Hyphal Elongation Is Regulated Independently of Cell Cycle in *Candida albicans*

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Submitted March 13, 2001; Revised October 16, 2001; Accepted October 24, 2001

Monitoring Editor: John Pringle

The mechanism for apical growth during hyphal morphogenesis in *Candida albicans* is unknown. Studies from *Saccharomyces cerevisiae* indicate that cell morphogenesis may involve cell cycle regulation by cyclin-dependent kinase. To examine whether this is the mechanism for hyphal morphogenesis, the temporal appearance of different spindle pole body and spindle structures, the cell cycle-regulated rearrangements of the actin cytoskeleton, and the phosphorylation state of the conserved Tyr19 of Cdc28 during the cell cycle were compared and found to be similar between yeast and serum-induced hyphal apical cells. These data suggest that hyphal elongation is not mediated by altering cell cycle progression or through phosphorylation of Tyr19 of Cdc28. We have also shown that germ tubes can evaginate before spindle pole body duplication, chitin ring formation, and DNA replication. Similarly, tip-associated actin polarization in each hypha occurs before the events of the G₁/S transition and persists throughout the cell cycle, whereas cell cycle-regulated actin assemblies come and go. We have also shown that cells in phases other than G₁ can be induced to form hyphae. Hyphae induced from G₁ cells have no constrictions, and the first chitin ring is positioned in the germ tube at various distances from the base. Hyphae induced from budded cells have a constriction and a chitin ring at the bud neck, beyond which the hyphae continue to elongate with no further constrictions. Our data suggest that hyphal elongation and cell cycle morphogenesis programs are uncoupled, and each contributes to different aspects of cell morphogenesis.

INTRODUCTION

Candida albicans is a polymorphic fungal pathogen that undergoes reversible morphogenetic transitions among budding, pseudohyphal, and hyphal growth forms (Odds, 1985). Its ability to switch between yeast and hyphal growth forms is directly related to its virulence, because mutants defective in hyphal growth are less virulent in mouse models than are their wild-type counterparts (Leberer *et al.*, 1997; Lo *et al.*, 1997; Gale *et al.*, 1998). Hyphae may be suited to breach barriers in the host, whereas the yeast form is more easily disseminated within the host. Therefore, understanding the mechanisms for this morphogenetic switch should provide insight into the pathogenicity of this fungus.

During hyphal growth in *C. albicans*, cell surface expansion is restricted to a small region at the hyphal tip. This apical growth zone is active during the entire hyphal growth

period (Staebell and Soll, 1985). In contrast, yeast-form cells expand from a small area in a mostly apical manner only at the initial stage of budding. When the bud has reached a critical size, apical growth shuts down and general (isotropic) expansion takes place (Staebell and Soll, 1985). The localization of the actin cytoskeleton in yeast and hyphal cells reflects these differences in morphogenesis. Polarization of the actin cytoskeleton to the hyphal tip is observed in all hyphal cells (Anderson and Soll, 1986). However, in yeast-form cells, the cortical actin patches are observed at the area of apical expansion only in small budded cells but not in large budded cells (Anderson and Soll, 1986). It has been suggested that the actin cytoskeleton is essential for polarized apical growth because chloropropham, a drug affecting actin microfilament organization, has been shown to inhibit hyphal growth (Yokoyama *et al.*, 1990, 1994). The mechanism for polarization of the actin cytoskeleton to the hyphal tips remains unknown, partially because *C. albicans* is an obligatory diploid with no sexual cycle, a fact that has hindered genetic studies in this organism.

Several lines of evidence from *Saccharomyces cerevisiae* indicate that cell morphogenesis and change in actin organization may be regulated by cyclin-dependent kinase (CDK) (Lew and Reed, 1993; Johnson, 1999). Increasing the levels of G₁ cyclins promotes apical growth, whereas increasing the

Article published online ahead of print. Mol. Biol. Cell 10.1091/mbc.01-03-0116. Article and publication date are at www.molbiol-cell.org/cgi/doi/10.1091/mbc.01-03-0116.

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Abbreviations used: DSPB, duplicated spindle pole body; GFP, green fluorescence protein; MS, mitotic spindle; SPB, spindle pole body; SS, short spindle.

levels of B-type cyclins leads to isotropic growth (Lew and Reed, 1993). Moreover, a delay in the activation of B-type cyclin/CDK activity, for any number of reasons, causes cell elongation. Based on this, Lew and Reed (1995) have proposed a model in which cyclin-CDK activities control apical and isotropic growth. They further hypothesized that cell elongation during pseudohyphal growth in *S. cerevisiae* might be caused by a delay in the apical-isotropic switch (Lew and Reed, 1993, 1995). In agreement with this, a *grr1* mutant, which has stable G₁ cyclins, has enhanced pseudohyphal growth (Barral *et al.*, 1995; Blacketer *et al.*, 1995), as do strains mutated for B-type cyclins (Lew and Reed, 1993; Ahn *et al.*, 1999). Furthermore, pseudohyphal *S. cerevisiae* cells exhibit symmetric cell division and have a longer G₂ phase than do yeast-form cells (Kron *et al.*, 1994).

In *S. cerevisiae*, activation of the morphogenesis checkpoint promotes cell elongation (Lew, 2000). This pathway, consisting of several protein kinases, can sense defects in the actin cytoskeleton and/or in the septin ring structure and up-regulate the Swe1 kinase (McMillan *et al.*, 1998; Barral *et al.*, 1999; McMillan *et al.*, 1999). In turn, Swe1 phosphorylates Cdc28 at Tyr19 and delays the activation of Cdc28/B-type cyclin and the cell cycle transition from G₂ to M (Sia *et al.*, 1996). This delay leads to cell elongation, and it has recently been proposed that the delay is a potential mechanism for regulating pseudohyphal growth in *S. cerevisiae* (Edgington *et al.*, 1999).

