Cell Polarity and Hyphal Morphogenesis Are Controlled by Multiple Rho-Protein Modules in the Filamentous Ascomycete *Ashbya gossypii*

J. Wendland and P. Philippsen

Lehrstuhl für Angewandte Mikrobiologie, Biozentrum, University of Basel, CH-4056 Basel, Switzerland

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ABSTRACT

Polarized cell growth requires a polarized organization of the actin cytoskeleton. Small GTP-binding proteins of the Rho-family have been shown to be involved in the regulation of actin polarization as well as other processes. Hyphal growth in filamentous fungi represents an ideal model to investigate mechanisms involved in generating cell polarity and establishing polarized cell growth. Since a potential role of Rho-proteins has not been studied so far in filamentous fungi we isolated and characterized the *Ashbya gossypii* homologs of the *Saccharomyces cerevisiae CDC42*, *CDC24*, *RHO1*, and *RHO3* genes. The *AgCDC42* and *AgCDC24* genes can both complement conditional mutations in the *S. cerevisiae CDC42* and *CDC24* genes and both proteins are required for the establishment of actin polarization in *A. gossypii* germ cells. *Agrho1* mutants show a cell lysis phenotype. Null mutant strains of *Agrho3* show periodic swelling of hyphal tips that is overcome by repolarization and polar hyphal growth in a manner resembling the germination pattern of spores. Thus different Rho-protein modules are required for distinct steps during polarized hyphal growth of *A. gossypii*.

THE establishment of cell polarity and the maintenance of cellular asymmetry are essential cellular properties that govern morphogenesis and development of unicellular and multicellular organisms. One of the most pronounced forms of cellular asymmetry coupled to growth is the unipolar hyphal growth of filamentous fungi (Harris 1997). In filamentous fungi growth is restricted to cell apices, the hyphal tips, to which the actin cytoskeleton is polarized, organizing the delivery of vesicles carrying enzymes and precursors for cell membranes and the cell wall. Unicellular yeast-like fungi are different from filamentous fungi in that polarized growth is limited to that phase of the cell cycle in which the bud is formed (Lew and Reed 1993, 1995; Kron and Gow 1995; Chant 1999). Rho-type GTPases regulate various aspects of cell growth, *e.g.*, through the reorganization of the actin cytoskeleton as well as through pathways leading to gene expression (Boguski and McCormick 1993; Symons 1996; Cabib *et al.* 1998; Eby *et al.* 1998; Hall 1998; Nobes and Hall 1999). GTPases can be viewed as molecular modulators cycling between an inactive GDP-bound and an active GTP-bound form. Switching of a Rho-protein between both states is controlled by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs; Tanaka and Takai 1998).

In *Saccharomyces cerevisiae* five Rho-like GTPases have been characterized, *CDC42* and *RHO1–4* (Schmidt and Hall 1998). ScCdc42p, and its GEF ScCdc24p are required for the establishment of a polarized actin cytoskeleton that is a prerequisite of bud emergence (Johnson 1999). ScRho1p is required to stimulate β (1-3) glucan synthase activity, which catalyzes the synthesis of the main structural compound of the yeast cell wall and is partially redundant in function with the nonessential ScRho2p (Cabib *et al.* 1998). ScRho3p and ScRho4p have been implicated in maintaining bud growth by coordinating polarization of the actin cytoskeleton and the secretory apparatus (Matsui and Toh-e 1992; Imai *et al.* 1996; Adamo *et al.* 1999).

Very little is known about the molecular mechanisms responsible for the establishment of cell polarity and the maintenance of hyphal growth in filamentous fungi. The influence of the small GTPase Ras on cell growth and development has been studied in *Aspergillus nidulans* (Som and Kolaparthi 1994). In *Phycomyces blakesleeanus* antibodies against RhoA showed the expression of a Rho-homolog during spore germination of this zygomycete (Ramirez-Ramirez *et al.* 1999). In screens of a temperature-sensitive mutant library of *A. nidulans* several mutant strains were isolated that showed defects in polarity establishment, polarity maintenance, and hyphal morphogenesis (Kaminskyj and Hamer 1998; Harris *et al.* 1999; Momany *et al.* 1999). However, characterization of these mutants on the molecular level is yet to be done.

The filamentous ascomycete *Ashbya gossypii* belongs to the family of Saccharomycetaceae on the basis of 18S rDNA comparisons (Wendland *et al.* 1999). The genome of *A. gossypii* is exceptionally small and encom-

Corresponding author: Jürgen Wendland, Friedrich-Schiller-Universität, Institut für Mikrobiologie-Mikrobielle Phytopathologie, Winzerlaer Str.10, D-07745 Jena, Germany. E-mail: juergen.wendland@uni-jena.de

TABLE 1

Strains used in this study

Strain	Genotype	Source
A. gossypii		
Wild type		ATCC10895
A. g. Δlt	*leu2 thr4*	P. Philippsen
AWE2	*rho3Δ1::GEN3 RHO3*	This study
AWE2b	*rho3Δ1::GEN3*	This study
AWE3	*rho1::GEN3 RHO1*	This study
AWE18	*rho3Δ2::GEN3 RHO3 leu2 thr4*	This study
AWE18b	*rho3Δ2::GEN3 leu2 thr4*	This study
AWE20	*cdc42Δ1::GEN3 CDC42*	This study
AWE21	*cdc24::GEN3 CDC24 leu2 thr4*	This study
AWE26	*cdc42Δ2::GEN3 CDC42 leu2 thr4*	This study
S. cerevisiae		
YEF1201	*MAT***a** *leu2 his3 lys2 trp1 ura3 cdc24::HIS3* pMGF5 (Gal1-*CDC24*)	Erfei Bi
DJTD2-16D	*MATα leu2 trp1 his4 ura3 gal2 cdc42-1*	Erfei Bi

passes only 8.85 Mb (S. Steiner, K. Gaudenz, J. Wendland, C. Mohr, R. Pöhlmann and P. Philippsen, unpublished results). On the basis of the very efficient homologous recombination in *A. gossypii*, one-step gene replacement via PCR-based gene targeting has been established in *A. gossypii*, allowing the facile functional analysis of genes (Steiner *et al.* 1995; Wendland *et al.* 2000). Recently, a rho-GAP homolog, encoded by the *A. gossypii BEM2* gene, has been shown to be involved in the determination and maintenance of cell polarity during distinct stages of germination and polar hyphal growth in *A. gossypii* (Wendland and Philippsen 2000). This analysis suggested that Rho-like GTPase modules are an important part of the regulatory network that controls polarized hyphal growth in filamentous fungi.

Therefore, we investigated the roles of three RHO-genes, *CDC42*, *RHO1*, and *RHO3*, and a gene coding for the guanine nucleotide exchange factor of Cdc42p, *CDC24*, during the establishment of cell polarity and maintenance of unipolar hyphal growth of *A. gossypii*.

