Conservation and Function of a Potential Substrate-Binding Domain in the Yeast Clb5 B-Type Cyclin

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Cyclin A contains a region implicated in binding to the p27 inhibitor and to substrates. There is strong evolutionary conservation of surface residues contributing to this region in many cyclins, including yeast B-type cyclins, despite the absence of a yeast p27 homolog. The yeast S-phase B-type cyclin Clb5p interacted with mammalian p27 in a two-hybrid assay. This interaction was disrupted by mutations designed to disrupt hydrophobic interactions (*hpm* mutation) or hydrogen bonding (*Q241A* mutation) based on the cyclin A-p27 crystal structure. In contrast, mutation of the Clb5p p27-binding domain only slightly reduced binding and inhibition by the Sic1p Clb-Cdc28p kinase inhibitor. Mutations disrupting the p27-binding domain strongly reduced Clb5p biological activity in diverse assays without reducing Clb5p-associated kinase activity. An analogous *hpm* mutation in the mitotic cyclin Clb2p reduced mitotic function, but in some assays this mutation increased the ability of Clb2p to perform functions normally restricted to Clb5p. These results support the idea of a modular, structurally conserved cyclin domain involved in substrate targeting.

Cyclin-dependent kinases (Cdks) drive key events in the eukaryotic cell cycle. Most eukaryotes contain multiple cyclin genes, expressed at different times in the cell cycle and appearing to control distinct cell cycle events. It has been proposed that simple oscillation of a single generic Cdk activity could account for cell cycle regulation (23, 35). However, recent observations (reviewed in reference 25) suggest that such models do not take into account strong differences in the efficiency with which different cyclins carry out different biological roles (for example, see references 8, 13, and 18). The molecular basis for such cyclin specificity remains unclear. We proposed recently that a candidate substrate “docking” site characterized in cyclin A (5, 29) might be evolutionarily conserved in many cyclins (8). Here we show that this docking site is functionally conserved in the yeast B-type cyclin Clb5p, both for the ability to bind specific proteins (the mammalian inhibitors p21 and p27) and for biological function in a range of assays presumably requiring interaction with diverse Clb5p substrates. These findings suggest that evolution of this region on the cyclin surface occurred during primordial eukaryotic evolution (predating the separation of yeast and metazoans) and probably arose as a substrate-binding domain rather than as a domain for binding inhibitors. In eukaryotic systems with multiple cyclins due to gene duplication, this region could then diverge to give different binding specificity to different cyclins, resulting in sharp differences in the biological efficiency of driving specific cell cycle events.

MATERIALS AND METHODS

Yeast strains. A *clb3,4,5,6 GAL::CLB5* strain in the W303 background (K3418 [31]; provided by K. Nasmyth) was converted to *ura3* by selecting *hisG::URA3-hisG* popouts on 5-fluoroorotic acid (5-FOA), to make K3418-1. $\times 1924$, a diploid *clb3,4,5,6 pGAL-CLB5* strain in the BF264-15D background, was constructed by crossing KL321 (*clb3,4,5,6 pGAL-CLB5/URA3 leu2 trp1* [8]), transformed with a *TRP1* vector, with strain MY21-6d (*clb3::LEU2 clb4,5,6 pGAL-CLB5/URA3 trp1*), followed by loss of the *TRP1* plasmid by growth on nonselective galactose medium. This diploid was constructed because of variability of results for *clb3,4,5,6* rescue between these two strains and because phenotypes with KL321

were variable in and between some experiments. Results with $\times 1924$ were quantitatively more consistent, perhaps because diploids are more stable to the generation of recessive modifiers. Results with all BF264-15D-derived *clb3,4,5,6* strains were qualitatively consistent with each other. A *clb5::ARG4 cdc28-4 pGAL-CLB5* strain (BF264-15D background) was constructed by crossing a *cdc28-4* strain carrying a *pGAL-CLB5* plasmid with a *clb5* strain carrying a *LEU2* vector and sporulating and dissecting tetrads. Two *cdc28-4 clb5 pGAL-CLB5/URA3* strains from this cross were mated. As expected from the work of Segal and Reed (32), the resulting diploid was completely inviable on glucose medium, unlike the parental haploids (data not shown), and was completely rescued by the introduction of either *CDC28* or *CLB5* on low-copy-number plasmids (see below). A *clb5::HIS3 pds1::URA3 cdc20::LEU2 ADE2::GALL-CDC20* strain was constructed in the W303 background by crossing a *pds1 cdc20 GALL-CDC20* strain with a *clb5::HIS3* strain (both provided by M. Shirayama), followed by tetrad analysis (the *GALL* promoter is an attenuated *GAL* promoter used in the *CDC20* construct to reduce toxicity [34]). A *clb1::URA3 clb2::LEU2 pCLB2/TRP1* strain was converted to *clb1::ura3* by brief UV light exposure followed by FOA selection, then transformed with a *CLB2/URA3* plasmid followed by loss of the *pCLB2/TRP1* plasmid. The resulting strain was FOA sensitive and could be used to test rescue of *clb1,2* lethality by transformation of plasmids followed by FOA selection. FOA-resistant plasmid loss segregants could then be tested for viability at various temperatures to examine the temperature sensitivity of rescue.

Transformants of various strains with *CLB5* plasmids were tested by growth on medium selective for strains carrying the plasmid. Transformants were first tested qualitatively for phenotype by replica plating, followed by serial dilution of stationary-phase cultures of two representative transformants, which were then plated under permissive or restrictive conditions. Occasional aberrant transformants were identified by replica plating and were not used for serial dilutions. Moderate variability in results between different transformants and/or between experiments may be due to a difference in plasmid copy number or to the accumulation of genetic modifiers in the transformants; the level of variability observed does not significantly affect any of the results reported (data not shown). In some experiments, pools of transformants (10 to 20 transformants per pool) were also tested, with similar results.

