Differential cellular localization among mitotic cyclins from *Saccharomyces cerevisiae*: a new role for the axial budding protein Bud3 in targeting Clb2 to the mother-bud neck

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Summary

The mitotic cyclin Clb2 plays a major role in promoting M-phase in budding yeast, despite its functional redundancy with three closely related cyclins Clb1, Clb3 and Clb4. Here, we further investigate the mechanisms controlling the cellular distribution of Clb2 in living cells. In agreement with observations recently made by Hood et al. [Hood, J. K., Hwang, W. W. and Silver, P. A. (2001) *J. Cell Sci.* 114, 589-597], we find that GFP-tagged Clb2 expressed from its natural promoter localizes to various cellular compartments, including the nucleus, the mitotic spindle, the spindle pole bodies as well as the mother-bud neck. The neck localization is specific to Clb2 as Clb1, Clb3 and Clb4 are never observed there, even when over-expressed. Mutational analysis identifies a central region of Clb2, comprising residues 213-255 and a phylogenetically conserved hydrophobic patch, as an essential cis-acting determinant. Clb2 co-localizes with the bud site selection protein Bud3. Consistent with a role of Bud3 in targeting Clb2 to the bud neck, we report a two-hybrid interaction between these proteins. Furthermore, Clb2 is shown to be specifically delocalized in Δ*bud3* cells and in a *bud3* mutant deleted for its C-terminal Clb2-interacting domain (*bud3*$^{\Delta 1221}$), but not in a Δ*bud10* mutant. Correlating with this phenotype, *bud3*$^{\Delta 1221}$ cells exhibit a pronounced (15-30 minutes) delay in cytokinesis and/or cell separation, suggesting an unanticipated function of Clb2 in these late mitotic events. Taken together, our data uncover a new role for Bud3 in cytokinesis that correlates with its capacity to target Clb2 at the neck, independently of its well established cell-type-specific function in bud site selection.

Key words: Budding yeast, Cyclins, Cell cycle, Bud3, Cytokinesis

Introduction

Proper coordination of cell cycle events is orchestrated by a family of protein kinases known as cyclin-dependent kinases or CDKs. To be active, CDKs have to be associated with a cyclin partner. In budding yeast, a single CDK, product of the *CDC28* gene, drives the entire cell cycle by successively complexing with a set of nine cyclins: three G1 or Cln cyclins and six B-type or Clb cyclins. They are thought to confer stage-specific functions to their common Cdc28 partner (for a review, see Nasmyth, 1996). Cln cyclins have functions required for commitment to a new cycle of division, including activation of Clb-Cdc28 kinase through phosphorylation of the CDK inhibitor Sic1 (Verma et al., 1997). The *CLB*s are expressed in three transcriptional waves: (1) *CLB5* and *CLB6* are first induced just before S phase in keeping with their implication in timely activation of DNA replication (Schwob and Nasmyth, 1993; Donaldson et al., 1998), (2) the expression of the mitotic cyclins *CLB3* and *CLB4* peaks upon completion of DNA replication and (3) induction of *CLB1* and *CLB2* peaks around the time of nuclear division (Fitch et al.,1992, Richardson et al., 1992).

Clb1-4 mitotic cyclins are involved in processes leading to G2/M transition, such as transcriptional regulation of numerous genes including *CLN*s (Amon et al., 1993), nuclear division (Richardson et al., 1992), regulation of protein degradation (Amon, 1997), formation of the mitotic spindle (Segal et al., 2000) and inhibition of polarized growth (Lew and Reed, 1993). However, the existence of specific functions for each mitotic cyclin remains elusive and their physiological targets unknown. Combined deletions of mitotic cyclins have shown they are at least partially redundant (Fitch et al., 1992; Richardson et al., 1992), but functionally, Clb2 seems to be especially important. Indeed, it is the only mitotic cyclin whose deletion shows a discernable phenotype with a delay in G2 and elongated cells.

All cyclins share a conserved structural domain, called the cyclin fold, dedicated to Cdc28 binding and activation (Brown et al., 1995; Jeffrey et al., 1995). B-type cyclins have in addition a highly divergent long N-terminal extension the function and structure of which have yet to be determined. Although B-type cyclins show some functional redundancy, they appear to convey specific function to their common Cdc28 partner. This is illustrated by experiments showing that Clb2 cannot efficiently replace the function of Clb5 in DNA replication even when expressed at the level and at the time of *CLB5* induction (Cross et al., 1999; Donaldson, 2000).

Table 1. Strains used

Strain	Relevant genotype	References
BF264-15Du	*MATa bar1 leu2 ura3 trp1 his2 ade1*	Richardson et al., 1989
MNY256	*MATa bar1 CLB2*-GFP2X::KAN^{R}	This study
MNY246	*MATa bar1* ura3:: *HIS3*p:*TUB*1-CFP::*URA3*	This study
MNY312	*MATa bar1* ura3::*SPA2*-GFP::URA3	This study
MNY252	*MATa bar1 bud3*::*TRP1*	This study
MNY313	*MATa bar1 bud3*::*TRP1* ura3::*SPA2*-GFP::*URA3*	This study
MNY314	*MATa*/α *BUD3*/*BUD3*-CFP::*TRP1*	This study
MNY264	*MATa bar1 bud3*$^{\Delta 1221}$::*TRP1*	This study
DHY 45	*MATa ade2 can1 his3 leu2 ura3 SPC29*-CFP::KAN	YRC Microscopy
Y524	S288c*MATa ura3 leu2 his3 lys2 ade2 trp1 cdc12-1*	Barral et al., 1999

Accordingly, cyclins have been suggested to contribute to the substrate preference of their CDK partner by direct binding (Schulman et al., 1998) (for a review, see Roberts, 1999). This is based on the identification, in mammalian cyclin A, of a conserved hydrophobic patch, spatially and functionally distinct from the cyclin/CDK interface that serves as a substrate docking site (Russo et al., 1996; Schulman et al., 1998). The 'hpm' mutation in cyclin A results from three alanine substitutions of conserved residues in this patch. It does not abolish histone H1 kinase activity but strongly interferes with the ability of CDK2 to phosphorylate physiological substrates thereby significantly lessening its biological activity (Schulman et al., 1998). The function of this hydrophobic patch seems conserved in yeast Clbs (Cross et al., 1999; Cross and Jacobson, 2000). However, the molecular determinants that modulate the selectivity of this general binding site in a specific cyclin remain to be identified.

Subcellular localization of CDK could introduce another level of specificity based on the modulation of the accessibility to specific substrates. The localization of CDK complex has been well-characterized in vertebrate cells (for a review, see Yang and Kornbluth, 1999). Although it has clearly been shown that control of nuclear localization of CDK-cyclin contributes to cell cycle regulation, the nuclear targets of the complex have not yet been identified. Controlled localization of cyclins could regulate both the kinase activity, through accessibility to specific kinase/phosphatase or to the degradation machinery (Heald et al., 1993; Takizawa and Morgan, 2000), and the targeting to specific substrates. In mammalian cells, it has recently been shown that the N terminus of cyclin B2 restricts not only its localization to the Golgi apparatus, but also its function relative to cyclin B1 (Draviam et al., 2001).

In a recent report, Hood et al. have described the mechanism of nuclear translocation of the cyclin Clb2 (Hood et al., 2001). In the course of their experiments, they also showed that the overexpressed protein localized to the mother-bud neck. We further analyzed this localization and show that it can also be detected when a Clb2-GFP fusion is expressed at physiological level from its own promoter. Furthermore, this localization appeared specific to Clb2 since it is not observed with the other mitotic cyclins. Finally, we show that the axial-budding landmark protein Bud3 is required for targeting Clb2 to the mother-bud neck in both haploid and diploid cells. Our data on *bud3*-deleted cells and cells expressing a truncated form of the protein shed light on a new role of Bud3 in cytokinesis that depends on its C-terminal domain required to target Clb2 to the bud neck but not for its function in bud site selection.

