Cdc5 Interacts with the Wee1 Kinase in Budding Yeast

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Development of a multicellular organism requires that mitosis and morphogenesis be coordinated. These processes must also be synchronized during the growth of unicellular organisms. In the yeast Saccharomyces cerevisiae, mitosis is dependent on the prior growth of a daughter cell in the form of a bud. Overexpression of wild-type Polo-like kinase Cdc5 or a catalytically inactive form resulted in the formation of multinucleate cells in budding yeast. Immunofluorescence analysis of these multinucleate cells showed that mitosis and bud formation were no longer linked. Others have shown that Swe1 is required for coupling mitosis to bud formation during a perturbed cell cycle. When the normal pathway of bud formation is perturbed, Swe1 functions to delay mitosis through negative regulation of Cdc5/Cdk. In cells lacking Swe1, multinucleate cells are formed in response to delays in bud formation. Affinity purification, two-hybrid analysis, and mutant characterization results suggested that Cdc5 and Swe1 interact. From these results, we conclude that multinucleate formation in response to Cdc5 overexpression is linked to titration of Swe1 function. These results also suggest that Cdc5 may be a negative regulator of Swe1.

The onset of mitosis in eukaryotic cells requires the activation of mitosis-promoting factor (MPF) (12). MPF is comprised of a cyclin-dependent kinase (Cdk) and B-type cyclin regulatory subunit (Clb/Cdk), and it is maintained in an inactive state during interphase through phosphorylation of Tyr15 on Cdk by the Wee1 kinase. MPF is activated in response to activation of the Tyr15 phosphatase Cdc25 and inactivation of the Tyr15 kinase Wee1 at the transition between G2 and mitosis (G2/M).

In addition to Clb/Cdk, Polo kinases are also key promoters of mitosis and have been implicated in coordinating the activation of Clb/Cdk. In vitro studies in Xenopus indicate that Cdc25 is activated when it binds to and is phosphorylated by the Polo kinase, Plk1 (23, 37). More recently, phosphorylation by the Polo kinase, Plx1, of a critical serine residue in the nuclear export signal sequence of cyclin B1 promotes accumulation of Clb/Cdk activity in the nucleus of vertebrate prophase cells (46). In Schizosaccharomyces pombe (3, 34), Drosophila (29), and vertebrate (15, 16) cells, Polo kinases have been localized to microtubule-organizing centers at the G2/M transition. Mutant studies in these systems show that Polo kinases play key roles in spindle pole duplication and formation of the bipolar spindle (15).

In Saccharomyces cerevisiae daughter cells originate as buds at the beginning of S phase (27). Bud formation is regulated by Cdc28, the yeast Cdk, in combination with the G1 (Cln) and mitotic (Clb) cyclins. The Cln-Cdc28 complex activates initiation of bud formation late in G1, while the Clb-Cdc28 complex inhibits re budding later in the cell cycle (2, 25, 26, 40). Perturbations of the actin cytoskeleton prevent the normal pathway of bud formation and result in delayed mitosis through negative regulation of Cdc5/Cdk by the Wee1 family kinase member Swe1 (33). In response to bud formation, Swe1 is negatively regulated by the Nim1-like kinase Hs1 (5, 24, 30, 31, 32). Swe1 is targeted to the bud neck after bud formation, and the neck localization requires Hs1 and its interacting factor, Hs17 (30, 42; S. H. Woo and C. F. J. Hardy, unpublished data). In this manner, morphogenesis is linked to cell proliferation in budding yeast.

Previous studies have shown that Swe1 overexpression prevents spindle pole body (SPB) separation but not duplication (28). This suggests that Swe1 plays a role at SPBs. Endogenous Cdc5 is also present at SPBs before they separate (Woo and Hardy, unpublished), consistent with it playing a role similar to other Polo kinases in regulating spindle pole separation (see above). In this study, we have found that overproduction of either wild-type Cdc5 or a catalytically inactive form uncouples mitosis from bud formation. This results in the formation of multinucleate cells. Cdc5 interacts with the N-terminal region of Swe1, and overproduction of the catalytically inactive form of Cdc5 suppresses Swe1-dependent phenotypes associated with unregulated Swe1. In response to Cdc5 overproduction, Swe1 is modified and localized to SPBs. Taken together, our results suggest that multinucleate formation in response to Cdc5 overexpression is linked to titration of Swe1 function. The results in this report also suggest that Cdc5 may be a negative regulator of Swe1, possibly playing a role in regulating Swe1 function at SPBs prior to SPB separation.

