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The chitin synthase genes *chsA* and *chsC* are not required for cell wall stress responses in the human pathogen *Aspergillus fumigatus*

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Abstract

Invasive aspergillosis is a leading cause of mortality in immunocompromised patients. The fungal cell wall is an attractive antifungal target, but it is dynamic and responsive to external stressors. The existence of multiple chitin synthases within *Aspergilli* is thought to reflect specialized functions in cell wall damage responses that facilitate continued growth and viability. We previously reported increased transcription of *Aspergillus fumigatus* chitin synthases *chsA* and *chsC* following echinocandin treatment, suggesting important roles for these chitin synthases in cell wall compensation. As only partial disruptions have been made of these genes, we generated deletion mutants of *chsA* and *chsC* singly ($\Delta chsA$ and $\Delta chsC$) and doubly ($\Delta chsA \Delta chsC$). The $\Delta chsA \Delta chsC$ strain displayed reduced total chitin synthase activity. Interestingly, deletion of these chitin synthase genes did not affect levels of chitin or β -1,3-glucan. The $\Delta chsA$, $\Delta chsC$ and $\Delta chsA \Delta chsC$ strains produced wild-type echinocandin-mediated chitin increases, consistent with unaltered cell wall compensation. Furthermore, transcript levels of the remaining chitin synthase genes were unchanged in the mutant strains. Taken together, these results indicate that *chsA* and *chsC* do not play a direct role in the cell wall stress response. Our findings support the existence of complex post-transcriptional regulatory mechanisms controlling chitin biosynthetic machinery in response to cell wall damage.

Keywords

Chitin synthesis; Echinocandin; Cell wall integrity; Antifungal; Paradoxical effect

1. Introduction

The fungal cell wall is a complex network of chitin, glucans, other polysaccharides and proteins that is necessary for normal growth and virulence in both yeasts and filamentous fungi. Chitin, a polymer of β -1,4-linked *N*-acetylglucosamine (GlcNAc), is essential for structural integrity, viability and virulence [1-5]. The chitin synthases (CHS) are integral

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi: 10.1016/j.bbrc.2011.06.180.

plasma membrane proteins that catalyze polymerization of UDP-GlcNAc into chitin fibrils. Phenotypes of single partial gene disruptions of the eight *Aspergillus fumigatus* chitin synthase genes have ranged from no phenotype to growth defects with diminished virulence, suggesting that individual chitin synthases have specialized roles [6-9].

The fungal cell wall integrity pathways encompass compensatory changes that occur with cell wall perturbation or cell wall gene mutations. In yeast and filamentous fungi, disruption of β -1,3-glucan synthesis results in cell wall changes including increased chitin deposition [10-12]. Treatment of *Candida albicans* with the β -1,3-glucan synthesis inhibiting echinocandin antifungals results in increased chitin synthase transcription [13]. Ectopic activation of echinocandin up-regulated pathways permits survival of *C. albicans* in the presence of lethal doses of echinocandins [11]. Understanding of the chitin-mediated cell wall responses is important for developing future antifungals.

We have previously reported echinocandin-mediated increases in chitin as well as transcriptional up-regulation of *A. fumigatus chsA* and *chsC* [12], suggesting that these chitin synthases act in the compensatory increase in chitin after cell wall stress. Mutation of the *A. fumigatus chsC* homolog in *Aspergillus nidulans*, *chsF*, has not been reported and its function is unknown [14]. Disruption of the *chsA* homolog, *A. nidulans chsC*, does not affect growth, however the protein is found in hyphal tips and developing septa [15,16]. In the model filamentous fungus *Neurospora crassa*, loss of the *chsC* homolog, *chs-1*, results in abnormal hyphal growth [17] and the proteins encoded by the *chsA* homolog, *chs-3*, and *chs-1* are localized to the hyphal tip and septa [18,19]. However, identification of the individual chitin synthases active in specific cell wall pathways has never been reported. To understand the roles of *A. fumigatus chsA* and *chsC* in cell wall biosynthesis and stress responses, we constructed and characterized complete deletions of *chsA* and *chsC* as well as the double deletion $\Delta chsA \Delta chsC$ strain. Our data suggest that *chsA* and *chsC* play only a minor role in cell wall biosynthesis and are not required for growth under cell wall stress. Thus, the molecular basis for increased cell wall chitin in response to echinocandins may consist of more complicated post-transcriptional regulation of chitin synthesis.

2. Materials and methods

2.1. Strains

All strains (Supplementary Table 1) are derived from AF293, a clinical isolate and sequence reference strain [20,21]. AF293.1 (*pyrG*⁻) is a uridine/uracil auxotroph [22], $\Delta argB$ (a gift from Nir Osherov) is an arginine auxotroph [23], and AF293.6 is deficient in both *pyrG* and *argB* [24].

