The Saccharomyces cerevisiae cyclin Clb2p is targeted to multiple subcellular locations by cis- and trans-acting determinants

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SUMMARY

The cyclin-dependent kinase Cdc28p associates with the cyclin Clb2p to induce mitosis in the yeast Saccharomyces cerevisiae. Several cell cycle regulatory proteins have been shown to require specific nuclear transport events to exert their regulatory functions. Therefore, we investigated the subcellular localization of wild-type Clb2p and several mutant versions of the protein using green fluorescent protein (GFP) fusion constructs. Wild-type Clb2p is primarily nuclear at all points of the cell. A point mutation in a potential leucine-rich nuclear export signal (NES) enhances the nuclear localization of the protein, and Δyb2 cells exhibit an apparent Clb2p nuclear export defect. Clb2p contains a bipartite nuclear localization signal (NLS), and its nuclear localization requires the α and β importins (Srp1p and Kap95p), as well as the yeast Ran GTPase and its regulators. Deletion of the Clb2p NLS causes increased cytoplasmic localization of the protein, as well as accumulation at the bud neck. These data indicate that Clb2p exists in multiple places in the yeast cell, possibly allowing Cdc28p to locally phosphorylate substrates at distinct subcellular sites.

Key words: Saccharomyces cerevisiae, Cyclins, Clb2p, Bud tip, Bud neck, Nuclear localization

INTRODUCTION

In all eukaryotic cells, the timing of cell cycle progression is regulated by the activities of cyclin dependent kinases (CDKs). To be active, a CDK must be associated with a cyclin partner, and must also be in a particular phosphorylation state (reviewed by Pines, 1995). The phosphorylation state of a cyclin/CDK complex may in part be determined by its localization to either the nucleus or the cytoplasm, and therefore its accessibility to specific kinases and phosphatases that are compartmentalized within the cell. Targeted localization – to the nucleus versus the cytoplasm, or to discrete subcellular structures – may also regulate the ability of a cyclin to interact with its CDK or with other regulatory molecules.

In vertebrate cells, the B-type cyclins regulate mitotic entry. Their timed association with active CDK1 and CDC2 is controlled through multiple mechanisms, including localization and targeted degradation. Beginning in metaphase and continuing through the next G1 phase of the cell cycle, the anaphase promoting complex (APC)/cyclosome targets cyclin B for efficient degradation by ubiquitin-mediated proteolysis (King et al., 1995; Sudakin et al., 1995; Brandeis and Hunt, 1996; Clute and Pines, 1999). When the APC/cyclosome is inactivated at the onset of S-phase, cyclin B accumulates in the cytoplasm. This distribution shifts into the nucleus in prophase of mitosis, before nuclear envelope breakdown (Pines and Hunter, 1991). This shift in cyclin B localization is primarily the consequence of regulated nuclear export rather than timed nuclear import. Prior to mitosis, cyclin B shuttles between the cytoplasm and the nucleus, but rapid nuclear export by the exportin CRM1 ensures its bulk cytoplasmic localization (Hagting et al., 1998; Toyoshima et al., 1998; Yang et al., 1998). CRM1 recognizes a leucine-rich nuclear export signal (NES) within an N-terminal domain of cyclin B. At the onset of mitosis, phosphorylation of cyclin B prevents its interaction with CRM1, thus trapping cyclin B in the nucleus (Yang et al., 1998).

The cell cycle of S. cerevisiae is regulated at both the G1/S and G2/M transitions through the activity of a single cyclin-dependent kinase, Cdc28p. Passage through the ‘START’ commitment point in G1 requires the G1 cyclins Cln1p, Cln2p and Cln3p, in association with Cdc28p; in mitosis, Cdc28p associates with the Clb proteins, Clb1p-Clb4p (reviewed by Nasmyth, 1993). Although none of the CLB genes are essential, CLB2 is the most important: Δclb1,3,4 null mutants are still viable, indicating that Clb2p alone can perform all essential mitotic functions; in contrast, a Δclb2 null mutation is synthetically lethal with either Δclb1 or Δclb3 (Fitch et al., 1992; Richardson et al., 1992). Clb2p proteolysis is required for exit from mitosis (Surana et al., 1993), and continues into the following G1 phase until the Cdc28p kinase is reactivated by Clns at START (Amon et al., 1994).

