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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 multinulceate 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 Clb/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 G_2 and mitosis (G_2/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, Plx1 (23, 37). More recently, phosphorylation by the Polo kinase, Plk1, 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 G_2/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 G_1 (Cln) and mitotic (Clb) cyclins. The Cln-Cdc28 complex activates initiation of bud formation late in G_1, while the Clb-Cdc28 complex inhibits rebudding 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 Clb/Cdk by the Wee1 family kinase member Swe1 (33). In response to bud formation, Swe1 is negatively regulated by the Nim1-like kinase Hsl1 (5, 24, 30, 31, 32). Swe1 is targeted to the bud neck after bud formation, and the neck localization requires Hsl1 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 *Xba*I (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 *Pst*I and *Bam*HI (a gift from R. Booher) (6). Yeast strains were grown in YPD at 30°C unless noted otherwise in the text.

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TABLE 1. Strains used

Strain	Genotype (source)
W303-1A	*MAT***a** *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100* (R. Rothstein)
W303-1B	*MATα ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100* (R. Rothstein)
AA1120	W303-1A *cdh1/hct1*::*HIS3 PDS1-HA-LEU2*::*pds1* (A. Amon)
IAY18	*MAT***a** *spc42Δ1*::*LEU2 TRP1*::*SPC42-GFP* (J. Kilmartin)
JC1195	*MATα leu2 ura3 his3-Δ200* (J. Cooper)
JC256	W303-1A *ura3*::*(GAL-CDC5-HA-URA3)*$_3$ (J. Charles)
JC278	W303-1A *ura3*::*(GAL-cdc5-N209A-HA-URA3)*$_3$ (J. Charles)
K3371	W303-A *clb2*::*LEU2 trp1*::*GAL-CLB2-TRP1* (Kim Naysmith)
K6451	W303-1A *cdc5-3(msd2-1*::*URA3) omns PDS1*::*LEU2* (K. Nasmyth)
MAY1	W303-1A *hsl1Δ1*::*URA3* (M. Grunstein)
MAY2	W303-1A *hsl1Δ1*::*URA3 swe1*::*LEU2* (M. Grunstein)
MAY3	W303-1A *hsl7Δ1*::*URA3* (M. Grunstein)
MAY4	W303-1A *hsl7Δ1*::*URA3 swe1*::*LEU2* (M. Grunstein)
NEY100X	*MATα hsl1*::*LEU2 ura3 leu2*
CH179	*MATα ura3*::*GFP-TUB1-URA3 his3 leu2*
CH199	W303-1A *bar1*::*URA3 CDC5-ProA-HIS3-URA3*
CH452	*MATα SWE1-GFP-HIS3 leu2 ura3 his3-Δ200* (GFP-tagged *SWE1* in JC1195)
CH459	*MAT***a** *bar1 ura3*::*(GAL-cdc5-N209A-HA-URA3)*$_3$ *SWE1-ProA-URA3-HIS3* (ProA tag of *SWE1* in JC278)
CH473	*MAT***a** *ura3*::*(GAL-CDC5-HA-URA3)*$_3$ *SWE1-ProA-URA3-HIS3* (ProA tag of *SWE1* in JC256)
CH474	W303-1A *swe1*::*GAL-SWE1-HA-LEU2 (GAL-SWE1-HA* integrated at the *SWE1* locus in W303-1A using pSWE1-41, from D. Lew)
CH499	W303-1A *ura3*::*(GAL-cdc5-N209A-HA-URA3)*$_3$ *swe1*::*(GAL-SWE1-HA-LEU2)* (*GAL-SWE1-HA* integrated at the *SWE1* locus in YJC278 using pSWE1-41, from R. Booher)
CH507	*MATα ura3*::*(GAL-CDC5-HA-URA3)*$_3$ *hsl1*::*LEU2* (segregant of JC256 × NEY100X)
CH508	*MAT***a** *ura3*::*(GAL-cdc5-N209A-HA-URA3)*$_3$ *hsl1*::*LEU2 ADE2 his3* (segregant of NEY 100X × JC278)
CH572	*MAT***a** *ura3*::*(GAL-cdc5-N209A-HA-URA3)*$_3$ *SWE1-GFP-HIS3 his3 leu2 ura3* (segregant of JC278 × CH452)
CH573	*MAT***a** *ura3*::*(GAL-CDC5-HA-URA3)*$_3$ *SWE1-GFP-HIS3 his3 leu2 ura3* (segregant of JC256 × CH452)
CH618	*MAT***a** *ura3*::*(GAL-cdc5-N209A-HA-URA3)*$_3$ *hsl7Δ1*::*URA3 his3 leu2 ura3 trp1-1* (segregant of CH696 × MAY3)
CH667	W303-1B *cdc5-3(msd2-1*::*URA3) swe1*::*LEU2* (SWE1 disrupted with pSWE1-10g, from R. Booher)
CH696	W303-1B *ura3*::*(GAL-cdc5-N209A-HA-URA3)*$_3$ (segregant of JC278 × W303-1B)
CH745	W303-1B *cdc5-3(msd2-1*::*URA3)* (segregant of KN6451 × W303-1α)
CH798	W303-1A *swe1*::*LEU2* (segregant of MAY4 × W303-1B)
CH810	W303-1B *cdh1(hct1)*::*HIS3* (segregant of AM1120 × W303-1B)
CH895	W303-1A *cdh1(hct1)*::*HIS3 swe1*::*LEU2* (segregant of CH798 × CH810)
CH896	W303-1B *cdh1(hct1)*::*HIS3 swe1*::*LEU2* (segregant of CH798 × CH810)
CH943	*MATα swe1*::*LEU2 leu2 ura3 his3-Δ200* (segregant of JC1195 × CH798)
CH964	W303-1A *cdh1(hct1)*::*HIS3 swe1*::*LEU2 pRS424* (CH895 transformed with *pRS424*)
CH965	W303-1A *cdh1/hct1*::*HIS3 swe1*::*LEU2 pRS424-SIC1* (CH895 transformed with *pRS424-SIC1*)
CH968	W303-1A *cdh1/hct1*::*HIS3 swe1:LEU2 ura3*::*GFP-TUB1-URA3* (GFP-tagged *TUB1* using pAFS92, from A. Straight, in CH895)
CH1039	*MAT***a**/α *ura3*::*GFP-TUB1-URA3/ura3*::*(GAL-cdc5-N209A-HA-URA3)*$_3$ (JC278 × CH179)
CH1173	W303-1A *cdh1/hct1*::*HIS3 swe1*::*LEU2 clb2*::*LEU2* (segregant of CH896 × K3371)
CH1175	*MAT***a** *swe1*::*LEU2 GAL-CLB2*::*TRP1* (segregant of CH943 × K3371)
CH1201	*MAT***a** *spc42Δ1*::*LEU2 TRP1*::*SPC42-GFP ura3*::*(GAL-cdc5-N209A-HA-URA3)*$_3$ (segregant of CH696 × IAY18)
CH1221	W303-1A *ura3*::*(GAL-cdc5-N209A-HA-URA3)*$_3$ *cdh1/hct1*::*HIS3* (segregant of JC278 × CH810)
CH1224	W303-1A *ura3*::*3(GAL-CDC5-HA-URA3)*$_3$ *cdh1/hct1*::*HIS3* (segregant of JC256 × CH810)

