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Cytolocalization of the Class V Chitin Synthase in the Yeast, Hyphal and Sclerotic Morphotypes of *Wangiella (Exophiala) dermatitidis*

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Abstract

Wangiella (Exophiala) dermatitidis is a polymorphic fungus that produces polarized yeast and hyphae, as well as a number of non-polarized sclerotic morphotypes. The phenotypic malleability of this agent of human phaeohyphomycosis allows detailed study of its biology, virulence and the regulatory mechanisms responsible for the transitions among the morphotypes. Our prior studies have demonstrated the existence of seven chitin synthase structural genes in *W. dermatitidis*, each of which encodes an isoenzyme of a different class. Among them, the class V chitin synthase (WdChs5p) is most unique in terms of protein structure, because it has an N-terminal myosin motor-like domain with a P-loop (MMD) fused to its C-terminal chitin synthase catalytic domain (CSCD). However, the exact role played by WdChs5p in the different morphotypes remains undefined beyond the knowledge that it is the only single chitin synthase required for sustained cell growth at 37°C and consequently virulence. This report describes the expression in *E. coli* of a 12 kDa polypeptide (WdMyo12p) of WdChs5p, which was used to raise in rabbits a polyclonal antibody that recognized exclusively its MMD region. Results from the use of the antibody in immunocytochemical studies supported our previous findings that WdChs5p is critically important at infection temperatures for maintaining the cell wall integrity of developing yeast buds, elongating tips of hyphae, and random sites of expansion in sclerotic forms. The results also suggested that WdChs5p localizes to the regions of cell wall growth in an actin dependent fashion.

Keywords

class V chitin synthase; black fungus; enzyme immunocytochemical localization; phaeohyphomycosis agent

1. Introduction

Wangiella (Exophiala) dermatitidis is a polymorphic, dematiaceous (melanized), fungal pathogen of humans, which is traditionally most associated with chronic dermatotropic forms of cutaneous and subcutaneous phaeohyphomycosis (Matsumoto et al., 1993; Brandt and Warnock 2003; de Hoog et al., 2005). Currently, however, it is being reported with increasing frequency as an agent of systemic disease in both immunocompetent and immunodeficient patients (Schnitzler et al., 1999; Graybill et al., 2004; Taj-Aldeen et al., 2006; Zheng et al.,

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2007). In infections caused by *W. dermatitidis*, a multiplicity of vegetative morphotypes has been observed, suggesting that in different host environments each may have a different survival potential. By manipulating nutritional and environmental conditions, each of the varied morphotypes of *W. dermatitidis* can be produced in vitro in a controlled fashion (Karuppayil and Szaniszlo, 1997; Wang and Szaniszlo, 2007). For example, in most rich media, a polarized budding yeast morphotype is most common, whereas hyphal and so-called sclerotic morphotypes are produced in less rich media or under conditions suboptimal for yeast growth. The extreme phenotypic variability of *W. dermatitidis* has been exploited for model studies that provide insights into the biology of the varied morphotypes expressed by the 100 or more other black fungi reported to cause human disease (Szaniszlo et al., 1993; de Hoog et al., 1994; Szaniszlo, 2002, 2006). Molecular genetic studies involving this fungus have mostly been aimed at discovering cell wall-related virulence and resistance factors, which may be targets for the development of new antifungal agents (Boyle et al., 1994; Wang et al., 2001; Feng et al., 2001; Liu et al., 2004; Zheng et al., 2006; Paolo et al., 2006; Dadachova et al., 2007).

The cell walls of fungi act as initial protective barriers that contact potential hostile environments (Latge, 2007). By using a variety of synthetic and hydrolytic enzymes fungi constantly remodel their cell walls during growth and sporulation (Klis et al., 2007). Chitin, a nonbranched $\beta 1 \rightarrow 4$ -linked homopolymer of *N*-acetylglucosamine (GlcNAc), is a structural component of the fungal cell wall. Together with $\beta 1 \rightarrow 3$ -linked glucan, chitin plays important roles in cellular development, structural morphogenesis, spore formation and the maintenance of cell wall integrity. Incorporation of chitin into the cell wall of a fungus is spatially and temporally regulated during its cell and life cycles (Bowman and Free, 2006). Chitin generally is also present in larger amounts in the cell walls of hyphae than in yeast cells and accounts for about 10-20% of the wall's dry weight in the former compared to about 1-2% in the latter (Bowman and Free, 2006; de Nobel et al., 2000; Klis et al., 2002, 2007; Latge, 2007). Nonetheless, the regulation of chitin metabolism is as critical for the growth of a budding yeast cell as it is for the extension of a hyphal tip and the cellular differentiation of a filamentous fungus (Bartnicki-Garcia, 1969; Bowman and Free, 2006; Cabib et al., 2001; Lesage and Bussey, 2006; Riquelme et al., 2007).

The synthesis of chitin is mediated by chitin synthases (Chsps). Found as integral membrane enzymes localized in fungal plasma membranes, and also associated with intracellular vesicles called chitosomes, Chsps catalyze the transfer of *N*-acetylglucosamine from uridine diphosphate (UDP)-*N*-acetylglucosamine (UDP-GlcNAc) to a growing chitin chain (Klis et al., 2007; Latge, 2007; Weber et al., 2006). In spite of the lack of direct structural data for the transmembrane nature of fungal chitin synthases, the biosynthetic mechanism of chitin synthesis is generally understood, at least for the yeast species *Saccharomyces cerevisiae* (Latge, 2007). In that fungus the consensus is that Chsps are transported from Golgi-vesicles in an inactive form to the plasma membrane, where they are arranged as complexes and activated in contact with resident activators (Latge, 2007). The situation is less clear in filamentous fungi, particularly as related to the source of the vesicles and chitosomes (Riquelme et al., 2007). Evidence nonetheless suggests that the chitin synthase catalytic domains (CSCD) of the Chsps contain the UDP-GlcNAc binding site facing the cytoplasm (Cabib et al., 1983; Rast et al., 2003). The cytoplasmic localization of the chitin synthase active site, and the lack of strong evidence for a mechanism of transport for UDP-GlcNAc, suggest that chitin is synthesized from intracellular precursors extruded through the plasma membrane (Cabib et al., 1983; Lesage and Bussey, 2006).

Three chitin synthase activities have been identified in *S. cerevisiae* membranes and are distinguished by their in vitro biochemical properties (Cabib et al., 2001; Lesage and Bussey, 2006). By contrast, the genomes of filamentous fungi encode up to 10 Chsps grouped usually

