The single Cdk1-G1 cyclin of Cryptococcus neoformans is not essential for cell cycle progression, but plays important roles in the proper commitment to DNA synthesis and bud emergence in this yeast

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Abstract

The cell cycle pattern of the pathogenic basidiomycetous yeast Cryptococcus neoformans differs from that of the ascomycetous budding yeast Saccharomyces cerevisiae. To clarify the cell cycle control mechanisms at the molecular level, homologues of cell cycle control genes in C. neoformans were cloned and analyzed. Here, we report on the cloning and characterization of genes coding for Cdk1 cyclin homologues, in particular, the C. neoformans G1 cyclin. We have identified three putative Cdk1 cyclin homologues and two putative Cdk5 (PHO85) cyclin homologues from the genome. Complementation tests in an S. cerevisiae G1 cyclin triple mutant confirmed that C. neoformans CLN1 is able to complement S. cerevisiae G1 cyclin deficiency, demonstrating that it is a G1 cyclin homologue. Interestingly, cells deleted of the single Cdk1-G1 cyclin were viable, demonstrating that this gene is not essential. However, it exhibited aberrant budding and cell division and a clear delay in the initiation of DNA synthesis as well as an extensive delay in budding. The fact that the mutant managed to traverse the G1 to M phase may be due to the activities of Pho85-related G1 cyclins. Also, that C. neoformans had only a single Cdk1-G1 cyclin highlighted the importance of keeping in order the commitment to the initiation of DNA synthesis first and then that of budding, as discussed.

Introduction

Cell cycle control has been studied extensively in Saccharomyces cerevisiae. START constitutes the most important regulatory point, wherein the cell is able to respond to various internal and external stimuli and commit to cell cycle progression, coordinating cell size, sensitivity to pheromone signaling and nutrient availability with the initiation of the cell cycle, leading to the landmark events of the initiation of DNA synthesis, bud emergence and spindle pole body duplication (Lew et al., 1997). This important late G1 event is mainly regulated by cyclin-dependent kinase 1 (Cdk1/Cdc28/Cdc2) associated with G1 cyclin (Wittenberg & Reed, 2005). Studies on Candida albicans and other ascomycetous yeasts are well in accord with the above paradigm (Bachewich & Whiteway, 2005; Berman, 2006; Whiteway & Bachewich, 2007).

The cell cycle control of the basidiomycetous yeast Cryptococcus neoformans is different. During exponential growth, budding and DNA synthesis occur simultaneously, similar to ascomycetous yeast species. However, as the growth phase progresses during the late exponential to the early stationary phase, budding is delayed toward the end of the cell cycle at the G2 phase, allowing cells to arrest either at the G1 or at the G2 unbudded state (Takeo et al., 1995; Ohkusu et al., 2001a). This phenomenon is also observed in response to stress conditions, e.g. oxygen depletion, pH and temperature changes, and is therefore believed to be an important stress-response mechanism (Ohkusu et al., 2001b; Takeo et al., 2003).
In order to elucidate the mechanisms for the *C. neoformans* cell cycle regulation, we isolated the main cell cycle control gene *CDK1* homologue in *C. neoformans*, *CDK1*, in a previous study (Takeo et al., 2004). *CDK1* was found to complement the *S. cerevisiae* temperature-sensitive *cdc28* mutations. Although CdK1 controls cell cycle progression, it has no activities without bound cyclins. Furthermore, different cyclins appear to direct Cdk1 to different substrates (Futcher, 1996; Lew et al., 1997). Thus, cyclins are key players of cell cycle control. This study reports on the identification of the cyclin homologues in *C. neoformans*. In addition to Cdk1 cyclins, Cdk5 (Pho85) cyclins in the Pcl1/Pcl2 subfamily (Pcl1, Pcl2 and Pcl9) were also implicated to play roles in cell cycle and polarized growth in *S. cerevisiae* (Moffat & Andrews, 2004; Huang et al., 2007).

Because the observed difference with *S. cerevisiae* and *C. albicans* occurs in the G1/S phase, we have particular interest in *C. neoformans* G1 cyclins that regulate the G1/S transition and budding in most yeast species studied (Hadwiger et al., 1989; Lew et al., 1992; Martin-Castellanos et al., 2000; Moffat & Andrews, 2004). Particularly, the observation that *C. neoformans* is able to delay bud emergence while proceeding with DNA synthesis under unfavorable conditions (Ohkusu et al., 2001b) suggests the existence of a G1/S transition and budding regulatory mechanism remarkably different from that of *S. cerevisiae*. This implies a more ‘flexible’ regulation at the G1/S transition or START in *C. neoformans*, whereby initiation of bud emergence and DNA synthesis are not only distinct and independently controlled, but can be temporally separated as a response to environmental conditions. If, like in *S. cerevisiae*, several G1 cyclins exist, the initiation of bud emergence and DNA synthesis may be under the regulation of these different G1 cyclins that have specialized functions for these two important processes in START. We therefore attempted to identify and clone the G1 cyclin genes in *C. neoformans*. Surprisingly, only a single Cdk1-related G1 cyclin homologue was found in the genome sequence. The gene of this single Cdk1-G1 cyclin homologue was cloned and the sequence analysis of its 5′ end-sequence information from 5′ rapid amplification of cDNA ends (RACE) using the GeneRacer Kit (Invitrogen) according to the manufacturer’s instructions. The RT-PCR products, 5′ and 3′ RACE products were cloned in pCR4-TOPO (Invitrogen) and sequenced using an ABI 3100 Genetic Analyser (Applied Biosystems). Using end-sequence information from 5′ and 3′ RACE products, primers to amplify the entire cDNA were constructed and PCR using Platinum Taq DNA Polymerase High Fidelity (Invitrogen) was performed to clone the full-length cDNA. Sequence data from the 5′ RACE product, RT-PCR product and 3′ RACE product was assembled to generate the sequence of the full-length cDNA. From this, the longest ORF was identified using Genetyx-Win v9 (Software Development Co., Tokyo, Japan). Primers to amplify the ORFs (Table S1) were constructed around the putative start and stop codons with appropriate restriction enzyme sites for cloning of *CDK1* cyclin homologues

