Properties of *Saccharomyces cerevisiae* wee1 and its differential regulation of p34^{CDC28} in response to G$_1$ and G$_2$ cyclins

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Wee1 is a protein kinase that negatively regulates p34^{cdc2} kinase activity. We have identified a *Saccharomyces cerevisiae* wee1 homolog encoded by the *SWE1* gene. *SWE1* overexpression arrests cells in G$_2$ with short spindles whereas deletion of *SWE1* did not alter the cell cycle but did eliminate the G$_2$ delay observed in *mih1$^-$* mutants. Swe1 immunoprecipitates were capable of tyrosine phosphorylating and inactivating p34^{CDC28} complexed with Clb2, a G$_2$-type cyclin, but not p34^{CDC28} complexed with Cln2, a G$_1$-type cyclin, consistent with the inability of Swe1 overexpression to inhibit the G$_1$/S transition. These results suggest that specific cyclin subunits target p34^{CDC28} for distinct regulatory controls which may be important for ensuring proper p34^{CDC28} function during the cell cycle.
Key words: cdc2/cell cycle/mitosis/*SWE1*/tyrosine kinase

Introduction

The *Schizosaccharomyces pombe cdc2* and the *Saccharomyces cerevisiae CDC28* genes encode functionally homologous 34 kDa protein kinases (Beach *et al.*, 1981; Hindley and Phear, 1984; Lörincz and Reed, 1984; Reed *et al.*, 1985; Booher and Beach, 1986; Simanis and Nurse, 1986). In both yeasts, p34$^{cdc2/CDC28}$ kinase activity is required for START and for the G$_2$/M transition, and is a major rate-limiting determinant of S phase and M phase initiation (Hartwell *et al.*, 1974; Nurse, 1975; Nurse and Thuriaux, 1980; Reed, 1980; Nurse and Bissett, 1981; Piggott *et al.*, 1982). In *S.pombe*, p34^{cdc2} kinase activity is regulated during the G$_2$/M transition both by its association with a mitotic (B-type) cyclin and by specific phosphorylation events on p34^{cdc2} (Booher and Beach, 1988; Hagen *et al.*, 1988; Booher *et al.*, 1989; Moreno *et al.*, 1989; Ducommun *et al.*, 1991; Gould *et al.*, 1991). The latter includes phosphorylation of Tyr15, which negatively regulates p34^{cdc2} kinase activity (Gould and Nurse, 1990; Lundgren *et al.*, 1991). Wee1 and mik1 protein kinases cooperate to repress p34^{cdc2} activity by promoting Tyr15 phosphorylation, and the cdc25 gene product, which is a tyrosine phosphatase, counteracts this repression (Gould and Nurse, 1990; Lundgren *et al.*, 1991). These interactions have been confirmed using purified components (Gautier *et al.*, 1991; Kumagai and Dunphy, 1991; Millar *et al.*, 1991; Parker

et al., 1991, 1992; McGowan and Russell, 1993). There are two proposed roles for negative regulation of p34^{cdc2} by tyrosine phosphorylation. First, fission yeast utilizes this mechanism to ensure that a critical cell size is attained prior to the completion of cell division (Nurse, 1975; Russell and Nurse, 1987). Second, Tyr15 phosphorylation is linked to a checkpoint mechanism which delays the initiation of mitosis when unreplicated or damaged DNA is present (Dasso and Newport, 1990; Enoch and Nurse, 1990; Lundgren *et al.*, 1991; Enoch *et al.*, 1992; Rowley *et al.*, 1992).

In *S.cerevisiae*, p34^{CDC28} kinase activity is controlled, in part, by its interaction with two functionally distinct classes of cyclins and by phosphorylation. The *CLN* genes (*CLN1, 2* and *3*) encode cyclin-like proteins that regulate p34^{CDC28} kinase activity at START (Cross, 1988, 1990; Nash *et al.*, 1988; Hadwiger *et al.*, 1989b; Richardson *et al.*, 1989). The four *CLB* genes (*CLB1, 2, 3* and *4*) encode B-type cyclins that regulate p34^{CDC28} mitosis-specific functions (Ghiara *et al.*, 1991; Surana *et al.*, 1991; Fitch *et al.*, 1992; Richardson *et al.*, 1992). Clb3, Clb4 and Clb5 also appear to play a role in S phase (Epstein and Cross, 1992; Richardson *et al.*, 1992). There is some evidence in *S.cerevisiae* that p34^{CDC28} is negatively controlled before mitosis by phosphorylation of the conserved tyrosine (Tyr19 in Cdc28), since deletion of the *MIH1* gene, a homolog of *S.pombe cdc25* (Russell and Nurse, 1986), delays the onset of mitosis; this delay is exacerbated by overexpressing the heterologous *S.pombe wee1* gene (Russell *et al.*, 1989). Additionally, phosphorylation of p34^{CDC28} on Tyr19 during the G$_2$ phase has been observed (Amon *et al.*, 1992; Sorger and Murray, 1992). However, unlike the situation in *S.pombe*, dephosphorylation of the Tyr19 does not appear to be the sole rate-limiting step controlling p34^{CDC28} activation (Amon *et al.*, 1992; Sorger and Murray, 1992).

The regulatory network controlling the onset of mitosis is partially described in several species and there is evidence that the rate of p34^{cdc2} phosphorylation and dephosphorylation on Tyr15 varies during the cell cycle (Solomon *et al.*, 1990; Kumagai and Dunphy, 1992; Smythe and Newport, 1992). This has not been fully explored in cells that show major regulation at G$_1$/S and which may use different kinase pathways to regulate the separate G$_1$/S and G$_2$/M transitions. In *S.cerevisiae*, where the G$_1$/S transition is best described, there is incomplete knowledge of the role of tyrosine phosphorylation in G$_2$/M and as yet no identification of the relevant tyrosine kinase. In this paper, we describe the isolation and characterization of *SWE1*, a *S.cerevisiae wee1* homolog. The results presented suggest that a phosphorylation/dephosphorylation regulatory pathway acting on p34^{CDC28} Tyr19 is actively engaged during the cell cycle and that the distinction between G$_1$/S and G$_2$/M controls is made by the type of cyclin protein complexed with p34^{CDC28}. We find specificity arises because B-type, but not G$_1$ cyclins, target p34^{CDC28} for tyrosine phosphorylation.

Results

Isolation of a S.cerevisiae wee1 homolog

To clone potential S.cerevisiae wee1 homologs, we utilized a pair of degenerate oligonucleotides to PCR-amplify DNA segments from S.cerevisiae genomic DNA. One of the primers corresponded to a region conserved in all Ser/Thr protein kinases, while the second primer corresponded to a region specific for the S.pombe wee1 and mik1 protein kinases (see Materials and methods). PCR amplification yielded a DNA fragment that encoded a protein sequence with significant similarity to the wee1 protein kinase. This fragment was then used as a probe to isolate a full-length clone from a library of S.cerevisiae genomic DNA. We determined the nucleotide sequence of the regions adjacent to and including the PCR fragment, which revealed an open reading frame (ORF) of 2457 bp predicted to encode a protein kinase of 819 amino acids with a calculated molecular mass of 92 kDa (Figure 1A). We named this gene SWE1, for S.cerevisiae wee1. While the Swe1 gene product shares some degree of similarity to all protein kinases, it is most similar to the S.pombe wee1/mik1 protein kinases based on computer comparison of a database of 193 protein kinase sequences. For example, Swe1 shared 64% sequence identity to S.pombe wee1 and 49% identity to S.pombe mik1 over a 150 amino acid segment within the kinase domain, while all other kinases shared <30% identity in the same region. In addition, Swe1 contains the Glu-Gly-Asp triplet motif which is found exclusively in kinase subdomain VIII of the wee1 protein kinase family (Figure 1B).

To test whether SWE1 encodes a wee1 homolog, we assessed its ability to rescue S.pombe wee1 mutations. The intact SWE1 gene was inserted into a S.pombe replicating plasmid and subsequently transformed into temperature-sensitive (ts) wee1-50^s cdc2-3w and wee1-50^s mik1::ura4 S.pombe mutants. Loss of wee1 function at 37°C in these mutants results in a lethal phenotype referred to as mitotic catastrophe (Russell and Nurse, 1987; Lundgren et al., 1991). As shown in Figure 2, SWE1 rescued the wee1-50^s defect in both mutant strains. Thus the Swe1 gene product is both structurally and functionally similar to the S.pombe wee1 protein kinase.

