracts of these cells were used to assay phosphorylation of added histone H1 in the presence of [γ-^{32}P]ATP. Histone phosphorylation was determined by autoradiography.

tional expression of *wos2* driven by the *nmt* promoter could not be sufficient to produce a significant effect in wild-type cells growing exponentially. To overproduce a significantly higher level of Wos2, a strain containing two copies of the *nmt:wos2* construction (2× *nmt:wos2*) was used. This 2× *nmt:wos2* strain behaved like a wild-type strain in media with thiamine, but in the absence of this vitamin, the overexpression of *wos2* was lethal, yielding ~75% multinucleate cells, most of them binucleate (Figure 9A). According to flow cytometry analysis of this strain, most of the cells were 2C (Figure 9B), suggesting that each individual nucleus of these cells was actually 1C in DNA content. The nuclei of binucleate cells were decondensed and the interphase microtubule array re-established (Figure 9C), indicating that cells had exited from mitosis, but the nuclei remained adjacent, indicating that exit from mitosis occurred prior to maximal microtubule elongation. Therefore, higher level of expression of *wos2* in a wild-type background allowed the exit from mitosis, but delayed progression through the early G1 phase.

The *SBA1* gene has been shown recently to code for the budding yeast p23 protein (Fang *et al.* 1998). Oligonucleotides were designed to PCR amplify to coding sequence of this gene, and its *nmt*-driven expression in the fission yeast demonstrated that *SBA1* was functionally identical to the *S. pombe wos2*, efficiently suppressing the cell cycle block caused by the overexpression of *Wee1-50*, showing the same allele-specific interactions with *cdc2* mutants, producing binucleate cells at higher levels of expression, and complementing the heat-shock sensitivity in a *S. pombe* strain with the endogenous *wos2* gene deleted. We therefore conclude that *wos2* and

