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Distinct Mechanisms Control the Stability of the Related S-Phase Cyclins Clb5 and Clb6†

Leisa P. Jackson,[1] Steven I. Reed,[2] and Steven B. Haase[1]*

Department of Biology, Duke University, Durham, North Carolina,[1] and Department of Molecular Biology, The Scripps Research Institute, La Jolla, California[2]

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The yeast S-phase cyclins Clb5 and Clb6 are closely related proteins that are synthesized late in G_1. Although often grouped together with respect to function, Clb5 and Clb6 exhibit differences in their ability to promote S-phase progression. DNA replication is significantly slowed in *clb5*Δ mutants but not in *clb6*Δ mutants. We have examined the basis for the differential functions of Clb5 and Clb6 and determined that unlike Clb5, which is stable until mitosis, Clb6 is degraded rapidly at the G_1/S border. N-terminal deletions of *CLB6* were hyperstabilized, suggesting that the sequences responsible for directing the destruction of Clb6 reside in the N terminus. Clb6 lacks the destruction box motif responsible for the anaphase promoting complex-mediated destruction of Clb5 but contains putative Cdc4 degron motifs in the N terminus. Clb6 was hyperstabilized in *cdc34-3* and *cdc4-3* mutants at restrictive temperatures and when S/T-P phosphorylation sites in the N terminus were mutated to nonphosphorylatable residues. Efficient degradation of Clb6 requires the activities of both Cdc28 and Pho85. Finally, hyperstabilized Clb6 expressed from the *CLB6* promoter rescued the slow S-phase defect exhibited by *clb5*Δ cells. Taken together, these findings suggest that the SCF^{Cdc4} ubiquitin ligase complex regulates Clb6 turnover and that the functional differences exhibited by Clb5 and Clb6 arise from the distinct mechanisms controlling their stability.

Progression through the eukaryotic cell cycle is mediated by cyclin-dependent kinase (Cdk) complexes. In the budding yeast *Saccharomyces cerevisiae*, nine cyclins can associate with and activate the kinase Cdc28 (Cdk1). Based on sequence, function, and timing of expression, these cyclins can be grouped into three categories: the G_1 cyclins Cln1, Cln2, and Cln3, the S-phase cyclins Clb5 and Clb6, and the mitotic cyclins Clb1, Clb2, Clb3, and Clb4. Cyclin binding is required to activate Cdc28, and cyclins are also thought to confer substrate specificity to the kinase complex. Thus, the expression of different cyclin subgroups at different times throughout the cell division cycle is thought to activate distinct events in each stage of the cycle.

Transition from G_1 into S phase is triggered by the S-phase cyclins Clb5 and Clb6 (1, 8, 23, 35). Clb5 and Clb6 are approximately 40% identical at the amino acid sequence level and are transcribed in a pulse in G_1 (8, 23, 35). Transcription is controlled by similar regulatory elements in the promoters of both *CLB5* and *CLB6* (28). Although *CLB5* and *CLB6* are transcribed in G_1, Clb5/Cdc28 (and presumably Clb6/Cdc28) complexes are not activated until the G_1/S border when the Clb-specific Cdk inhibitor, Sic1, is degraded by the action of the ubiquitin ligase complex, SCF^{Cdc4} (*S*kp1/*C*ullin/*F*-box) (33, 34, 41, 42).

Clb5 and Clb6 appear to play redundant roles in the initiation of S phase. Either Clb5 or Clb6 can promote entry into S phase on schedule (1, 8, 35). However, Clb5 but not Clb6 is required for timely progression through S phase (8, 35). Further studies showed that *clb5*Δ cells could not efficiently trigger DNA replication from replication origins that fire late in S phase but that replication initiation from late-firing origins in *clb6*Δ cells was unperturbed (5).

Phenotypic differences between *clb5* and *clb6* mutants were also observed in the regulation of spindle pole body duplication (17) and DNA replication (S. B. Haase and S. I. Reed, unpublished data). Moreover, Clb6 but not Clb5 is required to regulate the nuclear localization of the transcription factor Swi6 (12).

The differential phenotypes observed for *clb5*Δ and *clb6*Δ mutants could be explained by differences in posttranscriptional regulation, protein function, or both regulation and function. Here, we report that the stability of Clb5 and Clb6 is differentially regulated. Clb5 has been shown to persist throughout S phase and is marked for destruction in mitosis by the action of the ubiquitin ligase complex APC^{Cdc20}(*a*naphase-*p*romoting *c*omplex) (20, 21, 36). We present evidence that Clb6 is targeted for destruction at the G_1/S border by the activity of a functionally distinct ubiquitin ligase complex, SCF^{Cdc4}. We also show that the differential regulation of Clb5 and Clb6 protein stability can account for the differences observed in the ability of these S-phase cyclins to promote timely progression through S phase.

MATERIALS AND METHODS

Plasmid and strain construction. All strains used in this study are derivatives of BF264-15DU (*MAT***a** *ade1 his2 leu2-3,112 trp1-1 ura3*Δ*ns*) (31) except where noted. Relevant genotypes of strains are described in Table 1. *CLB6* and *CLB5* were tagged at their respective genomic loci by PCR-mediated homologous recombination (oligonucleotides are listed in Table 2) using pFA6KANMX2 (43) as a template. Haploid *CLB6-9MYC-KAN* cells (SBY464) were mated to haploid *CLB5-9MYC-KAN* cells (SBY466). Diploids were then sporulated to obtain haploid cells bearing both the *CLB5-9MYC* and the *CLB6-9MYC* alleles (SBY468).

CLB6-HA$_3$ was PCR amplified from the genome of SBY334, cloned into the pCRII vector (Invitrogen), and sequenced. *CLB6-HA*$_3$ was removed from PCRII

* Corresponding author. Mailing address: DCMB Group, Department of Biology, Box 91000, LSRC Bldg., Research Dr., Durham, NC 27708. Phone: (919) 613-8205. Fax: (919) 613-8177. E-mail: shaase@duke.edu.

† Supplemental material for this article may be found at http://mcb.asm.org/.

TABLE 1. Strains used in this study

Strain	Relevant genotype	Source
SBY147	*MAT***a** *bar1 clb6*Δ::*ADE1*	S. B. Haase
SBY317	*MAT***a** *bar1 GAL-CLB6*Δ*135-HA3-LEU2*	This study
SBY334	*MAT***a** *bar1 CLB6-HA-KAN*	This study
SBY464	*MAT***a** *bar1 CLB6-9MYC-KAN*	This study
SBY466	*MAT***a** *bar1 CLB5-9MYC-KAN*	This study
SBY468	*MAT***a** *bar1 CLB6-9MYC-KAN CLB5-9MYC-KAN*	This study
SBY476	*MAT***a** *bar1 GAL-CLB6-T39A-S6A-HA3-LEU2*	This study
SBY478	*MAT***a** *bar1 GAL-CLB6*Δ*100-HA3-LEU2*	This study
SBY502	*MAT***a** *bar1 GAL-CLB6-S6A-HA3-LEU2*	This study
SBY506	*MAT***a** *bar1 GAL-CLB6-T39A-HA3-LEU2*	This study
SBY510	*MAT***a** *bar1 GAL-CLB6-HA3-LEU2*	This study
SBY518	*MAT***a** *bar1 GAL-CLB6*Δ*41-HA3-LEU2*	This study
SBY523	*MAT***a** *bar1 GAL-CLB6-HA3-LEU2 apc2*Δ::*KAN apc2-4*	This study
SBY525	*MAT***a** *bar1 GAL-CLB6-T39A-S6A-HA3-LEU2 apc2*Δ::*KAN apc2-4*	This study
SBY557	*MAT***a** *bar1 CLB6-HA3-URA3*	This study
SBY573	*MAT***a** *bar1 GAL-CLB6*Δ*3P-HA3-LEU2*	This study
SBY577	*MAT***a** *bar1 GAL-CLB6-S147A-HA3-LEU2*	This study
SBY597	*MAT***a** *bar1 CLB6*Δ*3P-HA3-URA3*	This study
SBY603	*MAT***a** *clb5*Δ::*ARG CLB6*Δ*3P-HA3-URA3*	This study
SBY605	*MAT***a** *clb5*Δ::*ARG CLB6-HA3-URA3*	This study
SBY648	*MAT***a** *bar1 cdc4-3 sic1*Δ::*KAN GAL-CLB6*Δ*3P-HA3-LEU2*	This study
SBY653	*MAT***a** *bar1 cdc34-3 sic1*Δ::*KAN GAL-CLB6-HA3-LEU2*	This study
SBY654[a]	*MAT***a** *bar1 cdc28*Δ::*cdc28-as1 GAL-CLB6-HA3-LEU2*	This study
SBY696	*MAT***a** *bar1 GAL-CLB6-HA3-LEU2 pho85*Δ::*KAN*	This study
SBY702	*MAT***a** *bar1 GAL-CLB6-HA3-LEU2 sic1*Δ::*KAN*	This study
SBY715	*MAT***a** *bar1 cdc4-3 sic1*Δ::*KAN GAL-CLB6-HA3-LEU2*	This study
SBY722	*MAT***a** *bar1 GAL-CLB6-HA3-LEU2 pdr5*Δ::*KAN*	This study
SBY744	*MAT***a** *bar1 cdc28*Δ::*cdc28-as1*	This study
SBY752	*MAT***a** *bar1 GAL-CLB6-HA3-LEU2 pho85*Δ::*KAN cdc28*Δ::*cdc28-as1*	This study

