In *Candida albicans*, the Nim1 kinases Gin4 and Hsl1 negatively regulate pseudohypha formation and Gin4 also controls septin organization

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In the development of hyphal germ tubes of *Candida albicans*, a band of septin forms at the base of the germ tube (basal septin band). Later, a septin ring forms, which organizes the first septum within the germ tube (septin ring). We have investigated the role of the Nim1 kinases, Gin4 and Hsl1, in the formation of these septin structures. We show that during germ tube formation, Gin4 is required for the organization of the septin ring but not the basal septin band. Hsl1 is not required for the formation of either septin rings or basal bands. Unexpectedly, we found that both *gin4Δ* and *hsl1Δ* mutants form pseudohyphae constitutively, in a fashion that in the case of *gin4Δ*, is partly independent of Swe1. Gin4-depleted pseudohyphae are unable to form hyphae when challenged with serum, but this can be overcome by ectopic expression of Gin4 from the *MET3* promoter. Thus, Gin4 may regulate the developmental switch from pseudohyphae to hyphae.

Introduction

The human fungal pathogen *Candida albicans* can grow as yeast, pseudohyphae, or true hyphae (Odds, 1985; Gow, 1997; Brown and Gow, 1999; Berman and Sudbery, 2002). The unicellular yeast form closely resembles the budding yeast *Saccharomyces cerevisiae*. The pseudohyphal form consists of chains of cells that can display varying degrees of elongation, but retain a constriction at the septa that separate adjacent cellular compartments (Merson-Davies and Odds, 1989). The hyphal form consists of hyphae with parallel-sided walls with no constrictions at the septal junctions. It is thought that the ability to switch between the different morphological forms may be required for virulence because the different morphological forms are found in infected tissue and environmental signals that induce hyphae also coordinately induce a variety of known virulence factors such as the *SAP* family of aspartyl proteases (for reviews see Liu, 2001; Sudbery, 2001; Berman and Sudbery, 2002; Brown, 2002). Moreover, strains that are prevented from forming hyphae because of mutations in signal transduction pathways are avirulent (Lo et al., 1997).

*C. albicans* yeast cells can be induced to form hyphae or pseudohyphae by a variety of environmental signals such as the presence of serum, 35–37°C growth temperature, and neutral pH (Odds, 1985; Sudbery, 2001). The presence of serum at a growth temperature of 35°C, pH 6, results in a culture consisting almost entirely of the hyphal form, whereas in the absence of serum at 35°C, pH 6, cells develop as long pseudohyphae (Sudbery, 2001). Although pseudohyphae superficially resemble true hyphae, it is becoming increasingly clear that there are fundamental differences between the two forms in the organization of the cell cycle and the septin cytoskeleton (Sudbery, 2001). In the development of pseudohyphae, a septin ring forms at the junction between the mother cell and the daughter cell. The first mitosis and formation of the primary septum take place across the plane of this septin ring. Thus, the daughter cell behaves as a bud, modified by hyperpolarized growth. In hyphal development, germ tube evagination occurs before the cell cycle has initiated in the mother cell (Hazan et al., 2002). A band of longitudinal septin bars forms at the base of the germ tube, which later becomes faint and disorganized (Sudbery, 2001). A cap of septin is also present at the germ tube tip (Warenda and Konopka, 2002). A septin ring then
appears within the germ tube, 10–15 μm from the mother cell, probably marking the initiation of the cell cycle. The nucleus migrates out of the mother cell and the first mitosis takes place across the plane of this septin ring. After mitosis, the septin ring organizes the formation of the primary septum (for review see Berman and Sudbery, 2002). Throughout this paper the band of septin at the base of the germ tube will be referred to as the “basal septin band” and the ring within the germ tube, which organizes the formation of the primary septum, will be referred to as the “septin ring”.