2.2. Construction of the chitin synthase deletion and GFP-tagged strains

To generate the deletions, *chsA* (Afu2g01870) was replaced with *A. fumigatus argB* and *chsC* (Afu5g00760) with *Aspergillus parasiticus pyrG* (Supplementary Fig. 1A). Approximately 1.5 kb of *chsA* flanking sequence was cloned on either side of *argB* in the vector pArgB2 [24] and 1.5 kb of *chsC* flanking sequence on either side of *pyrG* in pJW24 [25] to generate the constructs. The amplified deletion cassettes were transformed into $\Delta argB$ or AF293.1 (*pyrG*⁻) protoplasts and selected for growth in the absence of arginine or uridine/uracil. Homologous integration was confirmed by Southern blot analysis (Supplementary Fig. 1). To generate the $\Delta chsA \Delta chsC$ strain, the $\Delta chsC$ construct was transformed into strain AF293.6. One $\Delta chsC \Delta argB$ strain was then transformed with the $\Delta chsA$ construct and screened by Southern blot analysis for proper integration of the two deletion constructs (Supplementary Fig. 1B).

Of note, the coding region of *chsA* is misannotated in the *Aspergillus* genome database (AspGD; www.aspgd.org). Based on homology to *A. nidulans* and *Aspergillus oryzae*, and the *chsA* cDNA from *A. fumigatus*, *chsA* contains two additional exons, one upstream of the currently defined start codon and the second at the 3' end due to omission of a small intron (Supplementary Fig. 1C).

For ChsC localization analysis, the *chsC* cDNA was cloned at the C-terminus of *egfp* in pUCnG-phleo to obtain an *egfp-chsC* fusion construct driven by the *otef* promoter. pUCnG-phleo was derived from pUCGH [26] via replacement of the N-terminal tag *egfp* with the C-terminal tag *egfp* and of the hygromycin resistance cassette with the phleomycin resistance gene (*ble*). pUCnG-*chsC* was transformed into the deletion strain to obtain a strain expressing amino terminal GFP-tagged ChsC.

2.3. Real-time PCR transcriptional analysis of the chitin synthase genes

For reverse transcription real-time PCR (RT-PCR) analysis, conidia (10^6 /ml) were grown in GMM broth [27] at 37 °C for 46.5 h, followed by addition of caspofungin (4 µg/ml) for 1.5 h. Primer sequences have been previously described [12]. The $2^{-\Delta\Delta C_T}$ analytic method [28] normalized to β -tubulin was used to calculate expression changes in each strain compared to wild type or within a strain after caspofungin treatment. Results are the mean of three technical triplicates $\pm$ standard deviation (SD).

2.4. Electron microscopy

For scanning electron microscopy (SEM), 10^4 conidia were grown in 20 ml GMM medium in a dish containing sterile coverslips for 24 h at 37 °C. The mycelia were fixed with PIPES buffer containing 3.5% formaldehyde and dehydrated in increasing ethanol concentrations. The fixed dried samples were gold sputter-coated and observed on an FE-SEM (FEI model XL-30) using an accelerating voltage of 7 kV.

For transmission electron microscopy (TEM), 10^7 conidia of each strain were inoculated into 10 ml GMM medium and grown for 24 h at 37 °C. The pellets were fixed in 5% glutaraldehyde in 0.1 M cacodylate buffer, embedded in low viscosity Spurr's resin, ultrathin sectioned and stained with uranyl acetate and lead citrate. Images were obtained on an FEI Tecnai G2 Twin transmission electron microscope.

2.5. Measurement of total chitin synthase activity

A nonradioactive activity assay was used [12,29]. Conidia (10^5 /ml) were grown in GMM broth for 24 h at 37 °C. Cultures were split and incubated for an additional 24 h with or without caspofungin (4 µg/ml). Microsomal membrane fractions were prepared as described [8,12]. Absorbance values were converted into chitin equivalents from the best-fit linear equation of the standard curve. Results of three technical replicates are presented as mean $\pm$ SD. Statistical analyses were performed using two-sample Student's *t* test assuming equal variances.

2.6. Measurement of chitin and β -glucan contents and galactomannan release

Conidia (10^5 /ml) were cultured in GMM broth for 48 h at 37 °C. Chitin assays were performed as described [30,31]. Glucosamine concentrations were calculated from the best-fit linear equation of the standard curve. Chitin levels are reported as glucosamine equivalents (µg/ml) per milligram tissue. Results are the average of three biological replicates $\pm$ SD.

The aniline blue assay method was used to quantify β -1,3-glucan [32-34]. β -1,3-glucan concentrations were calculated from the best-fit linear equation of the standard curve.