MATERIALS AND METHODS

Strains and media: The *A. gossypii* and *S. cerevisiae* strains used are listed in Table 1. Rich medium (AFM) was used as described (Wendland and Philippsen 2000). G418/geneticin was added to rich media for selection of antibiotic-resistant transformants at a final concentration of 200 μg/ml. Yeast media were prepared as described by Guthrie and Fink (1991). The *Escherichia coli* strains DH5α and XL1-Blue were used as hosts for plasmids.

Isolation of genes: DNA hybridization, colony screening, and standard recombinant DNA techniques were done as described (Sambrook *et al.* 1989). pAG-plasmids were derived from a plasmid library constructed by cloning genomic *A. gossypii* DNA into pRS416 (C. Mohr and P. Philippsen, unpublished results). Sequence information was generated as described previously (Wendland and Philippsen 2000). *AgRHO3* was isolated from a Yep352-based plasmid library (Maeting *et al.* 1999) using insert DNA from pAG1025 as a probe. *AgRHO1* was subcloned from a BAC library (provided by Rod Wing, Clemson University, Clemson, SC) using pAG2832 as a probe. The complete sequence of *CDC42* was determined by using the plasmids pAG12814 and pAG12854, which contain overlapping inserts. *AgCDC24*, initially identified on pAG1822, which contains only part of the gene, was completely sequenced using plasmid clones pAG13101 and pAG18216, which also contained overlapping inserts. Plasmid clones containing the complete *AgCDC42* (p*AgCDC42*) and *AgCDC24* (p*AgCDC24*) genes were constructed by ligating *Bam*HI-cleaved PCR fragments carrying these genes into the *Bam*HI-site of pRS415 (Sikorski and Hieter 1989). The following primers were used in the amplification of genomic *A. gossypii* DNA: to obtain *AgCDC42*, primer-1, 5′-CGA**GGATCC**GGGCGACGACTCGGGCGACGACTCCGATG-3′ and primer-2, 5′-TCA**GGATCC**GTGCCCACCAGTTACCCGGTCT CTTCTTTG-3′; and to obtain *AgCDC24*, primer-3, 5′-CTT**GGATCC**CGTCACCTACCACACGCAGCGTACTCTTATG-3′ and primer-4, 5′-GAA**GGATCC**GACGGTCCCAAATACACATTG ACATAGCCC-3′ (*Bam*HI restriction sites are in boldface). PCR was done using VENT-polymerase (New England BioLabs, Allschwil, Switzerland). Cloning of the correct gene sequences was verified by sequencing. All sequencing was done on ABI377 automated sequencers. Complete double-strand sequence contigs were assembled from overlapping fragments using the DNA-STAR software package. Sequence comparisons were done by BLAST searches (Altschul *et al.* 1990) and using Genetics Computer Group software from the University of Wisconsin. Profile searches were done on the ISREC profile server. GenBank accession numbers for the newly obtained *A. gossypii* sequences are AF210626–9; swiss-prot accession numbers for the *S. cerevisiae* proteins used for protein comparisons are Cdc42p, P19073; Rho1p, P06780; Rho3p, Q00245; and ScCdc24p, AAC04990.

Gene disruption: Insertion deletions or deletions of the complete open reading frames (ORFs) of *AgCDC42, AgRHO1, AgRHO3*, and *AgCDC24* were performed by PCR-generated *GEN3* disruption cassettes as described previously (Wendland *et al.* 2000). Primers used for the generation of the cassettes and for verification of the deletions are listed in Tables 2 and 3, respectively. *A. gossypii* and *S. cerevisiae* transformations were carried out as described (Becker and Guarente 1991; Wendland and Philippsen 2000). Disruptions were verified by PCR.

TABLE 2

Oligonucleotide primers used for gene deletions

Name of primer	Sequence of primer[a]
S1-*RHO3*Δ*1*	ACATCCATGACATCTTCGTGGACAACCAGCACATCACGCTGAGCCgctagggataacagggtaat
S2-*RHO3*Δ*1*	CAAGTCGCACTTCAGCGCCACTAGCACCAGCTTCACGCCCTCGCAaggcatgcaagcttagatct
S1-*RHO3*Δ*2*	CTGGCATCAGAGGAAGCTCCCACCACCAAGCTCTACAAACACAAgctagggataacagggtaat
S2-*RHO3*Δ*2*	ATTATATTAGTATAGTCTAAAGTTGCAGGCAGTGGGTATTAAAGTaggcatgcaagcttagatct
S1-*RHO1*	GTCGGAGATGGTGCATGCGGGAAAACATGTCTTTTGATTGTGTTTgctagggataacagggtaat
S2-*RHO1*	GCAGATCAACACAACATTGGAGTCTGGGTACGATAACGGCCGTAGaggcatgcaagcttagatct
S1-*CDC42*Δ*1*	GAGGACTACGACAGGTTGCGGCCGTTGTCGTACCCGTCGACGGACGTGTTTCTCGTGTGCTTCAGCG gctagggataacagggtaat
S2-*CDC42*Δ*1*	GCAGGGTACGCCCGGGCAGTGGTGATGTACCTCGGGGAACCACTTCTCCTTGACGTTCTCGAACGAC aggcatgcaagcttagatct
S1-*CDC42*Δ*2*	GCAGACATTGAAGTGCGTGGTCGTTGGGGACGGAGCTGTCGGCAAGgctagggataacagggtaatacag
S2-*CDC42*Δ*2*	CACAAGATGGTGCACTTCTTGCTCTTCTTGATGACCGGCGGTTCGActgcaggcatgcaagcttagatc
S1-*CDC24*	ACTTCTTCAAGCTTTATGAGCCGTGGTCTATTGGGCAAAATGCCGCAAgctagggataacagggtaat
S2-*CDC24*	CACAAGATGGTGCACTTCTTGCTCTTCTTGATGACCGGCGGTTCGActgcaggcatgcaagcttagatc

[a] Uppercase sequences correspond to those immediately upstream or downstream of the target locus. Lowercase sequences correspond to terminal regions of the *GEN3* selectable marker used in the PCR to generate the disruption cassettes. All sequences are written from 5′ to 3′.

