Sep7 Is Essential to Modify Septin Ring Dynamics and Inhibit Cell Separation during *Candida albicans* Hyphal Growth

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When *Candida albicans* yeast cells receive the appropriate stimulus, they switch to hyphal growth, characterized by continuous apical elongation and the inhibition of cell separation. The molecular basis of this inhibition is poorly known, despite its crucial importance for hyphal development. In *C. albicans*, septins are important for hypha formation and virulence. Here, we used fluorescence recovery after photobleaching analysis to characterize the dynamics of septin rings during yeast and hyphal growth. On hyphal induction, septin rings are converted to a hyphal-specific state, characterized by the presence of a frozen core formed by Sep7/Shs1, Cdc3 and Cdc12, whereas Cdc10 is highly dynamic and oscillates between the ring and the cytoplasm. Conversion of septin rings to the hyphal-specific state inhibits the translocation of Cdc14 phosphatase, which controls cell separation, to the hyphal septum. Modification of septin ring dynamics during hyphal growth is dependent on Sep7 and the hyphal-specific cyclin Hgc1, which partially controls Sep7 phosphorylation status and protein levels. Our results reveal a link between the cell cycle machinery and septin cytoskeleton dynamics, which inhibits cell separation in the filaments and is essential for hyphal morphogenesis.

INTRODUCTION

Candida albicans is an opportunistic human fungal pathogen that is used as a model to study morphogenetic changes in single-cell organisms. *C. albicans* is capable of growing with different morphologies ranging from the yeast form to elongated filaments in response to environmental signals such as pH, temperature, or the presence of serum (Odds, 1988; Brown and Gow, 1999; Sudbery, 2001). The ability to switch between the different morphologies is of key importance for the pathogenicity of this organism (Lo *et al.*, 1997; Zheng *et al.*, 2004).

Septins are GTP-binding proteins that assemble into homo- and hetero-oligomers and filaments, and they are an important element in morphogenesis in animals and fungi. They assemble a ring-like structure at the site of cytokinesis (for recent reviews, see Gladfelter *et al.*, 2001; Faty *et al.*, 2002; Douglas *et al.*, 2005; Versele and Thorner, 2005). The structure, dynamics and regulation of septin rings is well known in the budding yeast *Saccharomyces cerevisiae*, where the filaments form a collar at the bud neck that is composed of Cdc3, Cdc10, Cdc11, Cdc12, and Shs1/Sep7. Initially, these

septins form a ring at the bud site before bud emergence, which then rearranges into an hourglass-shaped collar that spans both the mother and the daughter sides of the neck (Gladfelter *et al.*, 2005; Kozubowski *et al.*, 2005). This collar persists until cytokinesis, when it splits into two rings (Cid *et al.*, 2001; Lippincott *et al.*, 2001). The functions of the septins are of at least two types: first, they act as scaffold for anchoring other regulatory proteins at the bud neck. Second, they form a diffusion barrier to control molecules trafficking between mother and daughter cells (for recent reviews, see Longtine and Bi, 2003; Douglas *et al.*, 2005; Versele and Thorner, 2005; Kinoshita, 2006; Spiliotis and Nelson, 2006).

The structure of the heterotrimeric human SEPT2–SEPT6–SEPT7 complex reveals that the basic polymerization unit is a trimer. Additionally, two heterotrimers associate head-to-head to form a hexameric unit containing two SEPT2 proteins in the center of the hexamer and with SEPT7 exposed on both ends, resulting in apolar filaments (Sirajuddin *et al.*, 2007). Interestingly, the yeast Cdc3–Cdc10–Cdc11–Cdc12 complex has a similar arrangement and forms an octameric complex, suggesting that apolar filaments could be a common design model.

In *S. cerevisiae*, the septin ring varies from a static (“frozen”) to a dynamic (“fluid”) state in a cell cycle-dependent manner. Septin rings are in the frozen state most of the cell cycle, in which the septin subunits are static, whereas during bud site selection and emergence, and at the onset of cytokinesis, the septin rings are in the fluid state, in which the septin subunits move inside the ring (Caviston *et al.*, 2003; Dobbelaere *et al.*, 2003). No septin subunit exchange with the cytoplasm occurs in any of these two states. Interestingly, the transition from hourglass to rings—one of the dynamic

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moments—involves a rearrangement of septin filaments (Vrabioiu and Mitchison, 2006). In the hourglass, the filaments are aligned parallel to the mother-bud axis, whereas in the ring the filaments rotate 90° in the membrane and become circumferential. The Shs1/Sep7 septin is not essential for septin filament formation in *S. cerevisiae* (Carroll *et al.*, 1998; Mino *et al.*, 1998), but plays a role in stabilizing the structure through its interaction with Cdc11, and it is a target for regulation by phosphorylation (Dobbelaere *et al.*, 2003). The change from the fluid to the frozen state is dependent on Shs1 phosphorylation by Cla4 and Gin4 kinases, resulting in the formation of the static structure, whereas its dephosphorylation by the PP2A phosphatase leads to the fluid state (Dobbelaere *et al.*, 2003).

