Myosin I Is Required for Hypha Formation in Candida albicans†

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The pathogenic yeast Candida albicans can undergo a dramatic change in morphology from round yeast cells to long filamentous cells called hyphae. We have cloned the CaMYO5 gene encoding the only myosin I in C. albicans. A strain with a deletion of both copies of CaMYO5 is viable but cannot form hyphae under all hypha-inducing conditions tested. This mutant exhibits a higher frequency of random budding and a depolarized distribution of cortical actin patches relative to the wild-type strain. We found that polar budding, polarized localization of cortical actin patches, and hypha formation are dependent on a specific phosphorylation site on myosin I, called the “TEDS-rule” site. Mutation of this serine 366 to alanine gives rise to the null mutant phenotype, while a S366D mutation, the product of which mimics a phosphorylated serine, allows hypha formation. However, the S366D mutation still causes a depolarized distribution of cortical actin patches in budding cells, similar to that in the null mutant. The localization of CaMyo5-GFP together with cortical actin patches at the bud and hyphal tips is also dependent on serine 366. Intriguingly, the cortical actin patches in the majority of the hyphae of the mutant expressing Camyo5S366D were depolarized, suggesting that although their distribution is dependent on myosin I localization, polarized cortical actin patches may not be required for hypha formation.

Polarized growth is a regulated cellular expansion which underlies many processes, such as phagocytosis in mammalian cells, morphogenesis of root hair and other specialized cell types in plants, cell locomotion in Acanthamoeba and Dictyostelium, and hypha formation in fungi (19, 24, 37, 49). Saccharomyces cerevisiae cells can elongate into pseudohyphae in response to specific environmental cues, and polarization of the actin cytoskeleton is essential for this differentiation (11). Similarly, hyphal morphogenesis in other fungi, such as Saprolegnia ferax, Neurospora crassa, and Aspergillus nidulans, requires filamentous actin, while microtubules play a secondary role (20, 51, 52). The pathogenic yeast Candida albicans can undergo a dramatic change in morphogenesis when round yeast cells form highly elongated filaments called hyphae, but little is known about the role of the actin cytoskeleton during hypha formation in this organism (25). Treatment of germinating cells with cytochalasin A prevents further hyphal growth, suggesting that filamentous actin is critical to hyphal growth (2). Nocodazole, in contrast, does not prevent apical cell elongation, suggesting that microtubules are not critical for polarized growth in C. albicans (61).

Two forms of actin appear to be important during polarized growth. First, actin cables serve as tracks for the vesicular transport of molecular components of the plasma membrane and cell wall toward the site of growth, i.e., the bud tip (23, 44, 45) and presumably the hyphal tip of C. albicans. These run along the longitudinal axis of yeast and hyphal cells in S. cerevisiae and C. albicans (4, 44). Second, cortical actin patches correlate with sites of targeted secretion and endocytosis, critical during cell wall biogenesis (44). These localize to the tips of emerging buds as well to growing hyphal tips in C. albicans (4). Proteins that modulate the structure of the actin cytoskeleton are key factors in determining cell polarity (44). Myosin I, one of these factors, is a single-headed molecular motor that functions in actin-based processes such as polarized growth, cell motility, phagocytosis, endocytosis and exocytosis, and contractile vacuolar activity in several organisms (12, 13, 32, 37, 40, 46, 50, 58). In S. cerevisiae, myosin I was shown to promote actin polymerization at cortical patches, which correlate with sites of growth (1, 14, 29). This myosin I regulation of actin polymerization was shown to be achieved by its interaction with and activation of the Arp2/3 complex, which nucleates the assembly of actin filaments (14, 29, 33, 34). Similarly, in Schizosaccharomyces pombe, myosin I is required for a polarized actin cytoskeleton and was shown to bind to the Arp2/Arp3 complex and activate its actin nucleation activity (32, 53). Jung et al. (22) also found that Dystostelium myosin I interacts with the Arp2-Arp3 complex via the CARMIL protein and may localize actin polymerization to sites of cellular growth. In accordance with its proposed role in polarized growth, myosin I colocalizes with cortical actin patches at the tips of buds in S. cerevisiae and of growing cells in S. pombe (3, 32, 53) and localizes as well to the tips of hyphae in Aspergillus (36).

The actin-dependent ATPase activity of Acanthamoeba myosin I and Dictyostelium myosin I is activated by the phosphorylation of a unique site, called the “TEDS-rule” site, by members of the p21-activated kinase (PAK) kinases (7, 9, 56). The corresponding phosphorylation site of S. cerevisiae myosin I is essential for its function in vivo and for its ability to polymerize...
actin in vitro (29, 57). It is also a target of the Ste20p and Cla4p kinases in vitro (57). These latter proteins are members of the PKA family of protein kinases and function to regulate cell morphology (6, 27, 47). Homologues of these kinases are involved in hypha formation in C. albicans (8, 26, 28).

In this study, we took a genetic approach to define the role of myosin I in C. albicans. We found that myosin I is required for hypha but not pseudohypha formation and that the PKA phosphorylation site (serine 366) is critical for myosin I function during budding yeast and hyphal growth.

