MOLECULAR AND CELLULAR BIOLOGY, Jan. 1996, p. 86–93 Vol. 16, No. 1
0270-7306/96/$04.00+0

Stockpiling of Cdc25 during a DNA Replication Checkpoint Arrest in *Schizosaccharomyces pombe*

ROBERT KOVELMAN† AND PAUL RUSSELL*

Departments of Molecular Biology and Cell Biology, The Scripps Research Institute, La Jolla, California 92037

Received 27 June 1995/Returned for modification 7 September 1995/Accepted 12 October 1995

The DNA replication checkpoint couples the onset of mitosis with the completion of S phase. It is clear that in the fission yeast *Schizosaccharomyces pombe*, operation of this checkpoint requires maintenance of the inhibitory tyrosyl phosphorylation of Cdc2. Cdc25 phosphatase induces mitosis by dephosphorylating tyrosine 15 of Cdc2. In this report, Cdc25 is shown to accumulate to a very high level in cells arrested in S. This shows that mechanisms which modulate the abundance of Cdc25 are unconnected to the DNA replication checkpoint. Using a Cdc2/cyclin B activation assay, we found that Cdc25 activity increased ~10-fold during transit through M phase. Cdc25 was activated by phosphorylations that were dependent on Cdc2 activity in vivo. Cdc25 activation was suppressed in cells arrested in G_1 and S. However, Cdc25 was more highly modified and appeared to be somewhat more active in S than in G_1. This finding might be connected to the fact that progression from G_1 to S increases the likelihood that constitutive Cdc25 overproduction will cause inappropriate mitosis.

Investigations of the fission yeast *Schizosaccharomyces pombe* have played a crucial role in the analysis of mitotic control (44). Central to this control are Cdc2, the cyclin-dependent kinase (CDK) required for both G_1-to-S and G_2-to-M transitions in *S. pombe*, and Cdc13, a cyclin B species that binds to Cdc2 and is essential for mitosis. Cdc2/Cdc13 kinase activity is primarily regulated by Wee1 and Cdc25. Wee1 inhibits mitosis by phosphorylating tyrosine 15 of Cdc2, whereas Cdc25 induces mitosis by dephosphorylating tyrosine 15 (6, 26). The importance of these enzymes was first demonstrated in studies showing that temperature-sensitive *cdc25* mutants arrest in G_2, while inactivation of Wee1 contracted G_2 and thereby caused cells to undergo mitosis at half the size of the wild type (43, 45). Conversely, overexpression of Cdc25 made cells wee, whereas overexpression of Wee1 caused G_2 arrest (49, 50). Later studies showed that the mitotic control has a degree of redundancy, with minor roles played by Pyp3 phosphatase and Mik1 kinase (30, 34). Homologs of most of the fission yeast mitotic control proteins have since been discovered in a wide variety of species. Of particular significance was the cloning of human homologs encoding Cdc2, cyclin B, Cdc25, and Wee1 and demonstration that the onset of M in cultured human cells was prevented both by overexpression of Wee1 and by antibody-mediated neutralization of Cdc25 (12, 21, 29, 32, 33, 41, 48, 51). The functional requirement for Cdc25 for the initiation of mitosis has also been proven with *Aspergillus nidulans*, *Drosophila melanogaster*, and *Xenopus laevis* (7, 28, 46).

Investigations have recently focused on Cdc25 and Wee1 regulation. One issue concerns the control which determines the length of G_2. In *S. pombe*, a role in size control is strongly suggested by studies showing that cell length at division is influenced by the doses of *wee1*$^+$ and *cdc25*$^+$ (49, 50). These observations, coupled with findings showing that the abundance of Cdc25 protein fluctuates during the cell cycle, peaking in M, suggest that the timing of mitosis in cycling cells is largely determined by a gradual change in the Cdc25/Wee1 ratio (5, 39). A similar view of cell cycle control has emerged from studies of embryonic divisions 13 to 15 of *D. melanogaster* (7–9). In contrast, studies of human cell lines and *Xenopus* eggs have not revealed a example in which the abundance of M-inducing Cdc25 varies during the cell cycle, nor is it clear whether the abundance of Cdc25 is rate limiting for the induction of mitosis in these systems (13, 16, 22, 23, 28, 33).

A gradual change in the Cdc25/Wee1 ratio provides a mechanism for coupling the onset of mitosis with cell growth, but it does not explain how Cdc2/cyclin B is swiftly activated at the G_2-to-M transition. Studies of *Xenopus* eggs and cultured mammalian cells have been informative in this regard. They have shown that arrest in M correlates with stimulation of Cdc25 activity and inhibition of Wee1; this would be expected to accelerate activation of Cdc2/cyclin B (18, 22, 23, 28, 31, 40, 53, 54). Cdc25 activation and Wee1 inhibition are caused by direct phosphorylation. The identity of the protein kinase(s) that carries out these phosphorylations in vivo is not known, although the latest studies suggest that Cdc2/cyclin B is one of at least two kinases that can carry out the phosphorylations in vitro (27, 40).

A third issue concerns the roles of Cdc25 and Wee1 in the DNA replication checkpoint. This checkpoint couples the onset of mitosis with completion of DNA replication. Mutants lacking Wee1 or Mik1 are not dramatically defective in this checkpoint, but simultaneous inactivation of both kinases abolishes the checkpoint (11, 30). Moreover, Wee1 activity is essential for viability in a variety of checkpoint-deficient mutants (1). This shows that maintenance of Cdc2 tyrosine 15 phosphorylation is essential for the DNA replication checkpoint. This conclusion is also supported by the fact that expression of *cdc25*$^+$ from a strong constitutive promoter overrides the DNA replication checkpoint (11). This finding suggested that the DNA replication checkpoint might function by inhibiting expression of *cdc25*$^+$ mRNA or Cdc25 protein in *S. pombe* (11). A second possibility is that the checkpoint increases the

* Corresponding author. Mailing address: Department of Cell Biology, Scripps Research Institute, 10666 North Torrey Pines Rd., La Jolla, CA 92037. Phone: (619) 554-8273. Fax: (619) 554-6165. Electronic mail address: prussell@scripps.edu.

† Present address: Signal Pharmaceuticals, San Diego, CA 92121.

threshold level of Cdc25 that is required to initiate the Cdc2 positive feedback loop(s). This could occur by inhibition of Cdc25 activity, direct intervention in the positive feedback loop(s), or general inhibition of Cdc2/cyclin B kinase activity, perhaps by a mechanism involving small protein inhibitors similar to those that inhibit CDK/cyclin complexes in mammalian cells (20).

Other very basic and important questions about the regulation of Cdc25 remain to be answered. For example, is Cdc25 activated during mitosis in cycling cells? Previous studies have used either *Xenopus* egg extracts that have been held in an M-like state or mammalian cells that have been arrested in mitosis by treatment with drugs that inhibit microtubule polymerization. Long-term arrest in M might cause exceptional hyperphosphorylation and activation of Cdc25, perhaps as part of a mitotic spindle checkpoint which ensures that Cdc2/cyclin B remains active until anaphase. Another unanswered question is whether Cdc25 activation is dependent on Cdc2 in vivo. Studies with *Xenopus* egg lysates have shown that Cdc25 can be activated and Wee1 can be inhibited in extracts depleted of Cdc2 and Cdk2 (6, 27, 40, 54); it was also reported that the increase in Cdc25 activity precedes Cdc2/cyclin B activation (23). In an organism such as *S. pombe*, in which the onset of mitosis is set to occur when cells reach a particular size, it is unknown whether the scheduled activation of Cdc25 is dependent on Cdc2. This report addresses these issues as they exist in *S. pombe*, with particular attention devoted to the regulation of Cdc25 protein abundance and activity in connection to the DNA replication checkpoint.

