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The Wee1 Protein Kinase Regulates T14 Phosphorylation of Fission Yeast Cdc2

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The Cdc2 protein kinase is a key regulator of the G1-S and G2-M cell cycle transitions in the fission yeast *Schizosaccharomyces pombe*. The activation of Cdc2 at the G2-M transition is triggered by dephosphorylation at a conserved tyrosine residue Y15. The level of Y15 phosphorylation is controlled by the Wee1 and Mik1 protein kinases acting in opposition to the Cdc25 protein phosphatase. Here, we demonstrate that Wee1 overexpression leads to a high stoichiometry of phosphorylation at a previously undetected site in *S. pombe* Cdc2, T14. T14 phosphorylation was also detected in certain cell cycle mutants blocked in progression through S phase, indicating that T14 phosphorylation might normally occur at low stoichiometry during DNA replication or early G2. Strains in which the chromosomal copy of *cdc2* was replaced with either a T14A or a T14S mutant allele were generated and the phenotypes of these strains are consistent with T14 phosphorylation playing an inhibitory role in the activation of Cdc2 as it does in higher eukaryotes. We have also obtained evidence that Wee1 but not Mik1 or Chk1 is required for phosphorylation at this site, that the Mik1 and Chk1 protein kinases are unable to drive T14 phosphorylation in vivo, that residue 14 phosphorylation requires previous phosphorylation at Y15, and that the T14A mutant, unlike Y15F, is recessive to wild-type Cdc2 activity. Finally, the normal duration of G2 delay after irradiation or hydroxyurea treatment in a T14A mutant strain indicates that T14 phosphorylation is not required for the DNA damage or replication checkpoint controls.

INTRODUCTION

Progression through the eukaryotic cell cycle requires a systematic chain of events that culminates in cell division. In the fission yeast, *Schizosaccharomyces pombe*, progression through the cell cycle requires the function of the *cdc2* gene product, both in G1 before the initiation of S-phase and at the G2-M boundary (Nurse and Bissett, 1981). The *cdc2* gene encodes a 34-kDa protein serine/threonine kinase (Hindley and Phear, 1984; Simanis and Nurse, 1986), which is conserved in all eukaryotes examined. Indeed, the human *CDC2* gene (Lee and Nurse, 1987) and *cdc2* genes from other organisms can functionally complement mutations in *S. pombe cdc2* (reviewed in Forsburg and Nurse, 1991).

The activity of the Cdc2 protein kinase oscillates throughout the cell cycle, peaking as cells enter M-phase (reviewed in Nurse, 1990; Solomon, 1993; Dunphy, 1994). This periodicity of Cdc2 is dependent on its cell cycle–specific association with various cyclins, a class of proteins that characteristically vary in their abundance throughout the cell cycle (reviewed in Hunt, 1991; Forsburg and Nurse, 1991; Nasmyth, 1993; Pines, 1993; Sherr, 1993). Although association of Cdc2 with a cyclin is required for cell cycle-dependent ac-

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tivation, Cdc2 is also subject to additional regulation by phosphorylation (reviewed in Solomon, 1993; Dunphy, 1994). *S. pombe* Cdc2 is phosphorylated on Y15 and T167 (Gould and Nurse, 1989; Gould *et al.*, 1991). Phosphorylation of T167, an activity recently attributed to CAK (CDK-activating kinase) in higher eukaryotes (Solomon *et al.*, 1992; Fesquet *et al.*, 1993; Poon *et al.*, 1993; Solomon *et al.*, 1993), is absolutely required for Cdc2 function (Gould *et al.*, 1991). Regulation of Y15 phosphorylation is critical for the timing of mitosis. A Y15F mutation results in premature activation of Cdc2 and as a result, cells expressing the Y15F mutant divide at a reduced size displaying a "wee" phenotype (Gould and Nurse, 1989). Phosphorylation at Y15 is governed by Wee1, a protein kinase (Russell and Nurse, 1987; Featherstone and Russell, 1991) that has been demonstrated to exert its inhibitory effects by directly phosphorylating Cdc2 on Y15 (Parker *et al.*, 1992). In addition to Wee1, the Wee1-related protein kinase Mik1 can also regulate Y15 phosphorylation in the absence of Wee1 (Lundgren *et al.*, 1991), and phosphorylate Cdc2 directly on Y15 (Lee *et al.*, 1994). The presence of either the Wee1 or Mik1 protein kinase is absolutely required for inhibition of Cdc2 by Y15 phosphorylation because removal of both activities results in rapid loss of tyrosine phosphorylation of Cdc2 and lethal premature advancement into mitosis (Lundgren *et al.*, 1991). The Cdc25 protein tyrosine phosphatase acts antagonistically to Wee1 to regulate the level of Y15 phosphorylation and can directly remove phosphate from Y15 (reviewed in Millar and Russell, 1992).

A block to DNA replication by either chemical or genetic means results in the activation of a complex feedback control pathway that prevents entry into mitosis. Several lines of evidence suggest that this feedback control mechanism, which involves several "checkpoint rad" genes (Enoch and Nurse, 1990; Enoch *et al.*, 1992; reviewed in Sheldrick and Carr, 1993), results in the inhibition of Cdc2 activity by the phosphorylation of Y15 (Al-Khodairy and Carr, 1992; Rowley *et al.*, 1992; Al-Khodairy *et al.*, 1994). *S. pombe* cells are also delayed in entry into mitosis after DNA damage, presumably to maximize survival by allowing time for repair to occur. Although the same checkpoint rad genes of *S. pombe* are required for this DNA damage checkpoint, the mechanism by which cell cycle arrest is affected can be distinguished from that used by the replication feedback control and appears to be independent of Y15 phosphorylation (reviewed in Sheldrick and Carr, 1993). The Chk1 protein kinase is required for cell cycle arrest specifically in the presence of damaged DNA. However, the mechanism by which Chk1 leads to cell cycle arrest is unknown (Walworth *et al.*, 1993; Al-Khodairy *et al.*, 1994).

The activity of CDC2 from higher eukaryotes is regulated by phosphorylation at sites homologous to Y15 and T167 of *S. pombe*, Y15 and T161 (reviewed in Solomon, 1993). However, a third phosphorylation event at T14 is always detected in Cdc2 from higher eukaryotes. T14 phosphorylation has been shown to collaborate with Y15 phosphorylation to inhibit Cdc2 activation (Krek and Nigg, 1991a,b; Norbury *et al.*, 1991; Solomon *et al.*, 1992). Here we report that T14 phosphorylation of Cdc2 can also occur in *S. pombe* under certain circumstances.

Moreover, we have found several additional properties of T14 phosphorylation in *S. pombe*. First, its level is modulated by the Wee1 protein kinase. This suggests that although Wee1 might directly phosphorylate only Y15 in Cdc2, it regulates both T14 and Y15 phosphorylation events. We also provide evidence that although Mik1 function overlaps with that of Wee1 to control Y15 phosphorylation, Mik1 is distinctly unable to regulate T14 phosphorylation. Finally, we show that T14 phosphorylation may be a late S-phase or early G2 event and we examine its involvement in replication and DNA damage checkpoint controls.

MATERIALS AND METHODS

Yeast Methods

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. *S. pombe* strains were grown in minimal medium with the appropriate supplements in the presence or absence of 5 μg/ml thiamine or in standard yeast extract (YE) medium (Moreno *et al.*, 1991). All temperature shift experiments were carried out by growing cells at 36°C for 4 h. *S. pombe* transformations were carried out by electroporation (Prentice, 1991) or by a lithium acetate procedure (Moreno *et al.*, 1991).

Survival Studies

Exponentially growing cells were harvested and resuspended in YE media. For ionizing radiation, aliquots of a cell suspension of 10^4 cells/ml were irradiated in a Gammacell 1000 Cs source (dose rate 12 Gray/min) and then plated on YE agar plates. For UV irradiation, cells were plated at a known density on YE agar plates and irradiated with UVC light (dose rate 12 J/m^2/min). The percentage of surviving cells relative to unirradiated controls was estimated by counting colonies after 4 days of incubation at 29°C.

Radiation Checkpoint Measurement

Exponentially growing cells were harvested and late G2 cells were isolated from a lactose gradient as described in Barbet and Carr (1993). Synchronous G2 cells were divided into 4 aliquots and irradiated in a Gammacell 1000 Cs source (dose rate 12 Gy/min) with either 0, 50, 100, or 250 Gy. From each aliquot, a sample was taken every 15 min and fixed in methanol. The percentage of cells that had passed through mitosis was estimated by counting the number of septated and binucleate cells after 4,6-diamidino-2-phenylindole (DAPI) and calcofluor staining (Al-Khodairy *et al.*, 1994).

DAPI Staining

S. pombe cells in liquid culture were collected by centrifugation, fixed with glutaraldehyde, and stained with a DNA-specific fluorescent dye (DAPI) as described previously (Lundgren *et al.*, 1991).