MATERIALS AND METHODS

DNA manipulations. The oligonucleotides used for cloning are listed in Table 1. The CaMYO5 gene was amplified by PCR from genomic DNA prepared from C. albicans strain SC5314 by using the UO5 and UO6 oligonucleotides. The PCR products were digested with BamHI and HindIII as well as with HindIII and XbaI. The 3.75- and 1.5-kb fragments were subcloned into pBluescript KS (Stratagene) to give pU14 and pU15, respectively. Several independent clones were verified by sequencing. The alignment of these sequences revealed the existence of two alleles for CaMYO5 in SC5314, as expected for a diploid organism. The allele that was used for subsequent cloning was different from the sequence available in the Stanford genomic database for amino acids R354K, T585A, and I954V and for a short deletion, available in the Stanford genomic database for amino acids R354K, T585A, and I954V and for a short deletion.

To construct the disruption cassette containing the S366A and S366D mutant alleles of CaMYO5, a 3.75-kb BamHI-HindIII fragment from pU55 was fused by PCR by using the reverse primer with UO26 and by using UO27 with UO6. The 1.5-kb PCR product was subcloned as a HindIII-XbaI fragment into pKS (pU50). A 700-base HindIII-PstI fragment encoding green fluorescent protein (GFP) from pGFP26 (38) and a 1-kbp KpnI-HindIII partial fragment from pU50 were subcloned into pU50 digested with KpnI and PstI (pU88). Several clones were verified by sequencing. Finally, a 2.2-kb XbaI-HindIII partial fragment from pU88 and a 3.5-kb HindIII-BamHI fragment from pU46A were subcloned together into pVEC digested with BamHI and XbaI to generate pU93. The S366A and S366D mutant alleles of CaMYO5 were obtained by site-directed mutagenesis by using a Quick Change kit from Stratagene. First, the mutations were introduced into pU14 by PCR with Pfu polymerase and by using primer UO28 with primer UO29 (S366A mutation; pU55) and primer UO30 with primer UO31 (S366D mutation; pU56). These clones were sequenced to ensure that the S366A and S366D mutations were introduced while no other mutations had occurred. A 3.75-kb BamHI-HindIII fragment from each of pU55 and pU56 and a 1.5-kb HindIII-XbaI fragment from pU5U were subcloned into pVEC digested with BamHI and XbaI (pU77 and pU78, respectively). Similarly, the 3.75-kb BamHI-HindIII fragments and a 2.2-kb HindIII-XbaI fragment from pU88 were subcloned into pVECT to create pU97 and pU98, which contain the S366A and S366D mutant alleles of CaMYO5 in frame with the GFP sequence, respectively. To express the S366A and S366D mutant alleles of CaMYO5 under the control of the PCK1 regulatable promoter, a 4.75-kb fragments were PCR amplified from pU77 and pU78 by using UO19 and UO20. These fragments were digested with BglII and BamHI and subcloned into pJA24 (PCK1 promoter in p5921; a kind gift from J. Ash) digested with BglII (pU50 and pU56, respectively). These clones were kept in which the fragments were oriented such that the ATG of CaMYO5 was immediately downstream of the PCK1 promoter.

Transformations in C. albicans and S. cerevisiae. The strains used in this study are listed in Table 2. Transformation of C. albicans and S. cerevisiae strains was done by the lithium acetate method (21). To create a mutant in which both copies of the CaMYO5 gene were deleted, the Ura + strain CAI4 (15) and the Ura - strain strain C4H-15 (15) were transformed with pU21 digested with KpnI and SacI. Genomic DNA was prepared from Ura + transformants, digested with SpeI, and analyzed for the correct integration event by Southern blotting with a digoxigenin system (Boehringer Mannheim). Positive transformants derived from strain CAI4 were plated on medium containing 5-fluoroorotic acid (Diagnostics Chemicals Ltd., Charlotte, NC, England, England). A drug which inhibits the growth of Ura - strains. Ura - strains thus obtained were analyzed similarly for the longest period that occurred by homologous recombination between the hisG direct repeats flanking the URA3 gene. The CaMYO5/Cam50::his5G strain was transformed with pU21 digested with KpnI and SacI. Ura - transformants were analyzed similarly for the correct integration event resulting in the disruption of the second CaMYO5 allele with the

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### Table 1. Oligonucleotides

| Oligonucleotide | Sequence |
|-----------------|----------|
| UO5             | GCGGATCCTTGAGTTACACCTTTGTTTAGG |
| UO6             | GCTCTAGAATGGCCATGTTGG |
| UO9             | CGGGGTACCAAGAGCTGTTG |
| UO10            | CGGCGATCTGAGCTGCTTTCATAAGG |
| UO11            | GGCCGTACCTGAGGAGTTGAAGGTG |
| UO19            | GCCGATTCGTGACAGCTTACCATG |
| UO26            | GCAAGCTTACGCCTGCAGTAGGTAAGAACTTGTTATATTTTG |
| UO27            | CGTACGGGTAGCTGGCCAAATCATCAGCAGTCCTTCC |
| UO28            | CGGGGTACGAGGTGAAGGTG |
| UO29            | CGGGGATCCGGTGAAGGTG |
| UO30            | CGGATGACAGGAGGTGACACTTATCATCACC |
| UO31            | GGTGAATGATAAGTGTCACCTGCTGTCATCC |
| UO36            | CCGGGGTACGAGGTGAAGGTG |
| UO37            | CCGGGGATCCGGTGAAGGTG |
| CB20F           | CCGGGGATCCGGGATGGG |
| CB20R           | CCGGGGATCCGAGGAGG |
| Reverse         | GGGGATCCGGGATCCGGGATGGG |

