Characterization of Novel Mutations at the *Schizosaccharomyces pombe* cdc2 Regulatory Phosphorylation Site, Tyrosine 15

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The cdc2 protein kinase family is regulated negatively by phosphorylation in the glycine ATP-binding loop at a conserved tyrosine residue, Y15, alone or in combination with T14 phosphorylation. In *Schizosaccharomyces pombe* and other systems, substitution of these residues with structurally similar but nonphosphorylatable amino acids has generated proteins (Y15F or T14AY15F) that behave as constitutively tyrosine-dephosphorylated proteins or threonine and tyrosine-dephosphorylated proteins. Here we report the characteristics of three additional mutants at Y15—Y15E, Y15S, and Y15T—in *S. pombe* cdc2p. All three mutant proteins are active in in vitro kinase assays, but are unable to functionally complement cdc2 loss-of-function mutations in vivo. Additionally, all three mutants are dominant negatives. A more detailed analysis of the Y15T mutant indicates that it can initiate chromosome condensation and F-actin contractile ring formation, but is unable to drive the reorganization of microtubules into a mitotic spindle.

INTRODUCTION

The cdc2⁺ protein kinase is a key regulator of eukaryotic cell-cycle progression. In the fission and budding yeasts, cdc2p (or CDC28p) protein kinase activity controls both the initiation of DNA replication and the entry into mitosis. In higher eukaryotes, a family of related enzymes (CDKs) is thought to control multiple transitions through the cell cycle (reviewed in Lees, 1995; Morgan, 1995; Pines, 1995).

Studies in a variety of cells and cell-free systems have shown that cdc2p protein kinase activity is periodic in the cell cycle, peaking at the G2/M phase transition. Furthermore, the regulation of its activation involves a series of posttranslational modifications and subunit associations. Activation of cdc2p requires that it is bound to a cyclin molecule, and inactivation of cdc2p requires cyclin destruction. In addition, the timing of cdc2p activation is regulated by the phosphorylation state of cdc2p (reviewed in Lees, 1995; Morgan, 1995; Pines, 1995).

cdc2p is phosphorylated primarily on two residues in fission yeast, Y15 and T167 (Gould and Nurse, 1989; Gould *et al.*, 1991), although T14 phosphorylation can also be detected under certain circumstances (Den Haese *et al.*, 1995). All three of these residues are normally phosphorylated in CDC2 and CDK2 from higher eukaryotes (reviewed in Lees, 1995; Morgan, 1995; Pines, 1995). Phosphorylation of T167 (or its orthologous site) is essential for cdc2p function, and phosphorylation at this threonine is mediated by CAK, also termed CDK7 (reviewed in Solomon, 1994). Phosphorylation at Y15 inhibits cdc2p activation, and substitution of phenylalanine for tyrosine at position 15 in *Schizosaccharomyces pombe* cdc2p results in premature activation of the cdc2/cyclin complex and consequent premature entry into mitosis (Gould and Nurse, 1989). Tyrosine 15 phosphorylation in *S. pombe* is mediated by the combined activities of the wee1p and mik1p protein kinases, whereas dephosphorylation of Y15 is controlled by the cdc25p protein phosphatase (reviewed in Coleman and Dunphy, 1994).

To address the specificity of the wee1p kinase for tyrosine at residue 15, Y15 was altered in this study to two other phosphorylatable residues, serine and

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threonine. In addition, Y15 was altered to glutamic acid in an attempt to mimic constitutive phosphorylation at this site. We report here a characterization of these three position-15 mutations. As expected, we found that the Y15E mutant protein was nonfunctional and behaved as a dominant-negative mutant when overproduced. We also found that although the Y15T and Y15S mutant proteins could not be phosphorylated at position 15, they were unlike the Y15F mutant protein. Although the Y15F mutant protein behaves as a gain-of-function mutation (Gould and Nurse, 1989), we found that the substitution of Y15 with threonine or serine produced dominant-negative mutations. Unlike several previously described dominant-negative mutants of *cdc2p*, the Y15T, Y15S, and Y15E mutant proteins retained protein kinase activity in vitro and were capable of initiating some, but not all, events of mitosis.

MATERIALS AND METHODS

Strains and Strain Culture

S. pombe strains used in this study are listed in Table 1. Strains were constructed by random spore analysis or by tetrad analysis when necessary. Media for vegetative growth (YE, EMM) and for matings of *S. pombe* were as described (Moreno *et al.*, 1991). For temperature-shift experiments, cultures were grown to early log phase at 25°C and shifted to 36°C for the indicated amounts of time. Transformations were carried out as described (Prentice, 1991). Repression of transcription from the *nmt1* promoter (Maundrell, 1990) was achieved by addition of thiamine to a concentration of 2 μ M. In vivo phosphate labeling of cells was performed as described previously (Gould *et al.*, 1991; Moreno *et al.*, 1991; Den Haese *et al.*, 1995). For cell synchronization, 4 l of cells were grown to a density of $\sim 5 \times 10^6$ cells/ml in YE media at 25°C. Small cells in G₂ were isolated using a Beckman JE-5.0 elutriation rotor and incubated at 25 or 36°C. The Y15*cdc2*-L7 strain was mutagenized using *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine to 50% survival. Cells were grown at 25°C for 24 h

and then placed at 36°C. Several hundred colonies were isolated at 36°C, but only 200 colonies chosen at random were analyzed further.

Cytology and Microscopy

To visualize DNA and F-actin, cells were fixed with freshly prepared formaldehyde for 30 min and stained with DAPI and rhodamine-conjugated phalloidin, respectively, as described previously (Moreno *et al.*, 1991). For indirect immunofluorescence, cells were fixed with paraformaldehyde and glutaraldehyde as described (Moreno *et al.*, 1991) and stained with an anti-tubulin monoclonal antibody (TAT-1) (Woods *et al.*, 1989) followed by Texas Red-conjugated anti-mouse IgG. Cells were viewed with a Zeiss Axioskop photomicroscope (Thornwood, NY), and photographs were taken with Kodak Tri-X film (Rochester, NY), or images were captured using a Zeiss ZVS-47DEC system.