### Materials and methods

#### Identification and cloning of genes coding for putative *CDK1* cyclin homologues

Using data available from the SGTC *C. neoformans* genome database (http://sequence-www.stanford.edu/group/C.neoformans/), cyclin ORFs were identified from the preliminary annotation. Seventeen ORF entries were found to contain cyclin domain motifs (Supporting Information, Table S1). Some ORFs were assumed to belong to the same gene when they were located considerably close to each other based on their position in the contig assembly. Sequences flanking the ORF coding sequences were downloaded and amino acid translations of the flanking regions and the coding sequences were deduced for all reading frames. Deducing amino acid sequences were BLAST searched in the (http://blast.ncbi.nlm.nih.gov/Blast.cgi) Gene Bank database and reading frame regions that had similarities to known cyclin sequences from other species were identified. Based on the location and degree of sequence similarity to known cyclin sequences, the 17 ORF entries were narrowed down to six cyclin candidates for cloning namely, ORF34, ORF56, ORF89, ORF1011, ORF1516 and ORF17. Primer pairs for reverse transcriptase RT-PCR were then constructed around these regions to amplify putative exon sequences, which were deduced based on reported intron splice site consensus sequence information for *C. neoformans* (Edman & Kwon-Chung, 1990). Primers were constructed to yield RT-PCR products < 1 kb in length. Total RNA was isolated using the FastRNA Pro Red Kit (QBiogene) and FastPrep FP120 (QBiogene) from *C. neoformans* (strain B4500 = JEC21, serotype D, mating type a) (Kwon-Chung et al., 1992). When RT-PCR products in the expected size range were obtained, the same primer sequences were subsequently used as gene-specific primers to amplify the ends of the entire coding sequence using 5′ and 3′ rapid amplification of cDNA ends (RACE) using the GeneRacer Kit (Invitrogen) according to the manufacturer's instructions. The RT-PCR products, 5′ and 3′ RACE products were cloned in pCR4-TOPO (Invitrogen) and sequenced using an ABI 3100 Genetic Analyser (Applied Biosystems). Using end-sequence information from 5′ and 3′ RACE products, primers to amplify the entire cDNA were constructed and PCR using Platinum Taq DNA Polymerase High Fidelity (Invitrogen) was performed to clone the full-length cDNA. Sequence data from the 5′ RACE product, RT-PCR product and 3′ RACE product were assembled to generate the sequence of the full-length cDNA. From this, the longest ORF was identified using Genetyx-Win v9 (Software Development Co., Tokyo, Japan). Primers to amplify the ORFs (Table S1) were constructed around the putative start and stop codons with appropriate restriction enzyme sites for cloning of the putative *CDK1* cyclin homologues.

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cloning into the yeast expression vector, pYES2 (Invitrogen). Putative amino acid sequences of the \textit{C. neoformans} cyclin candidates were compared with reported cyclin homologues from other fungi. Cyclin homologues from ongoing genome projects were obtained either by \textsc{blast} search (http://www.ncbi.nlm.nih.gov/genomes/leuks.cgi?p3=11:Fungi&taxgroup=11:Fungi\cite{[12]}) using these \textit{Cryptococcus} cyclin sequences or by searching genes or hypothetical proteins containing the Pfam Cyclin N-terminal domain motif that encompasses the conserved cyclin box region \cite{[1995]}

\textbf{Complementation in \textit{S. cerevisiae}}

Complementation experiments were attempted with an \textit{S. cerevisiae} \textit{cln}1, \textit{cln}2 and \textit{cln}3 triple-mutant strain using the pYES2-CLN ORF constructs for five of the cyclin candidates that were successfully cloned by RT-PCR and RACE. The pYES2-\textit{C. neoformans} CLN ORF constructs were transformed into the \textit{S. cerevisiae} \textit{cln1}, \textit{cln2} and \textit{cln3} triple-mutant strain [TK-209/K3413: \textit{MATa}, \textit{ade2-1}, \textit{trpl-1-can1-100}, \textit{leu2-3,112}, \textit{his3-11,15}, \textit{ura3}, \textit{SSD1}, \textit{cln1-1::hisG}, \textit{cln2-2::A}, \textit{cln3-3::LEU2}, \textit{CLN1}, containing YlpLac204-P\textsc{met}-CLN2 (Amon \textit{et al.}, 1994; Irninger & Nasmuth, 1997) using the lithium acetate/PEG method \cite{[2002]}. The pYES2 expression vector carries the \textit{URA3} marker and places the \textit{C. neoformans} cyclin ORF under the GAL promoter. The \textit{S. cerevisiae} strain carries the \textit{CLN2} gene under the \textit{MET} promoter and does not grow in a medium supplemented with methionine. Transformants were selected for growth in a uracil-deficient medium and tested for growth in a medium supplemented with methionine to suppress \textit{S. cerevisiae} \textit{CLN2} expression and with galactose as a carbon source, to induce the expression of each of the \textit{C. neoformans} cyclin candidates from pYES2.

