Testing Cyclin Specificity in the Exit from Mitosis

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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 overexpressors 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.

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 Clbp-Cdc28p kinase, although the CLN cyclins have additional cell cycle roles (6, 26).

It is likely that all of the CLB cyclins are descendents 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 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 at 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 (MATa 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 construct (GAL1::CLB5 URA3 CEN4 ARS1) (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 clb3,4,5,6 lethality (37), and GAL1::CLB5-Δdb-S399P is lethal with a phenotype
similar to that of the S399P 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 CE119-4R 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 S399P and S399P versions of GAL1::CLB5 (compare Fig. 2B and A).

We constructed GAL1::CLB5-HA and GAL1::CLB2-HA by digestion of galactose-induced cultures to inactivate GAL1::CLB5-HA and GAL1::CLB2-HA by incubating cultures with raffinose for 2.5 h to turn off GAL1::CLB5-HA, followed by galactose addition to turn on clb5p transcription (4). Addition of glucose to a final concentration of 3% to release the block, GAL1::CLB5-HA 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. (B) Clb5-HAp immunoblots and percent unbudded cells in a synchronized culture (top) and in synchronized cultures with clb5p transcription off (bottom). Clb5HAp is undetectable in raffinose-arrested cultures of this strain (data not shown).

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 Clb5Δdp protein and associated kinase (Fig. 2A). Glucose addition to such galactose-induced cultures to inactivate 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 Clb5Δdp 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 transcription and scored for clb5p transcription in yeast cultures synchronized with galactose and raffinose for 2.5 h. Glucose addition was followed by galactose addition to turn on clb5p transcription, and samples were collected either 40 or 80 min after release (bottom). In addition, we employed 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).

Glc functions as a signal for growth arrest in response to low nutrient levels. Arrest of cultures with YEP-raffinose (3%) (YEPRaf) cultures with histone H1 kinase assays were performed as described previously (22). For histone H1 kinase assays, we used a citrate buffer (17.4 g of K2HPO4 [anhydrous], 7 g of citric acid, 218.6 g of sorbitol, 2 ml of 1 M dithiothreitol [DTT] in 1 liter of water). The cell walls were digested in sorbitol-citrate buffer with 1 mM DTT, 0.01% zymolyase 20T (wt/vol), 10% glucose in a 30°C water bath for 2 h with occasional gentle mixing. Cells were washed three times in sorbitol-citrate buffer. Cells were deposited in wells on a microscope slide by completely aspirating off the buffer, dehydrating the slide for 5 min in methanol and 5 min in acetone, and quickly air drying the slide. Cells were rehydrated in blocking solution (PBS, 0.2% Tween 20, 0.01% wt/vol nonfat dry milk) at room temperature for 30 min and incubated with the primary antibody (9E10 mouse anti-Myc monoclonal antibody [Santa-Cruz Biotechnol- ogy] or YOL1-32 rat antitubulin monoclonal antibody [33]) in blocking solution at a 1:200 dilution for 2 h at room temperature in a humidified chamber. Cells were washed four times briefly and three times for 5 min in blocking solution before adding the secondary antibody (antimouse fluorescein isothiocyanate [FITC]-conjugated polyclonal antibody for Myc, antiantig- Conjugated polyclonal antibody for tubulin [Jackson ImmunoResearch Laboratories]) at a 1:200 dilution in blocking solution for 2 h at room temperature in the dark in a humidified chamber. Cells were washed as before and then washed three times briefly with PBS before being mounted under a slide cover with mounting medium (10% PBS in glycerol with 22.5 g of 4',6'-diamidino-2-phenylindole [DAPI] per ml and 1 mg of phenylendiamine per ml). DAPI staining in samples not processed for immunofluorescence was done as previously described (45). Fluorescence was visualized on a Zeiss Axiovert microscope, and images were captured with a Sony digital photo camera (DKC-5000) by using Photoshop software. Images were manipulated with Photoshop software. Comparable images were treated identically by Photoshop manipulations.

RESULTS

Clb5p degradation is cell cycle regulated. We constructed a cln1 cln2 cln3 GAL1::CLB5-HA strain, in which CLB5-HA substitutes for the CLN G1 cyclins in driving cell cycle initia- tion (12, 30). This strain was synchronized by raffinose block in G1, followed by release into the cell cycle with galactose addition to induce expression of the GAL1 promoter. Using timed glucose addition to repress GAL1::CLB5-HA expression, we saw a sharp increase in Clb5p instability approximately coincident with 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).