Oligonucleotide Mutagenesis and Plasmids

A 2.9-kb *Pst*I fragment containing the *cdc2*⁺ cDNA flanked by genomic 5' and 3' untranslated regions in the phagemid pTZ19R (Pharmacia, Piscataway, NJ) (Hindley and Phear, 1984; Gould and Nurse, 1989) was transformed into the dut- ung- bacterial strain CJ 236 and infected with M13KO7 helper phage to obtain uracil-containing single-stranded DNA. Oligonucleotide-directed mutagenesis was performed using the Bio-Rad Muta-Gene kit (Richmond, CA) according to the manufacturer's instructions. The following oligonucleotides were used: Y15E, 5'-GAAGGAACCGAAGGCGT-TGT-3', Y15T, 5'-GAAGGAACCACTGGCGTTGT-3', and Y15S, 5'-GAAGGAACCTCTGGCGTTGT-3'. The mutations of interest were confirmed by DNA sequence analysis. The mutants were subcloned into the yeast expression vector pIRT2 (Russell, 1989) in the context of the 2.9-kb genomic *Pst*I fragment such that their expression was controlled by the *cdc2* gene promoter. The mutant cDNAs were also subcloned as *Nde*I-*Bam*HI fragments (for details, see Gould and Nurse, 1989) into pMNS21L under control of the thiamine-repressible *nmt1* promoter (Maundrell, 1993).

Immunoblotting and Immunoprecipitations

S. pombe cells were lysed in a small volume of NP-40 buffer (10 mM sodium phosphate, pH 7.0, 0.15 M NaCl, 1% Nonidet P-40, 2 mM

Table 1. *S. pombe* strains used in this study

Strain	Genotype	Source
KGy28	<i>h</i> ⁻ 972	Paul Nurse
KGy155	<i>h</i> ⁺ <i>cdc25-22</i>	Paul Nurse
KGy265	<i>h</i> ⁺ <i>cdc13-117 his3-237 ura4-D18</i>	Paul Nurse
KGy621	<i>h</i> ⁺ <i>cdc13-A382V ura4-D18 leu1-32 his3-237 ade6-M210</i>	Berry and Gould, 1996
HY1	<i>h</i> ⁻ <i>cdc2-M63::CDC2Hs leu1-32 his3-237</i>	MacNeill and Nurse, 1993b
KGy19	<i>h</i> ⁻ <i>cdc2-33 leu1-32</i>	Paul Nurse
KGy72	<i>h</i> ⁻ <i>cdc2-M26 leu1-32</i>	Paul Nurse
KGy81	<i>h</i> ⁻ <i>cdc2-L7 leu1-32</i>	Paul Nurse
KGy37	<i>h</i> ⁻ <i>cdc2-M63::CDC2Hs his3-237 leu1-32 pMNS21Lcdc2-Y15E</i>	this study
KGy311	<i>h</i> ⁻ <i>cdc13-117 pMNS21Lcdc2-Y15T</i>	this study
KGy322	<i>h</i> ⁻ <i>cdc2-M63::CDC2Hs his3-237 leu1-32 pMNS21Lcdc2-Y15S</i>	this study
KGy344	<i>h</i> ⁻ <i>cdc2-M63::CDC2Hs his3-237 leu1-32 pMNS21Lcdc2-Y15T</i>	this study
KGy345	<i>h</i> ⁺ <i>cdc2-M63::CDC2Hs his3-237 leu1-32 pMNS21Lcdc2-Y15T</i>	this study
KGy546	<i>h</i> ⁻ <i>cdc2-L7 leu1-32 pMNS21Lcdc2Y15E</i>	this study
KGy547	<i>h</i> ⁻ <i>cdc2-L7 leu1-32 pMNS21Lcdc2Y15E ura4-D18</i>	this study
KGy548	<i>h</i> ⁻ <i>cdc2-L7 leu1-32 pMNS21Lcdc2Y15T ura4-D18</i>	this study
KGy569	<i>h</i> ⁻ <i>cdc2-M63::CDC2Hs his3-237 leu1-32 pREP41cdc2Y15E ura4-D18</i>	this study
KGy678	<i>h</i> ⁻ <i>cdc2-M63::CDC2Hs his3-237 leu1-32 pREP41cdc2Y15T ura4-D18</i>	this study

EDTA, 50 mM sodium fluoride, 100 μ M sodium orthovanadate, 5 μ g/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 2 mM benzamidine) or, for protein kinase assays, in HB15 buffer (25 mM 3-(*N*-morpholino) propanesulfonic acid, pH 7.2, 60 mM β -glycerophosphate, 15 mM *p*-nitrophenylphosphate, 15 mM EDTA, 15 mM MgCl_2 , 1 mM dithiothreitol (DTT), 100 μ M sodium orthovanadate, 1 mM PMSF, 5 μ g/ml leupeptin, 1% Nonidet P-40) with mechanical shearing. In some cases, this was followed by boiling for 2 min in SDS/lysis buffer (10 mM sodium phosphate, pH 7.0, 0.5% SDS, 1 mM DTT, 1 mM EDTA, and 50 mM sodium fluoride) and dilution in NP-40 buffer. Cell debris was removed by centrifugation. For immunoblotting, protein extracts were resolved by SDS-PAGE and electrophoretically transferred to polyvinylidene difluoride (PDVF) membrane (Millipore, Bedford, MA). Blots were probed with anti-*cdc2* serum PN24 (Simanis and Nurse, 1986), 4711 antibody (Gould *et al.*, 1991), or anti-PSTAIR monoclonal antibody (Yamashita *et al.*, 1991) followed by [125 I]protein A or by electrochemiluminescence (ECL) (Amersham, Arlington Heights, IL). For immunoprecipitation of *cdc2p*, lysates were prepared as above and incubated at 4°C for 60 min with anti-*cdc2p* 4711 antibody or the anti-*cdc13p* antibodies (our unpublished results), or, the anti-CDC2Hs AB-1 antibody (Calbiochem, La Jolla, CA) was used. Protein A Sepharose was added for an additional 30 min, and the immunocomplexes were recovered by centrifugation and washed extensively.

Phosphoamino Acid Analysis and Peptide Mapping

Slices of PDVF membrane containing ^{32}P -labeled samples were subjected to partial acid hydrolysis (Kamps and Sefton, 1989) or trypsinization as described (Boyle *et al.*, 1991; Den Haese *et al.*, 1995). Phosphoamino acids were separated in two dimensions by thin-layer electrophoresis at pH 1.9 and 3.5 (Cooper *et al.*, 1983), and phosphopeptides were separated in two dimensions by thin-layer electrophoresis at pH 1.9 followed by ascending chromatography (Boyle *et al.*, 1991; Den Haese *et al.*, 1995).

