Structure-Function Analysis of the *Saccharomyces cerevisiae* G₁ Cyclin Cln2

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Received 14 February 1997/Returned for modification 9 April 1997/Accepted 12 May 1997

We have generated 50 new alleles of the yeast *CLN2* gene by using site-directed mutagenesis. With the recently obtained crystal structure of cyclin A as a guide, a peptide linker sequence was inserted at 13 sites within the cyclin box of Cln2 to determine if the architecture of Cln2 is similar to that of cyclin A. Linkers inserted in what are predicted to be helices 1, 2, 3, and 5 of the cyclin box resulted in nonfunctional Cln2 molecules. Linkers inserted between these putative helix sites and in the region believed to contain a fourth helix did not have significant effects upon Cln2 function. A series of deletions in the region between the third and fifth helices indicate that the putative fourth helix may lie at the C-terminal end of this region yet is not essential for function. Two residues that are predicted to form a buried salt bridge important for interaction of two helices of the cyclin box were also mutated, and an additional set of 31 mutant alleles was generated by clustered-charge-to-alanine scanning mutagenesis. All of the mutant *CLN2* alleles made in this study were tested in a variety of genetic and functional assays previously demonstrated to differentiate specific cyclin functions. Some alleles demonstrated restricted patterns of defects, suggesting that these mutations may interfere with specific aspects of Cln2 function.

Cell cycle transitions in eukaryotic cells require the activity of one or more cyclin-dependent protein kinases (CDKs). These kinases are regulated by a family of molecules known as cyclins that share a region of sequence homology termed the cyclin box. Recently, it was shown that the cyclin boxes of human cyclin A, bovine cyclin A, and human cyclin H form five alpha helices that fold into a core domain termed the cyclin fold (3, 15, 16). Each of these cyclins also contains a second cyclin fold C terminal to that formed by residues of the cyclin box, although the primary sequence of the second cyclin fold contains little homology to either the first cyclin fold or similar regions in other members of the cyclin family. The cyclin box is a major component of the cyclin A-CDK2 interface. Binding of the cyclin molecule results in conformational changes in CDK2, resulting in activation of the kinase (15).

Transition through the start of the *Saccharomyces cerevisiae* cell cycle requires the action of one of three G₁ cyclins, encoded by the *CLN1*, *CLN2*, and *CLN3* genes, that complex with the CDK encoded by the *CDC28* gene. Although the three *CLN* genes are functionally redundant for cell cycle initiation, *CLN3* differs from *CLN1* and *CLN2* in structure and regulation (reviewed in reference 6). A number of recent studies have determined that the cellular roles of Cln2 and Cln3 also vary, as Cln3 is more efficient at stimulation of transcription at Swi4/Swi6 cell cycle box (SCB) sites and Cln2 is more important for initiation of bud formation and other start-specific cell cycle events (9, 20, 30). These differences are apparent in a number of genetic assays (20). For example, when expressed from the *CLN3* promoter, both *CLN2* and *CLN3* can support viability in a *cln1 cln2 cln3* mutant strain yet *CLN2*, but not *CLN3*, can support growth in a *cln1 cln2 cln3* mutant additionally deficient in *SWI4* or *SWI6*, which encode compo-

nents of the transcription factor SBF (SCB-binding factor; reviewed in reference 18; in the *CLB5-CLB6* pair of B-type cyclins (27); or in the *PCL1-PCL2* pair of cyclin homologs (21, 24). *CLN2* also suppresses the lethality caused when *SIC1*, the inhibitor of B-type cyclin-Cdc28 kinases (22, 26), is overexpressed (20, 31).

In this work, we generated a series of mutant alleles of *CLN2* with two aims: first, to test structure-function predictions based on the cyclin A structure and the Cln2-cyclin A sequence alignment; second, to examine the effects of mutations in multiple regions of Cln2 in a range of assays for Cln2 function.

MATERIALS AND METHODS

Plasmid constructions. Construction of the *CLN2* and *CLN3* plasmids (pT411 and pKL001), each containing a triple-hemagglutinin (HA)-tagged *CLN* gene under control of the *CLN3* promoter, have been previously described (20). *cln2-R74A*, *-D103A*, and the series of *cln2* alleles containing linker insertions within the cyclin box were constructed by splice overlap extension (13) by using pT411 as a template and Vent polymerase. Alleles

TABLE 1. Strains used in this study

Strain	Relevant genotype ^a
1254-14D	<i>MATa cln1 cln2 cln3 leu2::LEU2::GAL1::CLN1</i>
1607-2D	<i>MATa cln1 cln2 cln3 leu2::LEU2::GAL1::CLN3</i>
1446-10D	<i>MATa cln1 cln2 cln3 arg4 bck2::ARG4 leu2::LEU2::GAL1::CLN3</i>
1456-10.....	<i>MATa cln1 cln2 cln3 swi4::LEU2 pGAL1::CLN1/URA3</i>
2866-9D	<i>MATa cln1 cln2 cln3 swi6::LEU2 pGAL1::CLN1/URA3</i>
1591-10D	<i>cln1 cln2 cln3 HIS3 his3 pcl1::HIS3 pcl2::URA3 leu2::LEU2::GAL1::CLN1</i>
1581-2C.....	<i>MATa cln1 cln2 cln3 arg4 clb5::ARG4 clb6::ADE1 pGAL1::CLN1/URA3</i>
1238-11A-7	<i>MATa cln1 cln2 CLN3 ura3::URA3::GAL1::SIC1</i>
1255-3D	<i>MATa CLN1 cln2 CLN3 bar1::LEU2 far1::URA3</i>
1255-5C.....	<i>MATa CLN1 CLN2 CLN3</i>

^a All of the strains are congenic with BF264-15D (*trp1 leu2 ura3 ade1 his2*) unless otherwise noted.

