Candida albicans hyphal morphogenesis occurs in Sec3p-independent and Sec3p-dependent phases separated by septin ring formation

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Summary
The growing tips of Candida albicans hyphae are sites of polarized exocytosis. Mammalian septins have been implicated in regulating exocytosis and C. albicans septins are known to localize at hyphal tips, although their function here is unknown. Here, we report that C. albicans cells deleted of the exocyst subunit gene SEC3 can grow normal germ tubes, but are unable to maintain tip growth after assembly of the first septin ring, resulting in isotropic expansion of the tip. Deleting either of the septin genes CDC10 or CDC11 caused Sec3p mislocalization and surprisingly, also restored hyphal development in the sec3Δ mutant without rescuing the temperature sensitivity. Co-immunoprecipitation experiments detected association of the septin Cdc3p with the exocyst subunits Sec3p and Sec5p. Our results reveal that C. albicans hyphal development occurs through Sec3p-independent and dependent phases, and provide strong genetic and biochemical evidence for a role of septins in polarized exocytosis.

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Key words: Candida albicans, Exocytosis, Hyphal morphogenesis, Polarized growth, Septin

Introduction
Exocytosis plays an essential role in polarized cell growth by transporting molecules required for constructing new cell structures to the growth sites (Hsu et al., 2004). Precise temporo-spatial regulation of polarized exocytosis is important for diverse biological events such as bud formation in yeast (Drubin and Nelson, 1996; Longtine and Bi, 2003), epithelial cell polarization (Folsch, 2005) and neuron development (Clandinin, 2005). Polarized exocytosis occurs following at least three steps, which are evolutionarily conserved in eukaryotes. First, post-Golgi vesicles are transported through the secretory pathway to specific cortical regions via microtubule- or actin-based transport systems. Second, the vesicles are docked at specific sites of the plasma membrane (Pfeffer, 1999; Guo et al., 2000). Finally, the vesicle membrane fuses with the plasma membrane in a process mediated by interactions between the SNARE family proteins located both on the vesicles and at the target membrane (Rothman, 1994).

Targeting and docking of the secretory vesicles at the plasma membrane are assisted by a protein complex called exocyst (TerBush et al., 1996). In Saccharomyces cerevisiae (Sc) this complex comprises at least eight subunits, Sec3p, Sec5p, Sec6p, Sec8p, Sec10p, Sec15p, Exo70p and Exo84p, and localizes to sites of cell growth, such as the incipient bud sites, the tips of small buds, and the mother/daughter necks (TerBush and Novick, 1995; Finger and Novick, 1997; Finger et al., 1998; Guo et al., 1999). Sec3p is thought to serve as a spatial landmark for exocytosis (Finger et al., 1998) because it was found to localize to growth sites in each cell-cycle stage independently of other exocyst subunits, the actin cytoskeleton and the functioning of the secretory pathway (Finger et al., 1998). However, recent studies using antibodies raised against recombinant Sec3p showed that, like other exocyst subunits, Sec3p polarization requires ongoing secretion and polarized actin cables (Roumanie et al., 2005). Sec3p directly interacts with the key polarity regulators Cdc42p and Rho1p through its N-terminal region (Zhang et al., 2001) and with the exocyst subunit Sec5p through its C-terminus (Guo et al., 2001). Sec3p is required critically for the stability of the exocyst at 37°C. However, unlike all other exocyst subunits, Sec3p is not essential for cell growth at permissive temperatures (Wiederkahr et al., 2003).

Septins are filament-forming GTP-binding proteins and were first identified for their roles in cell-cycle progression in S. cerevisiae (Hartwell, 1971). These proteins are best known for their roles in cytokinesis in diverse organisms (Longtine and Bi, 2003; Kinoshita and Noda, 2001; Douglas et al., 2005; Versele and Thorner, 2005). In recent years, some septin members have also been found to have functions in postmitotic cells in animals, such as Caenorhabditis elegans and mammals (Hsu et al., 1998; Xue et al., 2004). In S. cerevisiae, septins assemble in a cell-cycle-dependent manner into scaffolds that recruit and organize proteins involved in various cell-cycle events including morphogenesis, spindle alignment, cytokinesis and bud site selection (Longtine et al., 1996; Longtine et al., 2000; Gladfelter et al., 2001; Faty et al., 2002; Longtine and Bi, 2003; Douglas et al., 2005). They also function as diffusion barriers, compartmentalizing cellular...
components (Takizawa et al., 2000; Barral et al., 2000; Dobbelare and Barral, 2004). Recently, Gladfelter et al. (Gladfelter et al., 2005) suggest that septins may play a role in defining the sites of cell growth, presumably by directing exocytosis towards them. Consistent with this hypothesis, several mammalian septins were found to associate with the exocyst (Hsu et al., 1998) and with the SNARE protein syntaxin (Beites et al., 1999; Kartmann and Roth, 2001). The septin CDCrel-1 directly regulates exocytosis in insulin-secreting cells in a syntaxin-dependent manner (Beites et al., 1999). Finger et al. (Finger et al., 2003) demonstrated that the two C. elegans septins UNC-59 and UNC-61 contribute to cell morphogenesis, migration and pathfinding in developing neurons, providing further evidence for a role for septin in polarity control.