In S. cerevisiae, septin rings at the bud neck provide a scaffold for proteins that are required for cytokinesis (for review see Longtine and Bi, 2003). The proper organization and function of the septin ring requires Gin4, a homologue of the Nim1 kinase of Schizosaccharomyces pombe (Russell and Nurse, 1987). In a gin4Δ mutant, septin is deposited in a series of longitudinal bars around the bud neck instead of a continuous ring (Longtine et al., 1998a, 2000). Gin4 localizes to the bud neck and this localization is septin dependent (Okuzaki et al., 1997; Longtine et al., 1998b; Barral et al., 1999). Therefore, the function and localization of septins and Gin4 are mutually interdependent. Mutations that disrupt septin organization cause an impairment of cytokinesis and bud elongation. This is due to the induction of the morphogenesis checkpoint that delays mitosis and prevents a switch from polarized to isometric growth of the bud (Lew and Reed, 1993, 1995a). The morphogenesis checkpoint is dependent on Swe1, the S. cerevisiae homologue of the S. pombe Wee1 kinase (Booher et al., 1993), which inhibits Cdc28 by phosphorylation of tyrosine 19 (Sia et al., 1996). A swe1Δ mutation abrogates the morphogenesis checkpoint in a temperature-sensitive cdc3 mutant and consequently exacerbates its growth defect (Barral et al., 1999). The morphogenesis checkpoint operates through control of Swe1 stabilization. When first synthesized in G1, Swe1 accumulates in the nucleus. After bud formation it is targeted to the mother side of the bud neck and it is degraded in the G2/M phase (McMillan et al., 1999). Swe1 also exerts a cell size checkpoint over the onset of mitosis (Harvey and Kellogg, 2003). This observation has given rise to an alternative model to the morphogenesis checkpoint in which it is postulated that abnormally prolonged bud growth occurs in cells lacking Gin4 because the signal indicating that cell size has passed the critical size threshold is not transmitted to Swe1. Apart from Gin4, the S. cerevisiae genome encodes two other Nim1-kinase homologues, Hsl1 and Kcc4. These also localize to the bud neck in a septin-dependent fashion (Barral et al., 1999). However, septin organization is not dependent on either kinase (Longtine et al., 2000), although Hsl1 is required for the transmission of the checkpoint signal to Swe1.

The C. albicans genome contains a single homologue of the S. cerevisiae SWE1 gene and two Nim1-kinases homologous to S. cerevisiae GIN4 and HSL1, respectively. In this paper, we have investigated the role of these genes in the organization of the basal septin band and the septin ring in hyphal germ tubes of C. albicans. We show that Gin4 is required for the organization of the septin ring but not the basal septin band. Thus, the difference in organization between these two structures may be due to the action of Gin4. Hsl1 is not required for the organization of either structure. Unexpectedly, we found that both gin4Δ and hsl1Δ mutants constitutively form pseudohyphae and that these pseudohyphae are unable to form hyphae when challenged with serum. Furthermore, as wild-type cells develop pseudohyphae, the level of Gin4 declines and they also become unable to form hyphae upon serum challenge. However, high levels of Gin4, ectopically expressed from the MET3 promoter, overcome this block. Thus, in addition to organizing the septin ring, Gin4 appears to determine the developmental potential of pseudohyphae.

Results

C. albicans homologues of Nim1 kinases

We identified two homologues of Nim1 kinases in the C. albicans database assembly 19 (http://www-sequence.stanford.edu/group/candida/): ORF19.663 and ORF19.4308. A structural comparison of these genes with the S. cerevisiae GIN4 and HSL1 genes is shown diagrammatically in Fig. 1 and the full CLUSTALW alignment, including RCC4, is shown in Fig. S1 (available at http://www.jcb.org/cgi/content/full/jcb.200307176/DC1). Neither C. albicans Nim1 kinase could be easily defined as homologous to one of the three S. cerevisiae Nim1 kinases on the basis of the overall degree of similarity. However, the CLUSTALW alignment reveals that C. albicans ORF19.4308 shares an NH2-terminal extension with S. cerevisiae Hsl that is not present in C. albicans ORF19.663, S. cerevisiae Gin4, or Kcc4. Moreover, C. albicans ORF19.663 shares three motifs that are uniquely shared with S. cerevisiae Gin4. Based upon these observations, ORF19.663 and ORF19.4308 will be designated as C. albicans homologues of GIN4 and HSL1, respectively. The results in this paper are consistent with this conclusion. Furthermore, annotations in the C. albicans databases at Stanford (URL provided above) and Galar Fungal (http://genolist.pasteur.fr/CandidaDB/) use the same designations for these ORFs.

Loss of Gin4 results in chains of elongated cells

Both copies of the GIN4 gene were sequentially deleted in C. albicans strain BWP17. The resulting homozygous deleted strain will hereafter be referred to as gin4Δ. In addition, a conditional gin4 mutant was constructed in which one copy of GIN4 was deleted and the remaining copy was placed under the control of the regulatable MET3 promoter (Care et al., 1999). This strain will be referred to hereafter as MET3-GIN4.
failed to form (Fig. 2 e). An identical phenotype was seen in the MET3-GIN4 strain when the MET3 promoter was turned off (Fig. 2, f–h). Calcofluor staining showed that primary septa fail to form (Fig. 2 g). Furthermore, immunocytofluorescence, using polyclonal antisera raised against S. cerevisiae Cdc11, showed that septin rings failed to form properly (Fig. 2 h). Cells were indistinguishable from wild type when the MET3 promoter was turned on (Fig. 2, i–k), indicating that the gin4Δ phenotype is due to loss of Gin4.

Cell elongation in the MET3-GIN4 strain after 6 h of growth in MET3-repressing medium was quantified by measuring the axial ratio (length/width). The results are presented in Table I, which also quantifies nuclear distribution. (This quantification was not performed in gin4Δ mutants because the strain grew in very large clumps that were difficult to separate sufficiently to count a large number of cells.) The axial ratio increased over 2.5-fold when Gin4 was turned off. The disturbance to septin ring formation and the consequent failure to form a primary septum explains the cytokinesis defect in gin4Δ cells. The distribution of nuclei was perturbed with a high proportion of the compartments containing either no nuclei, or more than one nucleus (Table I; Fig. 5 a).

