Cell Cycle Regulation of the Saccharomyces cerevisiae
Polo-Like Kinase Cdc5p

LIANG CHENG, LINDA HUNKE, AND CHRISTOPHER F. J. HARDY*

Department of Cell Biology and Physiology and Department of Genetics,
Washington University School of Medicine, St. Louis, Missouri 63110

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Progression through and completion of mitosis require the actions of the evolutionarily conserved Polo
kinase. We have determined that the levels of Cdc5p, a Saccharomyces cerevisiae member of the Polo family
of mitotic kinases, are cell cycle regulated. Cdc5p accumulates in the nuclei of G2/M-phase cells, and its levels
decline dramatically as cells progress through anaphase and begin telophase. We report that Cdc5p levels are
sensitive to mutations in key components of the anaphase-promoting complex (APC). We have determined that
Cdc5p-associated kinase activity is restricted to G2/M and that this activity is posttranslationally regulated.
These results further link the actions of the APC to the completion of mitosis and suggest possible roles for
Cdc5p during progression through and completion of mitosis.

Execution of the cell cycle is dependent on a cascade of precisely timed events, inherently linked to and critical for
ongoing cell proliferation and proper development. Progression through and completion of mitosis are regulated by at
least two key mechanisms: (i) modification of protein functions by the action of the cyclin-dependent kinase, Cdk, and (ii)
the ubiquitin-dependent proteolysis of specific substrates (19). Completion of mitosis is dependent on the degradation of
mitotic cyclins, and in turn (13, 19, 33, 35, 41), this stage-specific degradation is dependent on a 20S particle designated the
APC (anaphase-promoting complex) or cyclosome, a ubiquitin protein ligase (20, 34).

In addition to the APC, a number of other factors are required by budding yeast cells to complete mitosis. One of these factors,
Cdc5p, is a member of a conserved group of protein kinases called the Polo kinases (9, 24). Polo kinases have been
shown to be required for cytokinesis and also establishment of bipolar spindles (21, 24, 26). They have also been shown to
phosphorylate a number of mitotic regulatory proteins including CHO-1/mitotic kinesin-like protein 1 (MKLP-1) (25),
Xcde25 (23), and β-tubulin and microtubule-associated proteins (36).

Recently, Polo kinases have been implicated in budding yeast (3, 32), mammalian (22), and Xenopus (5) cells in the late
mitotic mechanism, which activates the cyclin-specific APC activity. In order to understand the role Cdc5p plays in this key
cell cycle event, we need to know more about the regulation of its protein levels and activity during the cell cycle. We report
here that Cdc5p accumulates in the nucleus of G2/M-phase but not G1 cells. We show that the level of Cdc5p drops dramatically as cells complete anaphase and that degradation of Cdc5p in G1 is sensitive to mutations in a key APC component, Cdc23p. We also report that Cdc5p-associated kinase activity is restricted to G2/M and is posttranslationally regulated. Taken together, these results provide more evidence that Cdc5p specifically and Polo kinases in general play key regulatory roles in the pathway leading to the completion of mitosis.

MATERIALS AND METHODS

Plasmids and strains. Saccharomyces cerevisiae strains used in this study are listed in Table 1. Plasmid DNA was transformed into yeast by the lithium acetate method as described previously (15). Yeast strains without plasmids were grown in YPD. Yeast strains bearing plasmids were grown in selective synthetic medium (SC) with 2% sugar (galactose or raffinose as indicated). Strains with plasmids to be induced with galactose were first grown in synthetic medium with 2% raffinose to an A600 of 0.2 to 0.4, pCH740 carries the Cdc5 open reading frame ligated into the BamHI site of pCH765 (pRS424-GAL1-URA3, where HA is the hemagglutinin epitope). Wild-type strains were grown at 30°C unless noted otherwise in the text.

Proa tagging of Cdc5p. The chromosomal copy of the CDC5 gene was tagged by a C-terminal, in-frame integration of a DNA fragment encoding the immunoglobulin G (IgG) binding domain of protein A (ProA) (40). The protein A gene and adjacent HIS3 and URA3 markers were amplified by PCR using pProA-HIS3-URA3 (a gift from Mike Rout and John Aitchison) (1). The following primers were used for the PCR: CDC5 sense primer (5′-GAG AAA CTA ACT TTT GAT ATA AAG GAA GTG TTG AAG CAG AAG TCA ATT GTG ACC GTA GAT GGT GAA GCT CAA AAA CTT ATT-3′) and CDC5 antisense primer (5′-TTC GTT AAG GGC AAG ACC ATT TAT TTT ATT TAG TAT TAG TTA TTA ATG GGG CCC AAT CAA TAT AAG GCA CTG AAT ACT-3′). The 5′ region of the sense primer encodes the carboxy-terminal 20 amino acids of Cdc5p (up to but not including the stop codon) and continues in frame to encode the 7 amino acids of ProA beginning with the glycine at amino acid residue 24 (40). The 5′ region of the antisense primer correspond to nucleotides 2225 to 2166 of the untranslated region of CDC5 (1 is the A of the initiation codon) and continues with 24 nucleotides, 1050 to 1072, of the reverse complement of the URA3 gene. The PCR product was transformed into yeast, and His− Ura− transformants were screened by PCR and Western blot analysis for expression of Cdc5p-ProA.