Protein Kinase Assays

Protein kinase assays of the 4711 immune complexes were performed in 20 μ l of HB buffer with 100 μ M unlabeled ATP, 0.2 μ Ci [^{32}P]ATP (3000 Ci/mmol), and 10 μ g of histone H1 (Boehringer Mannheim, Indianapolis, IN) for 10 min at 30°C. Reactions were stopped by the addition of 2 $\times$ gel sample buffer and loaded on SDS-15% polyacrylamide gels. Phosphorylated histone H1 was visualized by autoradiography and in some experiments was cut from the gel. The incorporated radioactivity was determined by scintillation counting of the gel slices.

Preparation of GSTsuc1 Beads

The *suc1*⁺ cDNA containing an *Nde*I site at the initiating methionine was subcloned into the *Escherichia coli* expression vector pGEX2T (Smith and Johnson, 1988), and the construct was transformed into the BL21(DE3)pLysS strain. Protein production was induced from cultures at midlog phase by the addition of 0.4 mM isopropyl thiogalactoside. The GSTsuc1 fusion protein was soluble. The soluble fraction of bacterial cell extracts was incubated with glutathione agarose beads for 30 min, and the beads were washed extensively with NP-40 buffer. They were stored at 4°C in NP-40 buffer before use. To isolate *cdc2* proteins, 20 μ l of a 1:1 slurry of beads:buffer were incubated for 30–60 min with yeast protein extracts and were then washed several times with NP-40 buffer.

RESULTS

Phosphorylation of Y15 Mutants

The *S. pombe cdc2*⁺ cDNA was altered by site-directed mutagenesis to encode three mutant *cdc2* proteins,

Y15E, Y15S and Y15T. To produce these proteins in vivo, the mutant cDNAs were placed under control of the thiamine-repressible *nmt1* promoter in the *S. pombe* expression vector pMNS21L (Maundrell, 1993) and transformed into the *S. pombe* strain, HY1 (MacNeill and Nurse, 1993b). In this strain, the *cdc2*⁺ gene has been replaced with the human CDC2 cDNA. Expression of mutant *S. pombe cdc2* proteins in the HY1 strain allows for their biochemical characterization regardless of whether they are functional, because antibodies are available that recognize only the *S. pombe cdc2* protein, and the CDC2Hs protein will perform all necessary cell-cycle functions.

HY1 strains containing integrated copies of the three mutant *cdc2* cDNAs were labeled with [^{32}P]orthophosphate. The *cdc2* proteins were recovered by immunoprecipitation with an antibody raised against a peptide corresponding to the C-terminal 7 amino acids of *cdc2p* (PN24; Simanis and Nurse, 1986) that does not cross-react with CDC2Hs. Like the wild-type protein (Gould and Nurse, 1989), the three mutant proteins incorporated ^{32}P (Figure 1A).

Wild-type *S. pombe cdc2p* is phosphorylated on Y15 and T167 (Gould and Nurse, 1989; Gould *et al.*, 1991). Phosphoamino acid analysis of the immunoprecipitated proteins revealed that the mutant proteins did not contain phosphotyrosine, but were phosphorylated on threonine, presumably because of T167 phosphorylation (Figure 1B). Wild-type *cdc2p*, prepared in a separate experiment, contained both phosphotyrosine and phosphothreonine (Figure 1B). To rule out the possibility that T15 was able to serve as a phosphoacceptor site in addition to T167, tryptic phosphopeptides from the ^{32}P -labeled proteins were examined. Only phosphopeptides corresponding to T167 phosphorylation were recovered in a cluster termed phosphopeptide 2 from the mutant proteins (Figure 1C). Wild-type *cdc2p* contained an additional phosphopeptide, No. 1, which has been shown previously to contain phosphotyrosine (Figure 1C; Gould and Nurse, 1989; Den Haese *et al.*, 1995). These data indicate that neither serine nor threonine can serve as phosphoacceptor residues in the place of Y15.

Function of the Y15 Mutants

To examine the functional capacities of the Y15S, Y15T, and Y15E mutants, each mutant was tested for its ability to rescue growth of three temperature-sensitive *cdc2* mutant strains and a *cdc2* null mutant. For these experiments, the *cdc2* cDNAs were expressed from the *cdc2* promoter itself or from the *nmt1* promoter, which allows low-level *cdc2p* production even in the presence of thiamine (Fleig and Nurse, 1991). As shown in Table 2, the Y15S, Y15T, and Y15E mutants were unable to complement either temperature-sensitive or null mutants of *cdc2* when expressed from

Figure 1. Phosphorylation of the Y15 mutants. The Y15 mutants were integrated into the genome of HY1 cells under the control of the *nmf1* promoter. After growth of these cells in the absence of thiamine for 16 h, they were labeled for 4 h with [³²P]orthophosphate and lysed under denaturing conditions in SDS/lysis buffer. (A) Cdc2 proteins were isolated by immunoprecipitation with PN24, resolved by SDS-PAGE, transferred to a PDVF membrane, and visualized by autoradiography. An arrowhead marks the position of cdc2p. (B) The bands detected in A and a sample of ³²P-labeled wild-type cdc2p were partially hydrolyzed in 6N HCl. The resultant phosphoamino acids were separated by two-dimensional electrophoresis on cellulose thin-layer plates. The positions of the phosphoserine, phosphothreonine, and phosphotyrosine standards are indicated with dashed circles. T, phosphothreonine; Y, phosphotyrosine. (C) The bands detected in A and a sample of ³²P-labeled wild-type cdc2p were digested with trypsin. The tryptic phosphopeptides were resolved on cellulose thin-layer plates by electrophoresis at pH 1.9, followed by ascending chromatography. Phosphopeptide 1 contains phosphorylated Y15, and the peptides denoted with the number 2 contain phosphorylated T167. Arrowheads indicate the positions where the samples were applied.

either promoter. In marked contrast, and as shown previously, the Y15F mutant was able to complement all four strains defective for *cdc2* function (Table 2; Gould and Nurse, 1989). The Y15F mutant is also capable of rescuing growth of a strain temperature-sensitive for *cdc25p* tyrosine phosphatase function (Gould *et al.*, 1990). The three new mutants, as might be predicted from their inability to rescue temperature-sensitive *cdc2* mutants, were unable to rescue growth of *cdc25-22* cells at the nonpermissive temperature (Table 2). Thus, there is a striking difference in the functional capacities of different Y15 mutants.