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A.

B.

FIG. 1. *CLN2* sequence. (A) Sequence alignment of residues of the cyclin box in human cyclin A, Cln1, Cln2, Cln3, and human cyclin H. The five bars above the sequence show the locations of the five alpha helices identified by the crystallization of cyclin A (3, 15) and the postulated positions of helices in Cln2 and Cln3 based upon structural modeling programs (1a). The four arrows indicate the locations of four conserved residues that were targeted for mutation in Cln2. (B) The complete Cln2 sequence with the locations of all of the mutations introduced in this study. The five shaded boxes represent the sequences predicted by sequence homology with cyclin A and structural modeling programs to contain the five helices of the cyclin fold. Filled arrows mark the sites at which linkers were inserted in the *cln2-2L* series of alleles. Open arrows denote the locations of the four conserved residues of the cyclin box (shown in panel A) that were replaced with alanines. Asterisks mark the charged residues of Cln2. The lines below the Cln2 sequence indicate the locations of clusters of charges that were targets for replacement with alanines; all of the charged residues in a given cluster were simultaneously changed to generate the *cln2-ala* allele indicated by the number below the line.

FIG. 2. (A) Plating efficiency assays for different *cln2-2L* alleles. Each graph shows the efficiency of growth when the allele indicated was introduced to a *cln* (*cln1 cln2 cln3 GAL1::CLN3* strain 1607-2D), *bck2* (*bck2 cln1 cln2 cln3 GAL1::CLN3* strain 1446-10B), *swi4* (*cln1 cln2 cln3 swi4 GAL1::CLN1* strain 1456-10), *swi6* (*cln1 cln2 cln3 swi6 GAL1::CLN1* strain 2866-9D), *pcl1 pcl2* (*cln1 cln2 cln3 pcl1 pcl2 GAL1::CLN1* strain 1591-10D), *clb5 clb6* (*cln1 cln2 cln3 clb5 clb6 GAL1::CLN1* strain 1581-2C), *GAL1p::SIC1* (*cln1 cln2 CLN3 GAL1::SIC1* strain 1258-11A-7), or *far1* (*CLN1 cln2 CLN3 far1* strain 1255-3D) strains. *CLN2* alleles (indicated at the bottom of each graph) were expressed from the *CLN3* promoter on plasmids in the strains indicated. Transformants were assayed for the ability to grow on YEPD and YEPGal (the first seven strains) or on YEPD and YEPD containing alpha factor (*far1*). The plating efficiencies indicated are averages of at least two independent transformants. The light gray, dark gray, and black columns indicate values obtained at 23, 30, and 37°C, respectively. (B) *BCK2*-independent, SCB-dependent transcription assay for *CLN* gene function. Strain 1446-10B (*cln1 cln2 cln3 bck2 GAL1::CLN3*) was cotransformed with a plasmid containing three SCB sites upstream of the *lacZ* gene and with a plasmid containing the *CLN* gene or the vector indicated. β -Galactosidase activity was measured after growth in glucose medium, when *GAL1::CLN3* expression is turned off. The values shown are averages from cultures of at least three independent transformants. Assays were performed at 30°C. (C) Cell volume assay for *CLN* gene function. The approximate cell volume at which budding is initiated is indicated. Strain 1607-2D (*cln1 cln2 cln3 GAL1::CLN3*) was transformed with a plasmid containing the *CLN* gene indicated. Cell volume analysis was performed as described in Materials and Methods. Cultures were grown at 30°C.

containing deletions of the fourth helix (*cln2-2L Δ 7-9*, *cln2-2L Δ 8-10*, *cln2-2L Δ 9-11*, and *cln2-2L Δ 7-11*) were formed by splice overlap extension by using the *cln2-2L7*, *-2L8*, *-2L9*, and *-2L10* plasmids as templates. Amplified products were subcloned into pT411 after *Cla*I-*Bam*HI digestion, and mutations were confirmed by DNA sequencing. Clustered-charge-to-alanine alleles were generated either by splice overlap extension or by site-directed mu-

tagenesis by using T4 DNA polymerase and the method of Kunkel (19). Plasmids pKL003, pKL004, and pKL005, containing alanine substitutions for either K129, E183, or the double mutation, were previously described (20). Plasmid pKH100, containing the HA-tagged *CLN2* gene driven from the *GAL1* promoter, was previously described (20). Plasmids with *cln2* alleles driven from the *GAL1* promoter were constructed by ligating the *Cla*I-

FIG. 2—Continued.

*Bam*HI insert from the *CLN3* promoter construct with a *Cla*I-*Bam*HI-restricted vector derived from pKH100. All of the plasmids used in this study are pRS314 derivatives. The vector control used in all of the experiments was pRS414.

Yeast strains and genetic procedures. Standard methods were used for all strain constructions and transformations (11). All of the strains or mutant alleles used have been previously described (20). All of the strains were congenic with BF264-15D (25) (Table 1).

