Apical localization of actin patches and vacuolar dynamics in Ashbya gossypii depend on the WASP homolog Wal1p

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Summary
Analysis of the Ashbya gossypii Wiskott-Aldrich syndrome-like gene AgWAL1 indicates that it is required for the maintenance of polarized hyphal growth. Growth and organelle dynamics of the wild type and of wal1 and other mutant strains were monitored by in vivo (fluorescence) time-lapse microscopy. Loss of WAL1 led to slow growth and defects in polarized growth that produced swellings in subapical regions, whereas formation of hyphal tips and dichotomous tip branching occurred as in the wild-type. Few actin cables in Agwal1 cells were found to insert into the hyphal tip, but specific clustering of cortical actin patches was observed in subapical regions of hyphal tips instead of at the hyphal apex. Distribution and movement of vacuoles was observed in vivo using FM4-64. In the wild type and in the slowly growing mutant strains bem2 and cla4, which lack a Rho-GTPase-activating protein and a PAK kinase, respectively, early endosomes appeared in the hyphal tip, whereas very few early endosomes and small vacuoles were found in the wal1 mutant hyphal tips, thus linking the cortical patch defect of wal1 hyphae with the distribution of endosomes. Vivid movement of vacuoles seen in the wild type and in the bem2 mutant in subapical regions was largely reduced in the wal1 and cla4 mutants. The tubular structure of mitochondria (as visualized by DIOC6 in vivo) was similar in the wild type and the wal1 mutant, although wall mitochondria appeared to be larger. Interestingly, mitochondria were found to insert into the hyphal tips in both strains. Our results indicate a function for Wal1p in filamentous fungi in coordinating actin patch distribution with polarized hyphal tip growth.

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Introduction
The dynamic reorganization of the actin cytoskeleton is essential for cell polarization, morphogenesis, the distribution of organelles and cytokinesis (Drubin and Nelson, 1996). Elaborate mechanisms ensure temporal and spatial control of these events and require a network of proteins that includes Rho-GTPase modules as central regulators of the actin cytoskeleton (Schmidt and Hall, 1998; Wendland, 2001; Etienne-Manneville and Hall, 2002). In the yeast Saccharomyces cerevisiae and the filamentous fungus Ashbya gossypii, homologs of the Rho-GTPase Cdc42p and its guanine-nucleotide exchange factor Cdc24 have been shown to be required for the establishment of a polarized cortical actin cytoskeleton that is a prerequisite for bud emergence in yeast and the formation of new hyphal tips in A. gossypii (Johnson, 1999; Wendland and Philippson, 2001). Rho-GTPases relay spatial and temporal information to effector proteins and are thus able to integrate multiple signalling inputs with the state of actin cytoskeletal organization (Schmidt and Hall, 1998). Among these effectors are multidomain adaptor proteins, such as members of the Formin, PAK (p21-activated kinase) and WASP (Wiskott-Aldrich syndrome protein) families, that can act as scaffolds (Frazier and Field, 1997; Ramesh et al., 1999; Mullins, 2000; Pollard et al., 2001; Sharpless and Harris, 2002; Wild et al., 2004). The S. cerevisiae formin Bni1p, which is activated by Cdc42p, has recently been shown to play a central role in the nucleation of actin cables, which is achieved in an Arp2/3 independent manner (Evangelista et al., 2002; Pryne et al., 2002; Sagot et al., 2002; Evangelista et al., 2003).

WASP family members are conserved from fungi to humans and are built in a modular fashion. They bind to components of the Arp2/3 complex and actin monomers via C-terminal WH2/C/A (WASP-homology-2/central/acidic) domains and activate actin nucleation (Machesky and Insall, 1998; Machesky et al., 1999; Madania et al., 1999; Rohatgi et al., 1999; Winter et al., 1999; Prehoda et al., 2000). Mammalian WASPs carry a GTPase-binding domain that allows direct interaction with Cdc42p. Such a domain is missing from members of the SCAR (suppressor of cAMP receptor) subgroup as well as from all known fungal WASP family members. In the N-terminal part, WASP family members share a WH1/pleckstrin-homology domain (lacking in SCAR proteins), which is involved in lipid binding, and a basic region (missing in Wsp1p of Schizosaccharomyces pombe) (Lee et al.,...
2000). Similar to WASP, which can link lipid (i.e. phosphatidylinositol-4,5-bisphosphate) signalling with the actin cytoskeleton, the PAK Cla4p in yeast can link lipid (in this case, via phosphatidylinositol-4-phosphate) and Cdc42p signalling (Rohatgi et al., 1999; Wild et al., 2004). The PAK-like kinase Cla4p in A. gossypii was found to be required for hyphal development and septation, and loss of CLA4 led to a partial disorganization of apical actin patch clusters (Ayad-Durieux et al., 2000).