\textbf{Construction and analysis of deletion mutants}

The \textit{C. neoformans} \textit{CLN1} deletion cassette was constructed using overlap PCR with primers that connect 1 kb fragment upstream and downstream of the \textit{CLN1} ORF with the \textit{NEO} (G418) marker amplified from plasmid pJAF1 (a gift from J. Heitman’s lab). Primers ORF171UP5DEL (\textit{5'-AAGCCCTTACTATTTAGTTCTG-3'}) and ORF171UP3 DELM13R (\textit{5'-gtcatagctttcctgCATCTTGGATAGATTTT-3'}) were used to amplify the upstream region (ORF171 UPDEL, 1001 bp) of the \textit{CLN1} gene and primers ORF171DOD5 DELM13F (\textit{5'-ctggcctgcttttacTAGTCTTTCC TCATCGTCA-3'}) and ORF171DO3DEL (\textit{5'-TCTCTG TTTGGACAGATTG-3'}) were used to amplify the downstream region (ORF171 DODEL, 1058 bp) of the \textit{CLN1} gene. Primers M13F (\textit{5'-GAAAACGACGAGCCAG-3'}) and M13R (\textit{5'-CAGGAAAACGCTATGAC-3'}) were used to amplify the G418 marker from the pJAF1 (2097 bp). Sequences in lower case represent the oligonucleotides for overlap PCR with the \textit{NEO} marker. PCRs were performed using KOD Plus DNA Polymerase (Toyobo, Japan) in an iCycler (Bio-Rad) thermal cycler. These three fragments for overlap were amplified separately and overlap PCR was performed by mixing equimolar amounts of the purified fragments (ORF171UP5DEL, \textit{NEO} marker, ORF171DO-DEL) and using the end primers ORF171UP5DEL and ORF171DO3DEL. Biolistic transformations were performed as described by \cite{[2000]}. Transformants were selected in a YPG medium supplemented with 200 mg L$^{-1}$ G418 (Geneticin). To confirm correct integration, two sets of PCR reactions using primers from both the 5\textsuperscript{'},\textsuperscript{'} upstream end (ORF171DELLP \textit{5'-AGCGTGACAT GTCCCTGAAC-3'}) and the 3\textsuperscript{'},\textsuperscript{'} downstream end (ORF171 DEIRP \textit{5'-GCTATCGGGAGAAGTTG-3'}) of the \textit{CLN1} ORF in combination with primers from within the \textit{NEO} marker (M13F, M13R) were performed.

To construct the \textit{cln1A+CLN1} gene reconstitution strain, genomic DNA from the wild-type parent B4500 containing the full-length \textit{CLN1} gene was amplified by PCR using KOD Plus Taq polymerase (Toyobo, Japan) using primers ORF17cDNA5endSpeI (\textit{5'-actagTAATCCA CACATTGGAAGGC-3'}) and ORF17cDNA3endKpnI (\textit{5'-ggattcGTATCGGTAGATCACAATG-3'}) located around 1 kb upstream and downstream from the genomic positions of 5\textsuperscript{'},\textsuperscript{'} and 3\textsuperscript{'},\textsuperscript{'} ends, respectively, of the full-length \textit{CLN1} cDNA end sequences identified previously by RACE. The 3.8-kb SpeI–KpnI fragment was cloned and sequenced in pCR4Blunt-TOPO (Invitrogen). After confirming sequence fidelity, it was subcloned into the SpeI–KpnI site of the pAI1 plasmid containing the nourseothricin (\textit{NAT}) dominant selection marker to yield plasmid pEV17 (Fig. S1a). The plasmid was linearized with Aor5H1 to cut along the \textit{CLN1} upstream flanking region at a position that provides around 900 bp on each side to allow for targeted homologous recombination at the site where the original \textit{CLN1} gene was replaced with the \textit{NEO} marker. Correct integration will thus generate a functional \textit{CLN1} copy upstream of the original \textit{CLN1} locus that was replaced with the \textit{NEO} marker, thus maintaining G418 resistance while gaining \textit{NAT} resistance. This targeted scheme ensures that \textit{CLN1} is reconstituted at the correct locus and provides a way to confirm that reconstituted strains are not wild-type contaminants, but those that have derived from the deleted strain. Linearized DNA was coated onto gold beads and biolistic transformation was performed as described above, except that transformants were selected in plates supplemented with 100 mg L$^{-1}$ nourseothricin. PCR with primers Recon Check LP1 (\textit{5'-GCGCTTGCTTACATA TCG-3'}) located in the 5\textsuperscript{'},\textsuperscript{'} upstream region of the cassette (to check for correct \textit{CLN1} locus) and Recon Check RP1 (\textit{5'-GCAAATTCGGTTGACAG-3'}) located within the \textit{CLN1} ORF (to check for the correct \textit{CLN1} coding
sequence) to yield a 2.5-kb product, which will also be detected in the wild-type B4500 strain, and with primers Recon Check LP2 (5'-CCGTTTacagATCCACCA-3') spanning the SpeI-CLN1 border at the pAI1 cloning site (to check for a reconstitution plasmid-specific sequence) and Recon Check RP2 (5'-GGAGACTGtagaacaagc-3') spanning the NEO-CLN1 downstream fragment border (to check for the original deletion sequence and locus) to yield a 3.8-kb product, were performed to identify correct transformants. The second PCR product was detected in neither the wild-type strain nor the deleted strain (Fig. S1b). Furthermore, deletion and reconstitution of CLN1 was also confirmed by Southern Blotting. Briefly, genomic DNA from the wild-type strain (B4500), the deleted strain and the reconstituted strain were digested with SphI and run on an agarose gel and blotted onto a nylon membrane. The 1-kb upstream fragment that was used in overlap PCR for the construction of the CLN1 deletion cassette was labeled using the PCR DIG Probe Synthesis Kit (Roche) and used as a probe. This yielded a 9.6-kb band common to the three strains, a 5.5-kb band unique to the wild-type B4500 strain, a 1.1-kb band common to the deletion and reconstituted strain and 4.6- and 2.5-kb bands unique to the reconstituted strain (Fig. S1c).