Immunofluorescence. Indirect immunofluorescence was carried out exactly as described previously (44). For localization of the epitope-tagged Cdc5p (Cdc5-ProA), wild-type strains were grown to early log phase and prepared for immunofluorescence microscopy. Cells from the various temperature-sensitive strains used in this study were grown to early log phase at 23°C and then transferred to 37°C for 3 to 4 h, at which time greater than 90% of the population exhibited the arrest morphology. α-Factor and nocodazole arrests were conducted on cells in early log phase as described previously (12) and as described by Jacobs and colleagues, respectively (16). When Cdc5-ProA was visualized, cells were fixed for 5 to 10 min. The fixed cells were first incubated with affinity-purified rabbit anti-mouse IgG (Cappel catalog no. 55480), and then with either fluorescence-conjugated donkey anti-rabbit (Chemicon catalog no. AP182F) or Texas red-conjugated goat anti-rabbit (Cappel catalog no. 55675) secondary antibodies. When tubulin was visualized, cells were fixed for 1 h. The fixed cells were first incubated with a monoclonal antibody recognizing rat tubulin (Chemicon catalog no. MAB065) and then with fluorescein-conjugated donkey anti-mouse IgG (Chemicon). Digital images were taken with a 100× objective on an Olympus microscope. Samples of the arrested cells used for immunofluorescence were also used for fluorescence-activated cell sorting (FACS), immunoblotting, and kinase assay analysis.

Immunoprecipitation and kinase assays. Cells were pelleted, washed once in water, and lysed or frozen in liquid nitrogen. Pellets were resuspended in 0.5 ml of lysis buffer (L buffer) containing 5% glycerol, 20 mM Tris-HCl (pH 8.0), 1 mM

* Corresponding author. Mailing address: Department of Genetics, Washington University School of Medicine, Box 8232, 660 South Euclid Ave., St. Louis, MO 63110. Phone: (314) 747-1808 Fax: (314) 362-7463. E-mail: chardy@cellbio.wustl.edu.
TABLE 1. Yeast strains used in this study

<table>
<thead>
<tr>
<th>Strain Description</th>
<th>Source</th>
</tr>
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<tbody>
<tr>
<td>W303-1A MATa ade2-1 his3-11,15 leu2-3,112 trpl-1 ura3-1 can1-100 LYS2</td>
<td>R. Rothstein</td>
</tr>
<tr>
<td>W303-1B MATa ade2-1 his3-11,15 leu2-3,112 trpl-1 ura3-1 can1-100</td>
<td>R. Rothstein</td>
</tr>
<tr>
<td>YC199 CDC5-PROA-HIS3-URA3 bar1-0</td>
<td>(segregant from YCH199 × YJC1203)</td>
</tr>
<tr>
<td>YC1203 W303-1B phsp2-LEU2 TRP1::SPC42-GFP</td>
<td>(segregant from YCH121 × YJC1203)</td>
</tr>
<tr>
<td>YCH301 CDC5-PROA-HIS3-URA3 bar1-0 spc2-LEU2 TRP1::SPC42-GFP</td>
<td>(segregant from YCH121 × YJC1203)</td>
</tr>
<tr>
<td>K1985 CDC5-PROA-HIS3-URA3 bar1-0 ura3 his3 TRP1</td>
<td>(backcrossed 4 times to W303) (K. Nasmyth)</td>
</tr>
<tr>
<td>YCH301 CDC5-PROA-HIS3-URA3 bar1-0 spc2-LEU2 TRP1::SPC42-GFP</td>
<td>(segregant from YCH121 × YJC1203)</td>
</tr>
<tr>
<td>K2034-CH-a cdc13-1 ade2 ura3 his3 TRP1</td>
<td>(backcrossed 5 times to W303) (K. Nasmyth backcrossed 3 times and then backcrossed it an additional 3 times to W303)</td>
</tr>
</tbody>
</table>