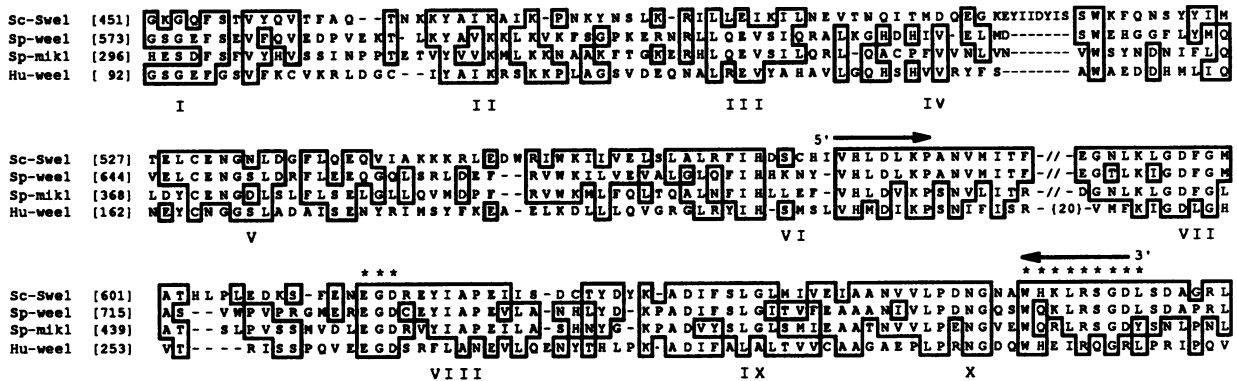In S.pombe, loss-of-function wee1 mutations shorten the G_2 phase of the cell cycle causing cells to divide at a reduced size, demonstrating that wee1 negatively controls mitotic initiation (Nurse, 1975; Nurse and Thuriaux, 1980; Russell and Nurse, 1987). To examine whether SWE1 plays a similar role in controlling the S.cerevisiae cell cycle, we constructed a strain deleted for the SWE1 gene (Figure 1C; see Materials and methods). Flow cytometric analysis showed that cultures of wild-type cells and swe1$^-$ cells have similar proportions of cells with 1N and 2N DNA content, indicating that the length of G_2 is unaltered in swe1$^-$ cells (Figure 3A and B). This suggests that either (i) Swe1 is inactive during normal cell division cycles, (ii) a secondary mechanism, independent of negative regulation
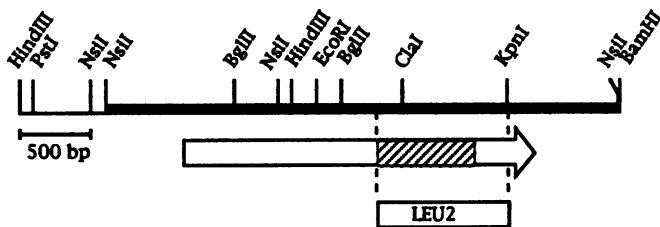
A

B

C

Fig. 1. Analysis of the *SWE1* gene. (A) Nucleotide and predicted amino acid sequence of the *SWE1* gene. The nucleotide sequence of a 3567 bp *Nsi*I—*Bam*HI DNA fragment is shown. This region contains a 2457 bp ORF encoding the 819 amino acid Swe1 product (nucleotides 1—2457). A repeated 12 bp element (CGACGCGA₅) in the 5' non-coding region is underlined. In addition, the *SWE1* coding region is close to the 3'-proximal exon of the ribosomal protein gene *RPL46* (residues 2792—2938, opposite strand) (A.deSilva and D.Goldfarb, personal communication; Leer *et al.*, 1985) and maps to chromosome X (unpublished data; Woolford and Warner, 1992). (B) Catalytic domain comparison of the Swe1 kinase with *S.pombe* wee1 and mik1 (Russell and Nurse, 1987; Lundgren *et al.*, 1991) and human wee1 kinases (Igarashi *et al.*, 1991). The numbers in brackets refer to the amino acid position in the proteins and the roman numerals correspond to catalytic subdomains as designated by Hanks *et al.* (1988). The asterisks designate amino acid residues which appear indicative of the wee1 kinase family. The arrows correspond to sequences used to design 5' and 3' degenerate oligonucleotides for PCR amplification. Protein alignment was by visual inspection. (C) Restriction map of *SWE1* and the *swe1::LEU2* gene disruption. The bold line is the sequenced region shown in panel A. The open arrow is the *SWE1* ORF with the internal catalytic domain striped (residues 451—673, see panel B). The 2.2 kb *LEU2* insert fragment is not drawn to scale.

via Cdc28 Tyr19 phosphorylation, maintains a minimal G₂ phase, or (iii) a functionally redundant *SWE1* homolog exists in *S.cerevisiae*.

To clarify whether the Swe1 kinase plays any negative regulatory role during the cell cycle, we examined its possible role in facilitating the G₂ delay observed in *mih1⁻* cells (Russell *et al.*, 1989). This was accomplished by using flow cytometry to assess the relative length of the G₂ phase in *mih1⁻* and *mih1⁻ swe1⁻* mutant strains. While *mih1⁻* cells have a significant G₂ delay, this delay was totally eliminated in *mih1⁻swe1⁻* double mutant cells (Figure 3C and D). Additionally, deletion of the *SWE1* gene eliminated the increased cell size observed in *mih1⁻* cells (unpublished data). We conclude that the inhibitory activity of Swe1 is normally counteracted by the activity of the Mih1 gene product during the *S.cerevisiae* cell cycle.

Previous studies of *S.cerevisiae* have shown that phosphorylation of p34^CDC28 on Tyr19 is not essential for the cell division arrest induced by DNA damage (Amon *et al.*, 1992; Sorger and Murray, 1992). Although similar results would be expected in *swe1⁻* cells, the possibility exists that Swe1 participates in additional inhibitory events, other than p34^CDC28 tyrosine phosphorylation, and thus *swe1* mutants might respond differently to treatments that cause DNA damage. To test this we first compared the terminal arrest phenotype of *cdc13* mutants and *cdc13 swe1* double mutants. The *cdc13* mutation was utilized because this defect induces a G₂ (*RAD9*-dependent) checkpoint arrest (Weinert and Hartwell, 1988). At the restrictive
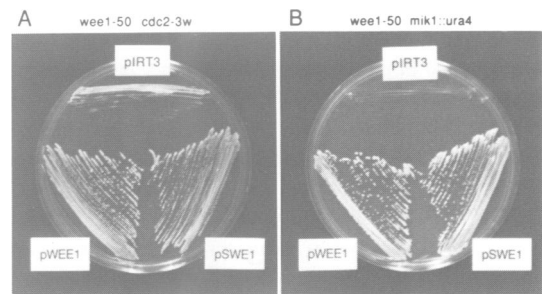
Fig. 2. *SWE1* rescue of *S.pombe* mutants with a ts wee1 mutation. Double mutant strains (A) *wee1-50 cdc2-3w* (SP669) and (B) *wee1-50 mik1::ura4* (SF3) carrying multicopy plasmids with the *S.cerevisiae SWE1* gene, the *S.pombe* wee1 gene or no insert (pIRT3). The plates were incubated at the non-permissive temperature of 37°C.

temperature of 37°C both *cdc13* cells and *cdc13 swe1* double mutant cells arrested as single large budded cells and did not continue to divide and form microcolonies (unpublished data). As a second test, we compared the sensitivity of wild-type and *swe1* cells with the DNA damaging effects of ultraviolet irradiation. As shown in Table I, the survival rate of *swe1* cells was not significantly different than wild-type cells following exposure to three different doses of UV irradiation, thus *swe1* mutants are not supersensitive to UV irradiation. Although these results do not exclude the possibility that the Swe1 kinase can participate in DNA damage checkpoint control, it is apparent that this checkpoint control is operative in the absence of Swe1 activity.
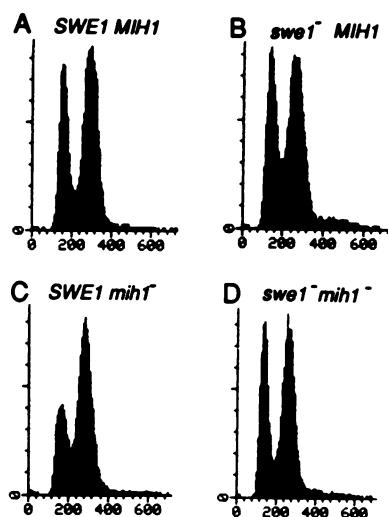
Fig. 3. Flow cytometric analysis. (**A**) Wild-type (204-4C), (**B**) *swe1* (SCY13), (**C**) *mih1* (Scy124) and (**D**) *swe1 mih1* (SCY177) strains were grown exponentially at 30°C in SD minimal medium prior to fixation and propidium iodide staining for FACS analysis.

