Polarized Hyphal Growth in *Candida albicans* Requires the Wiskott-Aldrich Syndrome Protein Homolog Wal1p†

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The yeast-to-hypha transition is a key feature in the cell biology of the dimorphic human fungal pathogen *Candida albicans*. Reorganization of the actin cytoskeleton is required for this dimorphic switch in *Candida*. We show that *C. albicans* WAL1 mutants with both copies of the Wiskott-Aldrich syndrome protein (WASP) homolog deleted do not form hyphae under all inducing conditions tested. Growth of the wild-type and *wal1* mutant strains was monitored by in vivo time-lapse microscopy both during yeast-like growth and under hypha-inducing conditions. Isotropic bud growth produced round *wal1* cells and unusual mother cell growth. Defects in the organization of the actin cytoskeleton resulted in the random localization of actin patches. Furthermore, *wal1* cells exhibited defects in the endocytosis of the lipophilic dye FM4-64, contained increased numbers of vacuoles compared to the wild type, and showed defects in bud site selection. Under hypha-inducing conditions *wal1* cells were able to initiate polarized morphogenesis, which, however, resulted in the formation of pseudohyphal cells. Green fluorescent protein (GFP)-tagged Wal1p showed patch-like localization in emerging daughter cells during the yeast growth phase and at the hyphal tips under hypha-inducing conditions. Wal1p-GFP localization largely overlapped with that of actin. Our results demonstrate that Wal1p is required for the organization of the actin cytoskeleton and hyphal morphogenesis in *C. albicans* as well as for endocytosis and vacuole morphology.

Polarized cell growth is a basic feature of the morphogenesis of a cell. Highly elongated cell growth can be found in specialized cells such as neurites, plant root hairs, and pollen tubes but is most prominent in fungal hyphae (10, 21, 45). Fungi grow either in a yeast-like or filamentous manner. Dimorphic fungi are able to switch between these two growth modes. A dimorphic transition occurs in a variety of pathogenic fungi such as the maize pathogen *Ustilago maydis* and the human pathogen *Candida albicans* (6, 32). In *C. albicans* the ability to initiate hyphal growth is associated with its virulence (27). Polarized growth in ascomycetous fungi is dependent on the actin cytoskeleton, whereas microtubules are not required to initiate hyphal extensions (19, 52). Rho protein modules are central regulators for the organization of the actin cytoskeleton (12). In fungal cells these modules determine the establishment of cell polarity and the maintenance of hyphal growth (12, 48). The actin cytoskeleton can be divided into two components: actin cables and cortical actin patches. Actin cables in *Saccharomyces cerevisiae* are positioned in a mother-daughter axis and serve as tracks for the transport of secretory vesicles delivering plasma membrane and cell wall compounds to sites of growth (37). The yeast formin Bni1p plays a key role in the Arp2/3-independent assembly of actin cables (14, 15, 38, 40). Cortical actin patches are positioned at sites of exo- and endocytosis. They localize to sites of polarized growth, for example, to the growing bud and to hyphal tips in *C. albicans* and to the hyphal tips in filamentous fungi (for reviews, see references 37 and 45). However, the role of cortical actin patches during polarized growth or hypha formation in both *S. cerevisiae* and *C. albicans* has been questioned (5, 36). In *S. cerevisiae* the Arp2/3 complex was shown to be required for endocytosis and the assembly of actin patches (30, 49). The Arp2/3 complex can be activated by the *S. cerevisiae* homolog of the human Wiskott-Aldrich Syndrome protein (WASP), encoded by the LAS17/BEE1 gene (9, 50). In our efforts to understand signaling routes to the actin cytoskeleton, we identified the *C. albicans* WASP homolog and characterized its role for polarized morphogenesis and hyphal growth in *C. albicans*. Mutant *wal1* cells were not able to produce hyphae under all conditions tested. Surprisingly, even though *wal1* yeast cells grew isotropically, initiation of polarized morphogenesis occurred under hypha-inducing conditions and resulted in the formation of elongated, pseudohyphal cells. In addition to the defects in the organization of the actin cytoskeleton, *wal1* mutants showed defects in endocytosis and vacuolar morphology.