Table 1. Yeast strains and genotypes

Strain	Genotype	Source
KGY 3	*cdc25-22 leu1-32 h*$^-$	P. Nurse
KGY 4	*wee1::ura4*$^+$ *leu1-32 ura4-D18 h*$^-$	P. Nurse
PN 588	*adh wee1-50 wee1-50::ura4*$^+$*ura4-D18 leu1-32 h*$^-$	P. Nurse
KGY 28	972 *h*$^-$	P. Nurse
KGY 78	*cdc2-L7 leu1-32 h*$^+$	P. Nurse
KGY 111	*cdc2-M63::CDC2 his3-237 leu1-32 h*$^-$	MacNeill and Nurse, 1993
KGY 246	*ade6-M210 leu1-32 ura4-D18* h$^-$	P. Nurse
KGY 247	*ade6-M210 leu1-32 ura4-D18 h*$^+$	P. Nurse
KGY 262	*cdc2/cdc2::ura4*$^+$*ade6-704/ade6-704 leu1-32/leu1-32 ura4-D06/ura4-D06 h*^{90}*/h*$^+$	P. Nurse
KGY 469	*mik1::ura4*$^+$ *leu1-32 ura4-D18 h*$^+$	Lundgren et al., 1991
KGY 475	*cdc2-L7 ade6-M216 ura4-D18his3-D1 leu1-32 h*$^-$	This study
KGY 495	*cdc2-L7::cdc2-T14A ade6-M216 his3-D1 leu1-32 ura4-D18 h*$^+$	This study
KGY 496	*cdc2-L7::cdc2-T14A ade6-M216 his3-D1 leu1-32 ura4-D18 h*$^-$	This study
KGY 511	*chk1::ura4*$^+$ *ade6-M216 leu1-32 ura4-D18 h*$^+$	Walworth et al., 1993
KGY 512	*cdc17-K42 h*$^-$	P. Nurse
KGY 527	*cdc2-M63::CDC2 ade6-M210 his3-D1 leu1-32 ura4-D18 h*$^-$	This study
KGY 556	*cdc2-L7::cdc2-T14S ura4-D18 leu1-32 his3-D1 ade6-M216 h*$^-$	This study
KGY 561	*cdc2-L7::cdc2-T14S h*$^-$	This study
KGY 562	*cdc2-L7::cdc2-T14S wee1::ura4*$^+$ *ura4-D18 his3-D1 h*$^-$	This study

Cells were viewed with a Zeiss Axioskop microscope (Hanover, MD) and photographs were taken using Kodak Tri-X pan film.

Site-specific Mutagenesis

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) 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 manufacturer's instructions. The following oligonucleotides were used: T14-to-A14, 5′-GGGGAAGGAGCCTATGGCGT-3′, T14-to-S14, 5′-GGGGAAGGATCCTATGGCGT-3′, and T14Y15-to-S14F15, 5′-ATTGGGGAAGGATCCTTTGGCGTTGTTTAT-3′. The mutations of interest were confirmed by the Sanger dideoxy-mediated chain termination technique of DNA sequencing with the Sequenase 2.0 kit (United States Biochemical, Cleveland, OH). The mutants T14A and T14S 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 T14S and T14SY15F cDNAs were released using the unique *Nde*I site, which had been introduced at the initiation codon (Gould and Nurse, 1989) and a unique *Eco*RV site in the 3′ noncoding region. This fragment was subcloned into *Nde*I- and *Sma*I-cut pREP1 under control of the thiamine-repressible *nmt1* promoter (Maundrell, 1993).

Construction of the Gene Replacement Strains

The 2.9-kb *Pst*I fragments containing the *cdc2*-T14A and *cdc2*-T14S cDNAs were isolated from the pTZ19R vector and transformed into the temperature sensitive *cdc2*-L7 *his3*-D1 *leu1*-32 *ade6*-M210 *ura4*-D18 *h*$^-$ strain. Transformants were plated directly at the restrictive temperature (36°C). Colonies that formed at the restrictive temperature and displayed a uniform morphology were isolated as possible gene replacement strains. Correct gene replacements were confirmed using both PCR and Southern blot analyses.

Epitope Tagging of wee1 and mik1

Oligonucleotide primers were used to amplify the coding regions of the *wee1* (Russell and Nurse, 1987) and *mik1* (Lundgren *et al.*, 1991) genes using PCR. The following primers were used: 5′-TACAGAACATATG$_{start}$AGCTC-3′, 5′-CAGATTTTGGATCCCATTCC-3′ for *wee1* and 5′-CAGTTACATATG$_{start}$GATTCATC-3′, 5′-GACCCGGGCCCTCAAGTTTCTAA-3′ for *mik1*. PCR amplification was for 34 cycles using 50 ng of wild-type *S. pombe* genomic DNA, 1 μM oligonucleotides, and 2 μM $MgCl_2$. Denaturation was at 94°C for 1 min, annealing was at 50°C for 2 min, and elongation was at 72°C for 2 min. Amplified fragments were first cloned into the phagemid pTZ19R. They were subsequently subcloned into the pETXHA vector (Elledge *et al.*, 1992) to fuse the coding region with the influenza hemagglutinin epitope HA1 at the 5′ end of the *wee1* and *mik1* genes. The epitope-tagged gene fragments were then subcloned into the yeast expression vector pREP3X (Forsburg, 1993) under control of the thiamine repressible *nmt1* promoter (Maundrell, 1993) as a *Xho*I-*Bam*HI fragment (*HA-wee1*) or a *Xho*I-*Sma*I fragment (*HA-mik1*).

In Vivo ^{32}P Labeling

In vivo labeling of cells was accomplished essentially as described previously (Gould *et al.*, 1991). Briefly, cells were grown overnight in phosphate-free minimal medium containing 50 μM NaH_2PO_4 and appropriate supplements to a density of 4×10^6-8×10^6 cells/ml. Cells were collected by centrifugation and resuspended at 6×10^6 cells/ml in phosphate-free minimal medium (Moreno *et al.*, 1991) containing 50 μM NaH_2PO_4 and 2–8 mCi of [^{32}P]orthophosphate. To label cells that were overexpressing a protein because of derepression of the *nmt1* promoter, cells were grown to mid-log phase in the presence of 5 μg/ml thiamine, rinsed three times in phosphate-free minimal medium lacking thiamine, and inoculated into a fresh culture of phosphate-free minimal medium. After 16 h in the absence of thiamine, the cells were pelleted and resuspended at 6×10^6 cells/ml in phosphate-free minimal medium containing 2–8 mCi [^{32}P]orthophosphate in the continued absence of thiamine. All strains were labeled for 4 h at the appropriate temperature.

Immunoprecipitation, Immunoblot, and Kinase Assays

S. pombe cell lysates were prepared as detailed previously (Gould *et al.*, 1991). Cdc2 was recovered from denatured cell lysates using PN24 antibodies (Simanis and Nurse, 1986) followed by incubation

with protein A-Sepharose beads. HA1-tagged proteins were immunoprecipitated using the 12CA5 antibody (Babco, Richmond, CA) and protein A-Sepharose beads (Field *et al.*, 1988). Immunoprecipitates were boiled for 3 min in 2× sodium dodecyl sulfate (SDS) sample buffer, resolved on 10% SDS-polyacrylamide gels, transferred to Immobilon-P membranes (Millipore), and bands were visualized by autoradiography. For immunoblot analyses, cell lysates or immunoprecipitates were resolved on 8–20% SDS-polyacrylamide gradient gels and transblotted to Immobilon-P. For detection of Cdc2, membranes were incubated for 2 h at room temperature with a 1:400 dilution of the 4711 anti-Cdc2 antibody followed by ^{125}I-protein A. HA1-tagged proteins were visualized by incubating membranes consecutively with 2 μg/ml 12CA5, 10 μg/ml rabbit anti-mouse antibodies, and ^{125}I-protein A followed by autoradiography. Autoradiography with Kodak XAR5 film was performed at −70°C with intensifying screens.

Phosphoamino Acid Analysis and Phosphopeptide Mapping

^{32}P-labeled Cdc2 bands were cut from immobilon-P membranes and slices were hydrolyzed in 6 N HCl for 60 min at 110°C (Kamps and Sefton, 1989) or subjected to trypsinization as follows: membrane slices were blocked for 30 min at 37°C with 0.1% Tween 20 in 50 mM ammonium bicarbonate. After two washes in 50 mM ammonium bicarbonate, two 10-μg aliquots of trypsin were added for 5 h incubations each (Boyle *et al.*, 1991). Partial acid hydrolysis products were separated by electrophoresis in two dimensions on thin-layer cellulose plates at pH 1.9 and pH 3.5 (Cooper *et al.*, 1983; Boyle *et al.*, 1991) using the Hunter thin-layer electrophoresis system (C.B.S. Scientific, Del Mar, CA). Two-dimensional phosphopeptide mapping was performed as described on thin-layer cellulose plates (Boyle *et al.*, 1991). Electrophoresis was performed at pH 1.9 for 30 min at 1 kV (88% formic acid:acetic acid:H_2O; v:v:v; 1:3.12:35.88) and ascending chromatography was performed in phosphopeptide chromatography buffer (*N*-butanol:pyridine:acetic acid:H_2O; v:v:v:v; 75:50:15:60). Phosphoamino acids and phosphopeptides were visualized by autoradiography on Kodak XAR5 film at −70°C with intensifying screens or with Molecular Dynamics PhosphorImager screens (Sunnyvale, CA).