Table I. Percent survival following UV irradiation[a]

Strain[b]	Dose (μjoules/cm^2)		
	2500	5000	7500
Wild-type	61	22	3
swe1	70	13	10

[a]Percent survival relative to unirradiated control plates. Surviving colonies were counted after 3 days at 30°C.
[b]Wild-type (204-4C) and *swe1* (SCY13).

The effects of SWE1 are limited to G_2 and M phase

Overexpression of the *S.pombe wee1* gene in either *S.pombe* or *S.cerevisiae* delays entry into mitosis and causes cells to divide at an increased size (Russell and Nurse, 1987; Russell *et al.*, 1989). To determine whether overexpression of *SWE1* has a similar inhibitory activity in *S.cerevisiae*, we constructed a strain containing a *GAL1:SWE1* fusion allele in which *SWE1* expression was controlled by the galactose-inducible *GAL1* promoter (Johnston and Davis, 1984). A culture of *GAL1:SWE1* cells was initially grown in raffinose-containing medium, a carbon source that does not activate the *GAL1* promoter. Galactose was then added to induce *SWE1* expression. Two hours after galactose addition the *GAL1:SWE1* cells ceased dividing and arrested largely as single, uninuclear cells with a bud-like protrusion which continued elongating with time (Figure 4A). Flow cytometry showed that these cells arrested with a 2C DNA content, indicating a G_2 arrest (Figure 4B). Thus, *SWE1* overexpression inhibits nuclear division but does not inhibit transit through START or DNA replication.

As a second test to confirm that Swe1 overexpression does not inhibit DNA replication, we added galactose to a culture of *GAL1:SWE1* cells which had been arrested in G_1 with the yeast mating pheromone α-factor. Two hours after galactose addition, the cells were shifted to galactose-containing medium lacking α-factor. Within 30 min after α-factor release, bud emergence occurred in nearly all of the cells. However, upon continued incubation these cells
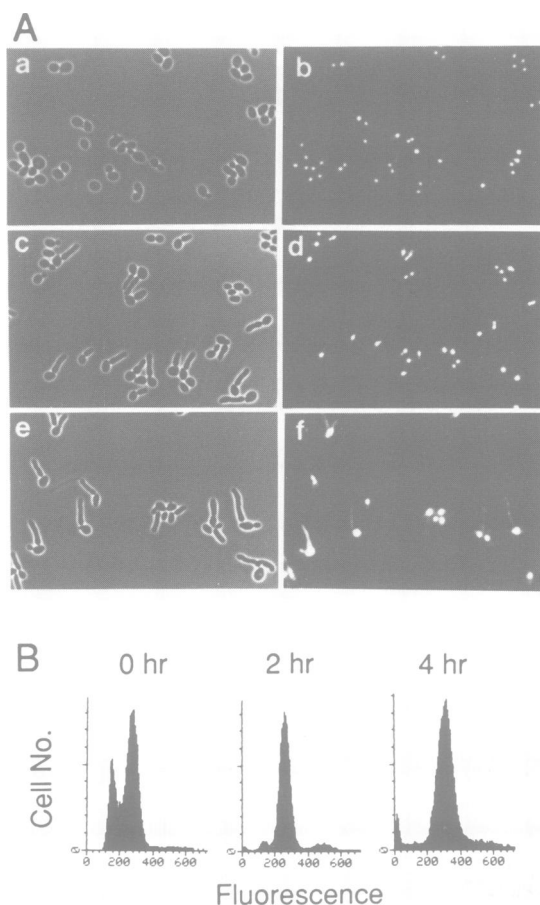
**Fig. 4.** Swe1-induced cell cycle arrest. An asynchronous culture of *GAL1:SWE1* cells (SCY169) was grown in YEP + Raf at 30°C. At 0 h, galactose was added to 2% and samples were withdrawn at 0, 2 and 4 h, fixed with EtOH and stained with DAPI or propidium iodide. (**A**) Phase-contrast images on the left and DAPI fluorescence on the right. Top, middle and bottom panels correspond to 0, 2 and 4 h. (**B**) Flow cytometric analysis of propidium iodide stained cells.

failed to divide and instead arrested as single cells with an elongated bud and undivided nucleus (unpublished data). Flow cytometry showed that these arrested cells had replicated their DNA, confirming that *SWE1* overexpression only inhibits the initiation of mitotic events (unpublished data).

The Gal−Swe1-induced terminal arrest phenotype is similar to that previously described for *cdc28-1N* arrested mutants, *clb1, 2, 3* and *4*-deficient mutants, and *S.cerevisiae mih1−* cells overexpressing *S.pombe wee1*, each of which arrests cells in G_2, presumably due to reduced Cdc28 mitosis-specific kinase activity (Piggott *et al.*, 1982; Russell *et al.*, 1989; Surana *et al.*, 1991; Fitch *et al.*, 1992; Richardson *et al.*, 1992). *cdc28-1N* mutants arrest with a short mitotic spindle [which normally forms during S phase in *S.cerevisiae* (Byers and Goetsch, 1975)], while *clb1,2,3,4*-deficient cells fail to form a spindle (Surana *et al.*, 1991; Fitch *et al.*, 1992; Richardson *et al.*, 1992). This has led to the proposal that Cdc28 activity is required for the formation, maintenance and elongation of the mitotic spindle, and that these functions are specified by particular subclasses of Clb-type cyclins (Fitch *et al.*, 1992; Richardson *et al.*, 1992). We therefore investigated whether Swe1 overexpression inhibited either of these Cdc28-dependent

Fig. 6. SWE1 overexpression induces bud emergence in nocodazole-arrested cells. A *GAL1:SWE1/GAL1:SWE1* diploid strain (SCY130), cultured in YEP + Raf medium, was arrested in mitosis by nocodazole treatment for 2 h at 25°C. The culture was split and galactose was added to one half while dextrose was added to the other half. Samples were withdrawn at 0 and 3.5 h post-galactose/dextrose addition. Photomicrographs of 0 h (**A**), 3.5 h galactose-cultured cells (**B**) and 3.5 h dextrose-cultured cells (**C**) are shown.

Fig. 5. Anti-tubulin staining of Gal:Swe1 arrested cells. Galactose was added to a log phase YEP + Raf culture of *GAL1:SWE1/GAL1:SWE1* diploid cells (SCY130). After growing for 4 h, the cells were harvested, fixed in formaldehyde and stained with the DNA binding dye DAPI and an anti-tubulin antibody. Micrographs of Swe1-arrested cells (**A**), DAPI staining (**B**) and anti-tubulin staining (**C**) are shown.

processes by immunostaining Gal:Swe1 arrested cells with an anti-tubulin antibody. Figure 5 shows that Gal:Swe1 arrested cells (cultured in galactose for 4 h) indeed formed a short mitotic spindle. We conclude that Swe1 overexpression does not inhibit Cdc28 activity associated with mitotic spindle formation, but instead inhibits the Cdc28 activity associated with spindle elongation.

