Polarized cell growth is important in all aspects of cellular development in both unicellular and multicellular organisms. Such growth requires the selection of a specific site, followed by rearrangement of the actin cytoskeleton to the site. A major regulator in these types of actin cytoskeletal rearrangements is the rho-type GTPase Cdc42, whose function and regulation have been studied extensively in Saccharomyces cerevisiae. Cdc42 is essential for budding, mating (1, 29), and pseudohyphal growth (19). Cdc42's subcellular localization correlates with its activity and is essential for its function.

During budding, green fluorescent protein (GFP)-Cdc42 clusters to the incipient bud site (22, 29), marked by the Bud1 GTPase module (6, 21), and later to the tips and sides of growing buds, until they reach medium size, at which point GFP-Cdc42 starts to diffuse. In large-budded cells postankar, GFP-Cdc42 clusters as a band at the mother-daughter neck region and persists as two distinct bands on mother-daughter cells following cytokinesis and cell separation (22). In S. cerevisiae, GFP-Cdc42 is also found at the plasma membrane of the entire periphery of the cell and on internal membranes. C-terminal CAAX and polylysine domains are sufficient for membrane targeting but not for clustering (22). During mating, Cdc42 is recruited to the tips of mating projections (shmoos) (29), possibly through the action of the pheromone receptor via Far1 and Cdc24 (12). In both cases, the activated Cdc42, in turn, activates downstream effectors that signal to the actin cytoskeleton. Studies done in S. cerevisiae with latrunculin A, a drug known to bind monomeric actin and therefore disassemble filamentous actin (F-actin) (8), have shown that Cdc42 accumulation at the prebud site is F-actin independent, whereas Cdc42 accumulation to the shmoo tip during mating is F-actin dependent (4, 5).

Candida albicans is a dimorphic fungus that can undergo reversible morphogenetic transitions between yeast and hyphal forms, and the ability to switch between these morphologies is linked to its pathogenesis (15). Hyphal elongation is an extreme example of polarized growth. The actin cytoskeleton is polarized to the tip of the hypha (3) and is required for hyphal elongation (28). A C. albicans CDC42 gene has been cloned, and it encodes a protein with a high degree of identity to S. cerevisiae Cdc42 (87.8%) and human Cdc42 (76.4%) (18). Functional analysis of cdc42 mutants of C. albicans suggested that Cdc42 is important for budding as well as polarized growth in hyphae (26).

Our previous studies suggested that polarized growth in response to hyphal induction in C. albicans is not mediated by altering the progression of the cell cycle (13), in contrast to pseudohyphal elongation in S. cerevisiae (14). Rather, hypha-associated polarization of the actin cytoskeleton at the hyphal tip is regulated independently of the cell cycle. We further postulated that the hypha-associated morphogenesis program and the cell cycle program converge to regulate a common signaling module (13), presumably Cdc42, which in turn controls the polarization of the actin cytoskeleton. In this report we show that Cdc42 is regulated, as reflected by its localization, by both the hyphal morphogenesis program and the cell cycle. We further demonstrate that GFP-Cdc42 accumulation at the tip of polarized growth during hyphal induction resembles mating in S. cerevisiae in that both rely on an intact actin cytoskeleton.

**MATERIALS AND METHODS**

**Plasmid and strain construction.** To construct a fusion of GFP to the N terminus of Cdc42 in *C. albicans*, we started with plasmid pHL471, which contains GFP and URA3 on a pBluescript SK (13). A second copy of GFP was

