**Candida albicans** Rho-Type GTPase-Encoding Genes Required for Polarized Cell Growth and Cell Separation

Alexander Dünkler¹ and Jürgen Wendland¹,²*

Department of Microbiology, Friedrich Schiller University, Jena, and Junior Research Group, Fungal Pathogens, Leibniz Institute for Natural Product Research and Infection Biology, Hans Knöll Institute, Jena, Beutenbergstr 11a, D-07745 Jena, Germany,¹ and Carlsberg Laboratory, Yeast Biology, Gamle Carlsberg Vej 10, DK-2500 Valby, Copenhagen, Denmark²

Received 26 June 2006/Accepted 20 February 2007

Rho proteins are essential regulators of morphogenesis in eukaryotic cells. In this report, we investigate the role of two previously uncharacterized Rho proteins, encoded by the *Candida albicans* RHO3 (CaRHO3) and CaCRL1/CaRHO4 genes. The CaRHO3 gene was found to contain one intron. Promoter shutdown experiments using a MET3 promoter-controlled RHO3 revealed a strong cell polarity defect and a partially depolarized actin cytoskeleton. Hyphal growth after promoter shutdown was abolished in rho3 mutants even in the presence of a constitutively active ras1(G13V) allele, and existing germ tubes became swollen. Deletion of *C. albicans* RHO4 indicated that it is a nonessential gene and that rho4 mutants were phenotypically different from rho3. Two distinct phenotypes of rho4 cells were elongated cell morphology and an unexpected cell separation defect generating chains of cells. Colony morphology of crl1/rho4 resulted in a growth-dependent smooth (long cell cycle length) or wrinkled (short cell cycle length) phenotype. This phenotype was additionally dependent on the rho4 cell separation defect and was also found in a Cach3 chitinase mutant that shows a strong cytokinesis defect. The overexpression of the endoglucanase encoding the ENG1 gene, but not CHT3, suppressed the cell separation defect of crl1/rho4 but could not suppress the cell elongation phenotype. *C. albicans* Crl1/Rho4 and Bnr1 both localize to septal sites in yeast and hyphal cells but not to the hyphal tip. Deletion of RHO4 and BNRI produced similar morphological phenotypes. Based on the localization of Rho4 and on the rho4 mutant phenotype, we propose a model in which Rho4p may function as a regulator of cell polarity, breaking the initial axis of polarity found during early bud growth to promote the construction of a septum.

There is a strong interest in studying the molecular basis of cell differentiation in *Candida albicans* based on the importance of the yeast-to-hypha transition as a virulence trait in this human fungal pathogen (5). Upon induction by environmental cues, *C. albicans* produces hyphal filaments that enable *C. albicans* to adhere to and penetrate tissues and thus conquer new territories or niches in the human host or escape from the host's cellular immune defense.

Recent progress has identified GTP-binding proteins of the Ras and Rho superfamilies as being key regulators of morphogenesis in *C. albicans*. RAS1 is required for hyphal morphogenesis by activating downstream cyclic AMP- and mitogen-activated protein kinase-signaling pathways (21). A dominant active allele of *RAS1* [ras1(GV13)] stimulates enhanced/constitutive hyphal growth in *C. albicans* (12). However, Ras1-induced hyphal morphogenesis requires actin cytoskeletal components that regulate exo- and endocytosis, since a deletion of either the formin *BNRI* or the Wiskott-Aldrich syndrome protein homolog *WAL1* blocked hyphal morphogenesis (25, 39). The cell polarity establishment Rho protein Cdc42 is essential for viability and is also required for polarized hyphal growth (35, 37). In contrast, the *C. albicans* RAC1 homolog plays a specialized role in the organization of the actin cytoskeleton and in hyphal morphogenesis under embedded conditions (1). Furthermore, the Ras-related GTPase encoded by BUD1 in *C. albicans* plays a role in hyphal growth guidance similar to the role of its homolog in the filamentous ascomycete Ashbya gossypii (3, 16). In *Saccharomyces cerevisiae*, it was shown that the essential function of Rho3 and Rho4 is to activate formin homologs (either Bni1 or Bnr1) (9). In *Ashbya gossypii*, a deletion of RHO4 did not result in any obvious phenotypes (43). In contrast, deletion of the RHO4 homologs in either *Neurospora crassa* or *Schizosaccharomyces pombe* showed an involvement of Rho4 in septation (28, 31, 32). The *C. albicans* RHO4 (CaRHO4) gene was previously identified in a screen that isolated *C. albicans* genes that interfere with the *S. cerevisiae* pheromone response and was termed CRL1 for *C. albicans* RHO-like protein 1 (45). Given the role of Rho proteins in the organization of the actin cytoskeleton and in morphogenesis, we aimed to elucidate the role of the *C. albicans* RHO3 and CRL1/RHO4 genes. We show via MET3 promoter shutdown experiments that Rho3 is essential for hyphal morphogenesis. Crl1/Rho4 is apparently not involved in this process. On the other hand, a deletion of CRL1/RHO4 leads to a cell elongation and cell separation defect. This cell separation defect is similar to that observed in a chitinase cht3 mutant. In contrast, the cell elongation phenotype resembles that of bnr1 cells with a deletion of one of the *C. albicans* formin genes. In *C. albicans*, specific functions for Rho3 at the hyphal tip and for Rho4 at septal sites were discovered. Due to its role in septation, CaRho4 is functionally more similar to *N. crassa* and *S. pombe* than to *S. cerevisiae* and *A. gossypii* RHO4 homologs.
The exchange of marker genes, e.g., to convert a C. albicans strain, was done by transformation of the corresponding PCR products obtained from pFA plasmids (for a list of plasmids used in this study, see Table S2 in the supplemental material) and selection of transformants on the LEU2 locus. For verification of correct integration at the target locus and the absence of the target open reading frame (ORF) in homozygous null mutants was done by PCR. Incubation periods varied between 3 and 5 days to identify transformants. For this study, at least two independent mutants were generated. Verification was either by the lithium acetate procedure or by electroporation (19, 38).