into seven classes, according to amino acid sequence similarities. Among them, enzymes of two classes (V and VII) possess an additional N-terminal so-called myosin motor-like domain (MMD) (Munro and Gow, 2001; Ruiz-Herrera et al., 2002; Mandel et al., 2006; Werner et al., 2007). Our previous reports documented that *W. dermatitidis* had at least five chitin synthases: WdChs1p, class II (Zheng et al., 2006); WdChs2p, class I (Wang et al., 2001); WdChs3p, class III (Wang and Szaniszlo, 2000); WdChs4p, class IV (Wang et al., 1999); and WdChs5p, class V (Liu et al., 2004). However, we now know it has at least two more: WdChs6p, class VI, and WdChs7p, class VII, (GenBank accession nos. ABZ91899 and ABZ91900, respectively; unpublished data). Their deduced protein sizes range from about 100 kDa for WdChs1p, 2p, 3p, 4p, and 6p to about 210 kDa for WdChs5p and 7p, with the majority of the added size of the latter two being contributed by their MMD. In terms of amino acids, WdChs5p is a protein of 1885 amino acids distributed between MMD (first 800 residues) and its CSCD (~ 600 amino acids). More importantly, unlike yeast cells of the wild-type and mutants with defects in each of its other six single Chsps, yeast cells of *W. dermatitidis* devoid of WdChs5p function and cultured at 37°C are hyperpigmented, produce smaller colonies, have abnormal morphologies and eventually die due to loss of cell wall integrity, whereas when cultured at 25°C they are identical to the wild type (Liu et al., 2004; Szaniszlo 2006; unpublished data). They are also less virulent, most likely because of their temperature-sensitive lethality. Although it is unclear why the lack of WdChs5p function so drastically affects cells cultured at the temperature of infection, it is hypothesized to be associated partly with the transcriptional up-regulation of WdChs5p production that accompanies a shift to that temperature (Liu and Szaniszlo, 2007). In a process to investigate this question further, we produced a specific antibody as a molecular tool for additional studies of this most important chitin synthase enzyme. The project was initiated by expressing in *E. coli* a cDNA fragment of the gene (*WdCHS5*) encoding the myosin motor-like domain of WdChs5p. The resulting recombinant protein (WdMyo12p) was then used to raise polyclonal antibodies in rabbits, which after purification were used to visualize the subcellular location of WdChs5p in the varied vegetative morphotypes of *W. dermatitidis* cultured at 37°C.

2. Materials and Methods

2.1. Strains, culture media, and plasmids

The laboratory strains of *Wangiella (Exophiala) dermatitidis* used in this study were as follows: the well characterized wild-type (WT) strain (Karuppayil and Szaniszlo, 1997) Wd8656 (ATCC 34100; CBS 525.76), which grows as a yeast at all temperatures when grown in most rich media; the chitin synthase mutant *wdchs5Δ11* (Liu et al., 2004), which grows as a yeast at 25°C but dies at 37°C when grown in rich liquid media in the absence of osmotic stabilizer; the temperature-sensitive (ts) mutant Hf1, which grows reproductively as yeast cells at 25°C in most rich media and produces hyphal-forms under the same culture conditions at 37°C (Ye and Szaniszlo, 2000); the ts mutant Mc3 (*cdc2*, ATCC 38716), which under similar or identical conditions also grows reproductively as yeast cells at 25°C but as isotropically enlarged, sclerotic forms at 37°C, the (Cooper and Szaniszlo, 1993). Culture of the strains was routinely on rich YPD agar (YPDA) or in YPD broth (YPDB) as reported previously (Wang et al., 1999). For the production of WT hyphal or sclerotic morphotypes (Fig. 1), the WT was first incubated in YPDB medium at 25°C and then the resulting yeast cells were subcultured at 37°C in pre-warmed potato dextrose broth (PDB; Difco Scientific, Detroit, MI, USA) or modified Czapek Dox broth (CDYB) (Roberts and Szaniszlo, 1978; Wang and Szaniszlo, 2007; Liu et al., 2008), respectively, whereas mutants Hf1^{ts} and Mc3^{ts} were first cultured in YPDB medium at 25°C and the resulting yeast then subcultured at 37°C to produce hyphal or sclerotic morphotypes, respectively (Roberts and Szaniszlo, 1978; Wang and Szaniszlo, 2000). The *Escherichia coli* strain DH5 α was used for cloning and maintaining plasmids. The truncated His-tagged recombinant protein (WdMyo12p) was overexpressed in *E. coli* strain BL21(DE3)

pLys (Invitrogen, Carlsbad, CA, USA). Recombinant bacterial strains were grown in Luria-Bertani broth (LB) supplemented with ampicillin (100 $\mu\text{g ml}^{-1}$).

2.2. Cloning of the WdMYO12-encoding epitope of WdChs5p, and overexpression and purification of the WdMyo12p antigen

A 348-bp PCR product encoding the WdMyo12p epitope of WdChs5p with a C-terminal 6×HIS-tag (indicated by bold letters) was amplified using primers, WdMyo12.1pr (5'-CGGAATTCGAGGAGAGGTACAATGGAAGAAGCGAAGGTCAGGCGG-3') and WdMyo12.2pr (5'-CCGGATCCCTATCAAT**TGGTGATGGTGATGGTG**ACGATCGCTCCCGACGACA CCGCCGTC-3'), which carried *Eco*RI and *Bam*HI restriction enzyme sites (underlined), respectively. The PCR reaction was carried out in a solution (50 µl) containing 0.5 U *Pfu* DNA polymerase (Promega, Madison, WI, USA), 0.2 mM dNTP mixture, 50 ng genomic DNA, and 0.2 mM of each primer in 10× manufacturer's PCR buffer. The reaction mixture was incubated at 94°C for 2 min to denature the DNA and then subjected to 35 cycles of PCR with the following conditions: pre-denaturation at 94°C for 30 s, annealing at 54°C for 1 min, polymerization at 72°C for 1 min, and a final incubation for extension at 72°C for 10 min followed by a 4°C hold until analysis. After the PCR product was electrophoresed, excised from the gel, purified, and digested with *Eco*RI and *Bam*HI, the resulting fragment was ligated into the corresponding sites of the expression vector pLM1 (Sodeoka et al., 1993). The nucleotide sequence of the insert region was determined by DNA sequencing (Institute of Cell and Molecular Biology Core Facility of the University of Texas at Austin, USA), which also confirmed that the reading frame of the WdMYO12-encoding epitope from the T7 promoter of pLM1 was correct. The TSS method (Chung et al., 1989) was then used to transform the *E. coli* BL21(DE3)pLys expression strain with the plasmid. Briefly, LB medium was inoculated with the strain for overnight culture in the presence of ampicillin (0.25 mg ml⁻¹) and chloramphenicol (50 µg ml⁻¹) and cultured at 37°C to OD₆₀₀ = 0.8. After induction with 0.5 mM IPTG for 4 h to express the WdMyo12p recombinant protein, cells were collected, resuspended in lysis buffer (50 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 0.1 M Na₂HPO₃, 1 mM PMSF) and sonicated at 30 sec. intervals for 30 min. The cell lysate was then centrifuged at 15 000 g for 20 min and the resulting pellet, including inclusion bodies, subjected to a series of additional centrifugal collections after suspension first in lysis buffer, next in lysis buffer with 1% Triton-X, then again in lysis buffer to remove any remaining detergent, and finally overnight in denaturing buffer (8 M urea, 50 mM Tris-HCl, 0.1 M Na₂HPO₄, 0.5 M NaCl). The treated WdMyo12p recombinant protein was then purified to homogeneity under denaturing conditions with Ni-NTA resin (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. Finally the eluted protein was refolded in renaturing buffer (10% glycerol, 2 mM GSH, 0.2 mM GSSP, Triton X-100, 5 mM EDTA, 0.2 M NaCl, 1 mM DTT, 10 mM Tris, 0.1 M Na₂HPO₄, pH 7.0), concentrated in an Amicon Ultra centrifuge filter (Millipore, Bedford, MA, USA) to final concentration at 0.5 mg ml⁻¹ and used for rabbit immunization.