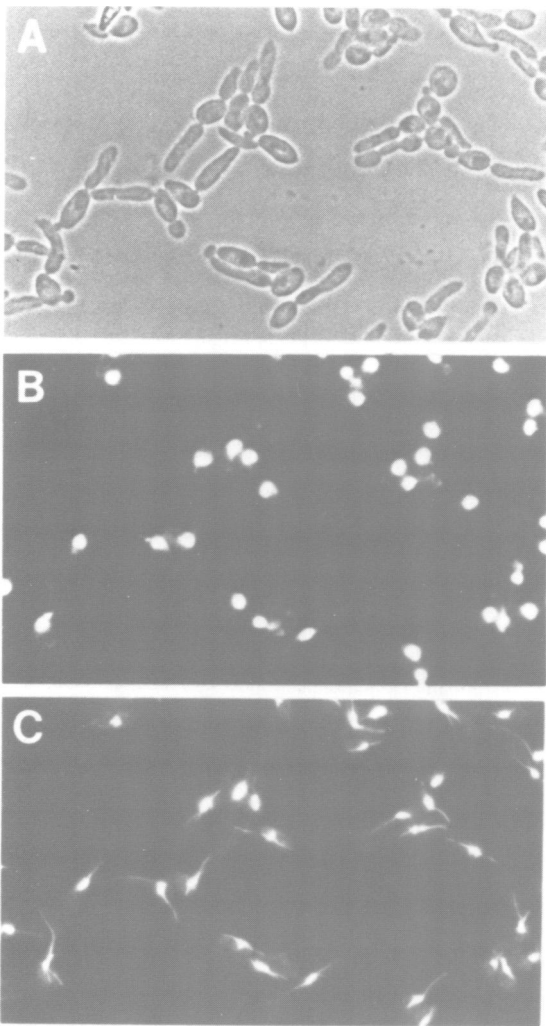As shown above, galactose-induced Swe1 overexpression caused asynchronously growing *GAL1:SWE1* cells to arrest as large cells with single elongated buds. However, a minority population actually arrested as large budded cells with an additional aberrant bud emerging from the daughter cell. This suggests that Swe1 is capable of functioning late in the cell cycle, perhaps during mitosis. To test this possibility, we determined whether Swe1 overexpression in mitotic cells exhibited a similar phenotype. *GAL1:SWE1* cells were arrested in mitosis by a 2 h treatment with the microtubule depolymerizing drug nocodazole. This culture was divided in half. Galactose was added to one half to induce *GAL:SWE1* expression, while glucose was added to the other half as a control. After 3.5 h of culturing in galactose, emergence of an additional bud from the daughter
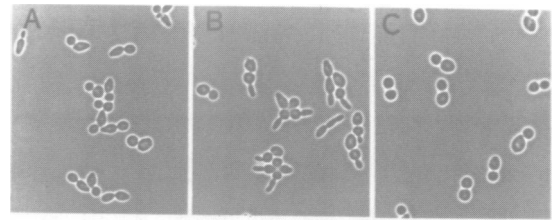
cell was apparent in nearly all of the cells (Figure 6). The cells in the control glucose culture remained arrested with a large budded morphology characteristic of M phase cells. DAPI staining of these cells, as well as cells from the galactose-containing culture, showed that nuclear division did not occur indicating that the nocodazole arrest remained in effect throughout the course of this experiment (unpublished data). These results show that mitotic cells are sensitive to Swe1 function, when *SWE1* is overexpressed. Moreover, elimination of the Swe1-sensitive Cdc28 activity in M phase-arrested cells uncoupled the budding cycle from the nuclear division cycle, implying that Cdc28 activity negatively regulates bud emergence during mitosis.

The activity of Swe1 on Cdc28 complexed with B-type cyclins

Just as *S.cerevisiae* cells containing a *CDC28-T18A,Y19F* allele (both Thr18 and Tyr19 residues are mutated) are resistant to *S.pombe* wee1 overexpression (Sorger and Murray, 1992), we found that cells containing either a *CDC28-Y19F* or *CDC28-T18V,Y19F* allele are resistant to the cell cycle inhibitory affect of *SWE1* overexpression (unpublished data). These results strongly suggest that the Swe1 inhibitory activity functions through a pathway which phosphorylates Cdc28 on Tyr19. To determine whether Swe1 directly phosphorylates Cdc28 or whether an intermediary, Swe1-activated tyrosine kinase is involved, we utilized an *in vitro* kinase reaction consisting of partially purified components. These components consisted of Swe1 immunoprecipitates and *E.coli*-synthesized Cdc28 which was activated by *E.coli*-produced GST−Clb2 in the presence of crude yeast extract and subsequently reisolated by glutathione affinity chromatography (see Materials and methods). To test the ability of Swe1 to phosphorylate directly Cdc28, Swe1 immunoprecipitates were incubated with various affinity-isolated Cdc28/GST−Clb2 complexes in a kinase reaction containing [^{32}P]ATP. After a 30 min incubation, the Cdc28/GST−Clb2 complexes were retrieved using p18^{CKS1}−agarose beads (*S.cerevisiae* homolog of p13^{suc1}; Hadwiger *et al.*, 1989a) and resolved by SDS−PAGE. Both wild-type Cdc28 and Cdc28-T18V proteins were ^{32}P-labeled in the kinase reaction (Figure 7A, lanes 3 and 6). In contrast, Swe1 failed to label the Cdc28-Y19F and Cdc28-T18V,Y19F mutants (Figure 7A, lanes 4 and 5). Phosphoamino acid analysis revealed that Swe1-treated Cdc28 contained only phosphotyrosine (Figure 7B). We conclude that Swe1 phosphorylates Cdc28 on Tyr19, but not on Thr18.
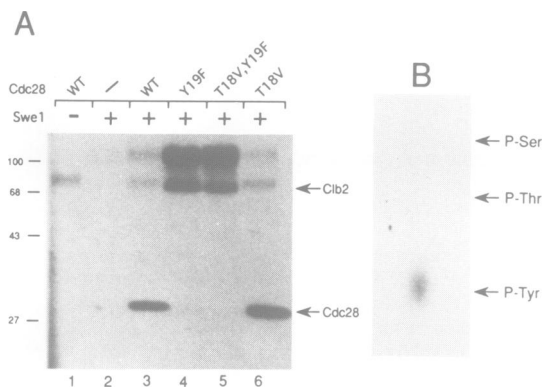
A

Fig. 7. *In vitro* phosphorylation of Cdc28 by immunoprecipitated Swe1 kinase. (A) Swe1 immunoprecipitates were isolated from extracts prepared from cells overexpressing Swe1-HA (SCY93) and incubated with various Cdc28−Clb2 complexes in a kinase reaction containing [^{32}P]ATP as described in Materials and methods. The Cdc28−Clb2 complexes were reisolated from the kinase reaction using p18^{CKS1} beads, resolved by SDS−PAGE and analyzed by autoradiography. The Swe1 (−) control (lane 1) corresponds to immunoprecipitates isolated from extracts prepared from a strain overexpressing non-HA-tagged Swe1 (SCY31). (B) Phosphoamino acid analysis of Cdc28 which had been ^{32}P-labeled by Swe1 as described in Materials and methods.

Besides Cdc28, additional ^{32}P-labeled proteins were observed after the labeling reaction. For instance, wild-type Cdc28 complexes alone contained a ^{32}P-labeled protein of ∼80 kDa. This is likely to be the GST−Clb2 subunit since it corresponds to this size and phosphorylation of cyclin B by p34^{cdc2} has been observed previously (Draetta and Beach, 1988; Booher *et al.*, 1989; Desai *et al.*, 1992; Solomon *et al.*, 1992). This is further supported by the fact that this protein is ^{32}P-labeled to a much lesser extent in the reactions containing Cdc28 and Cdc28-T18V, which lose kinase activity after phosphorylation by Swe1 (see below).