Two-hybrid analysis. 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, cell 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 gene (*GFP*)-*TUB1* fusions were obtained by transforming strain C895 (*cdh1/hct1 swe1*) with pAFS92, which integrates, at the *ura3* locus, a *GFP* fusion to the α-tubulin gene, *TUB1*, under control of the *MET3* promoter. 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.

Generation of fusion proteins in yeast. PCR was carried out to generate Swe1-protein A (ProA) and Swe1-GFP fusion sequences. The oligonucleotides are available on request. The *ProA* gene and adjacent *HIS3* and *URA3* markers were amplified by PCR using pProA-HIS3-URA3 (a gift from Mike Rout and John Aitchison) (1). *GFP* and the adjacent *HIS3* marker were amplified by PCR using pYGFP3 (a gift from Brendan Cormack) (10). The codons in the mutant *GFP* (pYGFP3) were optimized for expression in *Candida*. The resulting PCR constructs contained *SWE1* sequences fused in frame with sequence encoding GFP or ProA at their C-terminal ends. Strains were transformed with the resulting PCR products to generate a yeast strain expressing the desired fusion protein under its endogenous promoter. A strain expressing Spc42-GFP was previously obtained from J. Kilmartin (11). Strains lacking *HSL1* produce elongated buds in a *SWE1*-dependent manner. We determined that buds in *hsl1 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-thiogalactopyranoside 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], 100 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.

Affinity chromatography. Cells containing Cdc5 fused to ProA derived from strain C199 (*CDC5-ProA*) were pelleted, washed once in water, and lysed or frozen in liquid nitrogen. Pellets were resuspended in 0.3 ml of lysis buffer (L buffer) containing 5% glycerol, 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 10 mM $MgCl_2$, 0.3 M $N_2H_8SO_4$, 1 mM dithiotheitol, 1 mM benzamidine, 1 mM phe-