2.3. Production and purification of an anti-Myo12 antibody that targets WdChs5p

The WdMyo12p (2 mg) in Freund's incomplete adjuvant was used to immunize the New Zealand White Rabbit (Pacific Immunology, CA, USA) from which blood samples had been previously obtained for use as preimmune serum. Injections of the rabbit were carried out four times during a 13-week antibody production period. After the rabbit was bled, the serum (PAC990) was separated, precipitated and dialyzed against PBS buffer (20 mM Na₂HPO₄, pH 7.2, 150 mM NaCl). Subsequent purification of the anti-Myo12p antibodies was by an affinity procedure using WdMyo12p bound to nitrocellulose (NC) membrane (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and blocked for 1 h at room temperature with TBST buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 0.1% Tween-20) containing 3% bovine serum

albumin (BSA, Fraction V). Absorption of the antiserum to the membrane-bound WdMyo12p was overnight at 4°C. After one wash with TBS buffer and two washes with PBS, the anti-Myo12p antibody was desorbed from the membrane with 0.1 M glycine-HCl buffer pH 2.5, then immediately mixed with 1 M Tris-HCl pH 8.0 and finally dialyzed against PBS buffer. Endpoint titer calculations of anti-Myo12p antibody from ELISA data were used to estimate dilutions for the western blotting experiments – 1:500 for crude or 1:5000 for purified anti-Myo12p.

2.4. SDS/PAGE and Western blotting

Membrane protein extracts (MPEs) were prepared using the method described previously (Wang and Szaniszlo, 2000). Protein samples were separated by 10% or 15% SDS/PAGE under reducing conditions according to the method of Laemmli (1970) and then visualized with Commassie Brilliant Blue R-250 stain. For the Western blotting, proteins from the SDS/PAGE gel were first electrotransferred to PVDF or NC membranes (Bio-Rad, Hercules, CA, USA). The membranes were then incubated in TBST-blocking buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20, 10% defatted milk) for 1 h at room temperature, after which the blocked membranes were incubated with a 1:5000 dilution of purified anti-Myo12p antibody in blocking buffer for 1 h at room temperature, washed three times with TBST buffer, incubated with goat anti-rabbit IgG conjugated with alkaline phosphatase antibody or horseradish peroxidase (Sigma-Aldrich, St. Louis, MO, USA) at a dilution of 1:8000 in blocking buffer for 1 h at room temperature, and finally washed three more times with TBST. The proteins on the membranes were then visualized with NBT/BCIP chromagenic reagents (Sigma-Aldrich).

2.5. Chitin synthase assays

All chitin synthase assays were performed with membrane samples (100 µg protein) in 75-µl reaction mixtures containing 50 mM Tris-HCl, 4 mM magnesium acetate, 0.3 mg m⁻¹ phosphatidylserine, 16 mM N-acetylglucosamine (Sigma-Aldrich), 1 mM UDP-N-acetyl[¹⁴C] glucosamine (4 × 10⁵ cpm µmol⁻¹; GE Healthcare, Piscataway, NJ, USA) and trypsin (1 µg of 1 mg ml⁻¹) as described previously (Wang et al., 1999). After the reactions were stopped by the addition of 200 µl 10% TCA, insoluble material was collected on glass-fiber filter disks (Whatman, Sanford, MA, USA), washed with 10% TCA and measured in a liquid scintillation counter (Beckman Coulter LS6500).

2.6. Immunoprecipitation

The immunoprecipitation (IP) reactions were carried out by mixing pre-cleared MPEs diluted in IP buffer [50 mM Tris pH 8.0, 1 mM EDTA pH 8.0, 150 mM NaCl, 0.1% SDS, 0.1% Triton X-100, protease inhibitor cocktail (Sigma-Aldrich)] with the purified PAC990 antiserum and incubating overnight at 4°C. Rehydrated Protein A-Sepharose CL4-B beads (GE Healthcare), blocked in 5% BSA/PBS to 50% (v/v) slurry for 1 h at 4°C and washed with PBS to remove BSA, were then incubated at 4°C overnight with the pre-cleared lysate-antiserum mixture. After collecting and washing at least five times with IP buffer, the beads were resuspended in Laemmli's loading buffer containing 0.2 mM B-mercaptoethanol, boiled for 3 min and then microcentrifuged. Supernatants were finally loaded in SDS/PAGE gels and subjected to electrophoresis for analysis of anti-Myo12p reactivity with WdChs5p.

2.7. Indirect immunofluorescence microscopy

For most experiments, *W. dermatitidis* was cultured at both 25°C and 37°C as described above and sampled periodically as required by the experiment. Staining with 4', 6'-diamidino-2-phenylindole (DAPI, Accurate Chemical, Westbury, NY, USA) was performed as described previously (Liu et al., 2004). Immunostaining was performed as described by Amatrudda and Cooper (1992). Briefly, cells after fixing with formaldehyde (5% in PBS) for 2-3 h were washed

twice by centrifugation with PBS and incubated with $1 \mu\text{g ml}^{-1}$ Zymolyase (Zymo Research, Orange, CA, USA) in spheroplast buffer (0.9 M sorbitol, 0.1 M EDTA, 1% (v/v) beta-mercaptoethanol) for 2 h at 30°C . The enzyme-treated cells were then incubated for 10 min with a 1:50 dilution of normal goat serum in PBS/BSA buffer, washed again by centrifugation with PBS, incubated with the anti-Myo12p antibody (1:100), diluted in PBS with 1 mg ml^{-1} BSA, washed three times by centrifugation with PBS, reacted with goat anti-rabbit FITC-conjugated antibody (1:250; Sigma-Aldrich) diluted in PBS with 1 mg ml^{-1} BSA, washed three more by centrifugation with PBS buffer and finally subjected to DIC and fluorescent microscopic examination with a Zeiss Axio Imager AX10 microscope system (Carl Zeiss, Germany).

The nocodazole treatments were performed as described previously (Jacobs and Szaniszló, 1982). Briefly, to inhibit microtubule function, cells were incubated with nocodazole (Sigma-Aldrich) at $20 \mu\text{g ml}^{-1}$ final concentration and a final DMSO concentration of 1% (v/v), then sampled after 8 h and 16 h of incubation, and finally collected and in some cases re-cultured in the media in absence of the inhibitor for 12 h to release the nocodazole block. For the inhibition of actin polymerization, latrunculin A [(LAT-A; Biomol Research Lab, Plymouth Meeting, PA, USA)], 0.2 mM (final concentration) was added to cultures containing a density of about 1×10^7 cells ml^{-1} (the growth consisted of > 90% unbudded cells in case of the yeast morphotype or >75% moniliform and the true hyphae in the case of hyphal morphotypes). The LAT-A cultures were sampled every 10 min, washed with PBS, fixed with 5% formaldehyde. The nocodazole and LAT-A treated samples were both then prepared for immunofluorescence microscopy and observed as described above for yeast cells.

3. Results

3.1. Specificity of the anti-Myo12p antibody

The 12 kDa WdMyo12p epitope located in the C-terminal end of the MMD of WdChs5p is a conserved and unique polypeptide region of this class V chitin synthase (data not shown). A protein-protein blast showed very high homology of the epitope only with the MMDs of the class V chitin synthases of other fungi (62-70% identity) and only very low homology with myosins (less than 25% identity to such myosins as myosin-1, myosin-2 and myosin-5), suggesting its potential for high specificity. Furthermore, the WdMyo12p blast with the more recently described fungal chitin synthases of class VII, including WdChs7p of *W. dermatitidis* (unpublished data), was negative, giving a presumption that no non-specific interaction would occur between an anti-Myo12p antibody and WdChs7p (data not shown).