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generated by PCR from plasmid pH471 with primers 309 and 310 and cloned into the pNor site in pH471 to give pH584. The second GFP lacks the sequence for the first 8 amino acids and the stop codon but contains an Ala8 linker at the C terminus. We next generated two different GFP-Cdc42 fusions, one under its endogenous promoter and the other under the C. albicans ACT1 (CaACT1) promoter. A 500-bp PCR fragment from the CDC42 promoter region (with primers 315 and 316) was cloned into the XhoI and ClaI sites in pHL584 to give pH585. Then, for the CaACT1-regulated GFP-Cdc42, a 1,000-bp PCR fragment from the region upstream of CaACT1 (with primers 325 and 326) was cloned into the ClaI site of pH585 to give pH593. Next, a 500-bp PCR fragment from the CDC42 coding region immediately downstream of the start codon (with primers 319 and 320) was cloned in frame to the Ala8 linker into the CaACT1 locus. One copy of GFP is in frame with an Ala8 linker and CDC42 of primers used for PCR are indicated. Primers 217 and 344 are located outside of the CDC42 genomic region used in the construct. The second GFP is in frame with an Ala8 linker and CDC42. URA3 and one copy of GFP are excised by selecting for homologous recombination on 5-fluoro-orotic acid.

FIG. 1. GFP-CDC42 fusion construct. The region of plasmid pH598 used for C. albicans transformation is shown. The position and direction of primers used for PCR are indicated. Primers 217 and 344 are located outside of the CDC42 genomic region used in the construct. The second GFP is in frame with an Ala8 linker and CDC42. URA3 and one copy of GFP are excised by selecting for homologous recombination on 5-fluoro-orotic acid.

TABLE 1. Primers used in this study

| Primer | Sequence |
|--------|----------|
| 145....5'-ATA CCA TCC AAA TCA ATT CC-3' | 217....5'-AAA CAG CCC AAC ACA TCA AAG G-3' |
| 309....5'-ATA AGA ATG CCG CCG CAG TGG TGT TGT CCC AAT TTT GGT TG-3' | 310....5'-ATA GTT TAG CCG CCG CAG CCG CAG CCG CAG CTT TGT ACA ATT CAT CCA TAC CAT GG-3' |
| 315....5'-CCG TCT CAC CCG CTG TTA ATG GAT GAT GCC TTA GC-3' | 316....5'-CCA TCG ATG GGA TAT ATG GAT GAT ATT TAT GAG C-3' |
| 319....5'-TCC CCG CCG TGC AAA CTA TAA AAT GTG TTG TCG TC-3' | 320....5'-TCC CCG CCG CCG ACA CTG TTT CAC ATC GC-3' |
| 325....5'-CCA TCG ATC TAT TAA GAT CAC CAG CCT CG-3' | 326....5'-CCA TCG ATG TAT ATT TTT TTA GAT A-3' |
| 344....5'-AAT CTG CAC ACC CCG TGG CT-3' |
where latrunculin A was used, all samples were cultured in small volumes (75 to 500 µl) due to the cost of the reagent. Yeast-form cells were grown in 2-ml glass vials on a wheel at 25°C, and hyphal cells were grown in 1.8-ml plastic microcentrifuge tubes at 37°C in a shaking water bath.

Cell synchronization. Cell synchronization was performed as previously described (16). Unbudded G1 cells were released into YPD or SC at 30°C for yeast-form growth and YPD or SC with 10% serum at 37°C for hyphal growth, and aliquots of cells were taken for direct visualization by microscopy.

**Latrunculin A treatment.** Latrunculin A (Molecular Probes) was added directly to media from a 20 mM stock in dimethyl sulfoxide to a final concentration of 200 µM or 300 µM. In experiments where cells were treated with latrunculin A for longer than 3 h at 37°C, additional latrunculin A (one third of the initial amount) was added after 3 h.

**GFP-Cdc42 in S. cerevisiae.** GFP strain (HLY1541) were grown overnight in YPD. The C. albicans β-tubulin–GFP strain (HL1Y541) were grown overnight in YPD. The C. albicans 10-µl aliquots of the normalized overnight culture were added to 2 ml of 2× YPD, mixed with 2 ml of 1% top agar (cooled to 55°C), and poured over YPD plates, and 10-µl aliquots of latrunculin A in different concentrations (0, 0.5, 1.0, 2.0, 4.0, 8.0, and 16.0 mM) were prepared by diluting 20 mM latrunculin A stock into water and pipetted onto the center of a sterile 7-mm dish placed on the YPD plate. The plates were incubated at 30°C for 24 h.