GAL1::SIC1 strains were constructed by transforming the wild-type 1255-5C strain (BF264-15D background) with integrating plasmids containing *GAL1::SIC1-HA* (from E. Schwob) or *GAL1::SIC1* (from A. Amon). Stable transformants expressing high levels of Sic1p (presumably due to multiple integration) were identified on the basis of lethality on galactose medium, with the characteristic long-budded or multibudded phenotype associated with Sic1p accumulation (30).

Plasmid constructions. *CLB5* plasmids were based on HAdR1: RS314-*CLB5-HA*, described previously (8). Mutations were introduced into this plasmid by splice overlap extension PCR and gap repair in yeast, also as described previously (8). All *CLB5* mutants were completely sequenced to confirm the intended mutation and the absence of fortuitous mutations due to PCR misincorporation. In some cases, *CLB5* mutants with such extraneous mutations were repaired by gap repair using appropriate restriction fragments and digestion of the target plasmid. *URA3* versions of these plasmids were constructed by cotransformation of a *ura3* strain with the RS314-based (*TRP1*) plasmids and with *PvuII*-digested RS416; this results in gap repair of RS416 with the insert of the RS314-based

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plasmid. Myc-tagged versions of the *CLB5-HA* plasmids were constructed by replacing the *NotI* fragment containing the triple hemagglutinin (HA) tag with a *NotI* fragment containing nine copies of the myc tag (from a plasmid provided by A. Gartner).

For two-hybrid analysis, the system of James et al. (16) was used. *CLB5* and mutants were inserted as *BamHI-SalI* fragments from HADRI and derived plasmids into *BamHI-SalI*-digested pBDU-C1. *CDC28* was inserted from a *BamHI-XhoI* digest of *CDC28* contained in the polylinker of pFASTBAC-HTa (Bethesda Research Laboratories) into *BamHI-SalI*-digested pGAD-C3. P27-VP16-AD was obtained from T. Hunter (38), and p21-VP16-AD was obtained from J. Roberts. The VP16-AD vector and the GAD-C3 vector were used as controls.

Protein analysis. Immunoprecipitation, Western blotting, and an immune complex histone H1 kinase assay were performed on HA-tagged Clb5p, as described previously (8).

Sequence analysis and modeling. The protein database file for the cyclin A-Cdk2-p27 trimeric complex (26) was obtained from the Brookhaven database. Residues at the site of interaction between cyclin A and p27 were first determined by inspection using the program RasMol (documentation at <http://www.bernstern-plus-sons.com/software/rasmol/doc/rasmol.html>). Residues conserved between cyclin A and various yeast B-type cyclins were determined using the program CLUSTAL W (37). The surface representations of the van der Waals contacts between cyclin A and Cdk2 or p27 were produced using the program GRASP (24). The surface representations of conserved residues were produced using GRASP following replacement of B factors in the PDB file with a homology score derived from a CLUSTAL W alignment. For this analysis, bovine cyclin A, human cyclin E, Clb2p, and Clb5p were aligned. This set was chosen to avoid biasing the homology in favor of B-type-cyclin-specific residues (as would occur if more B-type cyclin sequences were included in the alignment). Sequence conservation was calculated from the alignment by averaging similarity scores at each position for all possible pairs of sequences (D. Jeruzalmi, unpublished software). Equivalence of nonidentical residues was established through use of the BLOSUM62 amino acid substitution matrix (14). Final renderings of the images in Fig. 1A were performed using the programs LOOSEGRASP (D. Jeruzalmi) and RASTER3D (22). A qualitatively similar profile of surface conservation was produced using RasMol (<http://www.bernstern-plus-sons.com/software/rasmol/>), by coloring of side chains that are identical in cyclin A and in a majority of yeast *CLB1-6* proteins and fission yeast *cig1*, *cig2*, and *cdc13* proteins, based on a CLUSTAL W alignment (36), using the PDB file 1JSU for the cyclin A-Cdk2-p27 structure (24). Therefore, the restriction to cyclins A and E, *CLB5* protein, and *CLB2* protein in Fig. 1A does not significantly affect the results.

Examination of the *Saccharomyces cerevisiae* genome sequence for candidate p27 homologs was performed using the BLAST search at the Saccharomyces Genome Data Base (<http://genome-www.stanford.edu/Saccharomyces/>).

RESULTS

Conservation of Clb5p residues involved in cyclin A-p27 binding. The inhibitor p27 binds to cyclin A-Cdk2 complexes by interacting at the p27 N terminus with a hydrophobic patch on cyclin A, on a distinct face from the Cdk binding interface. p27 also traverses the “top” face of the cyclin to interact with Cdk2, finally interacting with the ATP-binding region and displacing bound ATP (26). A comparison of the p27-interacting surface of cyclin A to surface residues of cyclin A that are conserved with yeast B-type cyclins demonstrates substantial overlap in the binding interface (Fig. 1A), where cyclin A interacts with the RXLF motif in p27 (26) (Fig. 1B). The degree of conservation at this surface is comparable to conservation of the Cdk binding interface (Fig. 1A). Thus, a region of cyclin A known to be required for interaction with the p27 inhibitor (26, 29) has been conserved throughout eukaryotic evolution, despite the absence of a p27 homolog in the yeast genome sequence identifiable by BLAST search (data not shown). Aside from the p27-binding surface and the Cdk-binding surface, there is very little conservation of surface residues (Fig. 1A and data not shown), suggesting that selective pressure for the p27-binding surface has been maintained throughout eukaryotic evolution. Most other residues conserved in the cyclin superfamily are internal residues presumably required for structural stability of the folded protein (data not shown).