Secretion in hyphal-tip cells of filamentous fungi is highly polarized and growth of the hyphae is restricted to the tip. Cortical actin patches are clustered at the hyphal tips during the hyphal growth phase (Wendland, 2001). In the ectomycorrhizal basidiomycete Suillus bovinus and the dimorphic fungus Candida albicans during hyphal growth stages Cdc42p was found to be localized to hyphal tips, indicating that Cdc42p is not only required for hyphal tip formation but might also play a role during polarized hyphal growth (Gorfer et al., 2001; Hazan and Liu, 2002).

Mutations of S. cerevisiae genes belonging to the Arp2/3 complex or deletion of the S. cerevisiae WASP LAS17/BEE1 led to a loss of cortical actin patch organization (Winter et al., 1997; Li, 1997). The yeast Las17p/Bee1 protein acts as an activator of the Arp2/3 complex and is involved in endosome/ vacuole motility (Winter et al., 1999; Yarar et al., 1999; Chang et al., 2003). N-WASP was also shown to promote the actin-dependent movement of endosomes and lysosomes in Xenopus eggs (Taunton et al., 2000). Although substantial progress has been made in the analysis of individual WASP-family-member domains, the cellular function of WASP homologs for polarized hyphal growth in filamentous fungi has so far not been elucidated. Recently, we could show that the WASP homolog of the dimorphic fungal pathogen C. albicans was required for hyphal growth. Yeast cells of C. albicans induced to form hyphae by serum at 37°C responded with an initial loss of cortical actin patch organization (Winter et al., 2000). G418/geneticin was added to rich media for selection of antibiotic-resistant transformants at a final concentration of 200 μg ml⁻¹. Yeast cells were transformed according to the lithium-acetate procedure (Gietz et al., 1995). Yeast media were prepared as described previously (Wendland and Philippsen, 2001). A. gossypii strains were transformed by electroperoration as described (Wendland et al., 2000). G418/geneticin was added to rich media for selection of antibiotic-resistant transformants at a final concentration of 200 μg ml⁻¹. Yeast cells were transformed according to the lithium-acetate procedure (Gietz et al., 1995). Yeast media were prepared as described (Wendland and Philippsen, 2001). The Escherichia coli strain DH5α served as host for plasmids.

Isolation of AgWAL1

AgWAL1 was isolated in a multicity suppressor screen using the temperature sensitivity of the las17/bee1 mutant yeast strains. Different plasmid libraries based on yeast episomal plasmids were used containing either A. gossypii or S. cerevisiae genomic DNA inserts. Transformated cells were plated on minimal medium plates and incubated for four days at 37°C, which is the restrictive temperature for bee1/las17 cells. Several yeast transformants could be isolated. Total DNA was extracted from these colonies and transformed into E. coli. Plasmids were recovered and retransformed into the yeast strain RLY157 to verify their ability to enable growth of RLY157 at elevated temperature. This resulted in the isolation of five independent plasmids from a CEN-ARS based S. cerevisiae plasmid library and one plasmid from a YEp352-based A. gossypii plasmid library (kindly provided by P. Stahmann, Fachhochschule Lausitz, Senftenberg, Germany), termed pST-WAL1. The complete double strand sequence of the A. gossypii DNA insert as well as the terminal sequences of the yeast DNA inserts were determined (MWG Biotech, Ebersberg, Germany). The GenBank accession number for A. gossypii WAL1 gene is AJ144115.

Construction of plasmids

A plasmid clone, pWAL1cc, containing the complete AgWAL1 gene was constructed by ligating a 3.5 kb SacI fragment of pST-WAL1 into the SacI site of pRS415. Plasmid pWAL1-XL was constructed making use of the in vivo recombination machinery of S. cerevisiae (Wendland, 2003). To this end, a polymerase chain reaction (PCR) fragment containing the complete AgWAL1 open reading frame (ORF) was amplified from pST-WAL1 using the primers XL1-WAL1, 5’-TCTGCTAGGCTAGCTGACATCCACATCCGAACATAAACAACCATGGTGCTTTTCAACACGGGAAGAGAATGCTGTGCTC-3’, and XL2-WAL1, 5’-ATGACAAAGTTCTTGAAAACAAGAAATCCTTTCATTTTATT-3’.
GTCAATCTGTATCAACATCTACAGAATTGTCG-3’. This PCR fragment contains terminal homology regions to the AgTEL promoter and terminator to direct homologous recombination. Plasmid pRS415-kanMX is a yeast CEN-ARS plasmid that carries the kanMX selectable marker, which consists of the kanamycin resistance ORF (kan) under control of the AgTEL1 promoter and terminator sequences. pRS415-kanMX was linearized using NreI, which cleaves a unique site within the kan ORF. This linearized plasmid cannot be propagated in yeast because S. cerevisiae cannot reseal blunt-end DNA breaks. Linearized plasmid and the AgWAL1 PCR product were co-transformed into yeast. By means of homologous recombination between the linearized plasmid and the PCR product, a new plasmid was generated, pAgWAL1-XL, in which the AgWAL1 ORF is fused to the AgTEL1 promoter and terminator. Correct integration was verified by sequencing.