First, we constructed a GAL1::CLB5-HA strain, in which CLB5-HA substitutes for the CLN G1 cyclins in driving cell cycle initia- tion (12, 30). This strain was synchronized by raffinose block in G1, followed by release into the cell cycle with galactose addition to induce expression of the GAL1 promoter. Using timed glucose addition to repress GAL1::CLB5-HA expression, we saw a sharp increase in Clb5p instability approximately coincident with 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).
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 (YPGal). We did this with cells with one or two copies of GAL1::CLB5-HA or one copy of GAL1::CLB5Δdb-HA. Clb5p was moderately stable in cells completing DNA replication (glucose addition 60 min after release), but by 90 min after release, Clb5p became highly unstable. After division (120 min), Clb5p became highly unstable, and stability decreased again later in the second cell cycle. Thus, Clb5p stability is cyclically regulated, with peak instability in dividing cells, consistent with the results in Fig. 1. The destruction box-deleted Clb5Δdb protein was degraded similarly slowly at each time point. We controlled for increased Clb5p levels due to destruction box deletion with the two-copy GAL1::CLB5-HA integrant; the pattern of instability with this strain was similar to that with the one-copy strain (Fig. 2C). Thus, we observed destruction box-dependent and cell-cycle-dependent Clb5p degradation, with peak instability in dividing cells. The GAL1::CLB5Δdb-HA strain did not complete nuclear division during the time course (Fig. 2C), and an accumulation of cells with divided nuclei was observed. A further examination of the effects of GAL1::CLB5Δdb-HA is presented below.

Clb5p, but not Clb5Δdbp, is degraded in the nucleus at mitosis. We replaced the HA tag with a Myc tag to allow immunofluorescent detection of Clb5p (Fig. 4). In unbudded cells, small budded cells, and most large budded cells with an undivided nucleus, Clb5p is concentrated in the nucleus. In large budded cells with an undivided DNA mass near or spanning the bud neck and in large budded cells with two DNA signals, Clb5p is distributed diffusely throughout the nucleus.
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) or released into YPD and rearrested with α-factor (+αf) 60 min later. 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::CLB5ΔdbHA. Protein levels were monitored by Western blotting against the HA tag. (B and C) Strains containing CLB5ΔdbHA under the control of its endogenous promoter (CLB5::CLB5ΔdbHA) and CLB5::CLB5ΔdbΔdbHA were synchronized in G1 with α-factor. Strains were either released into yeast extract-peptone-dextrose (YPD) or released into YPD and rearrested with α-factor (+αf) 60 min later. 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).

CLB5Δdb expression was not lethal, but expression of GAL1::CLB5Δdb was (8). GAL1::CLB5 expression results in the accumulation of cells with a 2C DNA content (see Fig. 8). GAL1::CLB5 expression becomes lethal in a stc1 background (data not shown). Deletion of the SWE1 inhibitory kinase (5)
had little additional effect on these phenotypes (data not shown).

In contrast, CLB5-Δdb 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-Δdb 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-Δdb (Fig. 2 and 3). It may be that overexpression of Clb5p-Δdb is required to saturate some means of Clb5p degradation that is independent of the identified destruction box. These observations on expression of CLB5-Δdb 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-Δdb 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-Δdb and GAL1::CLB5 yield nearly comparable levels of protein through most of the cell cycle, it seemed likely that the lethality of GAL1::CLB5-Δdb may require specific persistence of Clb5p through mitosis, when Clb5p but not Clb5-Δdbp 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-Δdbp 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-Δdb strain relieved lethality (8). Deletion of SIC1 made expression of GAL1::CLB5-KA,EA-Δdb lethal (data not shown), again confirming that destruction box-dependent degradation and Sic1p inhibition can coregulate 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-Δdb lethality. GAL1::CLB5-Δdb 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-Δdb cells appear to delay as binucleate large budded cells with a 2C DNA content, resembling a GAL1::CLB5-Δdb-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 most of the cells are arresting with postmitotic spindles (Fig. 6). The DNA signals (DAPI) are heterogeneous with respect to nuclear fluorescence: approximately half of the cells have an S-shaped DNA content, and the other half have a dumbbell shape. 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-Δdbp overexpression delays completion of mitosis, but many cells nevertheless ultimately divide in the presence of Clb5-Δdbp. These cells do not efficiently replicate DNA after division, 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).