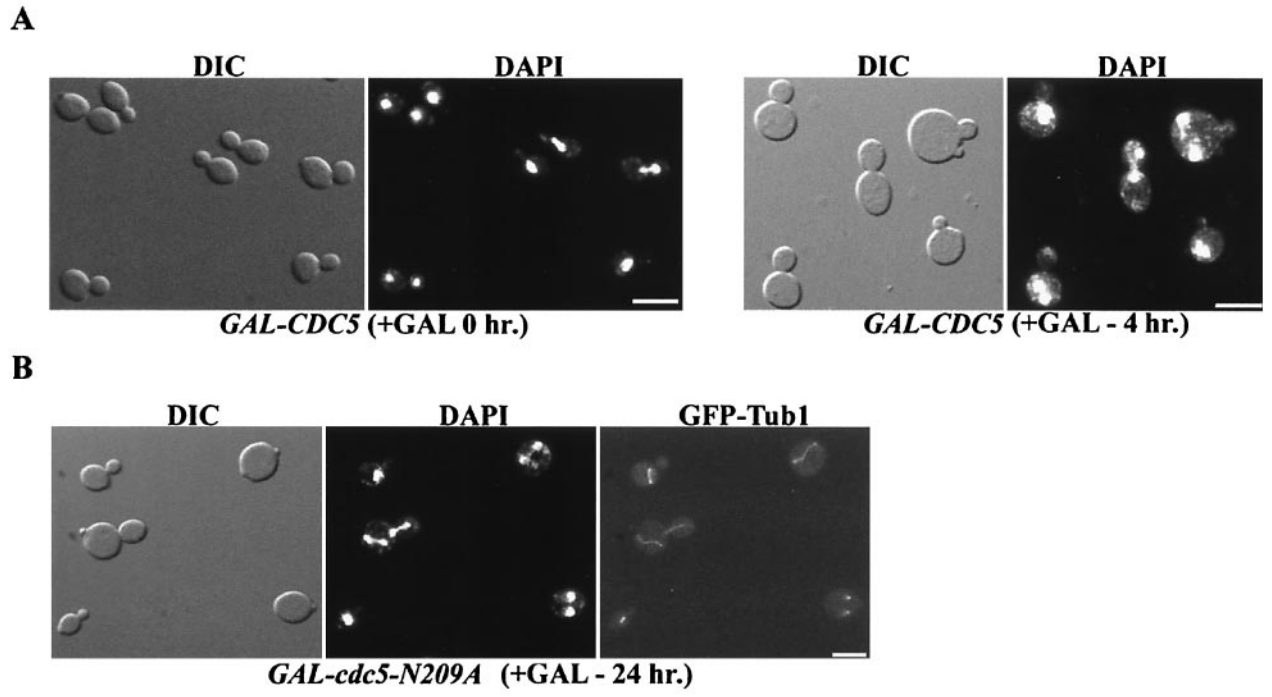


FIG. 1. Cells overexpressing *CDC5* or *cdc5N209A* are multinucleate. To induce Cdc5 (A) or cdc5-N209A (B), 2% galactose was added to mid-logarithmic-phase cultures of strains JC256 (*GAL-CDC5*) and C1039 (*GAL-cdc5N209A GFP-TUB1*) for the indicated times. To induce GFP-Tub1 expression, the same cells 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.

nylmethylsulfonyl fluoride 5 mg of leupeptin per ml, 2 mM pepstatin A, 50 mM NaF, 10 mM sodium pyrophosphate, and 0.5 mM $NaVO_4$. 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 $NaVO_4$, 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 h of incubation, beads were washed extensively with L buffer, and the bound proteins were resolved by SDS-PAGE and analyzed by immunoblotting.

Other methods. YEP medium contained 1% yeast extract and 2% Bacto Peptone. Carbon sources (glucose, raffinose, and galactose) were all used at a 2% final concentration. α-Factor and hydroxyurea 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-mg/ml stock solution in dimethyl sulfoxide. It was used at a final concentration of 20 μg/ml in 1% dimethyl sulfoxide as described by Jacobs et al. (19). The DNA content of cells was measured on a Becton Dickinson (San Jose, Calif.) FACSsan as described by Epstein and Cross (13). Movies were collected as described elsewhere (48). Briefly, mid-logarithmic-phase cells 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 5 min for 0.5 to 3 h. NIH image 1.62 (written by Wayne Rasband) was used for image acquisition. Cells were also visualized by differential interference contrast microscopy (DIC).

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* promotor, *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.

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

Strain	Distribution (%)[a]	
	1 nucleus/mother	2 nuclei/mother
WT	99.94	0.06
WT + Gal (4)	99.94	0.06
GAL-CDC5 + Gal (0)	99.7	0.3
GAL-CDC5 + Gal (4)	97.5	2.5
GAL-cdc5-N209A + Gal (0)	99.9	0.1
GAL-cdc5-N209A + Gal (4)	99.1	0.9
cdh1/hct1 + Gal (0)	98.2	1.8
cdh1/hct1 + Gal (4)	98.8	1.2
GAL-CDC5 cdh1/hct1 + Gal (0)	98.9	1.1
GAL-CDC5 cdh1/hct1 + Gal (4)	78.6	21.4
GAL-cdc5-N209A cdh1/hct1 + Gal (0)	99.0	1.0
GAL-cdc5-N209A cdh1/hct1 + Gal (4)	85.0	15.0

[a] 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.

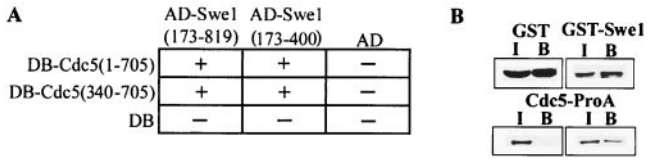


FIG. 2. Cdc5 interacts with Swe1. (A) Two-hybrid assay performed with pDB-Cdc5(1–705), pDB-Cdc5(340–705), pAD-Swe1(173–819), and pAD-Swe1(400–819). pDB (Ga14 DNA binding domain [DB] fusion vector) and pAD (Ga14 activation domain [AD] fusion vector) were used as negative controls. The *HIS3* and *ADE2* reporter strain PJ69-4a was used, and transformants were tested for growth on medium lacking uracil, leucine, histidine, and adenine. + and − indicates growth and no growth, respectively, after 5 days at 30°C. (B) GST and GST-Swe1 were incubated with yeast extract containing Cdc5 fused to ProA, derived from strain C199 (*CDC5-ProA*). After 2 h of incubation, beads were washed extensively and the bound proteins (lanes B) were resolved by SDS-PAGE and analyzed by immunoblotting; 10% of the extract added to each incubation was loaded in the input lane (I).

1B; Table 2). In contrast, the multinucleate phenotype occurs very infrequently in wild-type cells (Table 2). To visualize the spindles, a GFP-tagged version of Tub1, the major α-tubulin, was expressed. Strikingly, elongated spindles were observed in unbudded and small budded cells coincident with Cdc5 (data not shown) or cdc5-N209A (Fig. 1B) overproduction. The advanced stage of the cell cycle relative to bud growth in these cells suggested that mitosis had become uncoupled from bud formation.