Gene disruption and complementation

Insertion and deletion of AgWAL1 were performed via PCR-based GEN3 disruption cassettes as described previously (Wendland et al., 2000). The following primers were used for the generation of the disruption cassettes: S1-WAL1 (5’-TAAACAGGGAATCTCCCCA-AAGCATGACAAATTATAGATGGCTGATTAGGATAGACAG-GTAATGAC-3’) and S2-WAL1 (5’-CTCTTGGACAGAGGACCAG-ATGGCTTGTCCAATTGAGACTTGTCAACCTAGGCATGCAAG-CTTATGATC-3’). Disruption of AgWAL1 was verified by PCR using primers G1-WAL1 (5’-CGCGGATCCGCTGACATATTACCT-GACGCAC-3’), G2 (5’-GTTAGTCTGGACACATC-3’), G3 (5’-TCTCGCAGACCTGATATACGCATATTACCT-GTTTAGTCTGACCATC-3’) and G4-WAL1 (5’-CGCGGATCCGCTGACCATATTACCT-GTTTAGTCTGACCATC-3’). Primary transformants of A. gossypii were heterokaryotic (i.e. mycelia with multicellular 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. The Agwall1 mutant phenotype was complemented by plasmids carrying AgWAL1.

Cytological techniques

Septa were stained using calcofluor (0.1 μg/ml), with cells either grown in liquid rich medium or on slides covered with thin layers of rich medium, as described previously (Wendland and Philippson, 2000). Actin was stained from exponentially growing cultures. Cells were fixed in formaldehyde (3.7%) and PBS, and stained with rhodamine-phalloidin (Molecular Probes, Leiden, The Netherlands) as described elsewhere (Oberholzer et al., 2002). Endocytosis was visualized using the vital dye FM4-64 (Molecular Probes). Strains were grown in liquid rich medium or on slides covered with thin layers of liquid medium, harvested and fixed in formaldehyde (3.7%) and PBS. Mitochondria were stained using DIOC6 (Molecular Probes). Cells were mounted on deep-well microscopy slides and then either used directly for microscopy to allow the analysis of early endocytosis or incubated with the dye for up to 16 hours before microscopic observation. Fluorescence microscopy was done on a fully motorized Zeiss AxioplanII imaging microscope with the appropriate filter combinations for each dye (Chroma Technology, Rockingham, VT). Images were taken using a digital video imaging system (Princeton Instruments (Roper Scientific), Trenton, NJ, USA) as described previously (Wendland and Philippson, 2002).

Time-lapse microscopy

Strains were grown in either complete or minimal medium. Microscopy slides were prepared as follows. Deep-well slides were used and filled with 100 μl of YPD agar medium each, containing FM4-64 when required. This medium was heavily vortexed to increase the oxygen content. The deep well was then covered with an extra slide to generate an even surface; once the agar had solidified, the cover was removed, cells were applied and the well sealed with a coverslip. Microscopy was carried out at 26°C. Images were acquired in a fully automated fashion (2.5 second or 60 second intervals for FM4-64 or DIOC6 time-lapse, and 180 seconds for differential interference contrast (DIC) or Nomarski time-lapse series), collected into stacks and processed as video clips with a frame rate of 10 images per second using Metamorph Software (Universal Imaging Corporation, Downingtown, PA, USA).

Results

Isolation and protein comparison of fungal WASP homologues

A multicopy suppressor screen was performed on bee1/ls17 mutant yeast strains based on their temperature sensitivity and growth arrest at 37°C with the aim of isolating either extragenic suppressors or the WASP homologue of a filamentous fungus. With an S. cerevisiae genomic library, five plasmids were isolated containing different but overlapping inserts bearing in common the S. cerevisiae LAS17/BEE1 gene. No other isolate was obtained. Using an A. gossypii genomic 2μ-ARS plasmid library, a single plasmid was obtained several times. Sequencing of the insert revealed the presence of an A. gossypii WASP homologue, AgWAL1 (Wiskott-Aldrich syndrome like protein) and adjacent genes. Comparison of the A. gossypii WAL1 locus with S. cerevisiae showed syntenic arrangements to two S. cerevisiae loci on chromosomes XII and XV, which is an indication of ancient synteny potentially revealing the gene order in a common ancestor that was altered in yeast after its apparent genome duplication but retained in A. gossypii. Interestingly, homologues of two S. cerevisiae genes located on different chromosomes (XII and XV) that are positioned next to each other in A. gossypii, WAL1 and the A. gossypii homologue of YLR287c, are also neighbours in Candida albicans (see Fig. S1 in supplementary material).