Other techniques. Protein extraction, immunoprecipitation, immunoblotting, and protein kinase assays were performed as previously described (20), except that protein extraction was performed with LSHNN buffer (10 mM HEPES [pH 7.5], 50 mM NaCl, 10% glycerol, 0.1% Nonidet P-40) instead of TNN, and after washing three times with extraction buffer, a fourth wash in HNN (250 mM NaCl, 10 mM HEPES [pH 7.5], 10% glycerol, 0.1% Nonidet P-40) was included prior to washing with kinase assay buffer. To determine the protein levels and associated kinase activities of the various Cln2 mutants, plasmids encoding the different *CLN2* alleles expressed from the *GAL1* promoter were transformed into a wild-type strain (1255-5C). Transformants were grown first in synthetic medium lacking tryptophan (SC-Trp) and containing glucose and subsequently grown in SC-Trp medium containing galactose prior to protein extraction. Radioactivity in histone H1 was quantitated with a PhosphorImager (Applied Biosystems). Proteins were detected by enhanced chemiluminescence, and protein levels were quantitated by using a desktop scanner and ImageQuant software.

Plating efficiency assays were performed as previously described (20). Spot dilutions of strains containing the plasmids indicated were plated on a test plate (for all but the *GAL1::SIC1* and *far1* strains, a YEPD plate) and a control plate (for all but the *GAL1::SIC1* and *far1* strains, a YEPGal plate). For the *GAL1::SIC1* strain, the test plate was a YEPGal plate and the control plate was a YEPD plate. To determine pheromone resistance, strain 1255-3D (relevant genotype, *CLN1 cln2 CLN3 far1 bar1*) transformants were plated in 10-fold serial dilutions on YEPD plates (control plate) and on YEPD plates containing 0.33 μ M alpha factor (test plate). For all assays, the plates were incubated at the temperatures indicated in Results and efficiency of growth was calculated by comparing cell growth on the test plate with that on the control plate.

Cell volume assays were performed as previously described (20), by using strain 1607-2D (relevant genotype, *cln1 cln2 cln3 GAL1::CLN3*). Cultures were first grown in synthetic medium lacking tryptophan to select for the plasmid containing the *CLN2* allele to be assayed and subsequently grown in YEPD medium for 6 to 7 h prior to fixation, sonication, and cell volume measurement with a Coulter Channelyzer 256. To determine the volume at which budding is initiated, the percentage of budded cells for each culture was also determined. Because the Coulter Channelyzer determines the number of cells in a population with a specific cell volume, the percentage of budded cells can be used to approximate the volume at which budding occurs. A running summation of the number of cells smaller than volume X was determined, and this sum was used to determine a running value of the percentage of the population smaller than volume X. When this percentage was equal to the percentage of unbudded cells, as determined by microscopic measurement, volume X was considered to be the volume at which budding occurred. This calculation is an approximation because it makes the assumption that all cells bud at a uniform size.

Expression of β -galactosidase from an SCB-*lacZ* reporter plasmid was determined as previously described (20). Transformants containing an SCB-*lacZ* reporter plasmid and a plasmid containing the *cln* allele to be assayed were shifted from SCGal medium to SCDex medium (both lacking tryptophan and uracil to maintain plasmid selection) for 24 h and reinoculated into SCDex-Trp-Ura to allow growth to mid-log phase. β -Galactosidase liquid assays were carried out as previously described (1), with standardization by measuring the optical density at 600 nm of the culture before extraction.

RESULTS

Introduction of helix-disrupting sequences at 13 sites within the cyclin box. Sequence homology among Cln2, cyclin A, and cyclin H throughout the region of the cyclin box (Fig. 1A) suggests that Cln2 may similarly contain a cyclin fold comprised of five alpha helices separated by short linker regions (1a). To test these predictions, we designed oligonucleotides encoding the amino acid sequence Gly-Gly-Pro-Gly-Gly and inserted this sequence at 13 sites within the cyclin box coding region. Because this sequence contains residues antagonistic to the formation of alpha helices, insertion of this sequence within an essential helical region should disrupt Cln2 function while its presence in an interhelical region should be better tolerated. The sites of insertion of the sequence are shown in Fig. 1B. To assay these alleles, we first determined their ability to function in a number of genetic assays for specific cyclin function (20). In all of these assays, *CLN2* alleles are expressed from the *CLN3* promoter to remove any effect of positive feedback on *CLN2* expression and to allow *CLN2* expression in certain genetic backgrounds (such as *swi4* or *swi6* [20]). As shown in Fig. 2A, *CLN2* under control of the *CLN3* promoter is able to rescue a *cln1 cln2 cln3* strain, as well as a *cln1,2,3* strain additionally deficient in the transcriptional regulator *SWI4*, *SWI6*, or *BCK2*. *CLN2* also supports growth in strains with *CLB5* and *CLB6* or *PCL1* and *PCL2* deleted. An additional plating efficiency assay is based upon the lethality caused when *SIC1* is overexpressed in the absence of *CLN1* and *CLN2* (20, 31). (See reference 20 for justification for all of these assays for *CLN2* function.) The last assay shown in Fig. 2A measures the ability of *CLN2* to promote pheromone resistance in a *far1* strain (4).