Materials and Methods

Yeast strains

All strains were isogenic with BF264-15DU (*MATa leu2 ura3 trp1 his2 ade1*) (Richardson et al., 1989) unless otherwise stated and are listed with relevant genotypes in Table 1. Strains were constructed and analyzed by standard genetic methods. Cells were grown in rich medium (YEPD: 1% yeast extract, 2% bacto-peptone, 2% dextrose) or, for plasmid selection, in synthetic minimal SC medium (0.67% yeast nitrogen base, 2% glucose, raffinose or galactose) supplemented with the appropriate amino acids at 40 μg/ml. DNA transformations were done by the lithium acetate method. Deletion of the *BUD3* gene was achieved by replacing the entire open reading frames using a *TRP1* cassette amplified by polymerase chain reaction (PCR) according to Wach et al. (Wach et al., 1994). The deletion was checked by PCR and through the bipolar budding pattern of the deleted cells (Chant and Herskowitz, 1991). The *bud3*$^{\Delta 1221}$ and *BUD3-CFP* alleles were constructed by in vivo recombination of the appropriate PCR-amplified cassettes (Wach et al., 1994). The *bud3*$^{\Delta 1221}$ mutant encodes a 13-myc fusion protein lacking the 406-C-terminal residues of Bud3.

Plasmids

Standard protocols were used to manipulate DNA (Sambrook et al., 1989). GFP tagging at the *CLB2* locus was performed with a pKHA3-*CLB2*-derivatived plasmid (kindly provided by Steve Reed) in which a *Hin*cII/*Eco*RI *CLB2* fragment was cloned in frame with a GFP$_{2X}$ tag. The resultant plasmid, pK-*CLB2-GFP$_{2X}$*, was integrated at the *CLB2* locus after *Bgl*II digestion. The plasmid pK*TUB1-CFP* was from S. Jensen (Jensen et al., 2001). Hof1-GFP and Spa2-GFP fusion protein were expressed from the vectors pBM325 (kindly provided by Marc Blondel) and pRS406-*SPA2*GFP (Arkowitz and Lowe, 1997), respectively. Truncated forms of *CLB2*, full-length *CLB2* and *CLB3* were first amplified by PCR using the Finnzyme Taq polymerase and oligonucleotides described in Table 2 as primers. In vitro mutagenesis was performed with the ExSite™ PCR-Based Site-Directed Mutagenesis Kit from Stratagene on the plasmid pRS314 *CLB5p-CLB2*^{KA,EA} (Cross and Jacobson, 2000) to generate the F354/A substitution. CLB2Δ176-213 was generated after PCR amplification of the *CLB2* ORF upstream and downstream nucleotides 528 and 639, respectively, and ligation of the two fragments by using compatible *Xho*I and *Sal*I ends. This fusion introduced a leucine in place of amino acids 176 to 213.

For localization experiments, the products of PCR amplification, in vitro mutagenesis and the full-length *CLB2* were subcloned as *Bam*HI/*Eco*RI fragments in frame with the GFP in the pRS314 or pRS316-based vectors pMJ200 and pMJ300 (Blondel et al., 2000) (this study). To construct the YFP versions of these plasmids, pMJ200-*CLB2* and pMJ300-*CLB2* were digested with *Eco*RI and *Xho*I to remove the GFP fragment and blunt ended with the Klenow fragment of the T4 DNA polymerase. YFP was introduced as a *Bam*HI/*Bgl*II-blunted fragment from the vector pDH5 (Yeast Resource Center). *CLB3* was subcloned as a *Bgl*II fragment in the

Table 2. Oligonucleotides primers used in this study

Name	Sequence
clb2-0*,†	5′-GGATCCTATAGATGTCCAACCCAA-3′
clb2-1*	5′-CGCGGATCCGAATTC**ATG**AGTGAAACTGATAGTGGA-3′
clb2-2*	5′-CGCGGATCCGAATTC**ATG**GACCTAGATGCAGAAGATGT-3′
clb2-3*	5′-CGCGGATCCGAATTC**ATG**AATATTCATCAAAATCGAGATATC-3′
clb2-4*	5′-CGCGGATCCTCAGCGGCCGCCATGCAAGGTCATTATATCAT-3′
clb2-9†	5′-GCGCTCGAGTTCGGTATTTCTAACTTGAAGTTGACT-3′
clb2-10†	5′-GCGGTCGACCTAGATGCAGAAGATGTAAATGATCCA-3′
clb2-F/A‡	5″-CATCGGATTGGGATAATTTAGGTTAGCTTTCAATGTCTTTAAAATG-3′
clb3-1§	5′-CGCAGATCTGAATTCAAAATGCATCATAACTCACAGTC-3′
clb3-2§	5′-CGCAGATCTTCCGGGTTGGGTTTATACAGGTC-3′
clb3-3§	5′-CGCGGATCCGGATAAACAACCAGAGTTAAGA-3′
clb3-4§	5′-CGCGAATTCAGCGGCCGCTGTTAGATCTTTCTACTCTGTG-3′
bud3-1¶	5′-TTACGTGTTCGGGCTCTTATCTGGTTGCTAAAAGAGTATATTTACACCTCACCACGGATCCCCGGGTTAATTAA-3′
bud3-2¶	5′-AATGTATACATTGCATTAAATTAAAAAGAAAAAAAAAATCAATAAAACACGAATTCGAGCTCGTTTAAAC-3′
bud3-3¶	5′-TGGGAAACAGAACTGCGCTGTTGGTGGCCCAGAGAAACTGAAATTTTATCGGATCCCCGGGTTAATTAA-3′
Bud3-4**	5′-GCGCCGCCAGCTTCAAAAATTAACTTTCAAAGGTCACCATCCTATATTCGGATCCCCGGGTTAATTAA-3′
Bud3-5**	5′ATAATGGGCATCATGCTTATCTAAAACCACCCTCATTCCTTGAAAGAGGAATTCGAGCTCGTTTAAAC-3′

*Primers used for PCR amplification of truncated mutants of *CLB2* (Δ132, Δ176, Δ213, Δ255). Underlined nucleotides correspond to restriction sites used for cloning into the pMJ200 vector and pRS316GAL1-HA3.

†Primers used for the construction of the Δ176-213 internal deletion of *CLB2*. Underlined nucleotides correspond to the restriction sites used for the internal fusion (clb-9 and clb-10) and for cloning into the pMJ vectors and pRS316GAL1-HA3 (clb2-0 and clb2-14 or clb2-4).

‡Primers used for F354/A in vitro mutagenesis. Underlined nucleotides correspond to introduced mutations.

§Primers used for amplification of *CLB3* full-length, C-ter and N-ter domains. Underlined are restriction sites for chimeras construction and cloning into pMJ300, and pRS316GAL1-HA3.

¶Primers used for PCR-based *BUD3* deletion (1 and 2) and CFP tagging (3 and 2).

**Primers used for PCR-based construction of the *bud3*$^{\Delta 1221}$ allele.

*Bam*HI site of pMJ200. For protein expression from the *GAL1* promoter, *Eco*RI/*Not*I fragments were subcloned in frame with a triple HA tag in a pRS316-GALp1,10-HA3 (this study). Chimeras have been made at the level of the internal *Bgl*II restriction site of *CLB2*, which is located just before the beginning of the cyclin box-encoding sequence. The Clb2/3 chimera was constructed by PCR amplification of the *CLB3* ORF from nucleotide 571 to the end and subcloning it as a *Bam*HI/*Eco*RI in pMJ300-*CLB2* cut with *Bgl*II/*Eco*RI. This fusion introduced an extra Pro residue between Asp250 from Clb2 and Asp191 from Clb3. The Clb3/2 chimera was constructed by PCR amplification of the *CLB3* ORF from the start codon to nucleotide 561 and subcloning it as a *Bgl*II fragment in pMJ300-*CLB2* cut with *Bam*HI and *Bgl*II. This fusion introduced an extra Glu residue between Pro186 from Clb3 and Asp250 from Clb2.

Protein analysis

To determine their capacity to bind and to activate Cdc28, mutant and truncated proteins were overexpressed from the GAL promoter as (HA)3-tagged proteins. Total extracts were prepared as described previously (Bailly and Reed, 1999) from mid-log cultures in raffinose medium in which protein expression was induced by 2% galactose for 3 hours. For immunoprecipitation of HA-tagged cyclins, 500 μg of lysate protein were incubated with rabbit polyclonal anti-HA-specific antibodies and incubated with protein A Sepharose as previously described (Bailly and Reed, 1999). Immunoprecipitates were resolved on SDS-PAGE and transferred on nitrocellulose membranes. Membranes were cut and incubated with either anti-HA or anti-PSTAIRE antibodies. The associated kinase activity was assayed with histone H1 (Life technologies) as described previously (Richardson et al., 1992). Kinase activity was visualized by autoradiography.