A similar phenotype is observed in S. cerevisiae gin4Δ mutants, but only when the gin4Δ mutation is combined with null alleles of CLB1, 3, 4 or in other genes required for septin ring formation (Altman and Kellogg, 1997; Longtine et al., 1998a, 2000; Barral et al., 1999). Thus, Gin4 plays a more essential role in C. albicans than S. cerevisiae. Alternatively, there may be redundancy for Gin4 function in S. cerevisiae. This may be supplied by Kcc4, which is known to have partially overlapping functions with Gin4 (Okuzaki et al., 2003), but which has no C. albicans homologue.

C. albicans gin4Δ mutants are hyperinvasive

C. albicans gin4Δ cells invade the agar substratum. In contrast to wild type, gin4Δ colonies cannot be washed off a yeast extract peptone dextrose (YPED; 2% glucose, 2% Dico-Bacto Peptone, 1% Dico-Bacto yeast extract) agar plate after a 3-d growth at 30°C (Fig. 3 a). Microscopic examination of a transverse section of the plate shows extensive penetration of the agar by gin4Δ cells, but no penetration by wild-type cells (Fig. 3 b). Agar invasion is characteristic of hyphal and pseudohyphal cells. The degree of penetration of gin4Δ cells growing at 30°C in the absence of serum penetrate the agar at least as much as wild-type hyphal and pseudohyphal cells (Fig. 3 b). However, there is a clear difference in the pattern of invasion. A lower proportion of wild-type hyphal and pseudohyphal cells appear to become

**Table I. Axial ratio of Gin4 and Swe1-depleted cells**

| Axial ratio (n)          | 0  | 1  | 2  | 3  | n  |
|-------------------------|----|----|----|----|----|
| SWE1 MET3-GIN4 on       | 1.49 ± 0.05 (95) | 0.5 | 99.5 | 0  | 0  | 188 |
| swe1Δ MET3-GIN4 on      | 1.24 ± 0.03 (122) | 1.0 | 86.6 | 12.4 | 0  | 203 |
| SWE1 MET3-GIN4 off      | 3.87 ± 0.03 (103) | 17.7 | 35.9 | 24.8 | 21.5 | 181 |
| swe1Δ MET3-GIN4 off     | 2.36 ± 0.1 (107) | 11.1 | 35.5 | 29.6 | 23.7 | 152 |
invasive and they form structures with a feathery appearance that is not apparent in gin4Δ cells growing at 30°C. When grown at 37°C, gin4Δ cells are considerably less invasive than wild-type cells and gin4Δ cells growing at 30°C. Addition of serum at this temperature mildly stimulates invasion. The invasive growth defect is not due to a general temperature-sensitive growth defect because a comparison of growth of the gin4Δ strain on solid YEPD medium at 25°C, 30°C, 34°C, and 37°C, shows no obvious differences after a 5-d incubation. However, growth is significantly reduced in the mutant strain compared with wild type at all growth temperatures (unpublished data).

**The C. albicans SWE1 homologue**

In *S. cerevisiae*, the elongated cell-chain morphology of septin and gin4Δ mutants is dependent on Swe1 function. Therefore, we characterized the *C. albicans* Swe1 homologue in order to determine whether this was the case in *C. albicans*. We also wished to test the hypothesis that hypha formation is due to the operation of the morphogenesis checkpoint. We identified a single SWE1 homologue in the *C. albicans* database (ORF19.4867; note that the corresponding sequence in assembly 6 and the annotation in the Galar Fungal database is erroneous in the 3’ region of the sequence due to incorrect contig assembly). Alignments of ORF19.4867 with Swe1 homologues of other eukaryotes are shown in Fig. S2 (available at http://www.jcb.org/cgi/content/full/jcb.200307176/DC1).

To confirm that this sequence encodes the homologue of *S. cerevisiae* Swe1, we amplified it by PCR and expressed it from the GAL1 promoter on the multicopy pYES2 vector in *S. cerevisiae* Σ1278b wild-type and swe1Δ strains. Σ1278b responds to 0.5% isoamyl alcohol (IAA) by growing in filaments. This response is entirely dependent on Swe1 (Martínez-Anaya et al., 2003). When expressed on galactose, the *C. albicans* SWE1 completely complemented the filamentation defect of a Σ1278 swe1Δ mutant (Fig. 4, a–c) but had no effect in the absence of IAA (Fig. 4 d). The Mih1 tyrosine phosphatase reverses the inhibition of Cdc28 exerted by Swe1. Thus, mih1Δ strains are more sensitive to overexpression of SWE1. Overexpression of the *C. albicans* SWE1 gene in a Σ1278 mih1Δ background resulted in highly elongated cells, indicative of a strong G2/M cell-cycle delay (Fig. 4, d–f). Thus, ORF19.4867 encodes a functional homologue of *S. cerevisiae* Swe1.