Consistent with functional as well as structural conservation of this binding surface, we found that a p27-VP16 activation

domain (AD) fusion interacted with a Clb5-Gal4-DNA-binding domain (DBD) fusion (Fig. 2). p21 is a relative of p27 that also binds to cyclin A (33), and a p21-VP16 fusion also bound to Clb5p in the two-hybrid assay (Fig. 2).

To test the specificity of these interactions to the conserved region (Fig. 1A), we examined the effects of mutations in residues contributing to cyclin A interaction with RXLFG motifs (Fig. 1B) (5, 26). The “hydrophobic patch” mutation (*hpm*) (8, 29) changes three conserved hydrophobic residues in α -helix 1 of the cyclin box to alanine (M197A, L201A, W204A). The L and W are completely conserved between cyclin A and all yeast B-type cyclins, and the M is conserved between cyclin A, Clb5, and Clb6. This triple mutation changes residues central to the conserved region (Fig. 1A) and reduces hydrophobic interactions between p27 and cyclin A (29) (Fig. 1B). The Clb5 *hpm* mutation strongly reduced p27-Clb5p and p21-Clb5p interaction by the two-hybrid test (Fig. 2).

We also constructed a mutation of Q241 to alanine. In the Cdk2-cyclin A-p27 crystal structure, Q254 (in α -helix 3 of the cyclin box, equivalent to Clb5p Q241) is involved in two hydrogen-bonding interactions to main-chain p27 atoms in the conserved RXLFG region (26) (Fig. 1B). This glutamine is highly conserved in the cyclin superfamily, especially in A-, B-, and E-type cyclins (3) (Fig. 1A). Similarly to the *hpm* mutation, the Q241A mutation strongly reduced p27 and p21 interaction with Clb5p by the two-hybrid test (Fig. 2). These mutant Clb5p-DBD fusions interacted with a Cdc28p-Gal4 AD fusion (Fig. 2), which was expected since the predicted Cdk binding interface is intact in these mutants. This result is consistent with high Clb5p-associated kinase activity with these mutants (reference 8 and see below). Thus, p27-Clb5p binding probably specifically requires the conserved region (Fig. 1).

The *hpm* mutation and the Q241A mutation are at a significant distance from each other in the primary sequence and are present in different predicted α -helices (helix 1 and helix 3, respectively) (3, 4, 17). Their common effect on p27 and p21 binding is therefore most plausibly interpreted by the idea that the p27 binding interface is conserved from mammalian cyclin A to Clb5p in considerable molecular detail.

Glutamate 220 in cyclin A (homologous to Clb5p E207) forms a salt bridge to a highly conserved arginine in p27 (in the conserved RQLFG sequence) (27) (Fig. 1B). Mutation of E207 in Clb5p-DBD to lysine strongly reduced p27-AD and p21-AD binding, as might be predicted since this could introduce a clash of positive charges (although the effect was not as strong as the effect of the *hpm* or Q241A mutation) (Fig. 2). Mutation of this residue to alanine had no detectable effect on p27-AD or p21-AD binding. Interestingly, this residue is variable in yeast B-type cyclins: it is glutamate in Clb5p and Clb6p, glutamine in Clb3p and Clb4p, and lysine in Clb1p and Clb2p. Clb2p-DBD binds poorly to p27-AD, despite conservation of two of the three residues mutated in the Clb5p *hpm* mutation as well as conservation of the equivalent to Clb5p Q241. Thus, it is possible that the lysine residue at the equivalent of Clb5p E207 may account for some of the Clb2p-DBD defect in p27-AD binding.

The p130 retinoblastoma-related protein binds to cyclin A via sequences related to the binding motif in p27 (1, 6). A p130-Gal4 AD fusion (12) bound to Clb5-DBD in the two-hybrid assay, and this interaction was abolished by the *hpm* mutation (data not shown), although the p130-Clb5 interaction was weaker than the p21-Clb5p or p27-Clb5 interactions. This result is significant because p130 shares little homology with p27 outside the RXLF motif (i.e., Fig. 1B). The finding that Clb5p binds to p130 may confirm the speculation of Hannon et al. (12) that the original isolation of p130-AD as an interactor

FIG. 1. (A) Interaction surfaces and conserved surface residues in cyclin A. Images of the cyclin A molecular surface are shown. The van der Waals interaction surfaces on cyclin A for p27 (top row) and Cdk2 (bottom row) are indicated in blue (left), using the cyclin A-Cdk2-p27 trimeric crystal structure (26) (PDB file 1JSU). The perspective for the Cdk2 interface is obtained by an approximately 90° rotation of the top set of images from left to right. Degrees of evolutionary conservation on these same faces of cyclin A between mammalian cyclins A and E and yeast cyclins Clb5 and Clb2 are indicated (middle) in shades of yellow to green (green is highest conservation; dark green is 100% identity). The images on the right are overlays of the left and middle images, merged using Photoshop software, and the positions of side chains mutated in this study are shown. For ease of understanding the mutagenic experiments described here, the numbering is for Clb5p. Sequence alignments from cyclin A to Clb5p are as follows: D220–D207; M210–M197; L214–L201; W217–W204; Q254–Q241; K266–K253; E295–E282; and F304–F291 (3). (B) Schematic of interaction between residues in cyclin A and the conserved RXLF motif in proteins that interact with cyclin A (1, 5, 26). The numbering is for Clb5p (see above for translation to cyclin A).

with Cdk2-DBD might have been bridged by endogenous yeast cyclins.