Primary transformants of *A. gossypii* are heterokaryotic; *i.e.*, mycelia with multinucleate hyphal segments carry two kinds of nuclei with either wild-type or mutant alleles. Sporulation yielded homokaryotic null mutants, which allowed phenotypic analyses of the mutant strains. Specifically, spores were obtained from primary heterokaryotic transformants or in the case of *Agrho3* from null mutant strains after incubation and growth of mycelia for 1 wk on full medium selective plates supplemented with G418. Mycelial fragments could be destroyed by incubation with cell wall lytic enzymes (Zymolyase, Seikagaku, Japan). Spore preparations were incubated in selective full medium that restricts the germination of wild-type spores. Mutant spores of *Agcdc42* and *Agcdc24* were germinated for 24–36 hr, which in wild type results in the formation of small colonies. Spores together with remnants of heterokaryotic cell material of *Agrho1* strains were plated on selective full medium plates and incubated for 2–4 days to analyze *Agrho1* minicolonies. To analyze the effect of osmo-stabilizing media, spores of primary heterkaryotic *Agrho1* strains were isolated (since the homokaryotic *Agrho1* minicolonies lyse prior to the initiation of spore development) and appropriate dilutions were plated on selective full medium plates either with or without the addition of 1 M sorbitol and incubated for 3 days at 30°. For *AgCDC42* and *AgRHO3* two different disruption alleles were constructed using two sets of primer combinations resulting in either insertion deletion (Δ*1*) or complete ORF deletion (Δ*2*) generating the disruption alleles *Agcdc42*Δ*1*, *Agcdc42*Δ*2*, *Agrho3*Δ*1*, and *Agrho3*Δ*2*, respectively (see Table 1). Both disruption alleles of *Agcdc42* and *Agrho3* behaved phenotypically identically. For *AgCDC24* and *AgRHO1* independent transformants were analyzed carrying the respective gene deletion. Although the transformation frequency is rather low when using PCR-based gene targeting (resulting in 0–40 transformants/10 μg of disruption cassette produced by PCR and used in one transformation experiment; see also Wendland *et al.* 2000), in all cases the obtained primary transformants carried the correct deletion, which is due to the very high efficiency of homologous integration in *A. gossypii* (Steiner *et al.* 1995). Therefore, as a standard procedure two independent transformants of each strain were characterized.

TABLE 3

Oligonucleotide primers used for the verification of gene deletions

Name of primer	Sequence of primer[a]
G1-*RHO3*Δ*1*	CACGCGAGGGTACTTTCCG
G4-*RHO3*Δ*1*	GGCGTGATGGCGCTCTCGTT
G1-*RHO3*Δ*2*	GTCCGTGATCACCAGTGGTG
G4-*RHO3*Δ*2*	CGCCAACCAGTGAACGTTGGCAGC
G1-*RHO1*	GTATCAGGAGAAAATTGGTG
G4-*RHO1*	CAAGATGATCGGAACACCC
I1-*RHO1*	ATGCCCAGCCGTATCCCAAAGCG
G1-*CDC42*Δ*1*	GTCAGCCGTACACGTTGGGC
G4-*CDC42*Δ*1*	GGTCAAGGCCGAGCACTCC
G1-*CDC42*Δ*2*	GTTTCATTCAGAGCAGTAGAGTG
G4-*CDC42*Δ*2*	GGTCTCGCGCGGGTCCTGG
G1-*CDC24*	CGGATTGGTGCTCTTTTTATGC
G4-*CDC24*	CAGAGCAGTTTGTAACTCCTTAGT
G2	gtttagtctgaccatctcatctg
G3	tcgcagaccgataccaggatc

[a] Uppercase sequences correspond to regions upstream (G1-) and downstream (G4-) or within (I1-*RHO1*) the loci targeted for disruption. Lowercase sequences (G2 and G3) correspond to internal sites of *GEN3* used for verification PCR of the 5′ and 3′ novel joints generated upon integration.

Cytological techniques: Staining of septa and nuclei was done by calcofluor (0.1 μg/ml) and 4′6,-diamidino-2-phenylindole (DAPI; 1.0 μg/ml), respectively, with cells either grown in liquid rich medium or on slides covered with thin layers of rich medium as described (Wendland and Philippsen 2000). Actin staining was done with logarithmically growing cultures. Cells were fixed in formaldehyde (3.7%) and phosphate-buffered PBS. Cells were permeabilized with 0.2% Triton X-100 for 5 min, washed with PBS, and stained with rhodamine-phalloidin (Molecular Probes, Eugene, OR). Fluorescence microscopy was done on Zeiss (Thornwood, NY) Axioskop microscopes with the appropriate filter combinations.

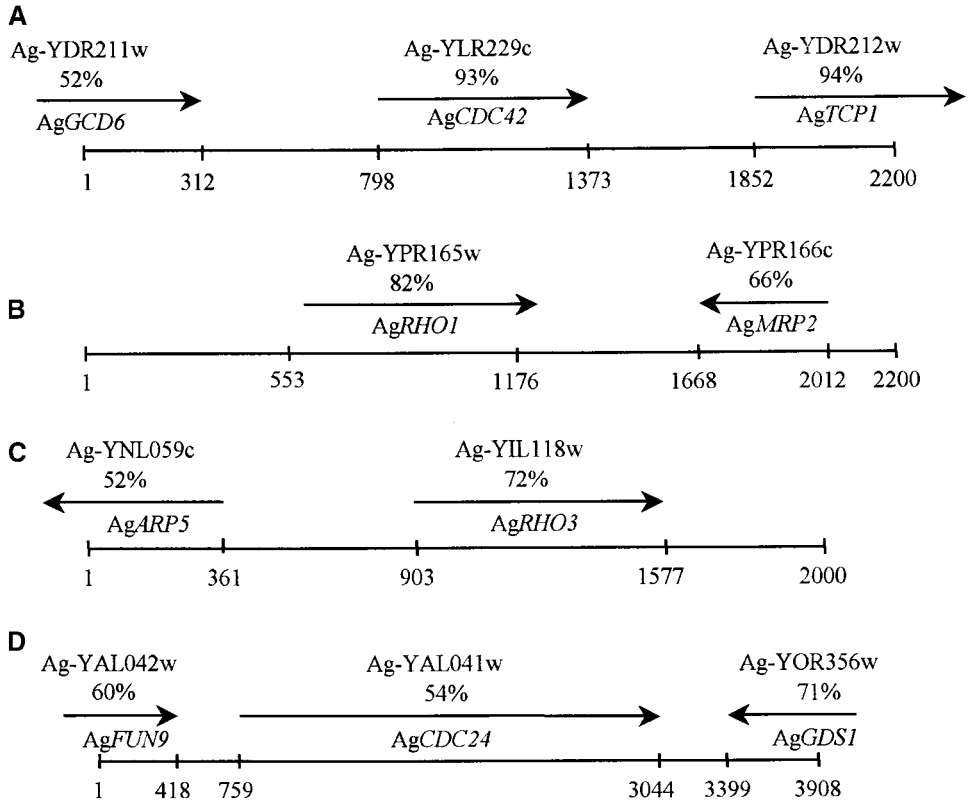

FIGURE 1.—Genomic loci of isolated Rho-genes and *AgCDC24*. Open reading frames are depicted by arrows indicating the transcriptional orientation. The nomenclature used is an Ag-prefix followed by the systematic name of the yeast gene bearing highest identity (above the arrow as percentage identity on the amino acids level) or an Ag-prefix followed by the standard three-letter code of the homologous yeast gene (below the arrow). The numbering on the scales depicts the length of the determined sequences and indicates either start or stop codons of the individual open reading frames.

