Cyclical inactivation of B-type cyclins has been proposed to be required for alternating DNA replication and mitosis. Destruction box-dependent Clb5p degradation is strongly increased in mitotic cells, and constitutive overexpression of Clb5p lacking the destruction box resulted in rapid accumulation of inviable cells, frequently multiply budded, with DNA contents ranging from unreplicated to apparently fully replicated. Loss of viability correlated with retention of nuclear Clb5p at the time of nuclear division. CLB2-Δdb overexpression that was quantitatively comparable to CLB5-Δdb overexpression with respect to Clb protein production and Clb-associated kinase activity resulted in a distinct phenotype: reversible mitotic arrest with uniformly replicated DNA. Simultaneous overexpression of CLB2-Δdb and CLB5-Δdb overexpressers similarly resulted in a uniform arrest with replicated DNA, and this arrest was significantly more reversible than that observed with CLB5-Δdb overexpression alone. These results suggest that Clb2p and not Clb5p can efficiently block mitotic completion. We speculate that CLB5-Δdb overexpression may be lethal, because persistence of high nuclear Clb5p-associated kinase throughout mitosis leads to failure to load origins of replication, thus preventing DNA replication in the succeeding cell cycle.

High Clb2-associated kinase activity blocks exit from mitosis

Cyclin-dependent kinase activity drives the eukaryotic cell cycle. In Saccharomyces cerevisiae, three G1, or CLN, cyclins and six B-type, or CLB, cyclins bind and activate the cyclin-dependent kinase Cdc28p. CLB function is required for initiation of DNA replication, spindle formation, and initiation of mitosis. With respect to DNA replication and mitosis, the main role of the CLN cyclins is to allow activation of Clnp-Cdc28 kinase, although the CLN cyclins have additional cell cycle roles (6, 26).

It is likely that all of the CLB cyclins are descendants of a single B-type cyclin-like ancestor, and it has been proposed (28) that a single B-type cyclin regulated both DNA replication and mitosis in a primordial eukaryotic cell. Multiple B-type cyclins derived from gene duplication have diverged in function. Functional divergence could simply reflect different timing of accumulation of functionally interchangeable cyclins; alternatively, specific cyclin cyclin coding sequences could have become intrinsically specialized for particular cell cycle roles. Recently, we showed that Clb5p is intrinsically specialized for activation of replication in comparison to Clb2p (8).

Clb5p-Cdc28p kinase drives some essential step(s) in replication, including the binding of Cdc45p and replication protein A (RPA) to the prereplicative complex (PRC) (44, 50). The PRC is formed by Cdc6p-dependent loading of minichromosome maintenance (MCM) proteins onto the origin recognition complex at origins of replication. PRC formation occurs in the absence of Cdk activity. B-type cyclin-associated kinase activity is thought to limit DNA replication to once per cell cycle by blocking loading of MCM proteins onto origins until B cyclin-Cdk inactivation at the end of mitosis (reviewed in references 26 and 29). There is conflict over whether inactivation of anaphase-promoting complex (APC) components allows rereplication in a single cell cycle even in the presence of Clb5p/Cdc28p kinase (17, 18, 32).

High Clb2-associated kinase activity blocks exit from mitosis: cells expressing high levels of Clb2p arrest with long spindles and separated chromosomes before cytokinesis (43). Thus, there is an additional requirement for the level of Clb-associated kinases to fall for the cell cycle to cycle. It is unclear if all Clb-associated kinases are efficient in inhibition of mitotic exit.

If Clb-associated kinases have both positive and negative roles in the cell cycle, their accumulation and degradation must be accurately regulated. Clb2p degradation is restricted to late mitosis after chromosome separation and the subsequent G1 period before initiation of the succeeding cell cycle. This is probably due to the requirement for Cdh1p to associate with the APC to allow Clb2p degradation. Cdh1p is inactive due to Cdk-mediated phosphorylation and is activated late in the cell cycle by the Cdc14p phosphatase (20, 41, 47). Clb5p is not under the control of Cdh1p, and relatively little cell cycle regulation of Clb5p degradation has been observed (39), although its degradation was reported to be destruction box dependent and dependent on components of the APC that are also required for Clb2p ubiquitination and degradation (19).