To explore the mechanisms for the regulation of cell polarity during hyphal development in *C. albicans*, we set out to examine whether regulation of the cell cycle is involved in hyphal elongation. Our results indicate that unlike the models proposed for *S. cerevisiae* pseudohyphal growth, hyphal elongation in *C. albicans* is not regulated primarily by cell cycle controls.

MATERIALS AND METHODS

Plasmid and Strain Construction

A *C. albicans* green fluorescence protein (GFP) integration plasmid, pHL471, was constructed by cloning a HindIII-PstI GFP fragment from pYGF3 (Cormack *et al.*, 1997) into the HindIII-PstI site of plasmid pHL159, which contains the *CaURA3* gene at the XbaI-BamHI sites of pBluescript SK (Liu *et al.*, 1994). The DNA sequence preceding the GFP in the polylinker of pHL471 is GGTACCGGGCCCCCTCGAGGTCGACGGTATCGATAAGC TTT ATT AAA **ATG**. The underlined region is from pYGF3, and the ATG in bold is the start codon for GFP. Polymerase chain reaction (PCR) primers p130-5'CGGGGTACCAACATTATAGAACTATGATGAGAG and p131-5'CCGGGTACCATCATGGCGGCATCTTCTAATCGGG were used to amplify *CaTUB2*, by using genomic DNA as template. The PCR fragment was cloned into the unique KpnI site of pHL471 by using the sites (underlined) included in the primers, resulting in plasmid pHL472, in which the *CaTUB2* was fused in frame to GFP. pHL472 was digested with BspMI (a unique site in *CaTUB2*) and transformed into *C. albicans* CAI4 (Fonzi and Irwin, 1993) for integration at *TUB2*. Each Ura⁺ transformant analyzed contained a functional *TUB2*-GFP fusion based on the characteristic appearance of microtubule spindles. These strains grew at a slightly slower rate than wild-type strains. One of the *TUB2*-GFP strains, HLY1541 was used in this study. Wild-type *C. albicans* strain SC5314 (Fonzi and Irwin, 1993), the progenitor of CAI4, was also used.

Cell Cultures

Routine culture of *C. albicans* was performed essentially as described for *S. cerevisiae* (Sherman, 1991). Cells were grown to log phase or to saturation in YPD medium at 30°C then diluted into YPD-based yeast-inducing (YPD at 30°C) and hyphal-inducing medium (YPD + 10% newborn calf serum [Sigma, St. Louis, MO] at 37°C), or into Lee's (Lee *et al.*, 1975; Buffo *et al.*, 1984) yeast-inducing (30°C, pH 4.5) or hyphal-inducing (37°C, pH 7.0) medium with 1% mannitol (Loeb *et al.*, 1999b).

Cell Synchronization

Cell synchronization was performed as previously described (Loeb *et al.*, 1999b). Unbudded G₁ cells were released into YPD-based yeast and hyphal-inducing media, and aliquots of cells were taken for direct visualization by microscope, fixed in formaldehyde for actin staining or in ethanol for DNA staining, or frozen in liquid nitrogen for biochemical studies.

Fluorescence-activated Cell Sorting (FACS) Analysis

Synchronous cells were fixed in ethanol, washed with Tris buffer (0.2 M Tris-HCl pH 7.5), sonicated, and then incubated overnight at 37°C in Tris buffer containing 1 mg/ml RNase. The cells were then stained with Tris buffer containing 0.05 mg/ml propidium iodide (Sigma) for 15 min on ice, washed with Tris buffer, and resuspended in Tris buffer containing 0.01 mg/ml propidium iodide. Flow cytometric analysis was performed using a Becton Dickinson FACScan fluorescence system equipped with Cell Quest acquisition and analysis software. Analysis was done on a collection of 10,000 gated events.

Kinase Assays and Western Analysis

Crude protein extracts were prepared as described previously (Surana *et al.*, 1993) by using buffer A (Wu and Russell, 1997) without pepstatin. The affinity precipitation of Cdc28 was done by incubating 20 µl of p13^{SUC1}-Sepharose beads (Calbiochem, San Diego, CA) with total protein extracts (50–150 µg) in buffer A for 2 h at 4°C with gentle agitation. Suc1 is known to bind to CDKs with high affinity (Ducommun and Beach, 1990). Kinase assays were performed essentially as described by Surana *et al.* (1993). Western analysis was performed on total protein extracts or the Suc1 precipitate (50–100 µg), separated on 12.5% SDS-PAGE. A 1:250 dilution of PSTAIRE primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) in phosphate-buffered saline (PBS) + 0.05% Tween + 5% dry milk was used. Tyr19 was visualized with a phospho-cdc2 (Tyr15) antibody (New England Biolabs, Beverly, MA). Membranes were blocked with TBS + 0.1% Tween (TTBS) + 5% dry milk overnight and incubated with a 1:1000 dilution of the phospho-cdc2 (Tyr15) antibody in TTBS + 5% bovine serum albumin overnight. Anti-rabbit-Ig-horseradish peroxidase (Amersham Biosciences, Piscataway, NJ) was used as a secondary antibody at 1:1000 in TTBS + 5% dry milk. All Western analyses were visualized using enhanced chemiluminescence (Amersham Biosciences).