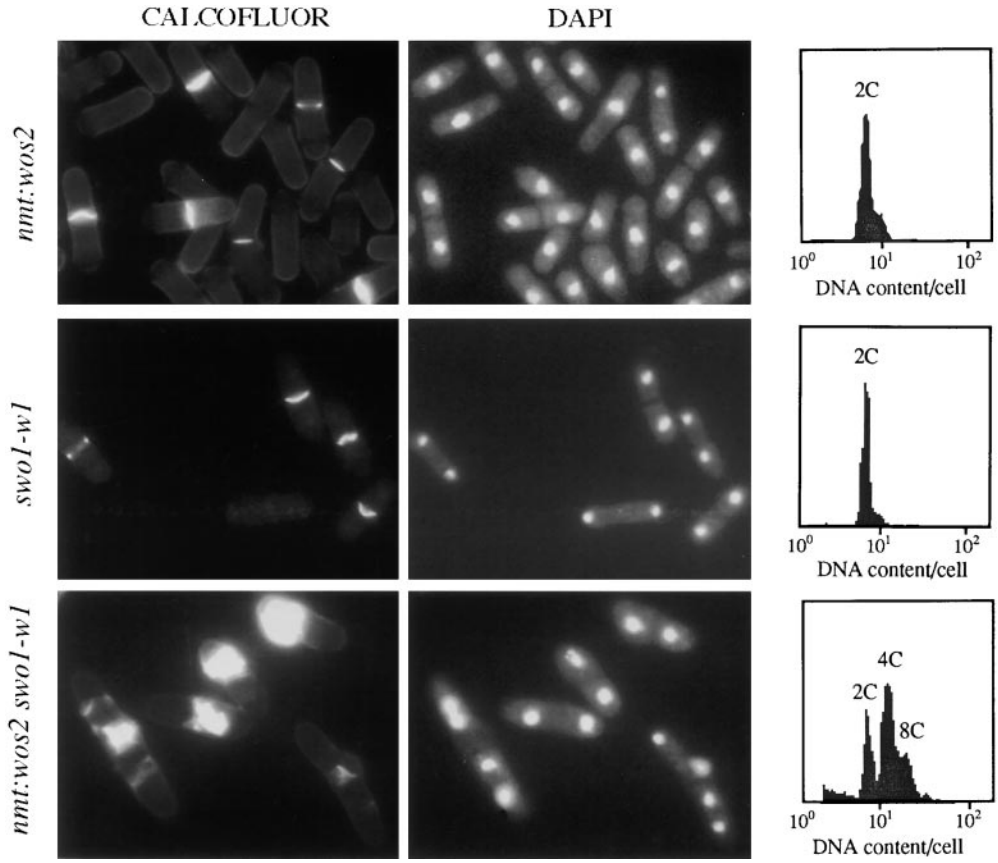

Figure 6.—Genetic interactions between *wos2* and *swo1*. Calcofluor and DAPI staining of cells overproducing Wos2 (*nmt:wos2* in media without thiamine), cells partially deficient for Hsp90 activity (*swo1-w1* at 25°), and cells overproducing Wos2 in a *swo1-w1* background (*nmt:wos2 swo1-w1* without thiamine at 25°). Flow cytometry analyses (DNA content per cell) of these cells are also shown. Populations containing 2C, 4C, and 8C represent mononucleate, binucleate, and tetranucleate cells, respectively.

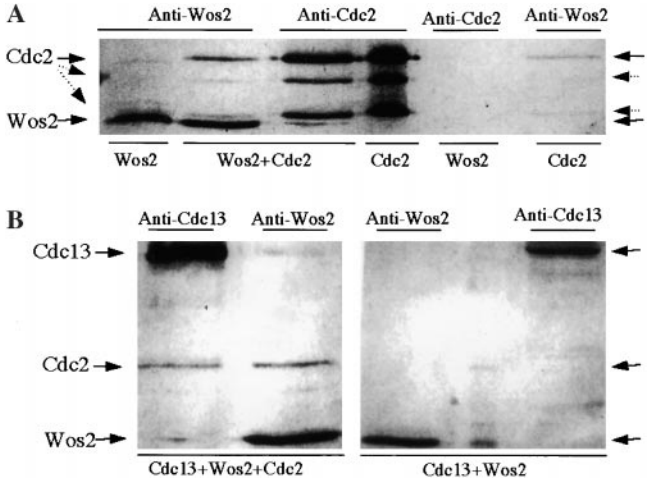

Figure 7.—Physical association of Wos2 and Cdc2 determined by coimmunoprecipitation assays from labeled proteins synthesized in reticulocyte extracts. (A) Immunoprecipitation of Wos2 and/or Cdc2 using anti-Wos2 or anti-Cdc2 from extracts containing Wos2, Cdc2, or both proteins, as indicated. The anti-Wos2 identified a single Wos2 protein. The anti-Cdc2 recognized Cdc2 and two additional truncated polypeptides, generated by translation of *cdc2* mRNAs from internal methionine codons. (B) Immunoprecipitation of Wos2, Cdc2, and/or Cdc13 proteins with anti-Wos2 or anti-Cdc13 from mixed extracts containing Wos2 and Cdc13 or Wos2, Cdc2, and Cdc13, as indicated.

SBA1 are functionally homologous. This fact, joint to the *in vivo* interaction of *wos2* with a Drosophila Cdc2 protein, suggests that the described function for *wos2* may be universal in eukaryotic cells.

DISCUSSION

Hsp90 is an abundant and highly conserved molecular chaperone. It exists in several discrete subcomplexes that together comprise the Hsp90 chaperone machinery. One of these subcomplexes contains several immunophilins and the acidic protein p23 (Johnson and Toft 1994). The *wos2* DNA sequence predicts a gene product homologous to this p23 cochaperone. In addition to sequence identity, both p23 and Wos2 migrate more slowly in denaturing gels than would be predicted from their size (21 kD for Wos2), suggesting some shared structural features (Johnson *et al.* 1994). Along with a significant sequence and structural similarity, Wos2 displayed biological properties previously described for p23 in higher eukaryotes. For instance, it is potentially regulated by phosphorylation (Figure 8); it is heat shock related (Figure 2); and it is very abundant and constitutively expressed in proliferating cells (Figure 3). Furthermore, the *S. cerevisiae* p23/Sba1 protein works in *S. pombe* as a functional homologue to Wos2. Taken together, these data indicate that Wos2 is a fission yeast homologue of vertebrate p23 proteins.