Images were taken using digital video imaging systems (Princeton Instruments, Princeton, NJ; Optronics; Diagnostic instruments).

RESULTS

Isolation of Rho-GTPases and *CDC24* of *A. gossypii*: Using initial sequence information provided by random sequencing of an *A. gossypii* plasmid library, four loci containing the *CDC42*, *CDC24*, *RHO1*, and *RHO3* genes were isolated and completely sequenced (Figure 1). Six additional genes were found in the flanking regions of the Rho-genes and *AgCDC24* that share homologies to known genes of *S. cerevisiae* (Figure 1). Two of the gene pairs isolated, *AgRHO1-AgMRP2* and *AgFUN9-AgCDC24*, show synteny, *i.e.*, conserved gene order, to the corresponding homologs of *S. cerevisiae*. Within the *AgCDC42* locus, interestingly, synteny between the homologous genes *GCD6* and *TCP1* of *A. gossypii* and *S. cerevisiae* is disrupted by *CDC42* in *A. gossypii*. Sequence identity between the *A. gossypii* and *S. cerevisiae* proteins ranged from 52 to 94% (Figure 1). Alignment of the *A. gossypii*

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1   MQ-----TL--------KCVVVGDGAVGKTCLLISYTTNQFPADYVPTVFDNYAVTVMIGDEPYTLGLFDTAGQEDYDRL A.g.Cdc42
1   MQ-----TL--------KCVVVGDGAVGKTCLLISYTTNQFPADYVPTVFDNYAVTVMIGDEPYTLGLFDTAGQEDYDRL S.c.Cdc42
1   MS-----QQMHNPSIRRKLVIVGDGACGKTCLLIVFAKGKFPQVYVPTVFDNYVADVEVDGRRVELALWDTAGQEDYDRL A.g.Rho1
1   MS-----QQVGN-SIRRKLVIVGDGACGKTCLLIVFSKGQFPEVYVPTVFENYVADVEVDGRRVEIALWDTAGQEDYDRL S.c.Rho1
1   MP-LCGSSSSSKHPIERKIVILGDGACGKTSLLNVFTRGYFPKVYEPTVFENYIHDIFVDNQHITLSLWDTAGQEEFDRL A.g.Rho3
1   MSFLCGSASTSNKPIERKIVILGDGACGKTSLLNVFTRGYFPEVYEPTVFENYIHDIFVDSKHITLSLWDTAGQEEFDRL S.c.Rho3

68  RPLSYPSTDVFLVCFSVVSPPSFENVKEKWFPEVHHHCPGVPCLIVGTQIDLRE-----NKMVIEKLQRQRLRPITPEQG A.g.Cdc42
68  RPLSYPSTDVFLVCFSVISPPSFENVKEKWFPEVHHHCPGVPCLVVGTQIDLRD-----DKVIIEKLQRQRLRPITSEQG S.c.Cdc42
76  RPLSYPDSNVVLICYSIDLPDSLENVMEKWISEVLYFCQGVPIILVGCKADLRN-----DPQVIEQLRQQGQQPVSQAQA A.g.Rho1
75  RPLSYPDSNVVLICFSIDLPDSLENVQEKWIAEVLHFCQGVPIILVGCKVDLRN-----DPQTIEQLRQEGQQPVTSQEG S.c.Rho1
80  RSLSYSDTHTIMLCFSVDSRDSLENVKNKWVSEIADHCEGVKLVLVALKCDLRSSDEYGNESAITPGSIQNQK------- A.g.Rho3
81  RSLSYSDTQCIMLCFSIDSRDSLENVQNKWVGEITDHCEGVKLVLVALKCDLRNNEN--ESNAITPNNIQQDNSVSNDNG S.c.Rho3

143 EKF-----------------ARELRAVKYVECSALTQRGLKNVFDEAIVAAL-----------EPPVIKKSKKCTIL   A.g.Cdc42
143 SRL-----------------ARELKAVKYVECSALTQRGLKNVFDEAIVAAL-----------EPPVIKKSKKCAIL   S.c.Cdc42
151 QEV-----------------ADQIGAVEYLECSAKTGFGVREVFEAATRASLMGK---QGKSKAKSDKKKKKKCVVL   A.g.Rho1
150 QSV-----------------ADQIGATGYYECSAKTGYGVREVFEAATRASLMGKSKTNGKAKKNTTEKKKKKCVLL   S.c.Rho1
153 ---YNGGGGNGLIPYDEGLAMAKQIGALRYLECSAKMNRGVNEAFTEAARCALTATPKG---ARDSAPEAESSSCTIM   A.g.Rho3
159 NNINSTSNGKNLISYEEGLAMAKKIGALRYLECSAKLNKGVNEAFTEAARVALTAGPVA---T--EVKSDSGSSCTIM   S.c.Rho3
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FIGURE 2.—Alignment of Rho-proteins from *A. gossypii* and *S. cerevisiae*. Identical amino acids residues in the *A. gossypii* (Ag) and *S. cerevisiae* (Sc) Rho-proteins are shaded.

TABLE 4

Percentage amino acid sequence identity of Rho-GTPases

	ScCdc42	AgRho1	ScRho1	AgRho3	ScRho3
AgCdc42	93.7	48.7	48.7	37.7	37.7
ScCdc42		48.7	49.7	37.7	37.7
AgRho1			81.6	43.0	44.9
ScRho1				44.0	45.0
AgRho3					72.3

and *S. cerevisiae* Rho-protein sequences revealed the most conserved regions to be those involved in GTP binding and hydrolysis [amino acids (aa) 5–20, 53–62, and 154–160 of ScCdc42p], effector protein interaction (aa 26–50 of ScCdc42p), and the isoprenylation consensus motif at the very C-terminal part of Rho-proteins (Figure 2; see JOHNSON 1999 for review). Pairwise comparisons were performed with the *A. gossypii* and *S. cerevisiae* Rho-proteins. This revealed highest sequence identity between *A. gossypii* and *S. cerevisiae* Cdc42 proteins and decreasing identity between the Rho1 and Rho3 proteins, respectively (Table 4). Comparison of the lengths of the homologous Rho-protein pairs showed that both Cdc42p proteins are 191 amino acids in length, whereas the AgRho1p (207 aa) is 2 and the AgRho3p (224 aa) is 7 aa shorter than its yeast homolog. The *A. gossypii CDC24* gene codes for a protein of 761 amino acids, which is 93 aa shorter than ScCdc24p. Both proteins contain GEF domains in their central parts followed by pleckstrin homology (PH) domains and share an overall sequence identity of 54% (Figures 1A and 3A). A dot-matrix comparison between AgCdc24p and ScCdc24p revealed that this homology is distributed over the entire lengths of the proteins except for a region encompassing the PH domains, which is 50 aa shorter in AgCdc24p compared to ScCdc24p (Figure 3B). Three lines of evidence indicated the correct isolation of the *A. gossypii* homologs of the *S. cerevisiae CDC42*, *RHO1*, *RHO3*, and *CDC24* genes: (i) the degree of amino acids sequence identity indicated that the corresponding *S. cerevisiae* proteins were the most closely related sequences in the databases; (ii) syntenic relationships of gene pairs suggested some evolutionary conservation of loci between *A. gossypii* and *S. cerevisiae*; and (iii) analyses of the protein sequences showed the presence of conserved domains, *i.e.*, GTP-binding/hydrolysis domains, effector domains, and membrane localization domains for the Rho-proteins, and PH and GEF domains for the CDC24 proteins. Additionally, the ability of the *AgCDC42* and *AgCDC24* genes to complement mutations in the corresponding *S. cerevisiae* genes was examined. To this end *S. cerevisiae* strains carrying either the *cdc42-1* temperature-sensitive allele or a deletion of the *CDC24* gene together with a *ScCDC24* gene under control of the GAL1-promoter were transformed with