Clb5p and the anaphase inhibitor Pds1p may both be targets of Cdc20p-directed APC degradation, because it has been observed that deleting CLB5 rescues cdc20 pds1 strains which would otherwise arrest in late anaphase, and deleting cdc20 stabilizes Clb5p (40)

Here we report on cell cycle dependence of Clb5p degradation. We also compare the effects of expression of stabilized Clb5p and Clb2p on cell cycle progression, to examine the issue of intrinsic Clb specialization in driving cell cycle events.

MATERIALS AND METHODS

Yeast strains. All strains are isogenic with 15Dau (MATα leu2 ura3 trp1 his2 ade1) (33). Strains were constructed and analyzed by standard genetic methods. DNA transformations were done by the lithium acetate method.

Plasmids. All plasmids were derived from CE119, a YCP50-based GAL1::CLB5 (100,000 base pairs) (11, 30), CE119-4, a hemagglutinin (HA)-tagged version of CE119, and the GAL1::CLB5-Δdb-HA construct DB4 were described previously (8). A point mutation, S399P, presumably generated during the PCR-based construction of CE119 (11), was present in CE119-4 and in the GAL1::CLB5-Δdb-HA construct. The S399P point mutation is relatively innocuous; it slightly reduces Clb5p function, but CLB5-S399P under control of the CLB5 promoter fully rescues the clb5 replication defect (12) and clb34,5,6 lethality (37), and GAL1::CLB5-Δdb-S399P is lethal with a phenotype
similar to that of the S399S version (data not shown). The experiments in Fig. 1, 2, 4, and 5 were performed with S399P versions of GAL1::CLB5. The remaining experiments were performed with constructs in which the S399P mutation was repaired by subcloning the wild-type fragment to make CE19-4R and DB4R. We assume that this does not significantly affect the results. The destruction box-dependent instability of Clb5p documented in Fig. 2 has been found to be similar to those of S399S and S399P versions of GAL1::CLB5 (compare Fig. 2B and A). The control GAL1-clb5fsHA construct is a product of the GAL1-CLB5fsHA plasmid, and one copy was inserted in the GAL1-CLB5dbHA plasmid. The Myc-tagged GAL1-CLB5 constructs all contained the S399P mutation. Integrating derivatives of these plasmids were constructed by digestion with SmaI and HpaI and religation to eliminate the CEN4 sequence and targeted for integration at ARS1 by BglII digestion. For the GAL1::CLB2 plasmids, integration was targeted to CLB2 by Xhel digestion.

**RESULTS**

**Clb5p degradation is cell cycle regulated.** We constructed a cln1 cln2 cln3 GAL1::CLB5-HA strain, in which CLB5-HA substitutes for the CLN1, CLN2, and CLN3 genes in driving cell cycle initiation (12). This strain was synchronized by raffinose block for 2.5 h to turn off GAL1::CLB5HA transcription, followed by galactose addition to 3% to release the block. GAL1-CLB5fsHA transcription was then turned off again at various times by the addition of glucose to a final concentration of 2%. (A) Graphs plotting the percent unbudded cells (% UB) against the time after release. The black bar represents the time at which glucose was added to the culture. (B) Clb5p immunoblots and percent unbudded cells in a synchronized culture (top) and in synchronized cultures with GAL::CLB5HA (bottom) released at various times post release. The black bar represents the time at which glucose was added to the culture. (C) Clb5p of Clb5p degradation was visualized on a Zeiss Axiophot microscope, and images were not processed for immunofluorescence. Images were manipulated with Photoshop software. Images were treated identically by Photoshop manipulations.

GAL1-CLB5-HA was constructed by digestion with SmaI and religation to eliminate the CEN4 sequence and targeted for integration at ARS1 by BglII digestion. For the GAL1::CLB2 plasmids, integration was targeted to CLB2 by Xhel digestion.

**Growth conditions.** Cells were grown at 30°C in yeast extract-peptone YEP medium. Arrest of cultures with α-factor was done by incubating log-phase cultures (22). For immunofluorescence, cultures were fixed at the time of nuclear division (Fig. 1). Consistent with this, shutoff of the GAL1 promoter at any time before 100 min (approximately the time of nuclear division) resulted in failure to bud in the next cell cycle (Fig. 1), consistent with a drop in Clb5p to a nonfunctional level during division (12, 30).