Characterization of the cln1 deletion mutant

Cells were grown in YNB [yeast–nitrogen base (Difco) supplemented with 1% glucose and 0.05 M MOPS buffer, pH 7]. To determine growth curves, cells were cultured in 20 mL of YPG liquid medium (1% yeast extract, 1% polypeptone and 1% glucose) in a 100-mL Erlenmeyer flask at 25 °C with reciprocal shaking at 100 r.p.m. The OD660 nm was measured using a UVmini-1240 spectrophotometer (Shimadzu, Kyoto, Japan) in a time-course experiment with a starting OD660 nm of 0.5. The doubling times of exponential-phase cells in liquid culture were determined by calculating the time required to double the OD660 nm. DNA of exponential-phase cells (25 °C, 100 r.p.m. shake culture) was quantitatively stained with propidium iodide (PI staining) as described in (Takeo et al., 1995), and then the DNA content was examined in each cell with regard to cell morphology using a laser-scanning cytometer, Olympus model LSC 101, as described in Ohkusu et al. (2001a). Cells having 1C (0.8–1.2), 2C (1.8–2.2) or 1.4–1.6 DNA content were photographed and photos were printed. Then, the sizes of each group of the cells were measured using a magnifier of ×10 with a scale. The volume of the cells was calculated based on their cell shapes whether ellipsoidal or spherical. Laser-scanning cytometry, aside from analyzing DNA quantity en masse, allowed us to determine DNA quantity in each cell located in the center of the microscopic field because the system records morphological images and allows them to be recalled so that each particular cell that was measured for DNA quantity can be examined more closely.

Cell wall chitin was stained with calcofluor and observed under a fluorescence microscope as described in (Yoshida et al., 2001). The morphology of the cells was examined under phase-contrast microscope Olympus model BX60 and photos were taken at direct magnification of ×100 by a digital camera model DP 11. To measure the size of the cells, photos were printed at a final magnification of X3.300. Calcofluor-stained cells were also examined using an LSM5 Exciter Laser Scanning Microscope (Carl Zeiss) to clarify septum formation in mutant cells. To test the mating capacity of cln1Δ mutants, they were crossed with B-3501 mating type a cells on Hay Cube Agar and grown for 1 week at 25 °C.

Results

Cloning and sequence analysis of genes coding for putative cyclin homologues

Six cyclin candidate ORFs were chosen from several sequence loci found to contain cyclin sequence similarities (See Materials and methods). Primers for RT-PCR were constructed around the ORFs. Upon obtaining RT-PCR products of the expected size, primer pairs that successfully amplified the target regions were then used as gene-specific primers in 5' and 3' RACE, respectively, to amplify the terminal regions of the entire cDNA. After the entire cDNA sequence was obtained from the assembled RT-PCR and RACE products' sequence data, the gene structure was determined by alignment with the genomic sequence to identify the location of the introns within the gene. Two to five introns were identified for the ORFs analyzed and most of them contained the typical 5' and 3' intron splice site sequences (5'-GTNNGY ... YAG-3') (Edman & Kwon-Chung, 1990).

The longest ORF within the entire cDNA of each cyclin candidate was identified using the GENETYX-WIN software, revealing the putative start and stop codons. For ORF 17, it is interesting to note that there were consistently two 5' RACE products of different sizes obtained with the same 5' end gene-specific primer (data not shown). Analysis of the assembled cDNA sequences, however, yielded only a single ORF occurring within the shorter transcript. Similarity search by TBLASTP in the NCBI protein database showed that putative amino acid translations from all the ORFs of the cyclin candidates carry significant similarities to cyclin domains such as the cyclin box regions, cyclin N-terminal and C-terminal domains and protein-binding domain characteristics of cyclins involved in cell cycle and transcription control (Kobayashi et al., 1992; Lees & Harlow, 1993; Gibson et al., 1994; Brown et al., 1995). Likewise, comparison with
Fig. 1. (a) This figure shows a comparison of the G1 cyclins of fungi. The sequences are aligned on their cyclin box regions (gray box); putative PEST regions are indicated as dotted boxes. Percentage values inside the box show the sequence identity with Cln1 cyclin box. ‘aa’ shows the amino acid length and percentage values representing the overall sequence identity with Cln1 are shown in parentheses. Putative G1 cyclin sequences of *Fusarium graminearum*, *Magnaporthe griseae*, *Histoplasma capsulatum*, *Neurospora crassa*, *Coprinus cinereus*, *Stagonospora nodorum*, *Uncinocarpus reesi*, *Sclerotinia sclerotiorum* and *Coccidioides imitis* were obtained from the Fungal Genome Initiative of the Broad Institute, MIT (http://www.broad.mit.edu/annotation/fgi/). (b) This figure shows an alignment of the cyclin boxes of certain G1 cyclins from fungi. α, residues that are highly conserved in cyclins; ▲, residues that distinguish the G1 cyclins; |, highly conserved residues that participate in Cdk interaction; ◦, conserved residues of the HPD. Numbers indicate the residues encompassing the cyclin box domain. The five bars above the sequence show the postulated positions of the five α helices of the cyclin box based on structural modeling in *Saccharomyces cerevisiae* G1 cyclins Cln2 and Cln3 (Huang et al., 1997; Miller et al., 2005).
amino acid sequences encoded by reported cyclin genes revealed that ORF34 and ORF56 could be grouped among the B-type cyclins. On the other hand, ORF 1516 and ORF 89 were grouped among the Pho cyclins, while ORF 17 was grouped among the G1 cyclins. Based on similarities, ORF34 and ORF56 were named CLB1 and CLB2, respectively, and ORF17, CLN1. For ORF1011, the 5′ RACE PCR product was not successfully obtained despite repeated attempts, and so it was not included in further analysis. Further characterization of the candidate homologues other than the G1 cyclin will be reported in a separate paper.