[a] In W303 (*ura3-1 trp1-1 leu2-3,112 his3-1 ade2-1 can1-100*) background.

by digestion with BamHI and ligated into the integrating *GAL1* expression vector YIpG2 (40). *CLB6* N-terminal deletions were constructed by PCR amplification using pCRII-CLB6-HA_3 as a template and oligonucleotides described in Table 2. PCR products were ligated into pCRII, sequenced, and then subcloned into YIpG2 as described above. Phosphorylation site mutations were generated by PCR-based site-directed mutagenesis of YIpG2-CLB6-HA_3 using primers described in Table 2. PCR products were then ligated into pDRIVE (QIAGEN) and sequenced before being subcloned into YIpG2 on 5′ BamHI and 3′ BglII ends. All strains containing *GAL-CLB6-HA$_3$* and its derivatives were constructed by EcoRV digestion of the YIpG2-based plasmids and integration by homologous recombination at the *LEU2* locus.

pCRII-CLB6-HA_3-URA3 was constructed by cloning the *URA3* open reading frame from pUCHURA3 into pCRII-CLB6-HA_3 on HindIII ends. pUCHURA3 was constructed by PCR amplification of URA3 from yeast genomic DNA and ligation into the HindIII site of pUCH (18). PCR-mediated gene replacement was used to replace the *CLB6* open reading frame with mutated *CLB6* alleles. Oligonucleotides described in Table 2 were used to amplify *CLB6-HA$_3$-URA3* and its derivatives from pCRII-URA3 vectors. The resultant PCR products were integrated into yeast cells by homologous recombination. Mutations were verified by sequencing PCR fragments generated from genomic DNA. All gene deletions were made by PCR-mediated gene replacement using oligonucleotides listed in Table 2. The kanamycin resistance gene was amplified from pFA6KANMX2 with oligonucleotides complementary to sequence flanking the gene being replaced on their 3′ ends. PCR products were then integrated into yeast cells by homologous recombination, and transformants were selected by screening for kanamycin resistance.

Cell growth and arrest conditions. Yeast cultures were grown in YEP medium (1% yeast extract, 2% Bacto peptone, 0.001% adenine, 0.001% uracil) supplemented with 2% sugar (dextrose, sucrose, galactose, or raffinose). Cells were grown at 30°C except as noted. Temperature-sensitive mutants were grown at 25°C and shifted to 34°C for arrest, except where otherwise noted. Mating pheromone synchrony experiments were performed by adding alpha factor (α-factor) (2 μg/ml α-factor for *BAR1* strains and 40 to 60 ng/ml for *bar1* strains) to the growth medium for 2 h. Synchronous populations were released from the arrest into medium without α-factor.

Protein stability assays. *GAL1*-driven expression of all *CLB6* alleles was induced by adding 10% galactose solution to mid-log-phase cultures in YEP-sucrose or YEP-raffinose to a final concentration of 2% galactose or by harvesting cells by centrifugation and resuspending them in YEP-galactose (except as noted). Galactose concentrations and pulse durations were adjusted as necessary to achieve protein expression levels similar to that of endogenous Clb6 at the G_1/S border. In order to terminate transcription and translation, cells were harvested and resuspended in equal volumes of YEP-dextrose medium containing 1 mg/ml cycloheximide at $t = 0$ min. Proteins were extracted from whole-cell lysates, and results were analyzed by Western blotting as described previously (17).

Phosphatase assay. Clb6-HA expression was induced in YEP-galactose medium for 1.5 h. Whole-cell lysates were prepared as described above and then treated with phosphatase. Phosphatase reaction mixtures were assembled as recommended by the vendor and either left on ice for 45 min or incubated at 30°C for 45 min. Where indicated, 400 U of lambda phosphatase was used per reaction, and 10 mM sodium orthovanadate and 50 mM sodium fluoride were used to inhibit phosphatase activity.

Flow cytometry. Flow cytometric analysis of DNA content was performed as described previously (16). The percentage of cells in S phase was determined by first fitting normal curves to the 1C and 2C peaks and integrating under these curves to quantitate the percentage of cells in G_1 and G_2/M phases. The remaining cells were judged to be in S phase.

RESULTS

Stability of Clb5 and Clb6 is differentially regulated through the cell cycle. In order to understand why *clb5*Δ and *clb6*Δ mutants exhibit distinct phenotypes, we began by examining the expression of the two proteins in a synchronous population of cells as they traversed the cell cycle.

Both Clb5 and Clb6 were fused at the C terminus with a nine-myc tag at their respective genomic loci. *CLB5-9MYC*

TABLE 2. Oligoucleotides used in this study

Purpose	Oligonucleotide	Sequence
Sequencing	CLB6 seq2	CACTGCTTACCTGAAACATT
	CLB6 seq2.5	TTCGATAGAGATGGATGATCC
	CLB6 seq3	CCCCCTAAATTTCATTAGGA
	CLB6revseq1	GATAGGGAGATTGCGTGTCC
	CLB6 5′ flank	CTCTGATATTCTCTCCCTCC
	CLB6 3′ flank	TGATATTTAAGATGCAGGGGG
Deletion	5′ CLB6Δ135	AGATCTATGTCCCTACCGACACATAACTATTTA
	5′ CLB6Δ41	GGATCCAAAATGTCTACGAATGAAAAAAAAGTTCTATCC
	5′ CLB6Δ100	AGATCTATGCATCAATGGAAAAATTTGGAT
	5′ SIC1_KAN	ATTTTGACCCTTGAAGCAGGGACTATTACACGAAAGCTTGCCTCGTCCCC
	3′ SIC1_KAN	TATAATCGTTCCAGAAACTTTTTTTTTTCATTTCTGACACTGGATGGCGGC
	5′ PDR5_KAN	TTCGTATCCGCTCGTTCGAAAGACTTTAGACAAAAGCTTGCCTCGTCCCC
	3′ PDR5_KAN	TAAGTTTCTTTTCTTAACCAAATTCAAAATTCTAGACAGTGGATGGCGGC
	5′ pho85_KAN	TAGCGCGGCAAACTGGGCAAACTTGAGCAATACCAGCTTGCCTCGTCCCC
	3′ pho85_KAN	ATACATGGCTACGGTTTTTCGCTGACGGGCTGCGGACACTGGATGGCGGC
Phosphorylation site mutation	5′ CLB6S6A	AGATCTATGAATTGTATCCCTGCTCCAATTAGTGAAAGGAAA
	CLB6HA 3′ BglII	AGATCTTCAGCGGCCGCACTGAGCAGCGT
	5′ CLB6T39A	GAGAAAGTTCAATTAACTTGGCACCTCACTCTACGAATG
	3′ CLB6T39A	CATTCGTAGAGTGAGGTGCCAAGTTAATTGAACTTTCTC
	5′ CLB6S147A	CTATTTAATGGACACGCAAGCTCCCTATCATTTGAAAAGC
	3′ CLB6S147A	GCTTTTCAAATGATAGGGACGTTGCGTGTCCATTAAATAG
Integration	5′ CLB6_replace	ATATTCTCTCCCTCCTTTTAAATTTTTAAAATGAATTGTATCCCTAGTCC
	5′ CLB6S6A_replace	ATATTCTCTCCCTCCTTTTAAATTTTTAAAATGAATTGTATCCCTGCTCC
	3′ CLB6flank_3′ URA3	CAGGGGGTTAGCTGGCTATAATTTTGATCTATGTTTTAGTTTTGCTGGCCG
Tagging	CLB6MYCKAN5′	ATGGTTTATTTCAAGGTTTTTGACTGGTGTAAACAAAAACGTGTCGACGGTGAACAAAAG
	CLB6MYCKAN3′	TAAGATGCAGGGGGTTAGCTGGCTATAATTTTGATCTATGTTGCTTGCCTCGTCCCCGCC
	CLB5MYCKAN5′	TCCGAAATGCATAGCAACTTTCAAAATCTATTTAATCTTAAGGTCGACGGTGAACAAAAG
	CLB5MYCKAN3′	ATGTAAAGAGTATGCGAATTCATGAGCATTACTAGTACTAATGCTTGCCTCGTCCCCGCC