To confirm these results in a wild-type background and to address the role of the Clb5p destruction box in Clb5p degradation, we constructed wild-type strains containing integrated GAL1::CLB5-HA or GAL1::CLB5-db-HA (lacking amino acids 56 to 64) (8). Addition of galactose to cultures of these strains yielded comparable initial accumulation of Clb5p and Clb5dbp protein and associated kinase (Fig. 2A). Glucose addition to such galactose-induced cultures to inactive GAL1-driven transcription showed that the destruction box-containing protein decayed much faster than the destruction box-deleted protein (Fig. 2B). A longer time course, however, revealed that Clb5p lacking its destruction box was still somewhat unstable (Fig. 3A). In addition, at best, minor stabilization of Clb5dbp protein was observed when it was expressed from the endogenous CLB5 promoter (Fig. 3B and C), consistent with a hypothesis that additional regions of Clb5p may contribute to its targeted degradation. Involvement of a Skp1-Cdc53-F box (SCF)-ubiquitinating activity in Clb5p degradation was suggested previously (4).

We employed timed glucose addition to shut off GAL1::CLB5-HA.
CLB5-HA transcription in cells synchronized by α-factor block-release, in which the cultures were blocked in raffinose plus α-factor medium and released into galactose medium lacking α-factor (YPαGal). (C) Following synchronization with α-factor, cultures were released into YPGal. The GAL1-driven transcription was turned off by the addition of glucose to a final concentration of 2% (time zero) at 60, 90, 120, and 150 min after release. Immunoblots for Clb5HA and Clb5ΔdbHA from one (1×) or two (2×) copies of GAL1::CLB5HA and for Clb5ΔdbHA from one copy of GAL1::CLB5ΔdbHA (top) are shown, as are DNA content profiles generated by FACS analysis, percent unbudded cells, and percent of divided nuclei (% DN) as assayed by the presence of two DAPI-stained spots for the 1× GAL1::CLB5HA (1), 2× GAL1::CLB5HA (2), and GAL1::CLB5ΔdbHA (d) cultures (bottom).
cell. This pattern indicates that Clb5p accumulates in the nucleus before budding and during DNA replication, but during mitosis, Clb5p nuclear abundance is strikingly reduced.

We compared the localization patterns of Myc-tagged protein throughout the cell cycle in GAL1::CLB5-MYC, GAL1::CLB5Δdb-MYC, and control strains blocked in raffinose plus α-factor medium and released into galactose medium lacking α-factor. Strains were either released into yeast extract-peptone-dextrose (YPD) (B) or released into YPD and rearrested with α-factor (+αF) 60 min later (C). Synchrony was gauged by counting the percentage of unbudded cells. CLB5Δdb expression was monitored by immunoblots (anti-HA) and associated histone H1 kinase blots (H1-P).

FIG. 3. Instability of Clb5Δdb protein when expressed from GAL1 and CLB5 promoters. All strains have the indicated constructs integrated in 1255-5C (wild type). (A) Cultures were grown overnight to log phase in YEP medium containing 3% raffinose (YPRaf). Following 3 h of incubation with 3% galactose to induce the GAL1 promoter, 2% glucose was added to repress expression of GAL1::CLB5HA and GAL1::CLB5ΔdbHA. Protein levels were monitored by Western blotting against the HA tag. (B and C) Strains containing CLB5HA under the control of its endogenous promoter (CLB5-CLB5 HA and CLB5-CLB5ΔdbHA) were synchronized in G1 with α-factor. Strains were either released into yeast extract-peptone-dextrose (YPD) (B) or released into YPD and rearrested with α-factor (+αF) 60 min later (C). Synchrony was gauged by counting the percentage of unbudded cells. CLB5Δdb expression was monitored by immunoblots (anti-HA) and associated histone H1 kinase blots (H1-P).
had little additional effect on these phenotypes (data not shown).

In contrast, CLB5-db expressed from its own promoter was not lethal (8), even in the absence of SIC1 (data not shown). Therefore, loss of transcriptional control (of periodicity, levels or both), in addition to loss of either proteolytic or Sic1p control, was required to elevate Clb5p activity sufficiently to achieve lethality. Clb5p-db expressed from the CLB5 promoter was moderately if at all stabilized in cell cycle time courses, in contrast to the strong stabilization observed with GAL1::CLB5-db (Fig. 2 and 3). It may be that overexpression of Clb5p-db is required to saturate some means of Clb5p degradation that is independent of the identified destruction box. These observations on expression of CLB5-db pose a paradox with respect to the results of Shirayama et al. (40). If Cdc20-dependent degradation of Clb5p is destruction box dependent, and if failure of Cdc20-dependent degradation of Clb5p (expressed from its own promoter) blocks mitotic exit, then CLB5-db expression should similarly result in significant persistence of Clb5p and a block to mitotic exit. There may be additional unidentified destruction boxes in Clb5p (S. Holloway, unpublished data), or there may be Cdc20-dependent but destruction box-independent means of Clb5p degradation. We are exploring these possibilities.