To test whether Swe1 could directly inactivate Cdc28, we assayed the histone H1 kinase activity of Cdc28/GST−Clb2 complexes that had been phosphorylated by Swe1. Various forms of Cdc28/GST−Clb2 complexes were incubated wth Swe1 immunoprecipitates in a kinase reaction. The histone H1 kinase activity of the Cdc28/GST−Clb2 complexes in this reaction mixture was then assayed. Figure 8A shows that Swe1-treated Cdc28/GST−Clb2 and Cdc28-T18V/GST−Clb2 complexes had greatly reduced histone H1 kinase activity (lanes 1−4). But surprisingly, the histone H1 kinase activity of Cdc28-Y19F/GST−Clb2 and Cdc28-T18V,Y19F/GST−Clb2 complexes was also reduced in the Swe1 reaction mixture (lanes 5−8). Since the labeling results above indicated that Swe1 only phosphorylated Tyr19, it seemed unlikely that a second, Swe1-specific negative regulatory site existed on Cdc28. Instead, we considered the possibility that the Cdc28-Y19F/GST−Clb2 complex was interacting with Swe1 in a pseudo-substrate manner and that this interaction hindered Cdc28-Y19F/GST−Clb2 kinase activity. To test this, the Swe1 kinase reaction was repeated, but afterwards, the reaction mixture was diluted with buffer and the eluted Cdc28/GST−Clb2 complexes were removed from the pelleted Swe1 immunoprecipitate beads by transferring the supernatant to another tube. The Cdc28/GST−Clb2 complexes were then isolated using p18^{CKS1} beads and subsequently assayed for histone H1 kinase activity. After this procedure, it was apparent that Swe1-treated Cdc28/GST−Clb2 and Cdc28-T18V/GST−Clb2
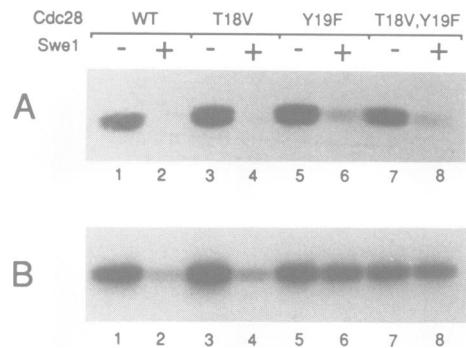
Fig. 8. Histone H1 kinase assay of Swe1-treated Cdc28−Clb2 complexes. Swe1 immunoprecipitates (+) or control immunoprecipitates (−), see Figure 7 legend for description, were incubated with the indicated Cdc28−Clb2 complexes in a kinase reaction as described in Materials and methods. Histone H1 kinase assays were performed either directly on the reaction mixture (A) or on Cdc28−Clb2 complexes that had been reisolated from the reaction mixture using p18^{CKS1} beads (B). In both cases the reaction products were resolved by SDS−PAGE and analyzed by autoradiography.
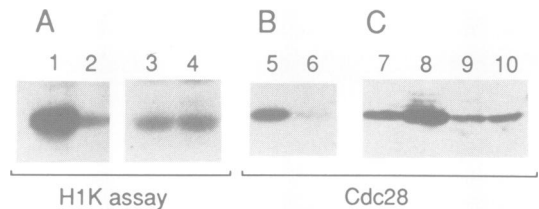
Fig. 9. Specificity of Swe1 for Cdc28/GST−Clb2 versus Cdc28/GST−Cln2 complexes. (A) Histone H1 kinase assays were performed on Cdc28/GST−Clb2 (lanes 1 and 2) and Cdc28/GST−Cln2 (lanes 3 and 4) complexes which had been reisolated, using p18^{CKS1} beads, from kinase reactions containing either control (lanes 1 and 3) or Swe1 (lanes 2 and 4) immunoprecipitates. The histone H1 kinase assay reaction products were resolved by SDS−PAGE and analyzed by autoradiography. (B) Swe1 labeling of Cdc28. Cdc28/GST−Clb2 (lane 5) and Cdc28/GST−Cln2 (lane 6) complexes were incubated with Swe1 immunoprecipitates in a kinase reaction containing [^{32}P]ATP. The Cdc28−cyclin complexes were reisolated from this reaction mixture using p18^{CKS1} beads, resolved by SDS−PAGE, and analyzed by autoradiography. (C) Anti-Cdc28 immunoblot analysis of Cdc28/GST-Clb2 and Cdc28/GST−Cln2 complexes before and after p18^{CKS1} bead purification. GST-eluted Cdc28−Clb2 (lanes 7 and 9) and Cdc28−Cln2 (lanes 8 and 10) complexes were resolved by SDS−PAGE either directly (lanes 7 and 8) or after p18^{CKS1} bead purification (lanes 9 and 10). The separated proteins were transferred to nitrocellulose and probed with an anti-Cdc28 antibody. In panels B and C, only the region corresponding to Cdc28 is shown.

complexes had drastically reduced histone H1 kinase activity (Figure 8B, lanes 1−4), while the Cdc28-Y19F/GST−Clb2 and Cdc28-T18V,Y19F/GST−Clb2 complexes retained nearly full kinase activity (Figure 8B, lanes 5−8).

Swe1 distinguishes G_1 cyclin complexes from G_2 cyclin complexes

We have previously demonstrated that Swe1 overexpression does not inhibit transit through START or the ensuing S phase. Since various genetic studies have shown that Cdc28−Cln functions, rather than Cdc28−Clb, are required for these events, a simple explanation would be that Swe1 did not recognize Cdc28 when it was complexed with a Cln-type cyclin subunit. To test directly this prediction, we utilized the *in vitro* system described above to ask whether

Swe1 immunoprecipitates would inactivate or phosphorylate a Cdc28/GST−Cln2 complex. Figure 9A shows that Swe1 treatment inactivated Cdc28/GST−Clb2 histone H1 kinase activity (lanes 1 and 2) but failed to inactivate Cdc28/GST−Cln2 complexes (lanes 3 and 4). Consistent with these results is the finding that Swe1 immunoprecipitates readily phosphorylated Cdc28 complexed with GST−Clb2 but not Cdc28 complexed with GST−Cln2 (Figure 9B). In addition, Swe1 was also unable to label Cdc28 complexed with GST−Cln3, another G_1-type cyclin (unpublished data). Immunoblot analysis revealed that the Cdc28/GST−Clb2 and Cdc28/GST−Cln2 complexes contained equivalent amounts of Cdc28 after p18^{CKS1} bead purification, ruling out the possibility that p18^{CKS1} beads fail to bind Cdc28/GST−Cln2 complexes (Figure 9C). Thus, an important determinant for Swe1 substrate recognition appears to be present on the Clb2 subunit and not the Cln2 subunit.

Discussion

The extended G_2 phase observed in *MIH1* (*S.cerevisiae cdc25* homolog)deleted strains led to the prediction that an opposing wee1-type kinase also existed in budding yeast (Russell *et al.*, 1989). In this paper, we present direct proof that this is indeed the case by the isolation of *SWE1*, a *S.cerevisiae* gene encoding a homolog of the *S.pombe* wee1 protein kinase. Four lines of evidence support the conclusion that *SWE1* encodes a wee1 homolog. First, *SWE1* encodes a polypeptide containing features common to all protein kinases, as well as certain motifs specific to the wee1 kinase family. Second, this gene was capable of rescuing *S.pombe* *wee1* mutants. Third, like *S.pombe* *wee1*, *SWE1* functions as a dose-dependent inhibitor of mitosis. Fourth, *in vitro* experiments showed that Swe1 directly phosphorylated p34^{CDC28} on tyrosine, resulting in drastically reduced p34^{CDC28} kinase activity.

The primary mode of Swe1 mitotic inhibitory activity is phosphorylation of p34^{CDC28} on Tyr19 since the Gal−Swe1 induced cell cycle arrest is largely suppressed in strains where the tyrosine is replaced with a phenylalanine. A secondary inhibitory mechanism could involve a direct physical interaction between Swe1 and Cdc28. *In vitro* studies demonstrated that Swe1 significantly inhibited Cdc28-Y19F but this inhibition could be relieved by dilution. One interpretation of these results is that Swe1 binds to Cdc28-Y19F in a pseudo-substrate manner and that this interaction hinders Cdc28-Y19F kinase activity. A similar inhibitory interaction may also occur in vivo, since galactose grown *CDC28-Y19F* strains containing two *GAL1:SWE1* loci exhibit a partial cell cycle arrest phenotype (unpublished data).

SWE1 overexpression causes cells to arrest with a 2C DNA content and a short mitotic spindle. This could imply that Swe1 does not recognize Cdc28 complexed with either Clb3 or Clb4, since cells lacking *CLBs1-4* fail to form a short spindle, whereas *clb1 clb2* double mutants arrest with a short spindle (Surana *et al.*, 1991; Fitch *et al.*, 1992; Richardson *et al.*, 1992). The observed functional differences between the Clb1/Clb2 pair and the Clb3/Clb4 pair is further supported by sequence comparison that showed Clb1/Clb2 and Clb3/Clb4 form distinct homology pairs (Fitch *et al.*, 1992). While this may explain the apparent inability of Swe1

to recognize Cdc28 complexed with Clb3 or Clb4, we are now in a position to test this directly. *CLB5* has recently been identified and it plays a role in S phase since loss of *CLB5* prolongs S phase (Epstein and Cross, 1992). We cannot formally role out the possibility that Swe1 inhibits Cdc28−Clb5 function since we have not examined that length of S phase during the Swe1-induced cell cycle arrest.

We have shown that Swe1 inhibits Cdc28 complexed with B-type cyclins by phosphorylating Tyr19. This inhibition does not occur with Cln2 (or Cln3), suggesting that different cyclins *trans*-target p34^{CDC28} for distinct negative regulatory controls. The differential recognition of Clb2−Cdc28 versus Cln2−Cdc28 complexes by Swe1 is likely to reflect the different factors that regulate p34^{CDC28} at START and G_2/M. For instance at START cell size, nutrients and pheromones are each important factors which regulate p34^{CDC28} activity, whereas at the G_2/M transition, factors such as DNA replication and spindle assembly probably regulate p34^{CDC28} activity. The observation that wee1 overexpression arrests *S.pombe* cells in G_2 (Russell and Nurse, 1987) also suggests that the cyclin−cdc2 complexes required for the G_1 to S phase transition are not substrates of the wee1 kinase. Additionally, studies utilizing *Xenopus* egg extracts have shown that cyclin B−cdc2 complexes, but not cyclin A−cdc2 complexes are inhibited by tyrosine phosphorylation (Devault *et al.*, 1992). Thus, differential cyclin-dependent targeting of p34^{cdc2} to tyrosine phosphorylation may be a general mechanism utilized by eukaryotic cells to restrict the activity of wee1-type kinases.