C. albicans (Ca) is a polymorphic fungus with significant medical importance (Berman and Sudbery, 2002), and has been used as an excellent model for unraveling molecular mechanisms for cell polarity control. Several groups have studied the role of septin in C. albicans hyphal growth (Gale et al., 2001; Sudbery, 2001; Warenda and Konopka, 2002; Gladfelter, 2006) and made an intriguing observation: septins localize to the sites of germ tube emergence in G1 cells at a very early stage, and later a fraction of the molecules appears to persist at the growing tips over extended periods. Crampin et al. (Crampin et al., 2005) recently reported polarization at hyphal tips of the Spitzenkörper body, which acts to concentrate the delivery of secretory vesicles to the tip. In this study, we have investigated the possibility that septins may contribute to the regulation of C. albicans hyphal growth by directing exocytosis.

Results
Identification of C. albicans SEC3 gene
Because of the role of ScSec3p as a landmark for exocytosis and the viability of Scec3Δ mutants, we first identified its orthologue in C. albicans for subsequent mutational disruption of exocytosis. A BLAST-search of the C. albicans genome database (CGD, www.candidagename.org) using the ScSec3p sequence identified a candidate CaSec3p containing 788 amino acids (a.a.) (orf19.2911). The protein is markedly shorter than ScSec3p, which is 1336 a.a. long. Sequence alignment revealed that the two proteins have ~37% identity over a 150-a.a. region near the N-terminus, whereas the rest of the sequences share insignificant homology. To determine whether the putative CaSEC3 is the orthologue of ScSEC3, we tested whether CaSEC3 can correct the temperature sensitivity of Scec3Δ mutants. Scec3Δ cells grew slightly more slowly than wild-type cells at 22°C on YPD plates but failed to grow at 30°C (Fig. 1A). Introducing a single CaSEC3 copy into the Scec3Δ mutant under control of the Scsec3Δ promoter enhanced cell growth at 22°C and 30°C but failed to support growth at 37°C, indicating a partial rescue of the temperature sensitivity of the Scec3Δ mutant by CaSEC3.

Accumulation of secretory vesicles is often used as an indicator of post-Golgi secretory mutants in S. cerevisiae (Wiederkehr et al., 2003). Next, we used electron microscopy to examine the cellular distribution of the secretory vesicles. We observed large numbers of secretory vesicles in small to medium-sized buds in many Casec3Δ cells, particularly in those grown at 37°C, whereas these vesicles were rarely seen in wild-type cells (Fig. 1B), indicating accumulation of secretory vesicles in Casec3Δ cells.

ScSec3p localizes to the sites of cell growth (Finger et al., 1998). To determine CaSec3p localization, the gene in a CaSEC3/Casec3Δ strain was tagged with GFP at the C terminus. This strain was indistinguishable from the wild type in morphology and growth rate (data not shown), indicating that the fusion protein is functional. Fluorescence microscopy revealed the concentration of Sec3p-GFP at the incipient bud sites (Fig. 1C) and the tips of small to medium-sized buds. The protein was also seen at the mother-bud necks either as a single band in cells with small or medium-sized buds or as two parallel bands in large-budded cells. This cell-cycle-dependent
localization pattern is very similar to that of ScSec3p (Finger et al., 1998). Under hyphal-induction conditions, Sec3p-GFP first localized to a single cortical site in G1 cells, presumably the site for germ tube growth (Fig. 1C), and later to the tips of almost all germ tubes and apical hyphal cells, suggesting persistent localization at the growing tips. Sec3p-GFP was also observed at the septa, co-existing with the tip fluorescence. Together, the data strongly support an orthologous relatedness of CaSEC3 with ScSEC3.