Microscopy

A Zeiss Axioplan2 microscope with a 100× objective and a digital camera (Sensys Photometrics, Tucson, AZ) were used for all microscopy. The temperature of the microscope stage was maintained at 37°C during time-lapse microscopy by Airtherm (World Precision Instruments, New Haven, CT). Visualization of β-tubulin-GFP was performed on cells in growth media or after resuspension in water. To stain DNA of live *C. albicans* cells, cultures were grown in the presence of 1 µg/ml 4'-6-diamidino-2-phenylindole (DAPI) for at least 16 h (40 h gave better results) then diluted into fresh medium containing 1 µg/ml DAPI and photographed directly in the medium. Calcofluor staining of the chitin ring was done as previously

described (Loeb *et al.*, 1999b). Rhodamine-phalloidin staining of actin was performed based on the protocol described by Adams and Pringle (1991). Cells were spun and then resuspended in 3.7% formaldehyde in PBS for 1 h, washed with PBS, and incubated with buffer B (100 mM sodium phosphate pH 7.4, with 1.2 M sorbitol) containing 2 μ l/ml β -mercaptoethanol for 20 min. Cells were then stained with 1 μ g/ml rhodamine-phalloidin (Sigma) or 1:20 dilution of Alexa488-phalloidin (Molecular Probes, Eugene, OR) in buffer B for 30–45 min in the dark, washed, and resuspended in freshly made mounting medium (1 $\times$ PBS, pH 9 with 1 mg/ml *o*-phenylenediamine) containing a 1:50,000 dilution of 1 mg/ml DAPI. For double labeling of chitin and actin, cells were stained first with calcofluor, washed with PBS, and then stained with phalloidin as described above.

RESULTS

Hyphal Germ Tube Emergence Can Occur before Cell Cycle Events of G₁/S Transition

Using GFP-tagged tubulin, four major spindle structures were observed in *C. albicans* yeast-form cells. The structures looked very similar to those seen in *S. cerevisiae* (Kilmartin and Adams, 1984; Carminati and Stearns, 1997) and were named accordingly. A spindle pole body (SPB) was observed as a very faint spot that colocalized with the nucleus (Figure 1A, a). The SPB had astral microtubules emerging from it. A duplicated spindle pole body (DSPB) was seen as a very bright, concentrated, nuclear-associated spot (Figure 1A, b). A short spindle (SS) was seen as a very bright, nuclear-associated rod (Figure 1A, c). The nucleus of cells containing a short spindle was usually located near the mother-bud neck (Figure 1A, c, bottom), with astral microtubules from one end of the spindle oriented into the bud. A long mitotic spindle (MS) spanned much of the length of the mother and daughter cells in large budded cells (Figure 1A, d and e) and was associated with elongating or separated nuclei (Figure 1A, d and e, bottom). Mitotic spindles were fainter than short spindles.

The appearance of these structures was correlated with other cell cycle events, such as DNA replication, budding and chitin ring formation, nuclear migration, and separation (Figures 1, A and C). Elutriated cells were initially unbudded and had an SPB with 2N DNA content (Figure 1C). After 90 min of growth in YPD, DSPBs started to appear. The appearance of DSPBs coincided with budding and chitin ring formation and was followed by a shift in DNA content toward 4N at 120 min, as seen from FACS analysis in Figure 1C. Therefore, in *C. albicans* yeast-form cells, SPB duplication, bud emergence, and DNA replication all happen at the G₁/S transition as in *S. cerevisiae* (Lew and Reed, 1995).

Similar spindle structures were observed in hyphal cells (Figure 1B), although some of the SS and MS were longer than in yeast-form cells, as previously reported (Akashi *et al.*, 1994). As in yeast-form cells, the temporal appearance of these structures during the cell cycle was correlated with other cell cycle events (Figure 1, B and D). Nuclear migration started before SPB duplication (Figure 1B, b). After 90–100 min in YPD + 10% serum, DSPBs began to appear (Figures 1B, c). This coincided with the appearance of the chitin rings in germ tubes and was followed by a shift in DNA content toward 4N at 120 min (Figure 1D).

Strikingly, hyphal germ tubes emerged at ~30 min, which was ~70 min before SPB duplication, chitin ring formation,

and DNA replication in hyphal cells (Figure 1D), events that coincided with budding in yeast-form cells. Therefore, germ tube formation is not the same as budding and is not an indication of the G₁/S transition but of hyphal induction. Closer observation of differential interference contrast (DIC) and GFP-tubulin images revealed that budding cells (with a duplicated SPB) have a constriction at the mother-bud junction (Figure 1E, b), whereas hyphal cells (with an unduplicated SPB) have no constriction at the mother-germ tube junction (Figure 1E, a).

Spindle structures have been visualized previously in *C. albicans* by using antibodies to tubulin. However, no SPBs were observed in unbudded cells. Thus, it was assumed that what we interpret herein as a DSPB was the SPB and that cells spend a substantial part of the cell cycle with no microtubule-organizing center (Barton and Gull, 1988). Our observations indicate that the spindle cycle in *C. albicans* is very much like the spindle cycle in *S. cerevisiae*.

It was reported previously that *C. albicans* hyphal cells only divide from the tip, whereas subapical cells seem to be cell cycle arrested (Gow, 1997). Consistent with this, all of ~5000 unbranched subapical hyphal cells we examined had a single nucleus and an unduplicated SPB (Figure 1B, g–j), whereas the apical cells exhibited dynamic changes in spindle structures.