MATERIALS AND METHODS

Strains. Yeast strains and sources are listed in Table 1. Plasmid DNA was transformed into yeast by the lithium acetate method as described (19). The open reading frame of the endogenous SWE1 was disrupted with LEU2 by transforming strains with the integrating plasmid pSWE1-10g restricted with XhoI (a gift from R. Booher) (6). The open reading frame of the endogenous SWE1 was placed under control of the GAL1 promoter by transforming strains with the integrating plasmid pSWE1-41 restricted with PstI and BamHI (a gift from R. Booher) (6). Yeast strains were grown in YPD at 30°C unless noted otherwise in the text.
Tub1-GFP was induced by growing cells for 1 h in SC lacking methionine. Digital images were taken with a 100× objective on an Olympus microscope. For localization of hemagglutinin (HA)-tagged proteins, indirect immunofluorescence was carried out as described (Rothstein) for T. b. brucei. Two-hybrid analysis was performed as described by James et al. (20) by using strain PJ69-4a, with the following variation. Following transformation of the reporter strain with the yeast library plasmids, the cells were washed and then resuspended into liquid synthetic complete medium (SC) lacking the amino acids histidine, uracil, and leucine but containing adenine; 200 μl of this solution was spread on 100-mm agar plates containing SC lacking histidine, uracil, leucine, and adenine. The extra initial adenine was essential to obtain transformants on the selective plates.

**Immunofluorescence.** Indirect immunofluorescence was carried out as described (49). Yeast cells were grown to early log phase and prepared for immunofluorescence microscopy. For localization of hemagglutinin (HA)-tagged Cdc5, cells were fixed for 5 min. The cells were first incubated with affinity-purified anti-HA antibody (BABCo) and then with rhodamine-conjugated donkey anti-mouse secondary antibody. For localization of Tub1, the cells were fixed for 30 min. The cells were first incubated with mouse anti-Tub1 and then with rhodamine-conjugated donkey anti-mouse secondary antibody. Green fluorescent protein (GFP)-TUB1 fusions were obtained by transforming strain CH696 (cdh1::hct1 swa1::URA3 swe1::LEU2) (SWE1 disrupted with pSWE1-10g, from R. Rothstein). The resulting GFP-TUB1 plasmids were transformed into W303-1A to produce elongated buds in a SWE1-dependent manner. We determined that buds in hsl1 GAL-SWE1-GFP and hsl1 SWE1-ProA strains are elongated and therefore the SWE1-ProA and SWE1-GFP constructs are functional.

**Purification of GST and GST-Swe1 from Escherichia coli.** E. coli BL21 cells were transformed with either pGEX-5X or pGEX-5X-Swe1 and induced with 0.4 mM isopropyl-β-D-thiogalactoside for 4 h at 28°C. Bacteria were pelleted and then suspended in NETN buffer (0.5% NP-40, 20 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1 mM EDTA) supplemented with protease inhibitors (Boehringer Mannheim) and were lysed with sonication. The lysate was clarified by centrifugation, and the clarified lysate was incubated with glutathione-agarose beads (Pharmacia) for 1 h at 4°C. Beads were pelleted and then washed extensively with NETN buffer with protease inhibitors, and bound proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by immunoblotting with anti-glutathione S-transferase (GST) antibodies.
nymethylsulfonyl fluoride 5 mg of leupeptin per ml, 2 mM pepstatin A, 50 mM NaF, 10 mM sodium pyrophosphate, and 0.5 mM NaVO4. Cells were lysed by adding 0.5 ml of acid-washed glass beads and vortexing in pulses until 90% lysis was achieved; 0.35 ml of immunoprecipitation buffer (1 M LiCl, 2% Triton X-100, 10% glycerol, 0.5 mM NaVO4, protease inhibitors as described for L buffer) was added, and the mixture was vortexed 1 min. The lysate was spun for 10 min at 3,000 rpm, and the supernatant was aliquoted and frozen in liquid nitrogen. Protein concentrations were determined with the Bio-Rad protein assay. Four hundred milligrams of lysate was incubated with 0.1 ml of GST and GST-Swe1 beads prepared as described above. After 2 ho f incubation, beads were washed extensively with L buffer, and the bound proteins were resolved by SDS-PAGE and analyzed by immunoblotting.