Since GAL1::CLB5-db and GAL1::CLB5 yield nearly comparable levels of protein through most of the cell cycle, it seemed likely that the lethality of GAL1::CLB5-db may require specific persistence of Clb5p through mitosis, when Clb5p but not Clb5-db is degraded. Since Sic1p protein accumulation is induced during late mitosis, this may explain the lethality of GAL1::CLB5 in sic1 strains: a low level of residual Clb5p escaping degradation may require Sic1p inhibition to avoid lethality due to active Clb5p complexes persisting through mitosis. Overexpressed Clb5-db may saturate the available Sic1p.

Confirming that high levels of Clb5p activity are required to induce lethality, the introduction of a double mutation (K253A, E282A) that partially interferes with Cdc28p kinase activation into the GAL1::CLB5-db strain relieved lethality (8). Deletion of SIC1 made expression of GAL1::CLB5-KA,EA-db lethal (data not shown), again confirming that destruction box-dependent degradation and Sic1p inhibition can correlate Clb5p activity, probably specifically in mitosis. GAL1::CLB5-KA,EA was not lethal even in the simultaneous absence of sic1 and swe1 (data not shown). The KA,EA mutation does not significantly affect degradation rates of Clb5p with or without its destruction box (data not shown).

**Characterization of GAL1::CLB5-db lethality.** GAL1::CLB5-db cells grown in raffinose medium arrest after several hours of galactose induction. Arrest is associated with lethality in that the plating efficiency of these cells on glucose medium drops approximately 1,000-fold by 4 to 6 h of galactose incubation (see Fig. 8C and 9C). After approximately 2 h of expression, the GAL1::CLB5-db cells appear to delay as binucleate large budded cells with a 2C DNA content, resembling a GAL1::CLB2-db-induced arrest (data not shown), but cells escape this mitotic block over the succeeding 2 h. By 4 to 6 h, the cells are heterogeneous with respect to DNA content: approximately half of the cells have a 2C (replicated) DNA content, and almost half have only 1C (Fig. 8B and 9B). There is usually some accumulation of cells that appear to have intermediate DNA contents. The cells are predominately large budded, and frequently they rebud after 4 h of galactose induction (Table 1). Tubulin staining by indirect immunofluorescence indicates that the DNA signals (DAPI) are heterogeneous with respect to DNA content: approximately half of the cells have a 2C (replicated) DNA content, and the other half have only 1C (Fig. 8B and 9B). There is usually some accumulation of cells that appear to have intermediate DNA contents. The cells are predominately large budded, and frequently they rebud after 4 h of galactose induction (Table 1). Tubulin staining by indirect immunofluorescence indicates that most of the cells are arresting with postmitotic spindles (Fig. 6). The DNA signals (DAPI) are heterogeneous in strength, compared to those of wild-type controls, possibly the result of an unequal distribution into the mother and daughter buds (Fig. 6). Combined with the FACS analysis showing a significant population of cells with approximately 1C DNA content, these results suggest that some cells have undergone abortive mitosis despite failure of DNA replication (31).

We interpret this phenotype as indicating that Clb5-db overexpression delays completion of mitosis, but many cells nevertheless ultimately divide in the presence of Clb5-db. These cells do not efficiently replicate DNA after division, accounting for the accumulation of cells with 1C DNA content. The GAL1::CLB5-db rebudding phenotype (Table 1) may be due to the initiation of a G1 cell cycle program without properly completing the later stages of mitosis and cytokinesis. The rebudding phenotype was significantly reduced when the cells were processed for indirect immunofluorescence. This most likely results from digestion of the cell wall during sample preparation and could indicate that cytokinesis (but not cell...
separation) is largely complete in many of the apparently re-
budded cells.