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FIG. 4. Loss of Clb5p nuclear localization in mitotic cells. Photographs of cell bodies (differential interference contrast [DIC]), nuclei visualized by DAPI staining (DAPI), and Myc-tagged protein detected by indirect immunofluorescence (Anti-Myc) from 1255-5C (wild type) strains carrying integrated copies of GAL1::CLB5ΔK,EA or GAL1::clb5fsΔK,EA. Samples were taken from cycling cultures.
separation) is largely complete in many of the apparently rebudded 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 differences 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 constructs 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 all 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,
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 α-factor and then released into galactose medium to induce GAL1::CLB5-db or GAL1::CLB2-Db. For reasons that we do not understand, in this protocol, GAL1::CLB5-db 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. 8) 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 GAL1::CLB5-db cells eventually escaped this block and divided their nuclei, and, overall, about 30% of the cells then rebudded (data not shown). In contrast, the GAL1::CLB2-Db 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 Clb5-Dbp, but that this is blocked by overexpressed Clb2-Dbp, 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 Cln5p-Cdc28p (1). If Clb5p-Cdc28p also stabilizes Clb2p, then some aspects of the phenotype due to overexpression of CLB5db could be indi-
rect, due to stabilized Clb2p-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-A were lethal in a clb2 background. clb2 deletion also enhanced the speed of induction of irreversible lethality due to GAL1::CLB5-D, although this effect was somewhat variable (data not shown). In almost all cases, GAL1::CLB5-D expression 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 CLB5-D strain overexpressers. We constructed a strain expressing both GAL1::CLB5-D and GAL1::CLB2-D. 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 a GAL1::CLB2-D-induced arrest. This is consistent with the idea that the accumulation of 1C DNA content in cells with GAL1::CLB5-D alone requires mitosis, which may be blocked by GAL1::CLB2-D expression. Similarly, GAL1::CLB2-D expression significantly reduces the number of cells displaying the characteristic GAL1::CLB5-D 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-D/GAL1::CLB2-D strain was reduced from that observed in GAL1::CLB2-D cells, viability was increased 10-fold from strains expressing GAL1::CLB5-D alone (Fig. 9). These results support the idea that Clb2p can restrain mitotic exit in GAL1::CLB5-D strain overexpressers and that the severity of GAL1::CLB5-D-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-D-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).

Clb5p 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
may be responsible, or there may be other unidentified destruction boxes in Clb5p. In either case, the overexpression of Clb5-Δdbp 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 stabilizing it, perhaps by phosphorylation of Cdh1p (1, 49). The idea that Clb5p restrains mitotic exit by phosphorylation of Cdh1p and Sck1p was also suggested by Shirayama et al. (40).

Accumulation of cells with unreplicated DNA in GAL1::CLB5-Δdb cells may be due to failure to license replication origins 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 shutoff 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::CLB2-Δdb 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 cig2) may be 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.

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**FIG. 9.** Overexpression of Clb2Δdbp in a GAL1::CLB5Δdb strain blocks cells in mitosis and partially suppresses lethality due to GAL1::CLB5Δdb. Wild-type (WT) 1255-5C transformed with vector (V) and 1255-5C with integrated GAL1::CLB5Δdb were grown overnight in synthetic complete raffinose-uracil medium. The cells were harvested by centrifugation and resuspended in galactose medium lacking a-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 medium lacking a db from the Clb5-Δdb.
at this activity than Clb2p (8). The results reported here suggest that Clb2p has a specific function of restraining mitotic exit, which is weak or absent in Clb5p. This difference is not due to defects in Clb5p 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 Clb5p, and late accumulation of M cyclins, including Clb2p, which are intrinsically specialized for appropriate roles. clb20 pds1 cells are blocked late in mitosis (25), and this block has been attributed to failure of Cdc20p-dependent Clb5p degradation (40). These observations lead to the prediction that expression of stabilized Clb5p in mitotic cells should permanently block mitotic exit. In contrast, upon expression of GAL1::CLB5-Δdlb, 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 Clb5p (40), may be more directly due to Clb5p-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 clb3Δ raf5Δ cells (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 Clb5p 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 CLB5 probably lacks this activity, and at least under some circumstances may actually drive expression of this class of genes (24, 30). This may partially explain the ability of GAL1::CLB5-Δdlb but not GAL1::CLB2-Δdlb cells to undergo extra rounds of budding (see above), since Cln1p and Cln2p are probably major activators of bud emergence (23). This could provide another example of restriction of late functions to late-expressed Clb proteins by intrinsic specialization.

Thus, the available data suggest that the budding yeast cell cycle is largely segregated into early functions promoted by S-phase cyclins and late functions promoted by M-phase cyclins. This segregation correlates with time of expression of these cyclins and also with the intrinsic functional capacities of these cyclins.

The linkage between these sets of functions is unknown, but it is an intriguing possibility that Clb5p may be important for stabilizing Clb2p through Cdh1p phosphorylation (1, 40, 49). Since Cdh1p is not involved in Clb5p degradation (35), this could allow early accumulation of Clb5p and performance of Clb5p-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 (16), indicating that 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 metazoans, it is likely that cyclins A and E are specialized for induction of S phase and that B-type cyclins 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