Table 1. Strains used in this study

| Strain* | Relevant genotype | Reference or source |
|---------|-------------------|---------------------|
| SC5314  | C. albicans wild type | 13 |
| BWP17   | ura3::imm34/ura3::imm34 his1::HisG/his1::HisG arg4::HisG/arg4::HisG | 46 |
| GC19    | bnr1::URA3/BNR1::HIS1 arg4 | 25 |
| CAA9    | cht3::URA3/cht3::HIS1 arg4 | 10 |
| CAA3MX  | cht3::ARG4/cht3::HIS1 ura3 | This study |
| CAA34   | RH04/his4::ARG4 ura3 his1 | This study |
| CAA35   | RH03/his3::URA3 his1 arg4 | This study |
| CAA41   | RH03/his3::URA3 his1 arg4 | This study |
| CAA43   | RH03/his3::ARG4 ura3 his1 | This study |
| CAA49   | rho4::HIS1/rho4::ARG4 ura3 | This study |
| CAA50   | rho3::URA3/HIS1-MET3p-RHO3 arg4 | This study |
| CAA51   | rho3::ARG4/HIS1-MET3p-RHO3 ura3 | This study |
| CAA57   | rho4::ARG4/rho4::URA3 his1 | This study |
| CAA57MX | rho4::ARG4/rho4::HIS1 ura3 | This study |
| CAA58   | rho4::ARG4/rho4::SAT1 ura3 his1 | This study |
| CAA58MX | rho4::ARG4/rho4::URA3 his1 | This study |
| CAA61   | rho4::ARG4/rho4::HIS1 leu2::URA3/LEU2 | This study |
| CAA62   | cht3::ARG4/cht3::HIS1 leu2::URA3/LEU2 | This study |
| CAA63   | rho3::ARG4/HIS1-MET3p-RHO3 ade2::URA3-MAL2p-ras1(G13V)/ADE2 | This study |
| CAA64   | ade2::URA3-MAL2p-ras1(G13V)/ADE2 his1 arg4 | This study |
| CAA66   | rho4::ARG4/rho4::URA3 HIS1-AgTEFP/ENG1/ENG1 | This study |
| CAA67   | rho4::ARG4/rho4::URA3 HIS1-AgTEFP/CHT3/CHT3 | This study |
| CAA69   | rho4::ARG4/rho4::URA3 leu2::HIS1-MAL2p-GFP-RHO4/LEU2 | This study |
| CAA74   | bru1::URA3/BRU1::HIS1 leu2::SAT1-MAL2p-GFP-BNR1/LEU2 arg4 | This study |

* All strains are derivates of BWP17 with the indicated genotypic alterations.

MATERIALS AND METHODS

Strains and media. The C. albicans strains used and generated in this study are listed in Table 1. The strains were grown either in complete YPD medium (1% yeast extract, 2% peptone, 2% glucose) or in complete supplement mixture (CSM) minimal medium (6.7 g/liter yeast nitrogen base with ammonium sulfate and without amino acids, 0.69 g/liter CSM, 20 g/liter glucose) or SD medium (6.7 g/liter yeast nitrogen base with ammonium sulfate and without amino acids, 20 g/liter glucose). Minimal media were supplemented with the strain-specific requirements for amino acids and uridine. Control of regulatable gene expression via the MET3 promoter was exerted by the presence or absence of methionine and cysteine (3.5 mM each) in the medium (7). Induced expression via the MAL2 promoter was achieved by growth on maltose (20 g/liter). Hyphal induction of C. albicans cells was done by adding 10% serum to the growth medium unless stated otherwise. Escherichia coli strain DH5α was used for plasmid propagation. Transformation of E. coli was done by electroporation.

Transformation of C. albicans. PCR products used in transformation were amplified from pFA cassettes using primers S1 and S2 as described previously (14, 34). Primers (see Table S1 in the supplemental material) were obtained from biomers.net GmbH (Ulm, Germany). Within these primers, approximately 100 nucleotides of the target homology region are incorporated at their 5' ends. Specific primer sequences will be provided upon request. Transformation was done either by the lithium acetate procedure or by electroporation (19, 38). Incubation periods varied between 3 and 5 days to identify transformants. For each new strain, at least two independent mutants were generated. Verification of correct integration at the target locus and the absence of the target open reading frame (ORF) in homzygous null mutants was done by PCR.

The exchange of marker genes, e.g., to convert a URA3/HIS1 strain into an ARG4/HIS1 strain, was done by transformation of the corresponding PCR products obtained from pFA plasmids (for a list of plasmids used in this study, see Table S2 in the supplemental material) and selection of transformants on the appropriate selective media. Reintegration of the URA3 marker at the CaLEU2 locus was done by using the cloned targeting cassette pSK-Cht3-CaURA3 (see below).

Promoter shutdown experiments. To deplete cells of Rho3, shutdown experiments with MET3 promoter-controlled RHO3 strains were performed. To this end, cultures of the strains were grown overnight in minimal SD medium with the appropriate amino acid supplements. Starter cultures were diluted to an optical density at 600 nm of 0.1 in the same type of fresh medium with the addition of 3.5 mM methionine and cysteine to allow for the shutdown of RH03 expression and then incubated for 4 h at 30°C (which results in the maximal repression of a gene expressed via a fungal MET3 promoter) (our unpublished observations) prior to further analyses. Cell cultures were then used for staining and imaging, or, in the case of hyphal induction, 10% serum was added to the cultures, which were incubated for an additional 4 to 5 h at 37°C prior to microscopy.

Molecular techniques. (i) Generation of pSK-Cht3-CaURA3. Two PCR fragments derived from the CaLEU2 locus were amplified using primers containing restriction sites at their 5' ends. This generated a 479-bp SacII/BamHI fragment via primers 1438 and 1439, used as a 5' homology region, and a 633-bp XhoI/KpnI fragment via primers 1440 and 1994, used as a 3' homology region. Both fragments were cloned into pBluescript SK(+) (+), generating pHEIb (where HEI is high-efficiency integration). CaURA3 was excised from pFA-URA3 as a BamHI/EcoRV fragment and inserted into pHEIb. The new cassette containing the URA3 marker and the LEU2 flanks was excised via SacII/KpnI and used for transformation.

(ii) Generation of GFP cassettes. To construct a fusion of green fluorescent protein (GFP) to the 5' end of RHO4, we amplified the RHO4 ORF with its terminator from a plasmid library (kindly provided by Joachim Ernst, Düsseldorf, Germany), added terminal restriction sites with the primers, and cloned the PCR fragment into plasmid pFDaHIS1-MAL2p-GFP(GA)6 using the unique EcoRI and Clal restriction sites. The CaHIS1-MAL2p-GFP(GA)6-CaRHO4 fragment was then excised as a BamHI/SacI fragment, the SacI site was blunted via Klenow treatment, and the fragment was inserted into pHEIb linearized with BamHI/EcoRV. The correct in-frame GFP-RHO4 fusion into the new plasmid 857 pHEIb-CaHIS1-MAL2p-GFP(GA)6-CaRHO4 was verified by sequencing. The integrative cassette was released by SacII/AatII cleavage and used for transformation of C. albicans.

To generate a GFP-BNR1 cassette, plasmid 857 was modified in that an EcoRI site in the 3' LEU2 flank was eliminated. This was done by amplifying a LEU2 fragment using primers 2007 and 2008 and replacing the previous 3' LEU2 flank of plasmid 857 to generate plasmid 869. Next, the HIS1 leu2::SAT1-MAL2p-GFP-BNR1/LEU2 arg4 fragment was amplified from pFA-SAT1 using primers 2013 and 2014, and the resulting fragment was cloned as BamHI/HindIII into pFA-SAT1. This resulted in plasmid 857 pHEIb-CaHIS1-MAL2p-GFP(GA)6-CaRHO4 with its terminator from a plasmid library (kindly provided by Joachim Ernst, Düsseldorf, Germany), added terminal restriction sites with the primers, and cloned the PCR fragment into plasmid pFDaHIS1-MAL2p-GFP(GA)6 using the unique EcoRI and Clal restriction sites. The CaHIS1-MAL2p-GFP(GA)6-CaRHO4 fragment was then excised as a BamHI/SacI fragment, the SacI site was blunted via Klenow treatment, and the fragment was inserted into pHEIb linearized with BamHI/EcoRV. The correct in-frame GFP-RHO4 fusion into the new plasmid 857 pHEIb-CaHIS1-MAL2p-GFP(GA)6-CaRHO4 was verified by sequencing. The integrative cassette was released by SacII/AatII cleavage and used for transformation of C. albicans.
TABLE 2. Defects in morphogenesis resulting from \textit{RHO3} shutdown\\n\begin{tabular}{lccc|ccc}