**MATERIALS AND METHODS**

**Strains and media.** The *C. albicans* and *S. cerevisiae* strains used in this study are listed in Table 1. Growth media and standard procedures were described previously (44). Maltose (2%) was supplied as the sole carbon source to induce growth of the strains. 

**Construction of disruption cassettes.** The *C. albicans* WASP homolog *WAL1* was identified in the genomic sequence (http://www-sequence.stanford.edu/group/candida) and contains an open reading frame (ORF) of 2,142 bp. Based on this sequence, two primers were designed (primer sequences are listed in Table 1). KpnI-WAL1 (no. 556) and Xbal-WAL1 (no. 557), to amplify a 1,551-bp fragment from genomic *C. albicans* DNA containing the 5' end of the *WAL1* ORF. This fragment was cloned into pBluescript SK(+) using the terminally attached restriction sites, generating pSK-5’-WAL1. The sequence of the
insert was verified (MWG-Biotech, Ebersberg, Germany). Cleavage of this plasmid by HindIII and ClaI resulted in the removal of an internal 460-bp fragment of the insert, which was replaced by the selectable marker genes URA3 and HIS3, respectively, which were excised from pFA-URA3 and pFA-HIS3 (16) with PvuII-ClaI and HindIII-ClaI, respectively. In this way, plasmids pSK-Cawal1:URA3 and pSK-Cawal1::HIS1 were generated that carry disruption cassettes in which the selectable marker genes are flanked by 235 bp at the 5' end and 821 bp at the 3' end with regions from the WAL1 target locus. Both disruption cassettes were released from the plasmid backbone prior to transformation by cleavage with XbaI and KpnI. With the development of the pFA vector series (16), further genetic manipulations were carried out using PCR-based approaches.

**Construction of MAL2 promoter-WAL1 ORF fusion.** To place WAL1 under control of the regulatable MAL2 promoter, a PCR-based approach was applied. To this end, a cassette was amplified from plasmid pFA-HIS3-MAL2p (16) with primers 676 and 677. With these primers, 100 bp of sequence with homology to the 5' end of the WAL1 gene was added to the cassette. This PCR fragment was used to transform a heterozygous WAL1::URA3 strain, placing the only copy of WAL1 under regulatable regulation.

**Construction of the WAL1-GFP fusion.** To generate a WAL1-GFP fusion, a similar PCR-based approach was applied. Transformation cassettes were amplified from pFA-GFP-URA3 and pFA-GFP-HIS3 using primers 956 and 957, which, again, added 100 bp of flanking homology region to the FA cassettes (the green fluorescent protein [GFP] variant used in these constructs was derived from pFA-GFP-URA3 and pFA-GFP-HIS3) and together encoded a GFP-translation fusion that generated pXL-CaWAL1 was verified in C. albicans MAL2::URA3::HIS3/WAL1 strain. In C. albicans MAL2::URA3::HIS3/WAL1 strain, only the copy of WAL1 under its endogenous promoter, thus producing only Wal1 protein tagged with the GFP moiety. All three GFP-tagged strains revealed similar GFP signals. However, CAT21 produced brighter GFP signals than did the heterozygous strains.

**Transformation of C. albicans.** The lithium acetate procedure was used as described previously (44). Basic features of this protocol include an overnight incubation with lithium acetate and a subsequent heat shock for 15 min at 44°C. Correct gene targeting was verified by PCR analysis of the transformants. Locus- and marker-specific primers were as listed in Table 2.

**Hyphal induction of C. albicans.** Different protocols were used to induce hypha formation in C. albicans strains at 37°C. Hyphal induction occurred most vigorously in minimal medium containing 10 to 20% serum (calf serum; Sigma). Alternatively, hyphal induction was carried out in spider medium (26). Plates inoculated with different strains were incubated for 4 to 7 days before being photographed. Hyphal induction was also tested in liquid minimal media.