CLB6-9MYC cells were synchronized in G_1 by treatment with the mating pheromone α-factor and then released into the cell cycle. Clb5 and Clb6 protein levels were then monitored at several time points by Western blotting (Fig. 1A). Cell cycle progression was assessed by monitoring budding index and DNA content (Fig. 1B).

As expected, Clb5 appeared in G_1 cells upon release from α-factor arrest and persisted until 60 min after release, when cells entered mitosis (Fig. 1A and B). Clb6 also appeared in G_1 but disappeared rapidly between 20 min and 30 min after release (Fig. 1A). Analysis of budding index and DNA content (Fig. 1B) indicates that Clb6 disappears near the G_1/S transition. Because *CLB5* and *CLB6* are transcribed simultaneously in G_1, these observations suggest that the turnover of Clb5 and Clb6 is regulated differently.

The N terminus of Clb6 confers instability on the protein. Clb5 and Clb6 are closely related at the protein sequence level. The proteins are approximately 40% identical throughout their lengths but show the highest degree of homology and identity in their C-terminal regions (Fig. 2A). The degron sequence motif recognized by the APC^{Cdc20} (RRALTDVPVN) has been mapped to the N-terminal region of Clb5 (20, 21, 36). Although Clb6 and Clb5 share some homology in this region, a critical amino acid change in the degron sequence (RXXL to KXXL) (Fig. 2A) is likely to prevent recognition of Clb6 by the APC. Since degradation signals for Clb5 are located in the N terminus and Clb5 and Clb6 protein sequences are the most divergent in this region, we reasoned that degradation signals specific for Clb6 might also reside in its N-terminal region.

In order to investigate this hypothesis, we made three N-terminal deletions of various lengths and examined their sta-

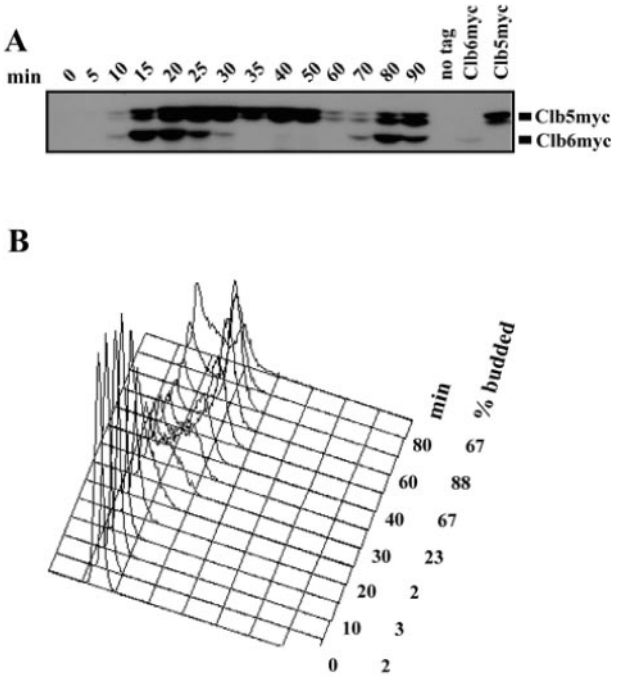

FIG. 1. Abundance of Clb5 and Clb6 during the cell cycle. (A) Clb5 and Clb6 protein levels over time in a synchronous population of cells released from α-factor arrest. (B) Budding index and flow cytometric analysis of DNA content for the same time points. Times indicated are minutes after release from α-factor.

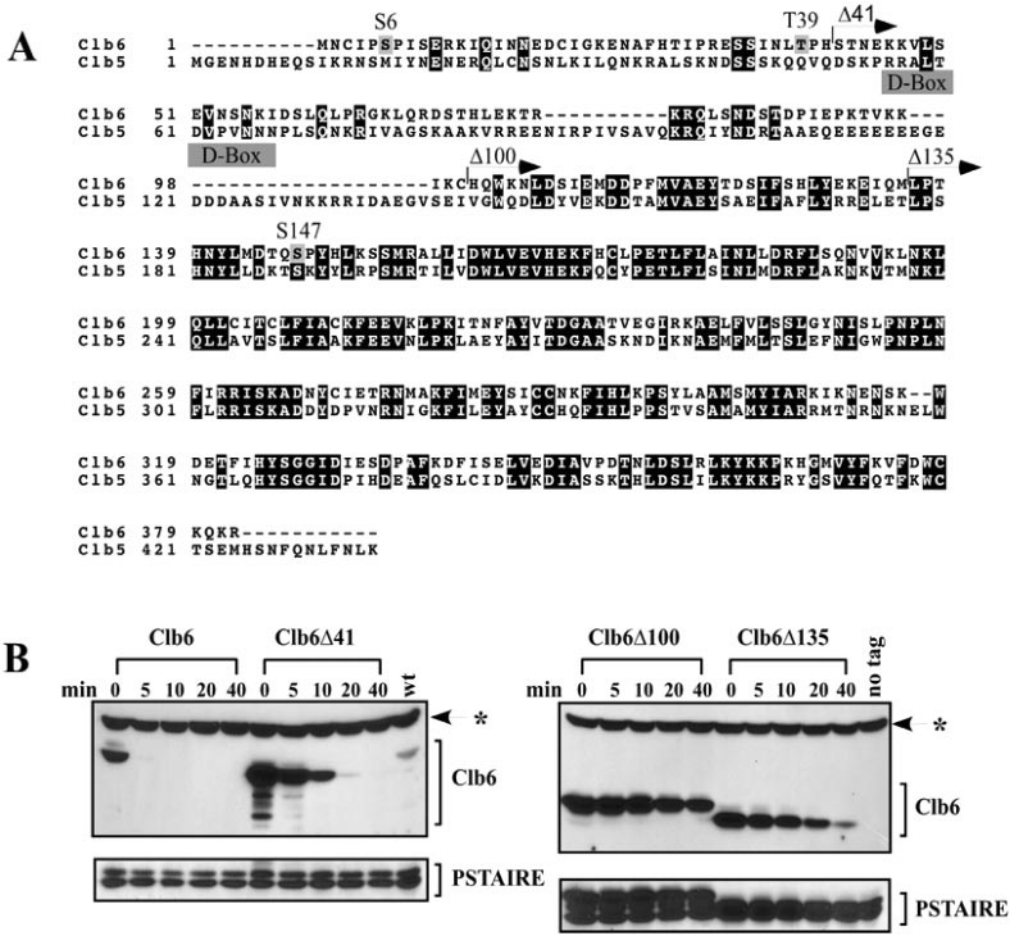

FIG. 2. N-terminal deletions hyperstabilize Clb6. (A) ClustalW alignment of Clb5 and Clb6. Black boxes indicate identities. Gray boxes indicate putative phosphorylation sites (S/T-P) in Clb6. Clb5 D-box is shown. Arrows demarcate the start of Clb6 N-terminal deletions. (B) The relative stability of HA-tagged Clb6 N-terminal deletions. *CLB6* transcription was induced from the *GAL1* promoter by treating asynchronous cultures (in YEP-raffinose) with 0.1% galactose for 25 min. Cells were then harvested, and at $t = 0$ min, cells were resuspended in YEP-dextrose containing 1 mg/ml cycloheximide. Wild type (wt), peak levels of endogenous Clb6 (cells collected from synchronous population expressing *CLB6-HA* from its own promoter, 15 min after release from α-factor); no tag, cell lysates from cells expressing Clb6 without an HA epitope tag; PSTAIRE, loading control. The asterisk indicates an HA background band.

bility by carrying out a promoter shutoff experiment (Fig. 2B). N-terminal deletions of *CLB6* were fused at the C terminus to a triple hemagglutinin (HA) tag and then expressed from the inducible *GAL1* promoter in asynchronous wild-type cells. Transcription was terminated by the addition of dextrose to the medium, and translation was blocked by the addition of cycloheximide. Cell populations were collected at several time points following shutoff and then examined for Clb6 protein levels (Fig. 2B).