**Microscopy.** A Zeiss Axioskop2 microscope with a 100× objective and a digital camera (SenSys Photometrics) was used for all microscopy. Visualization of GFP-Cdc42 (HLY3227) and β-tubulin–GFP (HLY1541) was performed as described by Hazan et al. (13).

**DNA replication.** To investigate if the actin cytoskeleton is required for GFP-Cdc42 localization to the hyphal tip in the events of the cell cycle, synchronous G1 cells were released into hyphal medium. GFP-Cdc42 localization at the tip coincided with germ tube emergence and appeared at approximately 30 min, which was 70 min prior to DNA replication, as analyzed by fluorescence-activated cell sorting (data not shown). In yeast-form cells, DNA replication, budding, and spindle pole body duplication all initiate at the G1/S transition, and the timing of DNA replication, spindle pole body duplication is the same between yeast and hyphal cells when synchronous G1 cells are transferred to yeast growth medium and hypha-inducing medium (13). The facts that GFP-Cdc42 localization to the tip coincided with germ tube emergence and appeared at approximately 30 min, which was 70 min prior to DNA replication, as analyzed by fluorescence-activated cell sorting (data not shown). In yeast-form cells, DNA replication, budding, and spindle pole body duplication all initiate at the G1/S transition, and the timing of DNA replication, spindle pole body duplication is the same between yeast and hyphal cells when synchronous G1 cells are transferred to yeast growth medium.

**Effect of latrunculin A on actin cytoskeleton and cell cycle progression in C. albicans.** In S. cerevisiae, GFP-Cdc42 localization during budding is F-actin independent, while its polarization during mating requires the actin cytoskeleton as shown by treatment of yeast cells with latrunculin A, a drug that causes a complete disassembly of F-actin within 2 min (4, 5). Latrunculin A acts on S. cerevisiae specifically through actin depolymerization because three act1 point mutants are resistant to latrunculin A treatment (5).

To investigate if the actin cytoskeleton is required for GFP-
Cdc42 localization during hyphal growth in *C. albicans*, we first examined the effect of latrunculin A on the actin cytoskeleton in *C. albicans*. No F-actin structures were detected by rhodamine-phalloidin staining in cells in 200 μM latrunculin A for 30 min or 4 h (Fig. 3A), indicating that latrunculin A caused a disruption of F-actin in *C. albicans*. Although budding in diploid wild-type cells of *S. cerevisiae* is mostly inhibited by 100 μM latrunculin A (5), the majority of *C. albicans* cells were able to bud in the presence of 200 μM latrunculin A (data not shown). At this concentration, latrunculin A only partially affected budding (Fig. 3B); 25.1 ± 9.8% of cells with duplicated spindle pole body cells were unbudded (Fig. 3Bg). In addition, 26.5 ± 9.4% of mitotic spindles were misoriented in latrunculin A-treated cells (Fig. 3Bh), likely due to the disruption of the actin cytoskeleton, which is known to be important for guiding astral microtubules into the bud in *S. cerevisiae* (25). It is likely that 200 μM latrunculin A does not completely depolymerize F-actin. The residual amount of F-actin was not detectable by rhodamine-phalloidin staining, but was sufficient to allow budding to happen. Consistent with this, *C. albicans* cells were unable to bud in the presence of 800 μM latrunculin A (Fig. 4A). Therefore, budding is F-actin dependant in *C. albicans*, but *C. albicans* is more resistant to latrunculin A in liquid medium than *S. cerevisiae*.