Casec3Δ mutants exhibit temperature sensitivity and hyphal growth defects

Next, we examined the Casec3Δ mutant under conditions for both yeast and hyphal growth. On plates, Casec3Δ cells grew only slightly more slowly than wild-type cells at 30°C, much more slowly at 37°C, and failed to grow at 42°C (Fig. 2A). Interestingly, Casec3Δ cells grew considerably better in GMM than in YPD under both solid (Fig. 2A) and liquid (Fig. 2B) conditions at 30°C with doubling times of 3.13 hours in liquid GMM and 3.89 hours in YPD. Most Casec3Δ yeast cells were round instead of the typical oval shape at temperatures below 30°C (Fig. 2C, top), whereas they appeared markedly larger than wild-type cells when grown at 37-42°C. Under hyphal-induction conditions, Casec3Δ cells initially grew normally-looking germ tubes (Fig. 2C, bottom), but at later times the apical tip growth abruptly switched to isotropic expansion, forming swollen tips. These cells did not divide further, indicating a terminal defect. Because the junction between the germ tube and its large globular end appeared quite sharp, we thought it might be the site of the first septum. To confirm this, we tagged the septin Cdc3p with GFP in Casec3Δ cells. Cdc3p-GFP exhibited a localization pattern very similar to that of Sec3p-GFP in both wild-type and sec3Δ cells during yeast growth (Fig. 3A,B, left). Upon hyphal induction, Cdc3p exhibited the same polarized localization during germ tube formation in both wild-type and sec3Δ cells (Fig. 3A,B, right). However, at later times in all sec3Δ cells with a swollen tip, a septin ring was invariably present at the exact position where the tips started to enlarge, indicating that the daughter cell had grown isotropically soon after the formation of the first septin ring. Calcofluor White staining also revealed a chitin ring at the same position (Fig. 3B, right). In addition, a fraction of Cdc3p remained at the hyphal tips in wild-type cells over extended periods, whereas it was not seen at the tips of the round daughter compartment in sec3Δ cells (Fig. 3B, center). Together, the data show that soon after the assembly of the first septin ring, sec3Δ cells lose its ability to maintain hyphal growth. The results reveal a previous unknown aspect of C. albicans hyphal development: when initiated from G1, it occurs through an initial Sec3p-independent phase and a later Sec3p-dependent phase. The former corresponds to germ tube growth, and the latter seems to start after the formation of the first septin ring. Reintegration of a copy of wild-type SEC3 into the sec3Δ cells fully rescued all the defects of the sec3Δ mutant (data not shown).

Cellular localization of markers for the exocyst and secretory vesicles in sec3Δ mutants

Because CaSec3p is thought to be the landmark for exocytosis, we next examined the effects of its deletion on cellular localizations of representative proteins of the exocyst and on the secretory vesicles. To do this, we GFP-tagged another exocyst subunit CaSec15p (orf19.1419) and the vesicle-resident GTPase CaSec4p (orf19.2571) under control of their native promoters. The two fusion proteins exhibited very similar localization patterns (Fig. 4). In wild-type yeast cells,
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they were seen at the incipient bud sites and bud tips, and at mother-daughter necks in most budded cells (Fig. 4A,B, left top). In wild-type hyphal cells, the proteins localized as a single small spot at the sites of germ tube evagination in G1 cells and at the tips of all germ tubes and hyphae (Fig. 4A,B, bottom left). These localization patterns are consistent with the expected roles of Sec4p and Sec15p in exocytosis. In sec3Δ yeast cells growing at 30°C, these proteins exhibited a localization pattern similar to that in wild-type cells except that they seemed to be less concentrated, occupying larger areas (Fig. 4A,B, left top). Under hyphal-induction conditions, the fluorescence was first localized to the sites of germ tube emergence and later at the tips throughout germ tube growth (Fig. 4A,B, right bottom). However, after the tip growth switched to isotropic expansion in sec3Δ cells, the fluorescence became increasingly diffuse. After 3 hours of induction, Sec15p-GFP was either undetectable or totally diffuse in >90% of the cells, whereas GFP-CaSec4p became evenly distributed in the round daughter compartment. Together, the data show the following. First, localization of the exocyst and secretory vesicles to growth sites can still occur in sec3Δ cells during yeast growth at permissive temperatures, although they are less concentrated than in wild-type cells except that they seemed to be less concentrated, occupying larger areas (Fig. 4A, right top). Under hyphal-induction conditions, the fluorescence was first localized to the sites of germ tube emergence and later at the tips throughout germ tube growth (Fig. 4A,B, right bottom). However, after the tip growth switched to isotropic expansion in sec3Δ cells, the fluorescence became increasingly diffuse. After 3 hours of induction, Sec15p-GFP was either undetectable or totally diffuse in >90% of the cells, whereas GFP-CaSec4p became evenly distributed in the round daughter compartment. Together, the data show the following. First, localization of the exocyst and secretory vesicles to growth sites can still occur in sec3Δ cells during yeast growth at permissive temperatures, although they are less concentrated than in wild-type cells except that they seemed to be less concentrated, occupying larger areas (Fig. 4A, right top). Under hyphal-induction conditions, the fluorescence was first localized to the sites of germ tube emergence and later at the tips throughout germ tube growth (Fig. 4A,B, right bottom). However, after the tip growth switched to isotropic expansion in sec3Δ cells, the fluorescence became increasingly diffuse. After 3 hours of induction, Sec15p-GFP was either undetectable or totally diffuse in >90% of the cells, whereas GFP-CaSec4p became evenly distributed in the round daughter compartment. Together, the data show the following. First, localization of the exocyst and secretory vesicles to growth sites can still occur in sec3Δ cells during yeast growth at permissive temperatures, although they are less concentrated than in wild-type cells except that they seemed to be less concentrated, occupying larger areas (Fig. 4A, right top). Under hyphal-induction conditions, the fluorescence was first localized to the sites of germ tube emergence and later at the tips throughout germ tube growth (Fig. 4A,B, right bottom). However, after the tip growth switched to isotropic expansion in sec3Δ cells, the fluorescence became increasingly diffuse. After 3 hours of induction, Sec15p-GFP was either undetectable or totally diffuse in >90% of the cells, whereas GFP-CaSec4p became evenly distributed in the round daughter compartment.