Wild-type Clb6 appears to be very unstable in asynchronous cells, exhibiting a half-life of less than 5 min. The Clb6Δ41 deletion is significantly stabilized compared to wild-type Clb6, indicating that sequences important for degradation are contained in the first 41 amino acids. However, Clb6Δ100 and Clb6Δ135 appear to be more stable than Clb6Δ41, suggesting that additional degron sequences exist downstream. We have shown that expression of Clb6Δ100 is capable of promoting S phase in Δ*clb1,2,3,4,5,6* cells, indicating that Clb6Δ100 can still bind Cdc28 and activate DNA replication (data not shown) and suggesting that hyperstabilization is not the indirect result of a failure to form normal cyclin/Cdk complexes.

Clb6 is hyperstabilized in SCF mutants and is ubiquitinated. Examination of the N terminus of Clb6 revealed sequences similar to SCF^{Cdc4} phospho-degron motifs found in the Cdk inhibitor Sic1 (29), including three potential Cdk phosphorylation sites (no SP or TP sequences are found in Clb5). Moreover, like Sic1 (34), Clb6 is destroyed at the G_1/S transition. Taken together, these observations suggest that SCF complexes may regulate Clb6 stability. Therefore, we tested the stability of Clb6 in cells bearing temperature-sensitive mutations in SCF components.

Using the *GAL1* promoter shutoff protocol described in the legend to Fig. 2, we found that Clb6 is significantly stabilized in *cdc34-3 sic1*Δ and *cdc4-3 sic1*Δ cells at restrictive temperatures, compared to that in wild-type cells at the same temperature (Fig. 3A and B). These findings suggest that SCF^{Cdc4} complexes regulate the turnover of Clb6.

We examined Clb6 stability in SCF mutants in which the

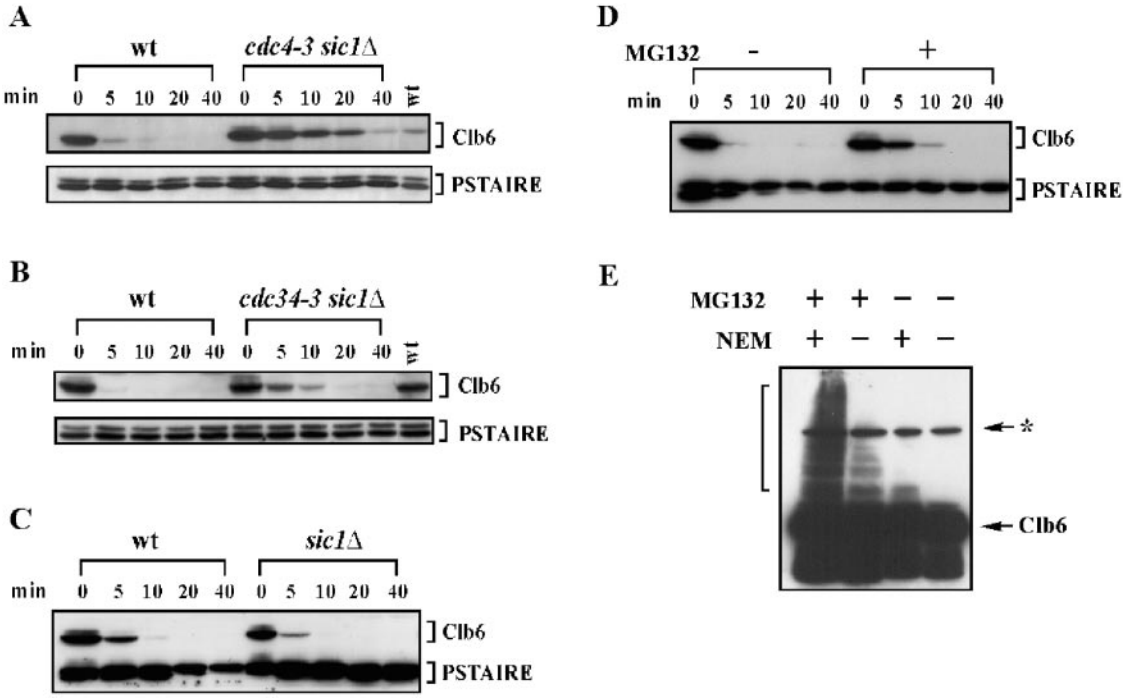

FIG. 3. Clb6p hyperstabilized in *cdc4-3* and *cdc34-3* mutant cells at restrictive temperatures and in the presence of a proteosome inhibitor. (A and B) The relative stability of HA-tagged Clb6 in (A) wild-type (wt) cells versus *cdc4-3 sic1*Δ mutants at 33°C and (B) wild-type cells versus *cdc34-3 sic1*Δ mutants at 35°C. (C) The relative stability of HA-tagged Clb6 in wild-type cells versus *sic1*Δ cells. The promoter shutoff experiments whose results are shown in panels A and B were performed as described in the legend to Fig. 2 except that cultures were shifted to restrictive temperatures for 30 min and then treated with galactose at restrictive temperatures for 30 min as follows: wt/*CLB6-HA* cells, 0.2% galactose; *cdc4-3 sic1*Δ/*CLB6-HA* cells, 0.8% galactose; and *cdc34-3 sic1*Δ/*CLB6* cells, 1% galactose. In panel C, cells were harvested from YEP-sucrose and grown in YEP-galactose for 30 min. Times indicated are minutes after resuspension in YEP-dextrose containing cycloheximide. wt, peak levels of endogenous Clb6. (D) Stability of HA-tagged Clb6 in cells treated or untreated with MG132. Mid-log-phase cultures in YEP-sucrose were treated with 50 μM MG132 in dimethyl sulfoxide (+) or dimethyl sulfoxide alone (−) and then grown at 30°C for 1 h. Galactose (0.5%) was then added to induce Clb6 expression from the *GAL1* promoter for 30 min. Times indicated are minutes after resuspension in YEP-dextrose containing cycloheximide. (E) Slow-mobility forms of Clb6 appear in the presence of the proteasome inhibitor MG132 and the deubiquitinase inhibitor NEM. Cells expressing an HA-tagged Clb6 from the *GAL1* promoter were untreated (−) or treated (+) with 100 μM MG132 in YEP-sucrose for 45 min. Clb6-HA expression was then induced by the addition of galactose for 45 min. Total protein was then isolated in the presence (+) or absence (−) of NEM. (D and E) Strains had the gene encoding the ABC transporter Pdr5 deleted to decrease the rate at which MG132 was pumped out of cells. PSTAIRE, loading control. The asterisk indicates an HA background band.

SIC1 gene was deleted because *cdc34-3* and *cdc4-3* cells arrest at the restrictive temperature at the G_1/S border due to hyperstabilization of Sic1 (27, 42). Thus, in the presence of the *SIC1* gene, any observed stabilization of Clb6 in these mutants is not necessarily due to direct stabilization of Clb6 but could reflect an indirect stabilization of Clb6, resulting from an arrest in a specific cell cycle stage or from binding of Sic1 to Clb6/Cdc28 complexes. By deleting *SIC1* from these strains, we eliminated the possibility that any observed stabilization in these SCF mutants is an indirect effect of Sic1 stabilization. We have also observed that the deletion of *SIC1* (*sic1*Δ) in otherwise wild-type cells does not affect the stability of Clb6 (Fig. 3C), indicating that the hyperstabilization of Clb6 in *cdc4-3 sic1*Δ and *cdc34-3 sic1*Δ mutants is not an indirect effect of the loss of Sic1.