Results of assaying the ability of the 13 linker insertion alleles of *CLN2* to support viability in a *cln1 cln2 cln3* strain support the hypothesis that Cln2 contains helices 1, 2, 3, and 5 in locations analogous to those of helices found in cyclins A and H. The *cln2-2L2*, *-2L4*, *-2L6*, and *-2L12* alleles all completely failed to support viability, presumably due to disruption of these four helices (data not shown). The *cln2-2L1*, *-2L3*, *-2L5*, *-2L7*, *-2L11*, and *-2L13* linker insertion alleles supported viability (Fig. 2A), suggesting that these insertions occur in flexible, interhelical regions, as predicted by sequence alignment (1a). The *cln2-2L8*, *-2L9*, and *-2L10* alleles were all functional and therefore failed to identify a fourth essential

helix in the cyclin box. Cln2 differs from cyclin A and cyclin H in that it contains a large insertion between residues proposed to comprise helices 3 and 5 (1a). Because of poor homology in this region, it is difficult to predict exactly where a fourth helix may occur in Cln2. In addition to the four alleles that failed to support viability, two other alleles showed a reduction of function in the plating efficiency assays. Strains carrying the *cln2-2L13* allele were temperature sensitive, failing to grow at 37°C in the *cln1 cln2 cln3* background and displaying reduced plating efficiency at 30°C in a number of more restrictive genetic backgrounds. In addition, *cln2-2L9* was unable to promote growth in the *GAL1::SIC1* strain at 37°C.

Linker insertion alleles that supported viability in a *cln1 cln2 cln3* strain were assayed for the ability to stimulate SCB-mediated transcription (Fig. 2B). The SCB sequence is responsive to the Swi4/Swi6 transcription factor (reviewed in reference 18). *CLN3* has been implicated in the activation of SCB-regulated genes, including *CLN1* and *CLN2*. *CLN2*, when expressed from the *CLN3* promoter, is also able to stimulate transcription from an SCB reporter construct (20, 30). An additional regulator of transcription at these loci is encoded by *BCK2* (8, 10), which contains a potent transcriptional activation domain (14). To measure *BCK2*-independent stimulation of SCB-mediated transcription in strains containing the different *CLN2* alleles, an SCB-*lacZ* construct was introduced into a *bck2 cln1 cln2 cln3 GAL1::CLN3* strain. Plasmid-borne *CLN2* alleles were subsequently introduced, and β -galactosidase activity was measured after growth in glucose medium (when *GAL1::CLN3* is turned off). The alleles with the greatest reduction of function in this assay were *cln2-2L10* and *-2L13* (Fig. 2B).

CLN function is essential for bud emergence, and *CLN* overexpression drives bud emergence and reduces cell volume (5, 9, 23, 25). As seen in Fig. 2C, cells containing a wild-type *CLN2* gene initiate bud formation when the cell volume is approximately 38 fl. *CLN3* is a less potent activator of budding (6, 9, 20); hence, cells are larger (approximately 75 fl under these conditions) when initiation of budding occurs. As shown in Fig. 2C, strains containing the *cln2-2L9*, *-2L10*, and *-2L13* alleles displayed the greatest increase in cell volume at the time of bud formation, although cells harboring the *cln2-2L9* and *-2L10* alleles were still more proficient at budding than those containing *CLN3*.

The ability of mutant Cln2 to bind to Cdc28 and activate its histone H1 kinase activity was also determined by coimmunoprecipitation (Fig. 3). *GAL1* promoter-driven constructs were used for these assays to increase assay sensitivity. As in previous assays, *cln2-2L13* had the most severely reduced ability to function, failing to promote phosphorylation at detectable levels. These results suggest that this allele generally reduces all Cln2 functions, rather than causing a defect in any one specific function. In cyclin A, a second cyclin fold is found C terminal to the first (3, 15). Computer modeling predicts that a similar fold is present in the Cln2 C terminus and that the *cln2-2L13* linker insertion may impinge on the first helix of this second cyclin fold (1a). This may account for the defects associated with the *cln2-2L13* allele.

Deletion of regions of Cln2 between the putative third and fifth helices. Because linker insertion constructs failed to suggest a location for the fourth helix of the cyclin fold in Cln2, we made a series of deletion mutants by removing residues between the sites of putative helices 3 and 5. Four deletion constructs were generated by removing residues between the sites of linker insertions in *cln2-2L7* and *-2L9* (*cln2-2L Δ 7-9*), between *cln2-2L8* and *-2L10* (*cln2-2L Δ 8-10*), between *cln2-2L9* and *-2L11* (*cln2-2L Δ 9-11*), and between *cln2-2L7* and

-2L11 (*cln2-2L Δ 7-11*). In each of these constructs, the residues indicated in Fig. 1B are removed and one copy of the linker sequence Gly-Gly-Pro-Gly-Gly is introduced. Our results indicate that the region of Cln2 proposed to contain a fourth helix (1a) is not essential for function. The *cln2-2L Δ 7-11* allele, containing only the linker sequence and six additional residues between what are proposed to be the third and fifth helices, was able to support viability in a *cln1 cln2 cln3* strain, although only at reduced temperatures (Fig. 4A). If a fourth helix exists in Cln2, our data support a location at the C-terminal region of the sequence between helices 3 and 5. While the *cln2-2L Δ 7-9* allele produced only a minor reduction in Cln2 function, *cln2-2L Δ 8-10* and *cln2-2L Δ 9-11* produced more significant defects in plating efficiency, transcription, budding, and kinase assays (Fig. 4 and 5). These results suggest that this region is more likely to contain an important structural element such as an α helix. This location is consistent with protein structure prediction programs (1a).