The localization of Clb2p has not been examined in detail, and it is possible that regulated localization may play a role in its function, as for cyclin B. We have therefore studied the
subcellular localization of Clb2p and have identified several cis- and trans-acting determinants of this localization. Clb2p contains a bipartite nuclear localization signal (NLS) and requires both importin α/Srp1p and importin β/Kap95p for its nuclear localization. There is also a cytoplasmic pool of Clb2p, a portion of which is concentrated at the bud necks of dividing cells. This bud neck localization is greatly enhanced by deletion of the bipartite NLS. We also present evidence that Clb2p is a substrate for nuclear export as well as import. We propose that concentration of Clb2p at specific sites may play a role in regulating the timing of cell cycle and morphogenic events.

MATERIALS AND METHODS

Yeast strains and plasmids

The yeast strains used in this study are listed in Table 1. The plasmids generated for this study were all based on pPS293 (GAL1 promoter inserted into EcoRI/HindIII sites of YEp52) and are listed in Table 2. To make the GAL1-GFP vector (pPS304), the GFP ORF (0.7 kb) was PCR amplified and cloned into HindIII site of pPS293. The CLB2 ORF (1.4 kb) was PCR amplified and cloned into the SalI site of pPS1304 to generate pPS2189. The L303A point mutation was engineered by site-directed PCR mutagenesis using primers JKH22 (5′-GGCCGCTAATTGTTGGCCACATC-3′) and JKH23 (5′-GGCCGGCTAATTGTTGGCCACATC-3′). The underlined bases introduced the mutation and an extra HaeIII restriction site, which was used to identify mutant clones. The PCR mutagenesis was performed on a Bluescript-based plasmid containing an EcoRV fragment of CLB2; this fragment was then swapped into pPS2189 and the resulting plasmid, pPS2190, was verified by sequencing.

Clb2ΔDbox was PCR amplified from pB536 (gift of D. Pellman) and cloned into the SalI site of pPS304, generating pPS2191. Clb2ΔNLS was created by PCR amplifying the fragments of the CLB2 ORF upstream (0.5 kb) and downstream (0.9 kb) of the bipartite NLS and joining them using KpnI ends. The resulting 1.4 kb fragment was cloned into the SalI site of pPS1304 to generate pPS2192. The KpnI site introduces a glycine and a threonine in place of the bipartite NLS.

Immunoblotting

To compare the expression levels of the four GFP fusion proteins, PSY580 cells transformed with pPS2189, 2190, 2191 or 2192 were grown first in synthetic complete glucose medium lacking uracil (SC-ura⁻), then grown to early log phase in SC-ura⁻ raffinose medium. GFP fusion protein expression was induced with 2% galactose for 1 hour. Cells from 50 ml cultures were harvested by centrifugation and lysed in PBSMT buffer (2 mM MgCl₂, 1 mM EDTA, 0.5% Triton X-100 in PBS) plus protease inhibitors (0.5 mM PMSF, 3 μg/ml each of pepstatin A, leupeptin, aprotinin and chymostatin) using glass beads in a FastPrep bead beater (Savant) as previously described (Hood and Silver, 1998). Protein samples were resolved in 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes using standard techniques (Ausubel et al., 1998). Blots were incubated for 1 hour at room temperature with anti-GFP antibody (Seedorf et al., 1997) diluted 1:2000. Blots were washed with PBS before incubation with secondary antibody (Jackson Immunoresearch), diluted 1:5000. Protein bands were detected using a Fluor-S Max MultiImager (BioRad). Band intensities were quantified using Quantity One software (BioRad).

Microscopy

GFP-tagged proteins were induced for 1 hour with 2% galactose and were observed using a Nikon fluorescence microscope fitted with a GFP-specific filter (Chroma Technology). Images were captured with a Princeton Instruments Micromax digital camera using Metamorph imaging software (Universal Imaging). For temperature-shift experiments, GFP fusion proteins were induced for 1 hour at 25°C, then cells were shifted to 37°C with continued induction for an additional hour. Where indicated in the Results, after the temperature shift, cells were fixed by adding 37% formaldehyde to 2% final concentration and incubating at 37°C for 15 minutes. The fixed cells were washed twice with potassium phosphate buffer (0.1 M, pH 6.5), then resuspended in P solution (1.2 M sorbitol in potassium phosphate buffer) before microscopy.