C. albicans is able to grow in different morphological forms, including unicellular budding yeast, pseudohyphae, or true hyphae. Septins are important for hyphal formation and virulence, but little is known about how septin ring structure and dynamics affect these processes (Warenda and Konopka, 2002; González-Novo *et al.*, 2006). The organization of septin structures is different in yeast and pseudohyphae compared with hyphae (Gale *et al.*, 2001; Sudbery, 2001; Warenda and Konopka, 2002). In yeast and pseudohyphae, a septin collar forms at the bud neck, which splits in two at cytokinesis as it organizes the formation of the primary septum, as it does in *S. cerevisiae*. During hyphal germ tube formation, a septin cap at the growing tip of the tube and a septin band at the base of the tube are formed. As the germ tube elongates, a septin collar forms at a place around 15 μ m within the tube, whereas the basal band and apical cap disappear (Sudbery, 2001). After mitosis, the collar splits into two rings and organizes the formation of the primary septum that divides the hyphal compartments. These double rings are maintained rather than disassembled immediately after cytokinesis, resulting in multiple rings along the length of the hypha, each marking a septation site. During yeast growth, five septin orthologues of Cdc3, Cdc10, Cdc11, Cdc12, and Sep7/Shs1 form the septin ring at the bud neck (Sudbery, 2001; Warenda and Konopka, 2002). Deletion analysis indicates that *CDC3* and *CDC12* are essential, whereas mutants lacking *CDC10* or *CDC11* are viable but they show defects in cytokinesis (Warenda and Konopka, 2002; González-Novo *et al.*, 2006). During hyphal growth, *cdc10 Δ* and *cdc11 Δ* mutants display abnormalities in septum assembly and form hyphae that are more curved than those of wild-type cells. Mutants lacking *SEP7* have previously been reported to have no phenotypes during yeast or hyphal growth (Warenda and Konopka, 2002).

The molecular basis of the differences between yeast and hyphal septin rings has not been elucidated, but such differences might explain the inhibition of cell separation seen in hyphae. Two known regulators of cell separation in *C. albicans* are Hgc1 and Cdc14. Hgc1 is a G1 cyclin-related protein essential for hyphal morphogenesis (Zheng *et al.*, 2004). *hgc1 Δ* mutants are severely impaired in the control of hyphal morphology and in the maintenance of cell–cell attachment after cytokinesis, suggesting that Hgc1 would be important for the inhibition of separation during hyphal growth. On the other hand, the Cdc14 phosphatase is required for cell separation during growth in the yeast form. In yeast cells, Cdc14 transiently localizes to the neck at the end of mitosis, but it is never found in the septa of the hyphae (Clemente-Blanco *et al.*, 2006).

Here, we report the characterization of septin ring dynamics during *C. albicans* hyphal growth. Induction of filamentation resulted in the conversion of the yeast septin rings to a hyphal-specific state, which was characterized by the pres-

ence of a stable core (composed by Sep7, Cdc3, and Cdc12) and a high exchange of Cdc10 between the ring and the cytoplasmic pool. Our results revealed that this modification was dependent on the Sep7 septin and the Hgc1 hyphal-specific cyclin and that alteration of septin ring dynamics in hypha is tightly correlated with the inhibition of cell separation.

MATERIALS AND METHODS

Strains and Growth Conditions

The strains used in this work are listed in Table 1. Cells were grown in YPD or in synthetic minimal (SC) medium at 28°C. Hyphae formation was induced by supplementing media with 10% fetal calf serum at 37°C. Strains were transformed using the lithium acetate protocol (Walther and Wendland, 2003). All transformants were checked for correct genome integration by polymerase chain reaction (PCR). Disruption of both alleles of *SEP7* was done in the BWP17 or CAG35 strains by using the PCR-mediated procedure described previously (Gola *et al.*, 2003). Different selectable markers were used, and the primers used to amplify the cassettes are listed in Supplemental Table 1. The absence of the *SEP7* open reading frame in the genome was checked by PCR. Generation of C-terminal fusions of *SEP7*, *CDC10*, *CDC3*, *CDC12*, and *CDC14* genes to different fluorescent proteins was performed as described previously (Gola *et al.*, 2003). Introduction of the hemagglutinin (HA) epitope at the C terminus of *SEP7* was done by transformation of different strains with linear pCaHA-*SEP7* plasmid. All the septin-green fluorescent protein (GFP) and septin-HA fusions generated were functional, because no growth defects or morphological differences were observed between heterozygous strains carrying the tagged and untagged alleles (data not shown). The Cdc14-yellow fluorescent protein (YFP) fusion used was described previously to be functional (Clemente-Blanco *et al.*, 2006).

Plasmid Constructions

To construct the pCaHA-*SEP7* plasmids, a 1-kb fragment containing the C-terminal end of *SEP7* without the stop codon was PCR amplified from genomic DNA. The PCR product was cloned in frame with the HA coding region of plasmid pCaHA (Peter Sudbery, University of Sheffield, United Kingdom) using an EcoRV restriction site introduced in the primer. The plasmid was linearized with XhoI before transformation in different strains. Correct integration of the plasmid was checked by PCR.

Microscopy

Cells were observed as wet mounts using a DMRXA microscope (Leica, Wetzlar, Germany) equipped with epifluorescence, Nomarski optics, and a Sensys charge-coupled device camera (Photometrics, Tucson, AZ). For fluorescence microscopy, cells carrying integrated versions of fluorescently tagged proteins were mounted on glass slides, and then they were observed with epifluorescence. Septum formation was determined by staining cells with calcofluor white as described previously (Sherman, 1991). For transmission electron microscopy, cells were fixed with 3% glutaraldehyde, postfixed with 2% potassium permanganate, and acetone dehydrated (González-Novo *et al.*, 2006). Samples were embedded in Spurr resin (Taab Laboratories, Reading, Berkshire, United Kingdom), stained with osmium tetroxide, and observed under a Zeiss EM900 microscope. For fluorescence recovery after photobleaching (FRAP) analysis, cells were grown as yeast or hyphae for 2 h, mounted on glass slides, and observed using a Zeiss LSM510 confocal microscope. Photobleaching was accomplished with a full-power laser until no fluorescence signal was observed. Pictures were taken every 40 s for 12 min to study the dynamics of Cdc10, and every 2 min for the other septins to be able to detect fluorescence recovery, given the bleaching effect of multiple images acquisition. Fluorescence intensity of the bleached areas was quantified with ImageJ (<http://rsb.info.nih.gov/ij/>), and it was normalized by subtraction of the background and correction for fluorescence loss using nonbleached control rings in the same image series.