To determine whether the Y15E, Y15T, and Y15S mutants might be able to rescue *cdc2^{ts}* mutants when present at elevated levels, they were overproduced using the *nmf1* promoter in the *cdc2-L7* strain. Under

these conditions, the cells elongated and died at 25°C, as well as at 36°C, indicating that these mutants were dominant negatives (our unpublished observations; also see below). Because the Y15T and Y15S mutants exhibited the same phenotypes in all of the above assays, Y15T was used as a representative of both throughout the remainder of this study.

Protein Kinase Activity of the Y15 Mutants

Because Y15 is within the ATP-binding domain of *cdc2p* (DeBondt *et al.*, 1993; Jeffrey *et al.*, 1995), the most straightforward explanation for the inability of the new Y15 mutants to function properly would be that the mutations had interfered with catalytic activity. To address this possibility, the Y15T and Y15E

Table 2. Rescue of mutant strains by <i>cdc2</i> alleles					
	<i>cdc2-33</i>	<i>cdc2-L7</i>	<i>cdc2-M26</i>	<i>cdc2::ura4⁺</i>	<i>cdc25-22</i>
vector alone	—	—	—	—	—
<i>cdc2⁺</i>	+	+	+	+	—
<i>cdc2-Y15F</i>	+	+	+	+	+
<i>cdc2-Y15S</i>	—	—	—	—	—
<i>cdc2-Y15T</i>	—	—	—	—	—
<i>cdc2-Y15E</i>	—	—	—	—	—

Shown is the ability (+) or inability (—) of plasmids pIRT2 and pMSN21L carrying the *cdc2* cDNAs indicated in the left column to promote colony formation of the strains indicated in the top vertical line.

mutants were expressed in HY1 cells from the *nmt1* promoter derepressed for 0 or 20 h and isolated from equal amounts of cell lysates by immunoprecipitation with the anti-*cdc2* antibody, 4711, which does not cross-react with human CDC2 (Gould *et al.*, 1991). Human CDC2 is also not a part of any complex precipitated by 4711, so that human CDC2 does not contribute to the kinase activity detected in these immunocomplexes (Gould *et al.*, 1991; Fleig *et al.*, 1992). These immunoprecipitates were divided in half. The abundance of *cdc2p* was determined using one half (Figure 2, top panel), and the other half was assayed for kinase activity toward the exogenous substrate, histone H1 (Figure 2, bottom panel). Both the Y15T and Y15E mutant proteins displayed protein kinase activity. In fact, in this conventional immunocomplex kinase assay, the activity of the two mutant proteins was equivalent to that of wild-type *cdc2p*. It is clear from this and other experiments (our unpublished results) that the Y15T protein is produced at a higher basal level than the Y15E protein. This could be because of differences in the stability of the two proteins, differences in the number of plasmid integration events in the two strains, and/or rearrangement of

one of the *nmt1* promoters during integration of the plasmid.

Y15T Promotes Partial Entry into Mitosis

To learn more about the defect of the Y15T protein, the HY1 strain containing the integrated Y15T cDNA was crossed to a *cdc2*-L7 strain, and a double mutant strain termed Y15T*cdc2*-L7 was isolated. This strain contained the *cdc2*-L7 mutation and the Y15T coding region under control of the *nmt1* promoter at a genetic locus unlinked to *cdc2*-L7. Y15T*cdc2*-L7 displayed a nearly wild-type phenotype at 25°C when Y15T production was kept low by the presence of thiamine in the media, although the strain grew slowly. Immunoblotting of total cell lysates followed by densitometry indicated that there was less than twice the amount of total *cdc2p* protein in Y15T*cdc2*-L7 as there was in *cdc2*-L7 (Figure 3A). Given that the Y15T protein was shown to have protein kinase activity in the HY1 strain (Figure 2), it was not surprising that a *cdc2p* immunocomplex from Y15*cdc2*-L7 displayed 3.5-fold more activity (determined by scintillation counting) at 36°C relative to that derived from *cdc2*-L7 (Figure 3B). Despite the presence of significant histone H1 kinase activity, the Y15T*cdc2*-L7 strain could not grow at 36°C, in accord with the inability of multicopy *cdc2*-Y15T to rescue *cdc2* mutant strains (Table 2).

To determine the arrest phenotype of Y15T*cdc2*-L7, a synchronized population of Y15T*cdc2*-L7 cells in the G2 phase was obtained by centrifugal elutriation at 25°C. A portion of the synchronized culture was shifted to 36°C, and the remainder was incubated at 25°C. Both cultures entered mitosis as judged by 4,6-diamidino-2-phenylindole (DAPI) staining of condensed chromosomes and began to form a septum as determined by light microscopy at approximately the same time (Figure 3C). The cells maintained at 25°C went on to divide, although progress through mitosis was slow. In the culture maintained at 36°C, the percentage of cells with a septum continued to increase, reaching 85% at 5 h, and these cells did not divide. When the *cdc2*-L7 strain itself was synchronized in the G2 phase by centrifugal elutriation and shifted to 36°C, the septation index remained at 0% for at least 6 h (Figure 3C).