Table 1. Yeast strains used in this study

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<tr>
<th>Name</th>
<th>Genotype</th>
<th>Reference</th>
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<tr>
<td>FY23 (PSY580)</td>
<td>MAT a, ura-52 trp1Δa3 leu2Δ1</td>
<td>Winston et al., 1995</td>
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<tr>
<td>PSY1102</td>
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<td>PSY962 (with pPS986)</td>
<td>MAT a, gsp1::HIS3 gsp2::HIS3 ura-3-52 leu2Δ1 trp1Δ63 with pPS986 (CEN TRP1 gsp1-1)</td>
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<td>PSY714</td>
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<td>PSY1237</td>
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<td>PSY967</td>
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<td>PSY1201</td>
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<td>This study</td>
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<td>PSY2153</td>
<td>MAT a, CLB2-GFP::LEU2 ura3 leu2 trp1</td>
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<td>PSY2155</td>
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<td>This study</td>
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Table 2. Plasmids generated for this study

<table>
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<th>Plasmid</th>
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<tr>
<td>pPS1304</td>
<td>2μ URA3 Gal-GFP</td>
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<td>pPS2189</td>
<td>2μ URA3 Gal-Cbl2-GFP</td>
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<td>pPS2190</td>
<td>2μ URA3 Gal-Cbl2/L303A-GFP</td>
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<td>pPS2191</td>
<td>2μ URA3 Gal-Cbl2ΔDbox-GFP</td>
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<td>pPS2192</td>
<td>2μ URA3 Gal-ΔNLS-GFP</td>
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RESULTS

Clb2p is nuclear throughout the cell cycle

To determine the localization of Clb2p, a construct with GFP fused to the C terminus of Clb2p was integrated into the genome of a wild-type strain at the CLB2 locus. Proper integration was assessed by PCR and Southern blotting. Clb2-
GFP was functional, since it could be integrated into the genome of a Δclb1,3,4 triple deletion strain, which requires Clb2p for viability. Immunoblotting with an anti-Clb2p antibody verified that the full-length fusion protein was expressed as the only form of Clb2p in the integrated strains (data not shown).

Clb2p is not an abundant protein. Owing to its low endogenous expression level, Clb2-GFP fluorescence could barely be seen by eye. Three-second camera exposures did allow the fusion protein to be visualized, however (Fig. 1a-h). Clb2-GFP was nuclear in all of the cells that had detectable signal. Background signal in the cytoplasm was presumably due to the cells’ intrinsic autofluorescence, since it was also present in G1 cells (e.g. Fig. 1c,e), which rapidly degrade Clb2p.

The difficulty of visualizing Clb2-GFP expressed from the genome led us to construct a plasmid containing Clb2-GFP under the control of the galactose-inducible promoter. Although galactose-induced overexpression of proteins may sometimes result in localization artefacts, we reasoned that using relatively short induction times should allow sufficient amplification of the Clb2-GFP signal while still providing relevant localization data, especially with regard to nuclear transport mutants. Increasing the Clb2-GFP signal to a level that was visible by eye was essential for examining Clb2-GFP in various nuclear transport mutant strains and comparing the effects of mutations in Clb2p itself.

When Clb2-GFP was induced with galactose for 1 hour in wild-type cells, the protein was strongly nuclear at all stages of the cell cycle (Fig. 1g-p). Weaker cytoplasmic signal was also observed. Very bright dots were sometimes visible in the cytoplasm (e.g. Fig. 1i,k), but did not consistently co-localize with any cellular structure. In addition, a small population of large-budded mitotic cells exhibited a faint signal at the bud neck (Fig. 1m,o, arrowheads). This localization was much more apparent with mutant versions of Clb2p, as discussed later. Clb2p is normally undetectable in G1 cells owing to the activity of the APC/cyclosome. The fact that ectopically expressed Clb2-GFP localized to the nucleus in G1 cells indicates that the signals that target Clb2p to nucleus are not specific to mitosis. Quantitative immunoblotting indicated that Clb2-GFP is approximately 100-fold higher when expressed from the GAL1 promoter as compared with the integrated Clb2-GFP (data not shown).