| Parameter                       | Wild type                      | \textit{rho3/MET3p-RHO3} | Value                    | Value                      |
|---------------------------------|--------------------------------|--------------------------|--------------------------|--------------------------|
|                                | 0 mM Met/Cys                   | 3.5 mM Met/Cys            | 0 mM Met/Cys              | 3.5 mM Met/Cys            |
| Cell shape                      |                                |                          |                          |                          |
| Length (\mu m)                  | 5.89 ± 0.5                     | 5.95 ± 0.6                | 6.02 ± 0.6                | 6.41 ± 0.7                |
| Width (\mu m)                   | 4.33 ± 0.4                     | 4.41 ± 0.4                | 4.26 ± 0.5                | 5.78 ± 0.6                |
| Length/width ratio              | 1.36                           | 1.35                      | 1.41                      | 1.10                      |
| No. of counted cells            | 132                            | 155                       | 221                       | 251                       |
| Polarized actin (%)             | 97                             | 95                        | 93                        | 54                        |
| Depolarized actin (%)           | 3                              | 5                         | 7                         | 46                        |
| No. of counted hyphae           | 100                            | 106                       | 111                       | 106                       |

\textsuperscript{a} Length and width of yeast cells were determined from cells out of exponential-phase cultures. Actin staining was done with germ tubes that were induced by 10\% serum at 37\°C for 4 h.

(iii) \textit{cDNA synthesis}. Total RNA was prepared from cells by acid phenol extraction. Poly(A)\textsuperscript{+} RNA was purified from the total RNA preparation by binding to oligo(dt) bound to a column according to the manufacturer's protocol (Oligotex; Qiagen, Hilden, Germany). First-strand synthesis of cDNA was done using SuperScript III and oligo(dt) as a primer (Invitrogen, Karlsruhe, Germany). PCR products were cloned into the pDrive plasmid vector (PCR cloning kit; Qiagen, Hilden, Germany). All sequencing was carried out by MWG-Biotech AG (Ebersberg, Germany).

Microscopy and staining procedures. All microscopy was performed using an AxiosplanII-Imaging fluorescence microscope (Zeiss, Göttingen, Germany) with the aid of Metamorph software tools (Molecular Devices Corp., Downington, PA) and a MicroMax1024 charge-coupled-device camera (Princeton Instruments, Trenton, NJ) as described previously (39). Fluorescence microscopy was done using the appropriate filter combinations. Chitin staining was done by directly adding calcofluor (1 mg/ml) to the cells. Actin staining with rhodamine-phalloidin was done as described previously (30).

Nucleotide sequence accession number. The \textit{CaRHO3} sequence was deposited in the GenBank database under accession number AY534886.

RESULTS

Sequence analysis of \textit{CaRHO3}. The initial sequence information in the \textit{Candida} genome database did not identify the correct sequence of \textit{CaRHO3} at the time that we started this project due to sequencing errors or misaligned contig information at the \textit{RHO3} locus. Therefore, we isolated this region from genomic DNA, cloned and resequenced this region, and identified the \textit{RHO3} ORF based on amino acid sequence identity to other Rho proteins (GenBank accession number AY534886). This required the placement of one intron in the 5' region of the gene (see Fig. S1 in the supplemental material). To verify the presence of this intron, we generated \textit{RHO3} cDNA from mRNA via reverse transcription-PCR. The sequence of the cloned cDNA corroborates the intron that contains standard intron splice sites as well as a standard “TACT AAC” lariat site. \textit{CaRHO3} thus encodes a protein of 210 amino acids with the conserved sequence features of a Rho-type GTPase including conserved GTP-binding domains and a C-terminal CAAX motif for posttranslational truncation and modification. Comparison of \textit{CaRho3} with other fungal Rho proteins showed the highest degree of sequence identity (>70\%) to the Rho3 proteins of \textit{Kluyveromyces lactis}, \textit{Yarrowia lipolytica}, and \textit{Ashbya gossypii}. Rho proteins of these organisms showed conserved N and C termini indicating the correct length of the CaRho3.

Depletion of \textit{CaRHO3} reveals a cell polarity defect. Deletion of the \textit{S. cerevisiae} \textit{RHO3} homolog has proven difficult to do, and thus, phenotypic analyses relied on the use of temperature-sensitive mutants (17, 27). Also, a number of \textit{SRO} genes were found clustered in the \textit{tip} region of the gene (see Fig. S1 in the supplemental material). To analyze the effect of \textit{CaRho3} depletion, we placed the single copy of \textit{RHO3} in a heterozygous \textit{RHO3}/\textit{rho3} mutant under the control of the regulatable \textit{CaMET3} promoter and performed shutdown experiments based on the presence of methionine/cysteine in the medium. Wild-type cells are ellipsoidal in shape, with a length/width ratio of about 1.36. Cells of strain \textit{CAA50} in which the single remaining \textit{RHO3} allele is expressed under \textit{MET3} promoter control are essentially like the wild type, with a length/width ratio of 1.41. Shutdown of \textit{RHO3} expression produces yeast cells of a round shape with a length/width ratio of 1.1 (Table 2). This is indicative of a cell polarity defect after shutdown resulting in nonpolarized growth.

To analyze the effect of a \textit{RHO3} shutdown under hypha-inducing conditions, the expression of \textit{RHO3} was blocked by the addition of methionine/cysteine to the medium prior to the induction of hyphal growth via serum at 37\°C. This procedure resulted in the initiation of germ tube formation and polarized hyphal growth with septum formation in the hyphal tube in most of the cells. However, soon after germ tube formation, hyphal tips began to swell, and polarized growth was blocked (Fig. 1A). Analysis of the actin cytoskeleton in \textit{Rho3}-depleted cells showed that after shutdown, the actin cytoskeleton became depolarized, and cortical actin patches were often not found clustered in the tip (Table 2).

Previously, we showed that the deletion of either the formin \textit{BN1} or the Wiskott-Aldrich syndrome protein homolog \textit{WAL1} abolishes hyphal growth in \textit{C. albicans} (25, 39). This hyphal growth deficiency could not be overcome by the expression of a constitutively active \textit{ras1} allele [\textit{ras1(G13V)}], indicating that both \textit{WAL1} and \textit{BN1} are essential components of the actin cytoskeleton downstream of the pathway that triggers hyphal growth. A constitutively active Ras1 triggered hyphal...
formation in the wild type already at 30°C in the absence of external inducing stimuli. Therefore, we went on to test whether ras1(G13V) could suppress the rho3 shutdown phenotype. To this end, we integrated the ras1(G13V) allele into strain CAA51. Expression of the ras1 allele was under the control of the MAL2 promoter. After the shutdown of MET3 promoter-controlled RHO3 expression, this newly generated strain, even with constitutively active Ras1, also stopped polarized hyphal growth (Fig. 1B). Thus, Rho3 is required for the polarized hyphal growth of C. albicans. Signaling via the Ras pathway is not sufficient to bypass or overcome a block in polarized cell growth posed by the deletion of RHO3. Rho3 is therefore part of a central network that regulates growth decisions.