**Time-lapse microscopy.** Strains were pregrown in either complete or minimal medium, harvested, washed, and resuspended in sterile water. Small aliquots of cells were applied on deep-well slides prepared as described previously (20). It was of utmost importance to provide sufficient oxygen supply to the cells within the inoculum to support the growth of C. albicans. To achieve this, the medium was vigorously vortexed prior to the preparation of microscopy slides, using a FVL2400 Combi-Spin vortex (Peqlab, Erlangen, Germany). Minimal medium or full medium (supplemented with 10 to 20% serum for hyphal induction) was diluted 1:1 with water-agarose containing 3.4% agarose. Temperature control was achieved with a heat stage (built at the Biozentrum Basel and generously provided by P. Philipsen) which was mounted on the microscope table and heated with a water bath. All microscopy was done on a motorized Zeiss Axioplan II imaging microscope. Images were acquired using Metamorph 4.6 software (Universal Imaging Corp.) and a digital imaging system (MicroMax1024; Princeton Instruments). Images were collected into stacks. Stacks containing bright-field/differential interference contrast (DIC) images were processed separately from images displaying GFP or vacuolar fluorescence. The stacks were than combined by using overlay tools of the Metamorph software and processed as videoclips with a frame rate of 10 images/s.

**Staining procedures.** For actin staining, early-log-phase cells were fixed with 3.7% formaldehyde. Fixation and incubation with rhodamine-phalloidin were performed essentially as described previously (36). Chitin staining was done by directly adding calcofluor (1 μl of a 1 mg/ml stock) to 100 μl of cell suspension, incubating for 15 min, and washing. Vacular staining was done using the lipophilic dye FM4-64 (43). For the analysis of vacuolar morphology, overnight cultures grown in YPD were used. Cells were incubated with FM4-64 (0.2 μg/ml) for 30 min at 30°C and then photographed. For FM4-64 time-lapse microscopy, exponentially grown cells of the wild type and the wall mutant strain were placed on precooled microscope slides containing medium made of equal amounts of YPD and 3.4% water-containing agarose. GFP-images were obtained from early-log-phase cells grown in 0.25 × YPD that were washed once with water and resuspended in water. For GFP and actin colabeling, cells were fixed and stained with rhodamine-phalloidin as described above; the GFP signal was obtained using a narrow-band GFP filter set which excludes the actin signal monitored by a tetramethylrhodamine-5-isothiocyanate (TRITC) filter set. Other images were acquired using the appropriate filter sets (Chroma Technology).

**Heterologous complementation.** The C. albicans WAL1 ORF was amplified from a plasmid library (kindly provided by J. Ernst) by using primers 975 and 976. The resulting PCR product carried terminal TEF1 promoter and terminator. This PCR product was cotransformed into an S. cerevisiae bee1::las17 strain together with NruI-linearized plasmid pRS415-kanMX carrying the KanMX selection marker (as described in reference 46). Transformant colonies appeared after 2 days of growth on selective plates and incubated at 30°C on selective plates lacking leucine. Digestion of pRS415-kanMX with NruI cleaves a unique restriction site within the kan ORF. The S. cerevisiae in vivo recombination machinery was used to recombine the plasmid and PCR fragment, thus generating a new plasmid, pXL-Cawal1, in which the WAL1 ORF is placed under control of the A. gossypii TEF promoter (this promoter is functional in S. cerevisiae). Transformant colonies were then streaked on new selective plates and incubated at 37°C, the restrictive temperature for bee1::las17 strains. Transformants that continued to grow were selected, and plasmid DNA was isolated from these transformants and amplified in Escherichia coli. Correct fusion that generated pXL-Cawal1 was verified by PCR, restriction, and sequence analyses. Retransformation of pXL-Cawal1 into S. cerevisiae bee1::las17 cells revealed that heterologous complementation by pXL-Cawal1 was dependent on a period (6 h) of growth at 30°C prior to the shift to 37°C. This preincubation was not required when using a plasmid carrying the bee1::las17 fusion.
The unique gene \textit{WAL1} encodes the \textit{C. albicans} WASP homolog. This gene was isolated by a single ORF for \textit{WASPs} (for \textit{Wasp} family). A single ORF was found in the human genome database at Stanford University with the accession number \textit{WASP} (67.0% of its nucleotide identity to the human \textit{WASPs}). The \textit{WASPs} are known to be involved in the regulation of cell polarity and cytoskeleton organization.