MATERIALS AND METHODS

Cells. Cultures were grown to mid-log phase (optical density at 600 nm [OD_{600}], 0.2 to 0.6) for all experiments. All cultures were grown in YES medium except for the *nda3-KM311* strain, which was grown in YEPD. Increases in culture temperature were performed by incubation in a water bath set to 65°C, and decreases in culture temperature were performed by incubation in an ice-water bath. Centrifugal elutriation was conducted essentially as described before (29) except that incubations and procedures were performed at 25°C. Cells were collected by centrifugation and washed once with either H_2O (for Northern [RNA blot] analysis) or STOP buffer (150 mM NaCl, 50 mM NaF, 10 mM disodium EDTA, 0.01% NaN_3) prior to freezing of the cell pellet in liquid N_2 and storage at −70°C.

Northern analysis. Cell pellets were resuspended in HE buffer (50 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid]-NaOH [pH 7.9], 5 mM disodium EDTA, 100 mM NaCl), and cells were lysed by vortexing at 4°C in the presence of glass beads. Supernatants were transferred to tubes containing an equal volume of HES buffer (200 mM HEPES-NaOH [pH 7.9], 10 mM disodium EDTA, 200 mM NaCl, 2% sodium dodecyl sulfate [SDS]) and incubated at 37°C for 1 h. RNA was purified by phenol-chloroform extraction and ethanol precipitation. Pellets were resuspended in H_2O, and the RNA concentration was determined by reading the absorbance at 260 nm. Equivalent amounts of total RNA were loaded onto agarose-formaldehyde gels, run, and transferred to nylon membranes. The *cdc25*$^+$ and *leu1*$^+$ mRNAs were visualized by hybridization with probes prepared by random-primed labeling of a PCR fragment containing the 1.7-kb open reading frame of *cdc25*$^+$ and, for the internal control, the *leu1*$^+$ gene prepared from pYK411 (25).

Immunoblots. Cell pellets were resuspended in lysis buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.5 mM disodium EDTA, 50 mM NaF, 60 mM β-glycerol-phosphate, 1 mM Na_3VO_4, 1 mM dithiothreitol [DTT], 1 mM phenylmethylsulfonyl fluoride [PMSF], and 12.5 μg each of leupeptin, aprotinin, and pepstatin per ml), and glass beads were added to the meniscus. Lysis was performed by vortexing at 4°C, and lysates were separated from the glass beads by centrifuging into new microcentrifuge tubes. An equal volume of 2× SDS sample buffer (128 mM Tris-HCl [pH 6.8], 4% SDS, 20% glycerol, 0.2 M DTT) was added, and samples were incubated at 100°C for 5 min prior to centrifugation in a microcentrifuge. Proteins in the supernatants were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membranes. Membranes were incubated in TBST (150 mM NaCl, 20 mM Tris-HCl [pH 7.5], 0.3% Tween 20) containing 6% milk prior to incubation in TBST containing 3% milk and affinity-purified anti-Cdc25 antiserum (39). Bands were visualized by sequential incubation of the membranes in anti-rabbit immunoglobulin G (IgG) (Fc)-horseradish peroxidase conjugate (Promega) and chemiluminescent detection reagents (Amersham). Membranes were washed at least three times with TBST between incubations. Cross-reaction with a background band served as an internal control (data not shown).

Histone H1 kinase assays. Cell pellets were resuspended in buffer F (25 mM MOPS [morpholinepropanesulfonic acid]-NaOH [pH 7.2], 60 mM β-glycerol-phosphate, 15 mM $MgCl_2$, 0.2 mM Na_3VO_4, 15 mM PNPP [*para*-nitrophenylphosphate], 1 mM DTT) also containing 15 mM EGTA (ethylene glycol tetraacetic acid), 1% Nonidet P-40, 0.5 mM PMSF, and 20 μg each of leupeptin, aprotinin, and pepstatin per ml, and lysis was performed as described in the previous paragraph. Lysates were centrifuged in a microcentrifuge at 4°C for 5 min, and anti-Cdc2 antiserum was added. After 3 h of incubation with constant rotation at 4°C, protein A-Sepharose CL-4B (Pharmacia) was added, and the incubation was continued for an additional 60 min. Beads were precipitated and washed four times with buffer F plus 5 mM EGTA. Kinase assays were performed by incubating beads in buffer G (1 mM DTT, 1 mg of histones [Calbiochem] per ml, 20 mM [γ-^{32}P]ATP [250 μCi/ml]) at 30°C for 15 min; 2× SDS sample buffer was added, and samples were electrophoresed in 11% polyacrylamide gels. Incorporation of ^{32}P into histone H1 was quantitated with a PhosphorImager (Molecular Dynamics).

Cdc25 activity assays. Cells were lysed as described above in buffer A (300 mM NaCl, 5 mM disodium EDTA, 10 mM EGTA, 25 mM MOPS-NaOH [pH 7.2], 50 mM NaF, 60 mM β-glycerol-phosphate, 1 mM Na_3VO_4, 1 mM DTT, 1 mM PMSF, 12.5 μg each of leupeptin, aprotinin, and pepstatin per ml). Unless otherwise indicated, single-point assays were done with 10 or 20 OD_{600} units of cells in 100 or 200 μl of buffer. Lysates were centrifuged at 4°C for 5 min, and the supernatants were transferred to tubes containing affinity-purified anti-Cdc25 antiserum and incubated on a rotator at 4°C for 30 min. Protein A-Sepharose beads were added, and incubation was continued for 30 to 45 min. Beads were precipitated and washed four times with buffer C (100 mM NaCl, 25 mM MOPS-NaOH [pH 7.2], 5 mM disodium EDTA, 10 mM EGTA, 50 mM NaF, 1 mM DTT), and lysates from *cdc25-22* cells containing inactive Cdc2 protein and lysed as described above except in buffer B (100 mM NaCl, 25 mM MOPS-NaOH [pH 7.2], 50 mM NaF, 5 mM disodium EDTA, 10 mM EGTA, 1 mM DTT, 1 mM PMSF, and 12.5 μg each of leupeptin, aprotinin, and pepstatin per ml) were added to the beads. Unless otherwise indicated, 2 OD_{600} units of cells were lysed. After incubation for 15 min at 30°C, supernatants were removed to fresh tubes, buffer D (100 mM NaCl, 25 mM MOPS-NaOH [pH 7.2], 60 mM β-glycerol-phosphate, 10 mM EGTA, 15 mM $MgCl_2$, 1 mM Na_3VO_4, 15 mM PNPP, 1 mM DTT, 1 mM PMSF, 12.5 μg each of leupeptin, aprotinin, and pepstatin per ml) was added to the beads, and after mixing and centrifugation, supernatants were added to the tubes containing the first supernatants. Anti-Cdc2 antiserum was added, and samples were incubated with rotation at 4°C for ~3 h (35). Protein A-Sepharose was added, and incubation was continued for 30 to 60 min. The beads were precipitated and washed four times with buffer E (25 mM MOPS-NaOH [pH 7.2], 60 mM β-glycerol-phosphate, 5 mM EGTA, 15 mM $MgCl_2$, 1 mM Na_3VO_4, 15 mM PNPP, 1 mM DTT). Kinase assays, electrophoresis, and quantitation were performed as described in the previous section. Cdc25 activity units are arbitrary.

For the experiment shown in Fig. 7, Cdc25 activity assays were performed as described above except that after immunoprecipitation of Cdc25, the beads were washed three times with buffer C and three times with buffer G (100 mM NaCl, 25 mM MES [morpholineethanesulfonic acid]-HCl [pH 6.0], 1 mM disodium EDTA, 1 mM DTT) prior to incubation at 30°C for 15 min in the presence of potato acid phosphatase (Boehringer Mannheim Biochemicals) and/or phosphatase inhibitors (50 mM β-glycerol-phosphate and 50 mM Na_3VO_4), as indicated in Figure 7. The beads were then washed three times with buffer G and three times with buffer C, and the remainder of the assay was performed as described above.