Construction of *cln2* alleles lacking residues that contribute to key structural features. Crystallization of the human cyclin A-CDK2 complex identified a number of residues that participate in key interactions either between these two proteins or between domains in the cyclin component of the complex. Four of these residues are shown in Fig. 1A. K129 and E183 are analogous to residues in cyclin A that form hydrogen bonds with CDK2 (15). *CLN2* alleles in which these residues were changed to alanines were described previously (20). The *cln2-R74A* and *-D103A* alleles alter residues homologous to Arg and Asp residues that in cyclin A hydrogen bond to each other to form a buried, interhelical salt bridge linking helices 1 and 2 in the first cyclin fold (3, 15). As seen in Fig. 6A, removal of the putative salt bridge resulted in Cln2 molecules that could still function in most genetic assays. The *cln2-R74A* allele produced a more severe phenotype than the *cln2-D103A* allele, an effect that is probably not related to loss of the salt bridge, since removal of either residue should abolish the salt bridge in an equivalent manner. The analogous arginine in cyclin A makes interdomain contact with the C-terminal end of the second helix in the second cyclin fold (3, 15). Although Cln2 shows little homology to bovine cyclin A in this region, it is possible that a similar structure exists.

The ability to promote transcription from an SCB-*lacZ* reporter plasmid and promote budding was also determined (Fig. 6). While strains containing the *cln2-R74A*, *-D103A*, and *-E183A* alleles showed only a moderate reduction in SCB-driven transcription, strains carrying the *K129A* and *K129A E183A* alleles had greatly reduced β -galactosidase activity, with levels only slightly above the background. As shown in Fig. 6C, all of the strains containing mutant *cln2* alleles displayed an increased cell volume at the time of bud formation although cells harboring the *R74A* and *D103* alleles were still more proficient at budding than those containing *CLN3*. Once again, strains carrying the *K129A* allele and the *K129A E183A* double mutant displayed the most profound defect in the assay.

Cln2-associated Cdc28 and histone H1 kinase activity were also measured. The *cln2-E183A* and *-K129A* alleles were designed to disrupt binding to Cdc28; the effect of this reduction has been previously reported (20) and is shown again in Fig. 7 for comparative analysis. The *K129A* mutation, which removes two hydrogen bonds from the cyclin-CDK interaction, results in a more significant reduction in binding than the *E183A* mutation, which removes only one. Cdc28 binding cannot be detected in the double mutant. It is notable that the defect in the Cln2-K129A mutant protein is exclusively a binding defect;

FIG. 3. Comparison for Cln2 protein levels, associated Cdc28 protein, and associated kinase activity for HA-tagged Cln2 proteins expressed from the *GAL1* promoter. Wild-type cells (strain 1255-5C) were transformed with plasmids containing the *CLN2* alleles indicated, and immunoprecipitates were prepared by using antibody 12CA5, which recognizes the HA epitope. (A) Cln2 protein and Cln2-associated Cdc28 levels determined by immunoblotting. Cln2-associated kinase activity was determined as described in Materials and Methods. (B) Relative levels of Cdc28 associated with Cln2. (C) Relative levels of Cln2-associated kinase activity. (D) Relative levels of Cdc28-associated kinase activity. The level of histone H1 kinase activity in the strain carrying the *cln2-2L13* allele was too low to be measured.

FIG. 4. Analysis of *cln2* alleles with deletions in the region of the putative fourth helix. (A) Plating efficiency assays. The strains used are indicated in Fig. 2A. The light gray, dark gray, and black columns indicate values obtained at 23, 30, and 37°C, respectively. (B) β -Galactosidase activity in strains containing the *CLN* plasmid indicated and an *SCB-lacZ* reporter construct. Strains and assay conditions were the same as in Fig. 2B. (C) Cell volume at the time of budding. Assay conditions were the same as in Fig. 2C. *, strains containing the *cln2-2LA9-11* and *-2LA7-11* alleles were more than 85% unbudded, and therefore cell volume at the time of budding could not be determined accurately.

the specific activity of the kinase complex is actually greater than that obtained from cells containing wild-type *CLN2*. The E183A mutant protein clearly shows reduced binding and probably also has moderately reduced specific activity (a fivefold reduction was observed in this study; we previously observed a twofold reduction [20]). Cln2 protein from strains containing the *R74A* and *D103A* alleles shows a less pronounced reduction in the ability to bind Cdc28, binding approximately 50% as well as wild-type Cln2. A reduction in the specific activity of the kinase complex (amount of kinase activity per Cdc28 molecule) is a greater factor in

the decrease in measured kinase activity for these two alleles.