Cdc5p was epitope tagged with the five IgG binding sites of ProA (for protein A) in both wild-type and cdc mutant cells (40). The Cdc5-ProA-tagged strains exhibited no growth defects, and therefore, the Cdc5-ProA fusions must be performing all the essential functions of Cdc5p. We initially examined

EDTA, 10 mM MgCl2, 0.3 M (NH4)2SO4, 1 mM dithiothreitol, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 5 mg of leupeptin per ml, 2 mM pepstatin A, 50 mM NaF, 10 mM sodium pyrophosphate, and 0.5 mM Na-VO4. Cells were lysed by adding 0.5 ml of acid-washed glass beads and vortexing in pulses until 90% lysis was achieved. Immunoprecipitation buffer (IP buffer consists of 50 mM HEPES, 10 mM MgCl2, and 5 mM MnCl2). Samples and washed two times with IP buffer and then two times with kinase buffer (K buffer consists of 50 mM HEPES, 10 mM MgCl2, and 5 mM MnCl2). Samples were resuspended in 50 μl of K buffer. For determination of kinase activity, 10 μg of casein, 1 μCi of [γ-32P]ATP, and cold ATP to 10 μM were added to 25 μl of this suspension. The reaction proceeded for 20 min at 37°C. To this suspension an equal volume of sodium dodecyl sulfate (SDS) gel-loading buffer was added, incubated for 5 min at 100°C, and resolved by electrophoresis on a SDS–15% polyacrylamide gel. Proteins were electrophoretically transferred to nitrocellulose membranes. Blots were incubated with primary antibody for 1 h at room temperature (rabbit anti-mouse IgG; Cappel catalog no. 55480) in 10 mM Tris-Cl (pH 7.5)–150 mM NaCl-0.5% Tween 20 (TBST) with 2% nonfat milk. Immunoblots were washed with TBST and then incubated for 1 h with secondary antibody in TBST (alkaline phosphatase-conjugated anti-rabbit IgG). Immunoblots were washed again in TBST and developed via color visualization with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl-1-phosphate (Promega). The blot of the kinase reaction was exposed to film at room temperature.

Other methods. YEP medium contained 1% yeast extract and 2% Bacto Peptone. Carbon sources (glucose, raffinose, or galactose) were all used at a 2% final concentration. o- Factor and hydroxyurea (HU) were obtained from Sigma and were used at final concentrations of 0.2 μM and 200 mM, respectively. Nocodazole was obtained from Aldrich and was added to medium from a 20-ml/20 ml stock solution in dimethyl sulfoxide (DMSO). It was used at a final concentration of 20 μg of nocodazole per ml and 1% DMSO, as described by Jacobs and colleagues (16). The DNA content of cells was measured on a Becton Dickinson FACScan (San Jose, Calif.) by the method of Epstein and Cross (8).

RESULTS

Cdc5p protein levels decrease as cells exit mitosis. We have previously determined that the levels of Cdc5p fluctuate throughout the cell cycle, suggesting that Cdc5p is present in both G2 and M cells (12). To further examine Cdc5p protein levels,

![FIG. 1. Decrease in Cdc5p levels as cells exit mitosis. CDC5-ProA cdc15-2 (YCH326) cells were synchronized in telophase by growth at 37°C for 3 h prior to release into fresh medium at 23°C. Samples for immunoblot analysis of Cdc5-ProA and actin, FACS analysis of DNA content, and detection of anaphase spindles by immunofluorescence staining of tubulin were taken at the times (in minutes) indicated. The percentage of cells with anaphase spindles is shown on the left side of the FACS analysis profile. Spindle morphology in cells was determined by indirect immunofluorescence staining with an antibulin antibody. Actin detection was used as an internal loading control. Cells released from a cdc15-2 block are delayed in cytokinesis which results in a 2h and 4h shift in DNA content after replication.](http://mcb.asm.org/Downloaded)
the fluctuations of Cdc5p levels in a population of cdc15-2 cells synchronously released from a temperature-induced (37°C) telophase arrest state. Progression through the cell cycle was followed by FACS analysis to determine DNA content and by indirect immunofluorescence using tubulin staining to determine the percentage of cells with anaphase spindles. These results (Fig. 1) show that Cdc5p levels are high in cells blocked in telophase and that the levels decline dramatically 40 min after release from the temperature block, when virtually all the cells have entered G1. Cdc5p begins to reaccumulate after 80 to 90 min when the cells have finished or are just finishing S phase but have not yet entered anaphase.