RESULTS

Cells overproducing Cdc5 or cdc5N209A are multinucleate. HeLa cells overproducing wild-type or catalytically inactive Plk1 are multinucleate (37). In an effort to further understand these results, we examined whether budding yeast became multinucleate in response to overproduction of Cdc5. To conduct the experiment, a strain expressing wild-type CDC5 from the inducible GAL1 promoter, GAL1-CDC5, was analyzed. The 4',6-diamidino-2-phenylindole (DAPI)-stained nuclei in GAL1-CDC5 cells were counted after inducing CDC5 expression for 4 h through addition of galactose. Strikingly, 2.5% of the cells were multinucleate (Fig. 1A; Table 2). In addition, 0.9% of cells overexpressing a mutant version of CDC5 that lacks kinase activity (cdc5-N209A) were multinucleate (Fig. 1B). To further confirm these results, we tested the effects of adding galactose to a strain overexpressing CDC5 in the presence of the cdc5-1 mutation, which encodes a temperature-sensitive CDC5 allele (cdc5-1) (12). In this case, 3% of the cells were multinucleate (Table 2). Collectively, these results indicate that CDC5 overexpression leads to cellular multinucleation in yeast cells. To rule out the possibility that the enhanced nuclear number is due to DNA loss, we also examined the DNA content of these cells. Cells that were multinucleate and grown in the presence of galactose had a DNA content equivalent to two nuclei/mother cell (Table 2). Therefore, cells that have been multinucleated in response to CDC5 overexpression are not haploid. This finding is consistent with previous observations that cells that have undergone nuclear fusion are diploid (37).

FIG. 1. Cells overexpressing CDC5 or cdc5N209A are multinucleate. To induce Cdc5 (A) or cdc5-N209A (B), 2% galactose was added to mid-logarithmic-phase cultures of strains JC256 (GAL-CDC5) and C1039 (GAL-cdc5N209A GFP-TUB1) for the indicated times. To induce GFP-Tub1 expression, the same cells were then resuspended in SC-methionine plus galactose for 2 h. The cells were fixed and stained with DAPI to visualize nuclei. It should be noted that the frequency of multinucleate cells as visualized here does not reflect the true distribution in these cultures. Rather, these particular fields were chosen in order to demonstrate the variety of multinucleate cells in the culture. Bars, 10 μm.

### TABLE 2. Multinucleate formation in CDC5 and cdc5-N209A overexpression strains

| Strain                                      | Distribution (%) |
|---------------------------------------------|------------------|
| WT + Gal                                    | 99.94 0.06       |
| GAL-CDC5 + Gal                             | 99.7 0.3         |
| GAL-CDC5 + Gal (4)                         | 97.5 2.5         |
| GAL-cdc5-N209A + Gal                       | 99.9 0.1         |
| GAL-cdc5-N209A + Gal (4)                   | 99.1 0.9         |
| cdh1/htc1 + Gal                            | 98.2 1.8         |
| cdh1/htc1 + Gal (4)                        | 98.8 1.2         |
| GAL-CDC5 cdh1/htc1 + Gal (0)               | 98.9 1.1         |
| GAL-CDC5 cdh1/htc1 + Gal (4)               | 78.6 21.4        |
| GAL-cdc5-N209A cdh1/htc1 + Gal             | 99.0 1.0         |
| GAL-cdc5-N209A cdh1/htc1 + Gal (4)         | 85.0 15.0        |