RESULTS

Overexpression of Wee1 Induces T14 Phosphorylation

While studying Y15 phosphorylation of *S. pombe* Cdc2, an experiment was designed to maximally induce this phosphorylation event in vivo by overexpressing the tyrosine kinase in the absence of the tyrosine phosphatase. For this purpose, the *wee1* gene was placed under the control of the thiamine repressible *nmt1* promoter in the pMNS21L vector (Maundrell, 1990) and transformed into a *cdc25*-22 *leu1*-32 h^- strain. Transformants were selected in the presence of thiamine to prevent Wee1 overexpression, consequent cell elongation, and cell cycle arrest (Russell and Nurse, 1987). These cells were then grown in liquid medium in the absence of thiamine and were labeled with [^{32}P]orthophosphate as the cells began to elongate because of derepression of the *nmt1* promoter. To concurrently inactivate the Cdc25 tyrosine phosphatase, the labeling was performed at 36°C. Contrary to expectation, the overall level of Cdc2 phosphorylation at the *cdc25*-22 block point did not change appreciably as a result of overproducing Wee1 (Figure 1A); however, phosphoamino acid analysis of Cdc2 from the Wee1-overproducing cells yielded a previously undetected partial acid hydrolysis product termed "X" as well as phosphotyrosine and phosphothreonine (Figure 1B, lower left panel). Only phosphotyrosine and phosphothreonine were detected in Cdc2 derived from the *cdc25*-22 strain (Figure 1B, upper left panel) as has been reported previously (Gould and Nurse, 1989). Further acid hydrolysis of X yielded phosphotyrosine and phosphothreonine (Figure 1B, lower right panel) indicating that X was a small phosphopeptide containing both phosphotyrosine and phosphothreonine. The abundance of Cdc2 did not change during the elongation and temperature shift (our unpublished results).

To determine whether X was due solely to the overexpression of Wee1 or whether it was a result of overexpressing Wee1 in the absence of functional Cdc25, the Cdc2 phosphorylation state was examined in wild-type cells expressing a temperature-sensitive *wee1* gene downstream of the *adh* promoter (Russell, 1989). Because the product of the *wee1*-50 allele can function at 25°C but is inactive at 36°C (Nurse and Thuriaux, 1980; Russell and Nurse, 1987), wild-type cells overexpressing Wee1–50 at 36°C can be maintained without lethal consequences. Logarithmically growing cells overexpressing Wee1–50 at 36°C were shifted to 25°C and labeled with [^{32}P]orthophosphate. Phosphoamino acid analysis of Cdc2 immunoprecipitated from these cells yielded phosphotyrosine, phosphothreonine, and X (Figure 1B, upper right panel). Phosphoamino acid analysis of X again yielded phosphotyrosine and phosphothreonine (our unpublished results). These data suggest that X is induced solely as a consequence of Wee1 overproduction.

To determine the identity of the partial acid hydrolysis product, X, Cdc2 immunoprecipitates from [^{32}P]orthophosphate labeled cells were subjected to tryptic phosphopeptide analysis. Tryptic digestion of Cdc2 from the *cdc25*-22 strain generated a series of phosphopeptides labeled phosphopeptide 1 and phosphopeptide 2 (Figure 1C, left panel) as reported previously (Gould and Nurse, 1989). Phosphopeptide 1 contains phosphotyrosine (position Y15) and phosphorylation at position T167 yields several distinctive phosphopeptides that are labeled as a group (phosphopeptide 2) (Gould and Nurse, 1989; Gould *et al.*, 1991). Tryptic phosphopeptides of Cdc2 from the Wee1-overproducing strain also yielded phosphopeptides 1 and 2, but an additional phosphopeptide termed phosphopeptide 3 was detected (Figure 1C, right panel). The phosphopeptides illustrated in Figure 1C (right panel) were analyzed to determine their phosphoamino acid composition. Phosphopeptide 1 contained phosphotyrosine only (Figure 1D, upper panel), phosphopeptide 2 contained phosphothreonine only (Figure 1D, middle panel), and phosphopep-

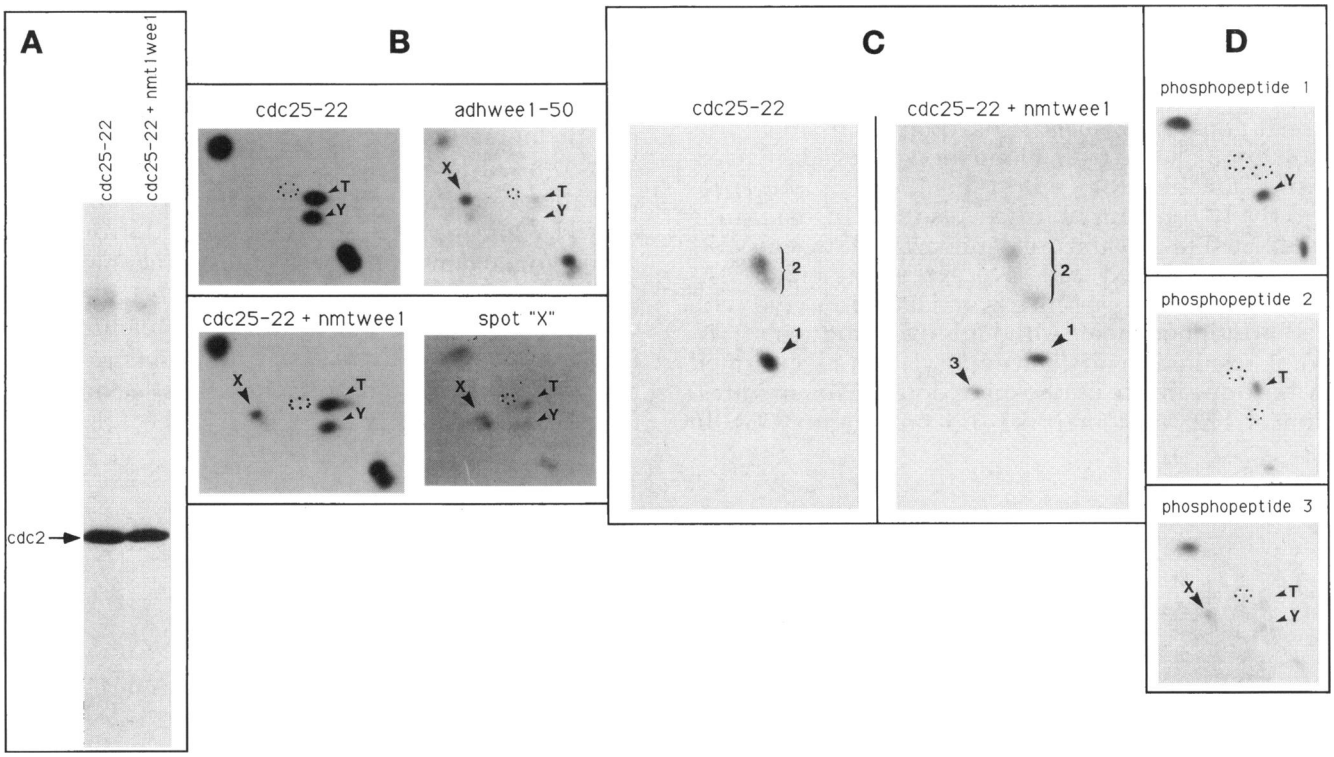

Figure 1. T14 phosphorylation of *S. pombe Cdc2*. The *cdc25*-22 strain, a *cdc25*-22 strain overexpressing Wee1 under control of the *nmt1* promoter, and a strain overexpressing Wee1–50 from the *adh* promoter were labeled with [^{32}P]orthophosphate. (A) Cdc2 was immunoprecipitated from lysates of the indicated strains, resolved by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to immobilon-P. Labeled Cdc2 was detected by autoradiography. (B) [^{32}P]Cdc2 proteins from the indicated strains were partially hydrolyzed with acid. The resultant phosphopeptides were separated by two-dimensional thin-layer electrophoresis. The partial acid hydrolysis product marked X from the *cdc25*-22 strain overexpressing Wee1 was isolated, subjected to further acid hydrolysis, and the resultant phosphopeptides were resolved as before. (C) Cdc2 immunoprecipitates shown in A were digested with trypsin and the resultant phosphopeptides were resolved in two dimensions by electrophoresis at pH 1.9 followed by ascending chromatography. (D) Phosphoamino acid analyses of tryptic phosphopeptides isolated from the map shown in part C, right panel. Dotted circles in B depict the location of the phosphoserine standard and in D circles depict the positions of the unlabeled phosphoamino acids. T, phosphothreonine; Y, phosphotyrosine; X, partial acid hydrolysis product containing both phosphothreonine and phosphotyrosine. In part C, phosphopeptide 1 and the bracketed phosphopeptides labeled 2 contain phosphorylated Y15 and T167, respectively. Phosphopeptide 3 contains phosphorylated T14 and Y15. Autoradiography was at −70°C with intensifying screens. Exposure times were: part A, 16 h; part B (left panels), 3 days; part B (top right panel), 1 wk; part B (bottom right panel), 2 wk; part C, 3 days; and part D, 10 days.

tide 3 contained phosphotyrosine, phosphothreonine, and X (Figure 1D, lower panel). This pattern of phosphopeptides was reminiscent of that observed for Cdc2 from higher eukaryotes where T14 as well as T161 and Y15 are phosphorylated (Krek and Nigg, 1991a; Norbury *et al.*, 1991; Solomon *et al.*, 1992). Similar patterns of phosphopeptides have also been generated from human CDK2, which is phosphorylated on the three homologous residues (Gu *et al.*, 1992). The observations that phosphopeptide 3 was more negatively charged and more hydrophilic than phosphopeptide 1 and contained both phosphotyrosine and phosphothreonine strongly suggested that the overexpression of Wee1 induced the novel phosphorylation of *S. pombe* Cdc2 on T14 as well as Y15. This conclusion was confirmed by mutagenesis studies presented below.