Clustered-charge-to-alanine mutants. An additional series of alleles was generated by clustered-charge-to-alanine scanning mutagenesis, a technique in which all of the charged residues within a small cluster of amino acids are simultaneously changed to alanines (2, 12, 28, 32). Clusters of charges are likely to lie on the surface of a protein and are potential sites of protein-protein interaction. Because alanine substitution removes the side chain beyond the β carbon without altering the main chain conformation or imposing severe elec-

FIG. 4—Continued.

trostatic or steric effects, these changes presumably introduce minimal disruption of the overall structure of the target protein (7). Therefore, we hoped that this approach might identify regions of Cln2 that regulate interaction with substrates or regulatory molecules. Because this series of mutations targeted regions outside of the cyclin box, it also had the potential to identify key elements of the Cln2 protein not identified by other alleles generated in this study. Thirty-one clusters of charges were targeted for replacement with alanines, at sites spanning most of the Cln2 protein (Fig. 1B). All of the alleles were assayed for their activity in cyclin-specific genetic assays, for their effect upon cell volume, and for their ability to stimulate SCB-mediated transcription (20). Those showing defects in these assays were further tested for Cln2-associated Cdc28 and histone H1 kinase activity.

Of 31 clustered-charge replacement alleles, only 1, *cln2-ala9*, failed to support viability in a *cln1 cln2 cln3* strain (Fig. 8A). This result suggests that Cln2 is surprisingly resilient when mutated, perhaps reflecting redundancy of protein folding determinants (as suggested by the results obtained with R74, D103, K129, and E183 discussed above). Of the remaining 30 alleles, 24 were indistinguishable from wild-type *CLN2* in terms of plating efficiency, even in restrictive genetic assays (20) (Fig. 8A). Six alleles, *cln2-ala7*, *-ala8*, *-ala10*, *-ala11*, *-ala15*, and *-ala23*, showed defects in specific genetic assays. In most cases, these defects were only clearly detected at 37°C; this temperature effect led to some difficulties in interpretation of the mutants (see Discussion). In terms of the ability to

promote SCB-mediated transcription, only *cln2-ala9*, *-ala15*, and *-ala31* showed a significant decrease in *CLN2* function (Fig. 8B). In the cell volume assay, the alleles showing the greatest defect were *cln2-ala8* and *-ala15*, which failed to promote budding until the cell volume was greater than that of cells containing *CLN3* (Fig. 8C).

Cln2 protein from strains containing the *CLN2*, *cln2-ala4*, *-ala7*, *-ala8*, *-ala9*, *-ala10*, *-ala11*, *-ala15*, and *-ala23* alleles was tested for associated histone H1 kinase activity (Fig. 9). Consistent with the inability of *cln2-ala9* to function in any of the assays in which it was tested, the Cln2 protein derived from a strain harboring this allele did not yield coprecipitated Cdc28 protein or kinase activity. It is also notable that the Cln2 protein from this mutant migrated rapidly as a single band, which suggests that it is primarily unphosphor-

FIG. 5. Cln2 protein levels, associated Cdc28 protein levels, and associated kinase activity for Cln2 protein derived from strains carrying *cln2-2LΔ* alleles. (A) Cln2 protein levels, Cln2-associated Cdc28 levels, and Cln2-associated kinase activity. (B) Relative levels of Cdc28 associated with Cln2. (C) Relative levels of Cln2-associated kinase activity. (D) Relative levels of Cdc28-associated kinase activity.

FIG. 6. Analysis of *cln2* alleles with mutations in residues important for key structural interactions. (A) Plating efficiency assays. The strains used are indicated in Fig. 2A. The gray and black columns indicate values obtained at 30 and 37°C, respectively. (B) β-Galactosidase activity in strains containing the *CLN* plasmid indicated and an *SCB-lacZ* reporter construct. Strains and assay conditions were the same as in Fig. 2B. (C) Cell volume at the time of budding. Assay conditions were the same as in Fig. 2C. *, strains containing the *cln2-K129A E183A* double mutant were more than 85% unbudded, and therefore cell volume at the time of budding could not be determined accurately.

ylated. Other impaired alleles in this study also displayed rapid migration, suggesting that phosphorylation is dependent upon Cln2 function and/or Cdc28 association. Strains containing *cln2-ala4*, *-ala7*, *-ala8*, *-ala11*, *-ala15*, and *-ala23* alleles yielded a Cln2 protein with reduced ability to bind Cdc28. The Cln2 protein derived from the *cln2-ala7* strain, the *cln2-ala8* strain, and particularly the *cln2-ala23* strain also displayed an additional reduction in specific activity once Cdc28 was bound (amount of kinase activity per Cdc28 molecule). *cln2-ala10* displayed little defect in Cdc28 binding or activation. The mutations in *cln2-ala4*, *-ala7*, *-ala8*, *-ala11*, and *-ala15* are in the cyclin box region, which may explain their effects on Cdc28 binding. The mutation in *cln2-ala23* is predicted to be in the second helix of the C-terminal cyclin fold (1a), so the drastic effect of this mutation on Cdc28 binding and activation is probably due to indirect conformational changes.

DISCUSSION

Cln2 has a cyclin fold. The recent crystallization of cyclin A prompted us to examine the effect of mutation on the ability of *CLN2* to function in vivo. Mutant *CLN2* alleles were designed to determine if it is reasonable to assume that a similar structure exists for Cln2. The low level of sequence homol-

ogy between Cln2 and cyclin A (~25% through the most highly conserved region) makes this a significant question for evaluation of cyclin folding throughout the cyclin superfamily (which is characterized by quite low sequence homology [1a]). The series of linker insertion alleles suggests that Cln2 has an overall structure similar to that of cyclin A through the majority of the cyclin box. The fourth helix of cyclin A is difficult to align with Cln2 based upon the primary amino acid sequence, especially since Cln2 contains a large insertion in this area. Our data suggest that the fourth helix (if one exists) is more likely to reside at the C-terminal end of the region, between helices 3 and 5, yet is largely dispensable for function.