Microscopy

Fluorescence and phase contrast microscopy were performed using a Zeiss Axiovert microscope with a 100× objective. Cell images were captured with an ORCA-ER Camera (Hamamatsu) using Axio-Vision software (Zeiss) and subsequently exported into Adobe PhotoShop F1-4.0 for preparation of figures. Iterative deconvolution was performed using The Huygens Essential software (Bitplane).

Induction of GFP fusion protein from the GAL1 promoter was performed as follows. Cells were grown to mid-log phase in SC-ura or SC-trp raffinose medium and approximately 10^7 cells were filtered onto 47 mm 0.45 μm GN-6 Metricel membrane (Gelman Science) as described previously (Jensen et al., 2001). Filters were then placed on YEP-Gal plates for 3 hours at room temperature. Cells were collected in sterile water before microscopic observation.

Results

Cellular localization of mitotic cyclins

A previous report showed that the cyclin Clb2 localizes to various cellular structures including the mother-bud neck (Hood et al., 2001). We wished to examine whether this pattern of localization was common to the four mitotic cyclins. A GFP tag was appended to the C terminus of Clb1, 2, 3 and 4 whose expression was under the control of the *GAL1* inducible promoter. Analysis of the distribution of the four GFP fusion proteins showed that the localization as a double ring at the mother-bud neck was specific to Clb2 and not found with Clb1, Clb3 or Clb4 (Fig. 1A). Besides this major difference, all four proteins were found to have a similar localization pattern, being primarily nuclear with a small but significant fraction located in the cytoplasm. Another common feature was their association with a line connecting the two DNA masses in late stages of the cell cycle as well as fluorescent foci situated at the edge of the nucleus. In order to determine whether these signals co-localize with specific cellular structures, Clb2 fused to the yellow variant of GFP (YFP) was introduced in strains expressing either *TUB1*-CFP fusion or *SPC29*-CFP, a known spindle pole body (spb) component. The signals showed a perfect coincidence of the fluorescence (Fig. 1B,C)

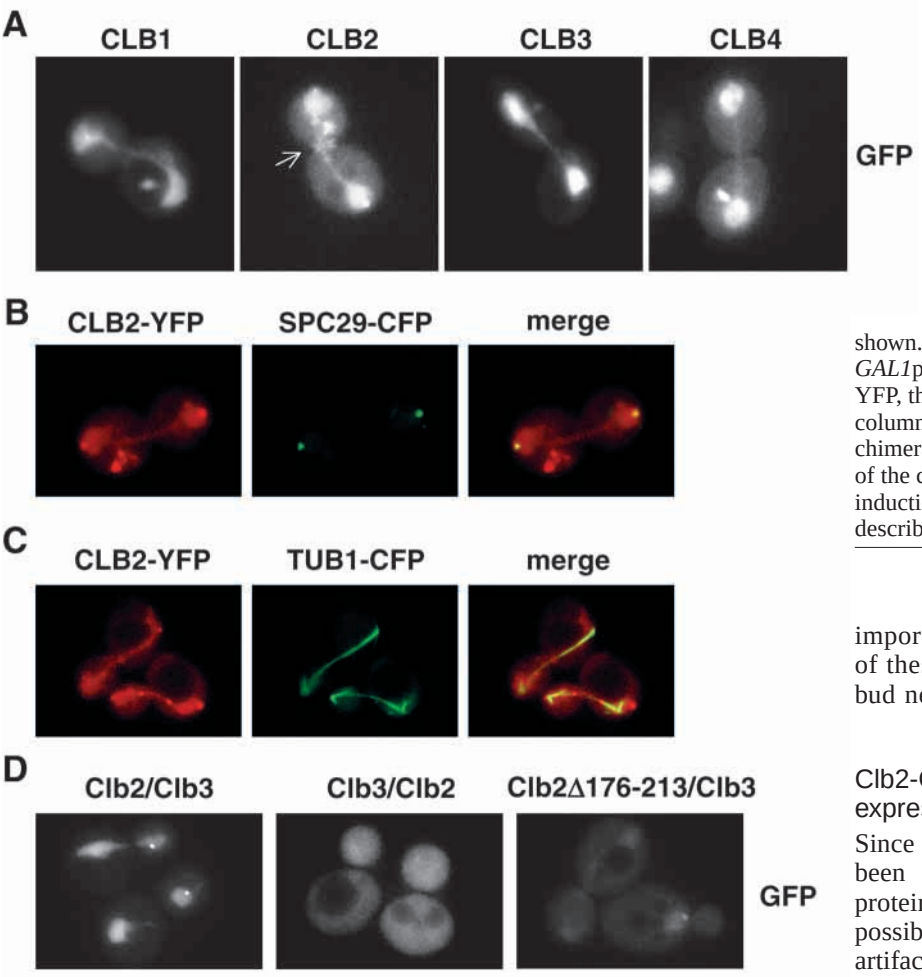

Fig. 1. Mitotic cyclin localization. (A) Localization of the Clb1, Clb2, Clb3 and Clb4 mitotic cyclins. The indicated mitotic cyclins were expressed as GFP fusions under the control of the *GAL1* promoter. Cells grown in SC-raffinose were collected on nitrocellulose filters and induced for 3 hours on YEP-galactose plates before microscopic observation. (B,C) Clb2 localizes both on the spindle pole bodies and along the mitotic spindle. Cells expressing Spc29-CFP and Clb2-YFP or Tub1-CFP and Clb2-YFP are shown. CLB2-YFP was overexpressed from the *GAL1*p as described below. The left hand column is YFP, the center column is CFP and the right hand column shows merged YFP/CFP. (D) Localization of chimeras between Clb2 and Clb3. Cellular localization of the chimeras fused to GFP is shown. Galactose induction of the GFP-fused chimeras was achieved as described in A.

demonstrating that Clb2 localizes along the mitotic spindle and to the spb.

The accumulation of Clb2 in the nucleus depends on a bipartite nuclear localization signal (NLS) located in the N-terminal part of the protein (Hood et al., 2001) (and see below). A close examination of Clb sequences revealed another important difference between Clb2 and Clb3, i.e. the absence of a consensus NLS in Clb3. This suggested that Clb3 could be imported into the nuclear compartment by a mechanism distinct from that of Clb2. To identify sequences in Clb3 that could mediate its nuclear import, we performed domain-swapping experiments between Clb2 and Clb3. A Clb3/2 chimera with a 170-aa (amino acid) N-terminal fragment of Clb3 fused to the cyclin fold of Clb2 (240 aa) remained cytoplasmic (Fig. 1D). Conversely, fusing the first 250 amino acids of Clb2 to the cyclin fold of Clb3 yielded a Clb2/3 chimeric cyclin that efficiently localized to nuclear compartment. However we found that a Clb2/3 chimera with an internal deletion (residues 173 to 216) which removes the NLS of Clb2 was defective in nuclear import, demonstrating that the nuclear targeting of Clb2/3 depends on the Clb2 NLS. Both chimeras appeared to interact with and activate Cdc28 at wild-type levels (see Fig. 3). We conclude from these experiments that none of the N- or C-terminal Clb3 fragments have the potential to promote nuclear import of this mitotic cyclin. Another important finding is the observation that none of these chimeras was localized to the mother-bud neck.

Clb2-GFP localizes to the bud neck when expressed from its own promoter

Since the bud neck localization of Clb2 has only been described upon overexpression of the protein, it was absolutely essential to rule out the possibility that this distinct localization was an artifact of protein overexpression. We introduced an in tandem GFP tag at the *CLB2* chromosomal locus to analyzed Clb2 cellular distribution when expressed at physiological level. This approach together with the use of deconvolution microscopy allowed us to confirm the bud neck localization of Clb2 in both haploid and diploid cells (Fig. 2) (data not shown). Moreover, this more physiological condition enabled us to observe this staining before anaphase up to late stages of mitosis. This is in contrast to overexpression of the GFP fusion protein which leads to a strong nuclear signal making the bud neck staining very difficult to distinguish until the two nuclei have separated in late anaphase cells.