DAPI staining of the Y15T*cdc2*-L7 culture shifted to 36°C, indicated that the nucleus in most cells underwent morphological changes between 2.5 and 3.5 h after shift to 36°C, and many cells with condensed chromosomes were observed at these times (Figure 3, D and E). The percentage of condensed chromosomes did not increase with prolonged incubation at 36°C (our unpublished observations), indicating that chromosome condensation did not occur in all cells or that it was transient, or both. Strikingly, mitotic spindles were not detected in any cells between 2 and 4 h after

Figure 2. Protein kinase activity of the Y15 mutants. HY1 cells, HY1 cells containing episomal copies of pMNS21L*cdc2*⁺, and the HY1 strains containing integrated copies of Y15E and Y15T were grown in media containing 2 μ g/ml thiamine. This provides low-level production of the mutant proteins. The integrant strains were also grown in the absence of thiamine for 20 h to produce higher levels of the mutant proteins. Equivalent numbers of cells were collected and lysed in HB15 buffer. *cdc2* proteins were immunoprecipitated with 4711, and the immunoprecipitates were divided in half. One-half was resolved by SDS-PAGE and transferred to PDVF membrane. The blot was incubated with 4711, followed by [¹²⁵I]protein A. It was then exposed to x-ray film for 16 h. Bands corresponding to *cdc2* proteins are indicated with the arrow. The other one-half of each immunoprecipitate was assayed for histone H1 kinase activity. After being heated to 100°C for 2 min in 2 $\times$ gel sample buffer, the reactions were resolved on an SDS-polyacrylamide gel, and the products were visualized by autoradiography. Exposure time was 6 h. The major band visualized corresponds to histone H1. The identity of the more slowly migrating species is unknown.

shift to 36°C. Cytoplasmic arrays of microtubules only were visualized. During septation, the cytoplasmic microtubule arrays were often “caught” by the septal material, sometimes giving the false appearance of postanaphase arrays (Figure 3E). These data suggest that a major defect in cell-cycle progression in this strain is the inability to reorganize microtubules into a mitotic spindle. Consistent with this interpretation, the Y15T*cdc2*-L7 strain grown at 25°C was more sensitive to the microtubule destabilizing drug, thia-bendazole, than either *cdc2*-L7 or wild-type cells. Whereas both wild-type 972 and *cdc2*-L7 cells grew well on plates containing thiamine and 12 mM thia-bendazole, Y15T*cdc2*-L7 cells did not form colonies (Figure 4).

The Y15E mutant was also crossed into the *cdc2*-L7 background to address its defect more carefully. In this case, however, the double-mutant cells were phenotypically indistinguishable from *cdc2*-L7 at 36°C in the presence of thiamine (our unpublished observations). Because the Y15E protein is undetectable in the presence of thiamine (see Figure 2), it is unlikely that the phenotype of this double mutant is relevant to the activity of the Y15E protein.

Subunit Associations of the Y15 Mutants

Because *cdc13*–cyclin B protein association is required for *cdc2p* protein kinase activity, it was likely that the Y15 mutant proteins were able to bind *cdc13p*. This notion was tested with a coimmunoprecipitation assay. HY1 protein lysates containing no *S. pombe* *cdc2p*, wild-type *cdc2p*, or mutant *cdc2* proteins were incubated with anti-*cdc13p* antibodies. The immunoprecipitates were resolved by SDS-PAGE and transferred to PDVF membrane. Both the Y15E and Y15T proteins were detected in the *cdc13p* immunoprecipitates by immunoblotting with anti-*cdc2* antibodies (Figure 5A).

To ensure that the *cdc2p* proteins were not coimmunoprecipitated indirectly as part of a complex that also contained the CDC2Hs protein, the following experiments were performed. First, we tested whether *cdc13p* was present in immunoprecipitates of the *S. pombe* *cdc2* proteins. This was done by immunoprecipitating *cdc2p*, Y15T, and Y15E, and any proteins in a complex with them, with the *S. pombe* *cdc2p*-specific antibody 4711 and probing these immunoprecipitates with antibodies to *cdc13p*. We found that in immunoprecipitates of HY1 cells expressing wild-type *cdc2p*,

Figure 3.

Figure 3 (cont). Y15T blocks formation of a mitotic spindle. The *cdc2*-L7 and Y15T*cdc2*-L7 strains were grown in thiamine-containing media at 25°C (A). They were then lysed in SDS/lysis buffer. Equal amounts (45 µg) of total cell protein, as determined by the BCA protein assay kit, were resolved by SDS-PAGE and blotted to a PDVF membrane. *cdc2* proteins were detected by ECL after incubation with PN24 and quantitated by densitometry or shifted to 36°C for 4 h (B), and lysed in NP-40 buffer. *cdc2* proteins were immunoprecipitated from equal amounts of cell protein with 4711 and assayed for protein kinase activity toward histone H1 in the presence of γ -[³²P]ATP. Labeled histone H1 was detected by autoradiography and quantitated by scintillation counting or synchronized by centrifugal elutriation (C-E). A portion of each synchronized strain was shifted to 36°C, and a portion of the Y15T*cdc2*-L7 strain was incubated at 25°C. (C) The percentage of cells containing a septum was determined in each culture. Samples of the Y15T*cdc2*-L7 strain

were also fixed with formaldehyde and glutaraldehyde at the indicated times following a shift to 36°C. They were either stained immediately with DAPI to visualize DNA (D) or were further processed for indirect immunofluorescence and stained with the anti-tubulin antibody TAT-1 (Woods *et al.*, 1989) and DAPI (E). The latter procedure causes many cells containing a septum to break apart. Therefore, the cells depicted in E contain a significantly lower percentage of septated cells than the cells illustrated in D.

Y15T, or Y15E, *cdc13p* was detected in the immunoprecipitates (Figure 5, C and D). We then confirmed that these immunoprecipitates did not contain CDC2Hs by probing the same immunoprecipitates with antibodies to the PSTAIR domain, which recognize both the yeast and human CDC2Hs proteins (Yamashita *et al.*, 1991). In the yeast *cdc2p* immunoprecipitates (Figure 5E, lanes 1–3), the band corresponding to CDC2Hs (Figure 5E, lane 5) is absent, indicating that the association between the mutant *cdc2p* proteins and *cdc13p* is direct.