Progression of Cell Cycle Is Similar in Yeast and Hyphal-Apical Cells

To investigate further possible cell cycle changes during hyphal morphogenesis, we compared the percentages of the different spindle structures among asynchronous yeast and hyphal-apical cells in two hyphal-inducing conditions (Figure 2). DSPBs and SS were counted together as one category (DSPB), corresponding to the S/G₂ phase of the cell cycle. If hyphal formation is due to modulating the length of a specific phase of the cell cycle, one would expect a difference in the percentage of cells in that phase when comparing asynchronous yeast-form and hyphal-form cells. Approximately 5% more cells in S/G₂ and 5% fewer cells in G₁ were observed in the apical cells of hyphae compared with yeast-form cells. The percentages of mitotic cells were identical (Figure 2). It has been reported that *S. cerevisiae* pseudohyphal cells have a 12–15% longer G₂ phase than that of yeast-form cells (Ahn *et al.*, 1999). Considering that hyphal elongation in *C. albicans* is much more dramatic than that of pseudohyphae in *S. cerevisiae*, the statistically significant 5% change in S/G₂ length is unlikely to account for the majority of apical growth observed in hyphal cells.

In addition, synchronous elutriated GFP-tubulin cells were released into yeast- and hyphal-inducing YPD-based media, and the percentage of cells with each spindle structure was counted. As shown in Figure 3A, for both yeast-form and hyphal-apical cells, the appearance of DSPBs peaked at 170 and 270 min after release, the appearance of mitotic spindles peaked at 200 and 290 min after release, and the reappearance of unduplicated SPBs peaked at 220 min after release. This suggests that cell cycle dynamics is similar for yeast-form and hyphal-apical cells. To examine cell cycle dynamics in an alternative hyphal growth condition, we also tried to release synchronous G₁ cells into Lee's medium. However, based on the counting of tubulin structures, elutriated G₁ cells did not enter the first cell cycle in synchrony,

Figure 1. SPB and spindle morphologies in yeast-form and hyphal-form cells of *C. albicans* and their temporal correlation to DNA replication and budding/hyphal formation. Elutriated HLY1541 cells (GFP fusion of β -tubulin) were released into yeast-inducing (YPD, 30°C) and hyphal-inducing (YPD + 10% serum, 37°C) conditions in the presence of 1 μ g/ml DAPI. (A and B) Examples of unduplicated SPBs (A, a; B, a and b), DSPBs (A, b; B, c), short spindles (A, c; B, d), and mitotic spindles (A, d and e; B, e and f) in yeast-form (A) and hyphal-form (B) cells. The corresponding DAPI staining of the same cells is shown in the lower panels. Spindle structures and nuclear localization are also shown for hyphal cells in their second cell cycle. (C and D) Living cells were counted for tubulin structures and were then fixed for FACS analysis and chitin staining. Each panel shows the DNA content, percentage of each tubulin spindle structure, percentage of chitin rings, and percentage of budding/germ tube formation for yeast-form (C) and hyphal-form (D) cells. (E) Constriction at the neck of budded cell (b) but not at the emerging hypha (a).

Figure 2. SPB and spindle structure distribution in asynchronous yeast-form (black) and hyphal-apical (gray) cells. Cells (HLY1541) were transferred from a log-phase culture into YPD-based yeast- or hyphal-inducing medium (A) or into Lee's yeast medium (30°C, pH 4.5) and hyphal-inducing medium (37°C, pH 7). Approximately 2500 cells were counted for each growth condition after 2–5 h of growth in YPD-based media and 5–8 h in Lee's media. In hyphal cells, only apical cells were counted. The differences in SPB and DSPB percentages are statistically significant but small.

although germ tubes did emerge before the appearance of DSPBs, as in YPD + serum (our unpublished data).

Hyphal Tip-associated Polarization of Actin Cytoskeleton Persists, whereas Cell Cycle-modulated Actin Assemblies Appear and Disappear during Hyphal Growth

Temporal changes in the actin assemblies of yeast and hyphal cells were studied by fixing and staining cells from the experiment of Figure 3. The actin organization observed in yeast-form cells was very similar to that described in *S. cerevisiae*. These included the concentration of cortical actin patches at the future bud site (Figure 4A, b), which often persisted for some time after the bud had emerged (Figure 4A, c); cortical actin patches concentrated in the emerging bud (Figure 4A, c and d); a fainter actin ring at the neck of large budded cells (Figure 4A, e); and a congregation of cortical actin patches on both sides of the neck during septation (Figure 4A, f) (Kilmartin and Adams, 1984; Bi *et al.*, 1998). The actin assemblies observed in hyphal cells were also similar. They included a bright ring of cortical actin patches close to the tip of a growing hypha (Figure 4B, c and d). This ring appeared before and at the initiation of nuclear migration (Figure 4B, c and d, bottom), and some probably persisted to SPB duplication (Figure 3, A and B, bottom). The actin ring colocalized with the chitin ring in the germ tube at the future septum site (Figure 4C, a). The ring of cortical actin

Figure 3. Similar cell cycles in yeast-form and hyphal-apical cells, and persistence of hyphal tip-associated actin throughout the cell cycle. An experiment similar to that shown in Figure 1 was extended through to the second cell cycle, monitoring SPB and spindle structures (A) and actin localization by staining with rhodamine-phalloidin (B). (A) Percentages of unduplicated SPBs (black squares), DSPBs (dark gray triangles), and MS (light gray circles) for 100–200 of yeast-form or hyphal cells were counted every 10 min. (B) Percentages of cells with a ring of cortical actin patches at the presumed future septation site (i.e., the incipient bud site in yeast-form cells or a site close to the hyphal tip in hyphal cells; open black circles), actin ring at the septation site (i.e., neck of large budded yeast-form cells or a site in the germ tube; gray open squares) were counted (see text; Figure 4). For hyphal cells, the percentage of cells with tip-polarized actin was also counted (black dashed line).

patches disappeared later in the cell cycle (Figure 4B, e). A faint actin ring located toward the middle of the hypha (Figure 4B, f and g) appeared at nuclear separation (Figure 4B, f, bottom) coincided with the appearance of mitotic spindles (Figure 3, A and B, bottom), and also colocalized with the chitin ring (Figure 4C, b). Thus, it was defined as a mitotic actin ring. This ring is followed by the appearance of actin repolarization around a dark gap that presumably corresponds to the septum (Figures 3B, bottom; and 4B, h). Actual visualization of these actin structures was more convincing under the microscope than what we were able to capture in the single-focus-plane images. The appearance of these different actin assemblies in both yeast-form and hyphal-form cells occurred with similar dynamics (Figure 3B), which supports the notion that the timing of cell cycle in the hyphal apical cells is not altered.