Clb6 does not appear to be fully stabilized in *cdc34-3 sic1*Δ mutants (compare Fig. 3B to Fig. 2B), which might suggest that other mechanisms are required for efficient Clb6 degradation. However, we have observed that *cdc34-3 SIC1* cells can enter S phase (data not shown), suggesting that the *cdc34-3* allele is partially functional even at 35°C. Furthermore, we have determined that Clb6 is significantly less stable at higher temperatures, even in wild-type cells (see Fig. S1 in the supplemental material). Thus, the general instability of Clb6 at high temperatures is likely to offset the stabilizing effects of the SCF^{ts} mutants. Consistent with these observations, the stability of Clb6 in *cdc4-3 sic1*Δ cells at 33°C (a partially restrictive temperature for the *cdc4-3* allele) is comparable to that in the N-terminal deletions (compare Fig. 3A to Fig. 2B).

If Clb6 is a direct target of SCF^{cdc4}, then it should be ubiquitinated and destroyed by the proteasome. Thus, inhibition of the proteasome should stabilize Clb6. Using the *GAL1* promoter shutoff protocol described above, we found that HA-tagged Clb6 is stabilized in cells treated with the proteasome inhibitor MG132 (Fig. 3D). Ubiquitinated proteins are highly unstable and normally appear as a "ladder" of slow-mobility species on sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels. A prominent ladder of slower-mobility Clb6 species, indicative of ubiquitination, was observed on long exposures of Western blots in extracts from cells treated with MG132 (Fig. 3E). Moreover, the intensity of slower-mobility bands was also increased in extracts that were treated with the

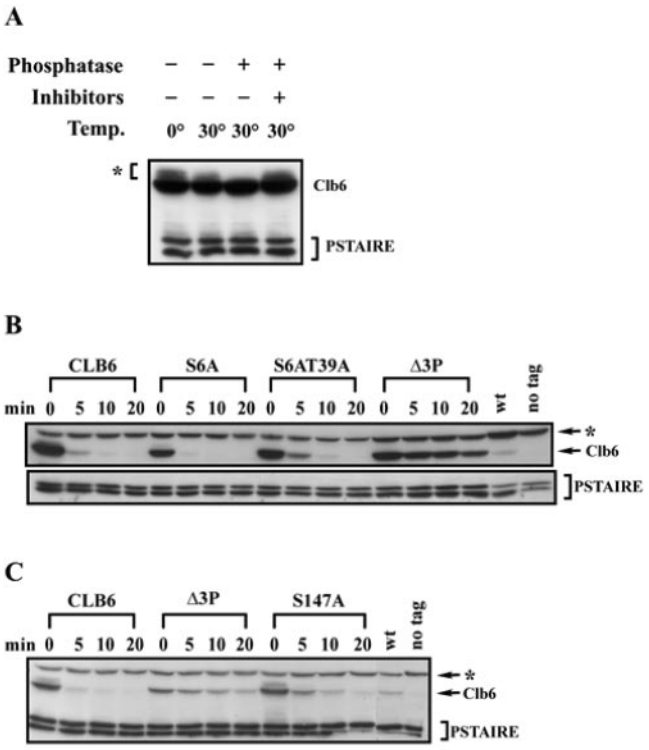

FIG. 4. Clb6 is a phospho-protein and is hyperstabilized when putative phosphorylation sites are mutated to nonphosphorylatable residues. (A) Multiple species of Clb6 are observed in whole-cell lysates. Lysates were untreated (−) or treated (+) with lambda phosphatase (phosphatase) in the presence (+) or absence (−) of sodium fluoride and of sodium orthovanadate (inhibitors). The asterisk indicates slow-mobility bands. (B and C) The relative stability of wild-type Clb6 and Clb6 proteins bearing phosphorylation site mutations. The experiment was carried out as described in the legend to Fig. 2 except that cultures were treated with 0.05% galactose for 20 min. Clb6 (wt) and the S6A, S6AT39A, and Δ3P mutants are shown (B). Clb6 (wt) and the Δ3P and S147A mutants are shown (C). Times indicated are minutes after resuspending cells in YEP-dextrose containing cycloheximide. wt, peak levels of endogenous Clb6; no tag, cell lysates from cells expressing Clb6 without an HA epitope tag; PSTAIRE, loading control. The asterisk in panels B and C indicates an HA background band.

deubiquitinase inhibitor *N*-ethylmaleimide (NEM). Slower-mobility forms were not observed in untreated cells (Fig. 3E).

Clb6 is a phospho-protein, and mutation of putative phosphorylation sites leads to hyperstabilization. The SCF complex normally recognizes phosphorylated substrates (37, 44). Thus, we first determined whether Clb6 is a phospho-protein. Slower-mobility forms of Clb6 were observed under a number of conditions (see Fig. 2 and 3). In order to determine whether the slower-mobility forms of Clb6 represented phosphorylated species, we treated cell lysates with lambda phosphatase. Slow-mobility forms of Clb6 disappeared in lysates treated with phosphatase but remained in untreated lysates and in lysates containing phosphatase and phosphatase inhibitors (Fig. 4A).

SCF targets are often phosphorylated by proline-directed serine/threonine kinases. We mutated the serines or threonines of the three (S/T)P sites in Clb6 to the nonphosphorylatable residue alanine and then tested the stability of Clb6 alleles bearing individual or multiple phosphorylation site mutations using a *GAL1* promoter shutoff assay (Fig. 4B and C). Mutation of either S6 to A (S6A) (Fig. 4B) or T39 to A (T39A) (data not shown) had no significant effect on the stability of Clb6. However, the allele bearing the S147A mutation was significantly stabilized (Fig. 4C). The S6A T39A double mutant was also significantly stabilized (Fig. 4B). Clb6 mutated at all three (S/T)P sites (Clb6Δ3P) was the most stable, exhibiting a half-life comparable to that of the N-terminal deletions (Fig. 4B and C). These results suggest that all three putative phosphorylation sites contribute to the instability of Clb6. SCF-regulated proteins, such as Sic1 in budding yeast (29) and cyclin E in human cells (39, 46), exhibit similar behaviors when putative phosphorylation sites are mutated. In both cases, mutation of a single phosphorylation site has some marginal stabilizing effect, but mutation of multiple sites is required for maximum stabilization.

Both Cdc28 and Pho85 contribute to the destruction of Clb6. Many SCF targets in both yeast and mammalian cells are phosphorylated by cyclin/Cdk complexes (19, 24, 37, 44, 46) and are hyperstabilized when cyclin/Cdk activities are inhibited (46). Therefore, we asked whether inhibition of cyclin/Cdk activity would stabilize Clb6. Using the *GAL1* promoter shutoff protocol described above, we examined Clb6 stability in cells bearing a *cdc28as-1* allele that can be inhibited by the ATP analogue 1-NM-PP1 (Fig. 5A) (2).

We found that Clb6 was partially stabilized in *cdc28as-1* cells, even in the absence of 1-NM-PP1 (compare Fig. 5A to Fig. 5B). The *cdc28as-1* allele is known to be hypomorphic, and the reduction in Cdc28 kinase activity may have contributed to the increased stability of Clb6 even in the absence of analogue. However, Clb6 was only marginally more stable in *cdc28as-1* cells treated with 1-NM-PP1 than that in untreated cells (Fig. 5A). We confirmed that this dose of 1-NM-PP1 rapidly arrested the cell cycle (data not shown) as previously reported (2). Moreover, Clb6Δ3P was significantly more stable than wild-type Clb6 in *cdc28as-1* cells treated with 1-NM-PP1 (data not shown), confirming that these putative phosphorylation sites are important for the degradation of Clb6. Taken together, these results suggest that an additional kinase is required for efficient degradation of Clb6.

Efficient degradation of the SCF^{cdc4} target, Sic1, requires the combined action of Cdc28 and Pho85 kinases (30). We therefore examined the stability of Clb6 in cells with the *PHO85* gene deleted, both alone (Fig. 5B) and in combination with the *cdc28as-1* allele. Again, Clb6 was only partially stabilized in *pho85*Δ cells (Fig. 5B) and in *pho85*Δ *cdc28as-1* double mutants in the absence of 1-NM-PP1 (Fig. 5C). However, Clb6 was significantly hyperstabilized in *pho85*Δ *cdc28as-1* double mutants in the presence of 1-NM-PP1 (Fig. 5C). These results suggest that Pho85 and Cdc28 have redundant functions in the regulation of Clb6 stability.