A search for upstream transcription regulatory motifs showed that Cln1 has some possible SCB, MCB and ECB elements, although no exact matches for these motifs were found other than for MCB (data not shown). These motifs are implicated in the transcriptional regulation of G1/S transition in S. cerevisiae via the SBF and MBF transcription regulatory network (Wittenberg & Reed, 2005). A similar transcriptional regulatory mechanism may be present in C. neoformans as these mechanisms were also found to be conserved in other eukaryotes (Costanzo et al., 2004). Cln1 also contains a PEST sequence (score 3.4) in the C-terminal region (Fig. 1a) (Berset et al., 2002). Sequence analysis showed that CLN1 encodes a putative protein of 428 amino acids and has two introns (GenBank accession number AY340660).

Because this work was started when the C. neoformans genome database was not yet completed, an exhaustive similarity search was performed again after the completion of the genome sequence (Loftus et al., 2005). Although additional cyclin candidates with similarities to cyclins of Pho85, Kin28, Ssn3 and CTDK1 were found, no candidates for homologues of CDK1 cyclins were found other than CLB1, CLB2 and CLN1. This suggests that C. neoformans has only three putative CDK1 cyclin candidates, namely, a single G1 cyclin and two B-type cyclins that might be involved in S/G2/M phases.

As shown in Fig. 1b, Cln1 possesses most of the conserved residues within the cyclin box, namely, those that are highly conserved among cyclins in general (Gibson et al., 1994; Brown et al., 1995), those that are involved in CDK interaction (Kobayashi et al., 1992; Lees & Harlow, 1993), residues within the hydrophobic patch domain (HPD) (Miller et al., 2005) and those that distinguish G1 cyclins (Forsburg & Nurse, 1991; Huang et al., 1997).

The alignment of the cyclin box (Noble et al., 1997) regions of the G1 cyclins revealed that the G1 cyclins can be grouped into two classes based on the presence or absence of an extra 20–40 amino acid region in the cyclin box (Castillo-Lluva & Perez-Martin, 2005). Cryptococcus neoformans Cln1 is grouped together with S. cerevisiae Cln3, Schizosaccharomyces pombe Puc1 and the C. albicans Cln1 and Cln3, and other cyclins from filamentous fungi, those lacking the aforementioned extra amino acid stretch (Fig. 1b). By comparing additional putative cyclin sequences of fungi from the ongoing genome database projects, it became apparent that the G1 cyclin class containing the additional 20–40 amino acid region in the cyclin box is unique in Saccharomyces and Candida. The other fungal G1 cyclins, including C. neoformans Cln1, did not contain the extra amino acid region in the cyclin box. These fungi were also found to have only a single G1 cyclin homologue. This particular insertion, which occurs at the spacer region between α helices 3 and 4 of the N-terminal cyclin fold, lies away from the conserved surface regions identified by comparative structure and sequence analysis and can easily be accommodated in a surface loop (Brown et al., 1995).

Complementation in S. cerevisiae

Cryptococcus neoformans CLN1 was cloned in the yeast expression vector pYES2 and tested for complementation of an S. cerevisiae G1 cyclin cln1, cln2 and cln3 triple-mutant strain. Cells transformed with the pYES2–CLN1 construct grew in a medium with methionine and galactose, where the expression of S. cerevisiae CLN2 was suppressed and the expression of C. neoformans CLN1 was induced (Fig. 2). Cells cultured in glucose, where the expression of C. neoformans CLN1 from pYES2 was suppressed did not grow. This indicates that C. neoformans CLN1 can replace S. cerevisiae G1 cyclin functions. On the other hand, the B-type cyclins, i.e., CLB1 and CLB2 and the other two Pho-cyclin-like sequences did not complement the S. cerevisiae G1 cyclin triple mutation (data not shown). Whereas Cln1 was found to have G1 cyclin function, the other putative cyclins may not operate as CDK1-related G1 cyclins. However, their roles as G1 cyclins cannot be definitely ruled out at this point.

Characteristics of CLN1 deletion mutants

The cln1Δ mutant cells devoid of CDK1-related G1 cyclin were viable. Furthermore, the doubling time as measured by OD660nm was 2.8 h, only a 10% increase from the wild-type B-4500 strain. Ninety percent of the cells did not show gross morphological abnormality, except for a larger cell size, and were increasing in cell number at a normal rate. However, defects gradually became severe, and eventually a smaller proportion of cells arrested (5% of cells), judging from their very severe morphological aberration. In the next cycle, the proportion of these cells is expected to be halved because of the multiplication defect. However, new severely affected cells arose from cells having more or less normal growth, reaching a steady state during the exponential phase.