Second, during hyphal growth, Sec3p is not required for the localization of the exocyst and secretory vesicles to the cortical sites of germ tube evagination in G1 cells and to the tips of germ tubes until the formation of the first septin ring. Third, soon after the assembly of the first septin ring, Sec3p becomes essential for maintaining the tip localization of the exocyst and secretory vesicles.

sec3Δ cells fail to maintain polarized localization of cell-polarity regulators during hyphal growth

The exocyst is spatially regulated by Rho-family GTPases, such as Cdc42p (Guo et al., 2001; Zhang et al., 2001). Actin structures and many other proteins, such as the polarisome components Bni1p and Spa2p, are well documented to play important roles in polarized growth (Pruyne and Bretscher, 2000). Using GFP-tagged Cdc42p, Wedlich-Soldner et al. (Wedlich-Soldner et al., 2003) demonstrated that vesicle delivery along actin cables is important for symmetry breaking, suggesting that membrane trafficking might mediate transport of the polarity regulators to growth sites. Cdc42p plays a central role in polarized growth and is among the earliest proteins to polarize to new growth sites before establishing the
actin-based vesicle transport (Pruyne and Bretscher, 2000; Caviston et al., 2003). Next we determined whether and how SEC3 deletion affects the cellular localizations of some of the polarity regulators. CaCdc42p was tagged at the N-terminus with GFP in BWP17 and sec3Δ cells. At 30°C, GFP-CaCdc42p could be observed over the entire plasma membrane and at some cytoplasmic membranes in wild-type cells (Fig. 5, left, top). However, intensity of the fluorescence was clearly stronger in buds, especially towards the tip region, and at the bud necks in large-budded cells. This localization pattern is reminiscent of ScCdc42p (Ziman et al., 1993; Wedlich-Soldner et al., 2003). However, in sec3Δ cells at 30°C, strong GFP-Cdc42p fluorescence appeared to fill up the entire body of most small buds (Fig. 5, right, top). Similar intense cytoplasmic fluorescence, sometimes as patches, was also seen in many medium-sized and large buds at 37°C. The results clearly indicate a marked increase of Cdc42p accumulation inside the buds of sec3Δ cells. Under hyphal-induction conditions, GFP-Cdc42p in sec3Δ cells exhibited normal tip localization only through germ tube formation but gradually lost the tip localization after the formation of the septin ring, and eventually became completely diffuse within the daughter cells (Fig. 5, right, bottom). The data suggest that Cdc42p requires a functional exocytic pathway to regulate its distribution between the plasma and cytoplasmic membranes and to maintain its polarized localization during hyphal growth.

We also determined the localizations of actin patches, Spa2p and Bni1p in sec3Δ cells. As previously reported by Li et al. (Li et al., 2005), in wild-type yeast cells, actin patches concentrated to the incipient bud sites in G1 cells, in small to medium-sized buds and at both sides of the neck in cells undergoing cytokinesis (Fig. 6A). By contrast, actin patches were evenly distributed in the majority of sec3Δ yeast cells except in a few small buds. Under hyphal-induction conditions, actin patches persistently localized toward hyphal tips in wild-type cells, whereas in sec3Δ cells they displayed this localization only during germ tube growth and became increasingly diffuse with the isotropic growth of the daughter cells. Similarly, in contrast to their persistent hyphal tip localization in wild-type cells, both Spa2p-GFP and GFP-Bni1p gradually lost their tip localization after the septin ring formation in sec3Δ cells (Fig. 6B, only Spa2p-GFP localization is presented). Together, the data demonstrate that Sec3p is not required for polarized localization of the polarity proteins during germ tube formation but is crucial for maintaining their localization after the formation of the first septin ring.

Fig. 5. Effect of SEC3 deletion on Cdc42p cellular localization. GFP-Cdc42p localization was examined in WYL35 (SEC3) and WYL36 (sec3Δ) cells under yeast (top) and hyphal (bottom) growth conditions. Bars, 5 μm.