The AgWAL1 locus represents the only WASP homologue of A. gossypii that could be determined by examination of the complete A. gossypii genome sequence. AgWAL1 (AGR285w) encodes a protein of 732 amino acids and is located on chromosome VII (Dietrich et al., 2004). The highest degree of amino acids sequence identity is found with the S. cerevisiae W ASP homologue, AgWAL1 was verified by PCR using primers G1-WAL1 (5’-ATGGCTTGTCCAATTGAGACTTGTCAACCTAGGCATGCAAG-CTTATGATC-3’) and G4-WAL1 (5’-ATGGCTTGTCCAATTGAGACTTGTCAACCTAGGCATGCAAG-CTTATGATC-3’). Disruption of AgWAL1 was achieved by PCR-based gene targeting. Deletion resulted in the removal of 87% of the
AgWAL1 ORF, thus generating a null allele (see Fig. S3 in supplementary material). Correct construction of the deletion was verified by PCR using standard verification primers. Primary transformants were wild-type-like in morphology owing to the heterokaryotic nature of the multinucleate hyphal compartments. Upon sporulation and clonal selection of mycelia containing only transformed Agwal1 mutant nuclei, a slow-growth phenotype became evident. Furthermore, growth of Agwal1 mutants at elevated temperature (above 35°C) was completely abolished (Fig. 1A). This temperature-sensitive growth inhibition could be rescued by reintroduction of plasmid-borne AgWAL1 (Fig. 1B). Complementation with plasmid pWAL1cc, which contains only the WAL1 gene, demonstrated that the mutant phenotype was due solely to the disruption of the genomic copy of WAL1. Heterologous complementation of the S. cerevisiae bee1/las17 defect with WAL1 was dependent on the high copy number of plasmid pST-WAL1 (containing a 2μ-ARS), because pWAL1cc (containing a yeast centromere and ARSH4) did not restore growth of the bee1/las17 strain at elevated temperature.

Fig. 1. Growth assay of wild-type and mutant strains. (A) Wild-type and wall mutant were grown on YPD plates at the indicated temperatures for 5 days before photography. Optimal growth conditions are at 30°C. Loss of WAL1 results in slow growth and temperature sensitivity (no growth) at elevated temperatures. (B) This temperature-sensitive phenotype can be complemented by introduction of a plasmid bearing the complete WAL1 gene (pWAL1cc) at 37°C. Complementation with plasmid-borne WAL1 at lower temperatures (30°C) results in increased growth of the mutant. However, owing to lack of selective pressure to maintain the plasmid growth rate is slower than in the wild type.

Agwal1 mutant reveals defects in the organization of the actin cytoskeleton.

During the polarized hyphal growth phase in A. gossypii wild-type hyphae cortical actin patches are concentrated in the hyphal tips (Fig. 2A,B). Defects in the organization of the actin cytoskeleton can be observed as isotropic growth phases and swollen hyphal morphologies and result in slow growth phenotypes as was shown for Agrho3 and Agbem2 mutants (Wendland and Philippsen, 2000; Wendland and Philippsen, 2001). Therefore, we analysed the distribution of the cortical actin cytoskeleton in the Agwal1 mutant (Fig. 2C,D). Localization of some of the cortical actin patches along the hyphae occurred in both wild-type and wall hyphae. Clustering of cortical actin patches in the hyphal tips, as found in the wild type, did not occur in wall hyphal tips (n=500 for each strain). Instead, cortical actin patches characteristically accumulated in subapical regions of wall hyphal tips, which was never observed in the wild type (Fig. 2). In the wild type, actin cables formed an abundant meshwork of cables that was tip directed in the hyphal apex. In the wall mutant, some actin cables were found to insert into the hyphal tip even when the tip did not contain any obvious cortical actin patches (Fig. 2C,D). Rhodamine-phalloidin staining also revealed the absence of actin rings at presumptive septal sites that can readily be found in wild-type hyphae. The presence and correct positioning of actin rings are, however, prerequisites for septation and chitin-ring formation in A. gossypii (Wendland and Philippsen, 2002). To explore this in more detail, we stained wild-type and mutant hyphae with calcofluor to analyse the distribution of chitin in the cell wall.

Agwal1 mutants show defects in septation

Wild-type hyphae are tube-like and form septa in regular intervals. Chitin is accumulated at these septal
Maintenance of hyphal growth sites and forms chitin rings. Furthermore, hyphal tips can be stained more brightly by calcofluor than subapical regions, indicating sites of active secretion (Fig. 3A). By contrast, hyphae of the wal1 mutant are wider than wild-type hyphae, irregular in shape and periodically swollen. Mutant hyphal cell walls were stained uniformly by calcofluor, corresponding to delocalized cell surface growth and the bulbous hyphal shape. As was suggested by the absence of actin rings at presumptive septal sites, chitin rings were only rarely observed in the mutant hyphae (n=200), indicating a severe defect in septation (Fig. 3B). This corresponds to observations in the Agcyk1 and Agcla4 mutants, in which the failure to form actin rings at presumptive septal sites has been shown to be accompanied by lack of chitin accumulation (Ayad-Durieux et al., 2000; Wendland and Philippsen, 2002). Formation of sporangia depends on septation. Thus, lack of septa (as in the Agcyk1 mutant) also resulted in the absence of sporulation in the Agwall1 mutant (not shown).