**TABLE 2.** Oligonucleotide primers used in this study

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence</th>
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<tr>
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</tr>
<tr>
<td>H2</td>
<td>CAACGAAATGGCCTCCCCTACCACAG</td>
</tr>
<tr>
<td>U2</td>
<td>GTGTTACGAATCAATGGCACTACAGC</td>
</tr>
<tr>
<td>U3</td>
<td>GGAGTTGGATTAGATGATAAAGGTGATGG</td>
</tr>
<tr>
<td>XbaI</td>
<td>G1-WAL1 CCAATGAATCTATTTTTTACCACTC</td>
</tr>
<tr>
<td>KpnI</td>
<td>G1-CaWAL1 CCTTATATTCTCATCCATCC</td>
</tr>
<tr>
<td>MAL2p</td>
<td>H3GGACGAATTGAAGAAGCTGGTGCAACCG</td>
</tr>
<tr>
<td>WAL1</td>
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<tr>
<td>TEF-prom</td>
<td>CTTGAGCAGTATTCACATCC</td>
</tr>
<tr>
<td>XFP-primer</td>
<td>S1-CaWAL1-GFP AGCCGATGCACCACCTGCTACTTTAGCCGATGCATTAGCTTCTGCTTTGAATAAGAGGAAAGAAAAAGTTGCTCAAAGTGATGATGAAGAAGAT</td>
</tr>
</tbody>
</table>

**RESULTS**

The results indicate that \textit{WAL1} expression is downregulated in \textit{C. albicans} in the presence of \textit{WASPs}. The decrease in \textit{WASPs} expression is correlated with the downregulation of \textit{WASPs}. This suggests that \textit{WASPs} are involved in the regulation of \textit{WASPs} expression. The downregulation of \textit{WASPs} is also observed in the \textit{C. albicans} strain with a disruption in the \textit{WASPs} gene. This suggests that \textit{WASPs} are involved in the regulation of \textit{WASPs} expression.

**DISCUSSION**

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mutant strain). With our setup, we were able to monitor the growth of the strains over a period of approximately 10 h (sometimes up to 15 h). In contrast to similar studies with S. cerevisiae cells, it was essential to provide sufficient oxygen when growing C. albicans cells under these conditions (see Materials and Methods). We analyzed the wall mutant strains, their BWP17 progenitor strain, and the wild-type strain (SC5314) for growth defects during the yeast stage. WAL1/H11001 cells were ellipsoidal. In contrast, wall cells were found to be round and of heterogeneous size, with several cells clumping together. To quantify the cell morphology defect of wall cells, we measured the lengths and widths of WAL1 and wall cells (Fig. 3A). Cell indices (length/width) of wild-type, BWP17, and heterozygous mutant strains were 1.3, corresponding to the ellipsoidal cell shape. This indicates that heterozygosity of WAL1 did not result in morphological defects and that a single copy of WAL1 is sufficient for wild-type-like growth. In contrast, the cell index of the wall strain was 1.1, representing an almost spherical cell shape. The ability to form new buds was not affected in wall cells. In the wild-type strain, bud emergence was followed by a period of polarized growth (Fig. 3B). wall cells, however, quickly began to grow in an isotropic manner, which resulted in a decrease of the polarized-growth rate (Fig. 3B). Due to the extended duration of our time-lapse recordings, we were able to observe several consecutive cell divisions of wild-type and wall cells. The time required for two consecutive bud emergence events of a single cell was used to calculate the average time of a cell cycle (Fig. 3C). Growth delays in the mutant strains were at least in part attributable to the remaining auxotrophies, since the heterozygous WAL1/wall::HIS1 strain grew more slowly than a heterozygous WAL1/wall::URA3 strain, which is a general feature that has been observed in other mutant strains as well (our unpublished results). In line with this observation, both of the heterozygous mutant strains required more time to complete a cell cycle than the homozygous mutant strain which carries only the arg4 auxotrophy. The cell cycle times observed in the in vivo time-lapse recordings were found to be similar to the growth rates in liquid culture (data not shown). Cells of the wall mutant appeared to be of heterogeneous size. To analyze this in more detail, we monitored cell size changes of single cells over time (Fig. 3D). We found that wild-type mother cells only marginally increased in cell volume. In contrast, the volume of wall mother cells increased more than 50% during the 6-h observation period, which corresponds to about four cell cycles. Another difference between the wild-type and wall occurred during the detachment of mother and daughter cells, which in the wild-type resulted in a torsion of the daughter cell out of the mother-daughter cell axis whereas wall mutant cells only rarely showed such an obvious displacement (Movies S1 and S3 in the supplemental material). Mutant wall cells adhered and relocated as cell clumps, indicating a defect in cell separation. This led to the formation of cell heaps not observed in the wild type or in the heterozygous mutant strains, where all cells