Mutation of R74 or D103 was predicted to abolish an interhelical salt bridge in the cyclin box (3, 15). Buried salt bridges are highly conserved evolutionarily, and R74 and D103 are highly but not absolutely conserved in the cyclin superfamily (1a). It is therefore surprising to find relatively minor functional defects upon mutation of either partner in the putative Cln2 salt bridge. It could be postulated that because the buried salt bridge is in a very hydrophobic environment, there are considerable hydrophobic interactions in this region that make the salt bridge nonessential. The second fold of cyclin A does not contain an equivalent salt bridge, although the orientation of its first helix differs from that found in the first fold (3, 15). While the salt bridge is likely to contribute to optimal packing

and precise arrangement of the helices, it is not required for Cln2 function. This contrasts with results obtained with *Xenopus* cyclin A, where mutation of the arginine and aspartate residues equivalent to R74 and D103 resulted in loss of function (17, 29).

Similarly, K129 and E183, residues that are almost completely conserved in the cyclin superfamily (1a), are salt bridged to each other in cyclin A, and appear central to the cyclin A-Cdk2 interface (3, 15), can be mutated to alanine in *CLN2* without inactivating function (20). The residual activity of *CLN2* when both K129 and E183 are removed could be attributed to the presence of additional residues at the cyclin-CDK interface. Twenty-five hydrophobic residues and additional hydrogen bonds contribute to the interface between cyclin A and CDK2 (15). It could again be postulated that, like R74 and D103, K129 and E183 are important for the formation of an optimal cyclin molecule yet may be structurally redundant. If the structure of Cln2 is redundantly specified by the sequence, this could perhaps explain the resiliency upon charge-to-alanine mutagenesis observed.

Quantitative versus qualitative interpretation of mutational analysis of Cln2. Many of the *CLN2* alleles in this study show a significant reduction in in vitro kinase activity yet resemble the wild type in most of the cyclin-specific genetic assays, even at increased temperatures, and display approximately wild-type transcription levels and cell volumes at the time of budding. This disparity between in vitro and in vivo results could be due to the protein extraction and washing procedure used in the kinase assays or to the fact that histone H1 is not an appropriate physiological substrate for this assay. Alternatively, it is possible that Cln2 activity is in excess, even when expressed from the weak *CLN3* promoter. Previously, we found that diploids carrying only one copy of *CLN2* executed bud emergence at a cell volume identical to that of isogenic *CLN2/CLN2* strains (20), suggesting by a different route that Cln2 could be present in excess for some biological functions.

For most of the alleles, rescue of *GAL1::SIC1* lethality and rescue of *cln1 cln2 clb5 clb6* lethality appeared to be the most stringent assays, especially at 37°C. These assays may therefore identify a function(s) of Cln2 requiring a high quantitative level of function. In contrast, rescue of *cln1 cln2 cln3 swi4*, *cln1 cln2 cln3 swi6*, and *cln1 cln2 cln3 pc11 pcl2* inviability did not show greatly increased requirements over rescue of the basic *cln1 cln2 cln3 SWI PCL* background. These results therefore suggest that Cln2 has at least two genetically separable functions. One class of functions, requiring high Cln2 activity, may have to do with the requirement for activation of B-type cyclin-dependent kinase activity (overcoming a block due to the Sic1 B-type cyclin-dependent kinase inhibitor or bypassing the *CLB5 CLB6* requirement). A second class of functions, requiring only low Cln2 activity, is identified by bypassing the *SWI4/SWI6/PCL1 PCL2* requirement(s). Allowing mating factor resistance in the absence of *FAR1* (4) shows a spectrum of requirements generally similar to those of the less stringent *SWI/PCL* assays. As described previously, Cln3 is defective in all of these functions (6, 20). The molecular complexity of these functions (how many Cln2-specific targets are involved and how they are regulated by Cln2) cannot be addressed at present.

The failure of certain *cln2* alleles in these assays could represent either quantitative loss of general function or a qualitative difference in the ability of the mutant Cln2-Cdc28 complexes to perform certain specific functions. It is difficult to distinguish between these two possibilities, but some results suggest that we have identified *cln2* alleles that cause loss of

FIG. 7. Cln2 protein levels, associated Cdc28 protein levels, and associated kinase activity for Cln2 protein derived from strains carrying *cln2* alleles containing mutations in key structural residues. (A) Cln2 protein levels, Cln2-associated Cdc28 levels, and Cln2-associated kinase activity. (B) Relative levels of Cdc28 associated with Cln2. (C) Relative levels of Cln2-associated kinase activity. (D) Relative levels of Cdc28-associated kinase activity. *, levels of Cdc28 and histone H1 kinase activity in the strain carrying the *cln2-K129A E183A* double mutation were too low to be measured.