Fig. 6. Effects of SEC3 deletion on actin (A) and Spa2p (B) localization. (A) Cells of SC5314 (SEC3) and WYL28 (sec3Δ) were grown under yeast (top) and hyphal (bottom) growth conditions and stained with rhodamine-phalloidin. (B) Cells of WYZ8 (SEC3 SPA2-GFP) and WYL43 (sec3Δ, SPA2-GFP) were grown under yeast (top) and hyphal (bottom) growth conditions. Bars, 5 μm.
Defective septin organization compromises sec3p localization but suppresses the hyphal defects of sec3Δ cells

The excellent temporal colocalization of the exocyst markers with septins and the sudden loss of polarized tip growth soon after septin ring formation in sec3Δ cells during hyphal development strongly suggest a role for septins in regulating exocytosis. To explore this possibility, we first determined whether disrupting septin organization will alter Sec3p localization. Warenda and Konopka (Warenda and Konopka, 2002) reported that the septin genes CDC10 and CDC11 are not essential for growth, but deletion of either one results in aberrant morphologies, suggesting defective septin structures.

Thus, we examined Sec3p-GFP localization in cdc10Δ and cdc11Δ cells. Fig. 7A shows that Sec3p-GFP localized at bud tips and bud necks in <30% of cdc10Δ and cdc11Δ cells at 30°C, in sharp contrast to their presence at these sites in nearly all wild-type cells. In addition, the fluorescence intensity of Sec3p-GFP in the septin mutants was markedly weaker than that in wild-type cells. Similarly reduced Sec3p-GFP localization at the tips and septa was also observed in hyphal cells (data not shown). The results indicate that well-organized septin structures are required for proper Sec3p localization.

Gladfelder et al. (Gladfelder et al., 2005) suggested that septins may direct the deposition of new cell materials at growth sites, causing localized expansion of the cell wall. One particular example is the requirement of the septin ring for constructing the hourglass-shaped bud neck. We reasoned that similar mechanisms might be responsible for the loss of apical growth in sec3Δ cells after septin ring formation, presumably by redirecting growth from hyphal tips to the neck region. If so, altering the septin ring structure might prevent this redirection of growth and thus allow the apical tip growth to continue. To test this hypothesis, we constructed sec3Δ cdc10Δ and sec3Δ cdc11Δ double mutants. The yeast cells of the double mutants appeared similar in morphology (data not shown) to those of the cdc10Δ and cdc11Δ single mutants previously described by Warenda and Konopka (Warenda and Konopka, 2002). However, under hyphal-induction conditions, ~80% of sec3Δ cdc10Δ and nearly all sec3Δ cdc11Δ cells developed largely normal-looking germ tubes and hyphae (Fig. 7B), showing that defects in septin structures can indeed prevent the cell from shifting growth from the tip to the neck in the absence of SEC3. Cell wall staining by Calcofluor White showed that the double mutant cells maintained normal apical growth beyond the septal chitin ring. Moreover, when Cdc3p was tagged with GFP in the double mutants, a fraction of the fusion protein was found to remain at hyphal tips after the formation of the first septin ring (Fig. 7C), resembling the pattern seen in the wild-type cells shown in Fig. 3. Although deleting CDC10 or CDC11 restored hyphal growth in sec3Δ cells, the cells remained unable to grow at 42°C (Fig. 7D), suggesting that the inability of sec3Δ mutants to maintain hyphal growth is unlikely to be a result of temperature sensitivity.

The protein kinases Gin4p and Cla4p play important roles in regulating septin organization in both C. albicans and S. cerevisiae, and deleting either gene causes significant cell elongation (Leberer et al., 1994; Longtime et al., 1998; Mortensen et al., 2002; Versele and Thorner, 2005; Wightman et al., 2004). We next tested whether mutating GIN4 or CLA4 would also restore hyphal growth in sec3Δ mutants. Probably because of synthetic lethality (see below), we were unable to obtain the gin4A sec3Δ mutant. Therefore we deleted one copy of GIN4 and controlled the other with the MET3 promoter in the sec3Δ background. Switching off GIN4 expression caused significant cell elongation in both gin4Δ/PMET3-GIN4 and sec3Δ/sec3Δ gin4Δ/PMET3-GIN4 cells. When tested for hyphal growth, the elongated cells of neither strain responded to hyphal induction. Wightman et al. (Wightman et al., 2004) has also reported that the pseudohyphae resulted from depleting Gin4p did not
switch to hyphal growth. We found that sec3Δ/sec3Δ gin4Δ/sec3ΔPMET3/GIN4 cells did not grow much in GMM containing methionine and cysteine, and many cells lost viability, whereas gin4Δ/sec3ΔPMET3/GIN4 cells continued to grow. This indicates synthetic lethality between sec3Δ and gin4Δ. The cla4Δ yeast cells exhibited elongated bud necks and failure in cell separation, whereas upon hyphal induction, a small number of cells appear to be able to grow short hyphae, which is similar to the phenotype described previously by Leberer et al. (Leberer et al., 1994). The cla4Δ sec3Δ double mutant was morphologically similar to the cla4Δ single mutant under yeast growth conditions. Although both cla4Δ and sec3Δ single mutants could undergo limited hyphal growth, cla4Δ sec3Δ cells did not seem to respond to hyphal induction at all. The inability to restore hyphal growth in sec3Δ cells by deleting GIN4 or CLA4 is most likely because gin4Δ and cla4Δ mutants are already severely impaired in hyphal growth; and septin functions are probably compromised both at the tip and the neck. However, the synthetic effects of deleting SEC3 with GIN4 or CLA4 are consistent with functional interactions between Sec3p and septins.