**Cdc5p degradation is APC dependent.** The pattern of CDC5 message and of Cdc5p levels is reminiscent of the message and protein levels of mitotic cyclins which decline sharply as cells complete anaphase (12, 19, 21). Mitotic cyclins are targeted for degradation by the APC as cells complete anaphase (19). During G1, the APC is active in Pds1p, Clb2p, and Ase1p degradation (2, 4, 18, 45). Moreover, the activity of the APC in G1 cells can be specifically inhibited by mutation of CDC23 encoding a subunit of the APC (4). To determine whether the cell cycle-regulated pattern of Cdc5p loss was a result of APC-mediated degradation, the stability of Cdc5p was examined in wild-type versus cdc23-1 G1-arrested cells. CDC23 and cdc23-1 cells containing plasmids expressing HA-Cdc5p under control of the GAL1 promoter were grown in raffinose at 23°C, synchronized in G1, with α-factor, and then shifted to 37°C to inactivate the cdc23-1 gene product. At this point, HA-Cdc5p expression was induced by the addition of galactose for 15 min, followed by the addition of glucose to turn off its expression. The restrictive temperature (37°C) was maintained while performing these steps. As shown in the immunoblot in Fig. 2, the majority of HA-Cdc5p was degraded after 60 min in the wild-type cells (CDC23) but was still present after 2 h in the cdc23-1 cells (Fig. 2). These results provided evidence that Cdc5p proteolysis might be APC mediated.

**Cdc5p accumulates in the nuclei of G2- and M-phase cycling cells.** To determine the spatial and temporal dynamics of Cdc5p in cells, we performed indirect immunofluorescence on Cdc5-ProA Spc42-GFP cells (YCH301). These experiments showed that in an asynchronous culture Cdc5-ProA was detected in only a subset of cells and that in these cells Cdc5-ProA was predominantly localized in the nucleus (Fig. 3). Multiple fields of such asynchronous cells were quantitated for the presence or absence of Cdc5-ProA staining (Fig. 4). Spc42p, a component of the spindle pole body (SPB) was tagged with the green fluorescent protein Spc42-GFP, which allowed detection of SPBs in this (YCH301) and other strains used in this report (6). Cdc5-ProA was rarely detected in unbudded cells. Of 75 unbudded cells in compiled asynchronous fields, 70 of the cells had the cytological phenotype shown in Fig. 4 (I), exhibiting no detection of Cdc5-ProA signal. Cdc5-ProA was also rarely detected in late mitotic cells, with only 6 of 97 large budded cells IVa having signal or 91 of 97 in class IVb with no signal (Fig. 4). These late mitotic cells were distinguished by two distinct nuclei and SPBs localized to the poles. Cdc5-ProA was detected in approximately one half of the budded cells containing a single nucleus with duplicated SPBs localized exclusively to the mother cell (Fig. 4, IIa and IIb). In contrast, the large fraction (47 of 50) of cells undergoing anaphase exhibited an intense Cdc5p staining pattern, where the nucleus was in the mother and daughter neck and SPBs were widely separated (Fig. 4, III). These results, combined with our previous immunoblot analysis of Cdc5p levels during the cell cycle, suggested that Cdc5p was not present during G1, did not begin to accumulate until at least late S, and disappeared as cells finish anaphase but before they completed mitosis or began cytokinesis.

FIG. 1. Cdc5p levels are high in cells blocked in telophase and decline sharply 40 min after release from the temperature block.

**FIG. 2.** The degradation of Cdc5p is dependent on Cdc23p function. Wild-type and cdc23-1 cells were transformed with a 2μm-based plasmid carrying GAL1-HA-CDC5 (PCH740). For both strains, an overnight culture was grown selectively at 23°C in SC lacking histidine. This was inoculated into YEP plus raf-finose, grown to early log phase at 23°C and arrested in G1 with α-factor. When >90% of the cells exhibited a schmoo-like morphology, as determined by microscopy, the temperature of the culture was shifted to 37°C (the restrictive temperature for cdc23-1). FACS analysis on these blocked samples also indicated that >90% of the cells had a 1n content of DNA and were in G1 (data not shown). After 30 min at the restrictive temperature, galactose was added for 15 min to induce expression of HA-CDC5 from the GAL promoter. Subsequently, at time 0, glucose was added to turn off expression of HA-CDC5 from the GAL promoter while maintaining the G1 arrest at the restrictive temperature. Samples of equal density were taken at the designated time points and assayed by immunoblotting for HA-Cdc5p and actin. Actin detection was used as an internal loading control.