Since the Clb cyclins diverge the most at their N-ter extension, we generated various mutant forms of Clb2 that progressively truncated its N terminus (Fig. 3A). We first checked the ability of these mutants to interact with and activate Cdc28. For this purpose, each of the truncated forms was tagged with a triple HA tag. As illustrated in Fig. 3B, all but one Clb2 mutants, namely Clb2-Δ255, appeared to bind Cdc28 and activate its histone H1 kinase activity as efficiently as the wild-type protein. The defective CDK binding of Clb2-Δ255 is in agreement with previous biochemical and structural studies showing the importance of the corresponding region of mammalian cyclin A in the cyclin/CDK interface (Kobayashi et al., 1992; Jeffrey et al., 1995; Brown et al., 1995).

We then analyzed the cellular distribution of the GFP-tagged truncated mutants (Fig. 3C,D). This cytological study revealed that a deletion up to amino acid 176 had no noticeable

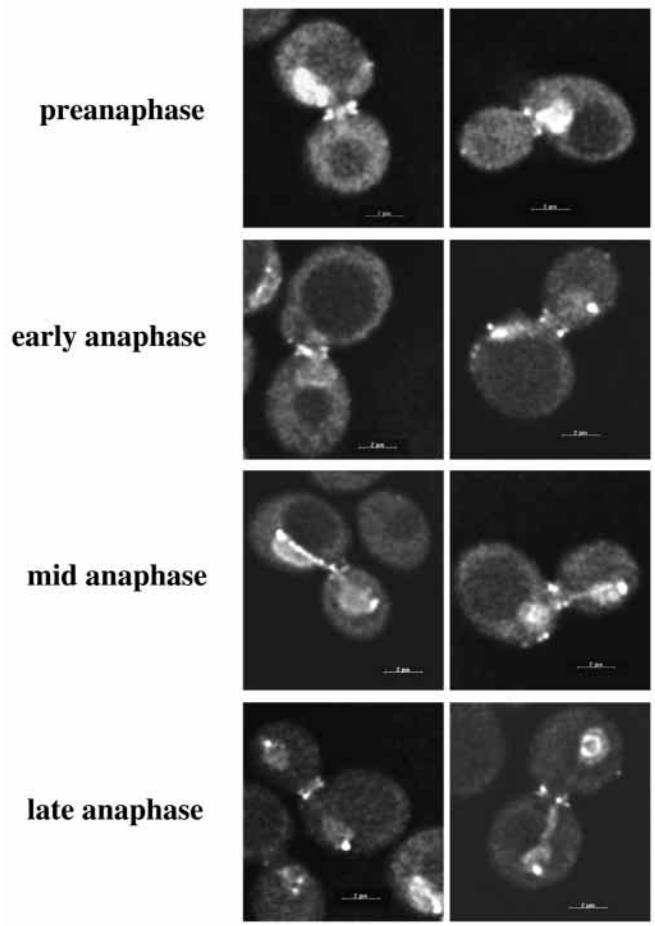

Fig. 2. Clb2-GFP shows similar localization when expressed from its endogenous promoter. 15Du haploid cells in which two GFP tags were introduced in tandem at the *CLB2* chromosomal locus were grown to midlog phase in YPD supplemented with 45 μg/ml adenine. Each frame was generated by projecting processed images (Hyugens software, Bitplane) of four focal planes taken at 0.15 μm intervals through the cell. Exposure times were 3 seconds for each plane, to be compared to a mean exposure time of 500 mseconds when the Clb2-GFP fusion is overexpressed.

effect on the cellular distribution of Clb2. Therefore, the region of aa 1-176, which harbors the destruction motifs of this mitotic cyclin does not appear to play a major role in Clb2 localization. A larger deletion that further eliminates the next 37 residues (Clb2 Δ213), as well as an internal deletion removing residues 173-213 (Clb2 Δ173-213) prevented targeting of Clb2 to the nucleus but not to the bud neck, the spb or the mitotic spindle (see also Fig. 4D). This strikingly contrasted with the effect of removing the 255 aa N-terminal region (Clb2 Δ255) as evidenced by the inability of this mutant to localize properly to the nucleus, to the bud neck, the spb and the mitotic spindle. Fusing the 255 aa N-terminal fragment of Clb2 (Clb2-Nter) to GFP was sufficient to drive the fusion protein into the nucleus, but not to the bud neck or the mitotic spindle (not shown). This domain cannot account on its own for the specific bud neck localization of Clb2, suggesting that additional determinants must exist elsewhere in the protein.

Clb2 localization to the neck is Cdc28 independent but needs a functional hydrophobic patch

We have shown that the N terminus up to aa 213 was dispensable for targeting Clb2 to the mother-bud neck, while a larger deletion up to residue 255 completely abolished the bud neck localization. Since Clb2-Δ255 was also defective in binding Cdc28, we wondered whether Cdc28 interaction was required for proper Clb2 localization. To address this question more directly, we used a mutant in which three highly conserved amino acids known to contribute essential interactions in the cyclin A/CDK2 interface (Jeffrey et al., 1995; Bazan, 1996) were mutated to alanine (K316A, E345, F354A). As described for a Clb5 mutant with the homologous substitutions (Cross and Jacobson, 2000), $Clb2^{KA,EA,FA}$ was found to be severely impaired in a Cdc28 co-immunoprecipitation assay as compared to the wild-type protein. Consistent with this result, no histone H1 kinase activity could be detected in the immunoprecipitate (Fig. 4A).

Despite its strong Cdc28 binding defect, the triple $Clb2^{KA,EA,FA}$ mutant exhibited an overall cellular distribution similar to that of wild-type Clb2. In particular, this mutant appeared to be retained at the bud neck to the same extent as the wild-type protein (Fig. 4B,C). $Clb2^{KA,EA,FA}$ is also detected along the mitotic spindle and at the spb. This result strongly suggests that Clb2 localization is independent of its Cdc28 partner. Therefore the loss of bud neck localization that was observed for Clb2-Δ255 is probably due to the deletion of key residues in the aa 213-255 region.

As mentioned above, the finding that a N-ter fragment, encompassing the first 255 residues of Clb2, was necessary but not sufficient to target GFP to the bud neck, suggested the need for additional signals that presumably map further downstream, i.e. to the cyclin box. The lack of bud neck localization of a Clb2/3 chimera, which contains the 255 residue N-ter region of Clb2 further supports this view. In a first step toward identifying these additional signals, we considered the possibility that the conserved hydrophobic patch located at the beginning of the cyclin box might be involved. In other cyclins such as cyclin A, this hydrophobic patch has been proposed to function as a substrate docking site. The hpm mutations resulting from the substitution of three residues in this motif (N260A, L264A, W267A) have been described to deeply impair the ability of Clb2 to rescue a *clb1,3,4Δ clb2*ts strain (Cross and Jacobson, 2000). Co-immunoprecipitation experiments indicated that the Cdc28 binding and activation capacity of a triply HA-tagged Clb2-hpm mutant was only moderately affected (Fig. 4A). In contrast to the wild-type protein, Clb2-hpm fused to GFP was undetectable at the mother-bud neck and in the cytoplasm, and an increased nuclear staining was observed instead (Fig. 4B,D). Because the lack of bud neck localization could be an indirect consequence of the massive nuclear accumulation of the mutant protein, we tested whether Clb2-hpm could be targeted to the neck when forced to reside in the cytoplasm. We therefore constructed a Clb2 mutant, Clb2-hpmΔNLS, that combines the hpm mutation and the deletion of the NLS-containing region aa 176-213. As expected, this mutant was trapped in the cytoplasm, yet it did not localize to the neck region. These data indicate that interactions involving the hydrophobic patch are required for bud neck localization of Clb2 and its normal nucleocytoplasmic shuttling.