Ovoid yeast cells are known to result from the initial apical growth phase, followed by the isotropic phase (Lew et al., 1997). The initial apical growth phase in the wild-type C. neoformans was very short and observed as a spike-like
protrusion (Fig. 3a, arrow), which is believed to represent the structure driving apical polar growth. Another important structure appearing initially was the neck between the mother and the bud with a width of around 1.2 μm (Figs 3 and 4). Soon after this, the isotropic growth phase was initiated and buds enlarged to take on an almost spherical shape (Fig. 3a, asterisks). The isotropic growth phase of bud continued until the end of bud formation when the cells divided (Fig. 3a). The neck between the mother and the bud was stained with calcofluor clearly, but not in the other parts of the bud (Fig. 3c). The neck stained brilliantly until the mother and the bud were separated at this part. Even after separation, this part was stained clearly as bud or birth scars (Fig. 3c). The width of the neck remained small even until the mother and the bud parts divided (Fig. 4a).

In deletion mutants, the typical spike-like structures found in the wild type that represent the initial buds were not observed, but atypical, much larger, initial bud structures were sometimes observed (Fig. 3b, arrowhead). We presumed that these also represent apical growth-driving structures despite the gross morphological alterations. In most cases, the sizes of the smallest buds observable were almost spherical and larger than those of the wild type (Fig. 3b, asterisk).

The neck in the deletion mutants widened more than double to 2.6 μm on the average compared with that of the wild type of around 1.2 μm (Figs 3 and 4a). The size distribution of the neck width also increased considerably in the deletion mutants, compared with the narrow distribution of the wild type (Fig. 4a). Similar to the wild type, the neck stained only faintly until the cells started to generate the septum at the neck.

To examine whether the increase in neck width results in serious defects in cell division, exponential-phase cells of the deletion mutant were stained with calcofluor and the number of single and attached budded and unbudded cells was counted. Eighty percent of the cells were found to show a delay of cell separation after cytokinesis within one cell cycle of the wild type (data not shown). Thus, the delay of cell separation after cell division was not serious.

In mutant cells having a neck width between 0.5 and 0.7 of the shorter diameter of the mother cell, a septum-like cell wall was often formed and cells often remained attached (Fig. 3d, arrowhead) for more than one cell cycle. Occasionally, large cell aggregates were observed. This made it easy to trace the increase in cell volume and order of birth (data not shown). It was observed that: (1) cells became much larger after three to five generations and (2) large cell aggregates showed the pseudomycelial type of multiplication, i.e., mother cells produce the next bud after the daughter cells bud (Yokoyama & Takeo, 1983), indicating that large mother cells became less reproductive after three to five generations.

Increase of the neck width, reaching > 70% of the shorter diameter of the mother, was found to be the border where cells exhibited drastic alterations in division. Namely, a few cells (4%) failed to complete this wall (Fig. 3d, upper arrow), resulting in seemingly elongated cells. However, the shape of each of the cells was always almost spherical, judging from the fact that cells that appeared elongated at a lower magnification were actually composed of a few cells whose division and cytokinesis were incomplete (see arrows in Fig. 3d). 3D imaging of cells sometimes revealed incomplete division wall formation, from seemingly complete division walls when only observed under a usual fluorescence microscope (data not shown). This made it difficult to estimate exactly the proportion of the cytokinesis defect, but it may be around 5%.

To summarize, the chIA mutant cells were found to be viable, but with an altered and larger shape than the wild type. The initial apical growth phase was altered, wherein the smallest observable buds were almost spherical and

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**Fig. 2.** This figure shows the results of complementation of *Saccharomyces cerevisiae* G1 cyclin mutation by CLN1. Here, it is shown that cells transformed with the pYES2-ORF17 (T) construct, but not those transformed with the empty pYES2 vector (V), can grow in the presence of methionine (where endogenous CLN2 expression is repressed) and only when supplied with galactose instead of glucose (wherein the expression of ORF17 from pYES2 is induced).
larger than the wild type. The size range of the neck between
the mother and the bud was increased, but the delay in cell
separation was not very severe. The proportion of cells that
exhibited severe defects in morphology and cytokinesis
eventually arrested was at around 5%.

To confirm whether the phenotypic changes exhibited by
the cln1Δ mutant were actually due to the absence of Cln1, a
functional copy of the gene was reconstituted back to the
deletion mutant (Fig. S1). Consequently, the reconstitution
strain was found to restore the morphological features of the

Fig. 3. Phase-contrast micrographs of (a) wild
type and (b) cln1Δ mutant. Wild-type cells show
spike-like initial bud protrusions [arrow in (a)] and
a small spherical bud [asterisk in (a)].
Morphologically altered initial bud protrusion
[arrowhead in (b)]. The smallest spherical bud
discerned [asterisk in (b)] was also enlarged much
wider than the wild type. In wild-type, necks are
narrow [arrow in (c)], and the buds are separated
from the mother. In cln1Δ mutant, necks are
wide. To show further abnormal morphology in
cln1Δ mutant, a seemingly elongated cell is
shown in upper right of (d). It is actually
composed of three cells, but due to the existence
of a very wide septum [lower arrow in (d)] and
an incomplete septum [upper arrow in (d)], cells
remained attached to each other. Scale
bar = 5 μm. Fluorescence images of
calcofluor-stained wild type (c) and deletion
mutant cells (d). Scale bar = 10 μm.

Fig. 4. (a) Neck width of the cln1Δ mutant (O) and the wild type (△) against the width of daughter cells. Both the average neck width and deviation
from average become much wider in the deletion mutant. Increase of the neck width with the growth of the daughter was small if at all. (b) In the wild-
type cells (△), the volume of the mother cell and neck width had a narrow size distribution. On the other hand, in the cln1Δ mutant (O), the variation of
volume of mother cell was considerably increased and the neck width became larger as the variation in size increased. The neck width did not correlate
strongly with the volume of the mother cell.
wild type (data not shown); thus, all the changes observed can be attributed to the loss of Cln1 function.