* Restriction sites used for cloning are underlined in UO5 to UO27 and UO36 to CB20R. Codons mutated for site-directed mutagenesis are underlined in UO28 and UO29.
C. albicans

Strain | Genotype | Reference or source
--- | --- | ---
SC5314 | CaMYO5/CaMYO5 CaURA3/CaURA3 | 15
CAI4 | ura3::iimm434/ura3::iimm434 | 15
RM1000 | CAI4 Cahis1::hisG/Cahis1::hisG | This study
COU13 | CAI4 CaMYO5/CaMYO5::hisG-U3A3::hisG | This study
COU42 | CAI4 Camyo5::hisG/Camyo5::hisG-U3A3::hisG | This study
COU186 | CAI4 Camyo5::hisG/Camyo5 (CamyOS-GFP) | This study
COU46 | CAI4 Camyo5::hisG/Camyo5::hisG | This study
COU190 | CAI4 Camyo5::hisG/Camyo5 (CamyOS::GFP) | This study
COU201 | CAI4 Camyo5::hisG/Camyo5 (CamyOS::GFP) | This study
COU232 | CAI4 Camyo5::hisG/Camyo5 (CamyOS::GFP) | This study
COU243 | CAI4 Camyo5::hisG/Camyo5 (CamyOS::GFP) | This study
CLJ4 | CAI4 Cacla4::hisG/Cacla4::hisG | 28

S. cerevisiae HA31-9c | MATa can1-100 ade2-1 his3-11 leu2-3, 112 ura3-1 trp1-1 myo3::HI15 myo5::TRP1 | 18

**TABLE 2. Strains**
albicans. In contrast, two such genes, MYO3 and MYO5, are found in S. cerevisiae (18). The presence of a single myosin I-encoding gene was confirmed by low-stringency Southern blotting of C. albicans genomic DNA probed with a sequence corresponding to the SH3 domain of CaMYO5 (data not shown). CaMYO5 was amplified from genomic DNA prepared from wild-type strain SC5314 by PCR with specific oligonucleotides designed according to the sequence in the database. The PCR products were cloned, and four independent clones were sequenced to confirm their identities. The amino acid sequence was aligned with those of the Myo3 and Myo5 proteins of S. cerevisiae. On average, the amino acid sequence of CaMyo5 is 40% identical with those of Myo3 and Myo5. The highest conservation is found for residues in the N-terminal head domain involved in motor activity and including the TEDS-rule site (data not shown).

To determine if CaMYO5 is a functional homologue of the S. cerevisiae MYO3 and MYO5 genes, the C. albicans gene was introduced into S. cerevisiae strain HA31-9c (18), which is temperature sensitive due to deletion of the MYO3 and MYO5 genes. This strain could grow at the restrictive temperature when carrying MYO5 on a centromeric plasmid (pVL62) but not when carrying the vector alone (pRS316) (data not shown). When this strain carried the CaMYO5 gene on a multicopy plasmid, it could grow at the restrictive temperature, but it barely grew when carrying CaMYO5 on a centromeric plasmid (data not shown). Thus, the overexpression of CaMYO5 can complement the growth defect of myo3 myo5 strains.

Myosin I is not essential for viability in C. albicans. To study the role of myosin I in C. albicans, we created a strain in which both copies of CaMYO5 were deleted (∆/∆ Camyo5). Figure 1 shows the sequential disruption of both alleles of CaMYO5 in C. albicans (for details, see Materials and Methods). A single ∆/∆ Camyo5 transformant was obtained starting from strain CAI4 (15), and 19 ∆/∆ Camyo5 transformants were obtained starting from strain RM1000 (39). The latter strain is auxotrophic for histidine as well as uracil, enabling the disruption of each allele with a different marker (CaHIS1 and CaURA3).
These 20 ΔΔ Camyo5 mutants displayed similar phenotypes, as described below. The ability to generate viable double-mutant strains indicates that the myosin I gene is not an essential gene in *C. albicans*.

Myosin I is required for the polarized distribution of cortical actin patches. We examined cells of the ΔΔ Camyo5 mutant and the wild-type strain (SC5314) microscopically for phenotypes that would indicate the role played by myosin I in budding yeast cells. Cells of the ΔΔ Camyo5 mutant viewed by Nomarski optics looked abnormally round, in some instances enlarged, and were clumped compared to wild-type cells (Fig. 2). To determine whether cortical actin patches were mislocalized in the ΔΔ Camyo5 mutant, we treated cells grown exponentially with rhodamine-phalloidin to stain filamentous actin.