Mik1 Overexpression Does Not Induce T14 Phosphorylation

The *mik1* and *wee1* gene products are redundant in their ability to cause Y15 phosphorylation of Cdc2 in vivo because loss of both but not either alone results in rapid loss of tyrosine-phosphorylated Cdc2 and lethal premature advancement of cells into mitosis (Lundgren *et al.*, 1991). We therefore examined whether Mik1, like Wee1, was able to induce T14 phosphorylation when overexpressed. To address this question, the influenza hemagglutinin HA1 epitope was added to the N-termini of Wee1 and Mik1 to allow detection of these proteins with the monoclonal antibody 12CA5. The epitope-tagged versions (HA-Wee1 and HA-Mik1) under control of the *nmt1* promoter were functional as judged by

their ability to rescue growth of a *wee1*-50 *mik1::ura4*$^+$ double mutant strain at the nonpermissive temperature of 36°C (Figure 2A). Furthermore, overexpression of HA-Wee1 and HA-Mik1 caused elongation of *S. pombe* cells (Figure 2B) that was indistinguishable from elongation induced by overexpression of either wild-type Wee1 or Mik1, respectively (our unpublished results). To determine whether T14 became phosphorylated when Mik1 was overproduced, cells overexpressing either HA-Wee1, HA-Mik1, or Mik1 were labeled in vivo with [^{32}P]orthophosphate. Although overproducing HA-Wee1 resulted in T14 phosphorylation as indicated by the appearance of phosphopeptide 3, overexpression of HA-Mik1 or Mik1 did not (Figure 2C). In these cases, phosphopeptide 1 contained only phosphotyrosine (our unpublished results). To confirm that the Mik1 and Wee1 proteins were being overexpressed in these cells, unlabeled cells expressing low or high levels of wild-type or HA-tagged versions of the proteins were subjected to immunoprecipitation and immunoblotting. Both the HA-Wee1 and HA-Mik1 proteins were detected from cells grown for 20 h in the absence of thiamine (high level expression) but were virtually undetectable when cells were grown in the presence of thiamine (low level expression) (Figure 2D). A few nonspecifically reactive bands were observed in cells lacking HA-tagged proteins (Figure 2D).

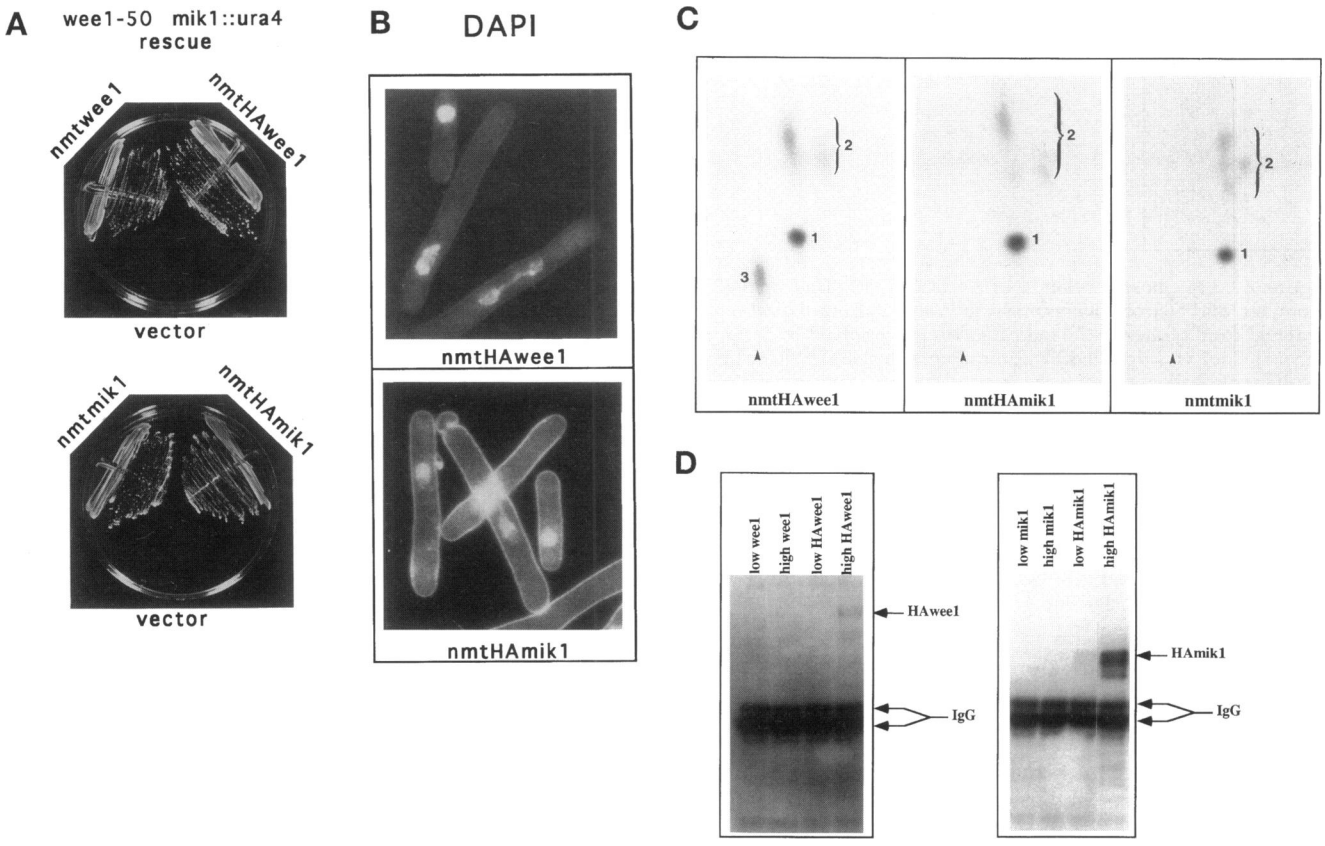

Figure 2. Mik1 overexpression does not induce T14 phosphorylation. The *wee1* and *mik1* genes with or without HA tag sequences at the 5′ end of the coding regions were placed under the control of the *nmt1* promoter in the pREP1 plasmid. (A) pREP1 vector and pREP1 vectors containing the indicated genes were transformed into the *wee1*-50 *mik1::ura4*$^+$ strain. After colony formation at 25°C, cells were streaked on fresh plates and placed at 36°C. (B) pREP1 vectors encoding the HA-tagged Wee1 and Mik1 were transformed into wild-type cells. Photomicrographs of DAPI-stained cells were taken after 16 h of growth in the absence of thiamine. (C) Wild-type cells harboring pREP1 vectors encoding HA-Wee1, HA-Mik1, or Mik1 were labeled in vivo with [^{32}P]orthophosphate for 4 h at 32°C (16–20 h in the absence of thiamine). Tryptic phosphopeptide maps of Cdc2 were generated as described in the legend to Figure 1 and tryptic phosphopeptides were detected by autoradiography after exposure for 3 days at −70°C. The identities of phosphopeptides are given in the legend to Figure 1. Arrowheads indicate where the sample was spotted. (D) Wild-type cells containing pREP1 plasmids encoding the tagged or nontagged version of Wee1 or Mik1 were grown in the presence (low) or absence (high) of thiamine for 16 h. HA-tagged proteins were immunoprecipitated from these strains with the 12CA5 antibody, immunoblotted with 12CA5, and detected by incubation with [^{125}I]protein A and autoradiography. Exposure time was for 16 h. The positions of the tagged proteins and the heavy chain of IgG are indicated with arrows.