*Fig. 3. gin4Δ cells are hyperinvasive.* (a) Wild-type (BWP17) and gin4Δ cells were grown on YEPD agar plates for 7 d. Colonies were photographed before and after the plates had been washed. (b) Wild-type and gin4Δ cells were patched over an area of a YEPD plate or a YEPD plate containing 20% serum and incubated at 30°C or 37°C, as indicated, and incubated for 7 d. After washing, transverse sections were cut from the plate and examined using a dissecting microscope.

*Fig. 4. The C. albicans SWE1 gene.* (a–f) *S. cerevisiae* cells of the indicated genotype were grown for 16 h in the presence of galactose to induce the GAL1 promoter. IAA, isoamyl alcohol. (g–i) *C. albicans* swe1Δ cells were grown to stationary phase as yeast and reinoculated for 2 h to form hyphae (g), pseudohyphae (h), or yeast (i). Bars, 10 μm.
control over the onset of mitosis, and in the absence of Swe1 daughter cells are born abnormally small (Harvey and Kellogg, 2003). Consistent with these observations we found that the swe1Δ allele also led to a 10% reduction in cell size in C. albicans: the mean volume of swe1Δ GIN4-on mother cells of the yeast form was 32.8 ± 0.66 fl (n = 122), whereas the mean volume in SWE1 GIN4-on mother cells was 36.7 ± 2.3 fl (n = 95). The swe1Δ mutant formed hyphae and pseudohyphae normally (Fig. 4, g and h). Therefore, neither hypha or pseudohypha formation is brought about by the activation of the morphogenesis checkpoint.

The pseudohyphal phenotype of gin4Δ cells is at least partially independent of Swe1

The pseudohyphal phenotype of the C. albicans gin4Δ strain could be due to the action of Swe1 acting to either exert a morphogenesis or cell size checkpoint. To address this issue, we constructed a MET3-GIN4 swe1Δ strain. When Gin4 was depleted in a swe1Δ mutant, cells were slightly less elongated than SWE1 cells (Table I), suggesting that some of the elongation was due to the action of Swe1. Further evidence for the operation of a Swe1-dependent checkpoint was a growth defect when Gin4 expression was turned off in swe1Δ MET3-GIN4 cells, that was not evident when Gin4 expression was turned off in SWE1 cells (Fig. 5 c). Together, these observations suggest that depletion of Gin4 triggers a Swe1-dependent checkpoint. However, SWE1 is not entirely responsible for the pseudohyphal phenotype observed because cells remained significantly elongated when GIN4 expression is turned off in swe1Δ strains. Furthermore, swe1Δ GIN4-off cells remained capable of agar invasion (Fig. 5 d). Thus, the pseudohyphal phenotype observed in gin4Δ cells is partly independent of a Swe1-dependent checkpoint.

Gin4 is required for the transition from pseudohyphae to hyphae

To investigate further the requirement of Gin4 for hyphal development, we made use of the conditional MET3-GIN4 allele. Cells were grown to stationary phase with the MET3 promoter induced. The resulting culture, consisting of over 95% unbudded yeast cells, was then reinoculated into serum-containing medium at 37°C with the MET3 promoter either induced or repressed (GIN4-on and GIN4-off, respectively). Germ tubes evaginated at the same rate in both cultures and the resulting hyphae were identical in appearance (Fig. 6, a and b). Hyphal growth continued normally in these cultures for at least 6 h.

One interpretation of this experiment is that pseudohyphal cells, but not yeast cells, require Gin4 to form hyphae when challenged with serum. To test this hypothesis, we designed an experiment in which MET3-GIN4 and the parental BWP17 cells were grown to stationary phase in derepressing medium. The unbudded yeast cells were then cultured as pseudohyphae for various times before being challenged to make hyphae by the addition of serum. Wild-type cells rapidly lost the ability to form hyphal germ tubes so that by 6 h <5% of elongated pseudohyphal cells formed hyphae (Fig. 6 c). In contrast, 50% of control cells, incubated in conditions that promote yeast growth, formed hyphae. This observation suggests that the pseudohyphal state is antagonistic to hypha formation. However, when the MET3-GIN4 strain was induced during pseudohyphal growth, the proportion of elongated cells forming hyphae was only slightly lower than the wild-type control cells grown as yeast, suggesting that high levels of Gin4 can overcome the inability of pseudohyphae to form hyphae.