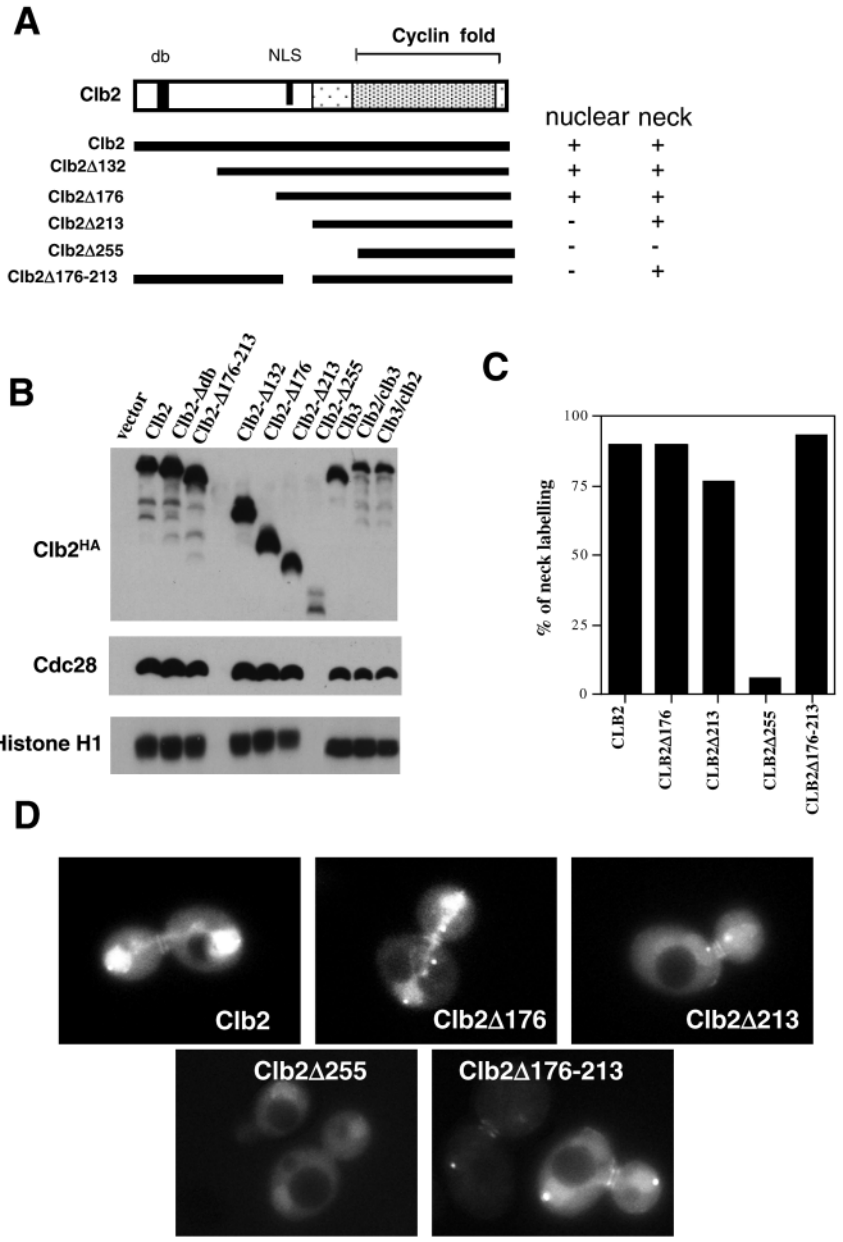

Fig. 3. Localization of truncated forms of Clb2. (A) Construction of truncated forms of Clb2. Positions of truncations and internal deletion are shown relative to the cyclin fold of Clb2 (started at residues 259). (B) Cdc28 binding and activation properties of truncated Clb2 mutants and chimera. Wild-type cells were transformed with a control vector or indicated constructs allowing expression of HA-tagged protein from the *GAL1* promoter and grown in SC-ura raffinose medium. Expression of the HA-tagged proteins was induced by addition of 2% galactose for 3 hours. Cell lysate were processed for immunoprecipitation with anti-HA antibodies. Clb2-HA (top panel) and Cdc28 (middle panel) in the immunoprecipitates were assayed by western blotting with anti-HA and anti-PSTAIRE antibodies respectively. The bottom panel shows an autoradiogram of the Clb2-associated histone H1 kinase activity present in the immunoprecipitates. (C) Bud neck localization of the truncated forms of Clb2. To make the comparison more accurate, the proportion of bud neck staining was estimated only in large budded cells with separated nuclei, a stage where the staining is best visualized with the wild-type protein. At least 150 cells were counted for each strain. (D) Cellular localization of truncated mutants fused to GFP. Expression of the GFP-fusion proteins from the *GAL1* promoter was as described in Fig. 1.

Bud3 is required for targeting Clb2 to the bud neck

In the course of a two-hybrid screen with Clb2 as a bait, we isolated a C-terminal encoding fragment of the *BUD*3 gene whose function in specifying the axial budding pattern in haploid cells is well established (Chant and Herskowitz, 1991). This two-hydrid interaction was specific of Clb2 as it was not observed with Clb3 (to be described elsewhere). Bud3 has been shown to assemble in a double ring encircling the mother-bud neck at the G2/M transition in both haploid and diploid cells (Chant et al., 1995). This observation, in the context of our two-hybrid and cytological data on Clb2, raised the possibility that Bud3 could play a critical role in mediating the Clb2 localization to the bud neck. To test this hypothesis, we first asked whether Clb2 could still localize to the neck in the absence of Bud3. Deleting the *BUD3* gene appeared to dramatically interfere with the ability of Clb2 to accumulate at the neck (Fig. 5A,B). This phenotype could be mimicked by truncating the 406 C-terminal residues of Bud3 (*bud3*$^{\Delta 1221}$) present in the two-hybrid fragment (Fig. 5B). This C-terminal deletion does not affect the axial budding pattern of *bud3*$^{\Delta 1221}$ cells (not shown) suggesting that the Clb2 targeting function of Bud3 is independent of its role in bud site selection. This hypothesis is reinforced by two observations: (i) the bud neck localization of Clb2 is also altered in diploid *Δbud3* cells in which bud site selection is not affect, and (ii) Clb2 localization is not affected by the deletion of *BUD10*, another gene required for the axial pattern of budding (data not shown). Interestingly, the effect of the *BUD3* deletion was quite specific in that neither the nucleocytoplasmic distribution of Clb2 nor its spb and mitotic spindle localizations were affected in the *Δbud3* or *bud3*$^{\Delta 1221}$ mutants.

We next wondered whether deleting *BUD3* would have deleterious consequence on the localization of other neck-localized proteins. We analyzed the localization of two proteins, Spa2 and Hof1, known to bind to the septin ring at different time of the cell cycle (Arkowitz and Lowe, 1997; Kamei et al., 1998; Vallen et al., 2000). Both Hof1 and Spa2 were detected as a double ring at the mother-bud neck of *Δbud3* and wild-type cells (Fig. 5A and data not shown). We conclude that the defect of Clb2 localization to the neck does not result from a gross disorganization of the neck structure.

It has been shown that assembly and maintenance of Bud3 rings are dependent upon the septin neck filaments (Chant et