Agwall1 mutants exhibit defects in the maintenance of polarized hyphal growth

To understand the growth defects of the Agwall1 mutant more clearly, we compared growth of the mutant strain with the wild type using in vivo time-lapse microscopy. First, we characterized growth of the wild type using in vivo time-lapse microscopy. First, we characterized growth of the wild type. Juvenile mycelia, approximately 10 hours after inoculation of spores in complete medium, grew initially at a slow extension rate (Fig. 4A; see Movie 1 in supplementary material). Lateral branches were formed and strong vacuolarization of the hyphal was initiated about 6 hours later at the centre of the mycelium. At about this time, an increase in growth speed was observed and dichotomous tip branching occurred, which is a hallmark of colony maturation in A. gossypii (Wendland and Philippsen, 2000). Movie 1 also showed that some newly formed hyphal tips (n=7 in this movie) stopped hyphal elongation and became vacuolarized, and that cytoplasmic flow was redirected out of these hyphae to other growing ends (Fig. 4A, arrowheads). Additionally, growth was found to be terminated in some instances upon contact of a hyphal tip with other hyphae that blocked further elongation (n=4 in this movie), which also resulted in vacuolarization and redirection of cytoplasmic streaming (Fig. 4A, asterisks). Spore germination of the Agwall1 mutant, in contrast to wild-type hyphae, was found not to result in the characteristic bipolar branching pattern (n=100), which, in the wild type, leads to the generation of two germ tubes at opposite ends of the germ cell (Fig. 4).

Most often a second hyphal tube was produced directly adjacent to the first hypha. Growth of the mutant hyphae was very slow (Fig. 4B; see Movie 2 in supplementary material). Under time-lapse growth conditions mutant hyphae often stopped growing and hyphal cells lysed, which made it difficult to obtain time-lapse movies at this early stage of growth from the wal1 mutant. Germination of heterokaryotic spores under selective conditions that favoured growth of only the wal1 mutant in liquid culture revealed similar defects in the bipolar germination pattern (n=200).

In adult stages, hyphal growth rate of the wild type was accelerated (at least tenfold) upon hyphal maturation and reached on average 170 μm hour⁻¹ (Fig. 5; see Movie 3 in supplementary material). The appearance of many vacuoles in adult wild-type hyphae occurred in subapical regions soon after hyphal tip growth. Using in vivo time-lapse microscopy, we were able to observe vacular movement (cytoplasmic streaming) in wild-type hyphae in the direction of the growing hyphal tip (Fig. 5; see Movies 1,3 in supplementary material). The speed of cytoplasmic streaming was dependent on the rate of tip growth. Interestingly, the direction of cytoplasmic streaming could also be influenced by septation. Once a septum had formed vacuoles could not pass across these sites (Fig. 5, asterisks; see Movie 3 in supplementary material). In the hypha shown, three consecutive septation events occurred. The first septum prevented backward flow of vacuoles. The second septum occurred at a dichotomous bifurcation and led to the direction of the vacuolar streaming into just the left branch. Finally, the third septum compartmentalized a hyphal segment.
and trapped the cytoplasm within. This also inhibited loss of cytoplasm out of this compartment. The septation event was soon followed by the formation of a new hyphal tip in this compartment (Fig. 5; see Movie 3 in supplementary material). Furthermore, retrograde flow was observed out of hyphae that stopped growing owing, for example, to contact inhibition (arrows indicate the direction of streaming in Fig. 5, whereas the arrowhead points to the relative positions of one of the large vacuoles).