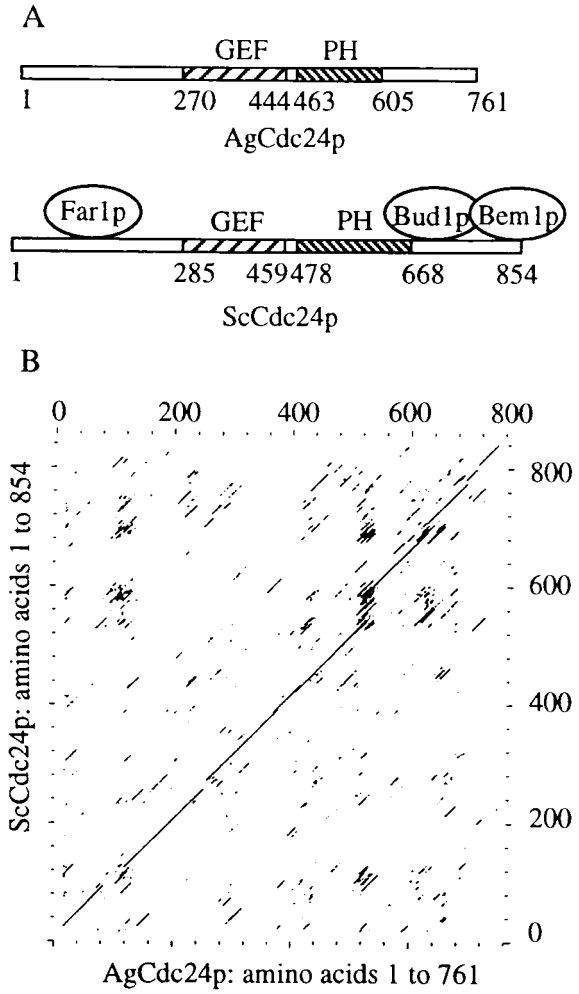

FIGURE 3.—Protein comparison of Cdc24p of *A. gossypii* and *S. cerevisiae*. (A) Schematic representation of the homologous Cdc24 proteins of *A. gossypii* (top) and *S. cerevisiae* (bottom). Guanine-nucleotide exchange factor (GEF) and pleckstrin-homology (PH) domains are shown as hatched boxes. Numbering corresponds to amino acids residues of the proteins and the length of the individual domains. In ScCdc24p protein-protein interactions with ScFar1p, ScBud1p, and ScBem1p to specific regions of ScCdc24p are indicated. (B) Dot-matrix comparison of AgCdc24p and ScCdc24p.

plasmids containing the *AgCDC42* or *AgCDC24* genes, respectively. A shift to the restrictive temperature in the case of *cdc42-1* or to media containing glucose only, which represses expression of the *CDC24* gene under GAL1-promoter control, indicated that both yeast strains continued to proliferate only when carrying the complementing *A. gossypii* gene (Figure 4).

Deletion of *AgCDC42* and *AgCDC24*: Using one-step gene replacement via PCR-generated disruption cassettes, primary heterokaryotic *Agcdc42/AgCDC42* and *Agcdc24/AgCDC24* strains were created. However, we were unable to generate homokaryotic mutants from spores of these strains germinated under selective conditions. To determine at which stage development is

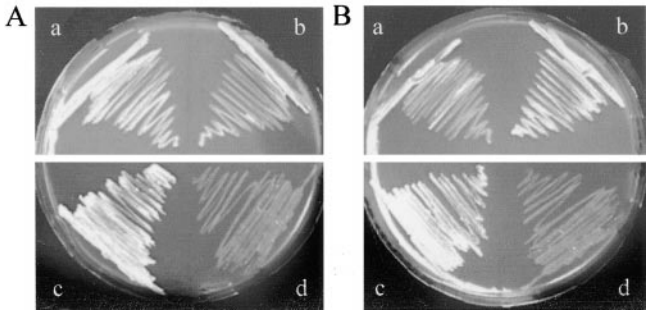

Figure 4.—Complementation of conditional *Sccdc24* and *Sccdc42* mutants. (A) YEF1201 carries a genomic deletion of *ScCDC24* and a plasmid with *ScCDC24* under *GAL1* promoter control. This strain was transformed with either a plasmid carrying *AgCDC24* (a and c) or a vector control (pRS415, b and d), and transformants were streaked onto full medium plates containing either 2% glucose and 2% galactose (a and b) or 2% glucose (c and d) and incubated for 3 days at 30° before photography. (B) DJTD2-16D harboring the temperature-sensitive *cdc42-1* allele carrying either p*AgCdc42* (a and c) or a vector control (pRS415, b and d) were streaked on full medium plates and incubated for 3 days at 30° (a and b) or 37° (c and d) before photography.