**Clb2p contains both NLS and NES motifs**

The steady-state nuclear localization of Clb2-GFP does not rule out the possibility that its position within the cell at any one time may be dynamic. For example, Clb2-GFP might be exported from the nucleus, then rapidly re-imported or degraded. We sought to identify sequences in Clb2p that might direct its nuclear import or export. A potential bipartite NLS with strong similarity to the canonical nucleoplasmin NLS was noted near the middle of the protein (Fig. 2A). Two potential leucine-rich NESs were also identified: one at the N terminus of the protein, overlapping the D box, and another in the C-terminal half of Clb2p.

To determine the role of these sequences in Clb2p localization, galactose-inducible GFP fusions of three mutant versions of the protein were constructed: Clb2p lacking the D box (ΔD box), Clb2p lacking the entire bipartite NLS (ΔNLS), and Clb2p with a leucine-to-alanine point mutation at amino acid 303 in the second NES motif (L303A). These three fusion proteins and wild-type Clb2-GFP were expressed in yeast

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**Fig. 1.** Clb2-GFP in living cells. (a-h) The localization of Clb2-GFP expressed from the genome under the control of the endogenous CLB2 promoter. The signal was too dim to see except with the camera. (i-p) Expression of the fusion protein was induced with 2% galactose for 1 hour prior to visualization by fluorescence microscopy. Cells shown at different points in the cell cycle are representative of the general population. Arrowheads indicate signal at the bud neck.
within 1 hour of galactose induction. Their relative expression levels were compared by immunoblotting of whole cell lysates with an anti-GFP antibody. The immunoreactive bands were quantitated using a fluorimager (Fig. 2B). For each sample, multiple amounts of total protein were loaded on the gel to verify that the signal detected by the fluorimager was in the linear range. Clb2(L303A)-GFP was expressed at approximately the same level as wild-type Clb2-GFP. As expected, owing to the stabilizing effect of the D box deletion, Clb2(ΔD box)-GFP showed between two- and threefold increased expression over Clb2-GFP. Clb2(ΔNLS)-GFP also showed a similar increase. This result suggests that the deletion of the Clb2p NLS may affect the protein’s turnover.

Clb2(L303A)-GFP shows increased nuclear localization

Clb2(L303A)-GFP was observed by fluorescence microscopy after one hour of galactose induction. The L303A mutation alters a key residue in the conserved NES sequence that has been shown to inactivate NES function in other proteins (Fridell et al., 1996; Meyer and Malim, 1994; Murphy and Wente, 1996; Wen et al., 1995). The fusion protein containing the L303A point mutation was more concentrated in the nucleus than was the wild-type protein, and no signal was visible at the bud necks of large-budded cells (Fig. 3A). This difference was not due to differences in protein expression levels, since the two fusion proteins were expressed at equal levels (Fig. 2B). This result corroborates the hypothesis that the deletion of the Clb2p NLS may affect the protein’s turnover.

To determine whether the L303A mutation affects Clb2p function, the mutant allele was integrated into the ∆clb1,3,4 strain using a LEU2-marked integrating plasmid. Proper integration was assessed by PCR, and the presence of the L303A mutation was verified by sequencing PCR product amplified from genomic DNA. LEU2+ integrants were viable and grew as well as the parental ∆clb1,3,4 strain, but had very elongated buds compared with the ∆clb1,3,4 parental strain and ∆clb1,3,4-expressing integrated wild-type Clb2-GFP (Fig. 3B). The fact that the L303A mutation did not affect the yeast growth rate suggests either that nuclear export is not essential for Clb2p function, or that the single mutation is not sufficient to completely disrupt its export. The elongated bud morphology does suggest that Clb2(L303A)-GFP is somewhat compromised for normal Clb2p function, however.