**FIG. 3.** Detection of Cdc5p by immunofluorescence in mitotic cells. Cells were transformed with a 2μm-based plasmid carrying GAL1-HA-CDC5 (PCH740). For both strains, an overnight culture was grown selectively at 23°C in SC lacking histidine. This was inoculated into YEP plus raf-finose, grown to early log phase at 23°C and arrested in G1 with α-factor. When >90% of the cells exhibited a schmoo-like morphology, as determined by microscopy, the temperature of the culture was shifted to 37°C (the restrictive temperature for cdc23-1). FACS analysis on these blocked samples also indicated that >90% of the cells had a 1n content of DNA and were in G1 (data not shown). After 30 min at the restrictive temperature, galactose was added for 15 min to induce expression of HA-CDC5 from the GAL promoter. Subsequently, at time 0, glucose was added to turn off expression of HA-CDC5 from the GAL promoter while maintaining the G1 arrest at the restrictive temperature. Samples of equal density were taken at the designated time points and assayed by immunoblotting for HA-Cdc5p and actin. Actin detection was used as an internal loading control.
Cdc5p accumulates in the nucleus of S- as well as M-phase-arrested cells. To more fully determine the subcellular pattern of Cdc5p localization during the cell cycle, the CDC5-ProA epitope-tagged gene was expressed in a panel of cdc mutant strains and immunofluorescence was performed on the temperature-arrested cells. Cdc5-ProA was not present in cells synchronized in late G1 (cdc4-1), Cdc5-ProA staining (Texas red), and Spc42-GFP using direct fluorescence. On the basis of these four characteristics, cells were placed into the following four groups. I (single G1 cells), II (budded cells with a single nucleus and duplicated SPBs; IIa without and IIb with Cdc5p staining), III (cells in anaphase with nuclei in the mother and daughter neck and separate SPBs present in both mother and daughter), IV (cells which have undergone anaphase, with separate nuclei and polar positioning of SPBs; IVa with and IVb without Cdc5p staining). For each class of cells at given points in the cell cycle, the number and fraction of cells with (Ib, III, and IVa) or without (I, IIa, and IVb) Cdc5p staining are indicated. All images were taken with a 100× objective and printed at the same magnification. Bar = 5 μm.

![Figure 4](http://mcb.asm.org/)
performed (23, 30, or 37°C; data not shown) did not alter these low levels. These results suggest that the cdc5-1 mutant protein may be unstable when isolated from cells grown under either permissive or restrictive conditions.

Following up on these results, we examined Cdc5p protein levels and associated kinase levels in populations of cells synchronized in specific phases of the cell cycle. As shown in Fig. 7, although Cdc5p was present in cells synchronized in G1 (cdc4-1), early S (α-factor arrest into HU) and S (HU) phases, the Cdc5p-associated kinase activity at these times was not above background levels (compare Fig. 7a, c, and d). Quantitation of the Cdc5p-associated kinase activity in these synchronized samples is shown in Fig. 7e. Cells synchronized in early (nocodazole [NZ], cdc13-1, and cdc23-1) and late stages of M (cdc15-2) all had high levels of both Cdc5p protein and associated kinase activity. The low level of Cdc5p-associated kinase activity in cells synchronized in S phase with HU could be due to the low levels of Cdc5-ProA in the immunoprecipitate (Fig. 7c). However, even when the level of Cdc5-ProA in the immunoprecipitate was increased fourfold, the Cdc5p-associated kinase activity was still not significantly above background (Fig. 7c and d, 4× HU). The results taken from Fig. 6 and 7 suggest that Cdc5p-associated kinase activity is not active until G2 or metaphase and may therefore be regulated.

Modification of Cdc5p in cdc13-1 cells requires Mec1p, Mec2/Rad53p, and Rad9p. During the immunoblot analysis of Cdc5p levels in cell cycle-arrested cells (Fig. 7), we observed that the electrophoretic mobility of Cdc5p was modified in cdc13-1 cells. The migration of Cdc5p in cdc13-1 arrested cells was notably slower. In contrast, the shifted form of Cdc5p was not observed in wild-type cells cycling through the cell cycle (Fig. 8) or in cells grown in either HU or nocodazole, which arrest in S phase and metaphase, respectively (Fig. 7). The appearance of the modified form of Cdc5p in cdc13-1 cells was

FIG. 5. Cdc5p is present in cells blocked in S and M phase as detected by immunofluorescence. Wild-type and cdc mutant cells were synchronized at different stages of the cell cycle using either chemicals or by growth of the cdc mutant at the restrictive temperature of 37°C and processed for immunofluorescence. Four views of each cell are shown: Nomarski optics (NOM), staining of DNA (DAPI), Cdc5p-ProA staining (Texas red), and Spc42-GFP (direct fluorescence). The cells were synchronized at the different stages as follows: late G1, with α-factor (YCH301) and cdc4-1 (YCH303); S phase, with HU (YCH301); Metaphase, cdc13-1 (YCH309), nocodazole (Na) (YCH301), and cdc23-1 (YCH305). Samples of the arrested cells used in this study were also used for FACS, immunoblot, and kinase assay analyses shown in Fig. 7. All images were taken with a 100× objective and printed at the same magnification. Bar = 5 μm.