Cdh1/Hct1 is a cofactor of the anaphase-promoting complex (APC) and is required for the ubiquitin-mediated destruction of the mitotic cyclins Clb1 and Clb2 at the end of mitosis (39, 47). In *cdh1/hct1* null cells, Clb1 and Clb2 inappropriately accumulate during G_1 and S (39, 47). We discovered that 1.8% of *cdh1/hct1* mutant cells are multinucleate (Table 2). Strikingly, the frequency of multinucleate cells in a culture of *cdh1/hct1* cells significantly increased in response to Cdc5 (21.4%) or cdc5N209A (15.0%) overproduction (Table 2).

Cdc5 and Swe1 interact. Because overexpression of either catalytically active or inactive Cdc5 resulted in multinucleate cells, we predicted that this phenotype might be due to titration of a Cdc5-interacting factor. We carried out a two-hybrid screen using Cdc5 as bait to isolate potential interacting factors (20). One of the positives contained an in-frame fusion to an open reading frame encoding the C-terminal region of Swe1 (amino acid residues 173 to 819) (Fig. 2A) (6). Further two-hybrid experiments showed that a C-terminal region of Cdc5 (between amino acids 340 and 705) is sufficient for interaction with Swe1 (Fig. 2A). This Swe1 interaction region in Cdc5 is distinct from the Cdc5 kinase domain (residues 82 to 337). Likewise, the region of Swe1 (between amino acids 173 and 400) that is sufficient for interaction with Cdc5 is distinct from the Swe1 kinase domain (residues 444 to 789). The Cdc5-Swe1 protein-protein interaction was confirmed by an affinity chromatography strategy (Fig. 2B). Cdc5 has also been independently isolated by another group in a two-hybrid screen using Swe1 as the bait (J. Harrison and D. Lew, personal communication).

***cdh1/hct1 swe1* double mutants have a high frequency of multinucleate cells resulting from an uncoupling of bud formation and mitosis.** Previous studies have concluded that Swe1 does not play a role during an unperturbed cell cycle (6). In agreement with these results, the frequency of multinucleate *swe1* cells was very similar to that observed in wild-type cells (Table 3). However, altering the levels of Clb/Cdk affected the accumulation of multinucleate *swe1* 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, *swe1* 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 *swe1* null cells resulted in 10.8% multinucleate cells, compared to only 0.08% multinucleate *GAL-CLB2 SWE1* cells (Table 3). The overexpression of the Clb/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. How-

TABLE 3. Multinucleate formation in *swe1* mutant strains

Strain	Distribution (%)[a]	
	1 nucleus per mother	2 nuclei per mother
Wild type	99.94	0.06
swe1	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

[a] See Table 2 for full description. Cells were grown in rich medium with two exceptions. Strains transformed with the *2*μm or *2*μm-*SIC1* plasmids were grown in synthetic medium lacking the amino acid tryptophan. The *GAL-CLB2* strains were grown in rich medium containing raffinose; where indicated, galactose was added for the time periods (hours) shown (in parentheses). The *swe1, cdh1/hct1*, and *clb2* mutations represent deletions of their respective open reading frames.