To confirm that levels of Gin4 are elevated when ectopically expressed from the induced MET3 promoter, we tagged Gin4 with GFP in the MET3-GIN4 strain (MET3-GIN4-GFP) and also in the wild-type strain so that GIN4-GFP expression was under the control of the GIN4 promoter (GIN4-GFP). The capacity of these strains to form hyphae, after incubation as pseudohyphae, was similar to that observed in the previous experiment (Table S2, available at http://www.jcb.org/cgi/content/full/jcb.200307176/DC1). Cellular Gin4-GFP levels were monitored by Western blots using a mixture of two mAbs to GFP. During incubation as pseudohyphae, Gin4-GFP levels were much higher in the induced MET3-GIN4-GFP culture compared with the GIN4-
Gin4 is required for the formation of the septin ring but not the basal septin band

Hyphal germ tubes that formed in Gin4-depleted cells induced from the yeast state were examined to determine the pattern of septin ring formation by immunocytofluorescence using polyclonal antisera to *S. cerevisiae* Cdc11 (aCdc11; Sudbery, 2001). The appearance of basal septin bands and septin rings in representative cells is shown in Fig. 7 (a–e) and quantified in Fig. 7 (h and i). The location of nuclei is also shown in Fig. 7 (a–e). By 90 min, a septin ring had formed in 30% of germ tubes regardless of whether Gin4 was turned on or off (Fig. 7 i). In Gin4-off cells, the proportion declined to 10% by 180 min and all septin rings had completely disappeared by 210 min (Fig. 7 i) and primary septa failed to form (Fig. 7 f). In contrast, when *Gin4* was turned on, the proportion of cells with a ring in the germ tube had increased to nearly 90% by 180 min (Fig. 7 i) and primary septa formed normally (Fig. 7 g). The first mitosis occurred at the normal time and in the normal position (Fig. 7 c) in *Gin4*-off cells. Thus, although in wild-type cells mitosis takes place across the plane of the septin rings.
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Figure 7. Properties of gin4Δ hyphae. MET3–GIN4 cells were grown to stationary phase under derepressing conditions. The resulting unbudded cells were reinoculated into derepressing (GIN4 on) or repressing (GIN4 off) medium containing 20% serum and stained with αCdc11 antibody (a–e, false color green) and DAPI (a–e, false color blue) or calcofluor (f and g). Arrows in b and d indicate examples of a basal septin band. Arrow in c indicates apical staining. Arrow in g indicates primary septum. All panels are at the same magnification. Bar, 10 μm. (h and i) Quantitation of cells with tubes and septin bands. A minimum of 70 cells was counted for each time point.

Figure 8. Properties of hyphae dependent on the MET3 regulated expression of GIN4-GFP. MET3–GIN4 cells were grown to stationary phase in derepressing medium, and then reinoculated into fresh medium containing serum at 37°C in repressing or derepressing conditions as described in Fig. 7. The percentage of evagination, germ tubes with septin rings, basal septin bands, and two nuclei were recorded. A minimum of 50 cells were counted for each time point. Levels of Gin4–GFP were monitored during the time course by Western blot analysis (e) and quantified (f) as described in supplemental material. Arrows indicate the Gin4-GFP band. The faint band beneath the Gin4 band is a nonspecific band present in non-GFP tagged strains. We have also used this as a loading control, with similar results to those presented.

(587)
taining the untagged MET3-GIN4 (compare Fig. 8 c with Fig. 7 h). Thus, although septin ring formation was delayed by the Gin4–GFP fusion, the appearance of basal septin bands was not affected. Again, nuclear migration and mitosis were not delayed and took place in the absence of septin rings (Fig. 8 d).

In the GIN4-off culture, no septin rings had appeared by 300 min (Fig. 8 b), but basal septin bands were again apparent in newly evaginated germ tubes (Fig. 8 c). Nuclear division was delayed by a small amount compared with the GIN4-on culture, but still took place in the absence of septin rings (Fig. 8 d). Gin4 levels were monitored by a Western blot (Fig. 8, e and f). Low levels of Gin4 were present at the start of the experiment but thereafter rapidly declined and Gin4 was undetectable by 300 min. In contrast, the level of Gin4 steadily increased when the MET3 promoter was turned on. Thus, expression of GIN4 from the MET3 promoter is tightly regulated and no Gin4 was detectable at the time (300 min) when second germ tubes form, which contain basal septin bands. Furthermore, fusing Gin4 to GFP delays the formation of septin rings, but has no effect upon the formation of basal septin bands. Together, these results suggest that Gin4 is required for the formation of the septin ring but is not required for the basal septin band or the apical cap.

**Gin4 localizes to the septin ring but not to the basal septin band**

To further test the hypothesis that Gin4 is required for the germ tube ring, but not the basal septin band, we examined the localization of Gin4-GFP in yeast, pseudohyphae, and hyphal germ tubes. We found that it localizes to the bud neck of pseudohyphae (Fig. 9 a), yeast (Fig. 9 b), and to a ring in the germ tube consistent with the position of the septin ring (Fig. 9 e). In striking contrast, we never observed any Gin4 localization to the base of developing hyphae (Fig. 9 c) where the basal septin band forms (Fig. 9 d).