* The number of nuclei per mother cell in haploid cells was determined by DAPI staining of asynchronous cultures at 30°C in mid-log phase. Greater than 1,000 cells were counted for each strain. All strains used were derivatives of W303 (wild type [WT]). All cells were grown in rich medium containing raffinose; where indicated, galactose was added for the time periods (hours) shown in parentheses.
Swel does not play a role during an unperturbed cell cycle (6). In agreement with these results, the frequency of multinucleate *swel* cells was very similar to that observed in wild-type cells (Table 3). However, altering the levels of Cib/Cdk affected the accumulation of multinucleate *swel* cells. Examination of *cdh1/hct1 swe1* double-mutant cells by DAPI staining revealed greater than 17% with multiple nuclei (Table 3). A representative image is shown in Fig. 3A. In contrast, *swel* and *cdh1/hct1* single-mutant cultures were only 0.06 and 1.8% multinucleate, respectively (Table 3). Interestingly, a genetic interaction between *CDH1/HCT1* and *SWE1* was established based on the slower growth of the *cdh1/hct1 swe1* double mutant compared to either single mutant at 37°C (Fig. 3B). Furthermore, overexpression of the mitotic cyclin Clb2 (*GAL-CLB2*) in *swel* null cells resulted in 10.8% multinucleate cells, compared to only 0.08% multinucleate *GAL-CLB2 SWE1* cells (Table 3). The overexpression of the Cdc/Cdc28 inhibitor Sic1 (2μm-*SIC1 cdh1/hct1 swe1*) or deletion of *CLB2* (*cdh1/hct1 swe1 clb2*) partially suppressed the *cdh1/hct1 swe1* multinucleate phenotype (Table 3).

To test whether mitosis was unlinked from bud formation in *cdh1/hct1 swe1* cells, spindle elongation was observed. In *cdh1/hct1 swe1* GFP-TUB1 cells, we found unbudded cells with nuclei positioned at the ends of an elongated spindle (Fig. 3A, arrow i) and small-budded cells with nuclei at the end of a disassembling spindle (Fig. 3A, arrow ii). Large-budded cells with an elongated spindle undergoing a normal mitosis were also present (Fig. 3A, arrow iii). These results were similar to the phenotype for cells overexpressing *cdc5N209A* or Cdc5. Live-cell video microscopy was used to monitor spindle formation in *cdh1/hct1 swe1* GFP-TUB1 cells. In Fig. 3C, an unbudded cell undergoing spindle elongation (interval from 34 to 68 min) and disassembly (interval from 68 to 74 min) is shown. This cell completed mitosis, as indicated by spindle breakdown at the 74-min time point, and formed a bud at the 100-min mark (data not shown). In contrast, in most cells that had formed a bud before elongating their spindle, the spindle was properly oriented through the neck into the bud (Fig. 3C, cell above and cell below the center cell in the cartoon). We did observe large-budded cells with misoriented spindles. However, these were not found in wild-type or *swe1* cells.

### Table 3. Multinucleate formation in *swel* mutant strains

| Strain | Distribution (%) | 1 nucleus per mother | 2 nuclei per mother |
|--------|------------------|----------------------|---------------------|
| Wild type | 99.94 | 0.06 |
| *swel* | 99.94 | 0.06 |
| *cdh1/hct1* | 98.2 | 1.8 |
| *cdh1/hct1 swe1* | 82.8 | 17.2 |
| *cdh1/hct1 swe1 clb2* | 96.5 | 3.5 |
| *cdh1/hct1 swe1 2μm* | 84.7 | 15.3 |
| *cdh1/hct1 swe1 2μm-SIC1* | 95.7 | 4.3 |
| *GAL-CLB2 SWE1 + Gal (0)* | 99.93 | 0.07 |
| *GAL-CLB2 SWE1 + Gal (4)* | 99.92 | 0.08 |
| *GAL-CLB2 swe1 + Gal (0)* | 99.94 | 0.06 |
| *GAL-CLB2 swe1 + Gal (4)* | 89.2 | 10.8 |