In contrast to the wild type, mutant *Agwal1* hyphae reached radial growth rates of a maximum of only 15 μm hour−1 and were thus more than ten times slower than adult wild-type hyphae (Fig. 6; see Movie 4 in supplementary material). Nevertheless, growth direction was still highly polarized and dichotomous tip branching occurred in *Agwal1* hyphae. *Agwal1* mutant hyphae were filled with cytoplasm and only few vacuoles were visible that occurred very late during these in vivo observations (Fig. 7, bottom row). Cytoplasmic streaming can be seen in Movie 5 (see supplementary material) but streaming is not directed and vacuoles remain localized in the same hyphal segments in which they were formed, in contrast to the wild type. Interestingly, at several positions in subapical regions, swellings of hyphae occurred very rapidly (within 3 minutes; i.e. a single interval of image acquisition during the time-lapse analyses) (Fig. 6, boxes, left to right) and shown (top row to bottom row) as enlarged images in Fig. 7 (and see Movie 5 in supplementary material). The positions at which these swellings occurred might represent attempts of the hyphae to produce lateral branches.
Wild-type hyphae showed rapid cytoplasmic streaming and vacuolar movement, in contrast to wal1 hyphae, during our DIC observations. Because defects in polarized growth might be the result of defects in exo- or endocytosis, we examined and compared the vacuolar dynamics of different strains in vivo using the vital dye FM 4-64. In wild-type hyphal tips, fast-moving small vesicles (endosomes) were found. There were often tubular structures linking these vesicles with each other and resulting in fusion of vesicles (Fig. 8A; see Movie 6 in supplementary material). Staining of the wild-type endosomes was very efficient, resulting in bright fluorescent images. Staining of wal1 hyphae was always more diffuse (Fig. 8B; see Movie 6 in supplementary material). Strikingly, whereas wild-type hyphae contained endosomal vesicles in the hyphal tips and, in subapical regions, had already fused these endosomes to smaller vacuoles, the wal1 hyphal tips contained very few visible endosomal vesicles and thus appeared relatively empty (compare Fig. 8A,B; see Movie 6 in supplementary material). Other A. gossypii slow-growth mutants deficient in genes that are also involved in actin cytoskeleton dynamics had already been analysed (Ayad-Durieux et al., 2000; Wendland and Philippsen, 2000). We stained hyphae of these cla4 and bem2 mutants with FM4-64 to analyse whether early endosomes were present in the hyphal tips of these mutants. Both of these mutant strains contained endosomal vesicles in their hyphal tips that showed movement and tubular structures similar to the wild type (Fig. 8C,D; see Movies 8,9 in supplementary material). To compare the morphology of larger vacuoles of the different strains, we performed high resolution in vivo time-lapse analyses of older (non-growing) hyphal segments under the identical conditions, running for approximately 4:30 minutes with recordings every 2.5 seconds (Fig. 8E-H; see Movies 7-9 in supplementary material). In the wild type, large vacuoles showed rapid changes in shape, became elongated
and (in two instances) fusion events were observed. Interestingly, not all vacuoles seen in the wild-type panel of Movie 7 (see supplementary material) are in motion at the same time. In the middle of the movie, two vacuoles appeared to be moving at the same time, coming to a stop and thus appeared round in shape, and afterwards simultaneously initiated motion again. By contrast, the vacuoles on both sides (tipward and farther back) exhibit phases of motion and pausing independently of the vacuoles in the centre. In Movie 7 (see supplementary material), a \textit{wal1} hypha is shown imaged under the same conditions as the wild type. Here, large vacuoles of the \textit{wal1} hypha showed drastically reduced motion compared with the wild type. (Fig. 8E,F; see Movie 7 in supplementary material). Whereas large vacuoles in the \textit{bem2} mutant readily changed shape, similar to the wild type, vacuoles of the \textit{cla4} mutant were rounder and thus resembled more closely the vacuoles of \textit{wal1} hyphae (Fig. 8G,H; see Movies 8,9 in supplementary material). Because these high-resolution recordings did not readily allow the analysis of vacuolar movement, we analysed wild-type and \textit{wal1} hyphae in a time-lapse series over 3.0-4.5 hours with images taken at 1-minute intervals (see Movies 10,11 in supplementary material). In these movies, the generation and tipwards movement of large vacuoles was evident in the wild type. Hyphae of the \textit{wal1} mutant grew much more slowly, did not contain endosomes in the hyphal tips, did not generate large vacuoles and displayed only oscillating, not tipwards, movement of vacuoles.

Because, in \textit{S. cerevisiae}, several organelles are dependent on the actin cytoskeleton for movement, we also wanted to elucidate whether, like vacuolar movement, the motility of mitochondria was affected in the \textit{wal1} mutant hyphae (Fig. 8I,J; see Movie 12 in supplementary material). To this end, we stained wild-type and \textit{wal1} hyphae with the vital dye DIOC6 and recorded in vivo fluorescence time-lapse series. In the wild type, mitochondria showed tubular structures, localized to the hyphal tip and appeared to be very motile. Mitochondria in \textit{wal1} hyphae appeared to be bigger than wild-type mitochondria. However, mitochondria in \textit{wal1} hyphae showed a tubular structure and wild-type-like motility. Strikingly, in \textit{wal1} hyphae mitochondria were localized along the hyphae.

**Fig. 6.** In vivo time-lapse analysis of growth of adult stages of \textit{wal1} hyphae. Mycelium from exponentially grown liquid YPD cultures was transferred to slides and growth was monitored. Images represent frames from Movie 4 (see supplementary material) at the corresponding time points (hours:minutes). Boxes indicate three regions processed for Fig. 7 and Movie 5 (see supplementary material). Scale bar, 40 \( \mu m \).
also including the hyphal tips. This finding is in sharp contrast to the distribution and movement of vacuoles in \textit{wal1} hyphae.