**Hsl1 also regulates pseudohypha formation, but Hsl1 is not required for septin ring organization or septum formation**

To investigate the role of Hsl1, we constructed a homozygous null mutant (hsl1Δ) and a strain in which one copy of HSL1 was deleted and the other copy expressed from the MET3 promoter (MET3-HSL1). The phenotype of the MET3-HSL1 strain under repressed conditions was indistinguishable from the hsl1Δ strain, demonstrating that the hsl1Δ phenotype was entirely due to loss of Hsl1 (unpublished data). When grown in liquid YEPD culture at 30°C, the morphology of the hsl1Δ mutant was dependent on cell density. At the beginning of the growth cycle, when the cell density was low, it grew with a constitutive pseudohyphal morphology (Fig. 10 a). Compared with gin4Δ cells, hsl1Δ pseudohyphae had shapes that are more regular, clear constrictions between cellular compartments and branching filaments typical of pseudohyphae. Unlike the gin4Δ mutant, septin rings formed normally in the hsl1Δ mutant (Fig. 10 b). Chitin rings appeared at the necks of new compartments and a primary septum subsequently separated adjacent compartments (Fig. 10 c). Three-dimensional restoration microscopy indicated that this septum was intact across the entire width of the bud neck (Fig. 10 d).

At later stages in the growth cycle, when cell density was high, the hsl1Δ mutant reverted to the yeast morphology.
characteristic of wild-type cells grown in these conditions. These yeast cells were able to respond to serum by developing germ tubes. Septin rings and the basal septin band formed normally in hsl1Δ hyphae (Fig. 10 e). Thus, in yeast and hyphae, Hsl1 is not required for septin ring formation. This closely parallels its function in S. cerevisiae, where it has been shown to act downstream of septin ring formation (Longtine et al., 2000).

Discussion

Gin4 is required for cytokinesis, but Nim1-kinases also negatively regulate pseudohyphal formation

Deletion of the GIN4 gene in C. albicans disrupted the formation of septin rings, caused a severe cytokinesis defect, and gave rise to chains of elongated cells, which resemble pseudo-hyphae. A priori, the most conservative interpretation of this pseudohyphal appearance is that it arises from the combination of the failure to complete septum formation combined with cell elongation caused by the action of Swel. The exact role of Swel in S. cerevisiae is currently unclear. The morphogenesis checkpoint model posits that it exerts a checkpoint in response to defects in bud formation (Lew and Reed, 1995a,b; Sia et al., 1996). The size control model posits that it delays mitosis until a critical cell size threshold is exceeded (Harvey and Kellogg, 2003). This model explains prolonged bud growth in gin4Δ mutants by supposing that they form part of a network that monitors cell size. Regardless of which model is correct, the action of Swel in gin4Δ mutants could result in a phenotype that misleadingly resembles pseudohyphae, i.e., they are "pseudo pseudohyphae".

Several observations suggest that gin4Δ mutants may form genuine pseudohyphae. First, gin4Δ cells vigorously invaded the agar substratum under conditions that do not stimulate invasive growth in wild-type cells. The extent of the invasion was at least as great as that observed during hypha and pseudohypha formation in wild-type cells, although the morphology of the invading structures was different. A cytokinesis defect was reported recently to result from mutations in genes encoding septins (Warenda and Konopka, 2002). These septin mutants are not constitutively invasive, in fact, they are defective in invasive growth (Warenda and Konopka, 2002). Therefore, invasiveness is not simply a consequence of the chains of cells that result from a failure of cytokinesis. Second, chains of elongated cells still formed in a swe1Δ mutant depleted of Gin4, although the extent of cell elongation was reduced. This observation suggests that although a gin4Δ mutation induces Swe1-dependent elongation, approximately half of the cell elongation is independent of Swe1. Moreover, these cells still invaded agar, thus agar invasiveness is also not dependent on Swe1. Third, gin4Δ mutants were unable to form true hyphae in response to serum. Again, this contrasts with septin mutants that formed germ tubes, although there were some abnormalities in their shape. Therefore, the failure to complete cytokinesis cannot explain the inability to respond to serum. Fourth, like gin4Δ mutants, hsl1Δ mutants also have a constitutive pseudohyphal phenotype, although septin ring organization is unaffected and septum formation takes place normally. Indeed, the hsl1Δ mutants were more convincingly pseudohyphal than gin4Δ cells, possibly because there was no additional complication of a failure to properly organize the septin ring and septate. Together, these results suggest that cells depleted of either Nim1 kinase become constitutively pseudohyphal and therefore that Nim1-kinases, Gin4 and Hsl1, act as negative regulators of pseudohyphal development.