Comparison between the GAL1::CLB5-Db and GAL1::
CLB2-Db phenotypes. GAL1::CLB2-Db was reported to
cause uniform arrest in late mitosis with replicated DNA (43),
unlike the GAL1::CLB5-Db phenotype we observed; these
cells also did not rebud, unlike the GAL1::CLB5-Db cells
(Table 1). These differences could be due to intrinsic differ-
ences in the ability of Clb2p and Clb5p to block exit from
mitosis; alternatively, levels of expression could differ between
the two GAL1::CLB-Db constructs. Therefore, we replaced
the CLB5 coding sequence with the CLB2 coding sequence in
the GAL1::CLB5-Db construct. We found that these con-
structs yielded comparable levels of Clb protein and associated
kinase activity (Fig. 7). As reported previously (43), GAL1::
CLB2-Db expression results in late mitotic arrest, with few or
no cells with unreplicated DNA detected (Fig. 8) and almost
cells displaying elongated spindles with the replicated DNA
separated into the mother cell and the bud (Fig. 6). This
phenotype was similar with or without the HA tag on GAL1::
CLB2-Db (data not shown). In addition, the rapid induction
of inviability by GAL1::CLB5-Db was not observed with
GAL1::CLB2-Db (Fig. 8). The multiple-budding phenotype of
GAL1::CLB5-Db was also not observed with GAL1::CLB2-
Db (Table 1). Thus, even at comparable levels of expression,

Table 1. Budding and rebudding percentages resulting from
GAL1-induced CLB2 and CLB5 constructs

<table>
<thead>
<tr>
<th>Genotype</th>
<th>% of cells⁶</th>
<th>Expt 1</th>
<th>Expt 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Budded</td>
<td>Re budded</td>
<td>Budded</td>
</tr>
<tr>
<td>Set I</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type</td>
<td>67</td>
<td>0</td>
<td>68</td>
</tr>
<tr>
<td>GAL1::CLB2</td>
<td>59</td>
<td>0</td>
<td>60</td>
</tr>
<tr>
<td>GAL1::CLB2-Db</td>
<td>88</td>
<td>0</td>
<td>90</td>
</tr>
<tr>
<td>GAL1::CLB5</td>
<td>67</td>
<td>2</td>
<td>68</td>
</tr>
<tr>
<td>GAL1::CLB5-Db</td>
<td>56</td>
<td>39</td>
<td>56</td>
</tr>
<tr>
<td>Set II</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type + vector</td>
<td>54</td>
<td>2</td>
<td>65</td>
</tr>
<tr>
<td>GAL1::CLB5-Db</td>
<td>68</td>
<td>27</td>
<td>69</td>
</tr>
<tr>
<td>GAL1::CLB2-Db</td>
<td>64</td>
<td>22</td>
<td>48</td>
</tr>
<tr>
<td>GAL1::CLB5-Db</td>
<td>54</td>
<td>4</td>
<td>53</td>
</tr>
</tbody>
</table>

⁶ Percentages of budded and rebudded cells were determined by counting 200
cells for each sample in two separate experiments. The samples were processed
by the methods described for Fig. 7 (set I) and 8 (set II) following 6 h of galactose
induction. Budded cells represent only singly budded cells, and rebudded cells
represent all variations of multiply budded cells (typically either three or four cell
bodies).
the phenotypes due to overexpression of stabilized Clb2p and Clb5p are different.

We also carried out experiments in which strains were synchronized in G1 by using alpha-factor and then released into galactose medium to induce \( \text{GAL1::CLB2-D} \) or \( \text{GAL1::CLB5-D} \) expression. For reasons that we do not understand, in this protocol, \( \text{GAL1::CLB5-D} \) expression resulted in a long preanaphase delay with apparently fully replicated DNA (data not shown); such a delay was not detected in the experiments (Fig. 6) in which galactose was added to asynchronous cultures. This difference makes it hard to directly compare results between the two protocols. Despite this, a significant population of the \( \text{GAL1::CLB5-D} \) cells eventually escaped this block and divided their nuclei, and, overall, about 30\% of the cells then rebudded (data not shown). In contrast, the \( \text{GAL1::CLB2-D} \) cells arrested stably late in mitosis with divided nuclei and without rebudding, as was seen in the experiments where galactose was added to asynchronous cultures (Fig. 6 and 8).

These results suggest that a new cell cycle is being initiated despite the presence of overexpressed Clb5p, but that this is blocked by overexpressed Clb2p, consistent with the results when galactose was added to asynchronous cultures (Fig. 8).