Western Blotting

For Sep7 detection by Western blot, a protocol kindly provided by Douglas Kellogg (University of California, CA) was used. Briefly, 1.6 ml of cells was collected and immediately frozen in liquid nitrogen. Then, 140 μ l of lysis buffer (65 mM Tris-HCl, pH 6.8, 3% SDS, 10% glycerol, 5% β -mercaptoethanol, 100 mM β -glycerol phosphate, 50 mM NaF, and 2 mM phenylmethylsulfonyl fluoride [PMSF]) was added, and cells were immediately broken with glass beads in a Fast-prep (FP120 Bio101; Thermo Electron, Waltham, MA), with two pulses of 45 s at top speed. Extracts were boiled 5 min and centrifuged at top speed another 5 min. Proteins (20 μ l) were separated on 7.5% SDS-polyacrylamide gel electrophoresis (PAGE) gels 3 h at a constant voltage of 140 V and transferred to polyvinylidene difluoride (PVDF) membranes (Hybond-P; GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom). Anti-HA (12CA5; Roche Diagnostics, Mannheim, Germany), anti-

Table 1. Strains used in this study

Strain	Genotype	Source
BWP17	<i>ura3::imm434/ura3::imm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG</i>	Enloe <i>et al.</i> (2000)
CAF2	<i>URA3/ura3::imm434</i>	Fonzi and Irwin (1993)
CAI4	<i>ura3::imm434/ura3::imm434</i>	Fonzi and Irwin (1993)
JC13	BWP17 <i>cdc14Δ::hisG/cdc14Δ::hisG</i>	Clemente-Blanco <i>et al.</i> (2006)
JC189	BWP17 <i>CDC14-YFP-HIS1/CDC14-YFP-ARG4</i>	Clemente-Blanco <i>et al.</i> (2006)
WYZ12	BWP17 <i>hgc1Δ::ARG4/hgc1Δ::HIS1</i>	Zheng <i>et al.</i> (2004)
YAW44	BWP17 <i>CDC12/CDC12-GFP-URA3</i>	Warenda and Konopka (2002)
YAW45	BWP17 <i>CDC3/CDC3-GFP-URA3</i>	Warenda and Konopka (2002)
BWP17 SHA	BWP17 <i>SEP7-3HA-URA3</i>	This study
CAG20	BWP17 <i>sep7Δ::URA3/sep7Δ::HIS1</i>	This study
CAG21	CAI4 <i>CDC10/CDC10-GFP-URA3</i>	Gonzalez-Novo <i>et al.</i> (2004)
CAG22	BWP17 <i>sep7Δ::URA3/sep7Δ::HIS1 CDC10/CDC10-GFP-ARG4</i>	This study
CAG23	BWP17 <i>sep7Δ::URA3/sep7Δ::HIS1 CDC14/CDC14-YFP-ARG4</i>	This study
CAG24	BWP17 <i>hgc1Δ::ARG4/hgc1Δ::HIS1 SEP7/SEP7-3HA-URA3</i>	This study
CAG29	BWP17 <i>CDC10/CDC10-GFP-ARG4</i>	This study
CAG30	BWP17 <i>SEP7/SEP7-3HA-URA3 CDC10/CDC10-GFP-ARG4</i>	This study
CAG35	BWP17 <i>cdc14Δ::hisG/cdc14Δ::hisG CDC10/CDC10-GFP-ARG4</i>	This study
CAG36	BWP17 <i>cdc14Δ::hisG/cdc14Δ::hisG sep7Δ::URA3/sep7Δ::HIS1 CDC10/CDC10-GFP-ARG4</i>	This study
CAG37	BWP17 <i>hgc1Δ::ARG4/hgc1Δ::HIS1 CDC10/CDC10-GFP-URA3</i>	This study
CAG38	BWP17 <i>hgc1Δ::ARG4/hgc1Δ::HIS1 SEP7/SEP7-GFP-URA3</i>	This study
CAI4 SHA	CAI4 <i>SEP7-3HA-URA3</i>	This study
CAG39	BWP17 <i>SEP7/SEP7-GFP-ARG4</i>	This study
CAG40	BWP17 <i>sep7Δ::URA3/sep7Δ::HIS1 CDC3/CDC3-GFP-ARG4</i>	This study
CAG41	BWP17 <i>sep7Δ::URA3/sep7Δ::HIS1 CDC12/CDC12-GFP-ARG4</i>	This study

tubulin (Sigma-Aldrich, St. Louis, MO), anti-PSTAIR (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-GFP (Clontech, Mountain View, CA) antibodies were used at 1:1000 dilution. Secondary antibodies conjugated with horseradish peroxidase were used at 1:4000 dilution. Membranes were developed with the ECL system (GE Healthcare). Phosphatase treatment was carried out incubating 15 μ g of total protein extracts prepared in lysis buffer without phosphatase inhibitors with 1000 U of λ -phosphatase (New England Biolabs, Ipswich, MA) for 30 min at 28°C.

Immunoprecipitation

Strains carrying *SEP7-HA*, *CDC10-GFP*, or both were grown for 150 min in yeast or hypha-inducing conditions, and then they were harvested by centrifugation. Cells were resuspended in 300 μ l of lysis buffer (50 mM Tris-HCl, pH 7.8, 100 mM KCl, 2 mM MgCl₂, 0.5 mM EGTA, 0.8% Triton X-100, 1 mM dithiothreitol, and 1 mM PMSF), and then they were broken in a Fast-Prep device using three pulses of 20 s at the 5.5 speed setting. Extracts were centrifuged at 13,200 rpm for 35 min. Five hundred micrograms of clarified extracts was preincubated for 20 min with protein A-Sepharose beads (GE Healthcare) to reduce nonspecific interactions, before the addition of 25 μ l of anti-Cdc11 antibodies (Santa Cruz Biotechnology) and subsequent incubation for 1 h at 4°C. Then, samples were incubated with protein A-Sepharose beads for 45 min. Finally, the immunoprecipitates were washed six times with wash buffer (50 mM Tris-HCl, pH 7.8, 250 mM KCl, 2 mM MgCl₂, 0.5 mM EGTA, and 0.2% Triton X-100) and loaded on 8% SDS-PAGE gels.