Pseudohyphae are in a developmental state that precludes the formation of hyphae

Pseudohyphae that form because of either Gin4 or Hsl1 depletion cannot form hyphae. However, using the conditional MET3-GIN4 allele, we showed that when yeast cells are produced in the presence of Gin4, they are able to form hyphae when Gin4 is repressed during the serum challenge. One explanation of this observation is that the pseudohyphal state blocks the formation of hyphae. We tested this hypothesis using the conditional MET3-GIN4 allele. Yeast cells formed in the presence of Gin4 were incubated in pseudohyphae-inducing conditions for various times before being challenged to make hyphae by the addition of serum. Wild-type and MET3-GIN4 off cells lost the ability to develop into hyphae. This suggests that it is the pseudohyphal state that prevents Gin4-depleted cells from forming hyphae. However, MET3-GIN4 cells cultured in derepressing conditions retained the ability to form germ tubes. We used a GIN4–GFP fusion to follow Gin4 levels during this experiment. The results of a Western blot using mAbs to GFP showed that these cells expressed high levels of Gin4 in a phosphorylated form. Thus, high levels of Gin4 and/or Gin4 phosphorylation overcomes the block that normally prevents pseudohyphae from forming germ tubes. This block may result from the low levels of Gin4 observed in wild-type pseudohyphae. The continued hyphal growth after Gin4 depletion in the MET3-GIN4 off culture shows that Gin4 is not required for the maintenance of the hyphal state. When wild-type cells are challenged to make hyphae in the yeast state when Gin4 levels are low, germ tubes quickly emerge, suggesting that high Gin4 levels are not required for the yeast-hyphal transition.

Gin4 is required for the organization of septin rings but not basal septin bands

Multiple lines of evidence strongly suggest that Gin4 is not involved in the formation of the basal septin band. First, basal bands resemble the striated appearance of the septin ring in S. cerevisiae gin4Δ mutants. Second, when GIN4 expression is turned off using the MET3 promoter, basal septin bands persist after there is insufficient Gin4 to maintain septin rings. The slow kinetics of Gin4 depletion do not allow us to completely rule out the possibility that Gin4 is required for the organization of the basal septin band on the basis of this experiment. However, as Gin4 was depleted, septin rings disappeared but basal septin bands persisted. Moreover, by 300 min Gin4 was undetectable, but second germ tubes formed that displayed a basal septin band, but no septin rings. Third, Gin4-GFP locates to septin rings, but not basal septin bands. Fourth, a Gin4-GFP allele delays the appearance of septin rings but not basal septin bands.
Septin rings are not required for nuclear migration and mitosis

Nuclear migration and mitosis occurred normally in MET3-GIN4-GFP cells and MET3-GIN4-off cells. In the latter case, mitosis also occurred normally in the second germ tube that formed in the mother cell when the MET3 promoter was turned off. None of the germ tubes displayed a septin ring when mitosis occurred. Thus, the septin ring is not required for mitosis and the position where mitosis occurs cannot be determined by the position of the septin ring. Importantly, the location of the septin ring cannot be determined by the position of mitosis, because in wild-type cells, the septin ring forms in the germ tube before nuclear migration commences (Sudbery, 2001). Therefore, the location of the septin ring and the position where mitosis occurs must be specified by independent markers.

Hyphae and pseudohyphae do not result from induction of the Swe1-dependent morphogenesis checkpoint

In S. cerevisiae, the Swe1-dependent morphogenesis checkpoint, which delays mitosis and prevents the switch from polarized to isotropic growth. This has led to the suggestion that the operation of the morphogenesis checkpoint is the mechanism by which hyphae and/or pseudohyphae form (Kron and Gow, 1995). We have tested this hypothesis by constructing a swe1Δ mutant. There was evidence that Swe1 is acting to trigger a morphogenesis checkpoint in C. albicans, because cells are longer in a gin4Δ mutant compared with a gin4Δ swe1Δ mutant. Moreover, swe1Δ mutants accumulate a small fraction of cells with two nuclei, which are never observed in wild-type cells. However, all aspects of hyphal and pseudohyphal formation are normal in a swe1Δ mutant and we conclude that the morphogenesis checkpoint is not important for hyphal or pseudohyphal growth. This is consistent with the recent report that there is no change in the amount or timing of tyrosine phosphorylation of Cdc28p in developing hyphae compared with yeast (Hazan et al., 2002). Furthermore, pseudohyphae form normally in an S. cerevisiae swe1Δ mutant (Ahn et al., 1999).

Materials and methods

Strains and growth conditions

Strains used are listed in Table II. All C. albicans strains were derived from BWP17 (Wilson et al., 1999). All S. cerevisiae strains were derived from Σ1278b. YEPD and synthetic-defined media were prepared as described previously (Care et al., 1999; Sudbery, 2001), except that all media were supplemented with 50 mg/ml uridine. Induction of the MET3 promoter was achieved in minimal medium consisting of 0.67% (wt/vol) Difco yeast nitrogen base, 2% (wt/vol) glucose 50 mg/l Difco yeast nitrogen base, 2% (wt/vol) glucose 50 mg/l uridine, and any amino acids required by the strain (40 mg/l). MET3 expression was repressed with 0.5 mM cysteine and 2.5 mM methionine as described previously (Care et al., 1999). Conditions for hyphal and pseudohyphal cultures were prepared as described previously (Sudbery, 2001). Induction of filamentation by IAA was prepared as described previously (Martínez-Anaya et al., 2003).