Clb6Δ3P expressed from the endogenous *CLB6* promoter is not destroyed at the G_1/S border and suppresses the S-phase defect of *clb5*Δ cells. Having established that mutation of putative phosphorylation sites stabilizes Clb6, we examined whether Clb6Δ3P was destroyed at the G_1/S border. We replaced the endogenous wild-type copy of *CLB6* with an HA-tagged *CLB6Δ3P* allele in a strain with the *CLB5* gene deleted (*clb5*Δ). *clb5*Δ *CLB6Δ3P* cells were arrested in G_1 with α-factor. The synchronized cell population was then released into the cell cycle and sampled at various time points after release

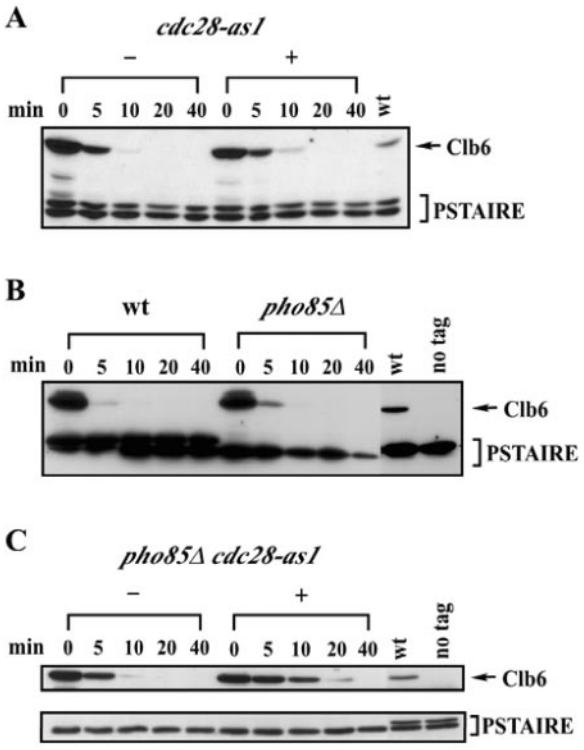

FIG. 5. Both Cdc28 and Pho85 regulate the stability of Clb6. (A) Stability of Clb6-HA in cells expressing the analogue-sensitive allele *cdc28-as1*. Log-phase asynchronous cultures in YEP-sucrose were harvested, and Clb6-HA expression was induced in YEP-galactose medium for 45 min. Cultures were then either untreated (−) or treated (+) with 5 μM 1-NM-PP1 for 30 min. Times indicated are minutes after terminating expression by resuspending cells in YEP-dextrose containing cycloheximide. (B) Stability of Clb6-HA in cells with the *PHO85* gene deleted. Cultures were treated as described in the legend to Fig. 2. (C) Stability of Clb6-HA in *cdc28-as1 pho85*Δ cells. *cdc28-as1 pho85*Δ cultures were either untreated (−) or treated (+) with 5 μM 1-NM-PP1 as described for panel A. wt, wild type; PSTAIRE, loading control.

for the analysis of both Clb6 protein levels and DNA content (Fig. 6B and C). Results were compared to those of *clb5*Δ cells bearing a wild-type allele of *CLB6* tagged with HA (Fig. 6A and C). In the *clb5*Δ *CLB6-HA* control strain, Clb6 protein appeared in late G_1, disappeared at the G_1/S border, and reappeared in G_1 of the next cell cycle (Fig. 6A). Clb6Δ3P also appeared in late G_1, but Clb6Δ3P levels persisted beyond the G_1/S border, remaining high throughout S phase (Fig. 6B). Thus, mutation of the phosphorylation sites bypassed the controls that normally trigger the destruction of Clb6 at the G_1/S border.

Although Clb6Δ3P persisted throughout the time course, protein levels dropped somewhat around the time of mitosis (Fig. 6B). From this experiment, we could not determine whether the decrease in Clb6Δ3P levels was cell cycle regulated (e.g., an APC-mediated event). Therefore, we performed a similar G_1 arrest/release experiment but released *clb5*Δ *CLB6*Δ*3P* cells into medium containing nocodazole in order to arrest cells before anaphase and prevent any APC-mediated destruction of Clb6Δ3P. We found that Clb6Δ3P levels declined in a continuous manner in nocodazole-arrested cells (see Fig. S2 in the supplemental material), suggesting that the decrease in Clb6Δ3P levels observed in cycling cells (Fig. 6B) is not due to a regulated mitotic destruction mechanism.

As mentioned above, cells with the *CLB5* gene deleted normally progress slowly through S phase compared to wild-type or *clb6*Δ cells. Because Clb6Δ3P persisted throughout S phase, we were able to determine whether the inability of Clb6 to promote normal S-phase kinetics on its own was related to its destruction at the G_1/S transition. S-phase kinetics of *clb5*Δ cells bearing wild-type *CLB6* or the *CLB6*Δ*3P* allele in the synchrony/release experiments shown in Fig. 6A and B were analyzed by flow cytometry (Fig. 6C).

A synchronous population of *clb5*Δ cells bearing wild-type *CLB6* moved slowly through S phase (Fig. 6C). Cells entered S phase on schedule (coincident with bud emergence), approximately 20 min after release from the α-factor arrest. Cells then progressed through the middle of S phase slowly, with DNA contents intermediate between 1C and 2C at the 25-min and 30-min time points. The majority of the population reached 2C content of DNA between 35 min and 40 min.

*clb5*Δ *CLB6*Δ*3P* cells entered S phase with similar kinetics, indicating that the phosphorylation site mutation is competent for initiating S phase on schedule. However, the *clb5*Δ *CLB6*Δ*3P* mutants completed S phase significantly faster than *clb5*Δ *CLB6* cells. Though *clb5*Δ *CLB6*Δ*3P* cells appeared to release from the G_1 arrest somewhat less synchronously than the *clb5*Δ *CLB6* cells, the bulk of the population had nearly completed S phase by 30 min. Furthermore, a significant fraction of *clb5*Δ *CLB6*Δ*3P* cells completed mitosis and reached G_1 (1C DNA content and unbudded) again at 60 min, while very few *clb5*Δ *CLB6* cells reappeared in G_1 until 75 min. These results suggest that the inability of Clb6 to promote normal S-phase kinetics in the absence of Clb5 is due to its destruction at the G_1/S border.

When Clb6Δ3P was expressed from the *CLB6* promoter in wild-type cells, we observed no increase in the speed of S phase (data not shown). Thus, the rescue of the slow S-phase defect observed in *clb5*Δ mutants is likely to be a suppression of the defect in the *clb5*Δ mutant rather than a general increase in S-phase velocity.

We also examined S-phase kinetics in asynchronous cell populations by flow cytometric analysis of DNA content (Fig. 6D). In all strains shown, Clb6 and Clb6 mutants are epitope tagged with HA. As shown previously (8, 23, 35), a significant increase in the population of S-phase cells is observed in *clb5*Δ cells compared to that in wild-type cells, indicative of a slow S phase. The cell cycle profile of an asynchronous population of *clb5*Δ *CLB6*Δ*3P* cells (13.6% S-phase cells) looks similar to that of the wild-type cells (12.6% S-phase cells) and has noticeably fewer cells in S phase than *clb5*Δ *CLB6* cells (22.9% S-phase cells). These results are consistent with the finding in synchronous populations (Fig. 6C) that Clb6Δ3P can suppress the slow S-phase defect of *clb5*Δ cells. Furthermore, asynchronous wild-type cells expressing Clb6Δ3P exhibit a cell cycle distribution (12.4% S-phase) similar to that of cells expressing wild-type *CLB6* (Fig. 6D), indicating that Clb6Δ3P does not accelerate cells through S phase in wild-type cells.