FIG. 6. Cdc5p is present in cells synchronized in telophase as detected by immunofluorescence. CDC5-ProA cdc15-1 cells (YCH307) were synchronized in telophase by growth at the restrictive temperature and processed for immunofluorescence. CDC5 and SPC42 are not tagged in cdc15-2* cells (YCH238). Four views of each cell are shown: Nomarski optics (NOM), staining of DNA (DAPI), Cdc5p-ProA staining (Texas red), and Spc42-GFP (direct fluorescence). All images were taken with a 100× objective and printed at the same magnification. Bar = 5 μm. ND, not done.
FIG. 7. Cdc5p is detected in late G1; S- and M-phase synchronized cells, but Cdc5p-associated kinase activity is restricted to cells arrested in M Phase. Wild-type and cdc mutant cells were arrested in specific stages of the cell cycle using either chemicals or by growth at the restrictive temperature of 37°C, respectively. The cells were synchronized at the various stages as follows: late G1, with α-factor (YCH301) and cdc4-1 (YCH303); G1/S, cells were initially blocked in G1 with α-factor (YCH199) and then released into fresh medium containing HU; S phase, with HU (YCH301); metaphase (Meta), with cdc13-1 (YCH309), nocodazole (NZ) (YCH301), and cdc23-1 (YCH305); and telophase (Tel.), with cdc15-2 (YCH307) and cdc5-1 (YCH214). Extracts from asynchronous (Async) cells were derived from cultures of Cdc5-ProA (YCH199) and strain W303a expressing an untagged Cdc5p. Samples for FACS analysis and extract preparation were taken when >95% of the cells in the culture were appropriately arrested, as detected by light microscopy. Cdc5-ProA (a) and actin (b) in the crude extract were detected by immunoblotting with anti-IgG and antiactin antibodies, respectively. (c) Cdc5-ProA was routinely immunoprecipitated from 400 μg of extract with IgG-Sepharose beads and detected as above by immunoblotting. (d) Kinase activity in these immunocomplexes was measured as described in Materials and Methods with casein as the substrate, except in the case of the 4× HU lane, where 1.6 mg of extract was used. (e) A bar graph of the [32P]-casein levels is shown. Levels were quantitated with a Molecular Dynamics PhosphorImager. (f) Cdc5-ProA kinase activity was determined for cdc5-1-proA (YCH214) cultures grown either asynchronously (Async) (23°C), in the presence of nocodazole (NZ) (3 h, 23°C) or in the presence of nocodazole (3 h, 23°C) followed by a 1-h shift to 37°C (NZ→37). Cdc5-ProA kinase activity was also determined for a wild-type strain expressing Cdc5-ProA (YCH199) arrested in the presence of nocodazole and loaded on the same gel. (g) FACS analysis of DNA content.
further monitored in a time course following a shift of the cdc13-1 culture to the restrictive temperature (Fig. 9a). The shift was apparent after only 1 h of growth of the cdc13-1 strain at the restrictive temperature. The cdc13-1 mutant cells are known to activate the DNA damage checkpoint when they are grown under restrictive conditions (7). Therefore, we next asked whether the MEC1, MEC2/RAD53, and RAD9 genes were necessary for the modification. These three gene products are required for the DNA damage checkpoint pathway (7). We monitored the shift of Cdc5p in cdc13-1 checkpoint defective strains by immunoblotting (cdc13-1 rad53-21, cdc13-1 rad9::URA3, and cdc13-1 mec1-1) following 3 h of growth at the restrictive temperature (Fig. 9b). In all three strains, the shifted form of Cdc5p was not observed. In addition, the shifted form was not observed after extended growth (7 h) of the double mutant strains at the restrictive temperature (Fig. 9c). Thus, accumulation of the shifted form of Cdc5p is dependent upon Mec1p, Mec2/Rad53p, and Rad9p function.