Clb2p nuclear localization requires importin α/β

Since Clb2p contains a candidate bipartite NLS, we hypothesized that it would most likely be imported by the importin α/β heterodimer. Taking advantage of the increased nuclear concentration of the L303A mutant protein, we expressed Clb2(L303A)-GFP in the temperature-sensitive rsl1-3 and srp1-31 strains, which contain mutations in yeast importin β/Kap95p and importin α, respectively. The fusion protein was induced for 1 hour with 2% galactose at 25°C, then the strains were shifted to 37°C or maintained at 25°C for an additional hour. As a positive control for the nuclear import defects of the rsl1-3 and srp1-31 strains, an NLS-GFP-b-galactosidase reporter was similarly expressed. Clb2(L303A)-GFP accumulated in the cytoplasm of both rsl1-3 and srp1-31 cells at the non-permissive temperature of 37°C (Fig. 4, upper panels). The Clb2(L303A) import defect was also visible in rsl1-3 at 25°C. This strain has a stronger NLS import defect
than does *srp1-31*, as indicated by the slight cytoplasmic accumulation of the NLS reporter protein in the unshifted *rsl1-3* strain, but not in the unshifted *srp1-31* cells (Fig. 4, lower panels). These results indicate that the importin α/β heterodimer is the primary nuclear import receptor for Clb2p.

The residual nuclear signal seen in *rsl1-3* and *srp1-31* cells at 37°C is likely due to protein that was imported prior to the shift, since it occurs to a similar extent with the NLS reporter.

**Clb2p nuclear localization requires yeast Ran/Gsp1p**

Wild-type Clb2-GFP was also expressed in a panel of nuclear import mutant strains. To facilitate microscopic examination of multiple strains at the same time, cells were fixed with formaldehyde for 15 minutes after the 1 hour temperature shift (see Materials and Methods). The results of these experiments are summarized in Table 3. As for Clb2(L303A)-GFP, cytoplasmic accumulation of wild-type Clb2-GFP was apparent in *rsl1-3* and *srp1-31* at the non-permissive temperature (data not shown). Receptor-mediated nuclear transport requires the GTPase Ran, which regulates the association of transport receptors with their cargoes on the appropriate sides of the nuclear envelope (reviewed in Görlich

**Fig. 4.** Clb2(L303A)-GFP requires importin α/β for nuclear localization. Clb2(L303A)-GFP was induced in wild-type, *rsl1-3* and *srp1-31* cells for 1 hour at 25°C, then cells were either maintained at 25°C or shifted to 37°C for 1 hour. GFP fluorescence was observed in living cells. Similar localization of the NLS-GFP-β-gal reporter construct is shown in the lower panels as a positive control for the nuclear import defects of *rsl1-3* and *srp1-31* cells.
and Kutay, 1999). The *gsp1-1* strain, which contains a temperature-sensitive mutation in the yeast Ran protein, also showed a nuclear import defect (Fig. 5), as did mutations in the genes that encode the Ran regulatory factors *Rna1p* (the GTPase activating protein for Ran) and *Yrb1p* (yeast Ran-binding protein 1). In contrast, mutations in genes that encode three other nuclear import receptors, *SXM1*, *KAP123* and *PSE1*, did not have a defect (Fig. 5 and data not shown). Thus, Clb2p is primarily, if not solely, imported into the nucleus by the Ran-dependent importin α/β pathway.

**A bipartite NLS affects Clb2p localization**

Clb2p amino acids 183-200 constitute a strong candidate NLS (Fig. 2A). To determine whether this sequence is required for nuclear localization of Clb2p, amino acids 183-200 were deleted in the context of the GFP fusion construct. As shown in Fig. 6A, Clb2ΔNLS-GFP exhibited increased cytoplasmic localization (compare with Fig. 1), but was still concentrated in the nucleus. This indicates that the bipartite NLS is not the only determinant of Clb2p nuclear localization. Although there is no other sequence in Clb2p that resembles a classical basic NLS, Clb2p might also be a substrate for another nuclear import pathway that is independent of importin α/β. Perhaps more likely, in the absence of its own NLS, Clb2p might be imported in complex with another NLS-containing protein such as Cdc28p.