*See Table 2 for full description. Cells were grown in rich medium with two exceptions. Strains transformed with the 2μm or 2μm-SIC1 plasmids were grown in synthetic medium lacking the amino acid tryptophan. The GAL-CLB2 strains were grown in rich medium containing rafinose; where indicated, galactose was added for the time periods (hours) shown (in parentheses). The *swel, cdh1/hct1,* and *clb2* mutations represent deletions of their respective open reading frames.*
ever, in these rare cases, both the mother and daughter cells were undergoing mitosis and each had a separate spindle (Fig. 3A, inset).

cdc5N209A overproduction suppresses Swel-dependent phenotypes. To test whether cdc5N209A overexpression inactivates Swel function, we investigated whether overexpression of cdc5N209A in hsl1 or hsl7 mutant cells would suppress the Swel-dependent defects of these mutants. The hsl1 and hsl7 mutants undergo a prolonged period of apical bud growth during G2 and have Swel-dependent elongated buds (31). We found that overexpression of cdc5-N209A suppressed the Swel-dependent elongated bud formation in hsl1 (Fig. 4A) and hsl7 cells. The hsl1 cells overexpressing cdc5-N209A were distinctly round. When the hsl1 and hsl7 cells were examined for DNA content by fluorescence-activated cell sorting (FACS), less than 10% of the hsl1 (Fig. 4A) and hsl7 cells were 1N. These results confirmed a G2 delay for hsl1 and hsl7 cells, as has been previously reported (31). However, a significant fraction of the hsl1 and hsl7 cells overexpressing cdc5-N209A were 1N (Fig. 4A, 3 h after the addition of galactose). The FACS profiles for the hsl1 cells overexpressing cdc5-N209A and the hsl1 swel mutant cells were very similar (Fig. 4A). We observed that

FIG. 3. Mitosis is unlinked from bud formation in cdh1/hct1 swel cells. (A) Visualization of elongated and disassembled spindles in unbudded and small-budded multinucleate cdh1/hct1 swel cells. Cultures of C968 (cdh1/hct1 swel MET3-GFP-TUB1) were grown to mid-log phase in YPD and transferred to SC lacking methionine for 1 h to induce GFP-Tub1. Arrows mark a multinucleate unbudded cell with an elongated spindle (i), a multinucleate small-budded cell with a disassembled spindle (ii), and a cell undergoing a normal mitosis (iii). (Inset) A rare cell type (0.1% of cdh1/hct1 swel GFP-TUB1 cells) in which the mother and its attached daughter are both undergoing mitosis. (B) The cdh1/hct1 swel double mutant grows slower than the single mutants at 37°C. Strains C895 (cdh1/hct1 swel), C798 (swel), C810 (cdh1/hct1), and W303a (wild type [WT]) were streaked on YPD plates and incubated at 30 or 37°C for 5 days. (C) Video sequence of spindle elongation and disassembly during mitosis in strain C968 (cdh1/hct1 swel MET3-GFP-TUB1). Cultures of C968 were grown to mid-log phase in YPD and transferred to SC lacking methionine for 2 h to induce GFP-Tub1. Following induction, they were placed on a slide with a thin agarose pad. A Z series of eight focal planes was collected over 8 s and projected onto a single two-dimensional image. Z series were collected every 2 min for 3 h. An unbudded cell undergoing spindle elongation (interval from 34 to 68 min) and disassembly (interval from 68 to 74 min) is shown (middle cell in the cartoon). The cell above and the cell below the middle cell in the cartoon elongated and aligned their spindles through the bud neck in a wild-type manner. Bars: (A) 10 μm; (C) 2.5 μm.
cdc5-N209A overexpression suppressed the lethality and the formation of elongated buds in response to Swe1 overproduction (Fig. 4B). Taken together, these results supported our model that cdc5-N209A was acting as an inhibitor of Swe1 function. As further evidence, we also found that the extreme slow growth or lethality of cdc5(msd2-1) at 30°C was suppressed in a cdc5(msd2-1) swe1 double mutant (Fig. 4C).