We also tested the growth inhibition of *C. albicans* by latrunculin A on solid media. Disks containing various concentrations of latrunculin A were placed on a lawn of cells (Fig. 3C). The latrunculin A concentration required to inhibit *S. cerevisiae* growth was similar to that reported previously (5). Similar concentrations of latrunculin A had no effect on *C. albicans* (data not shown). Nevertheless, halos were observed in the disks containing 10 μl of 8.0 and 16.0 mM latrunculin A.
Therefore, much higher concentrations of latrunculin A were required to arrest growth in C. albicans.

**Effect of latrunculin A on GFP-Cdc42 localization during budding and hyphal formation.** We first examined whether F-actin was required for GFP-Cdc42 localization in yeast-form cells. Elutriated G1 cells of the GFP-Cdc42 strain (HLY322) were transferred into yeast growth medium with and without latrunculin A. No GFP-Cdc42 patches or clusters were observed in the starting cells. In the presence of 200 μM latrunculin A, GFP-Cdc42 accumulation on the buds appeared at approximately 2 h (Fig. 4A). In the presence of 800 μM latrunculin A, cells were unable to bud, but a discrete cluster of GFP-Cdc42 was observed in unbudded cells after approximately 2.5 to 3 h of growth (Fig. 4A).

We believe that this GFP-Cdc42 clustering is not a remnant from a previous cell cycle but represents accumulation at the presumptive bud site, because it was not observed in these cells at earlier time points. Therefore, GFP-Cdc42 accumulation to the prebud site is not dependent on F-actin in C. albicans, as reported for S. cerevisiae (5). GFP-Cdc42 localization as a band at the mother-daughter neck was rarely seen in latrunculin A (200 μM and 800 μM)-treated cells, either because this event was out of the time frame of the experiment or because latrunculin A caused a cell cycle delay in late mitosis which affected the postanaphase GFP-Cdc42 localization.

To determine whether F-actin is required for hyphal tip-associated GFP-Cdc42 localization, synchronous G1 GFP-Cdc42 (HLY322) cells were put into hypha-inducing medium (SC with 10% serum at 37°C) in the absence and presence of 200 μM latrunculin A. At approximately 40 min, germ tube formation and GFP-Cdc42 clustering at the tip started to appear in the absence of latrunculin A (Fig. 4B, upper panel). Cells were longer in the later time points, and they all had GFP-Cdc42 accumulation at the tip. In contrast, in the presence of 200 μM latrunculin A, cells showed no GFP-Cdc42 clustering during the initial stage of hyphal induction and remained in their round shape with no buds or germ tubes (Fig. 4B, middle panel). Therefore, the hyphal tip-associated GFP-Cdc42 localization is F-actin dependent. It is clear that hyphal-associated polarized growth is much more sensitive to latrunculin A treatment than budding, since it required 800 μM latrunculin A to block budding. It is possible that cells need more F-actin to form polarized hyphae than they need to form a bud.

At approximately 130 min, discrete GFP-Cdc42 clusters were observed in what appeared to be small buds or germ tube evaginations (Fig. 4B, middle panels, 130 min). These “buds” appeared larger at later time points (Fig. 4B, middle panel, 160 and 200 min), and the discrete GFP-Cdc42 localization was more dispersed in large buds (Fig. 4B, middle panel, 230 min).
The formation of the bud-like morphology was not due to a decrease in the effectiveness of latrunculin A, because rhodamine-phalloidin staining at several time points did not detect reformation of the actin cytoskeleton. On the other hand, bud formation in this experiment is possible because 200 μM latrunculin A was sufficient to inhibit hyphal formation but not bud formation.