The *S.cerevisiae* Swe1 protein kinase shares many properties with the *S.pombe* wee1 kinase, yet the role of Swe1 and p34^{CDC28} Tyr19 phosphorylation is largely unknown. In *S.pombe* tyrosine phosphorylation of cdc2 is essential for cell viability, presumably acting to ensure that completion of S phase precedes initiation of M phase. However, our results utilizing *swe1⁻* mutants and the results of others utilizing *cdc28* mutants (Amon *et al.*, 1992; Sorger and Murray, 1992) indicate that tyrosine phosphorylation of Cdc28 is not crucial for normal cell division. This may be explained in part by the presence of additional, as of yet unidentified, rate-limiting factors of p34^{CDC28} activation that predominate or equally compensate Swe1 function during the G_2/M transition. However, the results presented here clearly show that Swe1 has G_2 inhibitory activity that is counteracted by the Mih1 gene product, which extends the conservation of the wee1/cdc25 control pathway in budding yeast (Russell *et al.*, 1989). The continued genetic and biochemical analysis of Swe1 should identify universally conserved mechanisms involved in size, feedback control and cell cycle regulation.

Materials and methods

SCY177, *MAT*a *swe1::LEU2 mih1::URA3*. The *sst1* mutation is a deletion of the *SST1(BAR1)* gene and was generated by transforming recipient cells with *SalI/EcoRI*-cut pJGsst1 (Reneke *et al.*, 1988). Standard *S.cerevisiae* genetic techniques were used for strain constructions (Sherman *et al.*, 1986). Transformations were performed using the lithium acetate method (Ito *et al.*, 1983). Rich medium consisted of 1% yeast extract, 2% peptone and either dextrose (YEPD), raffinose (YEP + Raf) or galactose (YEP + Gal) added to 2% as carbon source. Synthetic minimal medium (SD) was made and supplemented with amino acids as described by Sherman *et al.* (1986). In certain experiments, SD medium contained raffinose or galactose (each at 2%) rather than dextrose. Nocodazole (Aldrich) was added to prewarmed (65°C) medium to a final concentration of 20−30 μg/ml from a 10 mg/ml stock in dimethyl sulfoxide. A UV Stratalinker 1800 (Strategene) was utilized to UV irradiate cells plated on YEPD plates. Gene transplacements and deletion/disruptions were performed by the one-step gene disruption method (Rothstein, 1983).

S.pombe strains were derived from the wild-type strain 972h⁻. Strains used in this study were SP669, *h+N leu1-32 ade6-210 cdc2-3w wee1-50* and SF3, *h−S leu1-32 ura4-D18 ade6-210 wee1-50 mik1::ura4*. *S.pombe* was transformed by a spheroplast method and grown in standard medium (reviewed by Moreno *et al.*, 1991). *S.pombe* mutations *cdc2-3w* (Fantes, 1981), *wee1-50* (Nurse and Thuriaux, 1980) and *mik1::ura4+* (Lundgren *et al.*, 1991) have been previously described. The *mik1::ura4+* mutant was provided by Karen Lundgren.

Cloning the SWE1 gene and oligomutagenesis

An internal *SWE1* fragment was cloned by PCR amplification utilizing the following degenerate oligonucleotides: 5′ primer 5′-GCT*GGATCC*GTN-CAYYTNGAYSTNTTRCC-3′, corresponding to amino acid sequence VH(FL)D(FLV)LP; 3′ primer 5′-CGG*CTGCAG*TCNCCNMLNCKNAR-NYKYTGCCA-3′, corresponding to the amino acid sequence WQ (HQNKRS)(FL)(R/S)(STRCW)GD. Code for nucleotide mixes: L = A + T, R = A + G, K = G + T, Y = C + T, M = C + G, S = T + G + C, N = A + T + G + C. *Bam*HI and *Pst*I restriction sites are italicized. The 5′ primer recognizes a conserved sequence present in protein serine/threonine kinases, while the 3′ primer recognizes a putative wee1-specific motif (Figure 1B). PCR reactions consisted of *S.cerevisiae* genomic DNA as template (prepared according to Philippsen *et al.*, 1991), 50 pmol of each primer, buffer, dNTPs and AmpliTaq polymerase (Perkins Elmer Cetus), as recommended by the manufacturer. Samples were subjected to 35 cycles at 94°C for 1 min, 55°C for 1 min and 72°C for 1 min. An amplified fragment of the expected size (~270 bp) was purified from a 2% agarose gel, digested with *Bam*HI and *Pst*I, and cloned into *Bam*HI/*Pst*I-cut pUC119 (Vieira and Messing, 1987). Sequencing revealed one class of inserts with significant sequence identity with the catalytic domain of wee1 and mik1 protein kinases. The cloned fragment was radiolabeled and used to screen a *S.cerevisiae* genomic DNA library (partial *Sau*3A) carried in YCp50 (Rose *et al.*, 1987) (provided by Tim Stearns). Plasmid DNA was isolated from several positives and restriction enzyme analysis revealed four classes of plasmids (pSWE1-YC1, 2, 3 and 4) which differed only by the extent of overlapping insert DNA. Both strands of the DNA encompassing the Swe1 catalytic domain were sequenced by the dideoxy method (Sanger, 1977) using a Sequenase kit (United States Biochemical Corp.). This was facilitated by the creation of a series of overlapping deletions (Henikoff, 1987). A 3.5 kb *Pst*I−*Bam*HI fragment carrying the entire *SWE1* ORF was subcloned into *S.pombe* replicating vector pIRT3 (Booher and Beach, 1988), yielding plasmid pSWE1-14, referred to as pSWE1 in Figure 2. Plasmid pWEE1 is vector pIRT3 carrying a 3.7 kb *Spe*I−*Nhe*I, *wee1*-containing fragment (provided by Giulio Draetta).

Site-specific mutagenesis was performed by the method of Zoller and Smith (1984) using uracil-containing single-stranded DNA (Kunkel, 1985). A 14 residue extension containing a HA epitope [peptide YPYDVPDYA in the influenza hemagglutinin protein which is recognized by the monoclonal antibody 12CA5 (Wilson *et al.*, 1984; Field *et al.*, 1988)] was introduced at the Swe1 C-terminus using the oligonucleotide 5′-CAAAATTTTTT-AAAGGTGGTTACCCATACGATGTTCCAGATTACGCTAGCTTGG-GTTGATAAAGGAACA-3′. Alleles containing this epitope tag are designated *SWE1-HA*.

SWE1 and MIH1 gene disruption

SWE1 disruption. Plasmid pSWE1-10c, pUC119 containing a 2.3 kb *Hind*III−*Bam*HI fragment of *SWE1* (bases 749−3039), was PCR amplified using the 5′ primer 5′-TTC*TCTAGA*ATAGTACGATGGA-3′, corresponding to bases 2302−2317 and 3′ primer 5′-CTT*TCTAGA*ATGGAATG-CACATTC-3′, corresponding to the reverse complement of bases 1335−1346; *Xba*I restriction sites are italicized. The amplified fragment was gel purified, digested with *Xba*I and ligated, resulting in the substitution of the Swe1 catalytic domain (bases 1334−2318) with an *Xba*I restriction

site. A 2.2 kb *LEU2*-containing fragment was inserted at the *Xba*I site, yielding plasmid pSWE1-10g. The *swe1::LEU2* mutation was introduced into 204-4C haploid cells by transforming with *Hind*III/*Bam*HI-digested pSWE1-10g.

MIH1 disruption. The *MIH1* gene was PCR-amplified from *S.cerevisiae* genomic DNA using the 5′ primer 5′-GCT*GGATCC*AGGATTGAAG-TCAGCG-3′, corresponding to bases 509−526 (numbering according to Russell *et al.*, 1989) and the 3′ primer 5′-CGG*AAGCTT*GCGGGCCT-GGGTAAAT-3′ corresponding to the reverse complement of bases 2140−2155; respective *Bam*HI and *Hind*III restriction sites are italicized. The 1.65 kb amplified fragment was gel purified, digested with *Bam*HI and *Hind*III, and cloned into pUC119, yielding plasmid pMIH1-1. Bases 681−1877 within the coding region of *MIH1* contained in pMIH1-1 were replaced with a *Nhe*I restriction site by PCR amplification, creating pMIH1-4. A 2.2 kb *Xba*I, *LEU2*-containing fragment and a 1.1 kb *Nhe*I, *URA3*-containing fragment were ligated to *Nhe*I-cut pMIH1-4, yielding plasmids pMIH1-5 and pMIH1-8, respectively. *Bam*HI/*Nde*I-cut pMIH1-5 and *Bam*HI/*Hind*III-cut pMIH1-5 were transformed into RD204-4C, creating deletion/disruption *mih1::LEU2* and *mih1::URA3* alleles, respectively.