Strain construction

Full details of strain construction are given in the supplemental material and oligonucleotide primers used are specified in Table S1 (available at http://www.jcb.org/cgi/content/full/jcb.200307176/DC1). In brief, disruption cassettes, consisting of either URA3, ARG4, or HIS3 selection markers, flanked by a 60-bp sequence of the target gene, were generated by PCR. These were used to transform C. albicans strain BWP17 (Wilson et al., 1999). Fusion of GIN4 to YFP was achieved by PCR cassettes as described previously (Gerami-Nejad et al., 2001). Fusions of the MET3 promoter to the GIN4 and HIS3 coding sequences were generated by a promoter replacement strategy as described previously (Care et al., 1999).

Western blot analysis of Gin4-GFP

Details of protein extraction procedures and antibodies used for Western blotting are provided in the supplemental material.

Microscopy

Immunofluorescence using a Cdc11 (Santa Cruz Biotechnology, Inc.) was performed as described previously (Sudbery, 2001). Chitin was stained with calcifluor white (Sigma-Aldrich) as described previously (Sudbery, 2001). DNA was stained with DAPI. Cells were examined either with a fluorescence microscope (model DMLB; Leica) or a microscope (model DeltaVision Linux 4; Applied Precision Instruments). Digital images were captured by a CCD camera (model RTE; Princeton Instruments) controlled by an Apple Macintosh G4 computer running Open Lab software version 2.2.5 (Improvision). Images were exported as TIFF files and edited in Adobe Photoshop version 5.5. Images from the DeltaVision microscope were captured, deconvolved, and three-dimensional images were reconstructed using the Softworx software suite supplied with the microscope. Composite figures were assembled using Microsoft PowerPoint 2000.

Axial ratio measurements

Cell images were captured using a microscope (model DMLB; Leica) using DIC optics and the 100X objective. Cell length was determined by mea-

Table II. Strains used in this study

| Strain | Genotype | Source |
|--------|----------|--------|
| C. albicans | swe1Δ | This study |
| BWP17 | ura3Δ/ura3Δ arg4Δ/arg4Δ his1Δ/his1Δ | Wilson et al., 1999 |
| MET3-GIN4 | gin4Δ::ARG4/gin4Δ::HS1 ura3Δ/ura3Δ arg4Δ/arg4Δ his1Δ/his1Δ | This study |
| MET3-HSL1 | hsl1Δ::URA3/URA3-MET3-HSL1 ura3Δ/ura3Δ arg4Δ/arg4Δ his1Δ/his1Δ | This study |
| MET3-GIN4-GFP | gin4Δ::ARG4/GFP/gin4Δ::HS1 ura3Δ/ura3Δ arg4Δ/arg4Δ his1Δ/his1Δ | This study |
| BWP-17 GIN4-GFP | gin4Δ::ARG4/GFP/gin4Δ::HS1 ura3Δ/ura3Δ arg4Δ/arg4Δ his1Δ/his1Δ | This study |
| MET3-GIN4-CDC10-GFP | gin4Δ::ARG4/GFP/gin4Δ::HS1 ura3Δ/ura3Δ arg4Δ/arg4Δ his1Δ/his1Δ | This study |
| S. cerevisiae | MATα ura3-52 | Ahn et al., 1999 |
| Σ1278b | MATα ura3-52 | Ahn et al., 1999 |
| CMS50 | MATα swe1Δ::kanMX6 ura3-52 | Martínez-Anaya et al., 2003 |
| SKY903 | MATα mih1Δ::LEU2 | Ahn et al., 1999 |

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suring the distance between the constrictions using Open Lab Version 2.2.5 software (Improvement). The width was determined at the widest part of the cell. The axial ratio is defined as length divided by width. In yeast cells, the axial ratio is reported for mother cells only as buds may still be growing in an apical fashion. In cells forming pseudohyphae in MET3-repressing conditions, only the penultimate cell in a chain of four or more cells was measured as these have completed their apical growth.

Cell size determination
The long and short axes of mother cells were measured as described in the previous paragraph. Cell volume was calculated according to the formula 4/3πab² where a is the radius of the long axis and b is the radius of the short axis (Sudbery et al., 1980).

Online supplemental material
Supplemental material provides details of strain construction, and methods and antibodies used in Western blots. Table S1 specifies sequence of oligonucleotides. Table S2 provides quantitation of the proportion of each culture producing germ tubes in the experiment described in Fig. 6 d. Fig. S1 provides a Clustal alignment of the C. albicans and S. cerevisiae Nim1 kinases. Fig. S2 provides a Clustal alignment of the C. albicans and S. cerevisiae Swel kinases. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200307176/DC1.

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