The p27-binding domain in Clb5p is largely dispensable for Sic1p binding. The conservation of the p27-binding domain is

surprising, given that *S. cerevisiae* does not contain a p27 homolog detectable by BLAST search. The Clb-Cdc28p kinase inhibitor Sic1p has been proposed to bind to Clb5p via a C-terminal sequence with some distant similarity to the cyclin A-interacting regions of p27 and other cyclin A targets (15, 39). We tested the ability of Sic1p to bind to Clb5p with and without a functional p27-binding domain. Sic1p (HA-tagged and overexpressed from the *GAL1* promoter) could bind both wild-type and *hpm* mutant Clb5p, assayed by immunoprecipitating Sic1p-HA and assaying for myc-tagged Clb5p in the immunoprecipitates, although a moderate reduction in binding of the mutant protein was reproducibly observed (Fig. 3A).

FIG. 2. Clb5p interacts with p27 and p21 dependent on a conserved surface. Clb5-Gal4 DBD plasmids, wild type or mutant in conserved residues involved in p27-cyclin A binding (Fig. 1), and a vector control (all *URA3* plasmids) were transformed into PJ469- α (16). PJ469- α (16) was transformed with Gal4-AD vector and Gal4-AD-Cdc28 fusion plasmid and with VP16-AD fusions with p27 and p21 (all *LEU2* plasmids). (The VP16-AD vector was as negative for activation as the GAL4-AD vector; data not shown). Diploids between the DBD and AD transformants were selected by mixing and growth on yeast extract-peptone-dextrose (YEPD), followed by replica plating to medium lacking leucine and containing uracil, selecting for both plasmids. Activation was scored by growth on medium lacking histidine, on medium lacking histidine but containing 10 mM 3-aminotriazole (AT) (both scoring the same *GAL1* promoter-*HIS3* fusion, with differing stringency [16]), and on medium lacking adenine (scoring a *GAL2* promoter fused to *ADE2* [16]).

Similarly, when untagged Sic1p was expressed from the *GAL1* promoter in cells expressing wild-type or *hpm* Clb5p-HA, immunoprecipitated Clb5p-associated kinase activity was strongly inhibited in both cases, although a low level of residual kinase activity was detected with the *hpm* mutant but not with the wild type (Fig. 3B). Since both of these assays require stable Sic1p-Clb5p binding to persist through multiple washes of the immunoprecipitates, Sic1p-Clb5p-*hpm* binding must be reasonably tight.

Further work is clearly required to determine the basis and structural requirements for Sic1p-Clb5p interaction. The results presented here do not rule out the possibility that Sic1p interacts with the hydrophobic-patch region of Clb5p, but there must be other strong binding determinants for Sic1p binding that are independent of the hydrophobic patch.

Effects of mutations on Clb5p-associated kinase activity. Mutations affecting the putative p27-binding domain (*hpm* or Q241A) did not reduce Clb5p-associated kinase activity (8) (Fig. 4). This was expected, because these mutations are predicted to affect surface residues distant from the Cdk interface, based on the cyclin A-Cdk2 structure.

In contrast, the K253A (KA) E282A (EA) mutation in the Cdk interface strongly reduced Clb5p-associated kinase activity (8) (Fig. 4). In the Cdk2-cyclin A crystal structure, F304 of cyclin A (equivalent to Clb5p F291) interacts with hydrophobic residues of Cdk2 (17). This residue is highly conserved among cyclins and is found in all yeast B-type cyclins (3). To extend results with the K253A E282A mutation, we constructed the F291A mutation but found that it had only a minor effect on kinase activity; however, the triple K253A E282A F291A mu-

tation almost eliminated Clb5p-associated kinase activity (Fig. 4).

The simple concept of complete independence between Cdk interface mutations and p27-binding domain mutations is complicated by the finding that the F291A mutation moderately synergized with the Q214A mutation and the *hpm* mutation to further reduce kinase activity below that observed with F291A alone (Fig. 4 and data not shown). This is surprising because the Q214A and *hpm* mutations do not themselves detectably affect kinase activity, and the cognate residues in cyclin A are far from the region of Cdk2 binding. This fact complicates the interpretation of the biological interaction between these mutations; however, it is noteworthy that Clb5p-F291A, Q214A has significantly more kinase activity than Clb5p-K253A, E282A but much less biological activity (see below), suggesting that the main effect of the Q214A mutation in this context is

FIG. 3. The p27-binding domain makes only a marginal contribution to Sic1p-Clb5p interaction. (A) 1255-5C (wild type, BF264-15D background) and 1255-5C-3 (*GAL1::SIC1-HA*, multiple-copy integrant) were transformed with *TRP1* vector or *CLB5-myc/TRP1* plasmids (wild type or *hpm* [M197A L201A W204A], expressed from the *CLB5* promoter). Fresh stationary-phase cultures of transformants grown in medium lacking tryptophan were diluted in YEP-Gal medium and incubated for 6 h at 30°C, by which time most cells in the culture had adopted the long-budded morphology characteristic of Sic1p-mediated cell cycle arrest (30). Sic1p-HA was immunoprecipitated, and the immunoprecipitates were assayed for their content of Sic1p-HA and Clb5p-myc by Western blotting. An aliquot of the total extract was also assayed for Clb5p-myc. (B) 1255-5C and 1255-5C-6 (*GAL1::SIC1*, multiple-copy integrant) were transformed with *TRP1* vector or *CLB5-HA/TRP1* plasmids (wild type or *hpm*, expressed from the *CLB5* promoter). *GAL1::SIC1* was induced as for panel A. Following anti-HA immunoprecipitation, Clb5p-HA, associated Cdc28p, and histone H1 kinase activity were assayed.