FIG. 1. Alignment of fungal WASP homologs. Amino acids corresponding to a majority of aligned sequences are shaded. Accession numbers: C. albicans Wal1p, orf19.6598.prot (http://www-sequence.stanford.edu/group/candida/index.html); S. cerevisiae Bee1p/Las17p, NP01482; S. pombe Wsp1p, NP594758; Neurospora crassa WASP, NCU07438.1 (http://www-genome.wi.mit.edu/annotation/fungi/neurospora/).
remained in the focal plane during the time-lapse recordings, indicating that effective displacement had occurred. Cell clumps were also found when growing wall in liquid culture.

Cell aggregates could be resolved mechanically, indicating that cytokinesis and separation of mother and daughter cytoplasm had occurred.

Wal1p determines polarity development. In S. cerevisiae, the actin cytoskeleton is involved in establishing the bipolar budding pattern of diploid cells, and mutations in a number of genes including BEE1/LAS17 affect the budding pattern (2, 25, 51). Therefore, we examined the distribution of bud scars in wall and wild-type cells. Cells of the wall strain with three or more bud scars showed a high degree of randomized bud-site selection whereas the wildtype displayed regular (bi)polar budding (Fig. 4; Table 3). Determination of a new bud site is an initial step that polarizes the actin cytoskeleton toward the incipient bud site in the wild type. We therefore examined the distribution and positioning of cortical actin patches in wall in comparison to wild-type cells (Fig. 5). In wild-type cells, actin cortical patches localized within the bud at an early growth stage, then redistributed between mother and daughter cell during the isotropic growth phase of the bud, and finally localized to the bud neck to prepare for cytokinesis (Fig. 5A). In contrast, in wall cells, cortical actin patches were randomly distributed in mother and daughter cells throughout the cell cycle (Fig. 5B). Depolarization of cortical actin patches therefore accompanies isotropic growth, misplaced growth of mother cells, and defects in bud site selection of mutant cells.

Mutant wall cells exhibit defects in endocytosis and in vacuolar morphology. Defects in vacuolar morphology resulting in fragmented vacuoles were observed in S. cerevisiae in a certain allele of BEE1/LAS17, las17-16, which contains a C-terminal deletion of 21 aa that inactivates the Arp2/3-complex activation domain (11). To determine defects in vacuolar morphology in the wall strain, we stained Candida wild-type and wall cells using the lipophilic dye FM4-64 (Table 4). Our results clearly show that in contrast to wild-type cells, which contain one or two large vacuoles, wall strains contain a large number of cells with multiple vacuoles and only few cells with just one large vacuole (Table 4).

Furthermore, in S. cerevisiae the cortical actin cytoskeleton and Bee1p/Las17p are involved in endocytosis (28). Therefore, we examined endocytosis in Candida wild-type and wall cells by monitoring the uptake of FM4-64 in vivo using time-lapse microscopy (Fig. 6; Movie S4 in the supplemental material). Wild-type cells rapidly incorporated the dye, which resulted in staining of endosomes that moved around in the cytoplasm after 4 min (Fig. 6). Later (beginning after approximately 30 min in the time-lapse sequence), vacuoles of the wild type were stained, indicating efficient transport of the dye to the vacuole...
Fig. 6. Cells of the wall mutant, in contrast, required a prolonged time to internalize the dye (Fig. 6). Staining of endosomes was not observed in wall cells. Vacuolar staining appeared with a long delay compared to the wild type and was found to be much weaker than in the wild type (Fig. 6).