RESULTS

Stockpiling of Cdc25 during arrest in G_1 and S. Previous studies have not examined *cdc25*$^+$ mRNA or Cdc25 protein in cells arrested in G_1 or S; therefore, it was unknown whether periodic accumulation of Cdc25 depends on progression through early phases of the cell cycle (5, 39). This was first investigated by monitoring the level of *cdc25*$^+$ mRNA in *cdc10-129* and *cdc25-22* cells following a shift to the restrictive temperature, resulting in cell cycle arrests in G_1 and G_2, respectively. The level of *cdc25*$^+$ mRNA increased to a small extent during both arrests (Fig. 1). This finding shows that the periodic oscillation in the level of *cdc25*$^+$ mRNA that occurs in cycling cells does not depend on progression past the G_1/S boundary. Interestingly, in both experiments, the level of *cdc25*$^+$ mRNA decreased as the septation index increased (Fig. 1). This indicates that *cdc25*$^+$ mRNA levels drop upon exit from M phase, and it is this change which probably accounts for the oscillation of *cdc25*$^+$ mRNA abundance ob-

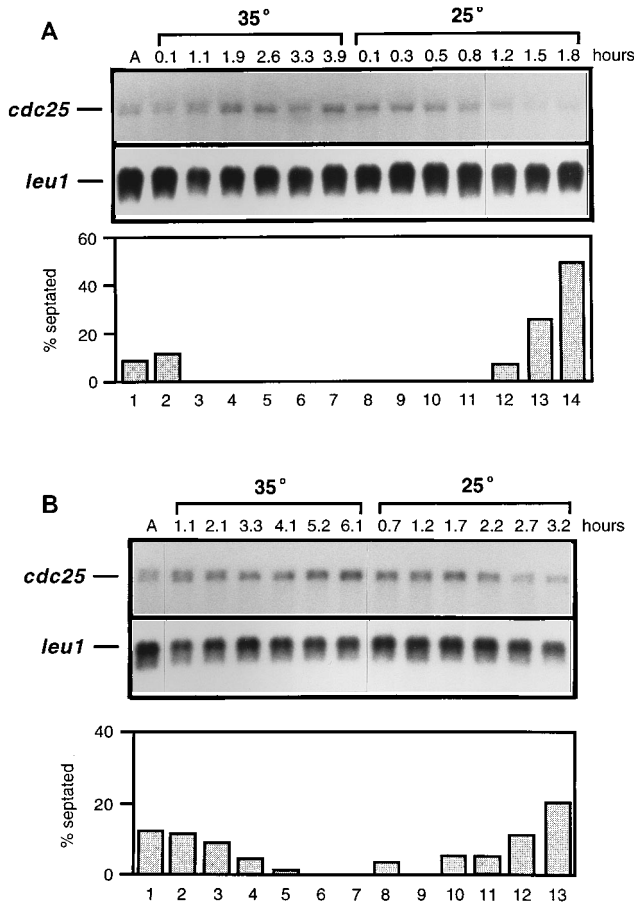

FIG. 1. Level of *cdc25*$^+$ mRNA increases less than twofold during cell cycle arrests in G_1 and G_2. *cdc25-22* (A) and *cdc10-129* (B) cells were grown to mid-log phase at the permissive temperature of 25°C. The culture was shifted to the restrictive temperature of 35°C for 4 h and then shifted back to 25°C. Samples were taken from the asynchronous culture prior to the shift to 35°C (A, lane 1) and at intervals during the block and subsequent release, as indicated above the top panel. Samples were processed for Northern analysis as described in Materials and Methods, and blots were probed for *cdc25*$^+$ and *leu1*$^+$ mRNAs as indicated in the top panel. Cell synchrony was monitored by determining the percentage of cells with visible septa at each time point, the graph of which is shown in the bottom panel.

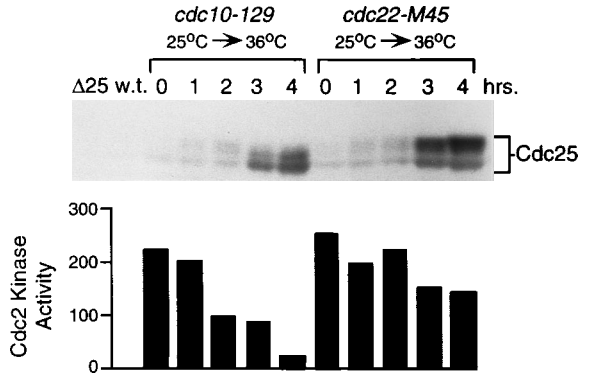

FIG. 2. Cdc25 protein accumulates to high levels during G_1- and S-phase arrests. SDS-PAGE and immunoblotting with anti-Cdc25 antibodies were performed on lysates of *cdc10-129* and *cdc22-M45* cells undergoing arrests in G_1 and S, respectively. The culture temperature was raised from 25 to 36°C at 0 h. Cdc2 H1 kinase activity (counts per minute) was also monitored. The controls are as follows: Δ25, Δ*cdc25* mutant; w.t., wild type.

served in synchronous cultures produced by centrifugal elutriation (3, 39).

The DNA replication checkpoint might operate by modulating the level of Cdc25 protein. This was investigated by monitoring Cdc25 protein in cells as they arrested at the execution points of the *cdc10-129* and *cdc22-M45* mutants, which occur in G_1 and S, respectively (42). In both strains, there was a large increase in the level of Cdc25 protein during the arrests (Fig. 2). This shows that the DNA replication checkpoint does not inhibit the accumulation of Cdc25 protein; in fact, it operates in spite of the stockpiling of a large amount of Cdc25. It should also be noted that Cdc25 from cells arrested at the *cdc22-M45* mutant execution point had reduced mobility in SDS-PAGE compared with Cdc25 from the *cdc10-129* mutant arrested cells (Fig. 2). The significance of this observation is explored below.

Periodic activation of Cdc25 during M phase in cycling cells. These findings suggested that Cdc25 activity was likely to be regulated by posttranslational modifications. An assay system to measure Cdc25 activity was developed (see Materials and Methods). Cdc25 was immunoprecipitated and then added to cell extracts of *cdc25-22* cells blocked in G_2. These extracts contain Cdc2/cyclin B in a latent tyrosine-phosphorylated form that can be activated by Cdc25 (35). Various serine/threonine phosphatase inhibitors were present in the lysis buffer in order to preserve the phosphorylation state of Cdc25 (see Materials and Methods). Cdc2 was subsequently immunoprecipitated from the lysates, and its kinase activity was measured with histone H1 as a substrate. As shown in Fig. 3, Cdc2 from G_2-arrested cells had low kinase activity that was not further increased by incubation with Cdc25 from G_2-arrested *cdc25-22* cells (Fig. 3, lanes 3 and 4). In contrast, lane 5 of Fig. 3 shows that Cdc2-dependent kinase activity was markedly increased after incubation with Cdc25 immunoprecipitated from cells carrying the *nuc2-663* mutation that had been arrested in mitosis (17). Under the experimental conditions employed for this particular assay, the resultant Cdc2 activity was slightly higher than when an equivalent amount of Cdc2 was immunoprecipitated from cells blocked in mitosis (compare lane 5 with lane 6). This is probably due to the fact that Cdc2/cyclin B accumulates to a higher level in a G_2 arrest than in an M-phase arrest (2). This Cdc25-dependent effect on H1 kinase activity was not due to any contaminating kinase activity in the Cdc25 immunoprecipitate, as no kinase activity was detected when Cdc2-containing lysate was not added (Fig. 3, lanes 1 and 2). This assay provided a sensitive and physiological probe for measuring endogenous Cdc25 activity.

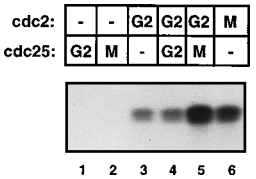

FIG. 3. Assay for Cdc25-dependent activation of Cdc2. As described in Materials and Methods, Cdc25 was immunoprecipitated from *cdc25-22* cells blocked in G_2 (lanes 1 and 4), from *nuc2-663* cells blocked in M (lanes 2 and 5), and from control *cdc25::ura4*$^+$ *cdc2-3w* cells (lanes 3 and 6). The immunoprecipitated Cdc25 was then incubated with a Cdc2-containing lysate of *cdc25-22* cells blocked in G_2 (lanes 3 to 5) or *nuc2-663* cells blocked in M (lane 6). As an additional control, immunoprecipitated Cdc25 was also incubated in the absence of any lysate (lanes 1 and 2). The Cdc2 protein was then immunoprecipitated from the lysates, and its kinase activity was determined with histone H1 as a substrate.