To determine whether Cln1 is involved in the commitment to the initiation of DNA synthesis, both the cell size and DNA content of individual cells were measured using a laser-scanning cytometer, Olympus model LSC 101 (Figs 5 and 6). Using this method, we were able to obtain both DNA and morphological data for all the cells analyzed. In contrast, simple FACS only yield cumulative data of cells analyzed. Thus, proper application of laser-scanning microscopy allowed us to examine and analyze cells without the need for arrest or synchronization experiments. Results showed that exponential-phase cells lacking Cln1 had more cells having 2C DNA content compared with the wild type (Fig. 5a). The sizes of the cells having 1C DNA content in the wild-type strain were ranked in increasing order (thick solid line in Fig. 5b). Cells having 1C DNA content indicate that these cells were mostly in the G1 state. Also, the size of the cells having 1C DNA content in the deletion mutant was measured and ranked in increasing order (thick solid line in Fig. 5b). The median volume of G1 cells in the wild type was 18 μm$^3$. The G1 cell volume of deletion mutants ranked in the first one-fiftieth exceeded the median volume of the wild-type strain of G1 cells (compare the thin solid line with the thick solid line in Fig. 5b). Furthermore, the G1 cell volume of deletion mutants ranked in the first one-tenth exceeded the median volume of the wild-type G2 cells (compare the thick solid line with the dotted line in Fig. 5b). In conclusion, the timing of the initiation of DNA synthesis was shown to be clearly delayed in the deletion mutants.

Likewise, the same thing was done to determine whether Cln1 is involved in the commitment to the initiation of bud emergence. The sizes of the cells having 2C DNA content in the wild-type strain were ranked in increasing order (dotted line in Fig. 5b). In the wild type, 2C cells, as expected, usually had buds (see also Fig. 6a). The size of the cells having 2C DNA content in the deletion mutant was also measured and ranked in increasing order (dashed line in Fig. 5b). The median volume of G2 cells in the wild type was 34 μm$^3$. The G2 cell volume of deletion mutants ranked in the first one-thirtieth exceeded the median volume of the wild-type G2 cells (compare the dashed line with the dotted line in Fig. 5b). Furthermore, 2C cells were mostly without or new buds as shown in Fig. 6a. These results show that the timing of initiation of bud emergence was shown to be extensively delayed in deletion mutants.

Both in wild type and in deletion mutants, G1 cells never had small or new buds (Fig. 6a). The wild-type G2 cells were
almost always budded as shown in Fig. 6a. This is a natural result because in wild-type cells, the bud emerges in the S phase during the exponential phase (Ohkusu et al., 2001a). If we assume that G2 cells are also budded in the deletion mutants, then the average size when cells initiate budding had increased more than double. Furthermore, more than two-thirds of G2 cells in deletion mutants were in fact not budded (Fig. 6a). These results show that the delay in the timing of bud emergence in the deletion mutant was much more severe than the delay in the timing of DNA synthesis. Also, the size distribution of deletion mutant cells having buds increased considerably compared with the wild type (Fig. 6b).

It was reported that the closely related basidiomycetous yeast Ustilago maydis cln1 mutant was found to have a mating defect (Castillo-Lluva & Perez-Martin, 2005), and so we tested the mating capacity of cln1Δ mutants by crossing with B-3501 mating type a cells on Hay Cube Agar and growing for 1 week at 25°C. The results of the mating test showed that Cln1 is not required for mating in C. neoformans, because its absence still allowed the mating and formation of basidiospores (Fig. 7). Furthermore, it was found that the mating ability of the mutant was found to become more pronounced in the absence of Cln1 (data not shown).

**Discussion**

The present paper shows evidence that C. neoformans has only a single Cdk1-related G1 cyclin, although all the ascomycetous budding yeasts reported so far are known to...
have multiple Cdk1 G1 cyclins (Sherlock et al., 1994; Futcher, 1996). *Cryptococcus neoformans* was found to complete the cell cycle without this Cdk1-G1 cyclin with mild morphological defects, compared with G1 cyclin-deletion mutants in other species (Richardson et al., 1989; Moffat & Andrews, 2004; Castillo-Lluva & Perez-Martin, 2005). This should be closely related to the physiological roles it plays.

The G1 cyclins of budding yeasts are generally presumed to play vital roles in (1) the commitment to the initiation of budding, (2) the commitment to the initiation of DNA synthesis and (3) budding itself (Lew et al., 1992; Kuntzel et al., 1994; Dirick et al., 1995). We show that these are also true in *C. neoformans*.

Measurement of the DNA content of the *cln1*Δ mutant showed that the timing of DNA synthesis was delayed, but not blocked in the absence of this cyclin. This result coincides well with the general roles cyclins play. Our observation that the *cln1*Δ cells were only delayed, but not blocked in DNA synthesis suggests that in *C. neoformans*, B-type or other cyclins can substitute the G1 cyclin in the initiation of the S phase. Despite the cytokinesis defects observed for *cln1*Δ mutants, there were no observable changes in ploidy associated with the increase in cell size (Fig. 5a).