The interaction between *cdc13p* and the Y15T mutant protein was also tested genetically. Because the *cdc13*-117 mutant strain is also sensitive to thiabendazole (Booher and Beach, 1988) and *cdc13*-117p is compromised in its ability to bind *cdc2p* (Booher *et al.*, 1989), we reasoned that there might be a synthetically

lethal interaction between *cdc13*-117 and strains expressing Y15T if Y15T was unable to effectively interact with *cdc13p*, particularly because the *cdc13*-117 mutant displays a synthetically lethal interaction with several temperature-sensitive mutations of *cdc2* (Booher and Beach, 1988; Berry and Gould, unpublished observations). The *cdc13*-117 mutant was crossed to both Y15T*cdc2*-L7 and HY1 containing integrated Y15T, and asci were dissected. In both cases, viable colonies containing both Y15T and *cdc13*-117 were obtained at 25°C. Double mutants could also be isolated, which contained Y15T and another temperature-sensitive mutation in *cdc13*, *cdc13*-A382V (Berry and Gould, 1996). During the course of these crosses, we also observed that a double mutant of *cdc2*-L7 and *cdc13*-117 was viable at 25°C. Finally, we found that overexpression of the *cdc13*⁺ gene could not rescue the

Figure 4. The Y15Tcdc2-L7 strain is sensitive to thiabendazole. Wild-type (972), *cdc2-L7*, and Y15Tcdc2-L7 strains were streaked onto plates containing thiamine, and either 12 mM thiabendazole (TBZ) or its vehicle (DMSO), and colonies were allowed to form for 4 d at 25°C. Then the plates were photographed.

temperature-sensitive lethality of either the *cdc2-L7* or the Y15Tcdc2-L7 strains. Taken together, these data indicate that the Y15T mutant protein is not defective in its interaction with cdc13p.

Another protein known to interact with wild-type cdc2p is p13^{suc1}. To determine whether the Y15 mutant proteins were able to associate with p13^{suc1}, a GSTsuc1 fusion protein was produced and coupled to glutathione-agarose beads. Both the Y15E and Y15T proteins were able to bind to the GSTsuc1 fusion protein (Figure 5B), suggesting that they are not defective in their interaction with p13^{suc1}.

Given the normal interactions with cdc13p and suc1p, we reasoned that the Y15T mutant might be defective in its interaction with an unknown protein. To identify it, we undertook two genetic screens. First, we attempted to rescue the temperature-sensitive lethality of the Y15Tcdc2-L7 strain with a multicopy genomic library. The strain was transformed with a *ura4*-based genomic library (Barbet *et al.*, 1992) and plated in the presence of thiamine. Of >20,000 transformants obtained, only six colonies were obtained that would grow at 36°C. Plasmids were isolated from these six colonies and shown to contain the *cdc2*⁺ gene by restriction mapping and Southern blotting. In the second screen, we mutagenized the Y15Tcdc2-L7 strain with nitrosoguanidine and isolated colonies that could grow at 36°C in the presence of thiamine. Backcrossing of a random sam-

pling of 200 such colonies to a wild-type strain indicated that all 200 suppressor mutations mapped within the *cdc2-L7* locus or suppressed the *cdc2-L7* mutation, but not the Y15T mutation.

Overexpression Phenotypes of the Y15 Mutants

As noted above, overexpression of the Y15 mutants was lethal. The terminal dominant-negative phenotypes of the Y15T and Y15E mutants are illustrated in Figure 6. In the case of Y15T overexpression, a large percentage of cells formed one septum, although some cells formed multiple septa around the first. This was visualized well by staining with rhodamine-conjugated phalloidin, which binds F-actin. F-actin in these cells was found predominantly alongside the septa; very few cells displayed phalloidin staining at the tips (Figure 6A). The high percentage of cells with septa and the lack of phalloidin staining at the tips suggested that the cells were blocked in mitosis even though the DNA did not seem to be condensed. DAPI staining showed that in septated cells, chromosomes had clearly been segregated unequally to the two cell compartments, and that in cells without a septum, the DNA was not in a half-moon configuration typical of interphase cells, but was present as elongated strands (Figure 6, A and B). Interphase microtubule arrays were observed by staining with anti-tubulin antibodies, but mitotic spindles were not detected (Figure 6B). Overexpression of Y15S produced a similar phenotype (our unpublished observations), and these phenotypes were similar to those of the Y15Tcdc2-L7 strain at the nonpermissive temperature (see Figure 3C).

Overproduction of the Y15E mutant protein generated elongated, swollen, and multiply septated cells. In most cases, the cells were also branched and contained multiple nuclei (Figure 6A). In contrast to the Y15T overexpression phenotype, there did not seem to be widespread difficulties with chromosome segregation; most compartments seemed to receive equal amounts of DNA as visualized by DAPI staining (Figure 6, A and B). Y15E-overproducing cells also did not contain mitotic spindles, but rather interphase or post-anaphase arrays of microtubules (Figure 6B). These data suggest that overproduction of the Y15E protein delays a late event in cell division.

Because the Y15T, Y15S, and Y15E mutants behaved as dominant negatives, and most dominant negatives are thought to exert their effect by titrating a component(s) away from the wild-type protein, we attempted to suppress the dominant-negative lethality of these mutants with multiple-gene copies of a putative titrated factor. We first tested known cdc2p-binding proteins. We found that overexpression of *cdc2*⁺, but not *cdc13*⁺ or *suc1*⁺, was able to restore viability to the HY1 strains overproducing Y15T and Y15S. Oddly, overexpression of *cdc13*⁺, but not *suc1*⁺ or *cdc2*⁺, re-

Figure 5. Subunit associations of the Y15 mutants. The same strains described in the legend to Figure 2 were grown in the same manner as described and lysed in NP-40 buffer; equal amounts of protein lysates were used for immunoprecipitations. (A and B) The protein lysates were divided in half. One-half (A) was incubated with anti-*cdc13* antibodies, followed by protein A Sepharose, and the other one-half (B) was incubated with GSTsuc1 beads. (C, D, and E, lanes 1–3) Lysates were incubated with 4711. (E, lanes 4 and 5) Lysates were incubated with anti-CDC2Hs-specific antibodies. The beads in all cases were collected by centrifugation and washed extensively. Proteins binding to the beads were resolved by SDS-PAGE and transferred to PDVF membrane. In A and B, the blots were probed with 4711 antiserum followed by [125 I]protein A. Exposure times were 16 h. The arrowhead indicates the position of bands corresponding to *cdc2p*. The position of the 30-kDa carbonic anhydrase marker in A and the position of GSTsuc1 in B that binds nonspecifically to [125 I]protein A are also indicated. In C and D, the blot was probed with *cdc13p* antibodies and bound proteins detected by ECL. C and D represent two different exposures of the same blot. The arrowhead indicates the position of *cdc13p*. The blot in E was probed with anti-PSTAIR monoclonal antibodies and

stored viability to the HY1 strain overproducing Y15E. Overexpression of *suc1*⁺ will rescue certain other dominant-negative *cdc2* mutants expressed from the *nmt1* promoter (Labib *et al.*, 1995; Fleig and Gould, unpublished results). This result indicates that *cdc13p* is likely to be the factor being titrated away from the wild-type protein by Y15E, but that neither *cdc13p* nor *suc1p* is being titrated away from the wild-type protein by Y15T. However, we were concerned that the full-strength *nmt1* promoter produced too much of the dominant-negative proteins to allow for compensatory overproduction of the titrated factor. Thus, we integrated Y15T and Y15S cDNAs under control of the attenuated *nmt1* promoter found in the pREP41 vector (Basi *et al.*, 1993) to produce less of the dominant-negative proteins, as was done with other dominant-negative proteins previously (Labib *et al.*, 1995). Identical results were obtained when these strains were transformed with *cdc2*⁺-, *cdc13*⁺-, and *suc1*⁺-containing plasmids (our unpublished observations).