**FIG. 2.** Cortical actin patch patterns in wild-type and mutant cells. Cells were fixed and stained with rhodamine-phalloidin. Nuclear DNA was stained with DAPI. Scale bar, 10 μm.
Localization of cortical actin patches in budding cells of *S. cerevisiae* and *C. albicans* has been described extensively (4, 44). In summary, cortical actin patches localize to the growing bud tip, redistribute evenly in an isotropic growing bud, and finally localize at the mother bud neck after cytokinesis. In our experiments, cortical actin patches in 69% of wild-type cells were entirely localized to the buds during early bud emergence and growth (Fig. 2 and Table 3). These patches remained in the buds of 18% of cells during and after nuclear division. These patches also were localized at the mother bud neck in 10% of cells. Strikingly, for the ΔΔ*Camyo5* mutant, only 3% of cells retained cortical actin patches exclusively in the buds. In this mutant, cortical actin patches were localized evenly in both the mother cell and the buds for 44% of cells before

### TABLE 3. Cortical actin patch patterns in yeast cells

| Pattern                            | Appearance | % of cells of the following strain with the indicated pattern: | | |
|------------------------------------|------------|---------------------------------------------------------------|---|---|
|                                    | DAPI       | RP               | SC5314 | ΔΔ (CaMYO5) | ΔΔ (CaMYO5S366D) |
| Polarized in bud                   |            |                  |        |             |                  |
|                                    |            |                  | 68.8   | 2.9         | 70.5             |
|                                    |            |                  |        |             | 6.9              |
| Loose                              |            |                  | 0      | 13.3        | 0                |
|                                    |            |                  |        |             | 16.0             |
| Depolarized                        |            |                  | 0      | 43.7        | 0                |
|                                    |            |                  |        |             | 41.5             |
| Polarized in bud; nuclear division |            |                  | 18.0   | 1.5         | 17.6             |
|                                    |            |                  |        |             | 4.8              |
| Depolarized; nuclear division      |            |                  | 1.2    | 31.1        | 2.8              |
|                                    |            |                  |        |             | 21.0             |
| Bud neck; cytokinesis              |            |                  | 8.1    | 0.7         | 7.7              |
|                                    |            |                  |        |             | 0.5              |
| Bud neck, loose; cytokinesis       |            |                  | 1.9    | 6.6         | 0.7              |
|                                    |            |                  |        |             | 7.4              |
| Depolarized; G1                    |            |                  | 1.9    | 0           | 1.4              |
|                                    |            |                  |        |             | 1.6              |

| Total no. of cells  | 161 | 135 | 142 | 188 |

*DAPI, nuclear staining; RP, rhodamine phalloidin, F-actin staining. Polarized in bud, cortical actin patches localize exclusively in bud; loose, cortical actin patches localize preferentially in bud but also in mother cell; depolarized, cortical actin patches localize equally in bud and mother cell; nuclear division, nuclei dividing; bud neck, cortical actin patches localize exclusively at neck; bud neck, loose, cortical actin patches localize at bud neck preferentially but also in daughter and mother cells; cytokinesis, nuclei have completed division, and mother and daughter cells are about the same size; G1, no apparent budding. Scale bar, 1 μm.*
nuclei had divided and in 31% of cells during nuclear division. Finally, in 7% of cells, the patches were localized at the mother bud neck while also localizing in daughter and mother cells. Overall, cortical actin patches are dramatically mislocalized in the \( /H9004 /H9004 /H9004 /H9004 \) Camyo5 mutant, suggesting that myosin I plays a role in the organization of the actin cytoskeleton.

Myosin I plays a role in chitin deposition in the cell wall and in the budding pattern. To determine whether myosin I also plays a role in cell wall biogenesis in \( C.\) albicans, cells of the mutant and wild-type strains were labeled for chitin with Calcofluor White. In the mutant, a small proportion of cells that were unusually enlarged and round showed aberrant chitin deposition in the cell wall (Fig. 3). These findings were not observed in the wild-type strain. At the same time, we found that nearly 20% of mutant cells exhibited a random budding pattern; the value for wild-type cells was 2% (Table 4). Mutations in actin or other cytoskeletal proteins lead to a high frequency of random budding patterns in diploid \( S.\) cerevisiae cells (11, 60). The higher incidence of random budding in the \( /H9004 /H9004 /H9004 /H9004 \) Camyo5 mutant than in the wild-type strain correlate with these data, suggesting that the actin-dependent mechanisms for bud site selection are similar in \( C.\) albicans and \( S.\) cerevisiae. In addition, \( C.\) albicans myosin I may be important for the regulation of cell wall biogenesis, based on the abnormal chitin deposition observed in the cell wall of the mutant.