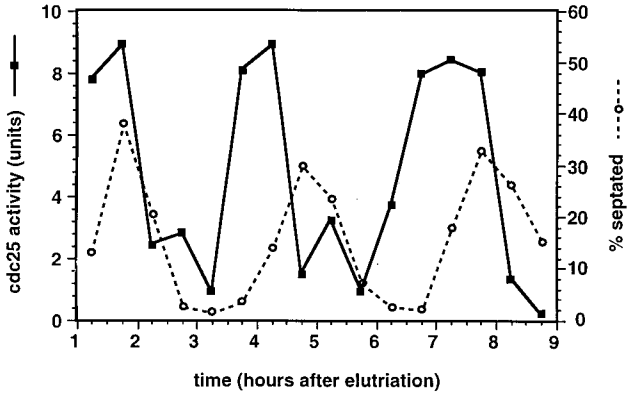

FIG. 4. Cdc25 activity peaks during M in a synchronous culture. Centrifugal elutriation was performed on wild-type cells grown to mid-log phase. The smallest cells were collected and allowed to continue growing for 9 h. The septation index was measured to monitor cell cycle progression, and samples were collected and processed to determine Cdc25 activity as described in Materials and Methods.

We then examined cell cycle regulation of Cdc25 activity in a synchronous population of wild-type cells obtained by centrifugal elutriation (Fig. 4). This technique selects cells on the basis of size, providing a method to study the cell cycle regulation of Cdc25 activity in cells that have not encountered a cell cycle blockade or the imposition of a checkpoint. Cdc25 activity was monitored during two complete cell cycles. In both rounds of division, Cdc25 activity oscillated once each cell cycle, being highest just before the peak in septation index, corresponding to M phase. This demonstrates that fission yeast cells have elevated levels of Cdc25 activity during M and that this increase occurs in cycling cells and is not dependent on activation of an M-phase checkpoint.

Activation of Cdc25 is dependent on Cdc2. Studies with *cdc25-22* cells have previously demonstrated that Cdc2 activation is dependent on Cdc25 activity in vivo. The development of a sensitive assay for endogenous Cdc25 activity provided the opportunity to formally determine whether the reciprocal relationship is also true, as predicted by positive-feedback models. Investigations were undertaken to monitor Cdc25 activity in a strain containing the temperature-sensitive *cdc2-33* mutation. A *cdc2-33* culture was shifted to 35°C for 4.5 h, causing the cells to accumulate in late G_2 and become highly elongated. As was shown previously (39), the Cdc25 protein accumulated to a high level in the blocked *cdc2-33* mutant (Fig. 5, top). Note, however, that Cdc25 activity remained low (Fig. 5, middle). As expected, Cdc2-dependent H1 kinase activity was also low in the blocked *cdc2-33* mutant (Fig. 5, bottom). Following release of the cells from the G_2 block by shifting the culture back to 25°C, Cdc2-dependent kinase activity rose rapidly and then began to subside (Fig. 5, bottom). There was a strikingly similar pattern of Cdc25 activation upon release into mitosis (Fig. 5, middle), and this activation coincided with the appearance of lower-mobility forms of Cdc25 (Fig. 5, top). Importantly, the large increase in Cdc25 activity occurred in the absence of any significant change in the amount of Cdc25 protein.

These findings demonstrate that Cdc25 activity is not stimulated in the absence of Cdc2 function even though cells have surpassed the cell size at which Cdc25 would normally be activated. Moreover, within the limits of experimental precision, Cdc25 and Cdc2 activations were coincident upon the shift to the permissive temperature. This proves that Cdc25 activation is dependent on Cdc2 in vivo, although it does not address whether Cdc2 is directly responsible for Cdc25 activation.

In order to make a direct comparison of Cdc25 activity during different phases of the cell cycle, an experiment was performed in which Cdc25 activity was measured in cells blocked in G_1 (*cdc10-129* mutant), S (*cdc22-M45* mutant), or M (*nuc2-663* mutant) phase (Fig. 6). Cdc25 activity was quite low in both the G_1 and S phase-arrested cells, although in comparing the two samples, it appeared that the activity was slightly higher in the arrested *cdc22-M45* cells. Cdc25 activity from these interphase cells was above background in this assay, since all of these assays resulted in higher levels of activity than were seen with control immunoprecipitations from Δ*cdc25* cells. These Δ*cdc25* cells are viable because they also harbor the compensatory *cdc2-3w* mutation (50). Strikingly, the activity of the Cdc25 protein from cells blocked in M phase (*nuc2-663*) was ~5- to 10-fold higher than in any other strain. Experiments with other strains (i.e., the *nda3-KM311* mutant) also showed that Cdc25 activation is tightly linked to Cdc2 activation (data not shown). Taken together, the data in Fig. 4, 5, and 6 demonstrate that (i) Cdc25 activity increases during the G_2-to-M transition with kinetics indistinguishable from those for Cdc2 activation, (ii) Cdc25 activation is dependent on Cdc2 in vivo, and (iii) activation correlates with posttranslational modifications that reduce the mobility of Cdc25 in SDS-PAGE.

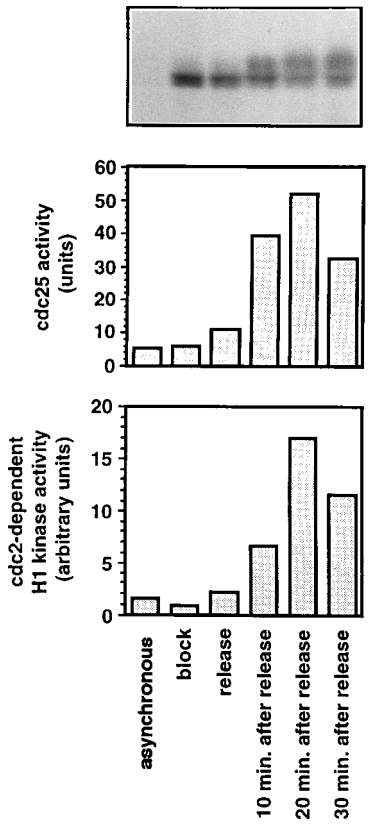

FIG. 5. Activation and mobility shift of Cdc25 are dependent on Cdc2. A *cdc2-33* culture grown to mid-log phase at 25°C (lane 1) was shifted to the restrictive temperature of 35°C for 4.5 h (lane 2) and released into mitosis upon a shift back to incubation at 25°C (lanes 3 to 6). Samples were processed to determine the mobility of Cdc25 (top panel), Cdc25 activity (middle panel), and Cdc2-dependent kinase activity (bottom panel).

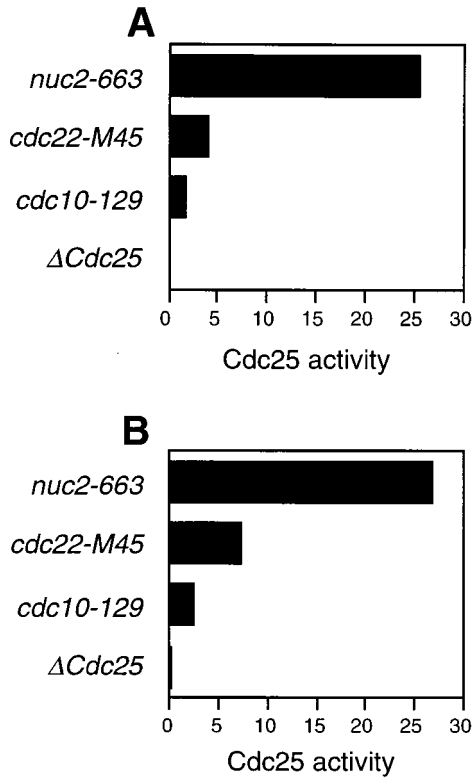

FIG. 6. Cells blocked in mitosis have the highest level of Cdc25 activity. Cells arrested by *cdc10-129* (G_1 arrest), *cdc22-M45* (S arrest), or *nuc2-663* (M arrest) mutations were used for Cdc25 activity assays. Cells with the genotype *cdc25::ura4 cdc2-3w* (Δ*cdc25*) were used as negative controls. Either 2×10^8 cells (A) or 4×10^8 cells (B) were used for the assay. Note that the activity from *nuc2-663* cells was saturating in the assay that used 4×10^8 cells.