We next attempted to identify a new Y15T-binding protein by transforming the HY1-Y15T strain with a genomic library on a multicopy vector and selecting for colonies that could grow in the absence of thiamine. From ~20,000 transformants, 18 such colonies were obtained. Plasmids that re-rescued the dominant-negative lethality could be isolated from only six of these. Four of these plasmids suppressed well but contained the *cdc2*⁺ gene as determined by restriction mapping and Southern blotting. The other two plasmids suppressed weakly. DNA sequence analysis of the genomic DNA insert from one of these plasmids revealed that it contained the 5' untranslated region and a portion of the coding region of the mitochondrial cytochrome-b-oxidase gene. The identity of the last suppressor is not known, but open reading frames were not detected by DNA sequence analysis. Although these two relatively weak suppressors did not suppress other dominant-negative mutations expressed from the *nmt1* promoter (our unpublished results), they presumably rescue HY1-Y15T by competing for factors used by the *nmt1* promoter, and thereby reduce production of the dominant-negative protein.

DISCUSSION

Tyrosine 15 is conserved and phosphorylated in all *cdc2p* family members. Threonine 14 is also phosphorylated at high stoichiometry in higher eukaryotes (re-

(Figure 5 cont.) bound proteins detected by ECL. Lane 1, HY1 cells containing episomal copies of pMNS21Lcdc2⁺ grown in presence of thiamine; lane 2, HY1 cells containing integrated Y15E grown in the absence of thiamine; lane 3, HY1 cells containing Y15T grown in the absence of thiamine; lane 4, 972 cells; lane 5, HY1 cells.

Figure 6. Overexpression phenotype of the Y15 mutants. HY1 with integrated pMNS21L plasmids expressing Y15T or Y15E were grown for 20 h in the absence of thiamine to derepress the *nmf1* promoter. Samples of cells were taken, fixed, and stained with (A) phalloidin and DAPI or (B) anti-tubulin antibody TAT-1, followed by Texas Red-conjugated goat anti-mouse IgG and DAPI.

viewed in Lees, 1995; Morgan, 1995; Pines, 1995). Tyrosine 15 has been altered in a number of studies by site-directed mutagenesis to the structurally similar but nonphosphorylatable amino acid, phenylalanine, alone or in combination with the substitution of alanine for threonine 14 (Gould and Nurse, 1989; Ducommun *et al.*, 1991; Krek and Nigg, 1991; Parker *et al.*, 1991; Amon *et al.*, 1992; Krek *et al.*, 1992; Pickham *et al.*, 1992; Solomon *et al.*, 1992; Sorger and Murray, 1992; Hemerly *et al.*, 1995; Lew and Reed, 1995). In *S. pombe*, prevention of tyrosine 15 phosphorylation by such alteration generated an enzyme that lost much of its negative regulation. It behaved as a dominant gain-of-function mutant, triggering entry into mitosis at a reduced cell size (Gould and Nurse, 1989). Satisfyingly, the phenotype generated by the Y15F mutant protein was very similar to that achieved by overexpressing a protein-tyrosine phosphatase that acts on cdc2p (Russell and Nurse, 1986; Gould *et al.*, 1990). Similar results have been obtained in higher eukaryotic systems when both tyrosine 15 and threonine 14 of cdc2p were altered to nonphosphorylatable residues (Krek and Nigg, 1991; Norbury *et al.*, 1991; Pickham *et al.*, 1992), and a cell morphogenesis checkpoint was partially overcome in *Saccharomyces cerevisiae* cells expressing the Y19F mutant protein (Lew and Reed, 1995). Three different alterations of Y15 were made in this study using *S. pombe* cdc2p—Y15E, Y15S, and Y15T—and two additional phenotypes resulted.

The protein kinases responsible for phosphorylating Y15 in *S. pombe* are wee1p (Russell and Nurse, 1987; Featherstone and Russell, 1991; Parker *et al.*, 1992) and mik1p (Lundgren *et al.*, 1991; Lee *et al.*, 1994). In vitro, wee1p is capable of autophosphorylation on both tyrosine and serine residues (Featherstone and Russell, 1991; Parker *et al.*, 1992) and capable of phosphorylating enolase on both tyrosine and serine residues (Parker *et al.*, 1992). Given this potential for dual specificity, the Y15T and Y15S mutants were generated to test the possibility that threonine or serine could substitute as phosphoacceptor sites for Y15. Neither amino acid could in vivo; only T167 became phosphorylated in these mutants. This result is consistent with the substrate specificities of *S. pombe* wee1p and mik1p toward cdc2p in vitro, which are restricted to Y15 (Parker *et al.*, 1992; Lee *et al.*, 1994).