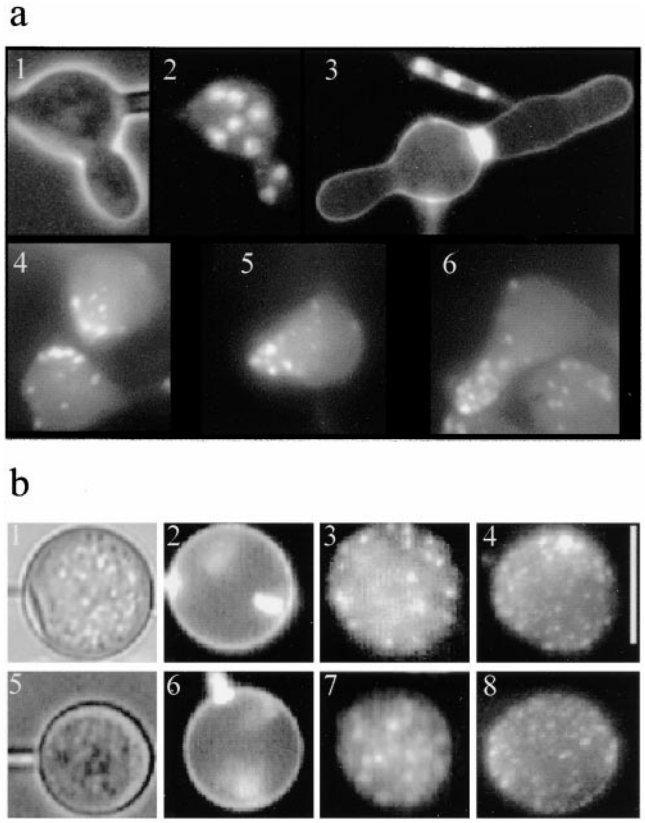

Figure 5.—(a) Germination of wild-type *A. gossypii* spores. (1) Phase contrast image of a germinated spore that switched from isotropic germ-cell to polarized hyphal growth. (2) DAPI staining of all nuclei of the cell shown in 1. (3) Chitin staining of a germ cell displaying the characteristic bipolar germ-cell branching pattern. (4–6) Actin staining of germ cells at various stages during the establishment of polar hyphal growth. (b) Photomicrographs of germ cells of *A. gossypii CDC42* and *CDC24* mutants. Micrographs show different spores imaged under phase contrast (1 and 5), after staining for chitin (2 and 6), after staining of nuclei (3 and 7), and after staining of cortical actin (4 and 8). Bar, 10 μm for a and b.

blocked in *Agcdc42* and *Agcdc24* mutants we reexamined spores germinated under selective conditions and compared these with germinated wild-type spores (Figure 5, a and b). Germination of wild-type spores is initiated by an isotropic growth phase generating spherical germ cells. After an incubation period of 7–8 hr in full medium at 30°, germ cells (containing 2 to maximally 8 nuclei) switch from isotropic to polar growth, which leads to the formation of the first hyphal tube (Figure 5a, 1 and 2). Later a second hypha is generated opposite of the first hypha producing the characteristic bipolar branching pattern of germ cells (Figure 5a, 3). Septation usually occurs at the bases of newly formed hyphae in *A. gossypii*, marking, *e.g.*, the transition between germ cell and hypha (Figure 5a, 3). Cortical actin in filamentous fungi were shown to be localized at the hyphal tips and at sites of developing septa (Torralba *et al.* 1998). We used rhodamine-phalloidin to stain cortical actin patches in germ cells of wild-type cells (Figure 5a, 4–6). Prior to the emergence of the first hyphal tube, cortical patches were found to cluster at the incipient branch site (Figure 5a, 4). At later stages actin patches were found to be localized within the hyphal tips (Figure 5a, 5 and 6). *Agcdc42* and *Agcdc24* spores were able to induce the isotropic growth phase (Figure 5b, 1 and 5), which was marked by uniform distribution of chitin throughout the cell wall (Figure 5b, 2 and 6). This isotropic growth phase continued beyond the wild-type size of germ cells (8 μm), resulting in *Agcdc42* or *Agcdc24* germ cells with diameters of up to twice that size (Figure 5b). Within the mutant germ cells nuclear cycles continued generating multinucleate cells containing >16 nuclei (Figure 5b, 3 and 7). Actin staining revealed delocalized cortical patches and *Agcdc42* and *Agcdc24* germ cells failed to induce the isotropic-polar switch, and thus did not produce hyphal branches (Figure 5b, 4 and 8).

Deletion of *AgRHO1* results in colony lysis: An *AgRHO1/rho1* deletion strain was constructed via PCR-based gene targeting and homokaryotic *Agrho1* mycelium could be obtained via clonal selection of spores, which was verified by PCR directly using these mycelial fragments (not shown). *Agrho1* null mutants formed colonies that showed a severely reduced growth rate compared to heterokaryotic strains that grew like the wild type (Figure 6A). The *Agrho1* colonies exhibited a high rate of cell lysis, which resulted in the death of these colonies within 4 days after germination (500/500 inspected colonies; Figure 6, B and C). This cell lysis phenotype could only partially be remedied by the addition of 1 M sorbitol to the medium. Germination on selective complete medium containing 1 M sorbitol

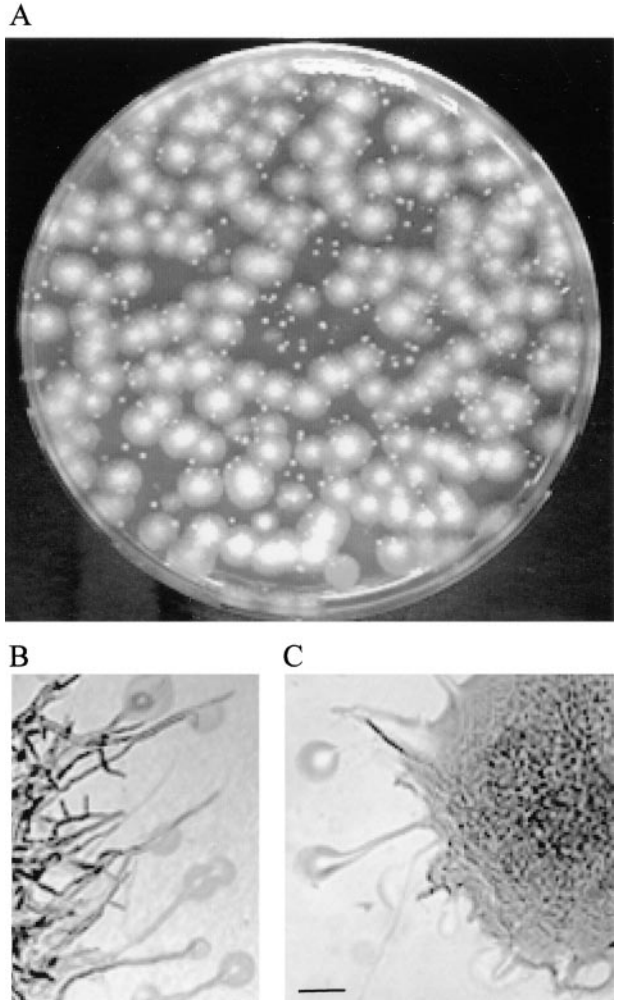

FIGURE 6.—Slow growth and lysis phenotype of *Agrho1* mutants. (A) Minicolony formation of *Agrho1* strains in comparison to heterokaryotic, wild-type-like growth. (B and C) Micrographs of 2- (B) and 4- (C) day-old *Agrho1* minicolonies. Bar for B and C, 100 μm.