**Clb5p is inefficient at blocking mitotic exit.** Clb2p is probably the major B-type cyclin active in mitosis (26, 27, 43). Previous studies have shown that Clb2p-associated kinase activity must be eliminated for completion of division and for proper loading of DNA replication origins during G1 (10, 15, 43). Clb2p can be stabilized by Clb2p-Cdc28p or Clnp-Cdc28p (1). If Clb5p-Cdc28p also stabilizes Clb2p, then some aspects of the phenotype due to overexpression of \( \text{CLB5-D} \) could be indi-
rect, due to stabilized Clb2-associated kinase activity. To test this, we constructed GAL1-CLB5 clb2::LEU2 strains, with or without the destruction box and with or without the KA,EA mutation in CLB5. clb2 deletion enhanced inviability due to CLB5, since GAL1::CLB5 and GAL1::CLB5-KA,EA-Db were lethal in a clb2 background. clb2 deletion also enhanced the speed of induction of irreversible lethality due to GAL1::CLB5-Db, although this effect was somewhat variable (data not shown). In almost all cases, GAL1::CLB5-Db induction in clb2 strains resulted in a significantly greater accumulation of cells with 1C DNA content than was observed in CLB2 strains (data not shown).

These results suggested that Clb2p might be restraining mitotic exit in the GAL1::CLB5-Db overexpressers. We constructed a strain expressing both GAL1::CLB5-Db and GAL1::CLB2-Db. The strain arrested as large budded cells with a 2C DNA content (Fig. 9) where the replicated DNA was separated into the mother and daughter cell bodies (data not shown), typical of GAL1::CLB2-Db-induced arrest. This is consistent with the idea that the accumulation of 1C DNA content in cells with GAL1::CLB5-Db alone requires mitosis, which may be blocked by GAL1::CLB2-Db expression. Similarly, GAL1::CLB2-Db expression significantly reduces the number of cells displaying the characteristic GAL1::CLB5-Db rebudding phenotype (Table 1), consistent with the inhibitory effects of Clb2p on cell polarization and bud emergence reported previously (2, 23). Although the reversibility of the arrest in the GAL1::CLB5-Db/GAL1::CLB2-Db strain was reduced from that observed in GAL1::CLB2-Db cells, viability was increased 10-fold from strains expressing GAL1::CLB5-Db alone (Fig. 9). These results support the idea that Clb2p can restrain mitotic exit in GAL1::CLB5-Db expressers and that the severity of GAL1::CLB5-Db-induced lethality is correlated with an accumulation of cells containing a 1C DNA content. Accumulation of 1C cells may not be the sole cause of irreversible arrest, because GAL1::CLB5-Db-expressing cells arrest with a heterogeneous population of DNA content. This is difficult to interpret fully, though, because these cells may ultimately divide upon plating for the viability assay.

**DISCUSSION**

**Clb5p degradation.** We find that Clb5p degradation is cell cycle regulated, and Clb5p is most unstable in dividing cells. These differences in stability correlate with loss of detectable nuclear accumulation of Clb5p in dividing cells (although significant cytoplasmic signal remains). We speculate that the specific loss of Clb5p from the nucleus may be due to nucleus-localized degradation, but further work is required to eliminate other possibilities (for example, that Clb5p is exported from the nucleus during mitosis and is independently rendered less stable at this time).

Clb5-Dbp expressed from the CLB5 promoter has at best minor phenotypes, even in the absence of sic1, and destruction box deletion from endogenously expressed Clb5p also results in, at most, minor stabilization of the protein, monitored with HA-tagged Clb5p in synchronous culture (Fig. 3 and data not shown). Destruction box-independent degradation of Clb5p...
FIG. 9. Overexpression of Clb2ΔdbHA in a GAL1::CLB5ΔdbHA strain blocks cells in mitosis and partially suppresses lethality due to GAL1::CLB5ΔdbHA. Wild-type (WT) 1255-5C transformed with vector (V) and 1255-5C with integrated GAL1::CLB5ΔdbHA (transformed with either vector, pGAL1::CLB2Δdb, or pGAL1::CLB2ΔdbHA) were grown overnight in synthetic complete raffinose-uracil medium. The cells were harvested by centrifugation and resuspended in galactose medium lacking α-factor (YPGal) to induce the GAL1 promoter. Samples were taken at 2-h intervals while the cultures were incubating at 30°C. The samples were analyzed for protein levels by an anti-HA Western blot (A), DNA content by FACS analysis (B), and viability by 10-fold serial dilutions on yeast extract-peptone-dextrose (YPD) and YPGal (C). Samples from all time points resulted in identical YPGal plating efficiencies, and therefore only a representative time point is shown.

may be responsible, or there may be other unidentified destruction boxes in Clb5p. In either case, the overexpression of Clb5Δdbp from the GAL1 promoter may swamp out these alternative means of degrading Clb5p.