Mik1 and Chk1 Protein Kinases Are Not Required for T14 Phosphorylation

Although Mik1 did not cause T14 phosphorylation when overexpressed, we tested whether the presence of Mik1 was necessary for Wee1 to cause this phosphorylation event. We also examined the requirement of a functional *chk1* gene product because Chk1 is another protein kinase implicated in the control of the G2/M phase transition (Walworth *et al.*, 1993; Al-Khodairy *et al.*, 1994). Wild-type cells overexpressing Wee1, *mik1::ura4*$^+$ cells overexpressing Wee1, and *chk1::ura4*$^+$ cells overexpressing Wee1 were labeled in vivo with [^{32}P]orthophosphate. Tryptic phosphopeptide mapping of Cdc2 from all three strains yielded phosphopeptide 3, indicative of T14 phosphorylation (Figure 3). Thus, neither the Mik1 nor the Chk1 protein kinases are necessary for the Wee1 protein kinase to induce T14 phosphorylation of Cdc2. The Chk1 protein kinase was also examined for its ability to induce T14 phosphorylation when overproduced and it was unable to do so (our unpublished results).

Phenotypes of T14A and T14S Mutations

To determine the role of Cdc2 T14 phosphorylation in *S. pombe*, the codon specifying T14 in the *cdc2* cDNA was altered to encode either alanine (T14A) or serine (T14S). The T14A cDNA was cloned into a multicopy vector under control of the *cdc2* promoter and transformed into three strains: a wild-type *cdc2*$^+$ strain, a temperature-sensitive *cdc2* strain, and the *cdc2* null mutant to determine the T14A mutation's effect on cell growth (Table 2). The temperature-sensitive *cdc2*-L7 strain grew at 36°C when expressing T14A and was "semi-wee" in phenotype. The T14A mutant also rescued growth of the *cdc2* null mutant and the cells were i-wee in phenotype. These data indicated that the T14A mutant was functional and that the T14A mutation resulted in a partial gain of function for Cdc2; however, T14A is a recessive mutation because expression of T14A in the presence of wild-type Cdc2 had no effect on the phenotype of the cells, quite unlike that of the Y15F mutant (Gould and Nurse, 1989). The T14S mutant was also capable of rescuing temperature-sensitive and null mutants of *cdc2* indicating that it too was a functional protein (see below).

Table 2. Expression of the T14A mutant cDNA in *S. pombe* strains

	Growth	Phenotype
wild-type	+[a]	wild-type
cdc2-L7	+[b]	semi-wee
cdc2::ura4$^+$	+[a]	semi-wee

[a] 32°C.
[b] 36°C.

To facilitate genetic and biochemical studies of the T14A and T14S mutants, a single-step gene replacement strategy was employed to create strains in which the temperature-sensitive *cdc2* allele *cdc2*-L7 was replaced by a single copy of the mutant *cdc2* cDNAs. The fidelity of these gene replacements was confirmed by polymerase chain reaction and Southern blot analysis (our unpublished results). The phenotypes of these mutant strains are shown in Figure 4A. The T14A strain was semi-wee in phenotype with an average

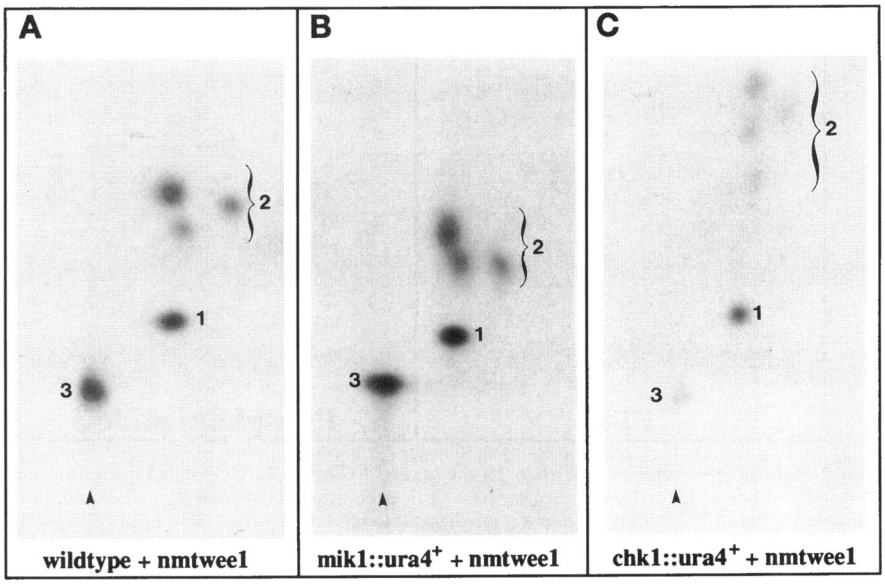

Figure 3. Mik1 and Chk1 are not required for T14 phosphorylation. Wild-type, *mik1::ura4*$^+$ and *chk1::ura4*$^+$ strains containing the pREP1 plasmid encoding Wee1 were labeled with [^{32}P]orthophosphate for 4 h at 32°C after 16 h in the absence of thiamine. Tryptic phosphopeptide maps of Cdc2 were generated as described in the legend to Figure 1 and tryptic phosphopeptides were detected by autoradiography. Exposure time was 4 days. Tryptic phosphopeptide maps of Cdc2 from (A) wild-type cells, (B) *mik1::ura4*$^+$ cells, and (C) *chk1::ura4*$^+$ cells. The identities of phosphopeptides are given in the legend to Figure 1. Arrowheads indicate the position of sample loading.

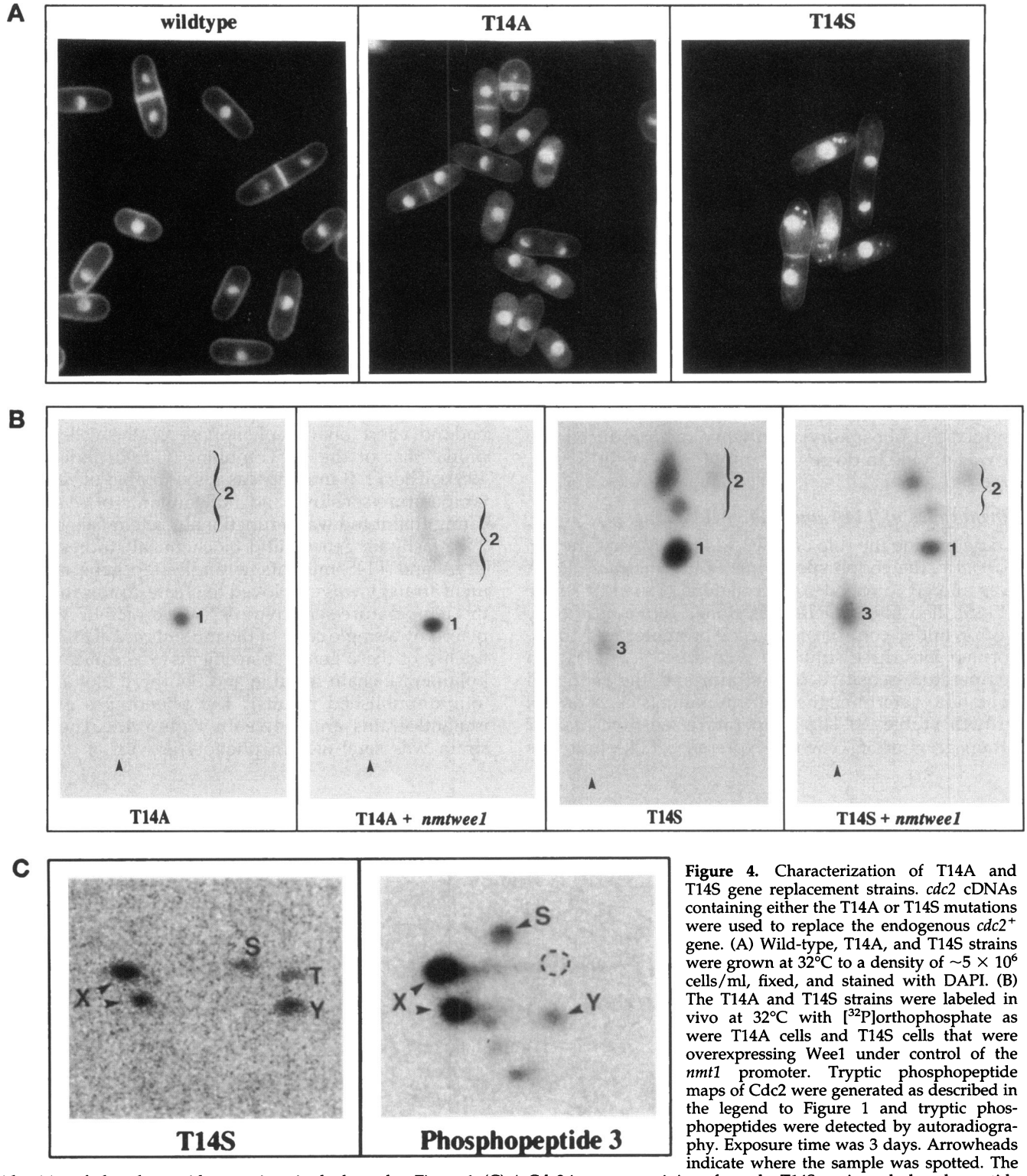

Figure 4. Characterization of T14A and T14S gene replacement strains. *cdc2* cDNAs containing either the T14A or T14S mutations were used to replace the endogenous $cdc2^+$ gene. (A) Wild-type, T14A, and T14S strains were grown at 32°C to a density of ~5 × 10^6 cells/ml, fixed, and stained with DAPI. (B) The T14A and T14S strains were labeled in vivo at 32°C with [^{32}P]orthophosphate as were T14A cells and T14S cells that were overexpressing Wee1 under control of the *nmt1* promoter. Tryptic phosphopeptide maps of Cdc2 were generated as described in the legend to Figure 1 and tryptic phosphopeptides were detected by autoradiography. Exposure time was 3 days. Arrowheads indicate where the sample was spotted. The identities of phosphopeptides are given in the legend to Figure 1. (C) A Cdc2 immunoprecipitate from the T14S strain and phosphopeptide 3 from the T14S map in B were subjected to phosphoamino acid analysis. Phosphoamino acids were visualized by exposing thin-layer cellulose plates to Molecular Dynamics PhosphorImager screens for 1 wk. S, phosphoserine; T, phosphothreonine; Y, phosphotyrosine; X, partial acid hydrolysis products containing phosphoserine and phosphotyrosine. Open dotted circle indicates the position of the phosphothreonine standard.