FIG. 4. Clb5p-associated kinase activity. Transformants of a wild-type strain (W303-background strain $\times$ 1907-19C) with vector or with the indicated *CLB5-HA* constructs, expressed from the *CLB5* promoter in RS316-based plasmids, were grown in medium lacking uracil to log phase, extracted, and immunoprecipitated with anti-HA antibody. Clb5-HAp in the immunoprecipitates was assayed by Western blotting (top). Associated histone H1 kinase activity was assayed by immune complex kinase assay (bottom). Most of these mutants were also tested in a wild-type BF264-15D strain (1255-5C) with similar results (data not shown).

not on levels of kinase activity but rather on targeting of the activity. Similar results for the kinase activity of these mutants were obtained in two strain backgrounds, W303 (Fig. 4) and BF264-15D (data not shown).

Mutations in the Clb5p p27-binding region interfere with biological activity. The p27-binding domain of cyclin A might also be important for the interaction of cyclin A with substrates and other regulators, providing targeting and specificity to cyclin A-Cdk2 kinase activity by allowing interaction with proteins containing the "RXL" motif (1, 28, 29, 40). Indeed, recent structural analysis shows that the p107 substrate binds to cyclin A in a manner structurally very similar to the binding of p27 (5), including involvement of the hydrophobic patch residues and Q254 (equivalent to Clb5p Q241). If the p27-binding domain in Clb5p plays a similar role, then loss of the ability to interact with targets due to mutations in this domain should reduce or eliminate Clb5p biological activity without significantly reducing associated kinase activity.

CLB3,4,5,6 are redundant for an essential function, since *clb3,4,5,6* quadruple mutants arrest the cell cycle with defects in spindle morphogenesis and some defects in DNA replication (31). We tested the ability of *CLB5* mutants to perform this function by plating strain K3418-1 (a *ura3* derivative of K3418: *clb3,4,5,6 GAL-CLB5*) (31) on glucose medium (where *GAL-CLB5* is off) after transformation with low-copy-number plasmids containing *CLB5* (Fig. 5A). Mutation of the hydrophobic patch in the p27-binding domain almost eliminated Clb5p function in this assay, and the Q241A mutation significantly reduced activity.

The K253A E282A Cdk interface mutation (8) severely reduced but did not eliminate *clb3,4,5,6* rescue (Fig. 5A). The F291A mutation did not detectably affect rescue, but it synergized strongly with either the K253A E282A mutation or the Q241A mutation to completely eliminate rescue. The F291A and K253A E282A mutations also eliminated residual activity of *CLB5-hpm* (barely detectable in Fig. 5A but observable with inoculation of larger numbers of cells) (Fig. 5A and data not shown). Thus, *clb3,4,5,6* rescue activity of Clb5p mutants with compromised associated kinase activity may have strongly enhanced dependence on a fully functional targeting domain.

In addition to these experiments with the *clb3,4,5,6 GAL-*

FIG. 5. Ability of *CLB5* mutants to rescue *clb3,4,5,6* inviability. (A) Transformants of K3418-1 (W303 background, a *ura3* derivative of K3418, *clb3,4,5,6 GAL-CLB5* [31]) with RS316-based plasmids containing the indicated *CLB5-HA* allele expressed from the *CLB5* promoter were selected on galactose-containing medium lacking uracil. Individual transformants were grown on this medium to stationary phase and suspended in water, and 10-fold serial dilutions were plated on yeast extract-peptone-Gal (YEPGal) (*GAL-CLB5* on) and yeast extract-peptone-dextrose (YEPD) (*GAL-CLB5* off). Plates were incubated for 3 days at 30°C. (B) Transformants of $\times$ 1924 (BF264-15D diploid, *clb3,4,5,6Δ pGAL-CLB5/URA3*) with RS314-based plasmids containing the indicated *CLB5-HA* allele expressed from the *CLB5* promoter were selected on ScGal-Trp. Individual transformants were grown on this medium to stationary phase and suspended in water, and 10-fold serial dilutions were plated on YEPGal (*GAL-CLB5* on) and YEPD (*GAL-CLB5* off). Plates were incubated for 3 days at 30°C or 35°C.

CLB5 strain K3418-1 (W303 strain background), we performed similar experiments in the BF264-15D background, using strain $\times$ 1924 (see Materials and Methods). In this strain background, the *hpm* mutant was significantly more active and

the F291A and K253A E282A mutants displayed no defects. The Q241A mutant had a very mild defect. The *hpm* mutation resulted in high sensitivity to interference with the Cdk interface, since the K253A E282A *hpm* and the F291A *hpm* mutants were inactive (Fig. 5B). The *hpm* mutant was temperature sensitive for rescue (Fig. 5B). The Q241A mutant exhibited a significant though partial reduction in activity at elevated temperatures. The basis for the quantitative differences in results between the two strain backgrounds (compare Fig. 5A and B) is unknown, but overall the results for both strains are consistent with a requirement for a functional targeting domain, especially under conditions where kinase activity may be limiting. Similarly, the E207K and E207A mutations strongly enhanced the defect of the K253A E282A mutation for *clb3,4,5,6* rescue in both strain backgrounds (data not shown), and E207 is implicated in full function of the p27-binding domain (Fig. 1B and 2). One peculiarity of this result is that the E207A mutation had no detectable effect on Clb5-p27 interaction, unlike the E207K mutation (Fig. 2), but these mutations were almost equivalent in their ability to enhance the K253A E282A defect (data not shown). This discrepancy could simply reflect subtle differences between the requirements for Clb5p binding to p27 and to a relevant target(s) for *clb3,4,5,6* rescue.

Synergy between a Cdk interface mutation and the *hpm* mutation of Clb5p was observed previously (8) and was interpreted as indicating that interference with substrate targeting enhanced the requirement for highly active kinase, while appropriately targeted kinase might be in excess. The present results extend this conclusion, using additional mutations in both interfaces.