Values are expressed as relative fluorescence units (RFU) per milligram of tissue. Results are the average of three biological replicates $\pm$ SD.

The Platelia *Aspergillus* enzyme-linked immunosorbent assay (Bio-Rad) detects the side chains of the cell wall polysaccharide galactomannan [35,36]. Conidia (10^6 /ml) were grown in GMM broth for 24 h at 37 °C. Cultures were split and incubated for an additional 24 h with or without caspofungin (1, 10 or 15 μ g/ml). The culture filtrate was diluted 1:5000–10,000 to achieve an absorbance between that of the controls. The diluted culture filtrate was processed and the adequacy of the controls was calculated as per the manufacturer's instructions. Absorbance was standardized, corrected for dilution, and divided by the total dry weight of tissue; the data are presented as galactomannan units per milligram tissue. Results are the average of three technical replicates $\pm$ SD.

3. Results and discussion

3.1. The double mutant has disorganized cell wall structure but normal septa

Transcription of *chsA* and *chsC* increases after echinocandin treatment [12], suggesting that these chitin synthases may play a major role in the compensatory response to β -glucan inhibition. To examine the function of these genes in the biosynthesis of the cell wall and hyphal growth, and also in mediating cell wall responses to stress conditions, we generated *A. fumigatus* Δ *chsA*, Δ *chsC* and Δ *chsA* Δ *chsC* strains (Supplementary Fig. 1).

We found no differences in colony diameter or growth rate between these deletion strains and the wild-type strain, establishing these genes as nonessential for normal growth (Fig. 1A). The *A. nidulans* ChsA homolog, ChsC, is found at developing septa and hyphal tips [16]. Using a GFP-tagged fusion construct, we found that *A. fumigatus* ChsC diffusely localized to the plasma membrane, though with hyphal tip and septal accentuation (Fig. 1B), thus we examined the cell wall and septal structures.

Whereas the wild-type hyphae were full and smooth, many of the Δ *chsA* Δ *chsC* hyphae appeared rough and flattened, suggesting decreased structural integrity (Fig. 1C). The single mutant hyphae appeared similar though less severely affected (data not shown). On TEM analysis, the double mutant cell wall appeared externally disorganized (Fig. 1D).

3.2. *A. fumigatus* *chsA* and *chsC* are not required for growth during cell wall stress

To determine if *chsA* and *chsC* function in cell wall stress response, we grew the mutants under temperature and cell wall stress conditions. We found that the growth of the mutants at 30 and 42 °C, on acidic or basic media, or following the addition of calcofluor white (1–100 μ g/ml) or congo red (1–100 μ g/ml), both of which interfere with chitin assembly, was unchanged (data not shown).

Given the previously reported increased chitin synthase transcription after echinocandin treatment, loss of these chitin synthases is expected to result in echinocandin hypersensitivity. We therefore compared growth on GMM agar containing a range of caspofungin concentrations (0.03–10 μ g/ml). Similarly to the wild-type strain, the three mutant strains demonstrated growth inhibition up to 1 μ g/ml caspofungin, and a paradoxical increase in growth at higher concentrations (Fig. 2A), yet at no concentration were the mutant strains more inhibited than wild type.

We also examined growth over a range of micafungin concentrations (0.03–10 μ g/ml), an echinocandin without the paradoxical effect in *A. fumigatus*, and did not note any differences in radial growth (data not shown). Finally, growth of the mutants on GMM agar containing the chitin synthase inhibitor nikkomycin Z (0.03–10 μ g/ml) was unaffected (data

not shown). Interestingly, the paradoxical response occurred in the wild-type strain grown on both nikkomycin Z and caspofungin (data not shown), thus increased cell wall chitin seen after echinocandin treatment may not explain the paradoxical growth increase.

To assay for more subtle growth defects, we examined growth of serial dilutions of conidia on media containing caspofungin, micafungin, nikkomycin Z, congo red or calcofluor white; all yielded no change (Fig. 2B and data not shown).

3.3. Biochemical analysis of the $\Delta chsA \Delta chsC$ cell wall reveals no changes in chitin or β -glucan content, though galactomannan release is increased

To ascertain if the cell wall differences noted on electron microscopy are due to changes in chitin, we assayed chitin synthase activity in the mutant strains. Total activity was unchanged in the $\Delta chsC$ strain, but was decreased 53% in the $\Delta chsA$ strain and 76% in the $\Delta chsA \Delta chsC$ strain when compared to wild-type strain (Fig. 3A).