Reintegration of wild-type myosin I in the null mutant leads to the recovery of wild-type characteristics. To determine whether the phenotypes of the mutant strain are due to the deletion of both copies of myosin I, the wild-type \( CaMYO5 \) gene was reintegrated at one of the \( CaMYO5 \) loci in a Ura\(^{-}\) strain (see Materials and Methods). Six transformants or “reintegrant” strains were thus obtained, and all exhibited normal cell shape. One reintegrant (COU73) was selected for more detailed analysis. This reintegrant exhibited normal cell shape (Fig. 2), cortical actin patch distribution (Fig. 2 and Table 3), and budding pattern (Fig. 3 and Table 4). Thus, the deletion of myosin I alone is responsible for the phenotypes of the \( /H9004 /H9004 /H9004 /H9004 \) Camyo5 strain.

Myosin I is required for the formation of hyphae on solid and in liquid media. We examined the ability of the \( /H9004 /H9004 /H9004 /H9004 \) Camyo5 mutant to form hyphae under different hypha-inducing conditions. The \( /H9004 /H9004 /H9004 /H9004 \) Camyo5 and wild-type (SC5314) strains were plated for single colonies on agar containing 10% FBS, Spider medium, SLAHD medium, or Lee’s medium and were grown for 4 to 5 days at 37°C. The wild-type strain could form extensive hyphae on all media (Fig. 4A and data not shown). In contrast, the \( /H9004 /H9004 /H9004 /H9004 \) Camyo5 strain did not form hyphae on any medium (Fig. 4A and data not shown). Occasional extensions could be observed on parts of a \( /H9004 /H9004 /H9004 /H9004 \) Camyo5 colony grown on agar containing 10% FBS, but microscopic examina-

### Table 4. Budding patterns

| Pattern | Appearance | SC5314 | Camyo5/ Camy05 | \( \Delta\Delta \) (Camyo5) | \( \Delta\Delta \) (Camyo5\(^{S366A}\)) | \( \Delta\Delta \) (Camyo5\(^{S366D}\)) |
|---------|------------|--------|----------------|--------------------------|--------------------------|--------------------------|
| Polar   |            | 97.7   | 80.6           | 98.6                     | 83.2                     | 90.2                     |
| Random  |            | 2.3    | 19.4           | 1.4                      | 16.8                     | 9.8                      |
| Total no. of cells |        | 177    | 139            | 145                      | 101                      | 133                      |

* Cells with more than two buds and/or bud scars were counted. Scale bar, 1 \( \mu\)m.
FIG. 4. Hypha formation in wild-type and mutant strains. (A) Hypha formation on solid media was determined by plating single colonies of each strain on 10% FBS (a) or Spider medium (b) plates. Plates were incubated for 4 days at 37°C. Scale bar, 1 mm. (B) Hypha formation in liquid YPD supplemented with 10% FBS was determined at 37°C after 2.5 h of incubation. Scale bar, 10 μm.
tion showed that these were formed by pseudohyphal cells (data not shown). To confirm that the deletion of myosin I alone was the cause of this nonhyphal phenotype, we tested the ability of the reintegrant strain to form hyphae under these conditions. This strain could form hyphae to the same extent as wild-type strain SC5314 (Fig. 4A and data not shown). These results indicate that myosin I is required for hypha formation on solid hypha-inducing media.

To determine cellular morphology under hypha-inducing conditions, cells were also incubated in liquid YPD medium containing 10% FBS or in Lee’s medium at 37°C. After 2 to 3 h of incubation, cells of the wild-type strain formed germ tubes that extended into hyphae (Fig. 4B and data not shown). In contrast, cells of the ΔΔ Camyo5 strain occasionally formed germ tubes but were unable to form hyphae after more than 6 h of incubation in YPD medium containing serum or overnight incubation in Lee’s medium, by which time the wild-type and reintegrant strains had formed extensive hyphae (data not shown). Pseudohyphal cells could be observed in the ΔΔ Camyo5 mutant instead (Fig. 4B). Because the ΔΔ Camyo5 strain could form pseudohyphae but not hyphae, it is possible that myosin I is required for maintaining polarized growth but not for initiating it.

Myosin I localizes to the bud and hyphal tips and partially colocalizes with cortical actin patches. To determine the localization of myosin I in C. albicans, we fused in frame the sequence encoding GFP (38) at the 3′ end of the CaMYO5 open reading frame. We then introduced CaMYO5-GFP into the Ura−ΔΔ Camyo5 strain, such that CaMyo5-GFP was the sole source of myosin I. Two independent transformants that were examined could form hyphae in the presence of serum. The CaMyo5-GFP protein in these transformants was detected by Western blot analysis as a band of 175 kDa (Fig. 5A). These results suggest that CaMyo5-GFP functions properly in the ΔΔ Camyo5 strain. Figure 5B shows the localization of CaMyo5-GFP in exponentially growing cells. CaMyo5-GFP clearly localized in patches at the tip of emerging buds. CaMyo5-GFP localization could also be observed at the mother bud neck after nuclear division was completed (Fig. 5B and data not shown). The localization of CaMyo5-GFP in hyphal cells was examined as well (Fig. 5C). CaMyo5-GFP localized in patches at the hyphal tip. The patch-like distribution of CaMyo5-GFP in budding and hyphal cells was similar to that of actin in cortical patches. To determine whether CaMyo5-GFP colocalizes with actin, we stained yeast cells and hyphal cells with rhodamine-phalloidin (Fig. 6). In most of the cells examined (108 of 120), there was either very good colocalization or partial colocalization of CaMyo5-GFP and cortical actin patches (two or more spots coinciding) either at the bud or hyphal tips or at the mother bud neck or septae (Fig. 6 and data not shown).