Very interestingly, in cells that had already undergone nuclear division, Clb2ΔNLS-GFP showed a striking localization to the bud neck in a double ring pattern (Fig. 6A, arrowheads). This was much clearer than for wild-type Clb2-GFP, and occurred in virtually all large-budded cells. The double-ring bud neck localization is characteristic of the septins, which are components of a ring of 10 nm diameter filaments that is positioned at the neck between mother and bud (Haarer and Pringle, 1987; Ford and Pringle, 1991; Kim et al., 1991). Upon close examination, concentration of Clb2ΔNLS-GFP at the tips of emerging buds was also visible (Fig. 6A, arrows). The septins also form a ring at the future site of bud emergence in unbudded cells, but they do not travel with the tip of the bud as it emerges (Ford and Pringle, 1991; Kim et al., 1991).

As for Clb2(L303A), Clb2ΔNLS was similarly integrated...
into the Δclb1,3,4 strain. The resulting cells showed no obvious defects in growth rate as compared with the Δclb1,3,4 cells. In addition, these cells accumulated Clb2ΔNLS-GFP at the bud neck, consistent with defective nuclear import of the mutant protein (data not shown).

We hypothesized that the bud neck-localized Clb2ΔNLS-GFP represented an amplification of a normally small population of Clb2p that exists at this site. The increased cytoplasmic Clb2p pool caused by the NLS deletion might simply augment the bud neck population to allow its easier detection. To test this hypothesis, we observed the localization of Clb2ΔDbox-GFP. The elevated protein level of this mutant compared with wild type Clb2-GFP (Fig. 2B) might be expected to increase the amount of protein at the bud neck enough to allow its visualization. Indeed, Clb2ΔDbox-GFP could be seen at the bud neck of many large-budded cells (Fig. 6B). The bud tip localization was not observed with Clb2ΔDbox-GFP, however.

We also expressed wild-type Clb2-GFP in rsl1-3 cells to determine whether increasing the cytoplasmic pool of Clb2-GFP was sufficient to enable visualization of the bud neck signal. Clb2-GFP did show bud neck localization in virtually 100% of large-budded rsl1-3 cells after a 1 hour shift to the non-permissive temperature of 37°C (Fig. 6C).

The results of these three experiments imply that the extent of bud neck localization is limited by size of the cytoplasmic Clb2p pool.

**Clb2ΔNLS-GFP accumulates in the nucleus in Δyrb2 cells**

The tight nuclear localization of Clb2(L303A)-GFP provides one piece of evidence in favor of Clb2p undergoing nuclear export. To further test this idea, we took advantage of the increased cytoplasmic localization of Clb2ΔNLS-GFP and expressed the fusion protein in Δyrb2 cells, which are defective for export of leucine-rich NES-containing proteins (Noguchi et al., 1999; Taura et al., 1998). The Δyrb2 strain is cold sensitive; therefore, wild-type and Δyrb2 cells were shifted to 15°C for 14 hours, then Clb2ΔNLS-GFP was induced by addition of galactose. Because of the low temperature, very little GFP signal was visible after 1 hour of induction. After 2 hours, the fusion protein could be seen in the nucleus and cytoplasm of both wild-type and Δyrb2 cells (Fig. 7, left-hand panels). In both strains, very bright dots were visible at the edges of the nuclei along the axis of cell division. This localization is consistent with the association of Clb2ΔNLS-GFP with spindle pole bodies. The spindle pole body signal was also noted in some cells expressing wild-type Clb2-GFP or Clb2ΔNLS-GFP at 30°C, but seemed to be stabilized in the cold. Fluorescence was also seen at bud necks and bud tips in both strains. After three hours of induction, however, the nuclear Clb2ΔNLS-GFP signal was greatly enhanced in the Δyrb2 cells (Fig. 7, right-hand panels). In addition, the bud neck and bud tip populations were no longer visible in these cells. In contrast, the wild-type cells showed no difference between the two timepoints. These data strongly suggest that Clb2p normally shuttles between the nucleus and the cytoplasm. The decreased nuclear targeting efficiency of Clb2ΔNLS-GFP coupled with the retarding effect of low temperature explains the slow onset of nuclear accumulation of the fusion protein in Δyrb2 cells.