Sequence comparison of RHO4 genes. In a recent paper on a RHO4 homolog in N. crassa, two groups of Rho4 proteins within the ascomycete clade were identified (31). One group, which includes the Rho4 homologs of the hemiascomycetes S. cerevisiae and A. glyceriae, does not reveal any striking defects upon the deletion of the respective RHO4 genes. The other group includes the archiascomycetes and euascomycetes S. pombe and N. crassa. In this group, deletion of RHO4 homologs yielded septation defects. Phylogenetic tree analyses did not place the C. albicans Cr1/Rho4 (or the sake of convenience, we will refer to CRL1/RHO4 solely as RHO4) clearly into one of these groups. A comparison of Rho4 proteins revealed a slightly higher degree of identity at the amino acid level between CaRho4 and S. pombe Rho4 (41.4%) than between CaRho4 and A. glyceriae Rho4 (37.1%). However, based on these analyses, we were not able to draw any conclusions regarding any functional similarities of CaCrl1/ Rho4.

Deletion of CaCRL1/CaRHO4 results in a cell elongation and cell separation defect. Next, we went on to generate deletion mutants of RHO4. In contrast to the efforts used to delete RHO3, we could readily obtain complete ORF deletions and rho4/rho4 mutant strains. Mutant cells did not show defects in polarized growth that were found in Rho3-depleted cells. Quantification of cell lengths and cell width measurements, however, indicated that in contrast to a ratio of 1.36 in the wild type, rho4 mutant cells showed a length/width ratio of 1.55. This ratio came about because rho4 cells are smaller in width than wild-type cells but more elongated. We also found that the budding pattern in the rho4 mutant was unipolar to a large extent compared to a bipolar budding pattern of the wild type (Table 3). The organization of the actin cytoskeleton was found to be similar to that of the wild type, and filament formation in the rho4 mutant cells occurred in a manner that was not distinguishable from that of the wild type under the conditions tested (Fig. 2). Interestingly, rho4 yeast cells showed a cell separation defect that resulted in the formation of chains of cells (Fig. 2A). This defect is URA3 status independent, as it does not occur in the progenitor strains used. Nuclear distribution between mother and daughter cells was not affected in rho4 strains (our unpublished results). This indicates that a deletion of RHO4 specifically interrupts the ability to finish the last stage of cytokinesis separating mother and daughter cells.

Cell separation defects coupled with fast growth result in altered colony morphology. In our efforts to construct rho4

FIG. 1. Hyphal growth defects of a down-regulated MET3p-RHO3 strain. (A) Wild-type hyphae showing polarized cell growth, septation, and a polarized actin cytoskeleton upon growth in medium supplemented with 10% serum at 37°C for 4 h in either the presence or the absence of methionine/cysteine in the medium. Regulated expression of RHO3 leads to wild-type-like hyphal development under inducing conditions, whereas hyphal growth is blocked and the hyphal tips swell under restrictive conditions. (B) Either strain BWP17 with its endogenous RAS1 or strain CAA64 [BWP17 with an additional ras1(G13V) allele] generates regular hyphae after 4 h in medium with maltose as the sole carbon source and containing 3.5 mM methionine and cysteine at 37°C. Under similar conditions that shut down RHO3, expression in MET3p-RHO3/rho3 strain CAA50 is not able to generate hyphae. Filamentation in strain CAA63 that carries an additional ras1(G13V) allele is also blocked. Cells were stained by calcofluor prior to photography. Bar, 10 μm.
deletion strains, we used different marker combinations. This resulted, for example, in deletion mutants that were genotypically rho4::ARG4/rho4::SAT1 (CAA58) or rho4::ARG4/rho4::URA3 (CAA57). When grown on full-medium YPD plates, these colonies showed different colony morphologies in that CAA58 showed smooth colony surfaces, while CAA57 had a wrinkled colony appearance. Independent of the marker combinations used, all rho4 mutants exhibited an identical cell separation defect (Fig. 3). We therefore analyzed these strains in more detail. The cell cycle durations of rho4 mutants in the BWP17 strain background were found to be dependent on the URA3 status. Strain CAA57, which carries one copy of the URA3 marker, showed faster doubling rates than mutants that were generated using the ARG4/SAT1 markers (Table 4). To demonstrate that the colony phenotype of these strains is dependent on the rho4 phenotype and on the cell cycle length, we exchanged the URA3 marker in CAA57 (rho4::URA3/rho4::ARG4) for HIS1 and the SAT1 marker in CAA58 (rho4::SAT1/rho4::ARG4) for URA3 and compared the growth phenotypes of these mutants. As expected, cell cycle durations changed due to the presence or absence of the URA3 marker. But, strikingly, the rho4 deletion strain that had become ura3 now formed smooth colonies, while the rho4 strain that had gained the URA3 marker could be converted to form wrinkled colonies (Fig. 3).

Recently, we identified CaCHT3 as encoding the functional homolog of the S. cerevisiae CTS1 chitinase gene (10). Deletion of CaCHT3 resulted in an even stronger cell separation defect than that observed in the Carho4 mutant. We therefore went on to analyze the colony morphology of cht3/cht3 strains of either the URA3 or ura3 genotype. Strikingly, as with the rho4 strains, we observed that cht3/cht3 URA3 strains were able to form wrinkled colonies, while cht3/cht3 ura3 strains formed smooth colonies only. To further corroborate that the change in doubling rates is solely responsible for the colony morphology phenotype in cell separation-defective mutants, we reintegrated the URA3 marker at the LEU2 locus in both rho4/rho4 ura3 and cht3/cht3 ura3 strains. As expected, the formerly smooth-colony-forming strains could be converted to the wrinkled phenotype (Fig. 3).

All of the strains tested exhibit smooth colony surfaces on minimal medium or on rich medium at lower growth temperatures (e.g., 20°C), both of which reduce doubling rates in all strains to a similar level (Fig. 3 and our unpublished results). Thus, we conclude that wrinkled colony morphology phenotypes can arise in mutant strains with delayed or inhibited cell separation when grown under optimal growth conditions allowing for fast cell cycle rates.