In addition to these cell cycle-modulated actin assemblies, cortical actin patches and cables are persistently polarized to the hyphal tip, a localization that appeared slightly before the evagination of germ tubes (Figures 3, A and B, bottom; and 4B). Polarization of the actin cytoskeleton at the tips of hyphae was reported previously and is probably required for hyphal elongation (Anderson and Soll, 1986; Yokoyama *et al.*, 1990). We observed that the actin polarization at the hyphal tip appeared 30 min after hyphal induction in G_1 cells, ~70 min before SPB duplication, DNA replication, and chitin ring formation, and that it persisted throughout the cell cycle (Figures 3B, bottom; and 4B). Such actin polarization was not observed in the yeast cells collected at the same time interval after release (Figure 4A). The persistent polarization of cortical actin patches and actin cables to the hyphal tip in the apical cell of each hypha was in sharp contrast to the dynamic rearrangement of cell cycle-regulated actin assemblies in the same cells (Figures 3B and 5B). These observations suggest that the hypha-tip-associated actin polarization is regulated independently of the cell cycle.

Cells in Phases Other than G_1 Can Be Induced to Form Hyphae

It has been reported that there is a point of phenotypic commitment in the cell cycle for setting the mode of mycelial outgrowth: small budded cells initiate hyphae as extensions of the buds, whereas large budded cells only evaginate in the next cell cycle (Mitchell and Soll, 1979). This suggests that later stages of the cell cycle may prohibit hyphal evagination. To address this question, time-lapse microscopy was used to follow hyphal emergence from log-phase yeast-form cells. Cells with a large bud were able to form polarized evaginations, which tapered off into long hyphae without constrictions as an extension of the bud (Figure 5A). However, the bud size alone could not inform us of the exact cell cycle stages at the point of hyphal induction. To further address this issue, we examined cell shape, spindle, and SPB structure of log-phase cells that had been transferred to a hyphal-inducing medium for 30–50 min. Distinct cell shapes seemed to associate with different spindle structures as soon as hyphal elongation was visible. Hyphae from unbudded G_1 cells had a narrow evagination from a single mother cell (Figure 5B, a). S/ G_2 cells had a small, elongated bud (Figure 5B, b). Mitotic cells exhibited large, elongated buds of taper-

ing appearance or dumbbell-shaped buds (Figure 5B, c). Budded cells with elongated G_1 daughter and mother cells were also observed (Figure 5B, d). The further along the cells were in the cell cycle at the time of hyphal induction, the wider the initial hyphal evagination seemed to be (Figure 5B). Because hyphal induction should happen before observable changes in cell shape, these cells might have been in M phase or just before M phase at the time of hyphal induction (Figure 5B, d). However, in hyphal-induced large budded cells containing a mitotic actin ring, tip actin polarization was visible presumably indicating the initiation of hyphal formation. Such polarization was never observed in similar cells grown in yeast medium (Figure 5C). Our data suggest that *C. albicans* can be induced to initiate hyphal formation in later stages of the cell cycle, perhaps even in mitotic cells.

The tapering appearance of an elongated bud with DSPBs (Figure 5B, b) is reminiscent of the elongated bud caused by a G_2 delay in *S. cerevisiae*. However, both FACS analysis and spindle counting of the hyphal cells did not reveal any cell cycle shift toward G_2 (our unpublished data). Furthermore, no new constrictions appeared in the hyphae evaginated from budded cells (Figure 5A). Thus, hyphal evaginations from cells past G_1 , although reminiscent of pseudohyphal cells, are actually true hyphae growing on already existing buds. It is interesting to note that although G_1 cells randomly initiate germ tubes, as previously reported (Chaffin, 1984), all budded cells we observed grow hyphal germ tubes at the tip of the existing bud rather than from the mother.

The chitin ring is a marker for the position of the septum between a mother and daughter in hyphal cells (Soll and Mitchell, 1983). We have observed that in elutriated G_1 cells, the distance of the first chitin ring from the base of the germ tube was relatively constant (Figure 5D, a), whereas in non-elutriated G_1 cells, the distance varied greatly (Figure 5D, c). Because the elutriated G_1 cells are mostly uniform in size, whereas nonelutriated G_1 cells are varied in size, the position of the first chitin ring possibly reflects the time elapsed between hyphal induction and formation of the first chitin ring (at the G_1 /S transition), which would be expected to occur earlier in cells that were larger at the time of hyphal induction. The observed variation suggests that the cell cycle program is responsible for septum positioning and is independent of the hyphal program. In cells that were presumably already budded at the time of hyphal induction, the first chitin ring was always at the site of the constriction from the mother cell (Figure 5D, b).