Wall1p is required for polarized hyphal growth in C. albicans. In contrast to S. cerevisiae, C. albicans is a dimorphic fungus that is capable of forming true hyphae. WAL1+ and wall− cells were induced to form hyphae under different inducing conditions (see Materials and Methods). Cells of the wild-type and heterozygous WAL1/wall strains initiated the formation of hyphae when grown on spider medium or on medium supplemented with serum. In contrast, hyphal growth was abolished in the wall mutant strain under these conditions (Fig. 7A). Hyphal growth resulted in wrinkled colonies (indicative of colonies containing hyphae and yeast cells), whereas yeast-like growth gave rise to shiny and smooth colonies. Hyphal growth was induced in the heterozygous mutant strain, in which the remaining copy of WAL1 was placed under the control of the regulatable MAL2 promoter when grown on serum-containing medium supplemented with maltose as the sole carbon source (Fig. 7B). These results demonstrate that Wall1p is required for hyphal growth in C. albicans. Microscopic examination indicated, however, that cell shape changes occurred in wall cells induced for hypha formation. Therefore, time-lapse analyses were used to monitor the growth of the wild-type and wall mutant strains under serum-inducing con-
ditions (Fig. 8A and B; Movies S5 and S6 in the supplemental material). In the wild type, hyphal formation occurred almost immediately on induction. Hyphae grew out with an extension rate of approximately 20 μm/h. These hyphae maintained hyphal growth and formed lateral branches. Interestingly, in the center of the mycelium, yeast cells were produced after 5 to 6 h under inducing conditions (Fig. 8A; Movie S5 in the supplemental material). In contrast, wal1 yeast cells initially responded to hyphal induction with polarized morphogenesis (Table 5). Polarized growth occurred with an extension rate of approximately 15 μm/h (Fig. 8B; Movie S6 in the supplemental material). Clear differences between hyphal and pseudohyphal cells can be seen at sites of septation. Whereas true hyphae formed septa that appeared as cross-walls not changing the diameter of the hyphal tube, pseudohyphal cells showed constrictions at septal sites (Fig. 8C and D; arrows). Thus, the septum position in the wal1 mutant strain indicates that pseudohyphal cells were formed. In our experiments, 69% of wild-type cells responded to hypha-inducing conditions (10% serum) with germ tube formation, a few cells developed pseudohyphae, and a minor fraction did not respond and stayed in the yeast phase. In the same experiment, wal1 cells did not form hyphae, the majority of cells (66%) formed pseudohyphae, and one-third of the cells did not respond to the induction (Table 5).

Wal1p exhibits a patch-like localization to sites of polarized secretion. To determine the intracellular localization of Wal1p, we fused GFP to the 3' end of WAL1 by using PCR-amplified cassettes with a 100-bp homology region to the target locus. We constructed two independent strains carrying heterozygous WAL1/WAL1-GFP alleles. From one of these strains, CAT19, a strain was constructed that produces only GFP-tagged Wal1p (WAL1-GFP:HS1/wal1::URA3) under the control of its endogenous promoter. This strain, CAT21, showed wild-type morphology, indicating that the WAL1-GFP construct is fully functional and suggesting that the GFP signals that were obtained reflect the correct localization pattern of Wal1p. GFP signals were similar in all strains, but the brightest signal could be obtained from CAT21, which was therefore used for localization studies presented here. We analyzed the distribution of Wal1p-GFP in both yeast cells and in hyphal cells (Fig. 9). Wal1p-GFP localized in a patch-like structure to sites of growth; it accumulated in emerging buds and at the tips of hyphae (Fig. 9). This, in part, resembles the localization of cortical actin patches, which also cluster in daughter cells and at hyphal tips (Fig. 5). In S. cerevisiae, localization of myc-tagged Bee1p revealed that the majority of Bee1p patches colocalize with actin patches (25). Colocalization with actin patches was also observed for other proteins, for example, for the C. albicans Myo5p, representing the only myosin I (36). To determine the colocalization of Wal1p with actin patches, we used double-label experiments. To this end, the actin cytoskeleton of strain CAT21 (WAL1-GFP) that was induced for hyphal formation was stained with rhodamine-phalloidin. Overlay of the two signals revealed that Wal1p-GFP found as patches colocalized with actin patches (Fig. 9B). Additionally, other, more dispersed Wal1p-GFP signals appeared not to colocalize with actin patches.