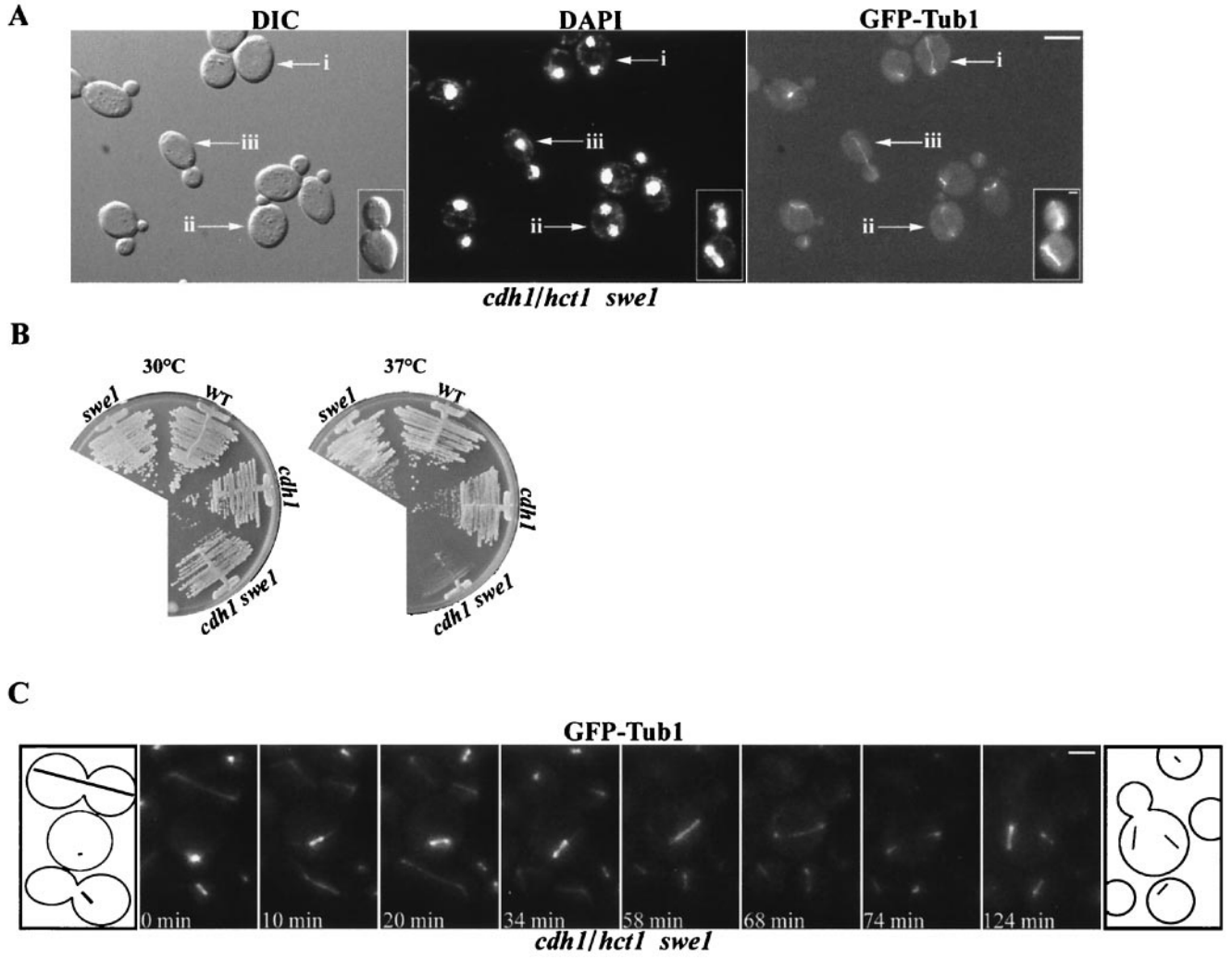
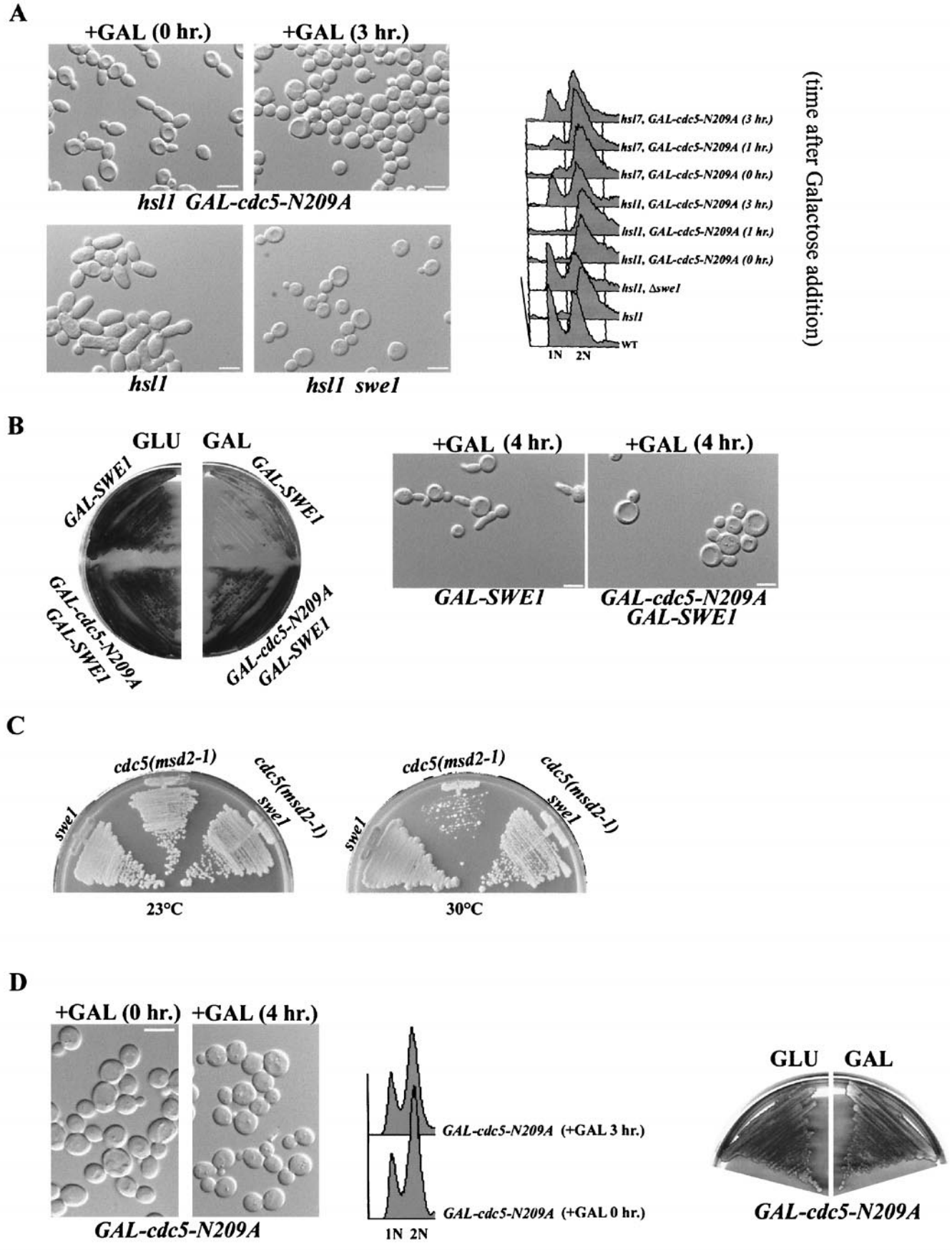