Northern Blotting

Wild-type and *sep7Δ* strains were grown overnight, and then they were transferred to yeast or hypha-inducing conditions. After 150 min, cells were collected, and total RNA was extracted with the acid phenol procedure. Poly(A⁺)-RNA was purified using the mRNA purification kit (GE Healthcare). Five micrograms of RNA was loaded on a gel and transferred to Hybond-N membranes (GE Healthcare). Probes were obtained by PCR, using genomic DNA as template and primers described in Supplemental Table 1.

RESULTS

Septin Dynamics Is Different between Yeast and Hyphal Growth in *C. albicans*

To examine septin ring dynamics during *C. albicans* hyphal growth, FRAP experiments were performed on a wild-type strain carrying GFP-tagged septins under the control of their own promoters. Dynamics of four of the five *C. albicans* septins, *CDC10-GFP*, *CDC3-GFP*, *CDC12-GFP*, or *SEP7-GFP*,

was analyzed in yeast cells, and after the induction of hyphal development. The entire septin ring was irradiated with a laser beam to irreversibly bleach its fluorescence, and recovery was monitored over time. Fluorescence recovery in the bleached area indicates the incorporation of unbleached molecules from the cytoplasm. FRAP analysis showed very low exchange with the cytoplasmic pool of three of the septins analyzed during hyphal growth. Thus, the half-time recovery for Cdc3, Cdc12, and Sep7 in unduplicated rings was higher than 600 s ($t_{1/2} > 600$ s; $n = 6$; Figure 1), and similar results were obtained when duplicated rings were analyzed (data not shown). In contrast, Cdc10 showed a rapid turnover in all rings analyzed, and the fluorescence was recovered with a half-time of 100 ± 20 s ($n = 10$; Figure 1). These results indicate that different septins in the ring have different dynamics during hyphal growth, and exchange of three of them, Cdc3, Cdc12, and Sep7, with the cytoplasm is very low, whereas Cdc10 is highly dynamic.

It has been shown that in *S. cerevisiae*, there is little or no exchange of the septin subunits with the cytoplasm (Dobelaere *et al.*, 2003). To analyze whether the high Cdc10 turnover observed in hyphae was a characteristic of *C. albicans* septin rings or it was specific for hyphal septin rings, FRAP experiments were performed in the same strains during yeast growth. The analysis was carried out in cells at different stages of the cell cycle, and the results resembled those described previously for *S. cerevisiae* (Caviston *et al.*, 2003; Dobelaere *et al.*, 2003). No Cdc10-GFP exchange with the cytoplasm was observed in cells with small buds or during septin ring duplication (Figure 2, top), and similar results were observed for duplicated rings (data not shown). Furthermore, similar results were obtained for Cdc3-GFP, Cdc12-GFP, and Sep7-GFP (Figure 2, bottom; data not shown). Thus, during yeast growth *C. albicans* septins behave similar to the pattern described in *S. cerevisiae*, and little or no exchange of septin subunits with the cytoplasm is detected at any stage of the cell cycle, although we did not

Figure 1. Septin dynamics during hyphal growth in *C. albicans*. The entire fluorescent ring was bleached in hyphal cells carrying Cdc10-GFP (CAG21), Cdc3-GFP (YAW45), Cdc12-GFP (YAW44), or Sep7-GFP (CAG38). Images were taken before (time 0) and every 120 s after photobleaching. The box in the first frame indicates the bleached region. The kinetics and average recovery half-times are shown for each protein. For Cdc10-GFP, recovery half-time was calculated from a series in which images were taken every 40 s. Bars, 2.5 μ m.

analyze septin dynamics when septins were being assembled before bud emergence. Together, these results indicate that induction of hyphal development triggers the conversion of the septin rings to a hyphal-specific state, characterized by a high Cdc10 turnover with the cytoplasm, whereas Cdc3, Cdc12, and Sep7 form a core with little or no exchange.

Sep7 Is a Phosphoprotein

Because septin ring dynamics in *S. cerevisiae* is controlled by phosphorylation of the Shs1/Sep7 septin (Dobbelaere *et al.*, 2003), we analyzed whether Sep7 might also be involved in the regulation of septin ring dynamics during *C. albicans* hyphal growth. First, Sep7 protein levels and phosphorylation status were analyzed during hyphal growth. To this end, the HA epitope was inserted at the C terminus of the *SEP7* gene, and samples were collected during yeast growth

and after hyphal induction. Western blot analysis revealed an increase in Sep7-HA levels after hypha induction in comparison with yeast cells (Figure 3A), a situation never found when the other septin levels were analyzed (data not shown). Interestingly, a fraction of Sep7 had lower electrophoretic mobility, and several bands could be detected under yeast and hyphal-inducing conditions. The presence of slow-migrating forms suggested the possibility that Sep7 might be modified by phosphorylation, similar to the *S. cerevisiae* Shs1/Sep7 (Dobbelaere *et al.*, 2003). When protein extracts from hyphal cells were treated with λ -phosphatase (PPase), a shift to the faster-migrating form was observed (Figure 3B), indicating that Sep7 had indeed been phosphorylated. Similar results were obtained during yeast growth (data not shown). Thus, Sep7 is a phosphoprotein that is up-regulated upon hyphal induction.

Sep7 Is Required to Convert Hyphal Septin Rings into the Hyphal-specific State

The change in Sep7 protein levels during hyphal growth suggested that this septin could perform a specific function during this stage of development. To test this hypothesis, FRAP experiments were performed on the *sep7* Δ mutant bearing *CDC10-GFP*. Disruption of *SEP7* had no effect on the rate of fluorescence recovery in yeast cells ($t_{1/2} > 640$ s; $n = 10$) compared with wild-type yeast cells ($t_{1/2} > 640$ s; $n = 10$; Figure 3, C and D, left). However, Cdc10 exchange in hyphal cells was significantly lower in the *sep7* Δ mutant ($t_{1/2} > 640$ s; $n = 10$) than in the wild-type strain ($t_{1/2} = 100 \pm 20$ s; $n = 10$, Figure 3, C and D, right). These results therefore indicate that Sep7 must be specifically required to increase Cdc10 exchange during hyphal growth.