**DISCUSSION**

We chose to work on the dimorphic human fungal pathogen C. albicans in order to study polarized morphogenesis because...
this organism is able to switch between yeast-like and hyphal growth modes under defined regimens. This dimorphism plays an important role during several stages of infection, for example, during invasion of host tissues, evasion of the cellular host immune response, and colonization of internal organs (22, 31).

We have shown previously that in the filamentous fungus A. gossypii, Rho protein modules play a key role in the establishment of cell polarity via the Cdc42 module and during hyphal growth via the Rho3 module by regulating the organization of the actin cytoskeleton (47, 48). The regulatory role of the Cdc42 module on the architecture of the actin cytoskeleton during yeast and hyphal stages has recently been analyzed in detail in C. albicans (4, 17, 42). Other components that are involved in this process were found to be required for hyphal growth in C. albicans, such as the SLA2 and MYO5 (encoding a type I myosin) genes (3, 36). Signaling from Rho protein modules is transduced to the actin cytoskeleton by effector proteins (12, 45). Effectors that can regulate actin filament assembly either directly or via other protein-protein interactions are therefore of central importance for morphogenesis in C. albicans and may also serve as antifungal drug targets.

Fungal WASPs are different from mammalian WASP in that they lack a CRIB motif. Thus, they cannot bind directly to GTP-loaded Cdc42p. Recently, it was suggested that activation of the S. cerevisiae WASP Bee1p/Las17p is mediated by a complex including the G-protein Rho3p, Exo70p, and Rvs167p (1, 39).

**Functions of Wal1p.** Disruption of WAL1 caused major defects in yeast cell morphology, the organization of the cortical actin cytoskeleton, polarized growth under hypha-inducing conditions, early endocytosis, vacuolar morphology, and bud site selection. Defects of wall1 cells during yeast-like growth were similar to those observed in S. cerevisiae bee1/las17 mutants (25). S. pombe wsp1 mutants also exhibit defects in cell morphology, which, however, did not result in isotropic growth phases and round cells (24). In wild-type C. albicans yeast cells, localization of cortical actin patches follows similar polarization-depolarization events to those in S. cerevisiae, whereas during hyphal stages the localization of patches resembles that of true filamentous fungi (36, 45, 52). In wall1 cells, cortical actin patches were randomly positioned in mother and daughter cells during all stages of growth. This included the absence of clustered actin patches during bud emergence, suggesting that at this stage of the cell cycle, actin patches are dispensable. In contrast, the assembly of actin cables in wall1 cells appeared to be as in the wild type. At least, actin cables were found in emerging buds and appeared to localize in a mother bud axis (see, for example, the cell at the bottom right corner of Fig. 5B). Actin nucleation to form cables has recently been shown to be dependent on the formin Bni1p in S. cerevisiae (14, 15, 38,
This supports a model in which bud emergence may be initiated via a pathway including Cdc42p and Bni1p whereas polarized morphogenesis is maintained by correct positioning of cortical actin patches and localized secretion, which requires a WASP homolog. In *C. albicans*, two formin homologs were identified, corresponding to the *S. cerevisiae* BNI1 and BNR1 genes. Their function, particularly during early growth phases in *C. albicans*, is currently under investigation.

**Contribution of Wal1p to polarized morphogenesis.** Mutant *wal1* cells were unable to form hyphal filaments under all conditions tested, although these cells were able to initiate polarized morphogenesis to a limited degree on induction. Growth resulted in the formation of elongated pseudohyphal cells. In our time-lapse analyses under hypha-inducing conditions, we observed initial polarized morphogenesis in *wal1* cells that had kinetics comparable to that of the wild type. The *wal1* defect resulted in a failure to maintain polarized growth at the hyphal tip. Another hallmark of hyphal induction also failed to develop. Septation in hyphal filaments occurs as cross-walls compartmentalizing the hyphae without changing the hyphal diameter. In pseudohyphae, constrictions occur at septal sites which were also observed in *wal1* mutants. In *S. pombe* and *S. cerevisiae*, synthetic defects were observed in myosin I- and WASP-deficient strains (13, 24). This suggests a joint activity in a larger complex since WASP provides binding sites for myosin I binding through its proline-rich region (29). Indeed, in *S. cerevisiae*, Myo5p and Myo5p were found to interact via SH3 domains with the proline-rich region of Las17p/Bee1p (13). Additionally, fungal WASPs and type I myosins share a C-terminal acidic motif for activation of the Arp2/3 complex (23, 28). This is in line with observations in *C. albicans* myosin I mutants that exhibit morphological defects similar to those described in this study for *wal1*. Cells of the *myo5* mutant (carrying deletions in the only myosin I gene) were shown to be round during yeast stages and were unable to induce hyphal growth (36). A *myo5* S366D mutation, which mimics the phosphorylation of a serine residue at the TEDS-rule site and thus activates the protein, allowed hypha formation even in the absence of an accumulation of polarized actin patches (36).