Swe1 is modified and localized to SPBs in response to Cdc5 or cdc5N209A overproduction. We found that Swe1 was localized to the mother bud neck in a strain expressing Swe1-GFP from the endogenous Swe1 promoter (SWE1-GFP) (Woo and Hardy, unpublished) (Fig. 5A). In contrast, overexpression of Swe1-GFP from the GAL1 promoter (GAL1-SWE1-GFP) resulted in Swe1-GFP localization in the nucleus, at SPBs as well as to the bud neck (Woo and Hardy, unpublished). Interestingly, we observed that endogenous Swe1-GFP localized to one or two bright spots in response to overproduction of Cdc5 (GAL1-CDC5-HA SWE1-GFP) or cdc5-N209A (GAL1-cdc5N209A-HA SWE1-GFP) (Fig. 5). In addition, Swe1-GFP signal was not observed at the neck in cells overexpressing CDC5 or cdc5-N209A. DAPI staining revealed that these spots were on the nuclear periphery of cells (data not shown).

The colocalization of Swe1 and Cdc5 in perinuclear spots suggested that this locale might correspond to SPBs. Cdc5 is present at SPBs when expressed under control of its endogenous promoter (41; Woo and Hardy, unpublished) or when overexpressed from the GAL1 promoter (43). Indirect immunofluorescence staining showed that the Swe1-GFP spots colocalized with cdc5-N209A-HA- (Fig. 6A) or Cdc5 (data not shown) in response to overproduction of cdc5-N209A or Cdc5, respectively. Combined with anti-Tub1 indirect immunofluorescence microscopy, we determined that Swe1-GFP colocalized to SPBs in response to cdc5N209A overproduction (Fig. 6B). Swe1-GFP also colocalized with SPBs in response to Cdc5 overproduction (data not shown).

To examine whether the level or state of Swe1 was altered in cells overexpressing Cdc5 or cdc5N209A, cell extracts were analyzed by immunoblotting. In these experiments, Swe1 was epitope tagged by a chromosomal in-frame integration of the immunoglobulin G binding domain of ProA at the sequence for the Swe1 C terminus. Overexpression of Cdc5 or cdc5N209A resulted in slower electrophoretic mobility for Swe1-ProA (Fig. 7A). The effect was less pronounced in cdc5N209A cells. Because localization of Cdc5 and Swe1 to the mother bud neck is dependent on Hsl1 and Hsl7, Hsl1 and Hsl7 might serve as potential adapters between Cdc5 and Swe1. Interestingly, Wee1 plays a similar role in S. pombe, where it functions to prevent premature mitosis in cells lacking Ste9, the homolog of Cdh1/Hct1 (21). The comparable responses of cdc5N209A cells to the deletion of SWE1 or the overexpression of Cdc5 suggest that Cdc5 overexpression is titrating Swe1 function in the cdc5N209A cells. In agreement with this proposal, Swe1-dependent phenotypes in hsl1 and hsl7 mutant cells are suppressed by overexpression of wild-type Cdc5 or a catalytically inactive form (Fig. 4). Because Cdc5 overexpression by itself triggers the formation of multinucleate cells whereas deletion of Swe1 does not, Cdc5 overproduction most likely affects the function of additional factors. It is also possible that high levels of Cdc5 prevent down-regulation of mitotic Cdk shift, and presumed modification of Swe1-ProA in response to Cdc5 or cdc5N209A overproduction was not dependent on Hsl1 or Hsl7 (Fig. 7B). Furthermore, the localization of Swe1 to SPBs in response to Cdc5 overproduction was also not dependent on Hsl1 or Hsl7 (data not shown).