Serine 366 in myosin I is essential for normal growth and hypha formation. In several organisms, the ATPase activity of myosin I is increased by phosphorylation of a conserved serine residue in the head domain (10, 31, 56). In S. cerevisiae, this phosphorylation is catalyzed by activated Ste20 and Cla4 kinases (57). Ste20 and Cla4 are members of the PAK family of protein kinases which may be activated by Cdc42p and function to regulate cell morphology (26, 27). To determine whether the phosphorylation of myosin I by C. albicans PAK kinases is important for hypha formation, we created mutant alleles of CaMYO5 that code for alanine or aspartate instead of serine in the unique PAK phosphorylation site (serine 366). These alleles were reintroduced into the ΔΔ Camyo5 strain at the Camyo5::hisG loci (see Materials and Methods). Proper integration and expression of these alleles in several transformants were confirmed by Southern and Northern blot analyses, respectively (data not shown).

To determine whether Camyo5S366D and Camyo5S366A can rescue the phenotypes of the ΔΔ Camyo5 strain, we measured the growth rates of strains carrying these alleles and characterized their budding patterns. We found that the wild-type strain and the strain expressing Camyo5S366D (COU201) divided every 55 min at 37°C in 2× YPD (Table 5). In contrast, the ΔΔ Camyo5 strain and the strain expressing Camyo5S366A (COU190) divided every 69 min under the same growth conditions (Table 5). We also found that the S366A mutation increased random budding 10-fold, similar to the complete deletion of myosin I, while the S366D mutation increased random budding 5-fold (Table 4). Although there was no abnormal chitin deposition in cells expressing Camyo5S366D, abnormal chitin staining could be observed in cells expressing Camyo5S366A (data not shown). These results suggest that the phosphorylation of serine 366 is important for optimal growth and for cell wall biogenesis. Furthermore, phosphorylation of serine 366 may also be important, albeit to a lesser extent, for localization of the bud site. We also observed that the mutant expressing Camyo5S366D did not form hyphal cells in YPD (Fig. 2), suggesting that other components of the actin cytoskeleton need to be activated to allow hypha formation under non-hypha-inducing conditions.

The mutant strains were plated for single colonies on agar containing 10% FBS and on Spider medium to determine their ability to form hyphae. All six transformants that expressed Camyo5S366D formed fuzzy colonies similar to those of the wild-type and reintegrant strains (Fig. 4A). Under liquid hypha-inducing conditions, these cells were able to form hyphae as well. However, the numbers and rates of growth of the hyphae formed were lower than those of the wild-type strain (Fig. 4B and Table 6). The majority of seven transformants that expressed Camyo5S366A formed fuzzy colonies with much shorter fringes than the wild-type and reintegrant strains on agar containing 10% FBS, but on the whole periphery of the colony, unlike the ΔΔ Camyo5 strain (Fig. 4A). On Spider medium, the colonies of the strain expressing Camyo5S366A were consistently larger than those of the wild-type and ΔΔ Camyo5 strains. Microscopic examination revealed that colonies of this strain consisted of pseudohyphal cells (data not shown). In liquid YPD supplemented with 10% FBS or in Lee’s medium, cells of the strain expressing Camyo5S366A were unable to form hyphae but formed pseudohyphae to a greater extent than cells of the ΔΔ Camyo5 strain (Fig. 4B and data not shown). These results suggest that CaMyo5S366A still retains some activity sufficient for more uniform pseudohyphal growth. Overall, the phosphorylation of serine 366 appears to be important for the proper function of CaMyo5 during hyphal growth.

To determine whether the TEDS-rule site serine 366 is important for the localization of myosin I, we tested the localization of CaMyo5S366A-GFP and CaMyo5S366D-GFP in normal
cells and elongated pseudohyphal or hyphal cells. These proteins were also detected by Western blot analysis as 175-kDa bands, and their levels were similar to the CaMyo5-GFP protein level (Fig. 5A). We found that the majority of CaMyo5S366A-GFP did not localize in cortical actin patches but localized in the cytoplasm (Fig. 5B and C). The majority of CaMyo5S366D-GFP did not localize to cortical actin patches either (Fig. 5B and C). Occasionally, some patches could be observed at the tips of buds, but these were not observed at hyphal tips. Mainly cytoplasmic staining and minor punctate staining at the periphery could be observed in these hyphae. These results suggest that serine 366 is important for the proper localization of myosin I and, surprisingly, that the proper localization of myosin I at the hyphal tip is not required for hyphal growth.