length of 11.6 μm at septation compared with 14.0 μm for wild type. Although the reduced cell size at septation indicated that the T14A protein was activated earlier than wild type, there was no increase in its specific activity as a histone H1 kinase when measured in an in vitro immune complex kinase assay (our unpublished results). The T14S strain was slightly elongated relative to wild type (14.0 μm) with an average length of 16.6 μm at septation, which suggests that the T14S protein was delayed in its activation relative to wild-type Cdc2. A 2C DNA content was observed for the T14S strain when subjected to flow cytometry (our unpublished results) suggesting that these cells are delayed in G2, also consistent with inhibition of kinase activation.

These gene replacement strains were used first to determine that the phosphorylation of Cdc2 that resulted in the appearance of phosphopeptide 3 when Wee1 was overexpressed was indeed due to phosphorylation at T14. The T14A and T14S strains were transformed with plasmids encoding Wee1 under control of the *nmt1* promoter. Overexpression of Wee1 resulted in elongation and cell cycle arrest in both strains (our unpublished results). The T14A and T14S strains with or without the overproduction of Wee1 were labeled with [^{32}P]orthophosphate and the phosphorylation states of the mutant proteins was determined by tryptic phosphopeptide analysis. The T14A mutant was phosphorylated on Y15 and T167 as indicated by the presence of phosphopeptides 1 and 2 (Figure 4B). Overexpression of Wee1 did not induce the appearance of phosphopeptide 3 (Figure 4B) confirming that T14 was indeed the phosphorylation site targeted by Wee1 overexpression.

Unexpectedly, phosphopeptide 3 was present in the T14S mutant protein even when Wee1 was not overexpressed (Figure 4B). Overexpression of Wee1 increased the stoichiometry of phosphopeptide 3 relative to phosphopeptide 1 (Figure 4B, our unpublished results). Phosphoamino acid analysis demonstrated that the T14S protein was indeed phosphorylated on serine (Figure 4C). Moreover, phosphopeptide 3 from the T14S mutant contained phosphotyrosine and phosphoserine (Figure 4C). Phosphopeptide 1 contained phosphotyrosine only and we did not observe phosphoserine alone in any phosphopeptide (our unpublished results). It appeared that the doubly phosphorylated S14Y15 was even more resistant to acid hydrolysis than T14Y15 as the ratio of X to the phosphoamino acids was very high (Figure 4C). These data indicated that serine could substitute for threonine as a phosphoacceptor site at residue 14, further establishing that the appearance of phosphopeptide 3 was due to dual phosphorylation at Y15 and residue 14.

Wee1 Is Required for Phosphorylation of Cdc2 at Position 14

The T14S protein, unlike wild-type Cdc2, was phosphorylated at position 14 even when the Wee1 kinase was not overexpressed, so we utilized this mutant to determine the requirement of Wee1 for the phosphorylation at residue 14. To this end, a *wee1::ura4*$^+$ *cdc2*-T14S double mutant was constructed by tetrad dissection. The phenotype of *wee1::ura4*$^+$ *cdc2*-T14S cells was wee (Figure 5A). When the T14S protein was labeled in this double mutant strain, phosphopeptide 3 was no longer present (Figure 5B). This result indicates that Wee1 not only can induce phosphorylation at residue 14 when overexpressed but is necessary for residue 14 phosphorylation as well.

Interdependence between T14 and Y15 Phosphorylation

In all of the tryptic phosphopeptide mapping experiments described above, T14 phosphorylation was not detected in the absence of Y15 phosphorylation in the

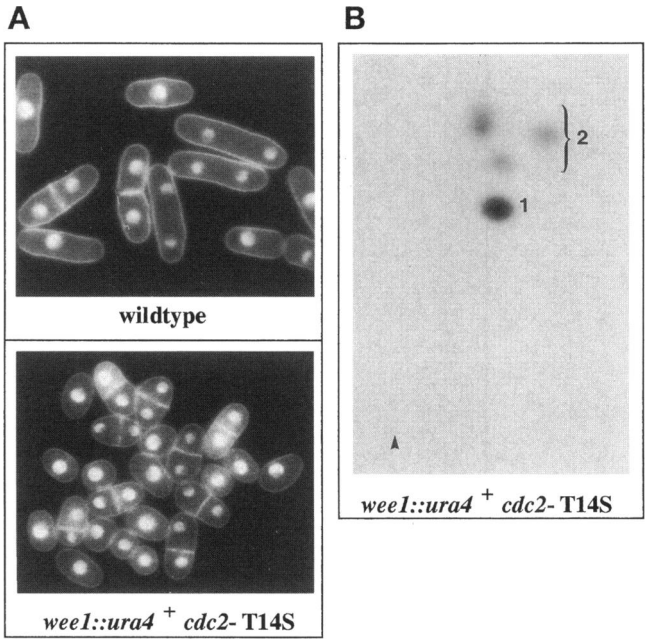

Figure 5. Wee1 is required for S14 phosphorylation. A double mutant strain was constructed containing both the T14S mutation and the *wee1* null mutation. (A) Wild-type and *cdc2*-T14S *wee1::ura4*$^+$ cells were grown at 32°C to a density of ~5 × 10^6, fixed, and stained with DAPI. (B) The double mutant strain was labeled in vivo at 32°C with [^{32}P]orthophosphate and Cdc2 was isolated by immunoprecipitation and SDS-PAGE. After tryptic digestion, the resultant phosphopeptides were separated in two dimensions as described in the legend to Figure 1 and visualized by autoradiography. Exposure time was 5 days. The arrowhead indicates where the sample was loaded. The identities of phosphopeptides were assigned in the legend to Figure 1.

same peptide; i.e., phosphopeptide 1 contained only phosphotyrosine and T14 phosphorylation was detected only in phosphopeptide 3 (see Figure 1 for example). Given that S14 phosphorylation in the T14S mutant strain occurred at readily detectable levels in the absence of Wee1 overexpression, we asked whether the Y15 residue was necessary for this phosphorylation event. To this end, a mutation was created in the *cdc2* cDNA that resulted in the substitution of S14F15 for T14Y15. The S14F15 mutant protein was expressed from the multicopy plasmid, pREP1 (Maundrell, 1993), in the HY1 strain. The HY1 strain contains a gene replacement of the human CDC2 cDNA for the endogenous *cdc2* gene (MacNeill and Nurse, 1993). Using the 4711 antibody, which does not cross-react with human CDC2 (Gould *et al.*, 1991), the T14SY15F protein was immunoprecipitated from the HY1 strain, which had been labeled with [^{32}P]orthophosphate. Tryptic phosphopeptide analysis demonstrated that S14 did not become phosphorylated in the absence of Y15 phosphorylation (Figure 6).

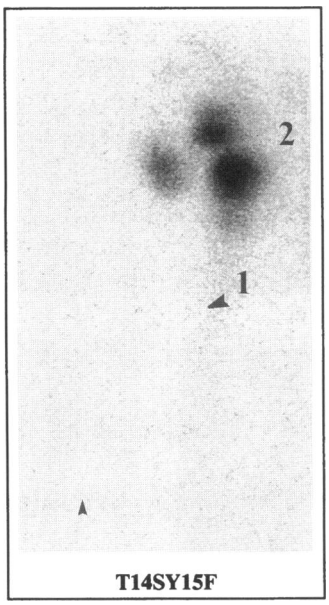

Figure 6. Y15 phosphorylation is required for residue 14 phosphorylation. The T14SY15F mutant protein was expressed in the HY1 strain under control of the *nmt1* promoter in pREP1 in the presence of thiamine (low level expression). The strain was labeled at 32°C with [^{32}P]orthophosphate and Cdc2 was isolated by immunoprecipitation and SDS-PAGE. After tryptic digestion, the resultant phosphopeptides were separated in two dimensions as described in the legend to Figure 1. The small arrowhead indicates where the sample was spotted. The phosphopeptide map was visualized after 10 days exposure to a Molecular Dynamics PhosphorImager Screen. Phosphopeptide 2 corresponds to phosphorylation at T167. The large arrowhead indicates the position where phosphopeptide 1 would be located if present.