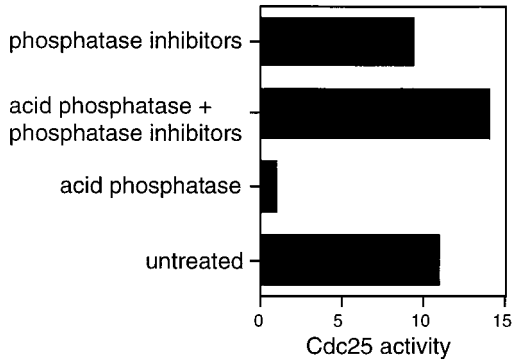

FIG. 7. Treatment with acid phosphatase reduces Cdc25 activity. Cdc25 activity assays were performed as described in the text except that an additional incubation of immunoprecipitated Cdc25 with acid phosphatase and/or phosphatase inhibitors was performed prior to the addition of Cdc2-containing lysate (see Materials and Methods). Cdc25 was derived from *nuc2-663* cells blocked in M phase.

Phosphatase treatment reduces Cdc25 activity. Work in other systems has shown that Cdc25 is activated by phosphorylation. To determine whether this was also true in *S. pombe*, M-phase Cdc25 protein was dephosphorylated and then assayed (Fig. 7). Treatment of active Cdc25 with acid phosphatase dramatically reduced its activity. Addition of a mixture of phosphatase inhibitors along with the phosphatase led to an elimination of this phosphatase-dependent decrease in Cdc25 activity, whereas the phosphatase inhibitors had no effect on Cdc25 activity on their own (Fig. 7). Immunoblotting confirmed that phosphatase treatment caused Cdc25 to migrate with increased mobility in SDS-PAGE (data not shown), as found in other systems (4, 23, 28). Thus, enhanced Cdc25 activity requires reversible phosphorylation.

Direct immunoblot comparison of the Cdc25 protein across the cell cycle. In order to visualize these differentially phosphorylated forms of the Cdc25 protein more clearly, immunoblots were performed on lysates from a panel of mutant strains either blocked at the relevant restrictive temperature or just after release from such a block. As shown previously (in Fig. 5), *cdc2-33* mutant cells blocked in G_2 accumulated a high-mobility form of Cdc25, and upon release into mitosis, there is a rapid upshift of the protein from forms migrating at 79 to 83 kDa to forms migrating at 80 to 88 kDa (Fig. 8). The forms of Cdc25 seen in the *cdc2-33* release sample were very similar to those seen when either *nuc2-663* or *nda3-KM311* cells were incubated at the restrictive temperature (Fig. 8). These mutants arrest in M phase with high Cdc2 kinase activity (17). Intriguingly, arrest of a *cdc22-M45* strain in S phase led to the predominant accumulation of forms of Cdc25 protein that were of slower mobility (80 to 85 kDa) than those seen in the blocked *cdc10-129* cells (Fig. 8), confirming the data shown in Fig. 2. At least one of these S-phase-specific forms was intermediate in mobility between the predominant forms seen in G_1 and the predominant mitotic forms, most likely indicating an intermediate level of phosphorylation. This is consistent with the slightly higher activity detected in arrested *cdc22-M45* cells compared with *cdc10-129* cells (Fig. 6). Thus, Cdc25 protein undergoes distinct cell cycle-specific changes in posttranslational modification in S as well as upon entry into M phase.

Cdc25 overexpression is lethal in blocked *cdc22-M45* cells. The comparative studies of samples from *cdc10-129* and *cdc22-M45* cells indicated that Cdc25 underwent differential modification upon entry into S phase and that this was accompanied by a small increase in Cdc25 activity. This could have physiological relevance, since studies of human cells have suggested that Cdc25A, one of three Cdc25 subtypes in human cells, is required for S phase (19, 24). This possibility was further enhanced by the recent demonstration that two B-type cyclins, Cdc13 and Cig2, collaborate with Cdc2 to bring about S phase in *S. pombe* (38). Previous studies showed that high Cdc25 overproduction led to the induction of mitosis in hydroxyurea-treated cells but not in cells arrested with the *cdc10-129* mutation, a result that can now be thought to be consistent with

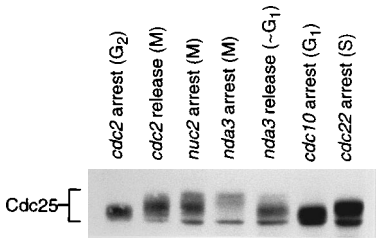

FIG. 8. Cell cycle-specific forms of Cdc25. Cdc25 protein in lysates from cell cycle mutants either blocked in or released into different phases of the cell cycle, as indicated above the panel, was visualized by immunoblotting. Lane 1, *cdc2-33* cells held at 35°C for 3.5 h; lane 2, *cdc2-33* cells 30 min after a shift to 25°C; lane 3, *nuc2-663* cells held at 35°C for 3 h; lane 4, *nda3-KM311* cells held at 20°C for 7.5 h; lane 5, *nda3-KM311* cells 20 min after a shift to 35°C; lane 6, *cdc10-129* cells held at 35°C for 4.5 h; lane 7, *cdc22-M45* cells held at 35°C for 4.5 h. Note that different amount of lysates were loaded on the gel in an attempt to normalize the amount of Cdc25 in each lane.

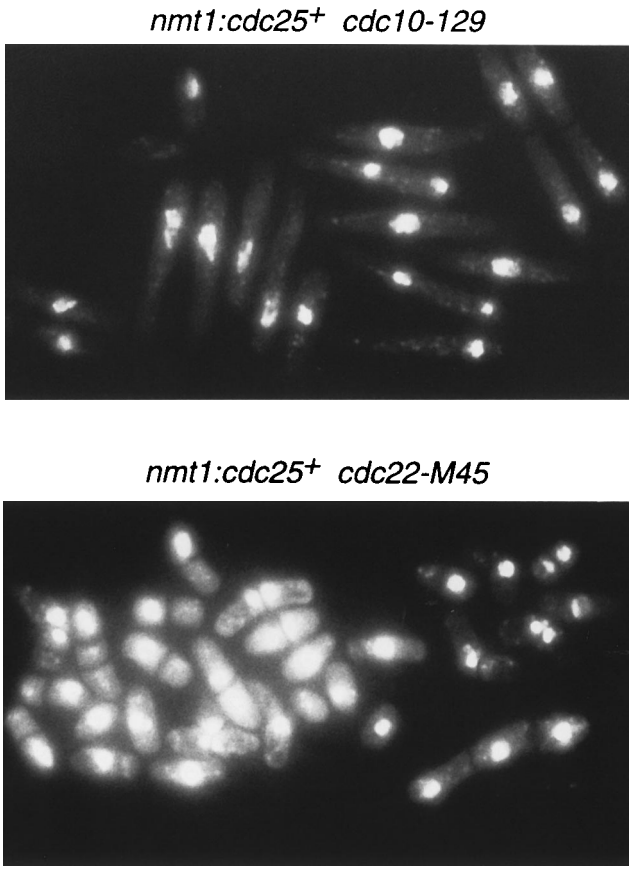

FIG. 9. Overexpression of Cdc25 during an S-phase block leads to mitotic catastrophe. Cultures were grown for 16 h at 25°C in medium lacking thiamine in order to activate high-level expression from the *nmt1* promoter. The cultures were then shifted to the restrictive temperature of 35°C for 6 h. Cells were fixed and stained with 4′,6-diamidino-2-phenylindole (DAPI). Cells arrested in G_1 (*cdc10-129*, top panel) exhibited a typical Cdc arrest phenotype, whereas cells arrested in S (*cdc22-M45*, lower panel) underwent mitotic catastrophe.

the idea that Cdc25 undergoes a small degree of activation upon entry into S phase (11). Since subsequent investigations have revealed that fission yeast cells leak through 10 mM hydroxyurea-induced arrests (52), experiments were performed to examine the impact of Cdc25 overexpression in cells with the *cdc22-M45* mutation arrested in S.