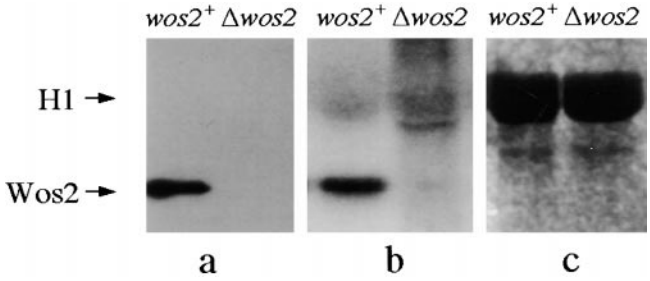

Figure 8.—*In vitro* phosphorylation of Wos2 protein. Histone H1 and [γ-^{32}P]ATP were added to anti-Wos2 immunoprecipitates from wild-type cells (*wos2*) or from cells deleted for *wos2* (Δ*wos2*) to assay for protein phosphorylation. Proteins were separated on SDS-polyacrylamide gel and the amount of Wos2 was determined by Western blot analysis (a), protein phosphorylation was determined by autoradiography (b), and Coomansie blue staining was used to visualize the added H1 histone (c).

The p23 cochaperone was first identified in association with the progesterone receptor (Smith *et al.* 1990), and was later shown to be an Hsp90-binding protein (Johnson *et al.* 1994). More recently, p23 has also been found to associate *via* Hsp90 with other proteins including transcription factors, heme-regulated eIF-2alpha kinase, and viral reverse transcriptase (Nair *et al.* 1996; Hu *et al.* 1997; Xu *et al.* 1997). The synthetic cell cycle defects caused by overproduction of Wos2 in specific Wee1 and Cdc2 mutants (Figure 1 and Figure 4) provide direct evidence that p23 also interacts with these two cell cycle kinases.

Such interaction led to loss of *wee1-50* activity but the effect on Cdc2 was different. In this later case, overexpression of *wos2* allowed for normal levels of Cdc2 kinase (Figure 5), but impeded Cdc2 functions required for the cell to undergo mitosis and cytokinesis in specific Cdc2 mutant backgrounds (Figure 4). Phenotypes of *nmt:wos2 cdc2-M26* synthetic lethal mutants resembled phenotypes displayed by dominant lethal mutants in *cdc2* that identify Cdc2 regulatory elements (Labib *et al.* 1995). We suggest that Wos2 interferes with regulatory functions in the Cdc2 complex. In agreement with this observation, other yeast Hsp90 cochaperones such as Ydj1, Cdc37, Cpr7, and Sti1 emerge as important elements in the cell cycle control and regulation of CDK activity (Xu and Lindquist 1993; Gerber *et al.* 1995; Kimura *et al.* 1995, 1997; Duina *et al.* 1996; Weisman *et al.* 1996; Yaglom *et al.* 1996; Chang *et al.* 1997).

The p23 protein has been characterized as a component of many Hsp90-containing heterocomplexes, and consequently, interactions of p23 with target proteins are thought to occur indirectly via its association to Hsp90 (Bohen 1998). However, it has been shown that purified p23 can bind independently to target polypeptides (Bose *et al.* 1996; Freeman *et al.* 1996). Our *in vitro* analysis is also consistent with a physical interaction between Wos2 and Cdc2 (Figure 7). Thus, p23 could exist in Hsp90 complexes, but this cochaperone itself could also interact with specific polypeptides. Interest-