Mutations in the putative targeting domain of the mitotic cyclin *CLB2* reduce Clb2-specific biological activity. Hydrophobic residues in helix 1 of the cyclin box are conserved in the cyclin superfamily (Fig. 1) (3). If this region forms a substrate-binding cleft, as proposed for cyclin A (5, 29), then the biological specificity of different cyclins might be due to interaction of the region with distinct targets based on the identity of these residues or flanking sequences. We mutated the three residues aligned with the cyclin A hydrophobic patch residues in the mitotic cyclin CLB2, N260, L264, and W267, to alanine. This *CLB2-hpm* mutation (expressed from the *CLB5* promoter) was tested for its ability to rescue *clb1,3,4Δ clb2-ts* strain inviability and was found to be severely defective (Fig. 6A). In contrast to these results with *clb1,3,4Δ clb2-ts* lethality, the *CLB2-hpm* mutant had no significant defect in rescuing *clb1,2Δ* lethality (in the presence of *CLB3,4*), although *CLB5* was completely unable to rescue (data not shown). We speculate that in the presence of *CLB3,4*, Clb2p may need to interact with a narrower range of substrates to provide rescue.

We constructed the mutation equivalent to *CLB5*-Q241A in *CLB2* (*CLB5::CLB2-Q304A*) but found that this mutation did not detectably reduce the ability of *CLB5::CLB2* to rescue *clb1,3,4Δ clb2-ts* lethality (data not shown). This residue may play a different role in Clb2p than in Clb5p; alternatively, this mutation may have too mild a phenotype to detect by this assay. The Q241A mutation in *CLB5* generally has more minor consequences than the *hpm* mutation (see above).

We also tested *CLB2-hpm*, expressed from the *CLB5* promoter, for the ability to rescue *clb3,4,5,6* lethality in the BF264-15D background strain $\times 1924$ (*clb3,4,5,6Δ pGAL-CLB5*). *CLB5::CLB2* exhibited very inefficient rescue of *clb3,4,5,6* lethality, as previously reported (8). Surprisingly, *CLB5::CLB2-hpm* rescued *clb3,4,5,6* lethality much better than did *CLB5::CLB2* (Fig. 6B). The KA EA Cdk interface mutation eliminated residual *clb3,4,5,6* rescue activity of *CLB5::CLB2* (Fig.

FIG. 6. The hydrophobic patch mutation (N260A L264A W267A) alters the biological activity and specificity of Clb2p. (A) Transformants of K3080-1A (*clb1,3,4Δ clb2-ts*; a *trp1::URA3* derivative of K3080 [2]) with the indicated plasmids (RS314 derivatives; all *CLB* coding sequences under control of the *CLB5* promoter) were selected on medium lacking tryptophan at 23°C. Individual transformants were grown on this medium to stationary phase and suspended in water, and 10-fold serial dilutions were plated on yeast extract-peptone-dextrose (YEPD). Plates were incubated for 2 days at 35°C (semipermissive) or 37°C (nonpermissive) and for 3 days at 23°C (permissive). (B) Transformants of $\times 1924$ (BF264-15D diploid, *clb3,4,5,6Δ pGAL-CLB5/URA3*) with RS314-based plasmids containing the indicated *CLB* coding sequences expressed from the *CLB5* promoter were selected on galactose-containing medium lacking tryptophan. Individual transformants were grown on this medium to stationary phase and suspended in water, and 10-fold serial dilutions were plated on yeast extract-peptone-Gal (YEPGal) (*GAL-CLB5* on) and YEPD (*GAL-CLB5* off). Plates were incubated for 3 days at 30°C. (Note that for unknown reasons, the defect in rescue by *CLB5::CLB2* seen in this strain background is not observed in the *clb3,4,5,6* W303 background strain K3418-1; see the text.)

6B), even though it only moderately reduced Clb2-associated histone H1 kinase specific activity (data not shown). The *hpm* mutation slightly reduced Clb2p levels but not histone H1 kinase specific activity in immunoprecipitates (data not shown).

As noted previously, Clb5p does not rescue *clb1,2,3,4* inviability, even when expressed from the same promoter as Clb2p (Fig. 6B) (8). Because the *hpm* mutation allowed Clb2p to rescue *clb3,4,5,6* inviability (a “Clb5p-specific” activity), we asked if the *hpm* mutation in Clb5p would allow it to rescue *clb1,2,3,4* inviability, but this was not observed (Fig. 6B).

Thus, a functional hydrophobic patch may actually interfere with rescue of *clb3,4,5,6* lethality by Clb2p (at least in the BF264-15D strain background; see below). While this result appears to indicate alteration of specificity by the Clb2p *hpm* mutation, this would be quite surprising given that the mutagenesis was simply intended to inactivate specific binding, as with the cyclin A and *CLB5 hpm* mutations (8, 29). It may be more likely that the loss of normal *CLB2* function is indeed due to lack of targeting, while increased ectopic rescue may be due to exclusion of appropriate Clb3,4,5,6p targets by the normal configuration of the Clb2p targeting domain. The *CLB2-hpm* mutation, by removing bulky side chains in this region, may simply allow a “sloppy fit,” improving ectopic rescue of

clb5 cdc28-4 pGAL-CLB5

FIG. 7. Cyclin- and targeting domain-restricted performance of Clb5p function in the *cdc28-4* background. A diploid strain of genotype *cdc28-4 cld5::ARG4 pGAL-CLB5/URA3* ($\times 1922$) was transformed with RS314-based plasmids containing the indicated *CLB* coding sequence under control of the *CLB5* promoter. SF19, an RS314-based plasmid containing *CDC28*, was included as a control. Transformants were selected on galactose-containing medium lacking tryptophan at 23°C. Individual transformants were grown on this medium to stationary phase and suspended in water, and 10-fold serial dilutions were plated on yeast extract-peptone-Gal (YEPGal) (*GAL-CLB5* on) and yeast extract-peptone-dextrose (YEPD) (*GAL-CLB5* off). Plates were incubated for 5 days at 23°C.

clb3,4,5,6 by *CLB2*. Another possibility is that Clb2p interacts with some targets or inhibitors that are deleterious to *clb3,4,5,6* rescue as well as with some targets that are helpful; if the *hpm* mutation preferentially blocks interaction with the former class, then the mutation could enhance *clb3,4,5,6* rescue. In preliminary results, the *CLB5::CLB2-Q304A* mutation (analogous to *CLB5-Q241A*) also increased *clb3,4,5,6* rescue over that observed with *CLB5::CLB2*, although probably not as efficiently as the *hpm* mutation (data not shown).