Strains of the *cdc10-129* and *cdc22-M45* genotypes were transformed with a plasmid containing *cdc25*$^+$ under the control of the repressible *nmt1* promoter. Following derepression of the *nmt1* promoter, cells expressing high levels of the Cdc25 protein became wee, as reported previously for the overexpression of *cdc25*$^+$ in other genetic backgrounds (49). The cultures were then shifted to the restrictive temperature for 6 h, a period of time sufficient to cause arrest of *cdc10-129* cells in G_1 and of *cdc22-M45* cells in S phase. The *cdc10-129* cells overexpressing Cdc25 exhibited a typical Cdc phenotype, becoming highly elongated with a single nucleus (Fig. 9). By contrast, the *cdc22-M45* cells overexpressing Cdc25 exhibited aberrant morphologies (Fig. 9). Many cells underwent improper cellular division, leading to anucleate cells as well as cells with a cut phenotype, in which the septum bisected the nucleus. This morphology is commonly referred to as mitotic catastrophe (30, 49) and can be caused by the initiation of mitosis before the completion of DNA replication. These phenotypes were not observed in a control culture in which *nmt1:cdc25*$^+$ expression was repressed by the addition of thiamine to the medium (data not shown). These data are consistent with a model in which Cdc25 has increased activity after entry into S phase and, when overexpressed, causes premature entry into mitosis.

DISCUSSION

A major aim of these studies was to understand how the regulation of Cdc25 is integrated with the DNA replication checkpoint. The motivation for these studies came from three key findings. First, in cycling cells, the cell size at which cells initiate mitosis is influenced by the abundance of Cdc25 protein (49). Second, the abundance of Cdc25 increases dramatically when *cdc2-33*, *cdc25-22*, or *cdc13-117* cells are arrested in G_2 phase (39). Third, constitutive overproduction of Cdc25 overcomes a hydroxyurea-induced DNA replication checkpoint arrest, forcing the onset of mitosis in cells that have not completely replicated their chromosomes (11). Taken together, these findings suggested that it might be important to prevent the accumulation of Cdc25 protein during an S-phase arrest. Perhaps the most surprising finding presented here is that Cdc25 protein accumulates to high levels during a DNA replication checkpoint arrest, precisely the opposite of what was expected. Moreover, Cdc25 also accumulates during a pre-START G_1 arrest.

These findings show that a level of Cdc25 protein that is sufficient to induce mitosis during G_2 in cycling cells is insufficient in cells arrested in S. This raises the question of whether controlling the level of Cdc25 activity is relevant during a checkpoint arrest, but clearly it is, since maintenance of Cdc2 tyrosine 15 phosphorylation is essential for operation of the checkpoint (10, 30). These findings can be understood if the induction of mitosis is normally promoted by posttranslational activation of Cdc25 that is in some way prevented by the checkpoint. Using a sensitive physiological assay of Cdc25 activity, we found that Cdc25 is upregulated during M phase in cycling cells. Importantly, Cdc25 activity remained quite low during an S-phase arrest even though cells had surpassed the cell size at which Cdc25 is normally activated and mitosis is initiated. These findings show that imposition of the DNA replication checkpoint prevents activation of Cdc25 and suggest that this is crucial for operation of the checkpoint.

Why does Cdc25 accumulate during a checkpoint arrest? The DNA replication checkpoint operates in spite of a dramatic accumulation of Cdc25. This result pointedly illustrates how the controls regulating the abundance of Cdc25, which appear to play an important role in coordinating the onset of mitosis with increase in cell size, are seemingly unconnected to the checkpoint. The inhibition of mitosis imposed by the checkpoint is completely dominant to the promotion of mitosis that is driven by the accumulation of Cdc25. Given the apparent sophistication of mitotic control networks, it is somewhat surprising that Cdc25 is allowed to accumulate at a time when the induction of mitosis could be a suicidal event. Therefore, it is tempting to speculate that there is a strategic purpose in the accumulation of Cdc25 during the checkpoint. It seems very likely that one effect of Cdc25 accumulation is to ensure that mitosis is initiated very rapidly after imposition of the checkpoint is relieved. This would be the situation during long-term imposition of the checkpoint, as happens when cells are exposed to hydroxyurea for several hours. Of course, a cell has no way of knowing how long the checkpoint will be imposed, and hence there is probably an advantage in keeping the cell size control operating in the background while the checkpoint is active. Indeed, during rapid growth, a brief imposition of the

checkpoint, say for one-half of a generation, would have little impact on the division rate, because a lengthening of S would be compensated for by a shortening of G_2. This compensation would occur because the cell size control, which in part involves the accumulation of Cdc25, would continue to operate during the checkpoint.

Cdc25 is activated ~10-fold during M in cycling cells. There have been a number of studies of the regulation of Cdc25 during the cell cycle, with a major focus on regulation during M phase. In most experimental systems, it is very difficult to obtain synchronous cultures of cells that have not experienced a cell cycle arrest regimen. Hence, all previous studies that have measured changes in the activity of Cdc25 isolated from cycling cells have relied on one or more methods to arrest cells in either M or some part of interphase. These include treatments with cycloheximide, sodium vanadate, okadaic acid, microcystin-LR, aphidicolin, hydroxyurea, nitrogen mustard, arabinoside, and nocodazole (4, 18, 23, 28, 47). Most of these studies have also relied on assaying PNPP hydrolysis, which is an excellent substrate for many types of phosphatases but is only very weakly dephosphorylated by Cdc25 (35). It has been presumed that these studies accurately indicate how the mitotic induction activity of Cdc25 is likely to be regulated in undisturbed cycling cells, but this had not been tested. Indeed, it is possible that arrest in M depends on a checkpoint which acts to prevent premature inactivation of Cdc2. This could involve hyperactivation of Cdc25.

S. pombe has been useful for cell cycle studies in part because it is possible to produce highly synchronous cultures from cycling cells by a selection method. The preferred method, centrifugal elutriation, relies on the fact that there is a very tight correlation between cell size and cell cycle position in *S. pombe* (36). A second advantage of *S. pombe* is genetics, which can be employed not only to identify genes but also to facilitate biochemical assays. In this case, a *cdc25-22* strain was used to produce cell extracts containing latent tyrosine-phosphorylated Cdc2/cyclin B, providing the physiologically relevant substrate with which to measure Cdc25 activity. The elutriation experiment presented in Fig. 4 is the first to determine whether Cdc25 becomes activated in the normal course of events as cells progress into mitosis. In this experiment, Cdc25 activity underwent a ~10-fold oscillation during both rounds of division. In both cycles, Cdc25 activity was maximal just prior to the peak in septation index, which occurs immediately after mitosis. These studies prove that Cdc25 undergoes a dramatic activation during the normal course of progression through M phase in *S. pombe*. Interestingly, Cdc25 is also ~10-fold more active in M-phase-arrested cells than in asynchronous cultures (Fig. 6), indicating that M-phase arrest does not cause activation of Cdc25 beyond the increase that normally occurs as cells transit M phase.