Physical interactions of septins with the exocyst components

The cell biology and genetic evidence above suggest that septins may interact with the exocyst in regulating proper hyphal morphogenesis in C. albicans. Although physical interactions between septins and the exocyst have been demonstrated in rat brain tissues (Hsu et al., 1998), it has not been reported in any other organisms. To determine whether septins associate with the exocyst in C. albicans, we constructed two strains: one co-expressing Myc-tagged Sec3p (Myc-Sec3p) and GFP-tagged Cdc3p (Cdc3p-GFP), and the other co-expressing Myc-Sec5p (orf19.75) and Cdc3p-GFP. We then carried out co-immunoprecipitation experiments, in which we used anti-Myc antibodies to pull down Myc-Sec3p or Myc-Sec5p from cell lysates and then used anti-GFP antibodies to detect Cdc3p-GFP in the precipitates by western blotting. Fig. 8 shows that Cdc3p-GFP could indeed co-precipitate with both Myc-Sec3p and Myc-Sec5p. The Cdc3p-GFP band is of the predicted size (~70 kDa) and was not precipitated by anti-Myc antibodies from lysates of the cells expressing Cdc3p-GFP alone. We also constructed a sec3Δ mutant co-expressing Cdc3p-GFP and Myc-Sec5p and again found co-immunoprecipitation of Cdc3p-GFP with Myc-Sec5p. Together, the data strongly suggest an association of the exocyst with septin complexes, for which Sec3p may not be critically required.

Discussion

To understand the role of exocytosis in C. albicans hyphal growth, we have investigated the effects of deleting the exocyst subunit gene SEC3. The sec3Δ mutant exhibited morphological defects in both yeast and hyphae. The majority of sec3Δ yeast cells are round instead of the typical ellipsoidal shape; and the exocyst subunit Sec15p and the vesicle-resident protein Sec4p are markedly less concentrated in the tip area than wild-type cells. These phenotypes are consistent with impairment of the secretory vesicle transport towards bud tips, limiting the apical growth of the bud. S. cerevisiae sec3Δ cells are of a similar round shape as a result of fusion of the vesicles over the entire bud surface (Wiederkehr et al., 2003).

Casec3Δ cells are severely defective in hyphal growth. Although the cells produced largely normal germ tubes, they later underwent a sudden apical-to-isotropic growth switch, producing globular tips. Concurrently, Sec4p, Sec15p, several polarity regulators, and actin patches all lost their initial polarized tip localization and became either dispersed or undetectable. The results reveal that C. albicans hyphal development initiated from G1 occurs in two phases: a Sec3p-independent phase followed by a Sec3p-dependent one. This finding suggests that distinct mechanisms control polarized exocytosis towards the growing tip in the two stages of growth.

What demarcates the two phases of hyphal growth? By GFP tagging of the septin Cdc3p and Calcofluor White staining, we observed that the sudden loss of polarized tip growth in sec3Δ cells always occurs immediately after the formation of the first septin ring. Does the septin ring suppress the polarized exocytosis towards hyphal tips? In S. cerevisiae, a well assembled septin ring at the neck was shown to attract the secretory vesicles to the bud side of the neck leading to expansion of the base of the bud (Gladel et al., 2005); and without the ring, secretion is directed to bud tips, resulting in the formation of tubular buds highly similar to the germ tubes of C. albicans. When the neck ring was first disrupted and then allowed to reassemble, new septin structures always appeared at bud tips but not at the neck. These results suggest that bud tips harbor factors that can attract septins, and this activity is normally suppressed when a ring is assembled at the neck. Hence, controlled distributions of septins between the tips and the necks appear to play an important role in determining where exocytosis is targeted. Based on the correlations between septin localization and sites of cell growth observed in S. cerevisiae and this study, we propose the following hypothesis to explain the inability of the sec3Δ mutant to maintain hyphal growth. During germ tube formation, septins are assembled at the site of germ tube emergence attracting exocytosis, in which Sec3p is not critically required. Later, when a germinating cell enters the cell cycle, a septin ring is
formed near the tip. In wild-type cells, hyphal development has a mechanism to continue to restrict exocytosis to the tip. This is a Sec3p-dependent mechanism that can override the intrinsic ability of the septin ring in attracting exocytosis. However, without Sec3p, the septin ring will dominate in attracting exocytosis, causing growth in the area near the daughter side of the ring as well as spreading growth over the entire cell surface of the daughter cell. In support of this model, we found that deleting one non-essential septin gene CDC10 or CDC11 in sec3Δ cells restored polarized hyphal growth. We interpret this as such: the septin rings assembled in the absence of Cdc10p or Cdc11p are compromised in its ability to attract exocytosis. Therefore, the Sec3p-independent mechanism that has directed exocytosis to the tip throughout germ tube growth continues to function after septin ring formation. These results provide strong genetic evidence for a role of the septins in guiding exocytosis in C. albicans.