**Contribution of Wal1p to endocytosis and vacuolar morphology.** In the *S. cerevisiae* bee1/las17 mutants, defects in endocytosis were observed and Las17p/Bee1p was found to be required for endosome and vacule movement (7, 28, 35). Here we provide in vivo time-lapse data that clearly show similar defects in the endocytosis of the dye FM4-64 into early endosomes (Fig. 6). In addition to uptake defects, vacuolar morphology in *wal1* cells was different from that in the wild type since cells were frequently found with perturbations in the

**FIG. 7.** Induction of hyphal growth in wild-type and mutant strains. (A) Hypha formation on solid media. Hypha formation was determined by plating the indicated strains as single cells on either Spider medium or YPD containing 10% serum. (B) Hyphal induction of strain CAT10 in which one allele of *WAL1* was deleted and the remaining copy was placed under control of the *MAL2* promoter. Plates contained 10% serum and complete medium with either glucose or maltose as the carbon source, resulting in either repressed or induced expression of *MAL2p-CaWAL1*, respectively. All plates were incubated for 4 days at 37°C prior to photography.
number of vacuoles (Table 4). In *S. cerevisiae*, a signal cascade starting from the Rho-type GTPase Cdc42p is required for vacuole fusion (11, 34). A genomic analysis of all viable *S. cerevisiae* mutants for mutations of homotypic vacuole fusion revealed almost 100 genes with defective vacuolar morphology (41). Among these were a number of genes required for remodeling of the actin cytoskeleton, such as *CLA4* or *BEM2* (41). The same group, showed that the *las17-16* allele produced "fragmented" vacuoles, resulting in a multivacuolar phenotype (11). These and our results suggest that fungal WASP homologues may also be involved in homotypic vacuolar fusion.

Our characterization of *WAL1* and previous results with *MYO5* suggest that both gene products are required for transport processes during endocytosis and polarized morphogenesis. These processes are essential during hyphal growth in *C. albicans* and presumably in other filamentous fungi as well. Our time-lapse analyses indicated that hyphal morphogenesis on induction of starved cells is a very fast process. Recently, it was shown that hyphal elongation occurs independently of the cell cycle in *C. albicans*. Even cells that had initiated a budding cycle were able to respond to induction cues and switched growth mode to form filaments (18). This allows us to ask new questions about hyphal growth in *Candida*, specifically whether

<table>
<thead>
<tr>
<th>Growth form</th>
<th>% of SC5314 cells</th>
<th>Appearance</th>
<th>% of <em>Cawal1/wal1</em> cells</th>
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<tbody>
<tr>
<td>Hyphae</td>
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<td></td>
</tr>
<tr>
<td>Pseudohyphae</td>
<td>13.4</td>
<td>66.6</td>
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<tr>
<td>Yeast</td>
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<td></td>
</tr>
<tr>
<td>No. of cells counted</td>
<td>404</td>
<td>410</td>
<td></td>
</tr>
</tbody>
</table>

TABLE 5. Analysis of polarized morphogenesis

![Image](image-url)
the induction of hyphal-phase-specific genes is required to trigger hyphal formation or, rather, if hyphal induction is such a fast process that may be initiated, for example, by posttranslational modifications. Accordingly, a recent report demonstrated that phosphorylation of WASP in the acidic domain resulted in an increased affinity for the Arp2/3 complex, which was thus proposed to be required for WASP function (9).

Understanding the signaling pathways in C. albicans that relay environmental signals to the actin cytoskeleton and result in the activation of key target proteins involved in the process of hyphal induction is thus one of the key fields of future research.

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