The decrease in the S-phase fraction observed for asynchronous *clb5*Δ *CLB6*Δ*3P* cells compared to that for *clb5*Δ *CLB6* cells (Fig. 6D) does not necessarily reflect a more rapid progression through S phase. A similar cell cycle profile could

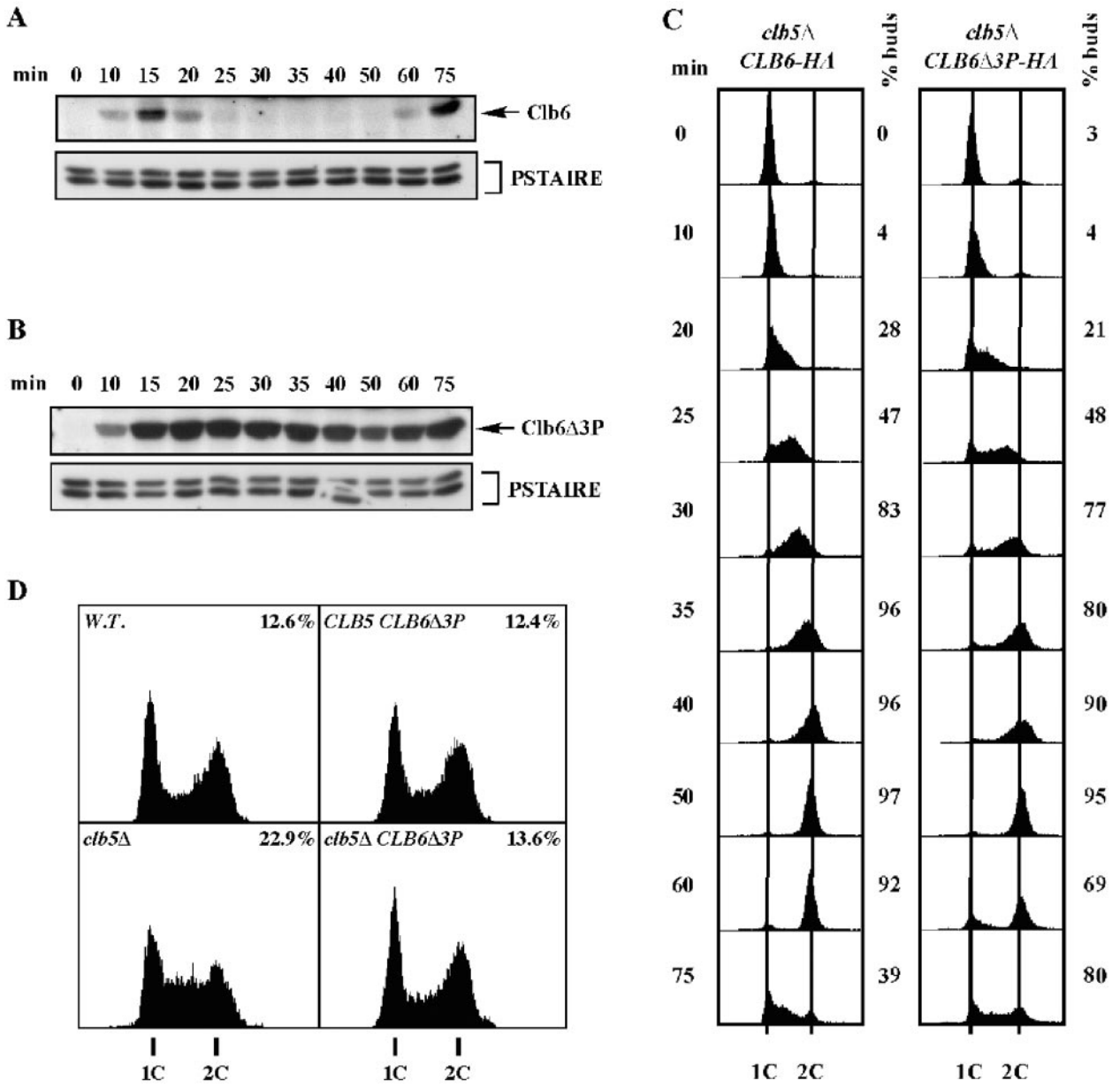

FIG. 6. Clb6Δ3P expressed from the endogenous *CLB6* promoter is hyperstable and suppresses the S-phase defect of *clb5*Δ cells. Wild-type Clb6 at its genomic locus was replaced by Clb6-HA or Clb6Δ3P-HA in *clb5*Δ cells. Clb6-HA (A) and Clb6Δ3P-HA (B) protein levels over time in a synchronous population of cells released from α-factor arrest. Times indicated are minutes after release from alpha factor. (C) Flow cytometric analysis of DNA content and budding index for the experiments whose results are shown in panels A and B. (D) Flow cytometric analysis of asynchronous, log-phase cultures. The percentage of cells in S phase is shown for each histogram. PSTAIRE, loading control.

result from extending the time required to traverse both G_1 and G_2/M phases. However, no evidence for slow progression through G_1 or S phase was observed in synchronized *clb5*Δ *CLB6*Δ*3P* cell populations (Fig. 6C). In fact, *clb5*Δ *CLB6*Δ*3P* cells entered S phase at the same time and returned to G_1 and S phase on the second cycle more rapidly than did *clb5*Δ *CLB6* cells (Fig. 6C), indicating no delay in progression through G_1 or G_2/M.

DISCUSSION

The stability of Clb5 and Clb6 is regulated by distinct mechanisms. Although Clb5 and Clb6 are often grouped together with respect to function, previous findings suggest that the S-phase cyclins are in fact functionally distinct (1, 5, 17, 35). We set out to determine whether the phenotypic differences observed for *clb5*Δ and *clb6*Δ mutants are related to differential posttranscriptional regulation of the two proteins. Our findings indicate that unlike Clb5, which persists until anaphase, Clb6 is destroyed at the G_1/S border via N-terminal motifs by the SCF^{Cdc4} complex. Three putative Cdk phosphorylation sites in Clb6 contribute to its instability, and both Cdc28 and the related kinase Pho85 promote the degradation of Clb6. The observation that Clb6 is not quite as stable in *pho85*Δ *cdc28as-1* double mutants (see Fig. 5C) as in the triple phos-

phorylation site mutant (see Fig. 5C and Fig. 4B and C) indicates that an additional kinase(s) may play a minor redundant role in the phosphorylation of Clb6.

The differential regulation of Clb5 and Clb6 stability appears to have arisen from the acquisition of SCF degron sequences in Clb6, as well as a mutation at a critical residue in the destruction box (D-box) (RXXL to KXXL). However, in *Drosophila melanogaster*, the Pim protein apparently has a functional D-box with a KXXL sequence (25), suggesting that the D-box in Clb6 could still be functional. Furthermore, the stability of the S-phase cyclin in *Schizosaccharomyces pombe*, Cig2, appears to be controlled collaboratively by the SCF and APC ligase complexes (47). We found that Clb6 was not measurably stabilized in cells treated with nocodazole or in cells bearing a temperature-sensitive allele of *APC2* (see Fig. S2 in the supplemental material). Taken together, these findings suggest that the APC plays a very minor role, if any, in regulating the stability of Clb6.

Differential regulation of S-phase cyclins and the progression of S phase. Entry into S phase is triggered by the destruction of Clb-specific Cdk inhibitor Sic1 at the G_1/S border (29, 33, 41, 42). Our findings indicate that Clb6 is also destroyed very close to the G_1/S border. Because Clb6 can promote timely S-phase entry in the absence of Clb5, active Clb6/Cdc28 complexes must be present at G_1/S, but such complexes are likely to exist for only a brief period.

Although Clb6-associated kinase is present for only a very short period at the G_1/S border, the conservation of Clb6 among the *Saccharomyces* species (see below) suggests an important function. Clb6 has been shown to be involved in the regulation of Swi6 localization (12), although this regulation does not appear to be critical for cell cycle progression. It has also been reported that *clb6*Δ mutants actually grow and divide faster than wild-type cells (1), which would suggest that cells bearing a wild-type allele of *CLB6* should be at a selective disadvantage. For now, the conserved function of Clb6 remains a mystery.