Genetic interactions were also observed between SEC3 and two protein kinase genes GIN4 and CLA4 which play important roles in regulating septin organization and functions in both C. albicans and S. cerevisiae (Leberer et al., 1994; Longtine et al., 1998; Mortensen et al., 2002; Versele and Thorner, 2004). However, unlike deleting CDC10 or CDC11, deleting GIN4 or CLA4 did not restore hyphal growth in sec3Δ cells. There are several explanations that are not mutually exclusive. First, gin4Δ and clavΔ mutants are already severely defective in hyphal growth. Second, Gin4p and Clavp are involved in other cellular functions (Holly and Blumer, 1999; Okuzaki et al., 1997; Tjandra et al., 1998), and thus the effect of deleting them may be less specific than deleting a non-essential septin gene on septin organization and functions. Third, septin functions may be compromised both at the tip and at the neck in gin4Δ and clavΔ mutants.

Sec3p is thought to be the spatial landmark for exocytosis (Finger et al., 1998). However, the dispensability of CaSec3p during germ tube growth suggests that other components at the tip can also serve as the landmark. Could this be a septin or a septin-associated protein? The precise temporal colocalization of septins and the exocyst components throughout germ tube formation and the suppression of sec3Δ hyphal defects by CDC10 or CDC11 deletion support this possibility. Co-immunoprecipitation of Cdc3p-GFP with both Myc-Sec3p and Myc-Sec5p suggests association of septins with the exocyst. Importantly, Cdc3p-GFP and Myc-Sec5p could be co-immunoprecipitated from sec3Δ cells, suggesting that Sec3p may not be required for this association. This explains why Sec3p is dispensable for germ tube growth. Owing to the multi-subunit nature of both the septin complex and the exocyst, identification of the proteins directly responsible for the interaction proved difficult. Several mammalian septins have been found to be present abundantly in postmitotic neurons and are localized to growth cones and the tips of growing neurites (Hazuka et al., 1999; Xue et al., 2004). Hsu et al. (Hsu et al., 1998) reported co-immunoprecipitation of SEC6 and SEC8 with four septins in rat brain tissues. Perhaps, the interaction between the two protein complexes is evolutionarily conserved in regulating polarized exocytosis in certain type of eukaryotic cells undergoing vigorous polarized growth or secretion.

In recent years, septins have been implicated in several neurodegenerative disorders, such as Parkinson’s disease (Dong et al., 2003), Alzheimer’s disease (Kinoshita et al., 1998) and hereditary neuralgic amyotrophy (Kuhlenbaum et al., 2005). There has been evidence that disruption of septin-exocyst interactions in neurons contributes to the development of some of these diseases (Dong et al., 2003). C. albicans hyphal growth may provide a relevant model in investigating the role of septin-exocyst interactions in polarized exocytosis and shed light on the mechanisms underlying the neurodegenerative disorders.

Materials and Methods

Strains, media and growth conditions C. albicans and S. cerevisiae strains used in this study are listed in supplementary material Table S1. Except where noted, the strains were grown in either YPD (1% yeast extract, 2% Bacto peptone, and 2% glucose) or GYM (2% glucose and 6.79 g/l yeast nitrogen base). For C. albicans yeast growth, cells were grown at 30°C; and for hyphal growth, the media were supplemented with 10% serum and incubated at 37°C.

Gene deletion

Gene deletion mutants were constructed by sequentially deleting the two copies of sec3Δ in BWP17 (Wilson et al., 1999). A gene deletion cassette was constructed by flanking a selectable marker gene, HIS3 or ARG4, with AB and CD DNA fragments (~400 bp each) corresponding to the 5’- and 3’-untranslated regions of the target gene, respectively. Transformants were selected using appropriate dropout GMM, and correct deletion was confirmed by Southern blotting or PCR (data not shown). To construct C. albicans strains deleted of SEC3 together with CDC10 or CDC11, a URA3 flipper cassette was constructed by flanking the 4.2-kb URA3 flipper (Morschhauser et al., 1999) with the AB and CD DNA fragments corresponding to the 5’ and 3’ UTR of SEC3. This cassette was used to disrupt both copies of SEC3 from BWP17. Then the two copies of CDC10 or CDC11 were deleted sequentially by using ARG4- and HIS-marked cassettes from the sec3Δ strain (WLY33).