Cdc5p-associated kinase activity is phosphorylation dependent. The phosphorylation state of a given kinase has often been found to play a key role in determining the activity of the kinase (27). To determine whether the Cdc5p-associated kinase activity was dependent on phosphorylation, Cdc5-ProA was immunoprecipitated from lysates of nocodazole-arrested...
cells and incubated with calf intestinal phosphatase (CIP) prior to the kinase assay. The kinase activity of the CIP-treated sample was dramatically lower than that of the untreated sample (Fig. 10a, right panel). In contrast, only a slight reduction was observed if the CIP was boiled for 10 min prior to use. The lack of full activity in the presence of boiled CIP may simply be due to incomplete inactivation by boiling. As a further control, the phosphatase reaction was repeated in the presence of β-glycerophosphate, a phosphatase inhibitor. Under these conditions, no reduction in Cdc5p-associated kinase activity was observed (Fig. 10b). It is interesting to note that Cdc5p may possess autophosphorylation activity as Cdc5p appeared to be phosphorylated in the untreated lane of Fig. 10a (right panel).

Further in vitro studies will be required to determine the nature of the phosphorylation that may be regulating Cdc5p-associated kinase activity.

DISCUSSION

Based on immunoblot and indirect immunofluorescence analyses, we report here that the levels of the conserved Polo kinase Cdc5p are strictly regulated during the cell cycle and steeply decline as cells exit mitosis. Cdc5p accumulates in the nuclei of G2 and early M-phase cells and disappears from cells as they complete anaphase. We show that the levels of Cdc5p are sensitive to mutations in a key APC component, CDC23, in a manner similar to that of other APC substrates including Pds1p, Ase1p, and Clb cyclins (4, 18, 45). The sensitivity of Cdc5p degradation during G1 to mutations in components of the APC (Fig. 2) and its disappearance from cells as they complete anaphase (Fig. 1) suggest that Cdc5p is targeted for degradation by the APC in the same manner as the mitotic cyclins are. While this research was being done, similar results were reported by other labs (3, 32), including one report that shows that Cdc5p is indeed ubiquitinated in an APC-dependent manner (32). Cdc5p is a member of the Polo family of kinases. Other members of the family including Plk1p from mammals exhibit the same cell cycle-regulated pattern of message and protein levels as Cdc5p, and therefore, their levels late in M may also be regulated by the APC (10, 25).

Recently, it has been shown that Cdc5p plays a positive role in regulating cyclin-specific APC activity (3, 32). The APC-associated cyclin ubiquitin ligase activity was reduced in cdc5-1 cells even at the permissive temperature and increased in wild-type cells which overexpress CDC5 (3). Our finding that cdc5-1 cells have low Cdc5p-associated kinase activity, at any temperature, suggests that Cdc5p-associated kinase activity is required to activate the APC. By analogy, studies on Polo kinases from mammals suggest that Polo kinases might play a direct role in activating the APC. The mammalian homologue of Cdc5p, Plk1p, interacts with and phosphorylates three components of the APC and this phosphorylation activates the APC to ubiquitinate cyclin B in vitro (22). Further support for a role of Polo kinases in this regulatory process comes from dominant negative and immunodepletion experiments with Xenopus, which indicate that Plk1 (Polo-like in Xenopus) is required for M-phase exit and destruction of mitotic cyclins (5).

It is interesting to note that a regulator of the APC, Cdc5p, is itself a target of the APC. Removal of Polo kinases late in mitosis may allow G1 cells to target a different or G1 class of factors for APC-mediated degradation. Alternatively, the removal of Cdc5p late in mitosis may serve to prepare cells for the eventual inactivation of the APC late in G1 (19). However, it is not clear from our study or any of the published Cdc5p analyses how the cell coordinates the APC-mediated degradation of Clb cyclins with that of Cdc5p or how it maintains its APC activity toward Clb cyclins in the absence of Cdc5p during...
Clearly, other factors, including perhaps Hct1p, may play roles in the activation and maintenance of APC function during G1 (30, 42). Further studies are required to understand the complex links between Cdc5p and the APC and their ramifications for progression through and completion of mitosis.