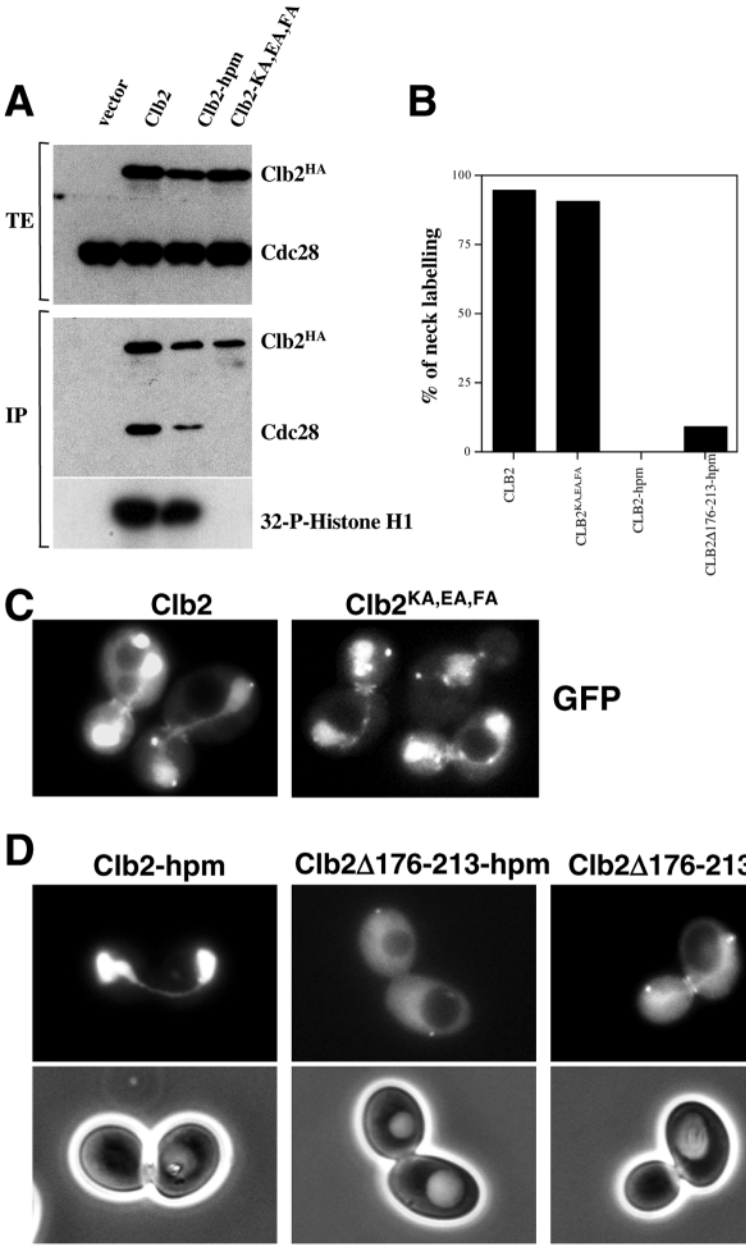

Fig. 4. Cdc28 binding activity and cellular localization of Clb2 mutants. (A) Cells were transformed with a control vector or the indicated constructs to direct expression of HA-tagged proteins under the control of the *GAL1* promoter. Transformants were grown in SC-ura raffinose medium, arrested in M phase with nocodazole (15 μg/ml) for 2 hours before induction of protein expression by 2% galactose for 3 hours. Nocodazole arrest was used to avoid potential bias due to differences in cell cycle progression upon overexpression of the various Clb2 mutants. Cells were processed for immunoprecipitation with anti-HA antibodies and the immunoprecipitates were analyzed for their content of Clb2-HA and Cdc28 by western blotting and for H1-kinase activity (IP) as described in Fig. 3. Aliquots of the total extracts were also assayed for Clb2-HA and Cdc28 (TE). (B) Proportion of bud neck staining was estimated as described in Fig. 2. (C) The *Clb2*^{KA,EA,FA} mutation was expressed from the *GAL1* promoter as GFP-fusion proteins and its cellular distribution followed by fluorescence microscopy as described in Fig. 1. (D) Cellular localization of Clb2-hpm, Clb2Δ176-213, Clb2Δ176-213-hpm mutants fused to GFP.

al., 1995). We thus wanted to test if Clb2 localization has the same requirement by examining the localization of Clb2-GFP in a *cdc12-1* temperature-sensitive mutant. Clb2-GFP was delocalized from the neck in most of the *cdc12-1* mutant cells incubated at restrictive temperature (Fig. 5C). The detection of bud neck staining in wild-type control cells similarly incubated at this temperature indicates that mislocalization of Clb2-GFP did result from the inactivation of Cdc12 at 37°C. This result demonstrates that bud neck localization of Clb2, like that of Bud3, requires an intact septin ring.

Finally, our hypothesis that Bud3 is directly involved in the Clb2 bud neck localization would predict that both proteins colocalize in the neck region. To address this point a *GAL1*p-*CLB2*-YFP construct was introduced in cells harboring a CFP tag at the *BUD3* chromosomal locus. Because tagging the C-terminal end of Bud3 was found to significantly interfere with the ability of Clb2 to localize to the neck (data not shown), we monitored Clb2-YFP distribution in a *BUD3*-CFP/*BUD3* diploid strain. As shown in Fig. 5D, both YFP and CFP fluorescence decorated exactly the same structure at the bud neck.

Cytokinesis is affected in *Δbud3* and *bud3*^{Δ1221} cells

To date, the only well established role of Bud3 is in the generation of an axial budding pattern in haploid cells. The two-hydrid interaction between Bud3 and Clb2 as well as the requirement of Bud3 for anchoring Clb2 to the neck suggested that *BUD3* could play a role in cell cycle progression. To address this question, we synchronized *Δbud3*, *bud3*^{Δ1221} and the wild-type isogenic strains by arresting cells with α-factor and releasing them in fresh medium at 25°C. Samples were taken at regular intervals and analyzed by FACS. Fig. 6A shows that DNA replication is not affected in *Δbud3* and *bud3*^{Δ1221} cells. In contrast, exit from mitosis, which started at 105 minutes in wild-type cells, was delayed by 15-30 minutes in both *Δbud3* and *bud3*^{Δ1221} cells. This delay temporally coincided with the accumulation of cells with a 4C DNA content. This suggests that entry into a new cell cycle is not prevented in either mutant. Consistent with this hypothesis, we found that at 120 minutes, a high percentage of *Δbud3* and *bud3*^{Δ1221} cells initiated a new budding cycle prior to cell separation (Fig. 6B,C). These rebudding cells are resistant to zymolyase treatment indicating that they still share cytoplasm (not shown).

To better characterize the mitotic stage of the cell cycle affected in *Δbud3* and *bud3*^{Δ1221} cells, we repeated the cell cycle experiment described above except that entry into a second round of DNA replication was prevented by adding back α-factor at 75 minutes after the release. The kinetics of nuclear and cellular division was followed by monitoring the percentage