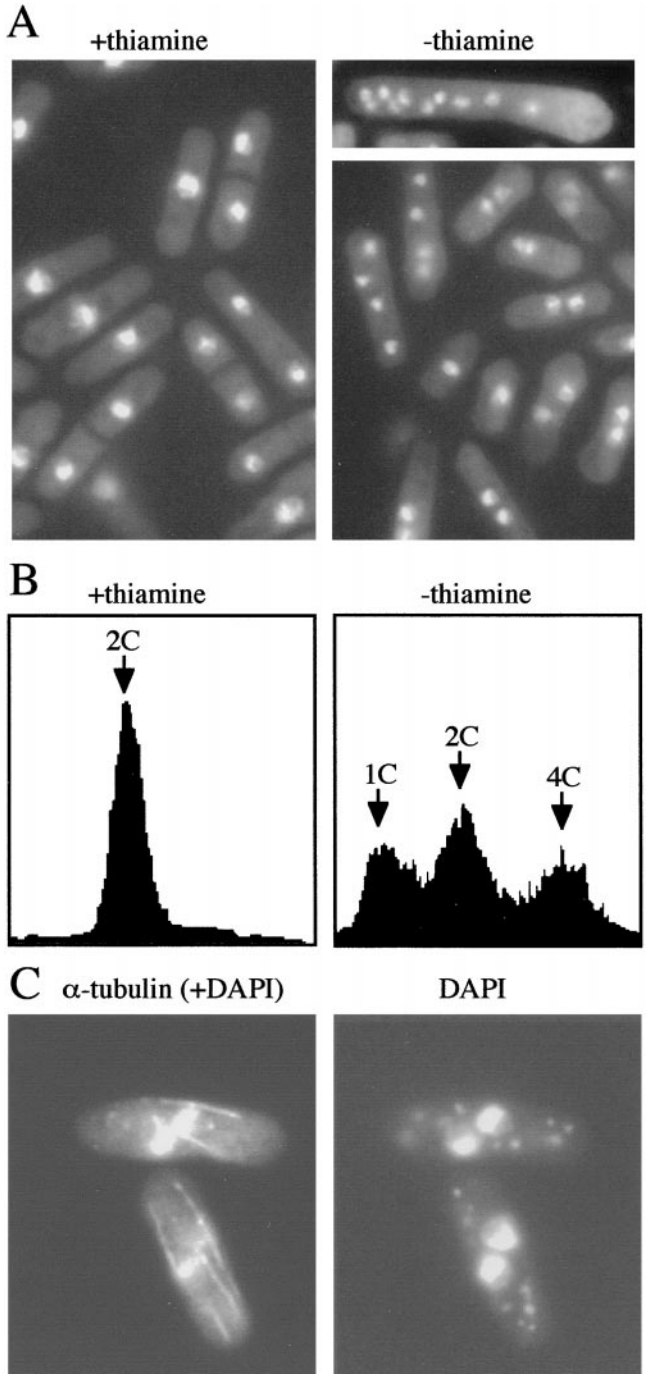

Figure 9.—DAPI staining (A) and flow cytometry analysis (B) of *S. pombe* cells harboring two copies of the *nmt:wos2* construction under repressed (+thiamine) and derepressed (−thiamine) conditions. Cells are as wild type in the presence of thiamine, but in media lacking this vitamin *wos2* produces abnormal cells containing 1, 2, or 4 nuclei with a 1C DNA content each. Cells with many nuclei may be also found (top micrograph). Microtubule array was also analyzed in these *wos2*-arrested cells (C) using monoclonal anti-α-tubulin serum to localize microtubule (left) and DAPI to visualize nuclei (right); (DAPI was also used in the α-tubulin staining).

ingly, the excess of Wos2 showed phenotypes and allele-specific interactions identical to mutants with reduced activity in the *swo1*-encoded Hsp90 chaperone (Aligue *et al.* 1994; Muñoz and Jimenez 1999), suggesting that Wos2 could compete binding of Hsp90 to common target substrates. Thus, the allele-specific interaction of Wos2 with Cdc2 may reflect a less tight interaction between mutant Cdc2-M26 and Cdc2-33 proteins with Hsp90 complexes (more easily perturbed by Wos2). The synthetic lethality caused by the excess of Wos2 in a Hsp90-deficient background is in agreement with this suggestion (Figure 6). Furthermore, incubation of these two *cdc2* alleles at 36° for 4 hr disassembles the mitotic Cdc2 machinery, while this treatment had no effect on other *cdc2 ts* alleles (Broek *et al.* 1991), indicating that Cdc2 complexes are particularly dependent on chaperone proteins in these two *cdc2* alleles. Both mutations lie in the central region of Cdc2 containing the regulatory thr167 residue (MacNeill *et al.* 1991). Phosphorylation of this residue is essential to enhance cyclin binding and drive the conformational changes that result in maximal kinase activation (Russo *et al.* 1996). Interestingly, certain mutations in this thr167 residue resemble the phenotype of yeast cells overproducing Wos2 in Cdc2-33 or Cdc2-M26 mutant backgrounds (Gould *et al.* 1998).