Three pieces of evidence suggested that the bud-like mor-
Phylogeny was indeed a bud but not a hyphal evagination. First, when these cells were fixed and stained with Calcofluor, the cells had chitin rings at the base of the bud-like structure (data not shown). Chitin rings typically appear at mother-bud necks but not at the base of hyphae. Second, the timing of their appearance in the cell cycle coincided with spindle pole body duplication. When synchronous G1 cells of the β-tubulin–GFP strain (HLY1541) were released into hypha-inducing conditions in the presence of 200 μM latrunculin A, they were not able to form hyphae but made similar bud-like structures at approximately 90 min. This coincided with the appearance of duplicated spindle pole bodies (Fig. 4C). Budding occurred earlier in this experiment than in the experiment in Fig. 4B because cells were released into YPD (instead of SC). Third, in a similar experiment in which hyphae were induced from synchronous G1 cells in the presence of 200 μM latrunculin A, hydroxyurea inhibited the bud-like structures from growing larger and arrested cells with duplicated spindle pole bodies, whereas in the absence of hydroxyurea, cells continued to enlarge their buds and progressed through the cell cycle (data not shown). Therefore, the discrete GFP-Cdc42 accumulation appearing at 130 min in the presence of latrunculin A reflected cell cycle-associated GFP-Cdc42 polarization at the site of budding rather than hyphal tip-associated GFP-Cdc42 localization.

To test the effect of latrunculin A on the maintenance of GFP-Cdc42 localization at the tip of hyphae, saturated cultures were first transferred into hypha-inducing medium for 80 min, and latrunculin A was then added to the hyphal cells to a final concentration of 200 μM. Clustering of GFP-Cdc42 at the tip was apparent in all hyphal cells in the starting culture (Fig. 5A, time zero). After 10 to 20 min of latrunculin A treatment, the GFP-Cdc42 tip polarization was not apparent anymore in many cells, and hyphal tips appeared swollen, indicating a switch from apical to isotropic growth (Fig. 5A, 20 min). At later time points, cells did not appear to have longer hyphae (in comparison to latrunculin A-free cultures), but had larger swollen tips (Fig. 5A, 40 min). At approximately 50 to 60 min, we observed GFP-Cdc42 clustering at the tip and side of buds evaginated from the end of these swollen enlargements (Fig. 5A, 60 min). When these cells were fixed and stained with Calcofluor, a chitin ring was apparent at the base of the bud-like structure (data not shown), supporting the notion that these were indeed buds evaginating off swollen hyphae. GFP-Cdc42 accumulation was no longer visible in cells with very large buds (Fig. 5A, 140 min), same as budding in yeast growth.

Based on the existence of a chitin ring at their bases, the cell morphology, and the timing of GFP-Cdc42 accumulation (130 to 140 min after the original release), we believe this was cell cycle-associated GFP-Cdc42 localization at the presumptive bud site, same as seen in Fig. 4B (130 to 200 min). In the control experiment, untreated hyphal cells all had constitutive GFP-Cdc42 localization at the tips of hyphae and had progressively longer hyphae at later time points (Fig. 5B). Therefore, sustained GFP-Cdc42 accumulation at the hyphal tip is also F-actin dependent.

**Role of actin cytoskeleton in transcriptional induction of hypha-specific genes.** Proper localization of the signaling machinery is often important for efficient signaling. In *S. cerevisiae*, latrunculin A causes a 60 to 70% reduction in pheromone-induced FUS1 transcription (an output of the pheromone-responsive mitogen-activated protein kinase pathway) compared to pheromone induction in the absence of latrunculin A (4). To examine whether the actin cytoskeleton is important for the hyphal transcriptional program, we examined the induction level of three hypha-specific genes (HWP1, ECE1, and

![FIG. 5. Effect of latrunculin A on sustained hypha-associated GFP-Cdc42 polarization. Saturated cultures of the GFP-Cdc42 strain were diluted into SC with 10% serum and grown at 37°C for 80 min, after which 200 μM latrunculin A (Lat-A) was either added (A) or not (B). Times indicated are incubation time in the presence of latrunculin A.](image-url)
HYR1) in cells grown in hypha-inducing medium in the absence and presence of 200 μM latrunculin A.