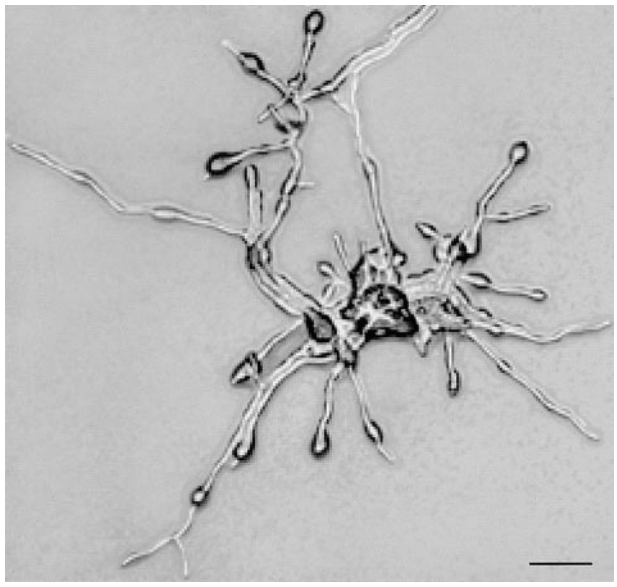

FIGURE 7.—Micrograph of a 2-day-old *Agrho3* colony incubated at 30°. Bar, 50 μm.

resulted in a 10-fold increase of minicolony formation compared to the colony formation frequency on complete selective medium without sorbitol. However, lysis of the cells of these minicolonies was not prevented on osmo-stabilized medium (not shown).

Deletion of *AgRHO3* results in a swollen tip phenotype: Viable *Agrho3* null mutant strains could be obtained after clonal selection of primary transformants. In growth assays at temperatures varying between 16° and 37° mycelia of *Agrho3* null strains were found to grow slower than wild type at all temperatures and reached only 35% of the diameters of wild-type colonies after 1 wk of growth (not shown). Interestingly, germination of spores at elevated temperatures, *i.e.*, 37°, totally abolished colony formation due to early lysis of emerging germ tubes. However, once a mycelium was established growth occurred also at elevated temperatures (not shown). *Agrho3* hyphae showed characteristic swellings at their hyphal tips (Figure 7). Isotropic growth phases in *Agrho3* tip cells could be overcome by the emergence of new hyphal tubes that continued growth in the direction established by the hyphae prior to the swelling (Figure 7).

Comparison of wild-type with *Agrho1* and *Agrho3* hyphae: Wild-type hyphae show chitin accumulation at sites of septation and at growing hyphal tips (Figure 8A). Actin cortical patches are clustered at hyphal tips and a network of actin filaments extends along the hyphae (Figure 8B). Nuclei are distributed with regular intervals in wild-type hyphae (Figure 8C). Calcofluor stained *Agrho1* hyphal tips with varying intensities and although sites of septation could be observed rather few septa were found along the hyphae of minicolonies (Figure 8E). During the growth phase of *Agrho1* hyphae actin cortical patches were found to localize at the hyphal tip (Figure 8G). However, within a minicolony some hyphae were observed that did not show a concentration of cortical actin at the tips (Figure 8D). The even distribution of nuclei was disturbed in *Agrho1* corresponding to the enlarged hyphae and irregular cell shape (Figure 8, F and H). Chitin staining of *Agrho3* hyphae revealed sites of septation within the hyphae (Figure 8I). Interestingly, hyphal tubes emerging from swollen tips were also found to be separated from areas of isotropic growth by septa (100/100 observed cases; Figure 8I). This phenotype bears a similarity to the germination process of spores in which the boundaries between germ cells and the emerging hyphal tubes are also marked by septa (Figure 5a, 3). Cortical actin distribution showed tip-localization during periods of polar growth and was found to be delocalized in swollen hyphal tips (Figure 8J). Accumulation of nuclei could be

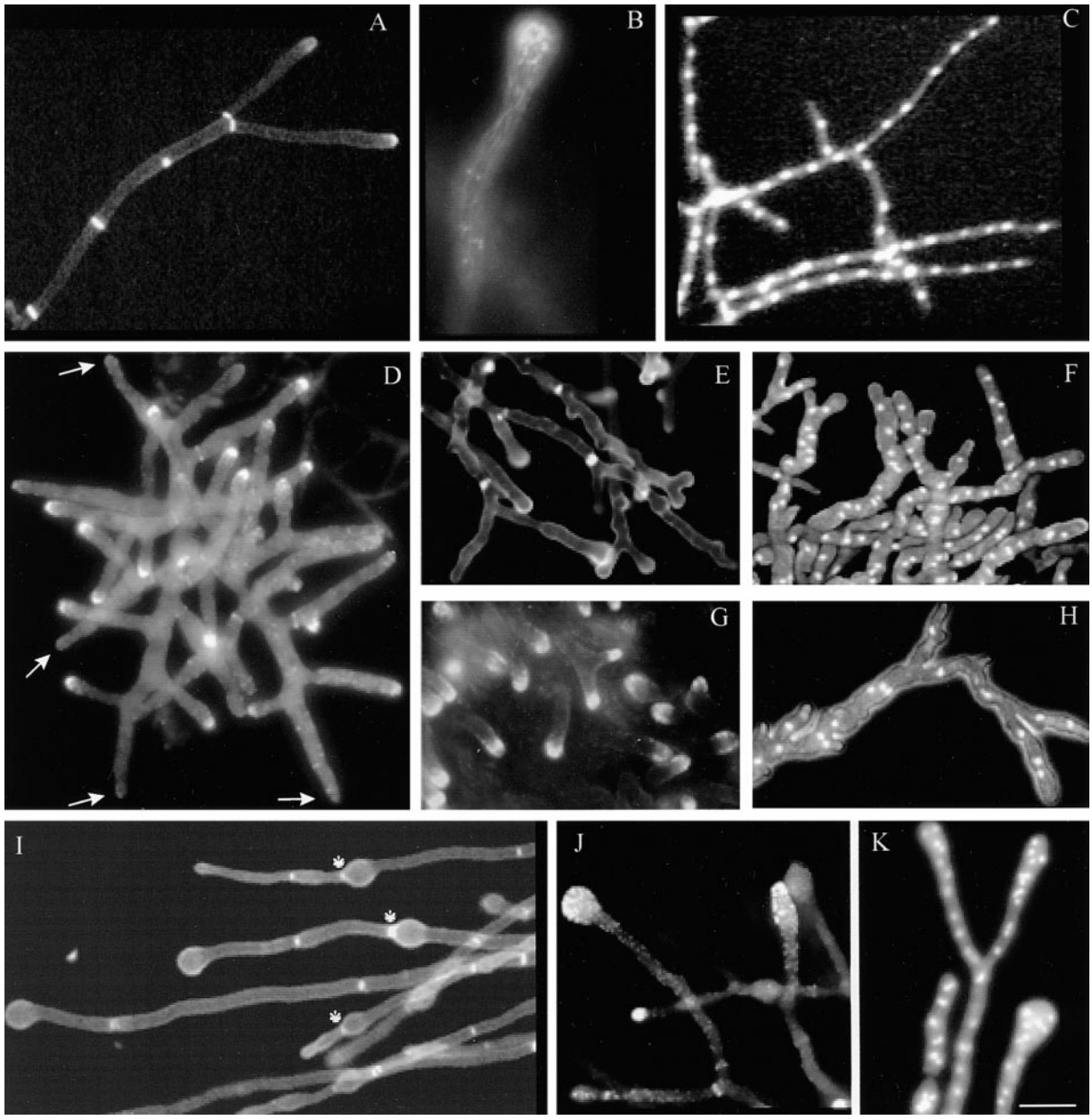

Figure 8.—Micrographs showing cytological features of the wild-type (A–C), *Agrho1* (D–H), and *Agrho3* (I–K) strains. Stainings show the distribution of chitin (A, E, and I), nuclei (C, F, H, and K), and the localization of actin (B, D, G, and J). Bar, 10 μm, except for B, 5 μm and C, 15 μm.

observed in swollen *Agrho3* tip cells (Figure 8K) as is generally the case in wild-type germ cells and was also observed in the *Agcdc42* and *Agcdc24* mutants (Figure 5b, 3 and 7).