SDS/PAGE analysis of the Myo12p recombinant protein fused with a His-tag and expressed in *E. coli* strain BL21DE3pLys showed a high expression level in the proteins extracted from the insoluble cell fraction and an undetectable level in those of the cytoplasmic soluble fraction (data not shown). Additional SDS/PAGE analysis of the WdMyo12p-His protein extracted from purified inclusion bodies and further purified by Ni-NTA chromatography confirmed it had the expected size of 12 kDa (data not shown). This purified and renatured WdMyo12p-His protein was then used to immunize rabbits to provide antiserum PA990, which was affinity purified. Analysis of the purified anti-Myo12p antibodies by Western blotting with membrane proteins from *W. dermatitidis* showed they reacted only with a protein band corresponding to the size estimated for WdChs5p (about 210 kDa) among the membrane proteins extracted from the WT strain cultured at 37°C (Fig. 2A, panel I). These results indicated that the purified antibody had good specificity for WdChs5p. Furthermore, no bands were visible when identical blots were probed with the preimmune serum (Fig. 2A, panel II). Nonetheless, because slight reactivity in the regions of gels associated with WdChs5p was also observed among the membrane proteins of the *wdchs5Δ11* mutant incubated at 25°C , an immunoprecipitation (IP) experiment was carried out. This experiment revealed a strong immunoreactivity of the anti-

Myo12p antibody with a similar size protein and no immunoreactivity with the preimmune serum or proteins of the *wdchs5Δ11* mutant incubated in 25°C (Fig. 3B). Taken together the specificity observed in the Western blotting and IP analyses for a 210 kDa protein suggested that the antibody preparation only recognized the WdMyo12p epitope present in WdChs5p.

3.2. Effect of the anti-Myo12p antiserum on the chitin synthase activity

Total chitin synthase activity of wild-type membranes of cells grown at 37°C and solubilized in 0.5% digitonin was strongly inhibited by addition of anti-Myo12p antiserum (31% average decrease at 10⁻¹ antibody dilution), whereas that from cells grown at 25°C were inhibited to a lesser degree (20% average decrease at 10⁻¹ antibody dilution). In addition, the levels of inhibition of chitin synthase activity in both cases were positively correlated with the amount of antibody added to each reaction mixture (data not shown). In contrast, only a minor reduction in chitin synthase activity was observed in control reactions of the same membrane samples tested with preimmune serum (6 to 3% average decreases at 10⁻¹ serum dilutions respectively). Furthermore, the anti-Myo12p reduction in total chitin synthase activity detected with the membranes of the *wdchs5Δ11* mutant grown at 25°C was essentially identical to those reductions associated with the same membranes or with the membranes of the WT grown identically at 25°C and treated with equivalent dilutions of preimmune serum (5% average decreases at 10⁻¹ antibody or preimmune serum dilutions, respectively). We suggest that the inhibitions by the anti-Myo12p antiserum observed in these assays indicated that the antibody preparation mainly or solely recognized WdChs5p and simultaneously inhibited its chitin synthase activity, possibly by inducing an allosteric change in enzyme conformation in the digitonin-solubilized WdChs5p which affected its catalytic domain. It is important to note here that assays of the anti-myo12p reductions in total chitin synthase activity associated with the membranes of the *wdchs5Δ11* mutant grown at 37°C were not carried out because this mutant dies at that temperature (Liu et al., 2004), which thus precluded the isolation of adequate membranes for assay from that strain at that temperature.

3.3. Immunocytolocalization of WdChs5p in the wild-type yeast, hyphal and sclerotic morphotypes

The subcellular localization of WdChs5p in the budding yeast morphotype of *W. dermatitidis* was studied using indirect immunofluorescence microscopy, the affinity-purified anti-Myo12p antibody and anti-rabbit antibody conjugated to FITC. Whereas the *wdchs5Δ11* yeast cells (Figs. 3A and B) and unbudded WT yeast cells cultured at 25°C (data not shown) revealed no strong FITC-signals respectively, significant bright punctuate fluorescence was observed in the periphery of unbudded, stationary-phase, yeast cells of the WT cultivated at elevated temperature (Figs. 3C and D). In contrast, in yeast cells in various stages of reproductive growth at 37°C, the brightest WdChs5p fluorescence, when observed, was most often localized in the apices of yeast buds and much less frequently as a band at septal regions between mother and daughter cells (Figs. 3E and F). An approximate quantification of the extent of fluorescence (n=100 cells) indicated that about 80% percent of the brightest signals was observed in small- and medium-size buds, with about 30% of those buds having the brightest WdChs5p fluorescence in the bud apex, 60% more widely spread and only about 10% at septal regions between a mother-cell and its daughter bud. This analysis also indicated that about 10% of the stationary-phase and log-phase, budding cells exhibited no fluorescence or only weak fluorescence, which we speculate is due to fluorescence quenching, less than perfect blocking with the normal goat serum, and variations in spheroplasting that hindered antibody penetration.

A similar analysis of the subcellular localization of WdChs5p in the hyphal morphotypes produced at 37°C of the WT showed that about 95% of FITC-WdChs5p signals were concentrated at the tips of main hyphae and hyphal branches (Figs. 4A-D). This finding was

consistent with those of others who found that the class V chitin synthases of *Aspergillus nidulans* and *Ustilago maydis* localized in growing hyphal tips (Takeshita et al., 2005; Weber et al., 2006). However in those studies the class V chitin synthase was also found to localize in hyphae in regions of septum formation, whereas in our study little or no staining was observed in those regions or other regions where septa might be expected to form. In contrast to the findings with yeast and hyphae, the WdChs5p fluorescence signals in the sclerotic morphotypes of the WT induced by acidic culture at 37°C were randomly distributed near sites of presumed new cell wall synthesis and septum formation (Figs. 4E and F). As expected, immunostaining of the hyphal morphotypes using preimmune serum instead of with the anti-Myo12p reagent produced no prominent signals (Figs. 4G and H). Surprisingly, the nonpolarized and isotropically enlarged sclerotic morphotypes produced at 37°C by subculture of Mc3^{ts} yeast cells grown at 25°C stained differently than did the sclerotic bodies produced by the wild type cultured under acidic conditions: in this case the signals in the former were now seen near discrete peripheral wall sites and not near putative septation sites between nuclei (Figs. 4I and J). Immunostaining of the hyphal morphotypes obtained with the Hf1^{ts} mutant cultured at the restrictive temperature produced results essentially identical to those obtained with the hyphae of the WT and showed again that WdChs5p predominantly localized in the tips of hyphae and not at regions where septa exist or might form (Figs. 4K and L). Collectively these findings suggest that at elevated temperatures WdChs5p localizes at sites where cell wall synthesis is required for new cell wall expansion.