Northern blotting showed that the transcription level of these genes was undetectable in yeast-form cells and very high in hypha-form cells (Fig. 6). In latrunculin A-treated cells, HWP1 transcription was partially induced (roughly a sixth of that of the hypha-form cells without latrunculin A). Transcription of ECE1 and HYR1 in cells treated with latrunculin A was similar to that of yeast-form cells. Thus, transcriptional induction of hypha-specific genes is affected by the disruption of the F-actin cytoskeleton. The requirement of the actin cytoskeleton in the induction of developmental genes in both hyphal induction of C. albicans and mating of S. cerevisiae indicates that the two developmental processes may have similar signaling mechanisms.

**DISCUSSION**

*C. albicans* Cdc42 is involved both in the cell cycle and in hyphal morphogenesis (26). In support of this, we show that GFP-Cdc42 is clustered to the tips and sides of buds and to a band at the mother-daughter neck region in yeast-form cells. In hyphal cells, both constant accumulation of GFP-Cdc42 to the tip of growing hyphae and cyclic localization as a band in postanaphase germ tubes are observed. This is consistent with our previous observation that the rearrangement of the actin cytoskeleton in hyphal cells is regulated by the cell cycle and hypha-associated polarized growth programs in parallel. Since Cdc42 is known to be a key regulator for the rearrangement of the actin cytoskeleton in eukaryotes, its localization in *C. albicans* suggests that both cell cycle and hypha-associated polarized growth programs converge on Cdc42 to regulate the rearrangement of the actin cytoskeleton.

Interestingly, while cell cycle-associated GFP-Cdc42 localization is independent of the actin cytoskeleton, hypha-associated GFP-Cdc42 localization at the tip requires an intact actin cytoskeleton. Similar results have been shown for the localization of Cdc42, Cdc24, and other polarity establishment proteins during budding and shmoo formation in *S. cerevisiae* (4, 5). The difference in F-actin dependence between budding and mating is probably because mating projection formation requires persistent positioning of growth polarity proteins at the growth site by the activated pheromone receptor, whereas a single point localization of the polarity proteins by the Bud1 GTPase module is sufficient for bud site selection (20). F-actin is required to constrain or transport the pheromone receptors to a discrete site at the cell cortex during mating (4). The clustering of the receptors to a discrete site by actin is thought to recruit Cdc42 and other polarity establishment proteins and signaling molecules, thus amplifying the signal of locally activated pheromone receptors, leading to both polarized growth and a full level of transcriptional induction. Our results are consistent with the idea that polarized hyphal growth in *C. albicans* is regulated through mechanisms similar to those for mating in *S. cerevisiae*.

In addition to the F-actin-dependent Cdc42 localization at the hyphal tip, polarized growth during hyphal induction in *C. albicans* is analogous to mating projection formation in *S. cerevisiae* in the following aspects: (i) neither shmoo nor hyphal germ tubes have constrictions at their base; (ii) shmoo are formed in G1-arrested cells, and polarized growth in hyphae can also occur before the G1/S transition (13); and (iii) both mating cells of *S. cerevisiae* and hyphal cells of *C. albicans* have a noncytokinetic septin ring around the base of the shmoo or hypha (10, 24). This ring is also much fainter and less organized than the cell cycle-associated septin ring at the site of septation.

The analogy between mating and hypha formation makes shmooing in *S. cerevisiae* an attractive model for hypha formation. This analogy implies that *C. albicans* may also use transmembrane receptors to respond to hypha-specific cues. However, such receptors have not been discovered to date, and chemotropism has not been demonstrated in *C. albicans*. In addition, hypha induction is probably more complex than the “one ligand-one receptor” model of mating. Not only serum and hormones (such as estrogen [27]) are hyphal inducers; growth conditions such as pH, heat, and starvation also induce hyphae. Despite this, it is still possible that less conserved receptors are used to signal hyphal morphogenesis in *C. albicans*, because the functions of the core regulatory molecules, such as Cdc42 and actin, are highly conserved. We favor the model which suggests that clustering of receptors by F-actin and other molecules mediates sustained Cdc42 localization, which then amplifies the signal for polarized growth during hyphal morphogenesis and allows efficient signal transduction for transcriptional induction of hypha-specific genes.

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