Analysis of *cln1*Δ mutant growth characteristics showed that the commitment to budding is also directed by Cln1 as expected. Cell size distribution in the wild type was narrow and relatively small during the exponential phase (Figs 3a, c, 4b, 6a and b). In the deletion mutant, the cell size not only became larger, but the cell size distribution also became much wider (Figs 3b, d, 4b, 6a and b). Thus, commitment to the initiation of DNA synthesis and budding are directed by Cln1. Furthermore, our results clearly showed that cell size control was disrupted in the deletion mutant. In *S. cerevisiae*, it was found that a late burst of G1 cyclin activity is needed for the proper timing of initiation of morphogenetic events at Start, in particular the recruitment of Cdc24 to the bud site and the formation of a septin ring (Moffat & Andrews, 2004). Cryptococcal septin mutants have phenotypes similar to the *cln1*Δ mutant (Kozubowski & Heitman, 2010), indicating that a similar mechanism may be involved in the initiation of bud morphogenesis in *C. neoformans*. The absence of Cln1 could lead to delays in division initiation, resulting in a larger cell size and extension of the growth phase. G1 cyclin activity is absolutely needed for bud emergence in *S. cerevisiae*. When both Cln1 and Cln2 activities are compromised, Pcl1 or Pcl2 plays roles as G1 cyclins in concert with Cdk5 (Pho85) (Moffat & Andrews, 2004; Huang et al., 2007). The fact that the *C. neoformans* *cln1*Δ mutant is viable in spite of several abnormalities as shown in Results prompted us to search for possible Pho85 G1 cyclin candidates and identified two homologues. Deletion of both genes did not significantly alter the yeast

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**Fig. 7.** Mating test of the *cln1*Δ mutant. Cells were grown in a Hay Cube Agar at 25°C for 1 week. (a) *cln1*Δ mutant grown singly showing filamentous growth from the colony, but no whitish spores present as confirmed by microscopy (b). Arrow shows clamp-connection-like structures. (c) *cln1*Δ mutant mated with a B3501 mating type a tester strain showing whitish hyphae-producing spores. Basidia visible in (d). Scale bar in (a, c) = 0.5 cm. Scale bar in (b, d) = 25 μm.
Molecular mechanisms of bud formation have been studied extensively in *S. cerevisiae* (Lew & Reed, 1993; Madden & Snyder, 1998; Moffat & Andrews, 2004; Huang et al., 2007). G1 cyclins guide the initial apical growth stages of budding, but G2 cyclins in concert with Cdk1 destroy the structure that drives apical growth, resulting in isotropic enlargement of the bud. G1 cyclins also play vital roles in the formation of the functional neck between the mother and the daughter cells, which restricts the movement of plasma membrane proteins and restricts growth in the bud. At division, the functional neck also directs cell wall material deposition in this region. In the following discussion, we presume that the *S. cerevisiae* model of cell wall formation is also applicable to the mitotic cell cycle of *C. neoformans*.

The shape of *C. neoformans* wild-type cells is actually one of the extremes among yeast species, being almost spherical in shape (Figs 3a, c and 6a). As such, because the apical growth stage of bud enlargement was very short as shown in Results, the G1 cyclin deletion mutant was not severely affected in the apical growth phase, except for the functional neck and initial bud formation. Most cells in the *cln1*Δ mutant appeared to have succeeded in traversing this very short apical growth phase. This process could be guided by Pho85-related G1 cyclins in the Pcl1/2 subfamily in concert with Pho85 CDK, which resulted in the formation of large initial bud structures and a large neck. The neck was formed at the beginning of budding, and this was shown to be directed by G1 cyclins in *S. cerevisiae* (Lew & Reed, 1995). The neck in the *cln1*Δ mutant appeared to have not completely retained the function of that of the wild-type strain, judging from the fact that several mutant cells failed to divide when grown at 32°C (data not shown). If the *C. neoformans* cells were elongated, they should have a longer apical growth phase and would probably be affected more severely by the deletion of the *CLN1* gene.

In the present paper, we showed that commitment both to the initiation of DNA synthesis and to budding is carried out by the single Cdk1-G1 cyclin, Cln1, although *C. neoformans* cells are able to enter G0 either through the G1 or the G2 phase (Takeo et al., 1995). Suppose that commitment to DNA synthesis and to budding occur in *C. neoformans* at the same time at START as in ascomycetous yeasts, then, for *C. neoformans* cells that enter G0 through the G2 phase, it would appear that commitment to budding occurs once and then is cancelled later under severe stress. This cancellation contradicts the notion of commitment. Thus, in *C. neoformans*, ‘Start’ should be separated into two events, namely, ‘Start-1DNA’ commits the cells to DNA synthesis and ‘Start-2Bud’ commits the cells to budding. Commitment to the initiation of DNA synthesis should occur before that of budding. Otherwise, cells that have once formed small buds, but enter G0 without cell division will have a weakened cell wall at the budding site. Also, to endure an unfavorable environment during dormancy, the whole cell wall including the extra budded wall should be thickened. Interestingly, during the course of our experiments, we never found budded stationary-phase cells with a 1C DNA content, suggesting that some mechanism exists that certifies the order of the commitments. The strong START in ascomycetous yeasts makes it unimportant whether commitment to initiation of DNA or that of budding occurs earlier, because budded cells do not enter G0. In *U. maydis*, the order problem is automatically solved because DNA synthesis occurs in late G1, whereas budding occurs in G2 (Snetselaar et al., 1996). The fact that *C. neoformans* has only a single Cdk1-G1 cyclin highlights the cryptic order problem that commitment to DNA synthesis should occur before that of initiation of budding. Studies to resolve this problem are currently being conducted.

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**References**


Supporting Information
Additional Supporting Information may be found in the online version of this article:

Fig. S1. Targeted reconstitution of the CLN1 deletion.
Table S1. Seventeen ORFs with cyclin sequence domains found in the preliminary annotation of the SGTC Cryptococcus neoformans genome database.

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