**Discussion**

Members of the WASP family regulate a range of cellular processes that require dynamic reorganization of the actin cytoskeleton (Caron, 2002). The role of WASPs has been analysed in great detail in various organisms and a functional understanding has been gained towards the role of WASP in activating the Arp2/3 complex, resulting in the generation of branched actin filaments (Higgs and Pollard, 2001; Weaver et al., 2003). WASP is regulated by phosphorylation and autoinhibition by intramolecular contacts that can be relieved through binding to Cdc42p (Cory et al., 2003; Buck et al., 2004). Fungal WASPs are different from mammalian WASPs in this respect, because they lack a G-protein-binding domain. Consequently, the \textit{S. cerevisiae} WASP Las17p/Bee1p was shown not to be autoinhibited but regulated by SH3-domain-containing proteins (Rodal et al., 2003).

Polarization of the actin cytoskeleton towards the hyphal tips is required for hyphal growth in filamentous fungi (Wendland, 2001). This results in the clustering of cortical actin patches at the hyphal tip and maintenance of polarized hyphal growth and tip-directed secretion in fungal hyphae (Momany, 2002). Previously, we have characterized the role of Rho-protein modules for polarized hyphal growth and the organization of the actin cytoskeleton in \textit{A. gossypii} (Wendland and Philippson, 2001). Because Rho-protein function is exerted via effector proteins, we are interested in elucidating the signalling route from Rho proteins to the actin cytoskeleton. In this effort, we recently analysed the \textit{C. albicans} WASP homologue \textit{WALL} (Walther and Wendland, 2004). We observed similarities between the yeast stages of \textit{C. albicans} and \textit{S. cerevisiae} of WASP mutant strains that include a partially delocalized cortical actin cytoskeleton, defects in bud-site selection and endocytosis (Li, 1997; Walther and Wendland, 2004). Particularly, in the \textit{C. albicans} WASP mutant, an increased number of vacuoles was found, suggesting a role in vacuole formation. \textit{C. albicans} is a dimorphic fungus that can be induced to form true hyphae by, for example, the presence of serum at 37°C. The \textit{C. albicans} WASP mutant, however, was not able to form hyphae under all laboratory conditions tested and thus we were not able to analyse hypha-specific defects in this organism. Therefore, we generated a WASP deletion mutant in the constitutively filamentous fungus \textit{A. gossypii}.

**Growth defects of the \textit{A. gossypii} \textit{wal1} mutant**

The \textit{Agwal1} mutant shows temperature-sensitive growth, which is completely abolished at elevated temperatures (e.g. 37°C). Polarized hyphal growth was drastically reduced in \textit{wal1} hyphae, resembling growth rates of \textit{bem2} and \textit{cla4}. Cla4p was shown to be required for a process termed hyphal maturation (Ayad-Durieux et al., 2000). This describes the phenomenon that growth speed during mycelium formation is slow in juvenile mycelia and increases dramatically – in \textit{A. gossypii} more then tenfold – in mature mycelia. The speed of polarized hyphal growth is dependent on the transport of secretory vesicles to the hyphal tip and the insertion of these vesicles into the membrane (Katz et al., 1972; Watters and Griffith, 2001). We found that fast polarized hyphal growth occurs at a time when large vacuoles are formed in subapical regions of hyphal filaments. With these vacuoles, transport processes in the direction of growing tips could be observed (see Movies 1.3 in supplementary material). Septation was found to block cytoplasmic streaming (corresponding to bulk tipwards organelle movement) resulting in a redirection of...
transport (see Movie 3 in supplementary material). This was surprising given that nuclear migration through septal pores was frequently observed in \textit{A. gossypii} (Alberti-Segui et al., 2000). Another surprising observation was retrograde flow of cytoplasm out of hyphae that stopped growing (e.g. owing to contact inhibition). This indicates that, in \textit{A. gossypii}, polarized hyphal growth can serve as a sink for cellular components of subapical regions not compartmentalized by septation. In the \textit{Agwal1} mutant, long-range transport of vacuoles was not evident in the time-lapse analyses. Large vacuoles were generated in \textit{wal1} hyphae, although this process appeared to be delayed in comparison to the wild type. The process of vacuolar fusion might be of importance for polarized hyphal growth. In order to maintain rapid polarized growth, large amounts of cytoplasm need to be produced. This requirement could be lowered if rear parts of hyphal compartments were filled with large vacuoles. Therefore, defects in vacuolar fusion per se might affect polarized growth rates. \textit{VAM4/YPT7} encodes a \textit{S. cerevisiae} gene that is required for vacuole fusion (Wichmann et al., 1992). Deletion of the \textit{VAM4/YPT7} homologue \textit{avaA} in \textit{A. nidulans}, however, was shown not to reduce the hyphal growth rate, although this mutant exhibited highly fragmented vacuoles (Ohsumi et al., 2002).