Although either Clb5 or Clb6 can trigger the timely entry into S phase, Clb6 cannot promote normal progression through S phase without Clb5. The basis for the S-phase defect in *clb5*Δ cells is not yet fully understood. Nonetheless, a defect in late origin firing in *clb5*Δ cells has been reported (5) and could account for the significant slowing of DNA replication in *clb5*Δ mutants. The fact that Clb6 is normally destroyed at the G_1/S border suggests that this defect is simply due to the lack of Clb6 availability late in S phase. Consistent with this hypothesis, Clb/Cdc28-dependent Cdc45 loading at late-firing origins occurs late in S phase (48, 49). Moreover, overexpression of Clb6 both promotes normal S-phase kinetics and restores the firing of late origins in *clb5*Δ mutants (13), suggesting that slow progression of cells through S phase in these cells is in fact linked to the defect in late origin firing.

Like Clb5 and Clb6, the related mitotic cyclins Clb1 and Clb2 also appear to have evolved divergent functions. Clb2 but not Clb1 is required for efficient mitotic progression in vegetative cells (10, 31), and Clb1 is essential for meiotic progression while Clb2 is dispensable (14). The basis for the functional diversity in this case appears to be differential transcription during mitosis and meiosis (14). It is interesting that both Clb1/Clb2 and Clb5/Clb6 diverged with respect to regulation of protein expression.

Gene deletion studies indicate a significant functional redundancy between the members of the B-cyclin family. In an extreme example, the essential functions of all six B-type cyclins in a sextuple deletion strain can be rescued by expressing Clb1 constitutively (15), arguing that differential expression patterns are the major distinguishing feature of B-cyclin functions. However, biochemical studies indicate that cyclin/Cdk complexes can phosphorylate a broad spectrum of Cdk targets but that distinct complexes vary in their intrinsic activities and substrate affinities (26). Thus, the specialization of cyclins for diverse cell cycle functions may reflect a suite of evolutionary changes that affect cyclin expression as well as localization, activity, and substrate affinity.

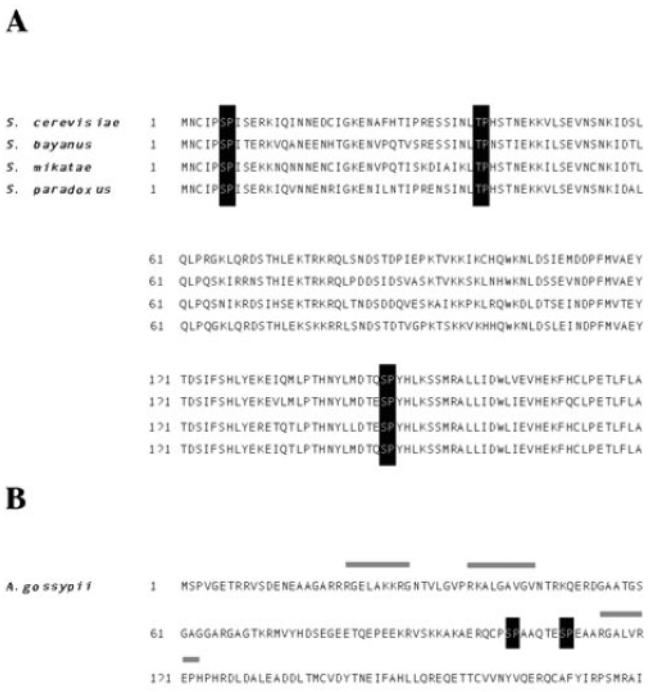

FIG. 7. Alignments of the N-terminal sequences of Clb6 for *Saccharomyces* species and the N-terminal sequences of *A. gossypii*. (A) The N-terminal amino acid sequence of Clb6 is shown for *S. cerevisiae*, *S. bayanus*, *S. mikatae*, and *S. paradoxus*. Sequences were downloaded from the *Saccharomyces* Genome Database (http://db.yeastgenome.org/fungi/YGR109C.html). Black boxes highlight putative S/TP phosphorylation sites. (B) The N-terminal amino acid sequence for AAR100C, the Clb5/Clb6 homolog from *A. gossypii*. The sequence was downloaded from the *Ashbya* Genome Database (http://agd.unibas.ch/Ashbya_gossypii/geneview?gene=AAR100C). Black boxes highlight putative S/TP phosphorylation sites, and gray bars indicate D-box-like sequences.

Why are Clb5 and Clb6 differentially regulated? Our observations suggest that Clb5 and Clb6 are functionally similar. Why then is it important for Clb6 to be destroyed at the G_1/S border while Clb5 persists until mitosis?

The destruction of Clb6 at the G_1/S transition may be conserved through the evolution of *Saccharomyces* species. Protein sequence alignments with Clb6 orthologs from other *Saccharomyces* species (*S. bayanus*, *S. mikatae*, and *S. paradoxus*) indicate that all three putative Cdk phosphorylation sites and the sequences surrounding them are highly conserved (Fig. 7A) (3, 22). Clb5 orthologs are highly conserved in the region of the D-box degron sequences that mediates destruction by the

APC. These observations suggest that the distinct regulatory processes for Clb5 and Clb6 are evolutionarily conserved.

Many duplicated genes in the *S. cerevisiae* genome, including the cyclin gene pairs *CLN1*/*CLN2*, *CLB1*/*CLB2*, and *CLB5*/*CLB6*, are thought to have arisen from an ancient whole-genome doubling event (45). *Ashbya gossypii*, a filamentous fungus, diverged from the budding yeasts before the genome duplication event and thus is likely to be a good model for the structure and function of ancestral yeast proteins before the genome duplication and evolutionary divergence of gene pairs (4). Interestingly, the Clb5/Clb6 homolog in *Ashbya* contains D-box-like sequences in the N terminus, as well as two putative (S/T-P) phosphorylation sites (Fig. 7B) (4). However, these phosphorylation sites are not embedded in consensus SCF^{Cdc4} degron sequences, suggesting that the ancestral S-phase cyclin may be degraded at mitosis and not at the G_1/S border.

It has been reported that Clb6 inhibits progression through G_2 (1). Thus, destruction of Clb6 at the G_1/S border may be required for timely progression through G_2. However, we have shown that expression of the *CLB6Δ3P* mutant from the endogenous *CLB6* promoter in *clb5*Δ cells (Fig. 6C and D) or wild-type cells (data not shown) causes no measurable change in cell division time or defects in cell cycle distribution (Fig. 6D) as measured by flow cytometry. It is likely that Clb6 levels in the constitutive overexpression experiment reported by Basco et al. are significantly higher than the levels of Clb6Δ3P expressed from the *CLB6* promoter (which are similar to wild-type levels) and that the significant G_2 delay they observed is a result of elevated Clb6 levels rather than the persistence of Clb6 late in the cell cycle. Furthermore, Basco et al. favored a model in which Clb6 exerts its inhibitory effect on G_2, at least in part, via regulation of *CDC6* transcription (1). Because *CDC6* is transcribed in G_1, our finding that Clb6 is destroyed at the G_1/S border is not necessarily inconsistent with the premise that Clb6 influences progression through G_2 by affecting *CDC6* transcription. However, our findings do indicate that destruction of Clb6 at the G_1/S border is not likely to be necessary for maintaining normal cell cycle kinetics through G_2/M.

Clues to the importance of destroying Clb6 at the G_1/S border may be found in studies of the mammalian S-phase cyclin cyclin E. In many respects, budding yeast Clb6 and mammalian cyclin E are strikingly similar. Like Clb6, cyclin E is first expressed in G_1 and then destroyed soon after cells traverse the G_1/S transition (7). Cyclin E contains two consensus Cdk sites, and its stability is also regulated by the SCF^{hCdc4} complex (39).

Improper regulation of cyclin E expression and turnover appears to be associated with tumorigenesis and genome instability. Mutations in hCdc4 in a breast cancer cell line have been correlated with high expression levels of cyclin E (39). In fact, cyclin E levels are elevated in many tumor types (6, 32), and deregulated cyclin E expression has been shown to promote chromosome instability (38). However, the mechanism for the induction of chromosome instability by deregulated expression of cyclin E has not yet been fully elucidated.

Reminiscent of Clb5, mammalian cyclin A is expressed at the G_1/S border (9) and persists until mitosis when it is degraded by the APC (11). Thus, there appear to be parallel regulatory mechanisms controlling the persistence of S-phase cyclins (Clb5 ≅ cyclin A and Clb6 ≅ cyclin E) in both yeast and mammalian cells. The biological significance of the differential regulatory mechanisms for cyclin E and cyclin A has yet to be determined.

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