To test whether Sep7 modifies the stoichiometry of the different components of the septin ring during hyphal growth, we used a strain carrying *CDC10-GFP*- and *SEP7-HA*-tagged alleles at their chromosomal locus for the immunoprecipitation of septin ring complexes by using anti-Cdc11 antibodies. The amount of Sep7 and Cdc10 in the immunoprecipitates was determined using anti-HA and anti-GFP antibodies, respectively. Quantification of the proteins levels indicated that the Cdc10/Sep7 ratios were similar during yeast and hyphal growth (Figure 3E). These results are in good agreement with previous findings indicating that the stoichiometry of the septin complex is not affected by cell morphology (Kaneko *et al.*, 2004). Thus, the composition of the septin ring does not substantially change during the switch from the yeast to hyphal form.

Deletion of SEP7 Activates Cell Separation during Hyphal Growth

It has been reported that deletion of *SEP7* results in no detectable phenotype during yeast or hyphal growth (Warenda and Konopka, 2002). However, the above-mentioned results clearly suggested that Sep7 was essential for the modification of the characteristics of the septin rings during this growth mode, which prompted us to reexamine the phenotype of mutants lacking this septin. To this end, we constructed a strain lacking both chromosomal copies of *SEP7* in the BWP17 background. Three independent clones were analyzed and the phenotypes described below were identical for all of them. In addition, introduction of a plasmid carrying *SEP7* was able to complement the mutant phenotypes (data not shown), confirming that they were indeed due to the absence of *SEP7*. As described previously (Warenda and Konopka, 2002), we could confirm that *sep7* Δ mutants had no growth defects in the yeast form and that they were able to develop filaments. Even though the *sep7* Δ

Figure 2. Septin dynamics during the cell cycle in *C. albicans* yeast cells. Exchange of septin subunits with the cytoplasm during yeast growth was analyzed by FRAP in wild-type cells bearing a chromosomal copy of *CDC10-GFP* (CAG21) or *CDC3-GFP* (YAW45). Images were taken before (time 0) and every 80 (for Cdc10-GFP) or 120 s (for Cdc3-GFP) after photobleaching. The small box in the first frame indicates the bleached region. Cells with small buds or during ring duplication are shown. The arrow indicates the first image in which the septum is visible in the transmitted light channel. The kinetics and average recovery half-time are shown for each stage. Bars, 2.5 μ m.

mutant was able to form hyphae, microscopic inspection of cells incubated in hyphal-inducing conditions revealed an abnormal phenotype. At short incubation times, normal hyphae were formed, but as the time in the presence of serum increased, many of the hyphal compartments of the hyphae became partially or totally separated from the rest of the hyphae (Figure 4A). This phenotype suggested that cell separation was taking place in the hyphal compartments of *sep7 Δ* mutants.

To confirm that this phenotype was due to cleavage of the separation septum rather than random breakage of the filaments, we used transmission electron microscopy. *sep7 Δ* mutants were able to assemble normal septa, in which both the primary and secondary septa could be seen, similar to the wild type (Figure 4B, left). However, the septum that separates hyphal compartments was usually degraded, as judged by the absence of the primary septum but not the secondary septa (Figure 4B, right). Thus, *sep7 Δ* hyphae are able to degrade the primary septum during filamentous growth in contrast to wild-type hyphae, in which cell separation is inhibited.

Degradation of the septum during yeast growth is an enzymatic process controlled by the Ace2 transcription factor (Kelly *et al.*, 2004; Clemente-Blanco *et al.*, 2006). Ace2 activates the expression of genes whose products are required for the controlled dissolution of the septum, such as the chitinase Cht3 or the endo- β -1,3-glucanase Eng1 (Dunkler *et al.*, 2005; Esteban *et al.*, 2005) and several other genes, such as *DSE1* (Mulhern *et al.*, 2006). To determine whether Ace2-dependent transcription was activated in *sep7 Δ* hyphae, the expression of some Ace2-target genes was

monitored by Northern blot. The expression of *ENG1* and *DSE1* was down-regulated in wild-type hyphae, as expected, because cell separation was inhibited (Figure 4C). In contrast, the expression of these genes was similar during yeast and hyphal growth in *sep7 Δ* , although no significant differences were detected in *ACE2* expression between wild-type and mutant cells. These results therefore indicate that *sep7 Δ* hyphae indeed activate the cell separation program.

Moreover, the septin rings in *sep7 Δ* filaments had other characteristics shared with those found in yeast cells. In wild-type cells, septin rings disassemble rapidly after cytokinesis and reassemble at the site of the next bud emergence in yeast cells, but during filamentous growth they are maintained at the sites of septum formation (Sudbery, 2001). Although wild-type septin rings were maintained along the hyphae, in *sep7 Δ* mutants the Cdc10-GFP signal of each septin ring started to disappear when the next one was assembled, as happens during yeast growth (Figure 4D). To gain further idea of the extent of this phenotype, the number of septin rings visible in filaments that had assembled at least three septa was counted. In wild-type cells, 100% of hyphae showed Cdc10-GFP at the septum region in all compartments. However, in the *sep7 Δ* mutant the first ring (closest to the mother yeast cell) was never observed; the second could be seen in around 20% of the cells and the third was present in all the filaments. Similar results were obtained when Cdc3-GFP and Cdc12-GFP were analyzed, indicating that septin rings, and not only Cdc10, disassemble after cytokinesis in *sep7 Δ* hypha. Thus, these data, together with FRAP analysis results, reveal that hyphal septin rings in *sep7 Δ* mutants are similar to wild-type yeast septin rings.

bearing a chromosomal copy of *SEP7-HA* (BWP17 SHA, lane 1), a chromosomal copy of *CDC10-GFP* (CAG29, lane 2), or both *SEP7-HA* and *CDC10-GFP* (CAG30, lane 3). Proteins in the immunoprecipitates were analyzed using anti-HA or anti-GFP antibodies. The GFP/HA ratio in each condition is indicated under each lane. The intensity of the signals was quantified using ImageJ.