Similar Dynamics of Cdc28-Tyr19 Phosphorylation and Dephosphorylation in Yeast and Hyphal Cells

In parallel to the cell biology studies, we also carried out biochemical experiments to address how CDK is regulated in yeast and hyphal cells. A *C. albicans* CDC28-like gene, whose deduced protein sequence is 79% identical to that of *S. cerevisiae* Cdc28, was identified by functional complementation in *S. cerevisiae* (Sherlock *et al.*, 1994). To assay CDK activity in *C. albicans*, p13^{Suc1}-agarose-conjugated beads were used to pull down the CDK/cyclin complex from cell extracts. H1 histone kinase activity associated with the precipitated fractions in synchronous cells showed cyclic levels during progression of the cell cycle, as expected for a CDK (Figure 6A). From total extracts, Suc1 precipitated only one of the several polypeptides recognized by an antibody

Figure 4. Two distinct actin-organization programs in hyphal cells: dynamic rearrangement of cell cycle-modulated actin and persistent polarization of hypha-tip-associated actin. An experiment similar to that of Figure 1 was carried out. Cells (HLY1541) were fixed and stained with rhodamine-phalloidin (A) or Alexa488 phalloidin and DAPI (B). (A) Actin organization observed in yeast-form cells: even distribution of actin cortical patches on cell cortex (a); polarized actin cortical patches at the presumptive bud site (b); a ring of actin cortical patches at the future septum (c); actin cortical patches filling the emerging bud (d); a potential actin contractile ring (e); repolarization of the actin cortical patches to the mother bud neck (f). (B) Actin organization observed in hyphal cells (top) and the corresponding DNA localization (bottom): polarization of actin cortical patches and filaments at the tip of hyphae before the G_1/S transition (a and b), persistent polarization at the

against PSTAIRE (Figure 6B, lanes 1 and 2). A phospho-specific antibody against human Cdc2-P-Tyr15 peptide recognized one major band in the total protein extract of *C. albicans* (Figure 6B, lane 4). This same polypeptide was also pulled down by Suc1 beads (Figure 6B, lane 3) and migrated, as a 34-kDa polypeptide, identical to the PSTAIRE-recognized polypeptide in the Suc1 precipitate (Figure 6B, lane 1). A search of the recently completed *C. albicans* genome sequence by using the CaCdc28 sequence indicates that there are no other kinases that are of the exact same size as CaCdc28 with a high degree of identity at both the conserved Tyr19 and PSTAIRE regions. Thus, it is likely that Cdc28 is the only CDK in *C. albicans*, and the Suc1-associated, anti-Cdc2-P-Tyr15 and anti-PSTAIRE-recognized 34-kDa protein is likely to be the *C. albicans* CDK, Cdc28. Western analysis of total protein extracts indicates that the phosphopeptide antibody can recognize the CaCdc28 only from active growing cells but not from stationary phase cells (Figure 6C), which is consistent with the phosphospecificity of the Cdc2-P-Tyr15 antibody if the phosphopeptide antibody recognizes only the Tyr19-phosphorylated form of Cdc28 from growing *C. albicans* cells.

Phosphorylation at Tyr19 of Cdc28 by Swe1p has been shown to increase cell elongation and filamentation in *S. cerevisiae* (Edgington *et al.*, 1999). To examine whether a change in Cdc28-Tyr19 phosphorylation is involved in hyphal induction in *C. albicans*, we first used the anti-Cdc2-P-Tyr15 antibody to follow the levels of CaCdc28-Tyr19 phosphorylation in asynchronous *C. albicans* cultures during the yeast-to-hyphal transition. Cells released into either a yeast- or hyphal-inducing medium from an overnight culture started to show detectable Tyr19 phosphorylation 2 h after release (our unpublished data), and levels of Tyr19 phosphorylation were fairly similar between yeast and hyphal cells (our unpublished data).

We then examined synchronous *C. albicans* cells released into a yeast- or hyphal-inducing YPD-based medium. In both populations, the Tyr19 phosphorylation state was cell cycle regulated, whereas the CaCdc28 protein level (as mon-

Figure 4 (cont). hyphal tips throughout the cell cycle (a–h); appearance of a ring of actin cortical patches at the eventual septum site (c and d); disappearance of cell cycle-regulated actin localization (e); probable mitotic actin rings (f and g); repolarization of the actin patches to the septation site (h). (C) Hyphae induced from asynchronous log-phase cells were stained with rhodamine-phalloidin and calcofluor. The ring of actin cortical patches at the future site of the septa colocalizes with the chitin ring (a), as does the later actin ring (b). (D) Illustration of dynamic changes in SPB/spindle structures and actin assemblies in G_1 cells transferred into yeast- or hyphal-inducing conditions (based on data from Figures 1, 3, and 4). Even distribution of actin cortical patches (a); appearance of tip actin polarization before other cell cycle events only in hyphal cells (b and c); a ring of actin patches at the future septation site (d); duplication of the SPB, initiation of nuclear migration and DNA replication (e); actin patches filling the bud, disappearance of the ring of actin patches from hyphae, nuclear migration and separation of DSPBs (f); MS formation (g); MS elongation and appearance of a mitotic actin ring (h); repolarization of actin patches to mother-daughter neck/septum site (i); and disappearance of the polarized actin from the septation site (j, black bar in hyphae) and cytokinesis in yeast-form cells. Hypha-tip-associated actin polarization persists through the cell cycle (b–j).

Figure 5. Morphologies of hyphae induced from different stages of the cell cycle. (A) Time-lapse microscopy of SC5314 cells on 2% agar containing 10% serum at 37°C. (B) Log-phase HLY1541 cells were transferred directly into YPD + 10% serum at 37°C and grown for 50 min. Differential interference contrast (DIC) and β -tubulin-GFP images of cell with visible hyphal evaginations were merged. G_1 cells (a); S/ G_2 cells (b); M phase cells (c); large budded cells with G_1 -type SPBs (d). The average widths of the germ tubes in a–d are 1.8, 2.32, 2.81, and 3.41 μ m, respectively. (C) Cells from the experiment of B were stained with rhodamine-phalloidin and DAPI. Cells induced to form hyphae show an actin ring and tip-associated actin polarization, whereas uninduced control cells (grown under yeast conditions) have no tip-associated actin polarization. (D) Log-phase HLY1541 cells were elutriated and transferred, or transferred directly into a hyphal-inducing medium (YPD + 10% serum, 37°C) and incubated for 180 min before fixing and staining the cells for chitin. The distance between the chitin ring and the base of the germ tube was measured in 100 cells for each sample: nonelutriated unbudded cells (a); nonelutriated presumably budded cells (b); elutriated G_1 cells (c).