Activations of Cdc25 and Cdc2 are mutually dependent in vivo. If the induction of mitosis is promoted by a Cdc2/Cdc25 positive-feedback loop, then activations of Cdc2 and Cdc25 should be mutually dependent in vivo. Half of the proof of this crucial test of the Cdc2/Cdc25 positive-feedback model was provided by Gould and Nurse (15), who showed that tyrosyl dephosphorylation and activation of Cdc2 were temperature sensitive in a *cdc25-22* strain. Moreover, the requirement for Cdc25 could be bypassed by ectopic expression of certain other tyrosine phosphatases (14, 34, 37). The studies reported here provide the other half of the test of the in vivo dependency of a Cdc2/Cdc25 positive-feedback loop. A *cdc2-33* mutant strain fails to activate Cdc25 when incubated at the restricted temperature even though cells have surpassed the cell size at which Cdc25 is normally activated. Upon shifting the *cdc2-33* culture to the permissive temperature, Cdc25 was activated within 10 to 20 min. Importantly, the kinetics of Cdc25 activation and appearance of Cdc2 activity were essentially the same. These findings prove that Cdc2 and Cdc25 M-phase activations are mutually dependent in vivo.

Regulation of Cdc25 activity prior to the onset of mitosis. With respect to determining how the onset of mitosis is triggered, the most interesting and important events have in a real sense occurred before large increases in Cdc25 and Cdc2 activity can be measured. For this reason, several of the experiments in this study have focused on the regulation of Cdc25 activity prior to the initiation of M. The first important point to make is that cells arrested in G_1 or S have Cdc25 activity that is significantly above background. This is known because Cdc25 activity in these cells can be compared with that in a *cdc25::ura4*$^+$ *cdc2-3w* strain, in which *cdc25*$^+$ has been deleted. Moreover, the data suggest that Cdc25 is slightly more active in *cdc22-M45* cells that have been arrested in S than in *cdc10-129* cells that have been arrested in G_1. This small difference in activity correlates with three other observations. The first is that the electrophoretic mobility of Cdc25 from S-phase cells is retarded relative to that of Cdc25 from G_1 cells. As noted here and as proven in other studies, reduced mobility of Cdc25 is caused by increased phosphorylation. These phosphorylations are believed to be the same ones that cause the activation of Cdc25, agreeing with data presented here showing that the M-phase form of Cdc25 is the most active and has the slowest electrophoretic mobility. The second correlation concerns Cdc2 activity, which is higher during a *cdc22-M45* arrest than during a *cdc10-129* arrest. Thus, higher Cdc2 activity correlates with both increased phosphorylation and activity of Cdc25.

The final noteworthy observation is that very high overexpression of Cdc25 causes induction of mitosis in cells arrested in S but not in cells arrested in G_1. As in previous studies, the implications of this result are that there is sufficient Cdc2/cyclin B kinase to cause the induction of mitosis during an S-phase arrest and that maintenance of this M-phase-inducing kinase in an inactive state is essential for the operation of the DNA replication checkpoint. Recent studies have indicated that Cdc25A, one of the three Cdc25 isoforms in human cells, is required for the induction of S (19, 24). The physiologically important target of Cdc25A is unknown, but it is likely to be one or more combinations of Cdk2 or Cdc2 bound to cyclin E or cyclin A. Recent studies with *S. pombe* have shown that either one of two CDK/cyclin B species, Cdc2/Cdc13 or Cdc2/Cig2, is sufficient to bring about the initiation of S phase (38). The tyrosine phosphorylation state of Cdc2/Cig2 is unknown, but it is clear that Cdc2/Cdc13 is a major target of the Wee1 and Mik1 tyrosine kinases. Since Cdc2/Cdc13 is able to promote the onset of S, this raises the possibility that Cdc25 or perhaps a related phosphatase might have a role in promoting cell cycle events other than the G_2-to-M transition in *S. pombe*. In this regard the differences between the G_1 and S forms of Cdc25 described here might be significant. Experiments are under way to explore this possibility.

ACKNOWLEDGMENTS

We thank Jonathan Millar for the affinity-purified anti-Cdc25 antibodies, Kazuhiro Shiozaki for helping with DAPI staining and microscopy, and Sabine Ottilie for assistance with the Cdc25 overexpression experiments and for many helpful discussions. We also thank Sabine Ottilie, Susan Forsburg, and Clare McGowan for critical readings of the manuscript.

R.K. was a recipient of a postdoctoral fellowship from the American Cancer Society. This work was supported by a grant from the NIH (GM46006) awarded to P.R.