**The Carho4 cell elongation phenotype can be suppressed by overexpression of CaENG1.** Previously, we had shown that a cht3 mutant, defective in the S. cerevisiae CTS1 homolog, has a strong cell separation defect. We went on to explore whether the rho4 cell separation defect could be suppressed by overexpressing a cell wall-degrading enzyme. To this end, we placed the C. albicans CHT3 gene, coding for a chitinase, and the ENG1 gene, which encodes an endo-1,3-beta-glucanase required for cell separation after division, under the control of the AgTEF1 promoter in the rho4 background (11) (Fig. 4). We then quantified the number of cells in aggregates in the strains used in this study (Fig. 5). Cells of the wild type occur

| Strain             | Length (μm) | Width (μm) | Length/width ratio | No. of counted cells | % Bipolar | % Unipolar | % Random | No. of counted cells |
|--------------------|-------------|------------|--------------------|----------------------|-----------|------------|----------|---------------------|
| Wild type          | 5.89 ± 0.5  | 4.33 ± 0.4 | 1.3                | 132                  | 66.9      | 31.7       | 1.4      | 326                 |
| rho4/rho4 (ura3)   | 6.22 ± 0.7  | 3.99 ± 0.5 | 1.5                | 178                  | 24.0      | 68.8       | 7.2      | 304                 |
| rho4/rho4 (URA3)   | 6.31 ± 0.7  | 4.05 ± 0.7 | 1.5                | 211                  | 22.3      | 72.1       | 5.6      | 343                 |

**FIG. 2.** Growth pattern of Carho4 mutants. Wild-type strain SC5314 and rho4 mutant strains CAA49 and CAA57 were grown in liquid SD medium at 30°C (A) or either on plates or in liquid medium at 37°C supplemented with 10% serum (B). (A) Yeast rho4 cells show a cell separation defect that leads to chains of cells connected at the septum, as seen in the images of calcofluor (ca)-stained cells. The actin cytoskeleton (ac) appeared to be normal in rho4 cells. (B) During the hyphal growth phase, no differences between wild-type and rho4 strains could be observed under all conditions tested. The bar for microscopic images is 5 μm.
which resulted in different generation times for the mutants. WT, wild strains (but not on the position of the URA3 or cht3 rho4 morphology was dependent on the cell separation defect of either LEU2 gene into the the exchange (MX) strains were generated by PCR-based gene targeting in 
medium or YPD rich medium and grown for 3 days at 30°C. Marker 
totions of the indicated strains were spotted onto either CSM minimal 
medium, on the growth medium, and on the 
ura3/ura3 his1/his1 arg4/arg4
BWP17
CAA58MX
CAA57MX
CAA3MX
CAA61
CAA62
CAA58MX
CAA57MX
CAA3MX
CAA61
CAA62
CAA58MX
CAA57MX
CAA3MX
CAA62
CAA58MX
CAA57MX
CAA3MX
CAA62
CAA58MX
CAA57MX
CAA3MX
CAA62

TABLE 4. Colony morphology is influenced by cell cycle lengths and cytokinesis

| Strain | Relevant genotype | Cell cycle length (min) | Colony morphology |
|--------|-------------------|------------------------|-------------------|
| SC5314 | URA3/URA3 HIS1/HIS1 ARG4/ARG4 | 73 ± 8 | Smooth |
| BWP17  | ura3/ura3 his1/his1 arg4/arg4 | 111 ± 10 | Smooth |
| CAA58  | rho4/ARG4/rho4/AT1 ura3/ura3 | 108 ± 12 | Smooth |
| CAA58MX| rho4/ARG4/rho4/URA3 his1/his1 | 92 ± 9 | Wrinkled |
| CAA57  | rho4/ARG4/rho4/URA3 his1/his1 | 88 ± 10 | Wrinkled |
| CAA57MX| rho4/ARG4/rho4/URA3 his1/his1 | 112 ± 15 | Smooth |
| CAA61  | rho4/ARG4/rho4/URA3 his1/his1 | 82 ± 13 | Wrinkled |
| CAA3   | chs3/URA3 chs3/HIS1 arg4/arg4 | 90 ± 11 | Wrinkled |
| CAA3MX | chs3/ARG4/chs3/HIS1 ura3/ura3 | 102 ± 16 | Smooth |
| CAA62  | chs3/ARG4/chs3/HIS1 | 87 ± 9 | Wrinkled |

* All strains were grown to logarithmic phase in full medium.

FIG. 3. Colony morphology of Ca rho4 and Cacht3 mutants. Dilutions of the indicated strains were spotted onto either CSM minimal medium or YPD rich medium and grown for 3 days at 30°C. Marker exchange (MX) strains were generated by PCR-based gene targeting in the RHO4 locus. Marker reintegration was done by targeting the URA3 gene into the LEU2 locus of marker exchange strains. Wrinkled colony morphology was dependent on the cell separation defect of either rho4 or cht3 mutants, on the growth medium, and on the URA3 status of the strains (but not on the position of the URA3 marker in the genome), which resulted in different generation times for the mutants. WT, wild type.

As we reinvestigated the bnr1 mutant phenotype, we found certain similarities to rho4. Thus, we went on to investigate the subcellular localization of GFP-Bnr1. Previously, it was reported that Bnr1 can be found at the hyphal tip and thus partially substitute for a loss of the second formin, BNI1. We generated a GFP-Bnr1 fusion under the control of the MAL2 promoter and integrated this cassette at the LEU2 locus of a bnr1/bnr1 mutant. We chose to produce an N-terminal GFP fusion since difficulties arose with nonfunctional Bni1 versions to which GFP had been fused to the C-terminal end. The GFP-Bnr1 construct appears to be fully functional, as it complemented the rho4 phenotype, we generated a GMT-Bnr1 construct that was shown to be functional, as the mutant phenotype could be suppressed, depending on the carbon source used, to allow for the expression of Rho4. This also demonstrated that the mutant phenotype observed is in fact due to the deletion of rho4. We found that only the overexpression ENG1 was able to suppress the cell separation defect, while the overexpression of CHT3 did not alter the mutant phenotype (Fig. 5). Interestingly, the bnr1 mutant, which carries a deletion of both alleles of the formin Bnr1, also exhibited a cell separation defect similar to that of rho4. Using the same strains, the cellular morphology was analyzed by determining the length/width ratios of the cell populations. The cell elongation phenotype of rho4 could be reversed only by reintroducing a functional RHO4 gene. Neither the overexpression of ENG1 nor that of CHT3 was able to suppress this phenotype, suggesting that Rho4 may act in two separate pathways (Fig. 5).

CaRho4 and CaBnr1 localize to septal sites. To study the subcellular localization of Rho4, we generated a cassette in which RHO4 was tagged at the 5′ end with GFP and placed under the control of the MAL2 promoter. This construct was flanked by sequences that allow homologous targeting to the C. albicans LEU2 locus. The construct, although not expressing RHO4 from its endogenous promoter, was able to complement the rho4 phenotypes. This suggests that it is functional and thus may reflect the endogenous situation. Under inducing conditions that allow the expression of GFP-RHO4, we observed a distinct localization of Rho4 to septal sites in both yeast and hyphal stages (Fig. 6). Rho4 was not found to localize to the bud tip or the hyphal tip and thus apparently specifically marks the septum. Rho4 was found to persist at septal sites after septation.