Construction of GAL1:SWE1 alleles

A cassette used for replacing the genomic *SWE1* gene with an allele controlled by the *GAL1* promoter was constructed by a four-part ligation. Fragment 1 is a 0.9 kb *Sac*I−*Xba*I fragment of 5′-flanking, non-coding *SWE1* DNA. This fragment was PCR-amplified using 5′ primer 5′-TTC*GAGCTC*A-AGCTTTTTACCGTATAA-3′ (sequence present at the 5′ most *Hind*III restriction site shown in Figure 1C, unpublished) and 3′ primer 5′-CTT-*TCTAGA*TTCTCGTGTGCGCCTGTG-3′, corresponding to the reverse complement of bases −21 to −4; respective *Sac*I and *Xba*I restriction site are italicized. Fragment 2 is a 670 bp *Xba*I−*Nco*I, PCR-generated fragment carrying the *GAL1* promoter (bases 146−816; numbering according to Johnston and Davis, 1981). The ATG codon within the *Nco*I site corresponds to the *GAL1* initiating methionine. Fragment 3 is a 2.2 kb *Xba*I, *LEU2*-containing fragment. Fragment 4 is a 3039 bp *Nco*I−*Bam*HI fragment carrying the entire *SWE1* coding region corresponding to bases 1−3039, where the ATG codon within the *Nco*I restriction site (generated by PCR amplification) corresponds to the *SWE1* initiating methionine. Derivatives of this cassette consisted of either substituting the *SWE1*-containing fragment 4 with the *SWE1-HA* allele or substituting the *LEU2*-containing fragment with a 1.1 kb *Nhe*I, *URA3*-containing fragment, or both. Plasmids carrying these cassettes were linearized prior to yeast transformation by digesting with *Pst*I and *Bam*HI in the case of the *LEU2*-containing cassettes and with *Sac*I and *Bam*HI in the case of *URA3*-containing cassettes.

Replacement of the genomic *HIS3* gene with the *GAL1:SWE1-HA* allele was accomplished by a two-step modification of the cassette described above. First, fragment 1 was replaced by a 425 bp *Bam*HI−*Xba*I fragment containing 5′-flanking, *HIS3* DNA (bases −447 to −25; numbering according to Struhl, 1985). Second, a fifth fragment was ligated to the *Bam*HI site of fragment 4. Fragment 5 is a 620 bp *Bcl*I−*Bam*HI fragment of 3′-flanking, *HIS3* DNA (bases +706 to +1323). These fragments were generated by PCR amplification. Transformation of this *Bam*HI-cut cassette into yeast generated the *his3::LEU2::GAL1:SWE1-HA* allele.

Construction of CDC28-T18V, -Y19F and -T18V,Y19F alleles

Mutagenesis of Cdc28 residues Thr18 and Tyr19 was accomplished by swapping a 430 bp *Xho*I−*Afl*II fragment (bases −339 to +90; numbering according to Lörincz and Reed, 1984) with a corresponding fragment which had been PCR-amplified using the T3 primer as the 5′ primer and the following 3′ 45mer primers,

T18V: 5′-AGGT*CTTAA*GTCTAACGCTTTATAAACAACACCGTAAA-CACCTTC-3′

Y19F: 5′-AGGT*CTTAA*GTCTAACGCTTTATAAACAACACCGAATG-TACCTTC-3′

T18V,Y19F:
5′-AGGT*CTTAA*GTCTAACGCTTTATAAACAACACCGAAAAC-ACCTTC-3′

The 3′ primer corresponds to the reverse complement of bases 46−90. The *Afl*II restriction site is italicized. Plasmid pRD47 (constructed by R.J.Deshaies) was used as template DNA and consists of an ~2 kb *Xho*I−*Pvu*II *CDC28*-containing fragment inserted into *Sal*I/*Sma*I-cut pRS316 (Sikorski and Hieter, 1989). The *Xho*I end of the insert was ligated to the *Sal*I site, while the *Pvu*II end was blunt-end ligated to the *Sma*I site. This *CDC28* clone also contains an *Nde*I restriction site at the initiating methionine. Swapping these amplified fragments with the wild-type fragment in pRD47 yielded plasmids pCDC28-T18Va, pCDC28-Y19Fa and pCDC28-T18V, Y19Fa. The *Xho*I−*Afl*II fragments were also swapped with the corresponding fragment in plasmid pSF19 (provided Peter Sorger), which carries a HA epitope-tagged *CDC28* gene (Sorger and Murray, 1992). The *CDC28* gene

from this series of plasmids was subcloned as a 1.8 kb *Xho*I−*Bam*HI fragment into the yeast integrating vector pRS305 (Sikorski and Hieter, 1989) creating plasmids pCDC28-T18Vd, pCDC28-Y19Fd and pCDC28-T18V,Y19Fd. These plasmids were linearized by digesting with *Xho*I and *Aat*II prior to transforming strain SCY22. Transplacement of the wild-type *CDC28* gene with mutant *CDC28* alleles was confirmed by restriction enzyme analysis.

Bacterial expression of Cdc28 and cyclins

For Cdc28 bacterial expression, a 980 bp *Nde*I−*Eco*RI *CDC28*-containing fragment, corresponding to bases +1−974, was cloned into *Nde*I/*Eco*RI-cut T7 expression vector pRK172 described in McLeod *et al.* (1987), a derivative of pAR3038 (Studier *et al.*, 1990), yielding plasmid pCDC28-5. The *Nde*I restriction site at the initiating ATG codon of *CDC28* and the *Eco*RI restriction site at base 974 were introduced by PCR amplification. Plasmid pCDC28-5 was modified to express mutant Cdc28 protein by swapping a 786 bp *Nde*I−*Kpn*I fragment (bases 1−786 of *CDC28*) with the corresponding *Nde*I−*Kpn*I fragment from plasmids pCDC28-T18Va, pCDC28-Y19Fa or pCDC28-T18V,Y19Fa. BL21(DE3)LysS cells (Studier *et al.*, 1990) carrying these plasmids were cultured to an A600 of 0.6−0.7, induced with 0.4 mM IPTG for 4 h at 22°C, and lysed by freeze−thawing and sonication. Extracts were clarified by ultracentrifugation, and the supernatant (25−45 mg protein per ml) was aliquoted, frozen in liquid nitrogen, and stored at −80°C. Details of Cdc28 expression and extract preparation will be published elsewhere by R.J.Deshaies and M.W.Kirschner (in preparation). GST−Clb2 is glutathione S-transferase fused to the N-terminus of Clb2 and involved ligating CLB2 into pGEX-1 (Smith and Johnson, 1988). The GST−Clb2 bacterial expression vector was constructed and provided by Douglas Kellogg. GST−Cln2 consisted of inserting CLN2 into pGEX-2T, generating glutathione S-transferase fused to the N-terminus of Cln2 (R.J.Deshaies, unpublished). GST−Clb2 and GST−Cln2 were purified from *E.coli* extracts by glutathione affinity chromatography.

Activation and purification of recombinant Cdc28 − cyclin kinase complexes

A ts *cdc28-4* strain was grown at 25°C in YEPD medium to an A600 of 2.6 and then shifted to 37°C for 4 h. Cells were collected, spheroplasted, lysed and the concentrated extract (48 mg/ml) was stored at −80°C. An *in vitro* activation reaction consisting of yeast extract, an ATP regeneration system, *E.coli* extract containing expressed Cdc28 protein and affinity-purified, *E.coli*-synthesized GST−cyclin was incubated at 24°C for 30 min, followed by purification of the Cdc28/GST−cyclin complexes using glutathione−agarose beads (Sigma). The details of extract preparation and the activation reaction will be published elsewhere by R.J.D. and M.W.Kirschner (in preparation). To purify and elute active Cdc28/GST−cyclin complexes, typically 1 ml of IPB (100 mM NaCl, 50 mM β-glycerophosphate pH 7.2, 5 mM EDTA, 0.2% Triton X-100, 2 mM DTT and standard protease inhibitors) and 200 ml of a 1:1 slurry of glutathione−agarose beads were added to a 100 µl activation reaction and mixed for 1.5 h at 4°C. The beads were pelleted, washed three times with IPB and twice with KAB (50 mM Tris pH 7.5, 10 mM MgCl₂ and 1 mM DTT). The Cdc28/GST−cyclin complexes were eluted with 100 µl KAB containing 10 mM glutathione (Sigma), 25% glycerol and 0.3 mg/ml ovalbumin. After a 5 min incubation at room temperature, with occasional mixing, the beads were pelleted and the supernatant was removed, aliquoted, frozen in liquid nitrogen and stored at −80°C.