GFP tagging

To express a Sec3p-GFP fusion protein under control of its native promoter, the C-terminal 1965 bp of SEC3 or was amplified by PCR with Km and XhoI sites added to 5’ and 3’ ends, respectively, cleaved with Km and XhoI, and cloned in frame with the GFP sequence at the Km-XhoI sites in the plasmid pSCEGFP (Zheng et al., 2003). The resulting plasmid was linearized by BglII at nt 814 and transformed into BWP17 or WLY27. For N-terminal GFP-tagging of Sec4p, the Sec4p was PCR-amplified with Clav and PstI sites added to 5’ and 3’ ends, respectively, cleaved with Clav and PstI, and cloned in frame with the GFP sequence at the Clav-PstI sites in pSCEGFP, yielding pSCEGFP-utr. A 1311-bp fragment of SEC4 promoter was PCR-amplified with Km and XhoI sites added to the 5’ and 3’ ends, respectively, cleaved with Km and XhoI, and cloned at the Km-XhoI sites in pGFP-utr-sec4. The resulting plasmid was linearized by HoxIII at nt ~880 and integrated at the SEC4 chromosomal promoter. To tag Sec15p with GFP, the C-terminal 810-bp fragment of SEC15 was PCR-amplified with Km and XhoI sites added to the 5’ and 3’ ends, respectively, cleaved with Km and XhoI, and cloned in frame with the GFP sequence at the Km-XhoI sites in pSCGFP. The resulting plasmid was linearized by EcoRV at nt 1317 and transformed into WLY27 and BWP17. To tag Sec4p, the entire SEC4 gene was PCR-amplified with Clav and PstI sites added to the 5’ and 3’ ends, respectively, cleaved with Clav and PstI, and cloned in frame with the GFP sequence at the Clav-PstI sites in the plasmid pSCEGFP (Li et al., 2005). The resulting plasmid was linearized by SphI at nt 204 and transformed into WLY27 and BWP17. To tag Sec3p, the entire CDC3 gene was amplified by PCR with Km and XhoI sites added to the 5’ and 3’ ends, respectively, cleaved with Km and XhoI, and cloned in frame with the GFP sequence at the Km-XhoI sites in pSCGFP. The resulting plasmid was linearized by BglII at nt 160 and transformed into WLY27. To tag Sec5p, the entire SEC5 gene was PCR-amplified with KpnI and PstI sites added to the 5’ and 3’ ends, respectively, cleaved with KpnI and PstI, and cloned in frame with the GFP sequence at the KpnI-PstI sites in pSCGFP. The resulting plasmid was linearized by EcoRV at nt 1317 and transformed into WLY27 and BWP17. To tag Sec15p, the entire SEC15 gene was PCR-amplified with Km and XhoI sites added to the 5’ and 3’ ends, respectively, cleaved with Km and XhoI, and cloned in frame with the GFP sequence at the Km-XhoI sites in pSCGFP. The resulting plasmid was linearized by BglII at nt 814 and transformed into WLY27 and BWP17. To tag Sec5p, the entire SEC5 gene was amplified by PCR with Km and XhoI sites added to the 5’ and 3’ ends, respectively, cleaved with Km and XhoI, and cloned in frame with the GFP sequence at the Km-XhoI sites in pSCGFP. The resulting plasmid was linearized by EcoRV at nt 1317 and transformed into WLY27 and BWP17.

Co-immunoprecipitation

Cells in 200 ml of overnight cultures were harvested by centrifugation, and then resuspended and lysed in 2 ml HEPE’s lysis buffer containing 50 mM HEPE’s (pH 7.5), 1 mM EDTA, 5% glycerol, 0.05% Tween 20, 105 mM KC1 and the protease inhibitor mix (Roche). Cells were broken by five rounds of 45-second beating at 5000 r.p.m. in a Micro SmashTM MS-100 bead beater (TOMY Medico, Minato-ku, Japan) with 1 minute of cooling on ice between rounds. The lysate was clarified by centrifugation at 10,000 g twice. Beads coated with anti-Myc antibodies were
Actin and cell wall staining and microscopy

Staining of nuclei, cell wall and actin structures was carried out as previously described (Zheng et al., 2003). A Leica DMR fluorescence microscope interfaced with METAMORPH software (Molecular Devices Corporation, Sunnyvale, CA) was used for imaging.

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