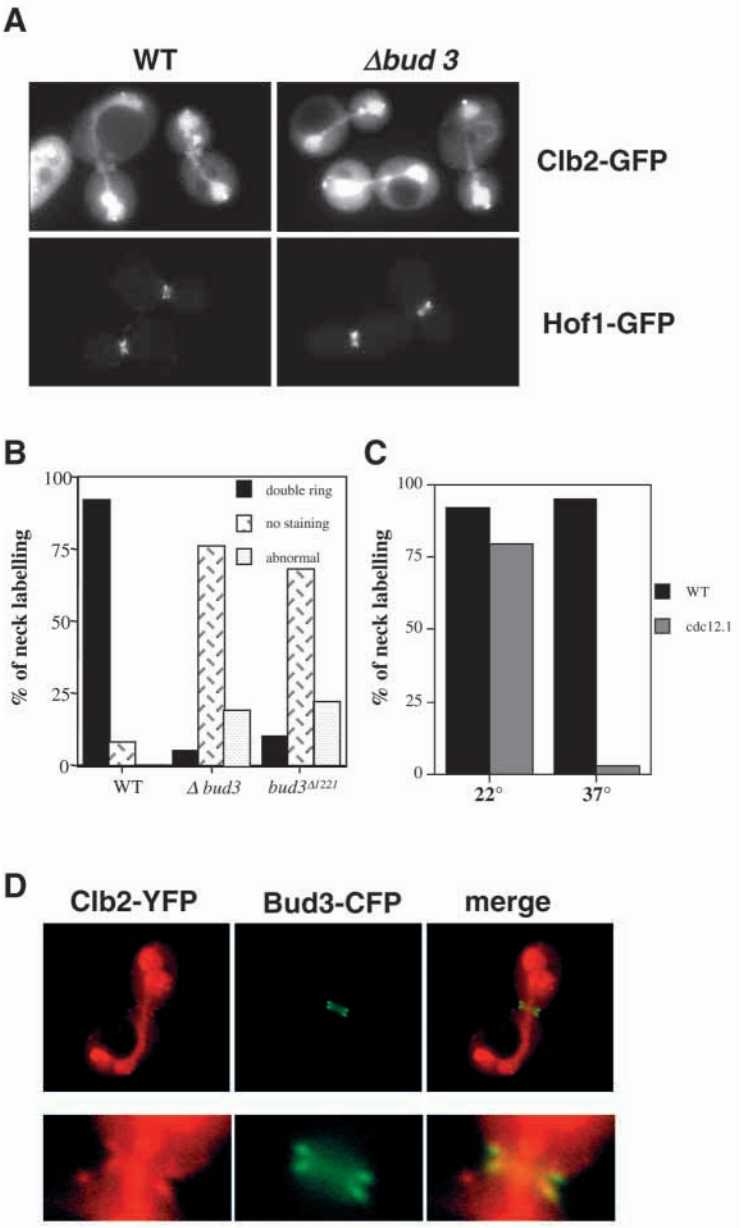

Fig. 5. Role of Bud3 in targeting Clb2 to the neck. (A,B) Localization of Clb2-GFP fusion protein expressed from the *GAL1*p promoter in 15DU haploid cells harboring a deletion of the *BUD3* gene or expressing a truncated form of Bud3 (*bud3*$^{\Delta 1221}$). The double ring labeling observed in wild-type cells is almost completely abolished in *Δbud3* cells although some cells (about 25%) show a faint single band at the bud neck junction. In contrast, the *BUD3* gene is not required for proper cell cycle-regulated localization of Hof1 to the mother-bud neck. (C) Clb2 localization to the neck depends on septin integrity. Cells were grown in SC raffinose at 22°C. Half of the culture was shifted to the restrictive temperature (37°C) for 2 hours and then induced for 3 hours on YEPGal plates prewarmed at the same temperature. The proportion of cells exhibiting bud neck staining was estimated in large budded cells with separated nuclei by microscopic observation and counting. At least 150 cells were counted for each strain and condition. (D) Clb2-YFP colocalizes with Bud3 at the mother-bud neck. CLB2-YFP was overexpressed from the *GAL1p* in a 15DU diploid strain in which a CFP tag was appended to the C terminus of one copy of the *BUD3* gene. The left hand column shows YFP, the center column shows CFP and the right hand column shows merged YFP/CFP signals.

of binucleate cells (Fig. 6C) and the budding index (Fig. 6D). The increase of budded cells with two separated nuclei followed the same kinetics regardless of the *BUD3* genotype, indicating that the onset of anaphase was not affected. In contrast, whereas cell separation began at 120 minutes in the wild type, it was delayed for 15-30 minutes in *Δbud3* and *bud3*$^{\Delta 1221}$ cells since the drop in the frequency of budded cells occurred at 135-150 minutes in these strains (Fig. 6D). A similar lag was observed in the disappearance of binucleate cells in *Δbud3* and *bud3*$^{\Delta 1221}$ compared to the wild type. Altogether our data strongly suggest that the delay in mitotic exit of *Δbud3* and *bud3*$^{\Delta 1221}$ cells stems from a defect in cytokinesis rather than initiation of anaphase.

Discussion

Differential localization of mitotic cyclins

In this report, we show that the bud neck localization of Clb2, first observed by Hood et al. (Hood et al., 2001) upon overexpression of the protein, can also be found under more physiological condition where GFP-tagged Clb2 is expressed from its own promoter. Moreover, this localization is unique among mitotic cyclins in *S. cerevisiae*. In both haploid and diploid cells, Clb2 was detected at the neck in medium to large budded cells, i.e. before the beginning of anaphase until telophase. Our co-localization data demonstrate that Clb2 is also targeted to the mitotic spindle and to the duplicated spb.

Besides the specific localization of Clb2 to the neck, mitotic cyclins also differ in their mechanism of nuclear translocation. Our results indicate that the nuclear localization of Clb2 primarily relies upon a region comprising amino acids 176-213. This region indeed contains a consensus bipartite NLS that has been characterized by others (Hood et al., 2001). In contrast, Clb3 lacks a classical NLS. Our data using chimeric cyclins indicate that neither the cyclin box nor the complementary N-ter fragment of Clb3 can mediate nuclear import on their own. We conclude that nuclear localization of Clb3 involves molecular determinants that are not conserved in Clb2 and not restricted to a single region of the protein. The reason for the existence of different nuclear import mechanisms for Clb2 and Clb3 remains to be elucidated. The functional implications are also difficult to address given the absence of a clearly defined role for Clb3 during the cell cycle.

Interestingly, this diversity in nuclear translocation mechanisms is reminiscent of what has been reported in other organisms. For example, in mammalian cells, cyclins A, E and B are imported by different pathways to the nucleus and show marked differences in their shuttling behavior between the nuclear and cytoplasmic compartments (Jackman et al., 2002). This raises the possibility that cyclin/CDK function could be regulated by modulating their import or export from the nucleus. Accordingly, it has recently been suggested that some function of the Cln2/Cdc28 complex at START would require active nucleocytoplasmic shuttling (Edgington and Futcher,

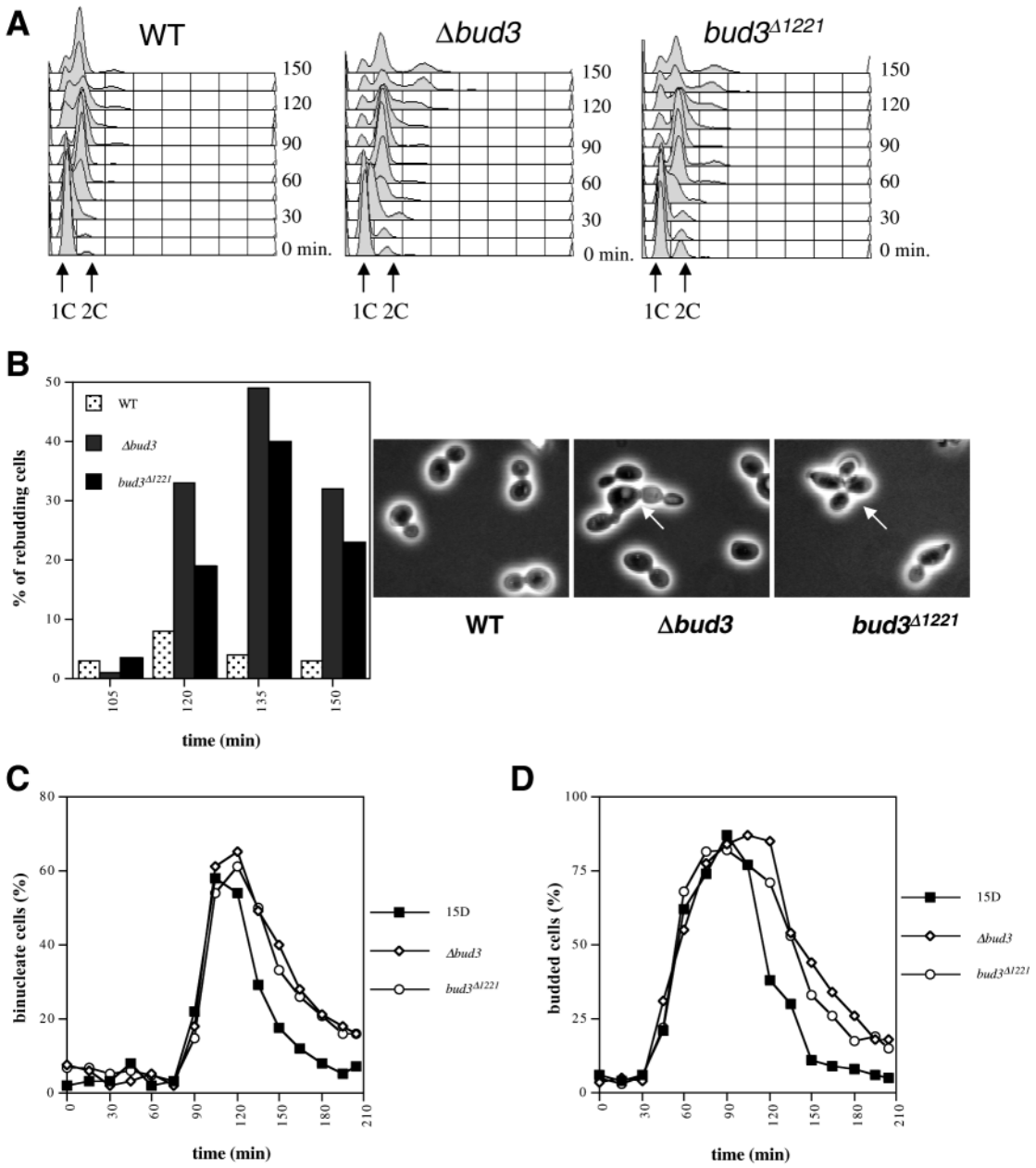

Fig. 6. Cytokinesis is delayed in *Δbud3* and *bud3*$^{\Delta 1221}$ cells. (A) Cells (15DU, MNY252, MNY264) were arrested with 60 ng/ml α-mating factor for 2 hours and released synchronously at 25°C. Aliquots were taken every 15 minutes and DNA content analyzed by flow cytometry. (B) The proportion of cells entering a second round of budding before cell separation (arrow) was scored at the indicated times. (C,D) Cells were synchronized as in A but a second cell cycle was prevented by adding back 60 ng/ml α-factor at $t_{75\ min}$. The percentage of binucleate cells (C) and budded cells (D) was determined microscopically after a brief sonication.