T14 Phosphorylation in a cdc17 Mutant

Y15 phosphorylation of *S. pombe* Cdc2 has been implicated as one mechanism that prevents Cdc2 activation in the presence of unreplicated DNA (reviewed in Enoch and Nurse, 1991; Sheldrick and Carr, 1993). Because the evidence suggests that T14 phosphorylation of Cdc2 in higher eukaryotes collaborates with Y15 phosphorylation to inhibit Cdc2 (reviewed in Solomon, 1993), we looked for T14 phosphorylation of Cdc2 in strains of *S. pombe* in which DNA replication had not been completed. We reasoned that under these circumstances, cells might utilize every means available for the inactivation of Cdc2 and cell cycle delay. One strain that arrests in late S phase is the DNA ligase mutant *cdc17*-K42. This strain was labeled in vivo with [^{32}P]orthophosphate at the restrictive temperature for 4 h and then released for 10 min to the permissive temperature. Cells were collected for Cdc2 phosphotryptic peptide analysis at both points. At the 4-h block point, Cdc2 was not detectably phosphorylated on T14 (Figure 7A). However, T14 phosphorylation at very low stoichiometry was observed at the release point as judged by the appearance of phosphopeptide 3 (Figure 7A). We confirmed by phosphoamino acid analysis of phosphopeptide 3 that it did correspond to double T14Y15 phosphorylation as it contained both phosphothreonine and phosphotyrosine (Figure 7B). We did not observe an appreciably higher stoichiometry of T14 phosphorylation by releasing *cdc17*-arrested cells for shorter or longer times (our unpublished results). Low levels of phosphopeptide 3 were also detected in another late S phase mutant, *cdc21*, (Coxon *et al.*, 1992; our unpublished results) but not in hydroxyurea-treated cells (Gould *et al.*, 1991; this study), which arrest earlier in S phase (Nasmyth, 1977). These results suggest that T14 phosphorylation might occur normally in the cell cycle during a short window of time in late S phase or early G2.

The T14A Mutant Exhibits a Normal G2 Delay in Response to Unreplicated or Damaged DNA

Phosphorylation of Y15 has been implicated in the checkpoint control pathway that couples the completion of DNA replication to the onset of mitosis. However, the checkpoint regulating the onset of mitosis in the presence of damaged DNA appears to be independent of the Y15 phosphorylation state (reviewed in Enoch and Nurse, 1991; Sheldrick and Carr, 1993). One possible rationale for negatively regulating Cdc2 by phosphorylation at two different sites is that one site responds to the presence of unreplicated DNA and the other to damaged DNA. We tested whether T14 phosphorylation was involved in either pathway by examining the behavior of the T14A mutant strain when challenged with

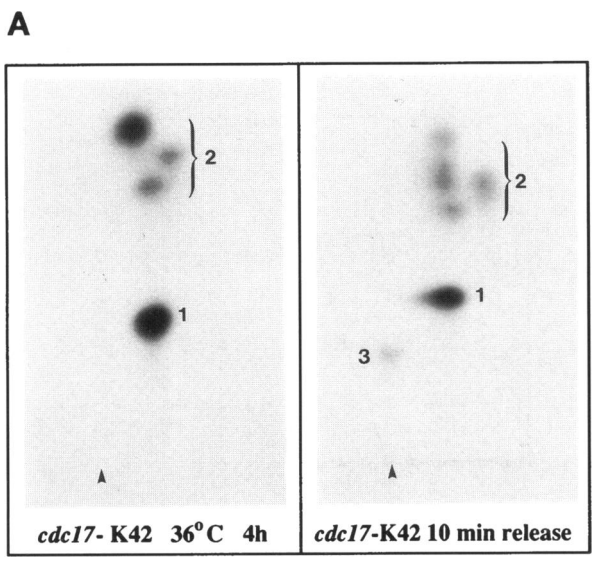

Figure 7. T14 phosphorylation in the *cdc17*^{ts} mutant. The *cdc17*-K42 strain was labeled with [^{32}P]orthophosphate for 4 h at 36°C and released to 25°C for 10 min. Cdc2 was immunoprecipitated from both the blocked and released cells, isolated by SDS-PAGE, and digested with trypsin. Tryptic phosphopeptides were separated in two dimensions as described in the legend to Figure 1 and detected by autoradiography on Kodak XAR5 film at −70°C for 6 days. The identities of phosphopeptides are given in the legend to Figure 1. Arrowheads indicate the spot where the sample was loaded. (B) Phosphopeptide 3 from A, right panel, was isolated and subjected to phosphoamino acid analysis. The dotted circle indicates the position of the phosphoserine standard. T, phosphothreonine; Y, phosphotyrosine; X, partial hydrolysis products containing both phosphothreonine and phosphotyrosine. Phosphoamino acids were visualized by exposing the TLC plate to a Molecular Dynamics PhosphorImager screen for 5 days.

hydroxyurea, ionizing radiation, or UV light. We found that the T14A strain, unlike a Y15F strain (Enoch *et al.*, 1991), elongated on plates containing hydroxyurea and displayed no increased lethality relative to wild-type cells (our unpublished results). When treated with ionizing radiation, the T14A strain delayed mitosis in an identical manner to wild-type cells and the length of G2 before mitosis depended on the dose of radiation (Figure 8, A and B). Also, the survival of the T14A strain when treated with ionizing (Figure 8C) or UV radiation (Figure 8D) was comparable to wild-type. These data suggest that T14 phosphorylation, unlike Y15 phosphorylation, is not required for either the DNA replication or the DNA damage checkpoint controls.

DISCUSSION

In the course of studying the regulation of Cdc2 Y15 phosphorylation in *S. pombe*, we observed a heretofore undetected phosphorylation event on Cdc2 from *S. pombe* cells, which overexpressed the Wee1 protein kinase. Evidence that this site was T14 included the observation that the new phosphorylation event occurred on the same phosphopeptide as did Y15 and was eliminated by mutating T14 to the nonphosphorylatable residue alanine. In addition to detecting T14 phosphorylation in cells overexpressing Wee1, we detected this phosphorylation event at low stoichiometry in certain mutants that blocked in S phase. We did not detect T14 phosphorylation in multiple other situations including nitrogen starvation, G2 phase arrest, M phase, and heat shock (our unpublished results). These results lead us to speculate that T14 phosphorylation might occur normally in a subpopulation of Cdc2 molecules during a brief time interval in S phase or early G2. This event appears to be transient in nature, so it is understandable that it was not detected in our previous studies, which examined Cdc2 phosphorylation in exponentially growing and G2 and M phase-arrested strains.

T14 phosphorylation has previously been identified on Cdc2 from vertebrate sources (Krek and Nigg, 1991a; Norbury *et al.*, 1991; Solomon *et al.*, 1992). It has been shown to collaborate with Y15 phosphorylation to inhibit Cdc2 activation during G2 (Krek and Nigg, 1991b; Norbury *et al.*, 1991; Solomon *et al.*, 1992); however, little is known about the regulation of T14 phosphorylation and the identity of the protein kinase responsible for it has not been established. The lack of detectable T14 phosphorylation on *S. pombe* Cdc2 had indicated that the regulation of *S. pombe* Cdc2 is uniquely less complex than that in higher eukaryotes (Gould and Nurse, 1989). Our finding of T14 phosphorylation in *S. pombe* demonstrates that this more complicated mechanism of regulating Cdc2 activation is present in *S. pombe* although it is not utilized to the same extent as in other organisms. The finding of this mechanism in *S. pombe*, however, has opened the possibility of dissecting the individual contributions of T14 and Y15 phosphorylation in the control of Cdc2 activity in a genetically tractable organism.

We observed T14 phosphorylation at relatively high stoichiometry in cells overexpressing the Wee1 protein kinase (up to 45% of the phosphorylated protein). The simplest explanation for this result is that Wee1, as a dual specificity kinase, is able to phosphorylate both T14 and Y15. However, Wee1 has been shown to phosphorylate human CDC2 in vitro on Y15 exclusively (Parker *et al.*, 1992). Additionally, a separate T14 protein kinase has recently been detected in membrane fractions of Xenopus