**DISCUSSION**

Multinucleate formation in response to Cdc5 overexpression is linked to Swe1. In budding yeast, the Cln/Cdk inhibitor Swe1 does not play a role in mitotic progression in an unperturbed cell cycle and is not required to link bud formation and mitosis (6). In contrast, Swe1 is part of the morphogenesis checkpoint and, in response to perturbations that prevent bud formation, inhibits mitotic progression through negative regulation of Cln/Cdk (24). The results in this paper suggest that the Polo kinase Cdc5 functionally interacts with Swe1. This conclusion is based on multiple pieces of evidence. Most interestingly, a population of cells overproducing Cdc5 has an increased, although low (2.5%), frequency of multinucleate cells. Mitosis appears to be unlinked from bud formation in response to Cdc5 overproduction (Fig. 1). Bud formation requires the action of G1 or Cln cyclins and is inhibited by the mitotic cyclins Cbl1 and Cbl2, in combination with Cdk (2, 25, 26, 40). Mutants lacking the APC cofactor Cdh1/Hct1 accumulate Cbl1/Cdk and Cbl2/Cdk activity in G1 (39, 47). We find that a population of cdh1/hct1 null cells has a low frequency of multinucleate cells (1.8%). Strikingly, Cdc5 overproduction or deletion of SWE1 in combination with a deletion in CDH1/HCT1 results in the formation of multinucleate cells at a dramatically higher frequency (18 and 20%, respectively). The cdh1/hct1 swe1 cells undergo mitosis in the absence of bud formation. Therefore, Swe1 prevents the mitotic Cdk from triggering mitotic progression in the absence of bud formation.

Interestingly, Wee1 plays a similar role in S. pombe, where it functions to prevent premature mitosis in cells lacking Ste9, the homolog of Cdh1/Hct1 (21). The comparable responses of cdh1/hct1 cells to the deletion of SWE1 or the overexpression of Cdc5 suggest that Cdc5 overexpression is titrating Swe1 function in the cdh1/hct1 cells. In agreement with this proposal, Swe1-dependent phenotypes in hsl1 and hsl7 mutant cells are suppressed by overexpression of wild-type Cdc5 or a catalytically inactive form (Fig. 4). Because Cdc5 overproduction by itself triggers the formation of multinucleate cells whereas deletion of Swe1 does not, Cdc5 overproduction most likely affects the function of additional factors. It is also possible that high levels of Cdc5 prevent down-regulation of mitotic Cdk synthesis.
FIG. 5. Swe1 is found at one or two distinct spots in cells overexpressing CDC5 or cdc5N209A. (A) Strain C573 (GAL-CDC5-HA SWE1-GFP) was grown to mid-logarithmic phase in raffinose. Galactose was added to 2% for 2 h. (B) Strain C572 (GAL-cdc5-N209A-HA SWE1-GFP) was grown to mid-logarithmic phase in raffinose. Galactose was added to 2% for 2 h. Bars: 10 μm.
by means other than down-regulation of Swe1 activity. It will be interesting to determine whether the interaction between Wee1 and Polo kinases is conserved in other systems. Intriguingly, overexpression of the wild-type or catalytically inactive form of the mammalian Polo kinase Plk1 in HeLa cells results in the formation of multinucleate cells (35).

Polo is a potential negative regulator of Wee1. In fission yeast, Wee1 is negatively regulated by the Nim1 kinase (9, 12, 36, 38, 51). The C-terminal region of Wee1 that forms the kinase domain is phosphorylated by Nim1 in vitro, resulting in complete inactivation of the Wee1 kinase activity (9, 36, 51). However, regulation of Wee1 most likely involves additional factors, as fission yeast cells lacking Nim1 activity are only slightly delayed in the G2 phase (38). A kinase activity in mitotic Xenopus extracts produces a 60-kDa change in the apparent molecular mass of recombinant fission yeast Wee1 (44). This hyperphosphorylated form of Wee1 is defective for phosphorylation of Clb/Cdk. In contrast to Nim1, the phosphorylation catalyzed by the mitotic Xenopus extracts is restricted to the N-terminal portion of Wee1 (44).

In budding yeast, Swe1 is negatively regulated by the Nim1-like kinase Hsl1, and there is a decrease in the level of Swe1 phosphorylated forms in hsl1 mutant cells (42). It is not known what region of Swe1 is targeted for phosphorylation by Hsl1. Interestingly, we find that Cdc5 interacts with the N-terminal region of Swe1 (Fig. 2). In addition, in cells overproducing Cdc5 or cdc5N209A, Swe1 is converted to a form with an apparent molecular mass increase of 60 or 30 kDa, respectively (Fig. 7). We have not been able to immunoprecipitate the modified forms of Swe1 to determine whether the changes in mobility are due to direct phosphorylation. The largest Swe1 mobility shift to the slowest-migrating form requires Cdc5 kinase activity. However, a shift is observed in cells overexpressing the catalytically inactive cdc5N209A. As Swe1 is targeted to SPBs in response to Cdc5 or cdc5N209A overproduction, other kinases associated with SPBs may be responsible for modifying Swe1. These include both Dbf2 and Cdc15 (14, 52); however, interactions between Swe1 and either Dbf2 or Cdc15 have not been reported.