In wild-type cells higher levels of *wos2* overexpression were required to display a cell cycle deficiency, but in this genetic background it caused an early G1 block and impeded cell separation. This Wos2-mediated interference suggests that G1 regulatory elements also require the activity of a p23-Hsp90 complex in *S. pombe* cells. In budding yeasts, Cdc37 is required for the association of Cdc28/Cdc2 with multiple cyclins (Gerber *et al.* 1995), and Ydj1 has a direct role in the phosphorylation of the G1 cyclin Cln3 by Cdc28 (Yaglom *et al.* 1996). Thus, Wos2 could also interfere with the assembly of Cdc2 with *S. pombe* G1 cyclins. Consistent with this idea, the G1 arrest caused by overexpression of *wos2* displayed a phenotype similar to that described for *S. pombe* cells disrupted for the *cig1* and *cig2* genes (Connolly and Beach 1994). Conflicting data for *cig1* and *cig2*/*cyc17* have obscured the function of these proteins, but *cig2*/*cyc17* emerges as a G1 cyclin (Martin-Castellanos *et al.* 1996; Mondesert *et al.* 1996).

Loss of the *SBA1*-encoded p23 protein in budding yeasts did not result in any growth phenotype, indicating that this Hsp90 cochaperone is dispensable under normal growth conditions (Fang *et al.* 1998). This result contrasts with the relative importance of Hsp90 for cell growth (Borkovich *et al.* 1989). In our study, we have shown that *S. pombe* cells lacking Wos2 were heat-shock sensitive (Figure 2), revealing for the first time an *in vivo* role for p23/Wos2 in the stress response. *In vitro* chaperone assays have shown that purified p23 maintains early unfolded proteins in a folding-competent state, but in contrast to Hsp90, this cochaperone is un-

able to refold them (Bose *et al.* 1996; Freeman *et al.* 1996). Surprisingly, p23/Sba1/Wos2 is a ubiquitous protein constitutively expressed at very high level (Figure 3 and Figure 5; Johnson *et al.* 1994; Fang *et al.* 1998), while expression of other chaperones such as Hsp90 requires time after stress induction (Aligue *et al.* 1994). Thus, from a physiological point of view, we suggest that Wos2 acts *in vivo* as a "first aid chaperone" that maintains cell proteins in a folding-competent state when environmental changes take place until Hsp90 and other chaperones accumulate and initiate the refolding tasks. Overall, Wos2 could play a dual role in living cells: at normal growth temperatures Wos2 could be involved in the regulation of key cell cycle kinases such as Wee1 and Cdc2. Conditions that cause general protein damage can divert Wos2 from its normal targets to other partially denatured proteins.

Although constitutively expressed, the biological function of Wos2 could be regulated by phosphorylation. Immunopurification of Wos2 led to the copurification of an associated protein kinase with efficient Wos2-phosphorylation activity (Figure 8). Interestingly, Hsp90 is able to autophosphorylate on serine and threonine residues (Csermely *et al.* 1995). Furthermore, purified Brassica Hsp90 protein could also phosphorylate other protein substrates (Park *et al.* 1998). Therefore, since p23 is known to be Hsp90 associated, we cannot exclude the possibility that p23 could be an *in vivo* substrate of this putative Hsp90 phosphorylation activity.

In conclusion, our results suggest that Wos2 is a p23 homologue. The genetic and biochemical analysis described here establishes a novel role for this p23 homologue in the control of the Wee1 and Cdc2 cell cycle kinases and also as a general chaperone in living cells. The functional conservation of the assayed homologue from budding yeasts supports the idea that roles for *wos2* described in this study may be of universal significance.

We thank Lucia Cruzado and Inmaculada Florido for excellent technical assistance, Andrés Garzón, Karin Labib, Mark L. Edgley, and Stuart MacNeill for useful discussions, Sergio Moreno, Jaime Correa, and Paul Nurse for providing antibodies, plasmids, and libraries, Juan Pérez for his help in preparing anti-Wos2 antiserum, and Elmar Maier for providing membranes for physical mapping of *wos2*. This work was supported by grants from the Spanish Ministerio de Educación y Cultura (PB96-0681) and Junta de Andalucía.

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Communicating editor: P. G. Young