Despite the lack of inhibitory phosphorylation at position 15, Y15T and Y15S behaved differently from Y15F. The Y15T and Y15S mutants were unable to rescue *cdc2^{ts}* mutants when expressed at low levels and were dominant negatives when overexpressed. A Y15T mutant in human CDC2p has been made previously. When assayed in *S. pombe*, it was also found to be a dominant-negative mutant (Krek *et al.*, 1992). We characterized the Y15T mutant more carefully because we found, unexpectedly, that its activity in an immunocomplex histone H1 kinase assay was equivalent to

that of wild-type cdc2p. Interestingly, this mutant protein, when expressed at low levels, seems able to induce chromosome condensation and actin ring/septum formation in a *cdc2*-L7 mutant background, but does not induce the reorganization of microtubules into a mitotic spindle. Further evidence for this conclusion comes from experiments with the drug, thiabendazole, which inhibits formation of a mitotic spindle. Strains producing Y15T at low levels are sensitive to thiabendazole, which seems to exacerbate the defect caused by Y15T production. Given that cdc13p has previously been connected with the process of microtubule reorganization at mitosis (Booher and Beach, 1988; Hagan *et al.*, 1988), we tested the interaction between Y15T and cdc13p by coimmunoprecipitations, by crossing Y15T into two different temperature-sensitive alleles of *cdc13* (*cdc13*-117 and *cdc13*-A382V), and by attempting to rescue the dominant-negative Y15T overexpression phenotype and the temperature-sensitive lethality of Y15T*cdc2*-L7 with high-level expression of cdc13p. No evidence suggesting that Y15T is altered in its ability to interact with cdc13p was obtained. Similarly, we found no evidence for a defective interaction with suc1p. This latter result might have been predicted from the recently determined position of the CKSHs1 binding site on CDK2 (Bourne *et al.*, 1996). If the Y15T and Y15E mutations did not, as we suspect, generate large changes in protein conformation, the suc1p binding site is predicted to be sufficiently far away from Y15 to make any interaction between it and residue 15 unlikely (Bourne *et al.*, 1996).

To test the possibility that the Y15T mutant protein was defective in its interaction with another protein component necessary for microtubule reorganization, we undertook a variety of genetic screens to identify such a component. We sought extragenic or intragenic second-site mutations, which would rescue the temperature-sensitive lethality of the Y15T*cdc2*-L7 strain. We did not obtain either type of suppressor mutation. Multicopy suppressors that would suppress either the dominant-negative phenotype of Y15T overexpression or the temperature-sensitive lethality of the Y15T*cdc2*-L7 strain were also sought. No such extragenic suppressors were identified. The inability to detect such interacting components can be interpreted in at least two ways. A likely explanation is that such components exist, but we were unable to identify them genetically. Simple overexpression of the component might not reconstitute an interaction with Y15T, and/or overexpression of the component might be lethal. It also might not have been possible to generate mutations in such proteins, which would enable them to interact with Y15T and still remain functional. A second possibility that we cannot rule out, however, is that, despite the results of in vitro histone H1 kinase assays, the defect of the Y15T mu-

tant might lie in altered kinetic properties and not in its ability to physically interact with another protein. If this is the case, the biological activity of the Y15T mutant would indicate that less cdc2p kinase activity is required for chromosome condensation and actin ring/septum formation than mitotic spindle formation.

The Y15E mutant was generated in an attempt to mimic constitutive phosphorylation at position 15 as had been done previously in human CDC2 (Krek *et al.*, 1992). Two of our results suggest that it failed to do so. First, Y15E was as active a protein kinase in a conventional immunocomplex histone H1 kinase assay as was wild-type cdc2p. Tyrosine-phosphorylated cdc2p is presumably inactive in such an assay (reviewed in Coleman and Dunphy, 1994; Lees, 1995; Morgan, 1995; Pines, 1995), although the mechanism by which tyrosine phosphorylation inhibits cdc2p is not completely understood. Steric interference with ATP binding has been ruled out as a possibility (Atherton-Fessler *et al.*, 1994). Second, cells overexpressing the Y15E protein went through repeated rounds of nuclear division and septation and did not arrest in G₂. Because either inactivation of the tyrosine phosphatase cdc25p or overexpression of the tyrosine kinase wee1p causes G₂ arrest, if Y15E behaved as a constitutively tyrosine-phosphorylated cdc2p, it would be predicted to block cell-cycle progression in G₂. Although it did not disable cdc2 function in the manner we had planned, the Y15E mutation did ablate the *in vivo* function of the protein. Interestingly, the phenotype of cells expressing Y15E was different from that of cells expressing Y15T or Y15S. For example, Y15E-overexpressing cells became swollen, multinucleate, and branched, whereas Y15T- or Y15S-overexpressing cells remained cylindrical in shape and could not complete nuclear division. Also, overexpression of *cdc2*⁺ could not rescue overproduction of Y15E, whereas it could rescue overproduction of Y15T. Conversely, overexpression of *cdc13*⁺ could rescue the lethality of Y15E overproduction but not Y15T overproduction. These data, coupled with previous results, indicate that quite distinct phenotypes can be obtained by altering Y15 to different residues, and they serve as another reminder that caution should be exercised when interpreting experimental results based on the behavior of site-directed mutants.

Many different dominant-negative mutants of the yeast cdc2 and CDC28 proteins have been made previously (Mendenhall *et al.*, 1988; Fleig and Nurse, 1991; Gould *et al.*, 1991; Fleig *et al.*, 1992; MacNeill and Nurse, 1993a; Labib *et al.*, 1995). In *S. pombe*, the majority of dominant-negative cdc2p mutants does not retain *in vitro* protein kinase activity (Fleig and Nurse, 1991; Gould *et al.*, 1991; Fleig *et al.*, 1992; MacNeill and Nurse, 1993a; Labib *et al.*, 1995). The exceptions to this include the Y15F mutant, which is lethal when over-

expressed (K.L. Gould, unpublished observations), and a deletion mutant of amino acids 240–242, which also induces premature entry into mitosis when overexpressed (Labib *et al.*, 1995). Two dominant-negative mutants that block cell-cycle progression at the metaphase/anaphase transition, P162S and P162L, exhibit very weak *in vitro* kinase activity (Labib *et al.*, 1995). Thus, the Y15T, Y15S, and Y15E mutants are unique among the collection of cdc2p dominant-negative mutants, because they exhibit high *in vitro* kinase activity, but, nonetheless, are unable to promote cell-cycle progression. That cdc2p protein kinase activity alone is insufficient for cell-cycle progression has been reported previously (Stueland *et al.*, 1993). In checkpoint-arrested *S. cerevisiae* cells, CDC28p seemed to be fully active as measured *in vitro*, and yet cells did not proceed into mitosis. The inability of CDC28p to promote entry into mitosis was correlated with a decrease in the size of CDC28p-containing protein complexes (Stueland *et al.*, 1993). It will be interesting to determine whether dominant-negative forms of cdc2p are unable to be incorporated into larger protein complexes necessary for complete *cdc2*⁺ function.

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