FIG. 3. Mitosis is unlinked from bud formation in *cdh1/hct1 swe1* cells. (A) Visualization of elongated and disassembled spindles in unbudded and small-budded multinucleate *cdh1/hct1 swe1* cells. Cultures of C968 (*cdh1/hct1 swe1 MET3-GFP-TUB1*) were grown to mid-log phase in YPD and transferred to 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 swe1 GFP-TUB1* cells) in which the mother and its attached daughter are both undergoing mitosis. (B) The *cdh1/hct1 swe1* double mutant grows slower than the single mutants at 37°C. Strains C895 (*cdh1/hct1 swe1*), C798 (*swe1*), 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 swe1 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.

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 Swe1-dependent phenotypes. To test whether *cdc5N209A* overexpression inactivates Swe1 function, we investigated whether overexpression of *cdc5N209A* in *hsl1* or *hsl7* mutant cells would suppress the *SWE1*-dependent defects of these mutants. The *hsl1* and *hsl7* mutants undergo a prolonged period of apical bud growth during G_2 and have Swe1-dependent elongated buds (31). We found that overexpression of *cdc5-N209A* suppressed the Swe1-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 G_2 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 swe1* mutant cells were very similar (Fig. 4A). We observed that


A
+GAL (0 hr.)
+GAL (3 hr.)
hsl1 GAL-cdc5-N209A
hsl1
hsl1 swe1
hsl7, GAL-cdc5-N209A (3 hr.)
hsl7, GAL-cdc5-N209A (1 hr.)
hsl7, GAL-cdc5-N209A (0 hr.)
hsl1, GAL-cdc5-N209A (3 hr.)
hsl1, GAL-cdc5-N209A (1 hr.)
hsl1, GAL-cdc5-N209A (0 hr.)
hsl1, Δswe1
hsl1
WT
1N 2N
(time after Galactose addition)
B
GLU
GAL
GAL-SWE1
GAL-cdc5-N209A GAL-SWE1
+GAL (4 hr.)
+GAL (4 hr.)
GAL-SWE1
GAL-cdc5-N209A GAL-SWE1
C
cdc5(msd2-1) swe1
cdc5(msd2-1) swe1
23°C
30°C
D
+GAL (0 hr.)
+GAL (4 hr.)
GAL-cdc5-N209A
GAL-cdc5-N209A (+GAL 3 hr.)
GAL-cdc5-N209A (+GAL 0 hr.)
1N 2N
GLU
GAL
GAL-cdc5-N209A

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 is 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 (30; Woo and Hardy, unpublished). However, electrophoretic 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 Clb/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 Clb/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 G_1 or Cln cyclins and is inhibited by the mitotic cyclins Clb1 and Clb2, in combination with Cdk (2, 25, 26, 40). Mutants lacking the APC cofactor Cdh1/Hct1 accumulate Clb1/Cdk and Clb2/Cdk activity in G_1 (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 Cdks

FIG. 4. cdc5N209A overproduction suppresses Swe1-dependent phenotypes. (A) Strains C508 (*hsl1 GAL-cdc5N209A* [DIC and FACS]) and C618 (*hsl7 GAL-cdc5N209A* [FACS only]) were grown to mid-logarithmic phase in raffinose. Galactose was added to 2% for the times indicated. Strains MAY1 (*hsl1* [DIC and FACS]), MAY2 (*hsl1 swe1* [DIC and FACS]), and W303a (wild type [WT] [FACS only]) were included for comparison. The cells were viewed by DIC (top); they were also stained with propidium iodide and analyzed by FACS (bottom) to evaluate cellular DNA contents. (B) Constitutive expression of *SWE1* is toxic and induces elongated buds. Both of these phenotypes were suppressed by *cdc5-N209A* overproduction. Strains C474 (*GAL-Swe1*) and C499 (*GAL-SWE1 GAL-cdc5-N209A*) were streaked on rich plates containing either 2% glucose (repressing conditions) or 2% galactose (inducing conditions) for 5 days (left). Galactose was added to mid-logarithmic-phase cultures of the same strains for 4 h to induce Swe1 (*GAL-SWE1*) or Swe1 and Cdc5 (*GAL-SWE1 GAL-cdc5-N209A*) and viewed by DIC (right). (C) Strains C798 (*swe1*), C745 [*cdc5*(*msd2-1*)] and C667 [*swe1 cdc5*(*msd2-1*)] were incubated on rich medium at 23 or 30°C for 5 days. (D) Strain JC278 (*GAL-cdc5N209A*) was grown to mid-logarithmic phase in raffinose. Galactose was added to 2% for the times indicated. The cells were viewed by DIC (left); they were also stained with propidium iodide and analyzed by FACS (middle) to evaluate cellular DNA contents. The same strain was also streaked on rich plates containing either 2% glucose (repressing conditions) or 2% galactose (inducing conditions) for 5 days (right). Bars: 10 μm.