Cyclin specificity. The six CLB B-type cyclins have distinct roles in vivo, although they overlap significantly in function. Distinct in vivo roles could be due to time of expression during the cell cycle or to intrinsic specialization of the different CLB coding sequences. Previously, we showed that Clb5p was much more potent at inducing DNA replication than Clb2p, even when time of expression and protein accumulation were made comparable, by placing both under control of the CLB5 promoter (8). Here, we examine the cell cycle-inhibitory activity of Clb5p compared to that of Clb2p. To make this comparison, we eliminated differential transcriptional control by using the GAL1 promoter and eliminated differential proteolytic control by removing the proteins’ destruction boxes. We find that while Clb2p is able to block exit from mitosis, as reported previously, Clb5p is relatively weak at this activity, even when strongly overexpressed. Even the limited apparent capacity of Clb5p to inhibit mitotic exit is likely to be dependent on endogenous Clb2p. Clb5p could recruit endogenous Clb2p for this role by stabilizing it, perhaps by phosphorylation of Cdh1p (1, 49). In addition, the potency of existing Clb2p may be increased due to Clb5p-dependent phosphorylation of Sic1p (46). The idea that Clb5p restrains mitotic exit by phosphorylation of Cdh1p and Sic1p was also suggested by Shirayama et al. (40).

Accumulation of cells with unreplicated DNA in GAL1::CLB5Δdb could occur due to high Clb5p-associated kinase (9), combined with the permissiveness of high Clb5p-associated kinase for completion of mitosis. The consequence may be that cells divide without being able to initiate DNA replication after division. If cells under these conditions pass the “point of no return” (31) when endogenous Clb-associated kinases block reloading of replication origins, then even after shut off of GAL1::CLB5-Δdb, irreversible lethality is predicted. It is also possible that some of these cells undergo anaphase without DNA replication, leading to unequal segregation of the haploid DNA content (31), a clearly lethal event.

The molecular basis for differences in cyclin specificity is unknown. The results reported here and previously (8) do not suggest that differential accumulation of the protein or associated kinase is responsible, although subtle differences in timing or levels are hard to rule out unambiguously. A candidate substrate-targeting domain (the hydrophobic patch [8, 34]) could function differently between Clb5p and Clb2p, potentially leading to differential substrate targeting. This region contributes to but is not essential for lethality of GAL1::CLB5-Δdb (8). The analogous region in Clb2p may be required for efficient blocking of mitotic exit in GAL1::CLB5-Δdb. GAL1: CLB2Δdb overexpressers in the assay shown in Fig. 9 (data not shown). Failure to detect a strong requirement for this region for some effects of Clb-Δdb overexpression may be due to masking the role for the region due to high expression levels. The region is required for efficient lethality of Clb5p in the absence of cdc20 and pds1 and is also required for efficient Clb2p mitotic function at lower levels of expression (7).

S and M cyclins and the organization of the yeast cell cycle. CLB5 and CLB6 have been called S-phase cyclins and CLB1 and CLB2 have been called M-phase cyclins, due to their time of expression and evident function as deduced from null phenotypes (12, 15, 26, 27, 37, 43). In fission yeast, cdc13 appears to be the predominant M-phase cyclin, and cig1 (with help from cig7) may be the major S-phase cyclin. Despite this differentiation of function among B-type cyclins, models have been proposed for both budding yeast and fission yeast in which a single generic B-type cyclin-dependent kinase activity could be sufficient to control both the S and M phases in proper alternation (13, 26, 42). These models suggest that functional differences in the roles of different B-type cyclins (as deduced from null phenotypes) are due solely to differential accumulation due to transcriptional or proteolytic controls. At least for budding yeast, this appears to be an oversimplification. Previously we showed that Clb5p is intrinsically specialized for induction of DNA replication, functioning much better
at this activity than Cdc2p (8). The results reported here suggest that Cdc2p has a specific function of restraining mitotic exit, which is weak or absent in Cdc5p. This difference is not due to defects in Cdc5p protein accumulation or kinase activation, and thus the difference is likely to be intrinsic to the protein. Thus, at least for these two activities, one early in the cell cycle and one at the end, the yeast cell cycle is driven by early accumulation of S cyclins, including Cdc5p, and late accumulation of M cyclins, including Cdc2p, which are intrinsically specialized for appropriate roles.