**Polarized distribution of cortical actin patches is dependent on serine 366.** Because myosin I appeared mislocalized in the phosphorylation site mutants, we also determined the localization of cortical actin patches in these strains. Cortical actin patches were dramatically mislocalized to the mother cells...
The cortical actin patch distribution in cells expressing Camyo5\textsuperscript{S366D} was quantified more precisely (Table 3). Cortical actin patches were localized uniquely in the buds in only 7\% of cells. They were also distributed in both the buds and the mother cell in 42\% of cells prior to nuclear division and in 21\% of cells after nuclear division. Finally, in 8\% of cells, cortical actin patches were localized at the mother bud neck, while in most of these cells, they also were localized in the daughter and mother cells. Overall, cortical actin patches are mislocalized when serine 366 is mutated to alanine or aspartate, and the patterns of actin distribution are similar to those of the ΔΔ Camyo5 strain.

We also compared cortical actin patch distribution in hyphal cells expressing Camyo5\textsuperscript{S366D}, in pseudohyphal cells of both the ΔΔ Camyo5 strain and the strain expressing Camyo5\textsuperscript{S366A}, and in wild-type hyphal cells. In wild-type hyphal cells of C. albicans, cortical actin patches always localize at the tip throughout hyphal elongation (4). In contrast, cortical actin patch distribution in pseudohyphal cells of S. cerevisiae and C. albicans resembles that found in yeast cells (11) (data not shown). Surprisingly, we found that cortical actin patches localized evenly throughout pseudohyphal cells of the ΔΔ Camyo5 strain and strains expressing Camyo5\textsuperscript{S366A} and Camyo5\textsuperscript{S366D} (Fig. 7 and data not shown). In these mutants, very few tips of emerging pseudohyphal cells contained more cortical actin patches than the mother cells, in accordance with cortical actin patch patterns in yeast cells (data not shown). Surprisingly also, we found that cortical actin patches were
localized throughout 66% of hyphal cells expressing Camyo5\textsuperscript{S366D} (Fig. 7 and Table 7). These patches localized at the tip of only 3% of hyphal cells in this mutant, while they localized at the tip of 75% of wild-type hyphal cells (Table 7). In addition, 14% of mutant hyphal cells showed an abnormal actin patch distribution rarely seen in wild-type cells. In these cells, cortical actin patches were found behind the hyphal tip (subapical). Overall, cortical actin patches were depolarized in the hyphae of the strain expressing Camyo5\textsuperscript{S366D}. These data indicate that cortical actin patch distribution is dependent on serine 366 in myosin I and suggest that a "tip-high" localization of cortical actin patches is not required for hyphal growth. Moreover, there is a strong correlation between myosin I localization and cortical actin patch distribution, because the strain expressing CaMYO5\textsuperscript{S366A}-GFP also exhibited a depolarized cortical actin patch distribution in 49% of hyphal cells; this value was 2% for the strain expressing CaMYO5-GFP (Table 7).

### DISCUSSION

We used C. albicans as a model system to study polarized growth because this yeast is a human pathogen and can switch from the yeast form to the hyphal form under defined conditions. Moreover, through genetic screens and manipulations, several components have been identified that reveal aspects of the mechanisms involved in this transition (8, 55). The structural roles of the actin cytoskeleton and associated regulatory proteins are critical to hypha formation; mutations affecting these components have dramatic effects on polarized growth in different organisms (24, 41, 44). For instance, a homologue of a cortical actin patch component in S. cerevisiae, CaSla2p, is required for hypha formation in C. albicans (5).

Several observations reported here support a role for myosin I during polarized growth in C. albicans. First, cells with a deletion of for myosin I (Δ/Δ Camyo5 strain) can form buds,
but they are abnormally round and some are enlarged. Moreover, the ΔΔ Camyo5 strain fails to form true hyphae under hypha-inducing conditions. Second, CaMyo5-GFP in wild-type cells colocalizes with cortical actin patches at sites of polarized growth at the tips of buds and of hyphae. This localization pattern has been found for Myo5p and Myo3-GFP in S. cerevisiae and for MyoA-GFP in Aspergillus (3, 14, 59). Finally, while buds of wild-type S. cerevisiae and C. albicans strains grow apically initially and have localized actin patches at the tips (4, 44), the ΔΔ Camyo5 strain exhibits mislocalized cortical actin patches. In this mutant, cortical actin patches are largely dispersed throughout the bud as well as in the mother cell. This distribution could account for excessive isotropic growth resulting in the round and enlarged shape of the mutant cells. Overall, these results suggest that C. albicans myosin I is required for polarized cortical actin patch localization and are consistent with the role of myosin I in other organisms (3, 18, 32, 48).