REFERENCES

1. **Al-Khodairy, F., and A. M. Carr.** 1992. DNA repair mutants defining G_2 checkpoint pathways in *Schizosaccharomyces pombe*. EMBO J. **11:**1343–1350.
2. **Booher, R. N., C. E. Alfa, J. S. Hyams, and D. H. Beach.** 1989. The fission yeast cdc2/cdc13/suc1 protein kinase: regulation of catalytic activity and nuclear localization. Cell **58:**485–497.
3. **Bueno, A., H. Richardson, S. I. Reed, and P. Russell.** 1991. A fission yeast B-type cyclin functioning early in the cell cycle. Cell **66:**149–159.
4. **Clarke, P. R., I. Hoffmann, G. Draetta, and E. Karsenti.** 1993. Dephosphorylation of cdc25-C by a type-2A phosphatase: specific regulation during the cell cycle in *Xenopus* egg extracts. Mol. Biol. Cell **4:**397–411.
5. **Ducommun, B., G. Draetta, P. Young, and D. Beach.** 1990. Fission yeast cdc25 is a cell-cycle regulated protein. Biochem. Biophys. Res. Commun. **167:**301–309.
6. **Dunphy, W. G.** 1994. The decision to enter mitosis. Trends Cell Biol. **4:**202–207.
7. **Edgar, B. A., and P. H. O'Farrell.** 1989. Genetic control of cell division patterns in the Drosophila embryo. Cell **57:**177–187.
8. **Edgar, B. A., and P. H. O'Farrell.** 1990. The three postblastoderm cell cycles of Drosophila embryogenesis are regulated in G2 by *string*. Cell **62:**469–480.
9. **Edgar, B. A., F. Sprenger, R. J. Duronio, P. Leopold, and P. H. O'Farrell.** 1994. Distinct molecular mechanisms regulate cell cycle timing at successive stages of *Drosophila* embryogenesis. Genes Dev. **8:**440–452.
10. **Enoch, T., A. M. Carr, and P. Nurse.** 1993. Fission yeast genes involved in coupling mitosis to the completion of DNA replication. Genes Dev. **6:**2035–2046.
11. **Enoch, T., and P. Nurse.** 1990. Mutation of fission yeast cell cycle control genes abolishes dependence of mitosis on DNA replication. Cell **60:**665–673.
12. **Galaktionov, K., and D. Beach.** 1991. Specific activation of cdc25 tyrosine phosphatases by B-type cyclins: evidence for multiple roles of mitotic cyclins. Cell **67:**1181–1194.
13. **Girard, F., U. Strausfeld, J.-C. Cavadore, P. Russell, A. Fernandez, and N. J. C. Lamb.** 1992. cdc25 is a nuclear protein expressed constitutively throughout the cell cycle in nontransformed mammalian cells. J. Cell Biol. **118:**785–794.
14. **Gould, K. L., S. Moreno, N. K. Tonks, and P. Nurse.** 1990. Complementation of the mitotic activator, $p80^{cdc25}$, by a human protein-tyrosine phosphatase. Science **250:**1573–1576.
15. **Gould, K. L., and P. Nurse.** 1989. Tyrosine phosphorylation of the fission yeast $cdc2^+$ protein kinase regulates entry into mitosis. Nature (London) **342:**39–45.
16. **Heald, R., M. McLoughlin, and F. McKeon.** 1993. Human Wee1 maintains mitotic timing by protecting the nucleus from cytoplasmically activated Cdc2 kinase. Cell **74:**463–474.
17. **Hirano, T., Y. Hiraoka, and M. Yanagida.** 1988. A temperature-sensitive mutation of the *Schizosaccharomyces pombe* gene $nuc2^+$ that encodes a nuclear scaffold-like protein blocks spindle elongation in mitotic anaphase. J. Cell Biol. **106:**1171–1183.
18. **Hoffmann, I., P. R. Clarke, M. J. Marcote, E. Karsenti, and G. Draetta.** 1993. Phosphorylation and activation of human cdc25-C by cdc2-cyclin B and its involvement in the self-amplification of MPF at mitosis. EMBO J. **12:**53–63.
19. **Hoffmann, I., G. Draetta, and E. Karsenti.** 1994. Activation of the phosphatase activity of human cdc25A by a cdk-cyclin E dependent phosphorylation at the G_1/S transition. EMBO J. **13:**4302–4310.
20. **Hunter, T., and J. Pines.** 1994. Cyclins and cancer. II: cyclin D and CDK inhibitors come of age. Cell **79:**547–550.
21. **Igarashi, M., A. Nagata, S. Jinno, K. Suto, and H. Okayama.** 1991. $Wee1^+$-like gene in human cells. Nature (London) **353:**80–83.
22. **Izumi, T., and J. L. Maller.** 1993. Elimination of cdc2 phosphorylation sites in the cdc25 phosphatase blocks initiation of M-phase. Mol. Biol. Cell **4:**1337–1350.
23. **Izumi, T., D. H. Walker, and J. L. Maller.** 1992. Periodic changes in the phosphorylation of Xenopus cdc25 phosphatase regulates its activity. Mol. Biol. Cell **3:**927–939.
24. **Jinno, S., K. Suto, A. Nagata, M. Igarashi, Y. Kanaoka, H. Nojima, and H. Okayama.** 1994. Cdc25A is a novel phosphatase functioning early in the cell cycle. EMBO J. **13:**1549–1556.
25. **Kikuchi, Y., Y. Kitazawa, G. Shimatake, and M. Yamamoto.** 1988. The primary structure of the $leu1^+$ gene of *Schizosaccharomyces pombe*. Curr. Genet. **14:**375–379.
26. **King, R. W., P. K. Jackson, and M. W. Kirschner.** 1994. Mitosis in transition. Cell **79:**563–571.
27. **Kuang, J., C. L. Ashorn, M. Gonzalez-Kuyvenhoven, and J. E. Penkala.** 1994. cdc25 is one of the MPM-2 antigenes involved in the activation of maturation-promoting factor. Mol. Biol. Cell **5:**135–145.
28. **Kumagai, A., and W. G. Dunphy.** 1992. Regulation of the cdc25 protein during the cell cycle in Xenopus extracts. Cell **70:**139–151.
29. **Lee, M. G., and P. Nurse.** 1987. Complementation used to clone a human homologue of the fission yeast cell cycle control gene *cdc2*. Nature (London) **327:**31–35.
30. **Lundgren, K., N. Walworth, R. Booher, M. Dembski, M. Kirschner, and D. Beach.** 1991. mik1 and wee1 cooperate in the inhibitory tyrosine phosphorylation of cdc2. Cell **64:**1111–1122.
31. **McGowan, C. H., and P. Russell.** 1995. Cell cycle regulation of human WEE1. EMBO J. **14:**2166–2175.
32. **McGowan, C. H., and P. Russell.** 1993. Human Wee1 kinase inhibits cell division by phosphorylating $p34^{cdc2}$ exclusively on Tyr15. EMBO J. **12:**75–85.
33. **Millar, J. B. A., J. Blevitt, L. Gerace, K. Sadhu, C. Featherstone, and P. Russell.** 1991. $p55^{CDC25}$ is a nuclear protein required for the initiation of mitosis in human cells. Proc. Natl. Acad. Sci. USA **88:**10500–10504.
34. **Millar, J. B. A., G. Lenaers, and P. Russell.** 1992. *Pyp3* PTPase acts as a mitotic inducer in fission yeast. EMBO J. **11:**4933–4941.
35. **Millar, J. B. A., C. H. McGowan, G. Lenaers, R. Jones, and P. Russell.** 1991. $p80^{cdc25}$ mitotic inducer is the tyrosine phosphatase that activates G_2-phase $p34^{cdc2}$ kinase in fission yeast. EMBO J. **10:**4301–4309.
36. **Mitchison, J. M.** 1970. Physiological and cytological methods for Schizosaccharomyces pombe. Methods Cell Physiol. **4:**131–146.
37. **Mondesert, O., S. Moreno, and P. Russell.** 1994. Low M_r protein-tyrosine phosphatases are highly conserved between fission yeast and man. J. Biol. Chem. **269:**27966–27999.
38. **Mondesert, O., C. McGowan, and P. Russell.** Submitted for publication.
39. **Moreno, S., P. Nurse, and P. Russell.** 1990. Regulation of mitosis by cyclic accumulation of $p80^{cdc25}$ mitotic inducer in fission yeast. Nature (London) **344:**549–552.
40. **Mueller, P. R., T. R. Coleman, and W. G. Dunphy.** 1995. Cell cycle regulation of a *Xenopus* Wee1-like kinase. Mol. Biol. Cell **6:**119–134.
41. **Nagata, A., M. Igarashi, S. Jinno, K. Suto, and H. Okayama.** 1991. An additional homolog of the fission yeast $cdc25^+$ gene occurs in humans and is highly expressed in some cancer cells. New Biol. **3:**959–968.
42. **Nasmyth, K., and P. Nurse.** 1981. Cell division cycle mutants altered in DNA replication and mitosis in the fission yeast *Schizosaccharomyces pombe*. Mol. Gen. Genet. **182:**119–124.
43. **Nurse, P.** 1975. Genetic control of cell size at cell division in fission yeast. Nature (London) **256:**547–551.
44. **Nurse, P.** 1990. Universal control mechanism regulating onset of M-phase. Nature (London) **344:**503–508.
45. **Nurse, P., P. Thuriaux, and K. Nasmyth.** 1976. Genetic control of the cell division cycle in the fission yeast *Schizosaccharomyces pombe*. Mol. Gen. Genet. **146:**167–178.
46. **O'Connell, M., A. Osmani, N. Morris, and S. Osmani.** 1992. An extra copy of $nimE^{cyclinB}$ elevates pre-MPF levels and partially suppresses a mutation of $nimT^{cdc25}$ *in Aspergillus nidulans*. EMBO J. **11:**2139–2149.
47. **O'Connor, P. M., D. K. Ferris, I. Hoffmann, J. Jackman, G. Draetta, and K. W. Kohn.** 1994. Role of the cdc25C phosphatase in G_2 arrest induced by nitrogen mustard. Proc. Natl. Acad. Sci. USA **91:**9480–9484.
48. **Pines, J., and T. Hunter.** 1989. Isolation of a human cyclin cDNA: evidence for cyclin mRNA and protein regulation in the cell cycle and for interaction with $p34^{cdc2}$. Cell **58:**833–846.
49. **Russell, P., and P. Nurse.** 1986. $cdc25^+$ functions as an inducer in the mitotic control of fission yeast. Cell **45:**145–153.
50. **Russell, P., and P. Nurse.** 1987. Negative regulation of mitosis by $wee1^+$, a gene encoding a protein kinase homolog. Cell **49:**559–567.
51. **Sadhu, K., S. I. Reed, H. Richardson, and P. Russell.** 1990. Human homolog of fission yeast cdc25 mitotic inducer is predominantly expressed in G2. Proc. Natl. Acad. Sci. USA **87:**5139–5143.
52. **Sazer, S., and P. Nurse.** 1994. A fission yeast RCC1-related protein is required for the mitosis to interphase transition. EMBO J. **13:**606–615.
53. **Strausfeld, U., A. Fernandez, J. P. Capony, F. Girard, N. Lautredou, J. Derancourt, J. C. Labbe, and N. J. Lamb.** 1994. Activation of $p34^{cdc2}$ protein kinase by microinjection of human cdc25C into mammalian cells: requirement for prior phosphorylation of cdc25C by $p34^{cdc2}$ on sites phosphorylated at mitosis. J. Biol. Chem. **269:**5989–6000.
54. **Tang, Z., T. R. Coleman, and W. G. Dunphy.** 1993. Two distinct mechanisms for negative regulation of the Wee1 protein kinase. EMBO J. **12:**3427–3426.