cdc20 pds1 cells are blocked late in mitosis (25), and this block has been attributed to failure of Cdc20p-dependent Cdc5p degradation (40). These observations lead to the prediction that expression of stabilized Cdc5p in mitotic cells should permanently block mitotic exit. In contrast, upon expression of GAL1::CLB5-Δdb, we observe only a transient mitotic delay followed by completion of mitosis (with concomitant loss in cell viability). From our results, it appears likely that restraint of mitotic exit in cdc20 pds1 cells (25), while genetically due to Cdc5p (40), may be more directly due to Cdc5p-dependent activation of mitotic cyclins, including Clb2p. We do not know why this effect is only transient under our conditions.

In addition to regulating mitotic exit, Clb activity must somehow lead to mitotic entry (for example, by activation of Cdc20p [21] leading to Pds1p degradation [48] and/or by some direct activation of spindle function). A specific role for Clb5p in some aspects of spindle function has been suggested based on the phenotype of clb3,4,5,6 diploid strains (37) and on analysis of clb5 cdc28-4 diploid strains (38). Thus, some aspects of spindle function may be more efficiently driven by early-expressed cyclins such as Cdc5p than by late-expressed cyclins such as Clb2p.

Clb1,2,3,4p activity has been implicated in down-regulation of expression of SBF-regulated genes, such as the G1 cyclins CLN1 and CLN2 (3), while Cdc5p probably lacks this activity, and at least under some circumstances may actually drive expression of this class of genes (24, 30). This may partially and at least under some circumstances may actually drive expression of late-expressed genes (24, 30). This may partially and at least under some circumstances may actually drive expression of late-expressed genes (24, 30).

The linkage between these sets of functions is unknown, but it is an intriguing possibility that Cdc5p may be important for stabilizing Clb2p through Cdh1p phosphorylation (1, 40, 49). Since Cdh1p is not involved in Cdc5p degradation (35), this could allow early accumulation of Cdc5p and performance of Cdc5p-specific early cell cycle functions, followed by accumulation of Clb2p. Transcriptional positive feedback control of CLB2 (3) could enhance temporal segregation of accumulation of different cyclins.

Fission yeasts are able to proliferate fairly normally with only one of the three identified B-type cyclins (cdc13), suggesting that this system may be significantly simpler than the budding yeast system. (It is worth noting, though, that the fission yeast sequencing project, while still incomplete, has identified a fourth B-type cyclin [EMBL locus SPBC16E9; accession no. Z99759.1] whose involvement in S phase and mitosis has not been explored to our knowledge.) Even in budding yeast, sufficient overexpression of the CLB1 B-type cyclin is sufficient for viability in the absence of CLB2-6 (16), indicating that it there is no absolute requirement for differentially targeted B-type cyclins for cell cycle progression. This is consistent with the evolutionary speculation (28) that the primordial eukaryotic cell cycle was driven by a single B-type cyclin capable of driving events in DNA replication and in mitosis. In metazonans, it is likely that cyclins A and E are specialized for induction of mitosis, and this situation may be closer to the budding yeast system when cyclins are expressed at ectopic levels.

REFERENCES

oehlen, l. j. w. m., d.-i. jeoung, and f. r. cross.

newlon, c. s.

schwob, e., t. bohm, m. d. mendenhall, and k. nasmyth.

nasmyth, k.

schulman, b. a., d. l. lindstrom, and e. harlow.

richardson, h. e., c. wittenberg, f. cross, and s. i. reed.

schulman, b. a., d. l. lindstrom, and e. harlow. 1998. substrate recruitment to cyclin-dependent kinase 2 by a multipurpose docking site on cyclin a. proc. natl. acad. sci. usa 95:10453–10458.


verna, r., r. s. annan, m. j. huddleston, s. a. carr, g. reynard, and r. j. deshaies. 1997. phosphorylation of Sic1p by G1 Cdk required for its degradation and entry into S phase. Science 278:455–460.

visintin, r., k. craig, e. S. hwang, s. prinz, m. tyers, and a. amon. 1998. the phosphatase Cdc14 triggers mitotic exit by reversal of Cdk-dependent phosphorylation. Mol. cell. 2:709–718.