Regulation of Cdc5p kinase activity. In addition to the regulation of Cdc5p levels by the APC, the timing of Cdc5p kinase activity is also posttranslationally regulated. In studies of synchronized populations of cycling cells, we determined that the peak in Cdc5p-associated kinase activity significantly lagged behind that of Cdc5p levels. Cdc5p was detected in HU- and nocodazole-arrested cells, which have activated the DNA replication and spindle assembly checkpoints, respectively. However, Cdc5p-associated kinase activity was detected only in the population of nocodazole-arrested cells. We also showed that this Cdc5p activity is sensitive to phosphatase treatment. These studies suggest that phosphorylation may play a role in the restriction of Cdc5p-associated kinase activity to G2/M. Further support for this hypothesis comes from studies of Cdc5p homologues in mammals, Drosophila, and Xenopus which show that the respective activation of Plk1-, Polo-, and Plx1-associated kinase activities are regulated by phosphorylation (11, 29, 36). Evidence for Cld/Cdk, as the Polo-activating kinase, comes from in vitro studies with recombinant Plk1 and Cld/Cdk (22). Interestingly, Cdc5p contains a single Cld/Cdk consensus phosphorylation site. An intriguing possibility suggested by the lack of Cdc5p-associated kinase activity in HU-arrested S-phase cells (Fig. 7) is that the DNA replication checkpoint, which is activated in these HU-treated cells (7), regulates Cdc5p kinase activity. In this model, the activation of Cdc5p-associated kinase activity may be dependent upon the prior completion of DNA replication. Alternatively, as suggested above, Cdc5p-associated kinase activity may simply be dependent upon an M-phase-specific activity, such as Cld/Cdk, for its activation.

Role for Cdc5p in adaptation to the DNA damage response. We observed that the electrophoretic mobility of Cdc5p is modified under conditions that induce the DNA damage checkpoint. In cdc13 mutant cells grown at the restrictive temperature, the mobility of Cdc5p is slower than that of Cdc5p derived from wild-type cells (Fig. 9a). In cdc13 arrested cells, Cdc5p-associated kinase activity is high and its modification is dependent upon the factors required to transduce the DNA damage signal including Mec1p, Mec2/Rad53p, and Rad9p (7). Budding yeast cells have a DNA damage-responsive checkpoint, which causes a transient metaphase arrest. Cdc5p is required for cells to adapt to or recover from the DNA damage checkpoint (37). A cdc5-ad mutant (for adaptation defective) fails to adapt and remains arrested in metaphase in response to DNA damage (37). It has recently been reported that although the kinase activity associated with the mutant cdc5-ad protein in cdc13-1 arrested cells is high, its stimulation of APC activity under these conditions is dramatically lower than that of wild-type Cdc5p (3). The failure of cdc5-ad cells to adapt may merely reflect its inabilities to specifically target and to activate the APC. It will be interesting to determine whether the Cdc5-ad mutant protein is modified under conditions, which activate the DNA damage checkpoint, and whether this modification is required for the adaptation response. In summary, Cdc5p, a regulator of adaptation to the DNA damage response, may also be regulated by the DNA damage response checkpoint.
Additional roles for Cdc5p. The results of our previous study suggests that Cdc5p may have a role in DNA replication (12). We suggested in our previously published study that Cdc5p could play a role in activating the degradation of the Clb-Cdk kinase activity that is implicated in the exit from mitosis (12). Proteolysis of the Cdk cyclins is required for the timely transition during anaphase/telephase, between the post- and prereplication complexes present at origins (28). The low level of cyclin-specific APC activity in cdc5-1 mutant cells late in mitosis may affect the efficient formation of prereplication complexes at origins at that time (3). Such a model could help explain the cdc5-1 mutant plasmid loss defect that can be suppressed by the addition to the plasmid of multiple replication origins (12).

Additional support for a Cdc5p role in regulating replication initiation is the interaction between Cdc5p and the origin interacting factor Dbf4p (12). Alternatively, the Cdc5p-Dbf4p interaction may reflect a role for Dbf4p during the late stages of mitosis. In support of this hypothesis, we have determined that Dbf4p is absent from cells during early G1, begins to accumulate in G1, persists through anaphase, and is degraded as cells finish mitosis (our unpublished data). Interestingly, we have found that like Cdc5p, the levels of Dbf4p are sensitive to mutations in a key APC component, CDC23 (our unpublished data). These results suggest that the APC-mediated removal of Dbf4p from cells late in mitosis may be important for the timing of the transition at origin complexes.

Additional factors required to complete mitosis. In addition to Cdc5p, there is a group of factors which are required to complete mitosis. These factors include the kinases Cdc15p (14) and Dbp2p (39), a Ras-like GTPase (Tem1p) (31), a nucleotide exchange factor (Lte1p), and a phosphatase (Cdc14p) (43). A number of genetic interactions between these genes have been reported, and they suggest a possible concerted role in initiation with the vertebrate homologue Nup155p and functional interactions in sister chromatid separation are needed for B-type cyclin proteolysis in budding yeast. Cell 81:269–277.


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REFERENCES


substrates of the anaphase promoting complex in *Saccharomyces cerevisiae*. EMBO J. 17:1336–1349.