FIG. 8. Analysis of *cln2* clustered-charge replacement alleles. (A) Plating efficiency assays. The strains used are indicated in Fig. 2A, except that the *cln* mutant strain used was 1254-14D (*cln1 cln2 cln3 leu2::LEU2::GAL1::CLN1*). The dark gray and black columns indicate values obtained at 30 and 37°C, respectively. (B) β -Galactosidase activity in strains containing the indicated *CLN* plasmid and an *SCB-lacZ* reporter construct. Strains and assay conditions were the same as in Fig. 2B. (C) Cell volume at the time of budding. Assay conditions were the same as in Fig. 2C. The strain containing the *cln2-ala9* allele was more than 85% unbudded, and therefore cell volume at the time of budding could not be determined accurately.

specific functions. For example, strains containing the *cln2-ala7*, *-ala11*, and *-ala23* alleles display less than 10% in vitro kinase activity yet initiate budding at 56 to 58 fl (versus 38 fl for those containing wild-type *CLN2*). Cells carrying the *cln2-ala4* allele show 50% of wild-type kinase activity yet bud at an average cell volume of 66 fl. These data therefore suggest the possibility that the *cln2-ala4* strain has a specifically reduced ability to promote bud initiation.

In previous work, we suggested that Cln3 might be specialized compared to Cln2 for activation of Bck2-independent, SCB-dependent transcription (20). This conclusion was based on the observation that Cln3 is fully active in *bck2* assays (i.e., rescue of *cln1,2,3 bck2* or the direct assay for Bck2-independent SCB-dependent transcription) but completely inactive in many other assays, such as rescue of *cln1,2,3 swi6*. In contrast, two partially functional Cln2 mutants were more defective than Cln3 in the *bck2* assays but less defective than Cln3 in the other assays. This general conclusion is supported by many of the mutants described here. All of the mutants displaying significant defects in the *bck2* assays retained significant rescue activity

in other assays (for example, rescue of *cln1,2,3 swi4* or *cln1,2,3 swi6*) in which Cln3 is completely inactive (20). (The only exception to this pattern is the completely defective allele *cln2-ala9*, which has no biological activity in any assay.) These results therefore support the conclusion reached previously on the basis of only two reduced-function *cln2* alleles (*cln2-K129A* and *cln2-E183A*) that Cln3 is intrinsically more specialized than Cln2, probably for transcriptional activation.

Two of the *cln2* mutants examined here appear to have an exaggerated version of this pattern of activity, suggestive of specific loss of function. *cln2-2L Δ 8-10* and *cln2-2L Δ 9-11* (alleles encoding deletions in the region of the putative fourth helix of Cln2) support growth in a *cln1,2,3 clb5 clb6* strain but completely fail to rescue the *cln1,2,3 bck2* strain (except at 23°C). In contrast, many other alleles, such as *cln2-ala7*, *-ala8*, *-ala10*, *-ala11*, *-ala15*, and *-ala23*, fail to support growth in the *cln1,2,3 clb5 clb6* strain yet retain at least partial rescue of the *cln1,2,3 bck2* strain. Rescue of the *cln1,2,3 clb5 clb6* strain may require a high quantitative level of Cln2 function, as discussed above, and therefore the *cln2-2L Δ 8-10* and *cln2-2L Δ 9-11* al-

FIG. 8—Continued.

leles may be specifically defective in *bck2* rescue as opposed to being generally low functioning. Although the *cln2-2LΔ8-10* and *cln2-2LΔ9-11* alleles are assayed at 30°C and the others are assayed at 37°C (due to complete loss of activity of the *cln2-2LΔ8-10* and *cln2-2LΔ9-11* alleles at 37°C), these comparisons are for a constant temperature for each *cln2* allele. Therefore, it seems reasonable to make the comparison, although the role of high temperature in making these assays more restrictive for the bulk of the alanine scan mutants (Fig. 8A) is unknown. The inviability of the *cln1,2,3 bck2* strain carrying the *cln2-2LΔ8-10* and *cln2-2LΔ9-11* alleles correlates with a defect in driving SCB-mediated transcription in the absence of *BCK2* (Fig. 4B). Although both of these alleles are highly defective in Cln2-associated histone H1 kinase activity, poor kinase activity is not sufficient to explain the defect: *cln2-ala23* results in similarly poor in vitro kinase activity yet is positive in *bck2* assays (indeed, it is higher than wild-type Cln2 in the transcription assay [Fig. 8B]). *cln2-2LΔ8-10* and *2LΔ9-11* may therefore identify a region of Cln2 important for transcriptional activation. A possible molecular interpretation of this result is that this region targets a specific substrate by protein-protein interaction; however, we cannot rule out the possibility that an indirect effect of the mutation on protein conformation is responsible. The fact that these alleles are capable of rescuing a *cln1,2,3 bck2* strain at 23°C could be more consistent with the conformational explanation.

FIG. 9. Cln2 protein levels, associated Cdc28 protein levels, and associated kinase activity for Cln2 protein derived from strains carrying the clustered-charge replacement alleles of *cln2*. (A) Cln2 protein levels, Cln2-associated Cdc28 levels, and Cln2-associated kinase activity. (B) Relative levels of Cdc28 associated with Cln2. (C) Relative levels of Cln2-associated kinase activity. (D) Relative levels of Cdc28-associated kinase activity. *, Cln2-associated histone H1 kinase activity and Cdc28 protein from the strain containing the *cln2-ala9* allele were too low to be measured.

ACKNOWLEDGMENTS

We thank Kristi Levine and Mary Miller for assistance in these studies and for comments on the manuscript and Ray Deshaies for providing the anti-Cdc28 antibody. We also thank Nikola Pavletich, Andrej Sali, and Roberto Sanchez for helpful discussions.

This work was supported by NIH grant GM47238. K.H. was supported by NRSA GM17092.

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