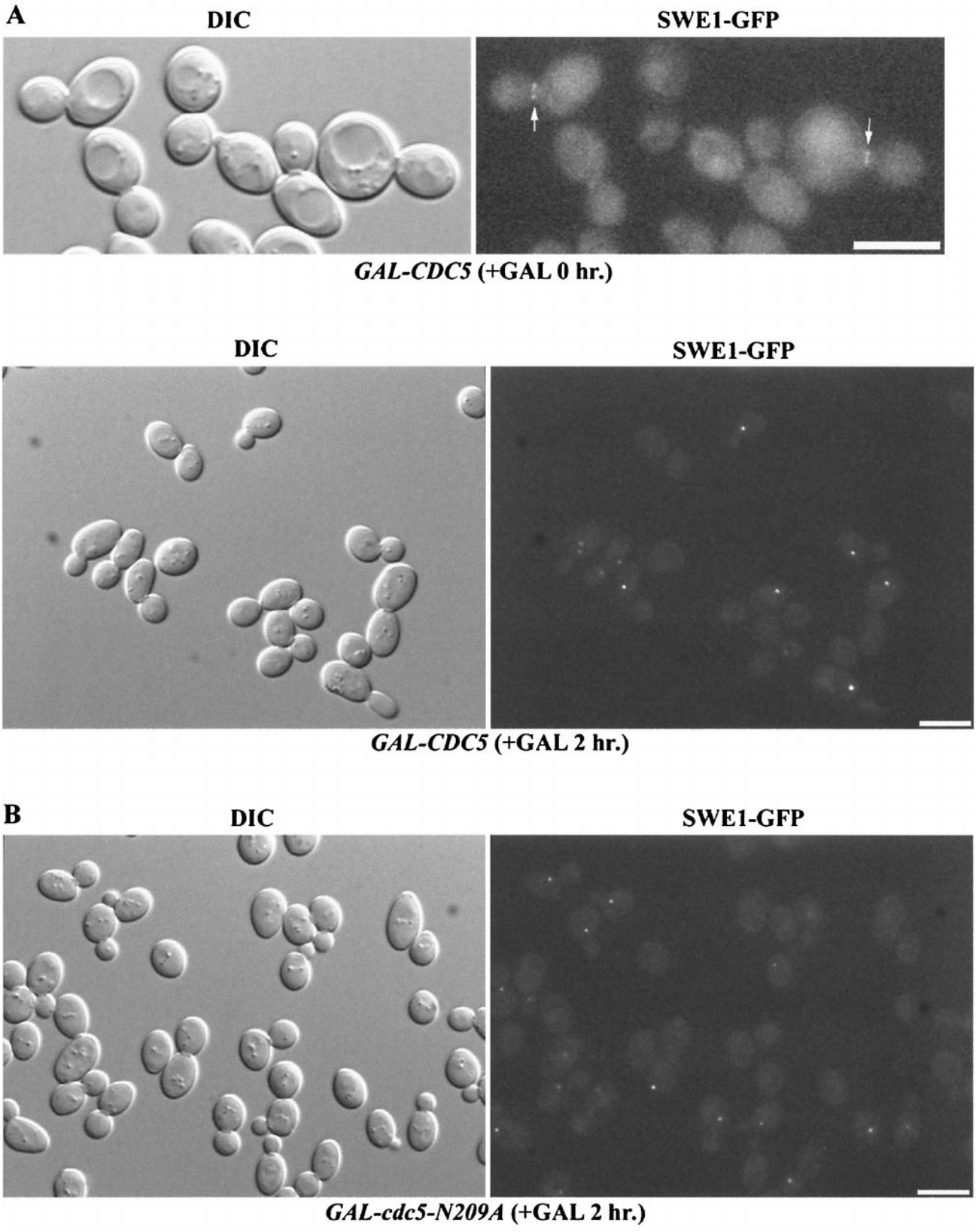


FIG. 5. Swe1 is found at one or two distinct spots in cells overexpressing *CDC5* or *cdc5N209A*. (A) Strain C573 (*GAL-CDC5-HA SWE1-GFP*) was grown to mid-logarithmic phase in raffinose. Galactose was added to 2% for 2 h. (B) Strain C572 (*GAL-cdc5-N209A-HA SWE1-GFP*) was grown to mid-logarithmic phase in raffinose. Galactose was added to 2% for 2 h. Bars: 10 μm.