Role of Cdc14 in Activating Cell Separation in *sep7Δ* Hyphae

The Cdc14 phosphatase is an activator of cell separation that transiently localizes to the bud-neck at the end of mitosis in

yeast cells. Interestingly, during hyphal growth, Cdc14 is never present at the septa of the hyphal compartments of wild-type filaments (Clemente-Blanco *et al.*, 2006). These observations prompted us to investigate whether activation

Figure 4. *sep7Δ* mutants form filaments that separate after cytokinesis. (A) Differential interference contrast images of wild type (BWP17) and *sep7Δ* cells (CAG20) grown for 3 h in filament-inducing conditions. Arrowheads indicate the points where cell separation is occurring after cytokinesis in the hyphae. The regions indicated with numbers are magnified in the lower panels. Bars, 5 μ m. (B) Transmission electron microscopy images of wild-type and *sep7Δ* hyphae 3 h after filament induction. The lower panels show magnifications of the septum region. ps, primary septum; ss, secondary septa. Bars, 0.5 μ m. (C) *sep7Δ* cells show increased expression of cell separation genes during hyphal growth. Polyadenylated RNA isolated from exponentially growing cells of the indicated strains grown as YF or HF for 2.5 h was probed with specific probes for *DSE1*, *ENG1*, and *ACE2*. *ACT1* was used as a loading control. The numbers below each pair of lanes indicate the hypha/yeast ratio for each gene after normalization using *ACT1*. ImageJ was used for quantification of the autoradiographs. (D) Persistence of septin rings in wild-type and *sep7Δ* filaments. Fluorescence images of wild-type and *sep7Δ* fila-

ments of strains bearing *CDC10-GFP*, *CDC3-GFP*, or *CDC12-GFP*. Cells were incubated for 2.5 h in the presence of serum. Arrowheads indicate the position of assembled septa along the filament and boxes mark the position of septa with no visible septin rings. Bars, 5 μ m.

Figure 5. Role of the Cdc14 phosphatase in activating cell separation in *sep7Δ* hyphae. (A) Cdc14 is present in the hyphal septa of *sep7Δ* mutants. Wild-type (JC189) and *sep7Δ* strains (CAG23) carrying Cdc14-YFP were used to analyze the localization of Cdc14 during hyphal growth after 2.5 h of induction. Cells were photographed using differential interference contrast (DIC) and YFP fluorescence. Arrowheads indicate the localization of Cdc14 in the hypha of the *sep7Δ* mutant and the position of the septa in the wild-type. Scale bars, 5 μ m. (B) DIC images of *sep7Δ* (CAG22) and *sep7Δ cdc14Δ* (CAG36) cells grown for 4 h in filament-inducing conditions. Arrowheads indicate the points where cell separation is occurring after cytokinesis in the filaments. Bars, 10 μ m. (C) Cdc10 septin rings in *sep7Δ cdc14Δ* filaments. DIC and fluorescence images of *sep7Δ cdc14Δ CDC10-GFP* (CAG36) filaments. Cells were incubated for 5 h in the presence of serum. Arrowheads indicate the position of assembled septa along the filament. Bars, 10 μ m. (D) Cdc10 dynamics in *sep7Δ cdc14Δ* and *cdc14Δ* mutants (CAG35). The stability of the septin ring was analyzed by FRAP experiments in strains bearing a chromosomal copy of *CDC10-GFP* during hyphal growth. The GFP fluorescence of the complete septin ring was photobleached and images were taken before (time 0), and at the indicated times (seconds) after photobleaching. Bars, 2.5 μ m. The graph shows the quantification of the fluorescence recovery for each strain. The kinetics and average recovery half-times are shown for each strain.

of the separation program in *sep7Δ* mutants was supported by the presence of Cdc14 in the septa of hyphal cells. To this end, we integrated a copy of *CDC14-YFP* into its chromosomal locus in the wild-type and *sep7Δ* strains. As described previously, Cdc14 was never found in the septa of filaments in the wild-type strain, but it was present in the septa of the *sep7Δ* mutant (Figure 5A). This result suggests that in the absence of Sep7, the characteristics of hyphal septin rings are different from those in wild-type cells, allowing the incorporation of Cdc14 to the septa of filaments.

The above-mentioned results suggest that the activation of cell separation observed in *sep7Δ* hyphae could be a consequence of the recruitment of Cdc14 to the hyphal septa. If this were the case, the deletion of *CDC14* in the *sep7Δ* mutant should suppress the separation phenotype. To test this possibility, a *sep7Δ cdc14Δ* double mutant was constructed and induced to form filaments. As expected, *sep7Δ cdc14Δ* hyphae were similar to wild-type hyphae; that is, no cell separation took place in the filaments (Figure 5B). Furthermore, deletion of *CDC14* did not alter Cdc10 dynamics as compared with that of the *sep7Δ* mutant, i.e., septin rings were not persistent and rapidly disassembled when the next ring was formed (Figure 5C), and Cdc10 turnover in the double mutant was similar to that of *sep7Δ* filaments ($t_{1/2} > 640$ s; $n = 6$; Figure 5D). As a control, we also analyzed Cdc10 turnover in the *cdc14Δ* mutant, and this proved to be similar to that of wild-type hyphae ($t_{1/2} = 130 \pm 15$ s; $n = 6$; Figure 5D). Together, these results indicate that Cdc14 is not required to convert the septin ring to the hyphal-specific state and that its recruitment to the hyphal septa in *sep7Δ* mutants activates the cell separation program.