We made several surprising observations relative to pseudohyphal and hyphal cells of C. albicans, suggesting that some differences in the mechanisms controlling polarized growth may exist between C. albicans and other organisms, including S. cerevisiae. First, we found that myosin I is not essential and does not significantly impair growth in C. albicans. In S. cerevisiae, the two redundant genes encoding myosin I are either essential or important for growth, depending on the strain background (18, 57). Second, we found that myosin I is not required for pseudohypha formation in C. albicans, in contrast to the situation for Aspergillus, where deletion of myoA causes the complete inability to form polarized structures (36). Third, pseudohyphal cells of the ΔΔ Camyo5 mutant show mislocalized cortical actin patches, in contrast to S. cerevisiae, where pseudohypha formation requires a highly polarized actin cytoskeleton (11). This delocalized pattern of cortical actin patch distribution is also observed in the majority of hyphal cells of the mutant expressing Camyo5S366D. This finding could explain the slow growth of these hyphae. Finally, we found that localization of myosin I to tips is not required for hypha formation, because hyphal cells were observed in the absence of polarized localization of myosin I in the strain expressing Camyo5S366D. Thus, the formation of pseudohyphae and hyphae does not necessarily depend on polarized cortical actin patch and myosin I localization. However, treatment with cytochalasin A of cells of the wild-type, the ΔΔ Camyo5 strain, and the mutant expressing Camyo5S366D inhibits germ tube formation.

FIG. 7. Cortical actin patch patterns in wild-type and mutant hyphal and pseudohyphal cells. Hypha formation was induced with 10% FBS at 37°C for 3 h. Cells were then fixed and stained with rhodamine-phalloidin. The Nomarski image and the corresponding rhodamine-phalloidin image are shown for each strain. Scale bar, 10 μm.
formation (data not shown). These results suggest that some form of the actin cytoskeleton is essential for polarized growth. To explain the formation of elongated cells despite the lack of cortical actin patch polarization, we suggest several possibilities. Perhaps only actin patches localizing at the hyphal tips of the mutant expressing Camyo5S366D are functional. This would be the case if cortical actin patch components required for patch function preferentially localize at the hyphal tips of the mutant. Together with the small proportion of CaMyo5S366D also localizing at the tips, this distribution may be sufficient to direct the growth of the hyphal form. On the other hand, the absence of myosin I and cortical actin patch components whose localization is dependent on myosin I may explain the hyphal defect of the /H9004/H9004/H9004/CaMyo5 mutant. In this regard, it would be interesting to determine the localization of key cortical actin patch components required for patch function in the various myosin I mutants. At least one cortical actin patch component, cofilin, is not found associated with actin patches in an S. cerevisiae myosin I mutant (48). Alternatively, other forms of actin that may specify polarized growth in C. albicans, such as actin cables, could be dependent on myosin I. The S. cerevisiae myo3 myo5 mutant shows defects in the organization of actin cables (18). It is therefore possible that the absence of hypha formation in the ΔΔ Camyo5 mutant can be explained by a defect in the organization of actin cables. Although the polarized localization of cortical actin patches is dependent on the presence of actin cables in S. cerevisiae and S. pombe (43, 45), the orientation of the cables may be restored and allow the formation of slowly growing hyphae in the mutant expressing Camyo5S366D in the absence of localized cortical actin patches. The importance of actin cables during polarized growth in C. albicans is presently uncertain, as we could not directly observe cables in the myosin I mutants, possibly because these were masked by cortical actin patches. Finally, it has been suggested that the transport of secretory vesicles occurs by dual microtubule- and actin-based systems (17). It is possible that when the actin-based system is impaired, vesicles are transported by a microtubule-based system.

**Regulation of C. albicans myosin I function.** We suggest that serine 366 of C. albicans myosin I is phosphorylated and that phosphorylation is required for the activity of myosin I, either by controlling the actin-dependent ATPase activity of the motor (56) or by controlling the interaction of myosin I with actin. That serine 366 is subject to phosphorylation is consistent with previous studies and is based on mutant phenotypes produced by mutations affecting this residue. Previous studies demonstrated that the phosphorylation of the TEDS-rule site in myosin I of different organisms is important for function. In one particular S. cerevisiae strain background (W303α), the phosphorylation of serine 357 in myosin I is essential (57). In A. nidulans, an S371E mutation introduces a residue that mimics a phosphorylated serine and leads to the accumulation of membranes in growing hyphae as a result of the hyperactivation of endocytosis. In contrast, an S371A mutation does not drastically affect this process (58). However, both of these mutations affect other aspects of cell growth and morphogenesis to the same extents, suggesting that the phosphorylation of serine 371 per se does not regulate all functions of myosin I. In

### TABLE 7. Cortical actin patch patterns in hyphae

| Pattern | Appearance | % of hyphae of the following strain with the indicated pattern: |
|---------|------------|---------------------------------------------------------------|
|         | SC5314     | ΔΔ (CaMYO5) | ΔΔ (CaMYO5-GFP) | ΔΔ (CaMYO5S366D) | ΔΔ (CaMYO5S366D-GFP) |
| Polarized | 74.3 | 77.7 | 87.0 | 3.1 | 7.8 |
| Loose | 20.0 | 18.4 | 12.2 | 18.8 | 30.0 |
| Depolarized | 4.3 | 3.9 | 1.7 | 65.6 | 48.9 |
| Subapical | 1.4 | 0 | 0 | 14.1 | 13.3 |

*Polarized, cortical actin patches localize exclusively at the hyphal tip; loose, cortical actin patches localize preferentially at the tip but also throughout the hyphae; depolarized, cortical actin patches localize throughout the hyphae; subapical, cortical actin patches localize beneath the tip. Scale bar, 1 μm.
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