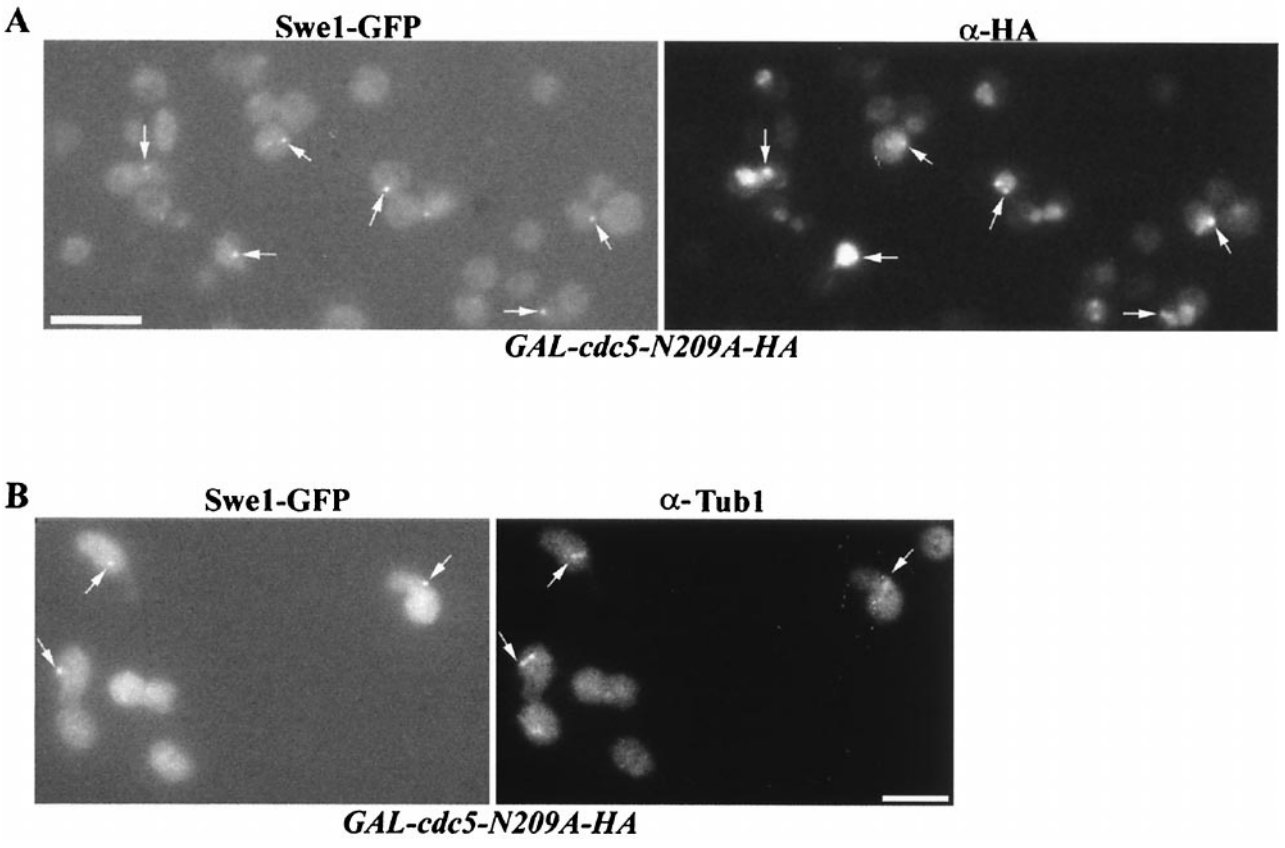


FIG. 6. Swe1 is found associated with cdc5-N209A at the SPBs of cells overexpressing *cdc5-N209A*. Strain CH572 (*GAL-cdc5N209A-HA SWE1-GFP*) was grown to mid-logarithmic phase in raffinose. Galactose was added to 2% for 2 h. The cells were fixed and stained for cdc5-N209A-HA (A) or Tub1 (B). Swe1-GFP was visualized by direct fluorescence. 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 G_2 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

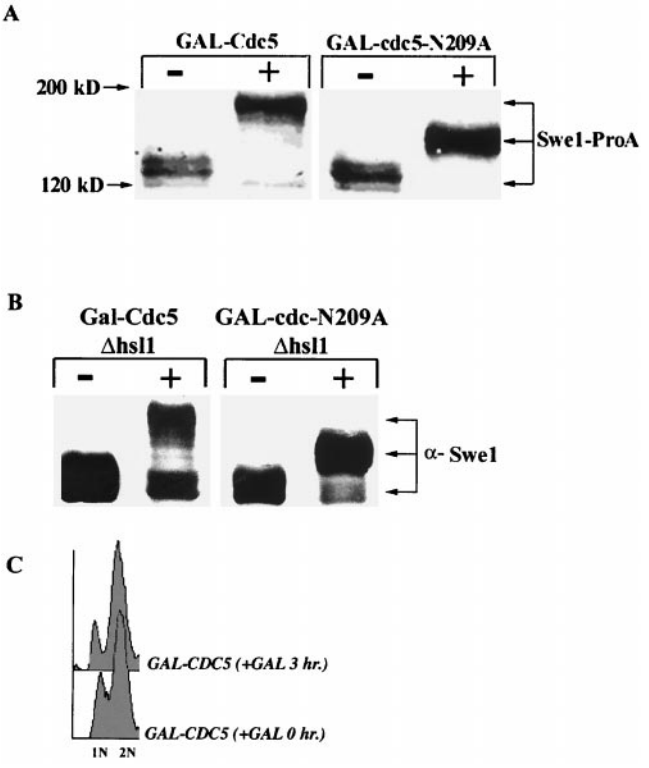


FIG. 7. Swe1 is modified in response to Cdc5 or cdc5N209A overproduction. (A) Strains CH473 (*GAL-CDC5 SWE1-ProA*) and CH459 (*GAL-cdc5N209A SWE1-ProA*) were grown in raffinose and shifted to 2% galactose. Samples for immunoblot analysis were taken just before (−) and 3 h after (+) addition of galactose. (B) Strains CH507 (*hsl1 GAL-CDC5*) and CH508 (*hsl1 GAL-cdc5N209A*) were grown in raffinose and shifted to 2% galactose. Samples for immunoblot analysis were taken just before (−) and 3 h after (+) addition of galactose. (C) Strain JC256 (*GAL-CDC5*) was grown to mid-logarithmic phase in raffinose. Galactose was added to 2% for the times indicated. The cells were stained with propidium iodide and analyzed by FACS to evaluate cellular DNA contents.

that *cdc55* 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 function. 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 places 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 expressed 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 Stf1/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 *stf1*/*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. Plx1, 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 G_2/M.

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