As we reinvestigated the bnr1 mutant phenotype, we found certain similarities to rho4. Thus, we went on to investigate the subcellular localization of GFP-Bnr1. Previously, it was reported that Bnr1 can be found at the hyphal tip and thus partially substitute for a loss of the second formin, BNI1. We generated a GFP-Bnr1 fusion under the control of the MAL2 promoter and integrated this cassette at the LEU2 locus of a bnr1/bnr1 mutant. We chose to produce an N-terminal GFP fusion since difficulties arose with nonfunctional Bni1 versions to which GFP had been fused to the C-terminal end. The GFP-Bnr1 construct appears to be fully functional, as it complements the bnr1 defects. We observed GFP-Bnr1 fluorescence only at septal sites in both yeast and hyphal stages (Fig. 7). GFP-Rho4 localizes to septal sites apparently in both

Table: Colony morphology is influenced by cell cycle lengths and cytokinesis

| Strain | Relevant genotype | Cell cycle length (min) | Colony morphology |
|--------|-------------------|------------------------|-------------------|
| SC5314 | URA3/URA3 HIS1/HIS1 ARG4/ARG4 | 73 ± 8 | Smooth |
| BWP17  | ura3/ura3 his1/his1 arg4/arg4 | 111 ± 10 | Smooth |
| CAA58  | rho4/ARG4/rho4/AT1 ura3/ura3 | 108 ± 12 | Smooth |
| CAA58MX| rho4/ARG4/rho4/URA3 his1/his1 | 92 ± 9 | Wrinkled |
| CAA57  | rho4/ARG4/rho4/URA3 his1/his1 | 88 ± 10 | Wrinkled |
| CAA57MX| rho4/ARG4/rho4/URA3 his1/his1 | 112 ± 15 | Smooth |
| CAA61  | rho4/ARG4/rho4/URA3 his1/his1 | 82 ± 13 | Wrinkled |
| CAA3   | chs3/URA3 chs3/HIS1 arg4/arg4 | 90 ± 11 | Wrinkled |
| CAA3MX | chs3/ARG4/chs3/HIS1 ura3/ura3 | 102 ± 16 | Smooth |
| CAA62  | chs3/ARG4/chs3/HIS1 | 87 ± 9 | Wrinkled |

* All strains were grown to logarithmic phase in full medium.

FIG. 3. Colony morphology of Ca rho4 and Cacht3 mutants. Dilutions of the indicated strains were spotted onto either CSM minimal medium or YPD rich medium and grown for 3 days at 30°C. Marker exchange (MX) strains were generated by PCR-based gene targeting in the RHO4 locus. Marker reintegration was done by targeting the URA3 gene into the LEU2 locus of marker exchange strains. Wrinkled colony morphology was dependent on the cell separation defect of either rho4 or cht3 mutants, on the growth medium, and on the URA3 status of the strains (but not on the position of the URA3 marker in the genome), which resulted in different generation times for the mutants. WT, wild type.

As we reinvestigated the bnr1 mutant phenotype, we found certain similarities to rho4. Thus, we went on to investigate the subcellular localization of GFP-Bnr1. Previously, it was reported that Bnr1 can be found at the hyphal tip and thus partially substitute for a loss of the second formin, BNI1. We generated a GFP-Bnr1 fusion under the control of the MAL2 promoter and integrated this cassette at the LEU2 locus of a bnr1/bnr1 mutant. We chose to produce an N-terminal GFP fusion since difficulties arose with nonfunctional Bni1 versions to which GFP had been fused to the C-terminal end. The GFP-Bnr1 construct appears to be fully functional, as it complements the bnr1 defects. We observed GFP-Bnr1 fluorescence only at septal sites in both yeast and hyphal stages (Fig. 7). GFP-Rho4 localizes to septal sites apparently in both

Table: Colony morphology is influenced by cell cycle lengths and cytokinesis

| Strain | Relevant genotype | Cell cycle length (min) | Colony morphology |
|--------|-------------------|------------------------|-------------------|
| SC5314 | URA3/URA3 HIS1/HIS1 ARG4/ARG4 | 73 ± 8 | Smooth |
| BWP17  | ura3/ura3 his1/his1 arg4/arg4 | 111 ± 10 | Smooth |
| CAA58  | rho4/ARG4/rho4/AT1 ura3/ura3 | 108 ± 12 | Smooth |
| CAA58MX| rho4/ARG4/rho4/URA3 his1/his1 | 92 ± 9 | Wrinkled |
| CAA57  | rho4/ARG4/rho4/URA3 his1/his1 | 88 ± 10 | Wrinkled |
| CAA57MX| rho4/ARG4/rho4/URA3 his1/his1 | 112 ± 15 | Smooth |
| CAA61  | rho4/ARG4/rho4/URA3 his1/his1 | 82 ± 13 | Wrinkled |
| CAA3   | chs3/URA3 chs3/HIS1 arg4/arg4 | 90 ± 11 | Wrinkled |
| CAA3MX | chs3/ARG4/chs3/HIS1 ura3/ura3 | 102 ± 16 | Smooth |
| CAA62  | chs3/ARG4/chs3/HIS1 | 87 ± 9 | Wrinkled |

* All strains were grown to logarithmic phase in full medium.
mother and daughter cell. In contrast, GFP-Bnr1 localizes to a septal site from the mother side only, as is apparent by the position of the septum in the calcofluor-costained cells (Fig. 7). Thus, both Rho4 and Bnr1 exhibit a distinct localization pattern at septal sites.

**DISCUSSION**

*C. albicans* is a human fungal pathogen that changes growth forms in response to environmental stimuli or host niches (5). Based on the yeast paradigm of polarized cell growth, a num-
ber of molecular analyses in recent years have identified GTP-binding proteins as being key regulators of polarized cell growth and/or hyphal morphogenesis in a variety of fungal species including, for example, \textit{A. gossypii}, \textit{C. albicans}, \textit{Ustilago maydis}, and \textit{S. pombe} \cite{2, 23, 41, 44}. In this report, we have analyzed \textit{CaRHO3} and \textit{CaRHO4} genes. Interestingly, there seems to be little functional overlap between both genes. \textit{CaRho3} is required for sustained polarized cell growth. Using DNA array technologies, \textit{RHO3} was found to be transiently up-regulated in the initial phase of germ tube induction \cite{29}. Our difficulties in obtaining a null deletion mutant suggest that \textit{RHO3} is an essential gene. Similar results have been obtained for \textit{S. pombe} \cite{36}. In \textit{A. gossypii}, \textit{Rho3} is required for germ tube formation at elevated temperatures. Freshly germinated spores lyse shortly after germ tube formation at 37°C \cite{44}. \textit{AgRho3} hyphae grown at 30°C show frequent depolarization at the hyphal tip, which results in swellings similar to those seen in \textit{CaRHO3}-depleted hyphae. In \textit{A. gossypii}, the resumption of polarized growth follows the direction of previous cell polarity \cite{44}.

\textit{Rho3} in \textit{S. cerevisiae} was shown to activate the formin \textit{Bni1} in inducing the polarized assembly of actin filaments, and \textit{AgRho3} was found to interact with \textit{AgBni1} using a two-hybrid approach \cite{9, 18}. We and others previously showed that a deletion of the \textit{BNI1} homolog in \textit{C. albicans} abolishes hyphal growth \cite{8, 22, 25}. This block in polarized morphogenesis could not be overcome by constitutively active \textit{Ras1} \cite{25}. Similarly, a shutdown of \textit{RHO3} expression resulted in a block of polarized hyphal growth that could not be suppressed by constitutively activating the Ras pathway. Shutdown of \textit{RHO3} expression using the \textit{MET3} promoter and on the other hand activating the transcription of the \textit{ras1} \textit{(G13V)} allele required a specific nutritional regimen. Due to the leakiness of the regulatable promoters, a terminal phenotype will not be established. Rather, at a given time, a majority of cells will display the shutdown phenotype, while other cells may have a partial phenotype or are already recovering from the block of expression. In particular, this led to the observation that upon \textit{Rho3} depletion, a certain amount of cells exhibited a delocalized actin cytoskeleton, while the actin cytoskeleton was apparently normal in others. Various attempts to localize \textit{Rho3} in \textit{C. albicans} using GFP were unsuccessful. The role in tip growth, however, suggests that in \textit{C. albicans}, \textit{Rho3} and the formin \textit{Bni1}, which is part of the \textit{C. albicans} Spitzenkörper in hyphal tips, act in a common pathway.