Figure 6. *C. albicans* Cdc28 kinase activity is cell cycle regulated. (A) Elutriated G₁ cells of strain SC5314 were released into YPD + 10% serum at 37°C, and proteins were extracted for precipitation with Suc1 beads. The precipitates were then incubated with [α -³²P]ATP and H1 histone, fractionated on SDS-PAGE, and autoradiographed. Western analysis of protein extracts with a PSTAIRE antibody was performed as a loading control. (The doubling time of strain SC5314 is slightly shorter than that of HLY1541.) (B) Western blot of Suc1 precipitates (lanes 1 and 3) and total protein extract (lanes 2 and 4) was probed with the anti-PSTAIRE (lanes 1 and 2) or with a phospho-cdc2 (Tyr15) antibody (lanes 3 and 4). (C) Extracts from an overnight culture (lanes 1 and 3) and a log-phase culture (lanes 2 and 4) were probed with the antiphospho-cdc2 (Tyr15) (lanes 3 and 4). The same filter was probed with the anti-PSTAIRE antibody as a loading control (lanes 1 and 2).

itored by anti-PSTAIRE) was roughly unchanged (Figure 7). The regulation of the Tyr19 resembles *Schizosaccharomyces pombe* more than *S. cerevisiae*. If hyphal cell elongation involves regulating Cdc28-Tyr19 phosphorylation, we would expect a delay in dephosphorylation of Tyr19 in synchronous hyphal cells compared with synchronous yeast cells. However, the yeast-form and hyphal cells displayed similar dynamics of phosphorylation and dephosphorylation of Tyr19 (Figure 7). That the cyclic pattern was more evident in hyphal cell than in yeast-form cells can probably be attributed to the different pattern of cell division: In yeast-form cells, mother cells always bud before daughters, whereas in hyphal filaments only daughters enter the cell cycle. These data suggest that *C. albicans* hyphal cells, unlike *S. cerevisiae* cells, do not use the Swe1 checkpoint to mediate cell elongation. These data also further support the view that the cell cycle timing in yeast and hyphal-apical cells is similar.

DISCUSSION

Subapical Cells in Hyphal Filaments Appear to Be Arrested in G₁, Leading to Linear Filament Formation

C. albicans cells can switch from unicellular yeast growth to an alternate growth program that generates linear chains of elongated cells with no constrictions at the site of the septa. Previous studies have shown that the subapical cells in a linear hypha are transiently arrested, probably in G₁, and

Figure 7. Similar timing of Tyr 19 phosphorylation and dephosphorylation in synchronous yeast-form and hyphal cells. G₁ cells (SC5314) from centrifugal elutriation were released into YPD at 30°C (A) or YPD + 10% serum at 37°C (B). Western analyses were performed with a phospho-cdc2 (Tyr15) antibody and PSTAIRE antibody. To follow the progression of the cell cycle, the percentage of cells budded was counted for yeast-form cells and the percentage of cells with chitin rings was counted for hyphal cells. The duration of one full cell cycle is indicated.

that they are highly vacuolated (Kron and Gow, 1995; Gow, 1997). Our studies suggest that the subapical cells are arrested, with an unduplicated SPB (Figure 1B, g-j). Only the apical cells continue to cycle as indicated by dynamic changes in the microtubule structure and rearrangement of the actin cytoskeleton. This type of asymmetric distribution of cell fate is a well known mechanism in development of multicellular organisms, as well as in the unicellular *S. cerevisiae*, where the asymmetric distribution of the *ASH1* mRNA to the daughter restricts mating-type switching to the mother cell only (Sil and Herskowitz, 1996). This asymmetric distribution of the *ASH1* mRNA depends on proteins of the actin cytoskeleton (Bobola *et al.*, 1996; Jansen *et al.*, 1996). By analogy, the constant actin polarization observed at the apical tip of the *C. albicans* hypha could be a means for asymmetric distribution of transcripts for factors important for cell fate determination.

Figure 8. Proposed relationship between cell cycle program and hyphal elongation program and their effects on final cell shape. Our data suggest that the cell cycle and hyphal morphogenesis programs are uncoupled. The cell cycle morphogenesis program contributes to cell cycle events such as budding and septum formation. The hyphal morphogenesis program contributes most of the apical growth during hyphal development. Different temporal combinations of the two programs determine the final cell shape. (A) Cells will grow in yeast-form if the hyphal program is not on. (B) No constriction will form at the base of the germ tube if the hyphal program is turned on before the G_1/S transition in unbudded cells. The first septum will be formed in the germ tube, as indicated by a black line. (C) Constriction and a septum will be visible at the base of the bud/germ tube if the hyphal program is on after the cell has budded.