3.4. Effects of inhibiting microtubule and actin functions on WdChs5p distribution in the yeast, hyphal and sclerotic morphotypes

To investigate whether the localization of WdChs5p is associated with microtubule and actin function, the WT morphotypes were treated with nocodazole and LAT-A respectively. Consistent with previous results, mitosis and nuclear migration, but not budding, were inhibited in yeast cells of *W. dermatitidis* incubated at 25° and 37°C by the presence of nocodazole (Jacobs and Szaniszlo, 1982). Irrespective of the number of buds associated with a mother cell, the WT yeast exposed to the drug showed only one nucleus, and thus served as an effective control for the absence of microtubule functions in such multiply budded cells (Figs. 5A-C). Nonetheless, the WdChs5p signals were localized to presumed multiple daughter buds. As expected, subculture of the nocodazole-inhibited yeast cells in the absence of the drug allowed the resumption of nuclear division and nuclear migration (Figs. 5D-F), even though the FITC-signals still were localized in yeast buds in a manner similar to those of the WT yeast incubated in the drug's absence. These results implied that actin instead of microtubules might be required for the normal localization of WdChs5p in budding yeast cells. Therefore we next used the actin polymerization inhibitor LAT-A to investigate that possibility. In contrast to the situation with WT yeast cultured in the absence of LAT-A, localized signals for WdChs5p in WT yeast cultured in its presence for 20 min were not observed in the bud apices of most cells, but instead the localized signals, when apparent, were aberrantly detected at the base of emerging buds and in the bud necks of about 95% cells (n = 100 cells) with buds (Figs. 5G and H). In these cases, the brightest FITC-signals were often seen as two bright bands oriented parallel to the yeast cell's long axis, indicating that WdChs5p had localized in a broad ring (Fig. 5H), instead of at bud apices and as the narrow bands associated with the septal regions of uninhibited WT cells (Fig. 3). However, when the yeast cells were exposed to LAT-A for periods longer than 40 min and then stained, no signals for WdChs5p were observed (Fig. 5J). This particular result may indicate that the extended treatment of the yeast cell with LAT-A prompted not only the abnormal localization of WdChs5p, but also resulted in stopping its production and promoting its degradation as suggested by prior Northern and Western analyses (Liu., 2004; Liu and Szaniszlo, 2007). Alternatively, this result may relate to technical variations in sample preparation.

The effects of nocodazole and LAT-A on WdChs5p localization in the yeast of *W. dermatitidis* suggested that similar studies with its hyphal and sclerotic morphotypes were warranted. Our analysis of the hyphal morphotypes produced at 37°C and exposed to nocodazole for 8 h, or even 16 h, revealed that no matter whether detected in the WT cultured in PDB (data not shown) or in the Hf1^{ts} mutant cultured in YPDB (Fig. 6), the WdChs5p-FITC signals were localized, in the manner of those in controls (Figs. 6A and C), at the tips of 90% of main hyphae and branches (Figs. 6D and F), in the lateral hyphal bud cells (Figs. 6G and I) that are often produced by the hyphae of this species (Oujezdsky et al., 1973), and in the tips of hyphae first inhibited with nocodazole and then subcultured in its absence (Figs. 6J and L). Staining of WdChs5p after release from the nocodazole block was infrequently also observed subapical to the hyphal tip (Fig. 6L). We speculate that this unusual staining pattern may be due to WdChs5p having localized to a site where a hyphal bud or branch would eventually form, or alternatively to an area where an injury had occurred in the hypha prior to subculture in the absence of the nocodazole. Staining the same hyphae with DAPI confirmed that mitosis and nuclear migration had been inhibited by the nocodazole, as evidenced by the presence of cells at the tips of hyphae devoid of nuclei (Figs. 6D and E and Figs., 6G and H). As noted with the budding yeast morphotype, subculture of such nocodazole-inhibited hyphae in media devoid of the nocodazole, allowed nuclear migration into areas devoid of nuclei (Figs. 6J and K), presumably through the central pores known to be present in the septa of moniliform and true hyphae of *W. dermatitidis* (Oujezdsky et al., 1973). In a similar manner, no effects of the nocodazole were apparent on the immunolocalization of WdChs5p in the sclerotic morphotypes of WT and Mc3^{ts} mutant cultured in the presence of the drug at 37°C in acidic CDYB and in-acidic YPDB, respectively (Fig. 7): both still exhibited randomly distributed FITC-WdChs5p signals (Figs. 7F and I), in the same manner as sclerotic forms incubated in its absence (Fig. 7C). DAPI staining again confirmed that nuclear division had been inhibited (Figs. 7D and E and G and H) and had recommenced after cells were released from the nocodazole block (Figs. 7J and K). In contrast, when the effects of the actin inhibitor LAT-A were evaluated in hyphae of the WT induced in PDB at 37°C (Fig. 8), as well as in hyphae of the Hf1^{ts} mutant in YPDB cultured at the restrictive temperature (data not shown), the WdChs5p FITC signals were reduced gradually from minor to very low levels in hyphal tips to the extent that by 20 to 40 min only about 5% hyphal forms showed organized fluorescent signals (Fig. 8B-J). These observations may indicate again that the LAT-A treatment was prompting a rapid turnover of WdChs5p in hyphae in the manner suggested above for its yeast morphotype. However when strong signals were observed, they were mostly associated with abnormally enlarged spherical cells that arose from somewhat distorted hyphae (Figs 8C and D and Figs. 8I and J), whereas those in the hyphae of LAT-A-minus control cultures appeared, as before, in morphologically normal hyphae and predominantly in hyphal tips (Figs 8K and L). Strong WdChs5p FITC signals were also only infrequently observed in the sclerotic morphotypes of the WT and the Mc3^{ts} mutant produced at 37°C and exposed to LAT-A. In general, the FITC-signal in these forms weakened shortly after initial exposure to the inhibitor (data not shown). We conclude from these inhibitor studies that WdChs5p localization at elevated temperatures is more likely to be dependent on actin rather than on microtubule functions.

3.5. Osmotic stabilization of the morphotypes of the *wdchs5Δ11* mutant produced at 37°C

Yeast cells of the *wdchs5Δ11* strain are protected from the loss of cell wall integrity at 37°C by osmotic stabilization (Liu et al., 2004). However no data were gathered previously about the loss of cell wall integrity in the hyphal and sclerotic morphotypes of that same mutant. Therefore, these alternate morphotypes, as well as yeast controls, were produced at 37°C in the appropriate liquid media (as described above) supplemented with 1.2 M sorbitol (Fig. 9). As reported previously, the yeast morphotype grew well in the YPDB medium that included the osmotic stabilizer, but in a multiply budded fashion that produced branched pseudohyphal-

like microcolonies (Fig 9A). Hyphae and sclerotic bodies also developed well in osmotically stabilized PDB and pH 2.5 CDYB respectively (Figs. 9B and G-I). Surprisingly, unlike the yeast and hyphal morphotypes, the sclerotic morphotypes also grew well in the absence of sorbitol, although much more slowly (data not shown). In addition, and in contrast to the WT sclerotic morphotypes, less than 1% of the osmotically stabilized sclerotic forms produced internal septa and then only a single septum (Figs. 9H and I), even after culture for two weeks (Figs. 9G-I). Subculture of the sorbitol-stabilized yeast in the absence of the sorbitol resulted in yeast buds that swelled and burst (data not shown) as reported previously (Liu et al., 2004). Cells of pseudohyphae and moniliform and true hyphae similarly burst but at the apices of hyphal tip cells (Figs. 9C-F). In contrast, the sclerotic cells treated identically remained alive and only infrequently were seen to show a bursting cell (Fig. 9J). We conclude from these studies that WdChs5p is more important for protecting the cell wall integrity of the yeast and hyphal morphotypes of *W. dermatitidis* than its sclerotic morphotypes at temperatures of infection.