\textit{RHO4} deletions in hemiascomycetes did not reveal a unique function for \textit{Rho4} in either \textit{A. gossypii} or \textit{S. cerevisiae} \cite{26, 43}. In contrast, deletion of \textit{RHO4} homologs in \textit{S. pombe} or in the euascomycete \textit{N. crassa} showed an involvement of \textit{Rho4} in septation \cite{28, 31, 32}. In \textit{N. crassa}, deletion of \textit{RHO4} abolishes septation, causing a failure in actin ring formation. On the other hand, a dominant active allele of \textit{N. crassa RHO4} showed
multiple rounds of septation at nearby distances (31). In *S. pombe*, rho4 cells were found to generate a cell separation defect at elevated temperatures (32). Thus, Rho4 may be involved either in septum construction or in septum degradation to allow cell separation. Consistently, Rho4 was recently shown to be involved in the secretion of the endoglucanase Eng1, and ENG1 overexpression was able to suppress the rho4 defect in *S. pombe* (33).

Deletion of RHO4 in *C. albicans* revealed a mutant phenotype of defective cell separation similar to was observed in *S. pombe rho4* cells. Thus, the role of Rho4 in cell separation/ septation is not confined to archaeascomycetes and euascomycetes but can also be observed in hemiascomycetes. In the basidiomycete *U. maydis*, the activation of the *U. maydis* Rho protein Cdc42 by the Rho guanine nucleotide exchange factor Don1 is required for cell separation. Deletion of either DON1 or CDC42 results in a defect similar to that with a deletion of the RHO4 homologs in *C. albicans* and *S. pombe* (23, 42). This suggests that conserved signaling functions required for cellular morphogenesis were bestowed on specific but different Rho-type GTPases in the fungal kingdom.

The cell separation defect in Carho4 cells could efficiently be suppressed by the overexpression of the CaENG1 gene. Thus, regulatory events requiring Rho4 during cell separation, e.g., at the level of endoglucanase localization, may be conserved between *S. pombe* and *C. albicans*. Overexpression of the chitinase gene *CHT3* did not suppress the cell separation defect. Interestingly, the overexpression of *CHT3* resulted in an increased sensitivity of the cells against cell wall-perturbing agents such as 0.1% sodium dodecyl sulfate, which was not observed in ENG1-overexpressing strains (our unpublished results).

Our studies on the CaCHT3 and CaRHO4 genes revealed a relationship between cellular morphology and colonial growth. With the use of different marker combinations (“URA3 status”), the resulting mutant strains showed differing cell cycle lengths. This indicated that optimal generation times in conjunction with a lack of cell separation lead to a wrinkled colony appearance. This may be due to the larger number of cells generated in URA3 strains than that generated the otherwise isogenic ura3 strains. Due to the failure in cell separation, mother and daughter cells cannot be moved apart from each other to make way for newly emerging buds. Thus, instead of dispersing laterally, an elevated colony morphology arises. Colony morphology, therefore, is an “open” structure that varies in a stochastic manner. This also suggests an explanation for the wrinkled colony appearance during hyphal growth of *C. albicans*, since the hyphal growth phase also results in non-separated cells that form filaments. Similarly, in *S. cerevisiae*, changes in colony morphology were observed between “fluffy” wild *S. cerevisiae* strains and “smooth” laboratory strains (20). Furthermore, structured colony morphology, i.e., a wrinkled appearance, was found to be linked to incompletely separated yeast cells that showed a monopolar budding pattern. Also, in *Cryptococcus neoformans*, an analysis of conserved genes in the RAM (regulation of Aces2 activity and morphology) signaling pathway revealed phenotypes similar to those that we observed in the Carho4 mutant strain. In that study, *C. neoformans cbk1, kic1, mob2, sog2, or tao3* mutants grown on YPD medium showed cell separation defects and a “crinkled” colony morphology (40). Fungal colony morphology is thus determined in a similar manner by cell separation defects or delays in cytokinesis.

A *MET3* promoter-driven RHO3 in a rho4 mutant background was not able to suppress the rho4 cell separation defect, providing further evidence that Rho3 and Rho4 fulfill separate cellular functions. We could localize *C. albicans* Rho4 at septal sides in both yeast and hyphal stages. Rho4 was found to persist at several septal sites in hyphae. This may allow for additional functions of Rho4 beyond vesicle delivery for septum formation or degradation during one cell cycle. Activated Rho4 could thus act to direct polarized cell growth to septal sites upon lateral branch formation, which regularly occurs at septa in *C. albicans* (25, 39). This notion may also provide an explanation for the altered budding pattern in rho4 yeast cells, which was found to be largely monopolar. The formin Bnr1 also was found to specifically localize to septal sites. However, the localization appeared to be in spots at the mother side of the neck rather than as a uniform ring as seen in GFP-Rho4. Thus, although mutant rho4 and bnr1 cells were elongated compared to the wild type, both proteins could act in different pathways. Elongated bud morphogenesis has been observed in a variety of mutants in both *C. albicans* and *S. cerevisiae*. Different processes could contribute to an elongated cell phenotype: (i) maintained polarized cell growth at the tip of the bud, e.g., as seen in mutants with prolonged G1, cyclin activity (4), (ii) delays in mitosis/nuclear distribution, e.g., as seen in dynein mutants or in mutants that activated a process collectively described as a “morphogenesis checkpoint” (24), and (iii) maintained polarization of the actin cytoskeleton at the tip of an emerging bud due to the prolonged activation of the cell polarity establishment Cdc42-Rho protein module (15). We hypothesize that cell elongation may also occur in the absence of a transport system that delivers polarity establishment functions to septal sites to redirect growth in preparation for septation. Rho4 could be well suited to elaborate such a mechanism by triggering actin cable formation, generating new tracks within the cell to promote secretion to septal sites. In its absence, this process may be taken over by other Rho proteins and Bni1, which also localizes to septal sites. However, a delay in this mechanism may prolong tip growth and thus lead to cell elongation (Fig. 8). In *S. cerevisiae*, polarized growth at the bud tip has been shown to depend on both positive and negative feedback loops (6, 15). This involves stabilizing Cdc24 at the sites of polarized growth by Bem1 or destabilizing the Cdc24-Bem1 interaction by phosphorylating Cdc24 via Cla4, which is itself an effector protein of Cdc42. Our model suggests a novel mechanism in which Rho4 helps to establish a new axis of polarity at the septal site. By establishing such a new site of polarity, both bud tip and bud neck may compete for either the same pool of vesicles or cellular components required for keeping up the established axis of polarity at the tip. This may finally result in breaking the tipward axis of polarity and redirecting secretion towards the bud neck. The lack of RHO4 could therefore avoid activation of a morphogenesis checkpoint and thus may not be Swe1 dependent. This model is supported by the localization of CaRho4 solely at septal sites after bud emergence, similar to what was found with *S. cerevisiae* (36). Furthermore, we could show, by two-hybrid analyses, that Rho4 and Bni1p interact, suggesting that a Rho4-Bni1
complex may compete with Cdc42-Bni1 (S. Seitz and J. Wendland, unpublished data). During hyphal growth, Rho4 was also observed at septal sites, suggesting that similar mechanism should operate. However, the distance between the hyphal tip and the septal site is greater than the distance between the tip of the bud and the septal site in yeast cells, which could allow both systems to operate at the same time in filaments without competing for the same set of polarity establishment proteins or secretory vesicles. Rho4 is not essential for the apical-isotropic switch but may be useful for the timely occurrence and regulation of cellular morphology. Future work will need to clarify the temporal localization pattern of Rho4 at septal sites, the mechanism of Rho4 activation and localization, the dependence of Rho4 on septin localization, and the role of Rho4 in actin filament assembly.