Preparation of p18^CKS1^ − Sepharose beads

The *CKS1* gene was PCR-amplified from *S.cerevisiae* genomic DNA using the 5′ primer 5′-TGG*CATATG*TACCATCACTATCACG-3′, corresponding to bases 243−262 (numbering according to Hadwiger *et al.*, 1989a) and the 3′ primer 5′-TAG*GAATTC*AATTTCAGTAATTA-3′, corresponding to the reverse complement of bases 710−729; respective *Nde*I and *Eco*RI restriction sites are italicized. The resulting 490 bp amplified fragment was gel purified, digested with *Nde*I and *Eco*RI, and cloned into *Nde*I/*Eco*RI-cut pRK172, creating vector pCKS1-1. BL21(DE3)LysS cells containing pCKS1-1 were induced at A600 of 0.65 with 0.4 mM IPTG for 4 h at 37°C. The cells were collected, washed with PBS, and frozen at −80°C. After thawing, the cells were lysed by resuspending and sonicating in lysis buffer (50 mM Tris pH 8.0, 2 mM EDTA, 10% glycerol and standard protease inhibitors). In general, the cell pellet from a 125 ml culture was resuspended with 1 ml lysis buffer. The extract was clarified by microfuging for 10 min at 4°C, followed by ultracentrifugation for 15 min at 50 000 r.p.m. in a TL100.3 rotor at 4°C. Ammonium sulfate was added to 30% and incubated on ice. After 20 min, the insoluble fraction was collected by ultracentrifugation as before. The pellet, essentially pure Cks1 protein, was resuspended in lysis buffer and clarified by ultracentrifugation. Cks1 purification by Sepharose CL6B column elution, coupling to CnBr-activated

Sepharose 4B (Pharmacia) and bead washing were performed as described for p13^suc1^ bead preparation by Solomon *et al.* (1990). The reaction resulted in the coupling of 3 mg Cks1 per ml of gel. The p18^CKS1^ beads were stored as a 1:1 slurry in 0.5 M NaCl, 5 mM EDTA and 0.1 M Tris pH 8.0 at 4°C.

Immunoblotting and immunoprecipitation of Swe1 and p34^CDC28^

Yeast cells were pelleted, washed once with cold PBS and either immediately lysed or stored at −80°C for later processing. Typically 1 ml glass beads and 0.5 ml buffer H2 [25 mM Tris (pH7.5), 15 mM EGTA, 15 mM MgCl₂, 1 mM DTT, 2 mM sodium orthovanadate, 0.1 mM NaF, 60 mM β-glycerophosphate, 15 mM sodium pyrophosphate and standard protease inhibitors] were added to the cell pellet of a 50 ml culture. Samples were vigorously vortexed for three 1 min periods, followed by addition of 0.5 ml H2 and a further 30 s vortexing. The glass beads were pelleted by centrifugation and the crude extract was clarified by a 5 min microfuge spin, followed by ultracentrifugation for 10 min at 50 000 r.p.m. in a TL100.3 rotor at 4°C. Extracts were aliquoted, frozen in liquid nitrogen and stored at −80°C. Small-scale total yeast extracts for Swe1 immunoblot analysis were prepared as described by Stearns *et al.* (1990b).

For immunoblot analysis, proteins were resolved on a 5−15% SDS−polyacrylamide gel and transferred to Immobilon membrane (Millipore). The blots were blocked in Blotto [5% nonfat dry milk in TBST (0.2 M NaCl, 0.1% Tween and 10 mM Tris pH 8)] and incubated with 12CA5 antibody (4.6 µg/ml) or anti-Cdc28 antibody (1.3 µg/ml) in Blotto. The anti-Cdc28 antibody used was raised against a C-terminal peptide and had been affinity purified from polyclonal rabbit serum (R.J.Deshaies, unpublished). After washing with TBST, the blots were incubated in Blotto containing HRP-conjugated goat anti-mouse (1:10 000 dilution, Cappel) or HRP-conjugated donkey anti-rabbit (1:4000 dilution, Amersham) antibodies, as appropriate. Immunodetection was performed using an enhanced chemiluminescence (ECL) system (Amersham).

For Swe1-HA immunoprecipitations, extracts were prepared from strain SCY93 and control strain SCY31 (non HA-tagged Gal−Swe1) which had been induced with 2% galactose for 6 h. A standard immunoprecipitation consisted of incubating 0.25 µl 12CA5 monoclonal antibody (~11 µg) with 0.25 mg of SCY93 or SCY31 extract in buffer H2 containing 0.1% NP40 for 1 h at 4°C, followed by addition of 20 ml of protein A−Sepharose (1:1 slurry, Pierce). After incubating for 30 min at 4°C, the beads were pelleted, washed three times with H2S (H2 containing 0.5 M NaCl and 0.1% NP40) and twice with KAB (50 mM Tris pH 7.5, 10 mM MgCl₂ and 1 mM DTT).

Kinase reactions and phosphoamino acid analysis

For Swe1 phosphorylation of Cdc28, a solution containing 2 µl of eluted Cdc28/GST−cyclin complex, 0.5 µl 0.5 mM ATP and 2.5 µl KAB was added to Swe1 immunoprecipitate beads and incubated at room temperature for 10 min with occasional agitation. The histone H1 kinase activity of this reaction was assayed by adding 10 µl KAB containing 0.5 mg/ml histone H1 (Boehringer), 50 µM ATP and 1 µCi [³²P]ATP (ICN, 4500 Ci/mmol). After incubating at room temperature for 15−20 min, 20 µl of sample buffer were added and the entire sample was resolved by PAGE, followed by autoradiography. In cases where Cdc28 was repurified prior to assaying histone H1 kinase activity, 0.5 ml IPB was added and the Swe1 immunoprecipitate beads were pelleted. 0.45 ml of the supernatant was transferred to a new tube containing 60 µl of a 1:1 slurry of p18^CKS1^ beads. After mixing for 1 h at 4°C, the p18^CKS1^ beads were pelleted, washed three times with IPB, twice with KAB and assayed for histone H1 kinase activity as described above.

³²P-labeling of Cdc28 by Swe1. Swe1 immunoprecipitate beads were incubated with 5 µl of eluted Cdc28/GST−cyclin complex and 10 µl KAB containing 1.5 µM ATP and 50 µCi [³²P]ATP (ICN, 4500 Ci/mmol) for 30 min at room temperature with occasional agitation. The Cdc28/GST−cyclin complexes were repurified as described above using 75 µl of p18^CKS1^ beads. After washing, the p18^CKS1^ beads were resuspended with 60 µl sample buffer and resolved by PAGE, followed by autoradiography. For phosphoamino acid analysis, the labeling reaction was modified such that 10 µl of eluted Cdc28/GST−Clb2 were incubated with twice the standard amount of Swe1 immunoprecipitate beads and 80 µl of p18^CKS1^ beads were used to repurify the Cdc28/GST−cyclin complexes. The ³²P-labeled Cdc28 protein was resolved on 5−15% polyacrylamide gel. Cdc28 was exised from the gel by digestion with trypsin and the identity of the phosphoamino acids were determined by one-dimensional electrophoresis in the presence of unlabeled phosphoamino acids as described by Ward and Kirschner (1990).

Microscopy and flow cytometric analysis

For general analysis of nuclear morphology, yeast cells were fixed in 70% EtOH, washed with H₂O and stained with 0.3 µg/ml DAPI. Microtubule

staining of yeast cells was performed according to the procedure of Kilmartin and Adams (1984) with the modifications described by Stearns et al. (1990a). Cells were viewed on a Zeiss Axioskop and photographed with Kodak T-Max 400 film. For flow cytometric analysis, yeast cells were fixed in 70% EtOH, washed with 20 × TE, treated with 1 mg/ml RNase for 4 h at 37°C, washed with PBS, stained with propidium iodide (50 μg/ml) for 30 min and then diluted 10-fold with PBS. The cells were briefly sonicated prior to FACS analysis.

Acknowledgements

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Note added in proof

The nucleotide sequence data have been deposited in the EMBL/GenBank/DDBJ databases under the accession number X73966.