4. Discussion

Numerous studies have shown that fungi have multiple Chsps distributed among at least seven classes and that high numbers of Chsps are typical of most filamentous eumycete and basidiomycete fungi (Choquer et al., 2004; Mandel et al., 2006; Weber et al., 2006). In contrast, some yeasts species, such as the archiascomycete *Schizosaccharomyces pombe* and hemiascomycete *S. cerevisiae* have only class I and class II and class I, II and class IV Chsps respectively (Cabib et al., 2001; Matsuo et al., 2004). The higher number of Chsps in the filamentous fungi is thought to reflect greater complexities in life styles and development (Weber et al., 2006). However, the ability to form hyphae alone does not explain the lower number of Chsps in some yeast species because many are vegetatively dimorphic or polymorphic and also produce hyphae. For example, the molecularly classified hemiascomycete yeast *Candida albicans* exhibits at least three different vegetative morphotypes, including two kinds of hyphae (Sudbery et al., 2004) but has only four Chsps distributed among the same three classes as those of *S. cerevisiae* (Munro and Gow, 2001). In contrast, the known dimorphic basidiomycete yeast *Cryptococcus neoformans* has eight chitin synthases distributed arguably among five classes (Banks et al., 2005). It has been proposed that the chitin synthases of classes III, V, VI and VII are present only in filamentous fungi and some dimorphic and polymorphic fungi that produce true hyphae (Munro and Gow, 2001; Weber et al., 2006). Based on functional analyses of mutants with disrupted and deleted genes, it is becoming increasingly clear that the class V Chsps are especially important to the biology of filamentous fungi because of their roles in maintaining the cell wall integrity of hyphae and conidia, participating in hyphal growth polarity by interactions with the actin cytoskeleton, sustaining growth at elevated temperature and for having relevance to pathogenicity and virulence (Amnuaykanjanasin and Epstein, 2003; Madrid et al., 2003; Liu et al., 2004; Ortoneda et al., 2004; Takeshita et al., 2005; Weber et al., 2006; Werner et al., 2007).

In the current study we used a part of the gene sequence that encodes mainly the MMD of WdChs5p (Liu et al., 2004) to produce a 12 kDa polypeptide in *E. coli* for raising antibody for additional studies of this class V chitin synthase of *W. dermatitidis*. Our previous work has shown that at least seven genes of this polymorphic fungus encode Chsps, among which five (WdChs1p, WdChs2p, WdChs3p, WdChs4p, WdChs5p) have been described in some detail by characterizing their genes and by studying the enzymatic, morphological and cytological properties of *wdchsΔ* mutant strains (Szaniszló, 2002; 2006). However, none of the WdChsps have been analyzed previously with respect to their subcellular localizations, because all of our attempts to optimize and express a localizing and fluorescently labeled form of the one of the enzymes in *W. dermatitidis* have failed (unpublished data). To fill this gap in knowledge, we instead used an anti-WdChs5p antibody approach to investigate the localization of one of

its seven known chitin synthases in each of its various morphotypes. We also employed this approach because the antibody could be used with multiple strains, thus circumventing the need to molecularly modify each strain identically. WdChs5p was chosen as the target for our initial immunocytolocalization studies because it is not required for growth of *W. dermatitidis* at 25°C, is the only single chitin synthase known to be required for its sustained growth at 37°C, and because it has a particularly important relationship to the virulence of this agent of phaeohyphomycosis (Liu et al., 2004).

Our analysis of the deduced amino acid sequence of WdChs5p showed that its N-terminal MMD domain has a 12 kDa unique region. Subsequent BLAST analysis of the 12 kDa WdMyo12p polypeptide showed no such match to any of the other known Chsps of *W. dermatitidis*, including that of the recently GenBank-deposited class VII chitin synthase WdChs7p, which also contains a motor-like-domain, but in this case one that is devoid of a P-loop and is shorter and, unlike those in some other conidiogenous molds, is not encoded in the genome in a head-to-head configuration with a gene that encodes a class V chitin synthase (Mandel et al., 2006; Takeshita et al., 2006; unpublished data). Overexpression of this region in a bacterial system then produced the WdMyo12p used for immunizing rabbits for the production the anti-Myo12p antibody. Tests of the anti-Myo12p specificity with membrane proteins of the WT and the *wdchs5Δ11* mutant of *W. dermatitidis* showed that it clearly detected a single 210 kDa protein (WdChs5p) in MPEs of cells grown at 37°C, detected a very much weaker signal of that size in the MPEs of the wild type cultured at a lower temperature and detected essentially no signal of the same size in the MPEs of *wdchs5Δ11* cells. A reduction of Chsps enzyme activity in assays of MPEs supplied with antibody was also clearly evident with MPEs of the WT cultured at 37°C, but was much less evident with MPEs of the WT grown at 25°C (data not shown) and was even less evident with MPEs of the *wdchs5Δ11* mutant grown at 25°C. We conclude from these results of differences in total Chsps activity between the WT strain cultured at 25°C and at 37°C in presence of the anti-Myo12p antibody that the almost 20% lower enzyme activity at higher temperature is caused by a specific direct blocking of WdChs5p activity by the purified anti-Myo12p antibody. We further suggest that these data confirm our previous conclusion that this membrane-bound protein is preferentially expressed at elevated temperature (Wang et al., 2002; Liu et al., 2004; Liu and Szaniszló, 2007).

The localization by immunofluorescence staining of WdChs5p to putative sites of cell wall expansion in all the morphotypes of *W. dermatitidis* produced at 37°C provides additional evidence for our previous suggestion that WdChs5p is required for the maintenance of cell wall integrity and viability during infections (Liu et al., 2004). With the WT yeast morphotype, the immunostaining revealed that in unbudded, stationary-phase, yeast incubated at 37°C WdChs5p is found in a punctuate fashion just under the cell wall surface. In contrast in yeast cells with smaller buds, WdChs5p was mostly localized at the bud apex, whereas in cells with larger buds WdChs5p was spread more widely toward the mother cell and less infrequently was localized to a septal region in the mother-bud neck when both were approximately equal size, presumably just before or concomitantly with cytokinesis. This result is similar to CaChs3p localization in budding yeast cells of *C. albicans*, but not that of CaChs1p, CaChs2p and CaChs8p which localize at septal sites (Lenardon et al., 2007). Our immunolocalization results with WT hyphae of *W. dermatitidis* similarly showed that WdChs5p is predominantly localized in areas of presumed cell wall expansion in hyphal tips in a manner documented by previous results concerning the class V chitin synthases of other fungi by use of different techniques (Amnuaykanjanasin and Epstein, 2006; Takeshita et al., 2005; Weber et al., 2006). However our findings that septa still were formed in the hyphae of the *wdchs5Δ11* mutant grown under osmotic stabilization conditions at 37°C and that signals for WdChs5p in the hyphae of *W. dermatitidis* were not visible in septal regions are contrary to results with some other filamentous fungi (Amnuaykanjanasin and Epstein, 2006; Weber et al., 2006). In this respect, our results are most consistent with those found with the *euscomycete* mold

Aspergillus nidulans in which large numbers of septa still formed normally in the class V chitin synthase-defective *csmA*-conditional mutant strain aAΔB1 grown under the restrictive condition (Takeshita et al., 2006). Identical results were also obtained in our studies for WdChs5p immunolocalization in the hyphae of the Hf1^{ts} mutant, which at the restrictive temperature produces the hyphal morphotype. We conclude from these results that WdChs5p at 37°C carries out an essential function during growth by maintaining the integrity of the hyphal tips of all the hyphal morphotypes of *W. dermatitidis*, but does not participate in the septation of those hyphae. Support for this conclusion is provided by our findings that the hyphal growth of the *wdchs5Δ11* mutant stopped after the removal of the osmotic stabilizer and that hyphal tips burst and leaked cytoplasm at exactly the same loci where the WdChs5p-FITC signals were localized in WT and Hf1^{ts} hyphal morphotypes. We further suggest that the specific chitin synthesized by WdChs5p at 37°C in elongating hyphal tips is essential for growth because without it that vulnerable cell wall expansion area is rendered too weak to resist the turgor pressure hypothesized to drive hyphal extension in *W. dermatitidis* (Brush and Money, 1999).