ACKNOWLEDGMENTS

We thank Gerry Fink for generously providing the ras1 allele clones. This research was supported by the Deutsche Forschungsgemeinschaft Priority Program 1111, Cell Polarity, the Friedrich Schiller University, and the Hans Knoll Institute.

REFERENCES

1. Bassilana, M., and R. A. Arkowitz. 2005. The small GTPase Cdc42 regulates yeast cell polarity without a role in cell division. Curr. Microbiol. 51:385–392.

2. Bodmer, Y., P. Knechtle, J. Wendland, H. Helfer, and P. Philippsen. 2006. The SH3/PH domain protein AgBni1 collaborates with the Rho-type GTPase Cdc42 to prevent nonpolar growth at hyphal tips of Ashbya gossypii. Eukaryot. Cell 5:1635–1647.

3. Leberer, E., D. Harcus, D. Dignard, L. Johnson, S. Ushinsky, D. Y. Thomas, and J. Wendland. 2005. A Rho-type GTPase, Cdc42, regulates yeast cell polarity without a role in cell division. Curr. Microbiol. 51:385–392.

4. Li, C., R. Y. Wang, X. D. Zheng, H. Y. Liang, J. C. Tang, and Y. Wang. 2005. The formin family protein Cba2p has a role in cell polarity control during both yeast and hyphal growth in Candida albicans. J. Cell Sci. 118:2637–2648.

5. Malsolt, M., L. Leveleki, A. Hlubek, B. Sandrock, and M. Bolker. 2003. Transcription profiling of Candida albicans cells undergoing the yeast-to-hyphal transition. Mol. Microbiol. 51:385–392.

6. Nakano, K., T. Mutoh, R. Araii, and I. Mabuchi. 2002. The small GTPase Rho4 is involved in controlling cell morphology and septation in fission yeast. Genes Cells 7:357–370.

7. Nanthel, A., D. Dignard, C. Bachewich, D. Harcus, A. Marcil, A. P. Bouin, C. W. Sensen, H. Hugues, M. van het Hoog, P. Gordon, T. Rigby, F. Benoit, D. C. Tessler, D. Y. Thomas, and M. Whiteway. 2002. Transcription profiling of Candida albicans cells undergoing the yeast-to-hyphal transition. Mol. Biol. Cell 13:3452–3465.

8. Oberhoffer, U., A. Marcil, E. Leberer, D. Y. Thomas, and M. Whiteway. 2002. Myo1 is required for hypha formation in Candida albicans. Eukaryot. Cell 1:213–220.

9. Rasmussen, N. A., and M. A. Tsay. 2005. A Rho-type GTPase, rho-1, is required for septation in Neurospora crassa. Eukaryot. Cell 4:1913–1925.

10. Santos, B., J. Gutierrez, T. M. Calonge, and P. Perez. 2003. Novel Rho GTPase involved in cytokinesis and cell wall integrity during the fission yeast Schizosaccharomyces pombe. Eukaryot. Cell 2:521–533.
P. Perez. 2005. Rho4 GTPase is involved in secretion of glucanases during fission yeast cytokinesis. Eukaryot. Cell 4:1639–1645.

34. Schaub, Y., A. Dünkler, A. Walther, and J. Wendland. 2006. New pFA-cassettes for PCR-based gene manipulation in Candida albicans. J. Basic Microbiol. 46:416–429.

35. Ushinsky, S. C., D. Harcus, J. Ash, D. Dignard, A. Marcil, J. Morchhauser, D. Y. Thomas, M. Whiteway, and E. Leberer. 2002. CDC42 is required for polarized growth in human pathogen Candida albicans. Eukaryot. Cell 1:95–104.

36. Vallen, E. A., J. Caviston, and E. Bi. 2000. Roles of Hof1p, Bni1p, Bnr1p, and Myo1p in cytokinesis in Saccharomyces cerevisiae. Mol. Biol. Cell 11:593–611.

37. VandenBerg, A. L., A. S. Ibrahim, J. E. Edwards, Jr., K. A. Toenjes, and D. I. Johnson. 2004. Cdc42p GTPase regulates the budded-to-hyphal-form transition and expression of hypha-specific transcripts in Candida albicans. Eukaryot. Cell 3:724–734.

38. Walther, A., and J. Wendland. 2003. An improved transformation protocol for the human fungal pathogen Candida albicans. Curr. Genet. 42:339–343.

39. Walther, A., and J. Wendland. 2004. Polarized hyphal growth in Candida albicans requires the Wiskott-Aldrich syndrome protein homolog Wal1p. Eukaryot. Cell 3:471–482.

40. Walton, F. J., J. Heitman, and A. Idnurm. 2006. Conserved elements of the RAM signalling pathway establish cell polarity in the basidiomycete Cryptococcus neoformans in a divergent fashion from other fungi. Mol. Biol. Cell 17:3768–3780.

41. Wang, H., X. Tang, and M. K. Balasubramanian. 2003. Rho3p regulates cell separation by modulating exocyst function in Schizosaccharomyces pombe. Genetics 164:1323–1331.

42. Weinzierl, G., L. Leveli, A. Hassel, G. Kost, G. Wanner, and M. Bolker. 2002. Regulation of cell separation in the dimorphic fungus Ustilago maydis. Mol. Microbiol. 45:219–231.

43. Wendland, J., Y. Ayad-Durieux, P. Knechtle, C. Rehischung, and P. Philippsen. 2000. PCR-based gene targeting in the filamentous fungus Ashbya gossypii. Gene 242:381–391.

44. Wendland, J., and P. Philippsen. 2001. Cell polarity and hyphal morphogenesis are controlled by multiple Rho-protein modules in the filamentous ascomycete Ashbya gossypii. Genetics 157:601–610.

45. Whiteway, M., D. Dignard, and D. Y. Thomas. 1992. Dominant negative selection of heterologous genes: isolation of Candida albicans genes that interfere with Saccharomyces cerevisiae mating factor-induced cell cycle arrest. Proc. Natl. Acad. Sci. USA 89:9410–9414.

46. Wilson, R. B., D. Davis, and A. P. Mitchell. 1999. Rapid hypothesis testing with Candida albicans through gene disruption with short homology regions. J. Bacteriol. 181:1868–1874.