\textbf{Septation defect of \textit{Agwal1}}

Deletion of \textit{AgWAL1} resulted in a septation defect similar to that observed in \textit{Agcla4} mutants: hyphae showed very few actin rings, and chitin accumulation at presumptive sites of septation was very rare. This is in contrast to the \textit{S. cerevisiae} \textit{bee1/las17} and the \textit{C. albicans wall1} mutant strains, which did not show defects in cytokinesis and septum formation but rather showed increased rates of random budding (Madania et al., 1999; Walther and Wendland, 2004). The failure to form actin rings might be due to either the direct involvement of Wal1p in the polymerization of actin or the requirement for Wal1p in transport processes that are necessary to achieve polarized secretion to septal sites. Although many of the key players involved in septum formation in \textit{S. cerevisiae} share homologues in filamentous fungi, our results indicate that there are mechanistic differences leading to septum formation between yeasts and filamentous fungi (Walther and Wendland, 2003). The \textit{wall1} mutant strain was also found to be defective in sporulation. This corresponds to the sporulation defect of the \textit{aseptate cyk1} strain (Wendland and Philippersen, 2002). In the wild-type strain, sporangia are formed by fragmentation of hyphae at septal sites. The failure to generate septa therefore results in the inability to produce sporangia and thus spores.

\textit{Wal1p plays a central role in the localization of cortical actin patches}

Loss of \textit{WAL1} resulted in the clustering of cortical actin patches in subapical parts of the hyphae, in contrast to the tipwards localization in the wild type. This represents a new actin cytoskeleton defect not previously observed in filamentous fungi. Delocalization of actin patches indicates that their presence in the hyphal tips is supporting polarized secretion and fast hyphal elongation that, in the \textit{wal1} mutant, is now converted into more isotropic growth, resulting in widened diameters and swollen hyphae. Surprisingly, we found that a few actin cables were able to extend into hyphal tips even without the presence of actin patches (Fig. 2). This could be of mechanistic importance for the establishment and maintenance of polarized hyphal growth in filamentous fungi. In \textit{S. cerevisiae}, Las17p/Bee1p is required for the activation of the
Arp2/3 complex and actin nucleation (Winter et al., 1999; Higgs and Pollard, 2001; Zalevsky et al., 2001). This activity might, however, be limited to the nucleation of branched filaments (Higgs and Pollard, 2001). By contrast, the formin family of proteins was shown to represent a class of actin nucleators that assemble straight filaments (Pruyne et al., 2002; Sagot et al., 2002). The wallI mutant phenotype displayed specific defects in the polarized localization of actin patches, whereas actin filaments were found to be tip localized in the absence of cortical patches in the tip. The reduced hyphal growth rate of wallI hyphae could therefore be the result of the basic activity provided by formin dependent assembly of actin filaments. This might occur in a Cdc42p-dependent manner, because CDC42 was shown to be required for the establishment of cell polarity in A. gossypii (Wendland and Philippsen, 2001). In such a model, the polarity establishment machinery, involving the Cdc42p/Rho-protein module and formin effectors, is required for choosing a site of growth and polarizing the actin cable network to result in an initial growth phase. Subsequently, activation of WASP leads to the coordination of polarized secretion, the polarization of cortical actin patches, and long-range transport to the hyphal tip, which is a prerequisite of fast hyphal elongation. In wallI hyphae, slow growth might therefore be due to shortcomings in either the sufficient delivery of secretory vesicles to the tip or in defective exo- or endocytosis.

Nevertheless, in the AgwallI mutant, cortical actin patches were still generated and localization was clearly enriched in subapical parts of the hyphae. Other proteins that can activate actin assembly might be responsible for the generation of actin patches, such as A. gossypii homologues of the S. cerevisiae ABPI and PAN1 genes. Currently, we do not know the mechanism for the accumulation of cortical actin patches at these specific subapical sites. However, localization of cortical actin patches to the hyphal tips requires Wal1p. Considering the protein domains of fungal WASPs, the N-terminal WH1/pleckstrin-homology domain can provide a link to plasma-membrane-associated lipids, specifically phosphatidylinositol-4,5-bisphosphate (Rohatgi et al., 1999). AgWal1p might act as a scaffold protein to coordinate the positioning of cortical actin patches with polarized morphogenesis by linking Rho-GTPase signalling with the ability to stimulate the Arp2/3 complex in combination with the use of lipids as positional information. Synergistic activation by lipid and Cdc42p signalling was shown for N-WASP and also the S. cerevisiae Cla4p, which thereby function as ‘coincidence detectors’ (Rohatgi et al., 1999; Wild et al., 2004).

Endocytosis and vacuolar fusion in A. gossypii

Loss of AgWALL might cripple the ability to generate actin filaments to provide tracks for vacuolar movement in the hyphae. A defect in vacuolar motility might therefore be a secondary consequence of the wallI mutation. In fact, the motility and directed movement of vacuoles in wallI hyphae was drastically reduced in comparison to the wild type. Whereas wild-type vacuoles displayed deformations and rapid shape changes, wallI vacuoles remained round and immobile, suggesting that relatively little motor-protein force influences these vacuoles. Our results further show that this phenotype of wallI hyphae is specific for vacuolar movement and distribution and does not affect the localization and movement of mitochondria.

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