Conclusions are less clear about the importance of WdChs5p to the cell wall integrity of the sclerotic morphotypes of *W. dermatitidis* produced at 37°C. In these nonpolarized growth forms, the WdChs5p-FITC-signals were mainly randomly dispersed, but sometimes seen also in septal regions of the WT but not in those of the Mc3^{ts} mutant. Interestingly, the *wdchs5Δ11* mutant converted to the sclerotic morphotype and grew almost as well as the WT in the acidic CDYB medium at 37°C regardless of whether or not the osmotic stabilizer was present. This implies that WdChs5p is not as critically important for protecting the cell wall integrity of the sclerotic morphotypes at 37°C as it is for protecting the yeast and hyphal morphotypes. This in turn may imply that one or more of the other WdChsp of *W. dermatitidis* are involved in protecting the cell wall stability of the sclerotic forms and may even suggest that the sclerotic forms are more resistant to host clearance pressures than are its polarized morphotypes. Support for this idea was previously suggested by experiments with *W. dermatitidis* and polyoxin (Cooper, et al., 1984). Those experiments showed that in the presence of this general inhibitor of chitin synthases, the sclerotic morphotypes of the *W. dermatitidis* Mc3^{ts} mutant induced by culture at 37°C became very fragile and tended to burst at discrete loci, presumably because all or most Chsps were inhibited in function by the polyoxin, instead of only by the absence of the single WdChs5p isozyme in the *wdchs5Δ11* mutant investigated in the current study. However, and in contrast to our findings in our current and past studies of the sclerotic morphotypes of the wild type and the Mc3^{ts} mutant, our studies of those morphotypes produced by the *wdchs5Δ11* mutant in this study showed they were mostly sclerotic cells and only rarely planate cells, neither of which developed further into multiply septated sclerotic bodies. These results may suggest that WdChs5p is directly involved in the septum formation that leads to the production of the planate cell and sclerotic body morphotypes. Alternatively the signals detected in the sclerotic forms of the WT, but not in those of the *wdchs5Δ11* mutant, may not be directly associated with actual septum formation but instead may be associated with areas of internal cell wall expansion or secondary wall thickening. However this latter scenario does not explain why septa were not seen in the sclerotic morphotypes of the *wdchs5Δ11* mutant. We propose that the resolution of this conundrum will require additional cytolocalization studies with our anti-Myo12p antibody coupled with transmission electron microscopy using methods such as those reported previously (Harris and Szaniszló, 1986).

The localization of WdChs5p in the yeast morphotype of *W. dermatitidis* was not inhibited by the culture in the presence of nocodazole, which as reported previously inhibits mitosis and nuclear migration but not bud formation in yeast cells of *W. dermatitidis* and *S. cerevisiae* (Jacobs and Szaniszló, 1982; Jacobs et al., 1988). In contrast, in the presence of the actin polymerization inhibitor LAT-A, WdChs5p was not localized to bud apexes but instead

localized aberrantly in bud necks. Moreover, when the WT yeast were exposed to LAT-A for periods longer than 40 min no signals for WdChs5p were observed. These results implied that WdChs5p was more likely to be transported to polarized cell sites in yeast cells of *W. dermatitidis* by an actin-based rather than a microtubule-based transport mechanism, and that actin may also have a role in maintaining the stability of WdChs5p. This suggestion is consistent with results with *S. cerevisiae* where the chitin synthase associations with actin-myosin trafficking are well established (Schmidt et al., 2001). However, because *W. dermatitidis* does not synthesize a chitin ring prior to yeast bud emergence (Harris and Szaniszlo, 1986) and *S. cerevisiae* has no class V chitin synthase and thus no chitin synthases with a MMD (Klis et al., 2002, 2007), it is unlikely that all aspects of such trafficking in the yeast cells of these two fungi are the same.

Unlike the situation in yeast species like *S. cerevisiae* and *C. albicans*, chitin synthases possessing a MMD have been found in a number of other filamentous fungi, where they are reported to be associated with an actin-based transportation mechanism (Takeshita et al., 2006; Weber et al., 2006). Previously, it had already been suggested, that an actin-motor system plays an important role in the determination of hyphal polarity and chitin synthase class V transportation in *A. nidulans* (Takeshita et al., 2005). Thus the possibility exists that the MMD of certain chitin synthases may act like myosins, which are mechanoenzymes that convert chemical energy liberated through ATP hydrolysis, into a mechanical force that is directed along polymerizing actin filaments (Kalhammer and Bähler, 2000). However, this scenario is dependent on the presumption that the putative MMD of the chitin synthase acts like a conventional myosin to localize the myosin-cargo protein among the vesicles in the apical cytoplasmic dome of hyphae, most likely represented by the Spitzenkörper (Steinberg, 2007; Weber et al., 2006; Riquelme, et al., 2007). Therefore, and since actin microfilaments and microtubules are required for the transport of various cargoes and the maintenance of polarity in hyphal growth (Xiang and Plamann, 2003), we also undertook to analyze the subcellular localization of WdChs5p in hyphae after inhibition of actin and microtubule network formation in the various hyphal morphotypes of *W. dermatitidis*. These studies showed that WdChs5p immunolocalization in hyphae treated with LAT-A had signals that continuously weakened from the time of exposure to the inhibitor. In contrast, no differences were detected in WdChs5p localization after treatment with the microtubule depolymerizing agent nocodazole, even though microtubule-dependent activities of mitosis and nuclear migration were clearly inhibited. We suggest these data indicate that an actin-based motor system also contributes to WdChs5p translocation in the hyphal morphotypes of *W. dermatitidis*. Unfortunately, our attempts to stain actin in the morphotypes of *W. dermatitidis* have for unknown reasons always failed (unpublished data). Nonetheless, the WdChs5p FITC-signal disappeared only after longer incubation with LAT-A, suggesting that the effect of the actin inhibitor was on WdChs5p supply to areas of cell wall expansion. Possibly WdChs5p uses an actin-based vesicle transport to be moved to hyphal tips during hyphal growth, after which it then is incorporated into the membranes in the apex to bring about hyphal tip extension and protection. However, it is important to note that we observed no evidence of such cytoplasmic localizations of WdChs5p in our studies. Should we eventually succeed in our efforts to express a fluorescently labeled and localizing form of WdChs5p, then such localizations in the cytoplasm of the varied morphotypes of *W. dermatitidis* may become evident.

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Fig. 1. Schematic representation of the media and temperature conditions used to induce the alternative morphotypes of the wild type (WT) and temperature-sensitive strains (Mc3^{ts} and Hf1^{ts}) of *W. dermatitidis* produced in vitro.