The Hgc1 Cyclin Is Required for the Conversion of the Septin Rings to the Hyphal-specific State

Hgc1 is a *C. albicans* hyphal-specific cyclin that plays an important role in hyphal development and in preventing cell

separation in this morphological state (Zheng *et al.*, 2004). To test whether Hgc1 was necessary for the conversion of the septin rings to the hyphal-specific state, *CDC10* was tagged with the GFP protein at the C terminus in the *hgc1Δ* mutant. Deletion of *HGC1* had no effects on Cdc10 localization (Figure 6A). Interestingly, *hgc1Δ* septin rings showed similar characteristics to those of *sep7Δ* mutants; i.e., they were not persistent and disassembled after cytokinesis (Figure 6A, bottom) and no Cdc10 turnover was observed ($t_{1/2} > 640$ s; $n = 6$; Figure 6B). Furthermore, Northern blot analysis indicated that the Ace2-dependent cell separation program was activated in *hgc1Δ* mutants, as suggested by the increased expression of *DSE1* and *ENG1* compared with the wild-type strain (Figure 6C). One possible explanation for these results is that the absence of Hgc1 might affect the incorporation of Sep7 to the septin ring, resulting in the low Cdc10 turnover. To check whether this was the case, *SEP7* was also tagged with the GFP in the *hgc1Δ* mutant. Sep7-GFP was present in the hyphal septa of *hgc1Δ* cells (Figure 6A) and its dynamics was similar to that of wild-type cells ($t_{1/2} > 640$ s; $n = 6$; Figure 6B). Thus, these results indicate that Hgc1 is also required for the conversion of the septin ring to the hyphal-specific state.

To test whether Sep7 phosphorylation during hyphal growth was dependent on Hgc1, we analyzed its phosphorylation state in an *hgc1Δ* mutant strain. In hyphal-inducing conditions, the slower migrating band was less abundant in the *hgc1Δ* mutant strain, indicating that Hgc1 is necessary, directly or indirectly, for Sep7 phosphorylation under these growth conditions (Figure 6D). In addition, treatment with PPase indicated that deletion of *HGC1* also produced a reduction in Sep7 protein levels. Interestingly, absence of the Hgc1 cyclin did not substantially reduced *SEP7* expression during hyphal growth, as revealed by Northern blot analysis (Figure 6E). Thus, the Hgc1 cyclin controls Sep7 protein levels and phosphorylation during hyphal growth.

Figure 6. Hgc1 is required for the conversion of the septin rings to the hypha-specific state. (A) Sep7 and Cdc10 are present in the hyphal septa of *hgc1Δ* mutants. *hgc1Δ* mutants carrying Cdc10-GFP (CAG37) or Sep7-GFP (CAG38) were used to analyze the localization of the septins during hyphal growth after 2 h of induction. Cells were photographed using DIC and GFP fluorescence. The lower panels show the localization of septins in hypha with two assembled septa. Bars, 10 μ m. (B) Cdc10 and Sep7 dynamics in the *hgc1Δ* mutant. The GFP fluorescence of the complete septin ring was photobleached and images were taken before (time 0), and at the indicated times (seconds) after photobleaching. Bars, 5 μ m. The graph shows the quantification of the fluorescence recovery for each strain. The kinetics and average recovery half-times are also shown. (C) *hgc1Δ* cells show increased expression of cell separation genes during hyphal growth. RNA isolated from the wild-type or the *hgc1Δ* mutant grown as YF or HF for 2.5 h was probed with specific probes for *DSE1* and *ENG1*. *ACT1* was used as a loading control. The numbers below each pair of lanes indicate the hypha/yeast ratio for each gene after normalization using *ACT1*. ImageJ was used for quantification of the autoradiographs. (D) Analysis of Sep7 phosphorylation in an *hgc1Δ* mutant. The wild-type (CAI4 SHA) and *hgc1Δ* mutant (CAG24) carrying a chromosomal copy of *SEP7-HA* were grown under filament-inducing conditions for 150 min, and samples were collected for Western blot analysis with anti-HA antibodies. Half of each lysate was pre-treated with PPase. Anti-PSTAIRE was used as a loading control. (E) Deletion of *HGC1* does not alter *SEP7* expression during hyphal growth. RNA isolated from the wild-type or the *hgc1Δ* mutant grown in hyphal-inducing conditions for 2.5 h was hybridized with specific probes for *SEP7* and *ACT1*.

DISCUSSION

When *C. albicans* yeast cells receive the appropriate stimulus, they undergo a change to a hyphal growth pattern, which seems to be important for the virulence of this organism (Lo *et al.*, 1997). Hyphal growth has special characteristics, such as continuous apical growth, the persistence of duplicated septin rings at the site of cytokinesis, and the inhibition of septum degradation after cytokinesis. How septin rings are maintained and cell separation is inhibited in hyphae has not yet been defined, despite the crucial importance of these issues for *C. albicans* morphogenesis. Here, we show that the properties of the septin ring are modified in hyphae in a Sep7- and Hgc1-dependent manner to inhibit cell separation in hyphal compartments.

Septin Ring Dynamics Changes upon Induction of the Developmental Switch in *C. albicans*

FRAP analysis revealed that in *C. albicans* the dynamics of the septin rings differed markedly, depending on the growth morphology. In yeast cells, septin ring dynamics was similar to that described for *S. cerevisiae* (Dobbela *et al.*, 2003). Thus, septin ring subunits showed no exchange of septins with the cytoplasm during yeast growth. However, we cannot rule out the possibility that under these growth conditions most of the septins are already incorporated in the

septin ring and the cytoplasmic pool is too low to observe recovery.