DISCUSSION

We chose the filamentous ascomycete *A. gossypii* to investigate the role of rho-like GTPase modules in the regulation of polarized hyphal growth. The attractiveness of this system is based on the regularity of filamentous growth and hyphal branching and the experimental accessibility of *A. gossypii* in combination with its small genome (Steiner *et al.* 1995; Wendland *et al.* 2000; S. Steiner, K. Gaudenz, J. Wendland, C. Mohr, R. Pöhlmann and P. Philippsen, unpublished results). In this study we have presented the characterization of a set of Rho-proteins, including AgCdc42p, AgRho1p, and AgRho3p, and *AgCDC24*, which encodes the GEF for AgCdc42p, and demonstrated their importance at distinct stages during development of cell polarity and the maintenance of hyphal growth. Deletion of *AgCDC42* and *AgCDC24* resulted in nondistinguishable phenotypes. Germ cells of *Agcdc42* and *Agcdc24* spores were unable to perform the isotropic-polar switch and thus did not induce hyphal growth. These mutant phenotypes in *A.*

gosyypii are strikingly similar to those observed in conditional mutants of the *S. cerevisiae CDC42* and *CDC24* genes in which cells at the restrictive conditions become enlarged, multinucleate, and do not polarize their actin cytoskeleton (Sloat *et al.* 1981; Adams *et al.* 1990). Additional evidence that the *CDC42*-Rho-module fulfills very similar functions in both organisms is derived from analyses that indicate that the *AgCDC42* and *AgCDC24* genes can complement the corresponding *S. cerevisiae* mutants without resulting in morphologically aberrant yeast cells. We can therefore conclude that (i) AgCdc24p acts as GEF for AgCdc42p, (ii) due to the similarity of the deletion phenotypes of *Agcdc42* and *Agcdc24* mutants *AgCDC24* is most likely the only Cdc42p-GEF present in the *A. gossypii* genome, (iii) the inability to induce polar growth in *A. gossypii cdc42* and *cdc24* mutants is likely to be the consequence of the failure to polarize the actin cytoskeleton, and (iv) the *CDC42*-module is not required to induce or maintain the isotropic growth phase during spore germination. Interestingly, it was shown that ScCdc24p is sequestered in the nucleus in a ScFar1p-dependent manner and is exported to the cytoplasm in G1 upon degradation of ScFar1p (Toenjes *et al.* 1999; Nern and Arkowitz 2000; Shimada *et al.* 2000). A similar nucleocytoplasmic shuttling has been shown for human ECT2, which is also a Rho-GEF (Tatsumoto *et al.* 1999), suggesting that this feature might be a more common theme and might also play a role in polarized hyphal morphogenesis.

We observed interesting similarities between the bipolar germ-cell branching pattern and the repolarization of swollen hyphal tips of *Agrho3* mutants. Isotropic growth of *Agrho3* hyphal tips could be overcome by generating a site of renewed polarized hyphal growth followed by growth in the same direction as was maintained prior to the swelling. Septation at the border between isotropic and polar growth in *Agrho3* mutants is indicative of a polarity establishment event. This is contrasted by the *Agbem2* mutant phenotype in which isotropic growth of tip cells resulted in the formation of multiple new cell polarities at random positions indicating (i) a loss of cell polarity in *Agbem2* mutants and (ii) a function of the *RHO1*-module in the preparation of the site at which a new cell polarity is to be established (Wendland and Philippsen 2000). The occurrence of tip swellings after certain periods of polar cell growth in *Agrho3* mutants indicates that the *RHO3*-module in *A. gossypii* plays a critical role for the maintenance of hyphal growth. In *S. cerevisiae* overexpression of *CDC42* suppresses defects in *Scrho3* indicating a genetic interaction between both Rho-modules (Matsui and Toh-e 1992).

Deletion of *AgRHO1* resulted in a penetrant phenotype of cell/colony lysis. Many conditional *S. cerevisiae rho1* mutants show defects in cell wall integrity resulting in the formation of cells with small buds that lyse at their tips (Cabib *et al.* 1998; Pruyne and Bretscher 2000). Growth on osmotically stabilized complete medium resulted in a 10-fold increase in the rate of colony formation from spores; however, lysis of these minicolonies was not prevented. Deletion of the putative AgRho1p-GAP, encoded by *AgBEM2*, generated viable mutants with severe defects in the determination of cell polarity during germination and hyphal growth (Wendland and Philippsen 2000). Hyphae of *Agrho1* mutants showed polarized localization of the actin cytoskeleton during their growth phase prior to lysis. Slow growth and the bulgy cell shape, however, indicated defects in the maintenance of polar hyphal growth. Therefore, deletion of *AgRHO1* or *AgBEM2*, which functionally should increase Rho1p signaling, might have provided some of the most extreme effects of the *RHO1*-module on polar hyphal growth in *A. gossypii*. In the zygomycete *P. blakesleeanus* antibodies against human RhoA indicated expression of a Rho1p-homolog during spore germination. This indicates a function of the *RHO1*-module during the isotropic growth phase of spore germination and is in line with our observation that cell lysis was observed already in *Agrho1* germ cells. This also suggests that signaling via RHO-modules is not following a linear hierarchy since the *CDC42*-module seems not to be required during the isotropic growth phase of spore germination.

Taken together, the distinct events of establishing cellular polarity (*CDC42*-module), the determination and maintenance of cell polarity and polarized hyphal growth (*RHO1*-module), and the regulation of growth at the hyphal tip (*RHO3*-module) appear to be controlled each by a set of rho-GTPase modules. This allows us, for the first time, to compare hyphal growth with cellular yeast-like growth on a mechanistic basis. Additionally, some of the *A. nidulans* morphogenetic mutants in the *swo*, *pod*, and *hyp* loci might turn out to be components of RHO-modules or accessory regulatory elements. A molecular and biochemical comparison of the morphogenetic networks of yeast-like and filamentous fungi will need to address the differences in both growth modes that might reside, *e.g.*, in distinct localization patterns of homologous proteins or in the molecular mechanisms involved in keeping the hyphal tip intact.

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