The inhibitory phosphorylation of the N terminus of Wee1 by the kinase activity in Xenopus mitotic extracts requires the addition of the phosphatase inhibitor okadaic acid (44). Okadaic acid is specific for protein phosphatase 2A (PP2A)-like phosphatases. Interestingly, purification from recombinant baculovirus systems of active Cdc5 and of Plx1 (the Xenopus Polo kinase) requires the addition of okadaic acid (23; Woo and Hardy, unpublished). This correlates with observations that Polo kinases including Cdc5 are activated by phosphorylation (8, 22, 37, 45). Taken together, these results suggest that Plx maybe the N-terminal inhibitory kinase for Wee1. Intriguingly, budding yeast cells that lack the PP2A regulatory subunit Cdc55 are defective in degradation of Swe1 (53). We speculate
that cdc5 mutant cells maybe defective in Swe1 degradation because misregulated PP2A targets Cdc5 for inactivation. Alternatively, the misregulated PP2A may target Swe1 or Hsl1.

Possible role for Cdc5 at SPBs. Mutant analyses in Drosophila, S. pombe, and mammals indicate that Polo kinases play a key role in the formation of bipolar spindles (15). Does Cdc5 play a role in spindle formation? The characterized mutant cdc5 alleles have no reported defects in bipolar spindle formation. However, the cdc5-1 mutant has been linked to microtubule destruction. After release of the cdc5-1 mutant from a temperature-induced block, the cdc5-1 cells become insensitive to the microtubule-depolymerizing drug methylbenzimidazole-2-yl carbamate (50). In budding yeast, formation of a short bipolar spindle takes place in S phase, and this step is inhibited by overexpression of Swe1 (28). Swe1 expressed from its endogenous promoter is localized to the mother bud neck (30; Woo and Hardy, unpublished). However, when overproduced, Swe1 is also found in the nucleus and at SPBs (Woo and Hardy, unpublished). In this report, we show that Swe1 from its endogenous promoter is targeted to SPBs in response to Cdc5 or cdc5N209A overproduction (Fig. 6). Cdc5 is localized to SPBs just prior to SPB separation, positioning it for a role in Swe1 regulation (Woo and Hardy, unpublished).

In mammalian cells, Wee1 has been localized to the nucleus but has not been found at the microtubule-organizing centers or centrosomes (4, 17). In addition, Wee1 has not been reported to interact with Polo kinases in other systems. However, it is interesting that in fission yeast cells with a mutation in the SPB component Stfl/Cut12, the requirement for Cdc25 is bypassed and Plo1, the Polo kinase in S. pombe, is prematurely recruited to the SPB (7). Cdc25 is a tyrosine phosphatase that opposes Wee1 function and activates Cdc2. The stfl/cut12 mutant cells may bypass Cdc25 by allowing Plo1 to prematurely access and negatively regulate Wee1.

Polo interacts with multiple regulators of mitotic Cdk activity. Plk1, the Polo kinase in Xenopus, was isolated as a Cdc25-interacting factor, and studies have suggested that Polo activates Cdc2 by phosphorylating and activating Cdc25 (23, 37). Recently it has been shown that the Polo kinase Plk1 in vertebrate cells regulates the nuclear localization of cyclin B1 (46). Plk1 phosphorylates an essential serine residue in the nuclear export signal sequence of cyclin B1 allowing the Cdc2-cyclin B1 complex to accumulate in the nucleus during prophase. Our work shows that Cdc5, the Polo kinase in budding yeast, interacts with Swe1. Further work will be required to determine the function of such a Polo-Wee1 interaction. However, combined with these earlier results, our results lead us to conclude that Polo kinase is a key coordinator for activation of Cdc2 during G2/M.

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