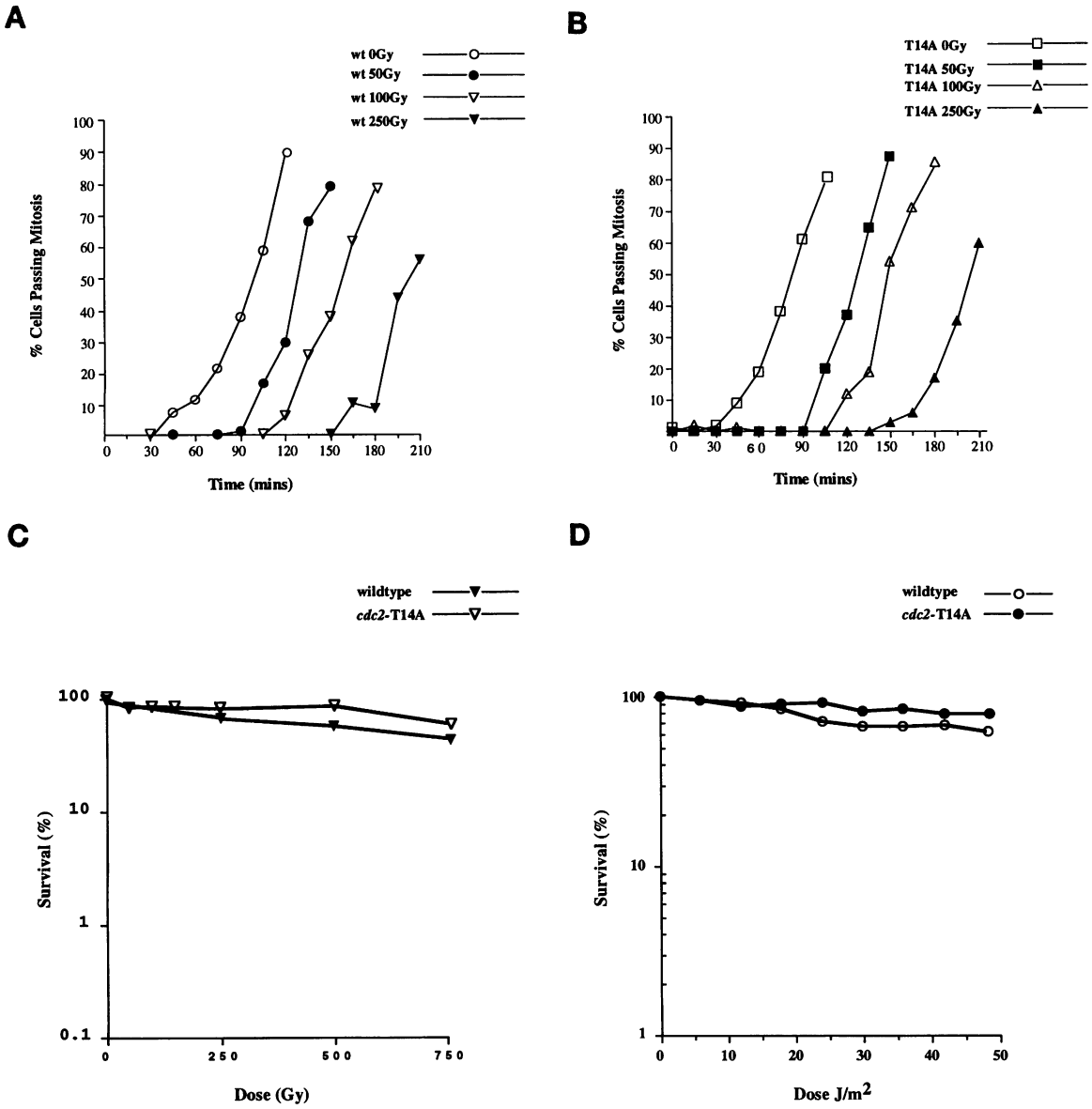

Figure 8. Behavior of T14A strain after ionizing radiation or exposure to UV light. (A) Wild-type and (B) T14A strains were synchronized in early G2 and treated with the indicated doses of ionizing radiation. The proportion of cells passing from G2 through mitosis were scored by counting binucleate and septated cells after staining with DAPI and calcofluor. (C) Wild-type and T14A were treated with the indicated doses of ionizing radiation. Survival was determined by counting the number of colonies relative to untreated controls after 4 days. (D) Wild-type and T14A strains were plated and immediately irradiated with the indicated doses of UV light. Percent survival was estimated by counting the number of colonies relative to untreated controls after 4 days.

cell lysates and HeLa cells (Kornbluth *et al.*, 1994; Atherton-Fessler *et al.*, 1994). These data indicate that Wee1 promotes T14 phosphorylation through an indirect mechanism either by stimulating the activity of the T14 protein kinase or by delaying cell cycle transit at the point where T14 phosphorylation normally occurs; however, we cannot rule out the possibility that Wee1 phosphorylates T14 directly.

As mentioned above, the identity of the T14 kinase is presently unknown. Two other protein kinases, Mik1 (Lundgren *et al.*, 1991) and Chk1 (Walworth *et al.*, 1993; Al-Khodairy *et al.*, 1994), are required to prevent entry into mitosis in the absence of Wee1 and might be considered potential T14 kinases. Because Cdc2 is phosphorylated when Wee1 is overexpressed in strains deleted for these kinases, we can conclude

that they are not T14 kinases. This is in agreement with the recent finding that Mik1 phosphorylates Cdc2 on Y15 only (Atherton-Fessler *et al.*, 1994).

Genetic evidence suggests that Mik1 plays a redundant role to Wee1 in the phosphorylation of Y15 because the deletion of Mik1 in the absence of Wee1 leads to complete dephosphorylation of Cdc2 on Y15 (Lundgren *et al.*, 1991). We have found here that the activities of Mik1 and Wee1 are not redundant with respect to T14 phosphorylation. Overexpression of Mik1 blocks cell cycle progression, as does overexpression of Wee1, but Mik1 overexpression does not lead to T14 phosphorylation. Thus, Wee1 possesses at least one function in cells that does not overlap with that of Mik1.

A T14A mutant is functional, as judged by its ability to rescue both temperature-sensitive and null mutants of Cdc2. It is also able to phosphorylate histone H1 in vitro with the same specific activity as wild-type Cdc2 (our unpublished results). The phenotype of the T14A mutant, semi-wee, is consistent with the inhibitory role of T14 phosphorylation proposed from studies in higher eukaryotic cells (Krek and Nigg, 1991b; Norbury *et al.*, 1991; Solomon *et al.*, 1992). Given that we do not detect phosphorylation of T14 in exponentially growing wild-type cells, the phenotype is actually more pronounced than we would have expected. There are several possible explanations for this. First, it might be that T14 phosphorylation occurs ordinarily in wild-type cells at significant levels but that we are unable to detect it because of phosphatase activity in our lysates. It is also possible that by mutating the protein in this sensitive ATP-binding region, we have altered the structure so that the protein kinase is less sensitive to inhibitory signals. Interestingly, cells expressing the T14A mutant are not semi-wee in the presence of wild-type Cdc2 but rather wild-type in length. This contrasts with the dominant wee phenotype of cells expressing a Y15F mutant protein (Gould and Nurse, 1989). It is possible that the T14A mutant competes ineffectively for cyclin binding partners although we have no evidence for this. So, although we do not understand why the T14A mutant is recessive, this property of the mutant might explain why effects on cell cycle progression were not observed when the T14A mutant protein was expressed in vertebrate systems that contained endogenous wild-type Cdc2 (Krek and Nigg, 1991b; Norbury *et al.*, 1991; Solomon *et al.*, 1992).

Cells that express the T14S mutant are elongated relative to wild type, and the T14S mutant is phosphorylated on S14 in an otherwise wild-type background. This is also consistent with position 14 being an inhibitory site of phosphorylation. Assuming that the identities of the kinase and phosphatase responsible for regulating S14 phosphorylation are the same as those which regulate T14, our observation that S14 does not become phosphorylated in strains lacking Wee1 leads us to conclude that Wee1 is necessary for phosphorylation at this site.

In all of our phosphorylation studies, we were unable to detect a phosphopeptide that contained only T14 or only S14. The only phosphopeptides in which we detected T14 or S14 phosphorylation also contained Y15 phosphorylation. A study of Cdc2 phosphorylation in mouse fibroblasts also did not detect a phosphopeptide containing only T14 phosphorylation (Norbury *et al.*, 1991). These observations suggest that Y15 phosphorylation might be a requirement for phosphorylation at position 14 or that the T14 kinase is a dual specificity kinase that phosphorylates a subpopulation of Cdc2 molecules on both T14 and Y15. The phosphorylation state of the T14SY15F mutant is consistent with either of these possibilities. In this mutant, only T167 becomes phosphorylated. Although this observation would tend to support the first interpretation, it is possible that this mutant does not reach the correct intracellular location for phosphorylation at S14 to occur. These results differ with certain phosphorylation studies of Cdc2 from higher eukaryotic cells. In chicken cells (Krek and Nigg, 1991a), Xenopus egg extracts (Solomon *et al.*, 1992), and HeLa cells (Gu *et al.*, 1992), a phosphopeptide containing only T14 was observed and protein extracts of HeLa and Xenopus are able to phosphorylate T14 and Y15 independently in vitro.

One possible role for T14 phosphorylation would be to more fully inhibit Cdc2 in cells undergoing some environmental stress in which entry into mitosis and cell division would be detrimental to cell survival. As discussed above, we did not detect T14 phosphorylation in wild-type cells as they responded to several different environmental stresses. The T14A mutant was not impaired in its ability to respond to damaged DNA and, unlike a Y15F mutant, was not impaired in its ability to delay cell cycle progression in the presence of unreplicated DNA. Thus, T14 phosphorylation might be used to inhibit Cdc2 for reasons other than those utilizing Y15 as an inhibitory mechanism. The nature of the signal that promotes T14 phosphorylation and how Wee1 is able to integrate both types of signals will be interesting subjects for future studies.

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