During *C. albicans* hyphal growth, three septin structures have been described: a diffuse, unstable band at the base of the germ tube; a double ring at septation sites; and a diffuse cap at hyphal tips (Sudbery, 2001; Warena and Konopka, 2002). We show that the induction of hyphal growth brings about important modifications in septin ring dynamics, because the rings were found in a different state from those of yeast cells. This hyphal-specific state contains a core of stable septins (Sep7, Cdc3, and Cdc12), and it shows a high Cdc10 turnover between the ring and the cytoplasm. At first sight, it seems paradoxical that persistence of the rings after cytokinesis is associated with high turnover of one of the septin subunits. However, the core of Cdc3, Cdc12, and Sep7 might be sufficient to guarantee the stability of the rings, whereas the increase in Cdc10 exchange might explain the different properties of hyphal rings. High Cdc10 exchange can substantially alter the surface and binding properties of the rings, modifying the scaffold function. Because the stoichiometry of the septins at the ring did not change between yeast and hypha, the high turnover rate observed in hyphal rings could be due to an increase in the dissociation rate of Cdc10 from the stable core.

Our results also indicate that the increase in Cdc10 exchange is Sep7 dependent. It is noteworthy that whereas in *S. cerevisiae* Shs1 acts in septin ring stabilization, in *C. albicans* it is required to maintain a high exchange rate of Cdc10 between the septin ring and the cytoplasm during hyphal development. It is possible that *C. albicans* septins might be organized in a different manner or stabilized by different factors. In fact, some key differences between septin rings in these two yeast have already been described: in *C. albicans*, *cdc11Δ* mutants are still able to form a septin ring, even at elevated temperatures, in contrast to the essential role proposed for *S. cerevisiae* Cdc11 in septin filament formation (Warenda and Konopka, 2002; Versele *et al.*, 2004). Alternatively, it is also possible that Sep7 function might be modified by hyphal-specific factors, such as the Hgc1 cyclin, to alter septin ring dynamics during this developmental state.

Septin Ring Dynamics and Cell Separation

Septin rings act as a scaffold for the recruitment of cellular factors required for cytokinesis and cell separation (Versele and Thorner, 2005). Both in yeast and hypha, cells are able to assemble separation septa, indicating that recruitment of the septation apparatus to the division plate is independent of the septin ring state. However, our results reveal a clear correlation between the modification of septin ring dynamics and the inhibition of separation observed during hyphal growth. First, yeast cells showed little or no exchange of Cdc10 subunits with the cytoplasm, whereas hyphal cells had a high Cdc10 turnover. Second, *sep7Δ* hyphae showed a low Cdc10 exchange and activated cell separation in subapical compartments. Third, deletion of *CDC14*, a key activator of cell separation (Clemente-Blanco *et al.*, 2006), eliminated the cell separation phenotype of *sep7Δ* mutants without altering septin dynamics.

One interesting possibility to explain how septin ring dynamics could control cell separation is that high Cdc10 exchange could interfere with the translocation of cell separation activators to septum. In fact, when Cdc10 turnover was eliminated by deletion of *SEP7*, the Cdc14 phosphatase localized to the division site in hyphae. Interestingly, deletion of *CDC14* in *sep7Δ* cells abolished the separation phenotype without altering septin ring dynamics, suggesting that Cdc14 translocation to the septum does not control septin ring dynamics, but that it is a signal required to activate the cell separation program mediated by the transcription factor Ace2 (Kelly *et al.*, 2004). These observations also suggest that Cdc14 recruitment to the septum could be required for the dephosphorylation of the proteins that control Ace2 function. In *S. cerevisiae*, the Cbk1–Mob2 complex localizes to the septum region, and it is required for the accumulation of Ace2 in the daughter nucleus (Weiss *et al.*, 2002; Nelson *et al.*, 2003). In *C. albicans*, Cbk1 localizes to the septum in yeast and hyphal cells (our unpublished results), and *cbk1Δ* mutants also have cell separation defects (McNemar and Fonzi, 2002). It is tempting to speculate that Cdc14 targeting to the septum would be the signal that activates cell separation through Cbk1–Mob2, although further experiments will be required to test this hypothesis.

Regulation of Sep7 by Hgc1

Hyphae are different from yeast cells in that their septin rings are not disassembled after the completion of cytokinesis (Sudbery, 2001; Warenda and Konopka, 2002). This suggests that hyphal-inducing signals control cellular factors that modify septin assembly/disassembly during this growth pattern. In fact, it has been recently shown that the *C. albicans* Cdk Cdc28, through its association with the Ccn1

cyclin, rapidly establishes phosphorylation of the Cdc11 septin and that this is necessary for hyphal morphogenesis (Sinha *et al.*, 2007). Interestingly, the Cdc28–Hgc1 complex is necessary to maintain Cdc11 phosphorylation during hyphal growth and to ensure proper hyphal development. Our results indicate that the Hgc1 hyphal-specific cyclin (Zheng *et al.*, 2004) also plays an important role in the conversion of the septin rings to the hypha-specific state. It is interesting that the phenotypes of mutants lacking either *HGC1* or *SEP7* were similar in the sense that they recruited Cdc14 to the septum region and activated cell separation when normally it should be inhibited. In addition, mutants lacking *HGC1* failed to convert the septin rings to the hyphal-specific state, suggesting that Hgc1 might control Cdc10 turnover during hyphal development. Sep7 protein levels and its phosphorylation were reduced in the *hgc1Δ* mutant during hyphal growth, whereas no changes in *SEP7* expression were observed, indicating that the role of Hgc1 in the control of hyphal septin rings dynamics might be to modulate Sep7 protein stability and/or its phosphorylation status. However, we do not know whether the Hgc1–Cdc28 complex phosphorylates Sep7 directly or whether it activates another kinase that would phosphorylate the septin. It will be interesting to map Sep7 phosphorylation sites during yeast and hyphal growth and analyze the effects of their mutation on septin ring dynamics.

Highly conserved mechanisms or structures between yeast and mammals cells may be specifically modified in different cell types or organisms to control different processes. Characterization of Sep7 function during hyphal growth suggests that *C. albicans* might have converted a mechanism normally used in *S. cerevisiae* to control septin ring dynamics during the cell cycle to modify the stability of the septin ring in response to the special growth characteristics during hyphal development. The inhibition of cell separation that is essential for hyphae morphogenesis relies on the modification of septin ring dynamics dependent on the hyphal-specific cell cycle machinery.

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