**DISCUSSION**

In *S. cerevisiae*, the activation of G2 cyclin-Cdc28p complexes is essential for inducing the switch from apical to isotropic growth as well as entry into mitosis (Amon et al., 1993; Lew and Reed, 1993; Richardson et al., 1992). The complex network of regulatory molecules that participate in or respond to G2 cyclin-Cdc28p activation is still poorly understood.
however. In an effort to shed light on the potential role of intracellular targeting on this regulation, we studied motifs in the G2 cyclin Clb2p that affect its localization.

Clb2p is primarily nuclear, but is not excluded from the cytoplasm. It contains a bipartite NLS, and the classical importin α/β transport receptor is primarily responsible for Clb2p nuclear import. The existence of additional minor Clb2p nuclear import pathways cannot be ruled out, however. This is in contrast to vertebrate cyclin B, which does not contain a classical NLS. Two different mechanisms have been proposed for cyclin B nuclear import: a piggyback mechanism that relies on interaction with the NLS-containing cyclin F (Kong et al., 2000), and an importin α-independent mechanism wherein cyclin B binds directly to the N terminus of importin β (Moore et al., 1999).

Clb2p also contains two potential leucine-rich NES motifs, and a point mutation in one of them causes the protein to be restricted to the nucleus. Clb2ΔNLS-GFP also accumulated in the nucleus of cells that have a defect in the nuclear export of NES-containing proteins. The biological role of Clb2p nuclear export remains uncertain, but it could be important for segregating different populations of Clb2p to their site of function. For example, Clb2p might be modified in the nucleus, then exported to the cytoplasm to be degraded or to associate with other cytoplasmic regulatory molecules. It remains a formal possibility that mutations in the NES affect interactions with Cdc28.

The most intriguing finding of the Clb2p localization studies was the identification of a subpopulation of Clb2p at the bud neck. The Clb2ΔNLS-GFP construct first allowed us to see this localization pattern, but the subsequent experiments with Clb2ΔDbox-GFP and wild-type Clb2-GFP in rsl1-3 cells indicate that this result is not an artefact of the NLS deletion. Moreover, faint bud neck signal could occasionally be seen for wild-type Clb2-GFP in wild-type cells. The double ring of Clb2p at the bud neck is indistinguishable from the localization of the septin proteins. Clb2ΔNLS-GFP was also visible at the cortex of unbudded cells and at the tip of newly emerging buds. The septins form a ring at the future site of bud emergence on unbudded cells, but once the bud starts to form they remain at the base of the bud.

The use of galactose-inducible protein expression in these experiments introduces some obvious caveats to their interpretation. Because the Clb2-GFP fusions are expressed ectopically, our data cannot be used to draw any firm conclusions about distinctly localized populations of Clb2p at specific points in the cell cycle. We emphasize, however, that the Clb2p import and export experiments could not have been carried out with the low level of GFP fluorescence generated by the integrated Clb2-GFP, and that the bud neck-localized Clb2p population would not have been identified without overexpression of the fusion proteins. The Clb2p bud neck localization concurs with the known biochemical association of Clb2p with Nap1p, a protein that also interacts with a kinase that localizes to the bud neck, Gin4p (Altman and Kellogg, 1997; Kellogg et al., 1995). The observance of Clb2ΔNLS-GFP at the emerging bud tip may be a consequence of uncoupling its expression from the cell cycle, but it seems unlikely that simple overexpression would cause such a specific localization. It may be possible that a very small population of Clb2p at the bud tip is protected from the APC/cyclosome. If this is true, Clb2p-Cdc28p may function in some capacity at the early stages of cell polarity establishment.

The studies presented here have identified both cis- and trans-acting determinants of Clb2p subcellular localization. They suggest several particularly intriguing avenues of further

Fig. 7. Clb2ΔNLS-GFP accumulated in the nucleus in Δyrb2 cells. Early log phase raffinose cultures of wild-type or Δyrb2 cells harboring pPS2190 were shifted to 15°C for 14 hours, then Clb2(L303A)-GFP expression was induced at 15°C by the addition of 2% galactose. GFP fluorescence images of live cells are shown for 2 and 3 hours post-induction.
investigation. First, what other proteins direct Clb2p to the bud neck, and is there any physiological role for Clb2p at the tips of emerging buds? Second, what is the significance of Clb2p nuclear export, and is it a regulated process? The answers to these questions will undoubtedly shed new light on the elaborate control mechanisms of the cell cycle.

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