2001). We did not observe significant variations in Clb3 nuclear localization during the cell cycle (data not shown), suggesting that Clb3 might be constitutively imported into the nucleus throughout the cell cycle.

A complex set of molecular determinants is required for Clb2 localization to the neck

Various mutations, either deletions or point mutations, were introduced to identify cis-acting domains within the *CLB2* coding sequence that could be essential for its neck distribution. First, the triple mutant *Clb2*^{KA,EA,FA} that shows a strong defect in Cdc28 binding and activation, localizes to the neck as efficiently as does the wild-type protein. The Cdc28 independency of Clb2 localization further supports the idea that cyclins play a role in targeting the kinase to different cellular compartments. We next tested the implication of a conserved hydrophobic patch present at the beginning of the cyclin box in this mechanism. Analysis of point mutations within this region revealed that the corresponding Clb2-hpm mutant is strongly affected in bud neck localization. The hydrophobic patch is well conserved among yeast B type

cyclins and it is likely that additional residues are required for proper localization of Clb2 to the neck. This hypothesis is strengthened by our finding that neither Clb2-Δ255 nor the Clb3/Clb2 chimera localizes to the neck although they contain an intact hydrophobic patch. Additional key residues in the region upstream of the cyclin box act in concert with this motif to confer Clb2 selectivity. Since Clb2-Δ213 still localizes to the neck, this region can be restricted between amino acids 213 to 255. All together, our data defined a region, comprising the hydrophobic patch and upstream sequence, required for bud neck localization of Clb2. Whether this region is sufficient remains to be determined. Unfortunately, all other chimeras constructed at a point upstream or downstream amino acid 255 of Clb2 were deeply deficient in binding Cdc28, suggesting that their overall structure was affected. However, our preliminary data on a new mutant allele of Clb2 suggest that residues in the C-terminal region of Clb2 would also be required for proper Clb2 localization to the neck (data not shown).

The lack of neck localization of Clb1 is at first sight surprising given its high sequence homology with Clb2 and its supposedly redundant functions. However, the Clb2 region from amino acids 213 to 255 contains specific residues that could be essential for correct targeting of the protein to the neck. Alternatively, the highly divergent sequence upstream of this region could negatively modulate bud neck localization of Clb1.

A dramatic consequence of the hpm mutation in Clb2 is the strong increase of its nuclear localization. One interesting possibility to explain this phenotype is that binding to the neck is required to maintain a fraction of the protein in the cytoplasm. This does not seem to be the case because the Clb2/Clb3 chimera, which does not localize to the neck, still exhibits a nuclear and cytoplasmic distribution. Also, in a *cdc12*-1 strain, Clb2 was delocalized from the neck at restrictive temperature and yet was still detectable in the cytoplasm. Alternatively, the massive nuclear accumulation of Clb2-hpm could reflect its inability to be exported from the nucleus. Consistent with this possibility, Hood et al. (Hood et al., 2001) have presented evidence for the existence of an active nuclear export of Clb2 mediated by a Yrb2-dependent pathway. The recent finding that hpm mutations had a very disturbing effect on the nuclear distribution of human cyclin A (Jackman et al., 2002) further argues in favor of this hypothesis. Our results indicate that, whatever the exact role of the hydrophobic patch is, it is not restricted to interactions with Clb2/CDK substrates.

A new role of Bud3 in targeting Clb2 to the neck

The most striking result of this study is the requirement of Bud3 for Clb2 localization to the neck as evidenced by the facts that: (i) the C-terminal part of Bud3 interacts with Clb2 in a two-hydrid assay, (ii) the neck localization of both proteins is indistinguishable and in both cases dependent upon the integrity of the septin ring and (iii) Clb2 localization to the neck is specifically disrupted upon *BUD3* deletion in both haploid and diploid cells. It should be also noted that the neck localization of Clb2 temporally coincides with the cell cycle-regulated expression of *BUD3*, i.e. in G2/M (Fig. 2) (Lord et al., 2000). Finally, we found that truncation of the Clb2-interacting domain of Bud3 strongly affects Clb2 localization to the neck without disrupting axial budding. Altogether, these observations are in favor of two distinct functions for Bud3. One is specific to haploid cells and mediated by the N-terminal part of the protein (Chant et al., 1995) (and this study). The second function acts in both haploid and diploid cells and relies on the C-terminal region of Bud3. To date, the only well-established role of Bud3 is in promoting the axial budding pattern of haploid cells of *S. cerevisiae* in which both mother and daughter cells form new buds adjacent to the preceding division site (Chant and Herskowitz, 1991). Our cell cycle analysis of *Δbud3* cells reveals an additional role of Bud3 in cytokinesis. This function is consistent with the conserved pattern of expression and localization of Bud3 in diploid versus haploid cells as reported by Chant et al. (Chant et al., 1995). Elucidating the nature of the defect will need further investigations. Nevertheless, the fact that *Δbud3* cells can initiate a new budding and nuclear cycle without prior cell separation indicates that the defect most probably lies in the process of cytokinesis itself rather than in an earlier stage of mitosis. The role of the *BUD3* orthologue from *Ashbya gossyppi* in septum construction is also in favor of this hypothesis (Wendland, 2003).

Remarkably, the delay in cytokinesis correlates with the absence of Clb2 at the neck since a similar phenotype was observed in a strain with the *bud3*$^{\Delta 1221}$ allele as the sole source of Bud3. Whether the delocalization of Clb2 is sufficient to account for the cytokinesis delay in *Δbud3* and *bud3*$^{\Delta 1221}$ remains to be established. Nevertheless, this correlation suggests that Clb2/Cdc28 complexes localized to the neck have an unanticipated function in positively regulating cytokinesis. Such a putative role has already been suggested in the context of the synthetic lethality between the *cdc28-1N* allele, which alleviates the function of Cdc28 in G2/M, and a hypomorphic mutation in *ELM1* which encodes a protein kinase involved in cytokinesis (Bouquin et al., 2000). Presently, it is difficult to infer a cytokinesis defect from the phenotype of *Δclb2* cells given the multiple roles of Clb2 in M phase. Obviously the isolation of mutant alleles of *CLB2* affecting specifically the bud neck localization of this cyclin will be of fundamental interest to further address this issue.

Such mutants will be also useful to investigate the role of the neck localization of Clb2 in other processes. Previous genetic studies have established that among the four mitotic cyclins, Clb2 is the most potent at inhibiting the Cln-induced apical growth in vegetatively growing cells (Lew and Reed, 1993). In this context, one attractive model is that the switch activity of Clb2 depends on its property to be targeted to the neck. The finding that Clb2-hpm mutant is strongly defective in the switch activity supports this hypothesis (our unpublished results). However, Clb1 can complement, at least when overexpressed, the switch defect of Δ*clb2* cells (Lew and Reed, 1993) strongly suggesting the existence of additional pathways acting redundantly to reverse the apical growth of the bud in G2/M. One probable mechanism is the well documented transcriptional repression of *CLN*s mediated by the mitotic cyclins (Amon et al., 1993). To what extent this pathway contributes to the isotropic switch and whether the nuclear pool of Clb2 plays an essential role remain to be determined. The weak elongated cell phenotype exhibited by Δ*bud3* cells in which Clb2 is no longer targeted to the neck could well be

accounted for by such a dual mechanistic model. Our observation that deleting *BUD3* in a Δ*clb1, 3, 4* strain resulted in a more pronounced elongated phenotype is also consistent with this model.

Finally, a growing set of evidence shows that a complex pattern of cytoplasmic microtubule attachments with both the mother and the bud cortex as well as the bud neck is required for the orientation of the mitotic spindle along the mother-bud axis and ultimately for correct nuclear division (for a review, see Segal and Bloom, 2001). Cdc28 has been recently shown to interact with the plus end of cytoplasmic microtubules (Maekawa et al., 2003; Liakopoulos et al., 2003). It is possible that Clb2 contributes to the spindle orientation through its specific localization to the mother bud neck. Addressing this question should help in further defining the roles of the mitotic cyclins.

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