To generalize our results, we wanted to perform similar experiments in the W303 background *clb3,4,5,6* strain K3418-1 (31). Surprisingly, in this background *CLB5::CLB2* rescued *clb3,4,5,6* lethality quite efficiently (data not shown), in contrast to the results obtained with the BF264-15D background strain $\times 1924$ (Fig. 6B) and with the BF264-15D strain KL321 (8). The basis for the difference in results between the two strain backgrounds is not known. Sufficient overexpression of the close *CLB2* relative *CLB1* from the *GAL1* promoter can rescue inviability due to deletion of the remaining five *CLB* genes even in the BF264-15D background (11), and even the *CLB5::CLB2* construct gives very low but detectable rescue in the BF264-15D background (reference 8 and data not shown). Perhaps in the W303 background, expression of *CLB2* from the *CLB5* promoter, in addition to endogenous *CLB1* and *CLB2* expression, provides sufficient overall *CLB1,2* overexpression to give a result similar to strong *CLB1* overexpression in the BF264-15D background. The defect of *CLB5::CLB2* in complementing the *clb5* replication defect (8), previously demonstrated in the BF264-15D background, was reproduced in the W303 background, with or without the Clb2p destruction box (M. Yuste-Rojas and F. Cross, unpublished data).

Additional assays for *CLB5* function. The results presented so far indicate that the essential functions shared by Clb3,4,5,6p require a substrate targeting domain in Clb5p for efficient performance. Lack of these four cyclins results in a defect in spindle assembly, as well as some defect in performance of DNA replication (31). We showed previously that efficient performance of the DNA replication function of Clb5p (9, 10, 31) requires a functional targeting domain (8).

Other assays for Clb5p function were needed to determine if this requirement was general.

An additional assay for specific Clb5p function is provided by the observation of Segal and Reed (32) that *cdc28-4 cld5* diploids are inviable, due to a defect in spindle positioning; ectopic expression of other *CLBs* could not rescue this phenotype. A *cdc28-4 cld5 GAL-CLB5* diploid was inviable on glucose medium; introduction of a wild-type *CDC28* or *CLB5* plasmid efficiently rescued the strain, consistent with the results of Segal and Reed (32) (Fig. 7). *CLB5::CLB2* could not rescue this strain; the *hpm* mutation significantly improved rescue by *CLB5::CLB2* (Fig. 8). Thus, for this assay, the defect in rescue by Clb2p (32) may be due in part to the Clb2p targeting domain (as seen also in Fig. 6B for *clb3,4,5,6* rescue; see above).

Cdc20p is a factor required for efficient ubiquitination and degradation of Pds1p, an anaphase inhibitor; it is also thought to be needed for destruction of other proteins, because *cdc20 pds1* double mutants are inviable, arresting in late anaphase (19). Recently, Shirayama et al. (34) found that *cdc20 pds1* cells are inviable because of the retention of high levels of Clb5p, which may be degraded under Cdc20p control. Thus, *cdc20 pds1* null strains are inviable, but they are rescued by further deletion of *clb5*. This background thus provides an assay for a specific cell cycle-inhibitory function of Clb5p. We tested *CLB5* mutants for this function by transforming a *cdc20 pds1 cld5 GALL-CDC20* strain with *CLB5* plasmids (Fig. 8). Introduction of wild-type *CLB5* resulted in a relative plating efficiency of this strain of 0.003 on glucose medium (*GALL-CDC20* off) compared to galactose medium (*GALL-CDC20* on), while vector transformants had a relative plating efficiency of around 1.0, confirming the observations of Shirayama et al. (34) that *cdc20 pds1* strains are inviable specifically due to Clb5p function (Fig. 8 and data not shown). The K253A E282A mutation inactivated Clb5p in this assay (relative plating efficiency of about 1.0), and the *hpm* mutation strongly reduced Clb5p activity (relative plating efficiency of about 0.4, where the colonies were significantly smaller than the vector-containing colonies (Fig. 8 and data not shown). Thus, this assay for Clb5p function essentially reproduces the structural requirements for rescue of *clb3,4,5,6* lethality, in that both a functioning targeting domain and high kinase activity may be required for maximal function. Consistent with the idea that Clb2p is inefficient at carrying out this role of Clb5p,

cdc20Δ pds1Δ cld5Δ GALL-CDC20

FIG. 8. Structural requirements for a cell cycle-inhibitory function of Clb5p. A *cdc20Δ pds1Δ cld5Δ GALL-CDC20* strain (1896-1; W303 background) was transformed with the RS314-based plasmids containing the indicated *CLB5* alleles. Transformants were selected on galactose-containing medium lacking tryptophan (Gal-trp) at 23°C. Individual transformants were grown on this medium to stationary phase and suspended in water, and 10-fold serial dilutions were plated on Gal-trp (*GAL-CLB5* on) and dextrose-containing medium lacking tryptophan (Dex-trp) (*GAL-CLB5* off). Plates were incubated for 6 days at 23°C.

CLB5::CLB2 introduction resulted in only partial reduction of viability in the *cdc20 pds1 clb5* strain (data not shown).