Fig. 2.

Immunoreactivity of the purified anti-Myo12p antibody. (A). Western blotting analysis of membrane proteins resolved by electrophoresis in a 10% SDS/PAGE gel. Panel I - immunoblot with the anti-Myo12p as the primary antibody of membrane proteins (100 μ g) isolated from wild type (wt) and from *wdchs5 Δ -11* (Δ 5). Panel II - immunoblot of the membrane proteins from wild type with preimmunoserum as the primary antibody (wt-pre). Both Westerns were developed with NBT/BCIP Alkaline Phosphatase substrate solution. (B). Immunoprecipitation (IP) of membrane protein (200 μ g) lysate from the wild type (wt) and the *wdchs5 Δ -11* mutant (Δ 5) with the purified anti-Myo12p antibody, and IP of the wild-type membrane protein extract with preimmune serum (wt-pre) as a negative control. Immunocomplexes were resolved by electrophoresis in a 10% SDS/PAGE gel and stained with CBB R-250.

Fig. 3.

WdChs5p immunolocalization in the yeast morphotype of *W. dermatitidis*. Budding yeast cells of *wdc5Δ11* produced in YPDB at 25°C (A, B); please note absence of any strong FITC signals for WdChs5p in B. WT unbudded stationary-phase yeast cells (C, D) and actively budding yeast cells (E, F) cultured at 37°C for 24 h; respectively; please note the arrows in latter that point to sites where the brightest WdChs5p signals were detected in enlarging buds, and at septal regions (E, F). Yeast cells (1×10^7 cells ml^{-1}) of the WT were first grown at 25°C in YPDB, next subcultured in pre-warmed YPDB at 37°C and sampled and treated with preimmune serum or anti-myol2p antibody as indicated and finally stained with FITC-anti-rabbit goat antibody and the same cells viewed and photographed using DIC (A, C, E) and fluorescent microscopy (B, D, F). Bars equal 10 μm .

Fig. 4.

WdChs5p immunolocalization in the hyphal and sclerotic morphotypes of wild-type *W. dermatitidis*. The WT hyphal morphotypes were produced at 37°C in PDB and immunostained as described in the legend of Fig. 3, after treatment with the anti-Myo12p antibody (A-D) or the pre-immune serum (G, H) as a negative control, whereas the WT sclerotic morphotypes were produced in CDYB acidified to pH 2.5 (E, F). The sclerotic morphotypes of Mc3^{ts} (I, J) and the hyphal morphotypes of Hf1^{ts} (K, L) were both produced in YPDB at restrictive temperature (37°C) and similarly stained after treatment with the anti-Myo12p antibody. Please note that the arrows in A, C and K identify septal positions in the DIC images and that no WdChs5p signals are apparent at those positions or other areas where septa might be expected to be forming in the same hyphae in the fluorescent images B, D and L. Bars equal 10 μm.

Fig. 5.

WdChs5p immunolocalization in budding WT yeast cells cultured at 37°C in YPDB, inhibited in microtubule function with nocodazole and in actin function with LAT-A. Budding yeast cells (1×10^7 cells ml^{-1}) after shift from 25°C to 37°C were first cultured in YPDB with nocodazole (20 $\mu\text{g ml}^{-1}$) and sampled 16 h (A-C), after which time some of the multiply budded yeast were recultured in the absence of nocodazole for an additional 12 h (D-F) to release the mitotic block; please note that in the latter both the mother and daughter buds have nuclei whereas in the former only a single nucleus is evident in the putative mother yeast cells. In contrast, the WdChs5p fluorescent signals, as indicated by the arrows in C and F, in those same cells are not associated with the putative mother yeast cells but only with one or more of the daughter buds. Before reaction with the anti-myo12p antibody and staining with the FITC-conjugated anti-rabbit goat antibody, the nuclei were stained for 10 min with 5 $\mu\text{g ml}^{-1}$ DAPI in PBS buffer, followed by washing with PBS buffer. For actin function inhibition (G-J), budding yeast cells after shift to 37°C were exposed to LAT-A (final conc. 200 μM) for 20 min (G-H) and for 40 min (I-J) prior to staining and photomicroscopy as described in the Fig. 3 legend. Please note that the arrows in H identify areas of abnormal WdChs5p localization in septal regions; please also note the general lack of WdChs5p signals at the tips of growing buds. Bars equal 10 μm .

Fig. 6.

WdChs5p immunolocalization in hyphal morphotypes produced by the temperature-sensitive mutant strain Hf1^{ts} cultured with nocodazole at the restrictive temperature in YPDB. Budding yeast cells (1×10^7 cells ml⁻¹) after shift from 25°C to 37°C were cultured first for 24 h (A-C) in the absence of nocodazole, then in the presence of nocodazole (20 µg ml⁻¹) for an additional 8 h (D-F) or 16 h (G-I), after which time some cells were re-cultured in the absence of the nocodazole for an additional 12 h (J-L). Please note that the thin arrows in B, E, H and K identify positions of nuclei, the thin arrows in D, G, and J identify septa in the DIC images of those same hyphae, and the thick arrows in C, F, I and L point to WdChs5p localized in the hyphal tips, a branch or the hyphal bud cell shown in the DIC images. Also please note the

abnormal subapical staining of WdChs5p in L, which may have resulted from its localization at a site of potential bud or branch formation or at a site of injury. In all cases, the staining of the nuclei and WdChs5p and the photomicroscopy was carried out as described in the legend of Fig. 5. Bars equal 10 μ m.

Fig. 7.

WdChs5p immunolocalization in sclerotic morphotypes produced by the temperature-sensitive mutant strain Mc3^{ts} cultured with nocodazole at the restrictive temperature in YPDB. Budding yeast cells (1×10^7 cells ml⁻¹) after shift from 25°C to 37°C were cultured first for 24 h in the absence of nocodazole (A-C), then in the presence of nocodazole (20 μg ml⁻¹) for an additional 8 h (D-F) or 16 h (G-I), after which time some cells were re-cultured in the absence of the nocodazole for additional 12 h (J-L). In all cases, the staining of the nuclei (B, E, H, K, N) and of WdChs5p (C, F, I, L, O) was carried out as described in the legend of Fig. 5. For a negative control, Mc3^{ts} was incubated for 16 h at elevated temperature with preimmune antibody (M-O). Bars equal 10 μm.

Fig. 8.

WdChs5p immunolocalization in WT hyphae inhibited in actin function with LAT-A. The hyphal morphotypes produced at 37°C in PDB were cultured in the presence of LAT-A (final concentration 200 μ M) for 10 min (A-D); 20 min (E-H); 40 min (I, J) or in its absence (K, L) prior to staining as described in the legend of Fig. 3. Bars equal 10 μ m.

Fig. 9.

Unstained DIC images of the morphotypes of the *wdchsΔ511* mutant strain cultured at 37°C initially in YPDB for yeast cells (A), in PDB for hyphae (B-F) and in pH 2.5 CDYB for sclerotic forms (G-J) supplemented with 1.2 M sorbitol (A, B, G, H, I) and subsequently after reculture in its absence (C, D, E, F, J). Please note that arrows in C, D, E, F, and J identify empty cells or cells extruding cytoplasm, whereas arrows in H and I identify septa in rare planate cells among the much more numerous sclerotic cells.