Hyphal Cell Elongation Does not Appear to Be Regulated through Alterations of Cell Cycle Progression

It has been proposed that cell polarity during hyphal morphogenesis is regulated by a change in cell cycle (Kron and Gow, 1995; Lew and Reed, 1995). Several recent publications on the relationship between pseudohyphal growth and Cdc28 activity in *S. cerevisiae* are supportive of this view (Ahn *et al.*, 1999; Edgington *et al.*, 1999; Loeb *et al.*, 1999a). However, several lines of evidence in this study suggest that the model does not apply to the elongation of true hyphae in *C. albicans*. Counting microtubule structures in asynchronous cells showed that there were only 5% more cells with DSPBs in hyphal-apical cells than in yeast-form cells (Figure 2), a difference that is too small to account for the dynamic hyphal-associated apical growth. Moreover, in synchronous cultures, the dynamics of the spindle cycle in yeast-form and hyphal-apical cells is almost identical (Figure 3A). Because SPB duplication coincides with DNA replication and budding and/or chitin ring formation, the dynamics of these cell cycle events must also be almost identical in yeast and hyphal-apical cells. In addition, the timing of cell cycle-regulated actin assemblies, such as the ring of cortical actin patches at the presumptive mother-bud neck and the mitotic actin ring, are almost identical between yeast-form and hyphal-apical cells (Figure 3B). Comparison of these cell cycle

events between yeast-form and hyphal cells is summarized in an illustration (Figure 4D). Finally, we have shown that phosphorylation and dephosphorylation of the conserved Tyr19 of CaCdc28 are cyclic, and the dynamics of these modifications is similar in yeast and hyphal cells (Figure 7). All these data suggest that yeast and hyphal tip cells exhibit similar cell cycle dynamics. Thus, hyphal elongation does not appear to be regulated through altering cell cycle progression, at least in the two conditions examined. Consistent with this, deletion of a G_1 cyclin gene did not affect hyphal initiation or development in a serum-containing medium, and it affected only sustained hyphal elongation, but not initial germ tube formation, in Lee's medium (Loeb *et al.*, 1999b). Our conclusion herein does not exclude the possibility that alterations in cell cycle through mutations or reagents that alter cell cycle progression can cause cell elongation in *C. albicans*. In fact, *C. albicans* cells became elongated when treated with hydroxyurea under yeast growth conditions (our unpublished observation).

Cell Cycle-regulated Morphogenesis and Hyphal Morphogenesis Programs Are Uncoupled, and Each Contributes to Different Aspects of Cell Morphology

Several lines of evidence suggest that cell cycle-regulated morphogenesis programs and hyphal morphogenesis are uncoupled. The timing of several cell cycle events examined in this study is not altered in hyphal tip cells compared with yeast-form cells. Germ tube formation can occur before the G_1/S transition, or in other stages of the cell cycle, and hyphal elongation persists throughout the cell cycle. In addition, cell cycle-modulated actin organization comes and goes, whereas hyphal-associated actin tip-polarization persists in hyphal cells. Consistent with the model of separate pathways, the distance of the first chitin ring from the base of each hypha differs greatly in asynchronous G_1 cells but is relatively constant in synchronous G_1 cells.

Hyphae initiated from G_1 cells did not have a constriction at their bases, and their first chitin ring is in the germ tube, whereas hyphae formed from budded cells inherit a constriction and the chitin ring at the mother-daughter neck. In budded cells, the shape of the daughter, whether it is tapered or dumbbell shaped, is also correlated with the cell cycle stage at the time of hyphal induction. Furthermore, evaginations on budded cells are initiated at the distal end of the daughter, whereas G_1 cells evaginate randomly in relation to the cell axis (Chaffin, 1984). These observations suggest that strong hyphal-associated apical growth is probably added onto already existing cell cycle-regulated structures, such as the bud neck and the actin cytoskeleton that determines apical or isotropic growth, at the onset of hyphal induction. The combination of the cell cycle-regulated apical and isotropic growth with the hyphal-associated apical growth may have given rise to the different cell shapes we observed (Figure 5B). After the initial cell cycle, hyphal-associated apical growth takes over in shaping the cells. Despite this, other cell cycle-regulated structures such as chitin rings continue to appear in hyphal cells in a timely manner. The addition of a chitin ring to a germ tube or a hyphal filament supports, from a different view, the concept that both programs contribute to cell morphogenesis in hyphae (Figure 8).

Potential Mechanisms for Hyphal-associated Polarization of Actin Cytoskeleton

Hyphal-associated polarization of the actin cytoskeleton is located exclusively at the tip of each hypha (Anderson and Soll, 1986). It coexists with the cell cycle-regulated actin assemblies in the apical cells of each hypha (Figure 4B), and both contribute to cell shape. It is likely that both cell cycle and hyphal-associated programs can regulate a common signaling module, which in turn controls the polarization of the actin cytoskeleton. This setting is reminiscent of that in *S. cerevisiae* where the polarization of the actin cytoskeleton during both mating and budding is mediated by altering the distribution of Cdc24, the guanine-nucleotide exchange factor of the RhoGTPase Cdc42, whose activation is required to orient the actin cytoskeleton toward the incipient bud site or toward pheromone during mating (Johnson, 1999; Gulli and Peter, 2001). Germ tube formation in *C. albicans* resembles cell polarity establishment during mating in *S. cerevisiae* in that both establish cell polarity in the absence of an active G₁ cyclin/CDK. Given that Cdc42 plays a vital role in cell morphogenesis and hyphal development in *C. albicans* (Whiteway, 2000), it is likely that *C. albicans* may use mechanisms similar to that of forming mating projections in *S. cerevisiae* for regulating hyphal elongation.

ACKNOWLEDGMENTS

We are in debt to the anonymous reviewers and the monitoring editor for critiques. We thank Drs. Steve Kron, David Pellman, and Chris Greer for comments on the manuscripts; Dr. Cormack for providing the *C. albicans* GFP construct; Dr. Melanie Oakes for assistance with microscopy; Amber Neben for help with FACS analysis; Dr. Giora Maymon for help with statistical analysis; and Avi Hazan for help with graphics. This work was supported by grants from the Burroughs Wellcome Fund (BWF-0462) and from the National Institutes of Health (GM-55155).

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