DISCUSSION

Specific cyclin requirements for the budding yeast cell cycle.

Of the nine budding yeast cyclins (three *CLN* and six *CLB* cyclins) known to activate the Cdc28p protein kinase, no single one is essential, and most double (and some triple and quadruple) mutants are viable as well (7, 21, 23), indicating substantial functional overlap among cyclins. In addition, Haase and Reed recently described a strain in which *clb1,2,3,4,5,6* were deleted and replaced with *CLB1* under the control of the strong *GAL* promoter (11). The viability of this strain is *prima facie* evidence that multiple B-type cyclins are not essential, provided that a single B-type cyclin is sufficiently overexpressed. However, this result does not imply that any B-type cyclin will similarly substitute for *CLB1-6*, and it appears likely from previous work that *CLB5*, at least, will not (8, 31). We showed that at comparable levels of expression, Clb2p was inefficient at substituting for Clb5p in the activation of DNA replication (8). We also recently found that the ability of Clb2p to block mitotic exit when overexpressed without its destruction box (36) could not be provided by Clb5- Δ dbp at comparable levels of overexpression (15a). Thus, it appears likely that the apparent degeneracy of the cyclin system may hide a significant level of cyclin-specific targeting, which is detectable only at appropriate expression levels and in specific assays.

A conserved region of cyclins that may provide interaction with targets. Although many residues in cyclins exhibit significant conservation across the cyclin superfamily, most of these residues do not contribute significantly to the cyclin molecular surface (Fig. 1 and data not shown). These residues generally contribute to the buried hydrophobic core of the cyclin and are presumably required for adoption of the double 5-helix bundles that are characteristic of the entire cyclin superfamily (3, 4, 17). Conserved surface residues are mainly in the Cdk binding interface and in a portion of the surface distinct from the Cdk binding interface that contributes part of the p27 binding interface in cyclin A (Fig. 1). These residues are conserved across eukaryotic evolution, although interestingly they are frequently not as well conserved in distantly related cyclins with distinct functions such as the yeast *CLN* G1 cyclins (3). Budding yeast lacks an identifiable p27 homolog in the genome, but despite this, the ability of this region to bind to p27 has been conserved since divergence of metazoans and yeast, even though p27 may not have existed at the time of this divergence. Therefore, the "p27-binding" domain may have had interaction with RXL motif cyclin-Cdk targets (1) as its original and conserved role, and p27 may have evolved to fit this region. A dual role for this region in p27 binding and substrate interaction in the case of cyclin A was proposed by Schulman et al. (29), and interaction of this region with the p107 substrate was indeed demonstrated by recent structural analysis (5). The budding yeast Cdk inhibitor Sic1p has little or no homology to p27, and a functional p27-binding domain is not required for Clb5p-Sic1p binding to Sic1p and kinase inhibition (Fig. 4). We cannot rule out the possibility, though, that p27 homologs were present in a primordial eukaryote and then lost in the yeast lineage. Also, our data do not rule out the idea that Sic1p may occupy the hydrophobic patch region in Clb5p. Indeed, the slight reduction in binding that we detected (Fig. 3) is consistent with this idea, although such an interaction is clearly not required for binding and kinase inhibition. Candidate RXL-like sequences in an important C-terminal region of Sic1p have been described (15, 39).

Clb5p localizes to the nucleus (15a, 34), and failure of nuclear accumulation could account for loss of biological activity. In preliminary experiments, though, we found little or no defect in nuclear localization of Clb5p-hpm compared to wild type, by immunofluorescent detection of myc-tagged Clb5p (data not shown).

Mutation of a specific cyclin targeting domain is predicted to lower or eliminate target interaction, without affecting associated kinase activity. It is not possible at present to assess specific target interaction directly because the relevant targets are unidentified, but diverse Clb5-specific biological assays reveal a requirement for an intact p27-binding domain for efficient function, and mutations in this domain do not affect associated kinase activity. There is a requirement for the p27-binding domain of Clb5p for efficient promotion of DNA replication (8), for rescue of *clb3,4,5,6* inviability (Fig. 5), for a potential spindle morphogenesis function of Clb5p identified in the *cdc28-4 clb5* background (Fig. 7) (32), and for a cell cycle-inhibitory function of Clb5p identified in the *cdc20 pds1* background (Fig. 8) (34). Normal Clb2p function may require a similar putative targeting domain (Fig. 6A), while biologically abnormal performance by Clb2p of some normally Clb5p-specific functions may be restricted by the Clb2p targeting domain (Fig. 6B and 7). It seems likely that these results reflect interaction of diverse substrates of both Clb5p- and Clb2p-associated kinases with the p27-binding domains. In preliminary results (data not shown), we found targeting domain-dependent two-hybrid interactions between Clb5p and proteins from a yeast genomic library, and we hope that this approach will provide candidate Clb-specific phosphorylation targets.

We speculate that divergence in the p27-binding domain among the B-type cyclins could account for some of the differences in biological specificity of different Clb proteins (for example, the enhanced ability of Clb5p over Clb2p to drive DNA replication, and the opposite specificity for mitosis [8]). A specific example of this could be the substitution in Clb2p of lysine for the equivalent of Clb5p glutamate 207, since this substitution strongly reduces p27 binding to Clb5p (Fig. 2), and Clb2p binds p27-AD poorly if at all (data not shown). Differences in this and other residues flanking the p27-binding domain could confer binding specificity upon a core of conserved residues recognizing the minimal RXL motif. The ability of different SH2 domains to sharply differentiate different phosphotyrosine-containing binding targets, while relying on core SH2 homology domains for phosphotyrosine binding, provides a precedent (20). This idea would be supported by the identification of cyclin-specific binding partners whose binding requires the core conserved residues.

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