Although no significant decrease in cell wall integrity was noted in the mutant strains, we hypothesized that the decreased chitin synthase activity of the double mutant could lead to altered cell wall composition. To test this, we examined the cell wall content of chitin and β -glucan. Despite the decreased total activity, total cell wall chitin content was unaltered (Fig. 3B) and distribution of the chitin binding dye calcofluor white was unchanged (data not shown) in all three mutant strains. As we had previously shown that echinocandin treatment increased chitin content and transcription of *chsA* and *chsC* [12], we hypothesized that ChsA and ChsC may be responsible for the increased chitin and therefore assayed chitin content after echinocandin treatment in the $\Delta chsA$, $\Delta chsC$ and $\Delta chsA \Delta chsC$ strains. As shown in Fig. 3A, similar increases in cell wall chitin content were noted both in the single and double mutants when compared to wild type. This indicates that the transcriptional increase in *chsA* and *chsC* after echinocandin exposure is not related to the increase in cell wall chitin, or that following deletion of these genes *A. fumigatus* recovers via up-regulation of other chitin synthases. However, the decreased chitin synthase activity in the $\Delta chsA$ and $\Delta chsA \Delta chsC$ strains (Fig. 3A) suggests that the former is more likely.

As different cell wall polysaccharides are functionally connected, we also examined β -glucan content. β -1,3-glucan levels were not significantly increased in the mutants (Fig. 3C) and distribution appeared unaffected via aniline blue staining (data not shown). Therefore, despite the absence of two chitin synthase genes, the polysaccharide content of the cell wall of the $\Delta chsA \Delta chsC$ strain is similar to the wild type.

Though the biochemical assays did not identify differences in cell wall polysaccharides, electron microscopy suggested cell wall defects. There may be an alteration in the balance of chitin fibril types that could in turn change the externally-associated polysaccharides, thus we measured release of the cell wall polysaccharide galactomannan (GM) in the mutants. Following growth in unsupplemented media, the amount of GM released was similar between wild type and $\Delta chsA \Delta chsC$ (Fig. 3D). Interestingly, growth in the concentration of caspofungin that produces maximal inhibition of radial growth (1 μ g/ml) resulted in 31% more GM release in $\Delta chsA \Delta chsC$ than wild type, suggesting cell wall weakness or disorganization. However, under concentrations of caspofungin that produce paradoxical growth on solid media (10 and 15 μ g/ml), the absolute amount of GM released by both the wild type and the double mutant is similar (Fig. 3D and data not shown). This pattern was mirrored in cultures treated with micafungin (data not shown) even though there is no paradoxical growth response with micafungin in *A. fumigatus*, suggesting that GM release may occur from β -1,3-glucan synthesis inhibition [37].

3.4. Transcriptional analysis does not show compensatory increases in chitin synthase gene expression

Despite increased transcription of *chsA* and *chsC* in response to echinocandins [12], deletion of both of these genes did not yield growth defects under cell wall stress. To test whether this lack of phenotype was due to compensatory transcription of other chitin synthases, we assayed mRNA levels of the remaining chitin synthase genes, both under basal growth as well as after treatment with echinocandins. As shown in Fig. 4A, chitin synthase transcription was mostly unchanged. Transcription of *chsF* was increased slightly (approximately two-fold) in all three mutants, but no gene exhibited a larger increase in the double mutant versus the singles (Fig. 4A). In addition, transcriptional analysis after echinocandin treatment did not demonstrate consistently increased transcription of any chitin synthase gene (Fig. 4B). Thus, if the lack of phenotypes in the $\Delta chsA$, $\Delta chsC$ and $\Delta chsA \Delta chsC$ strains is due to other chitin synthase expression changes, this compensation is not mediated via increased transcription and must occur due to more downstream effects.

In summary, based on the results from our previous work we deleted two of the eight *A. fumigatus* chitin synthase genes to evaluate their role in the fungal cell wall biosynthesis, particularly in the compensatory deposition of chitin in response to β -glucan inhibition. We found that loss of either *chsA*, *chsC*, or both genes does not produce defects in hyphal growth, even under conditions that induce cell wall stress, and that the cell wall of the double mutant is only mildly affected. *A. fumigatus chsC* has previously been disrupted, and was shown to not have any growth defects [7], consistent with the phenotypes observed in our deletion mutant strain. *A. fumigatus chsA* has also been mutated and described as having no phenotypes, but further details have not been published [8]. Disruption of the closest *chsA* homolog in *A. nidulans*, *chsC*, which is nearly 80% identical and 90% similar, did not affect morphology or growth rate [15]. This suggests that either these genes play minor roles in growth and stress responses, or alternatively that the fungus is able to compensate via altered regulation of remaining chitin synthases.

The molecular basis of increased cell wall chitin in response to echinocandin treatment is thought to be due to increased chitin synthesis arising from higher chitin synthase gene transcription. We showed that intact function of two *A. fumigatus* chitin synthase genes with echinocandin-responsive transcription is not essential for this response, and that the cell wall changes may instead depend on post-transcriptional processes. Studies from many fungal species have shown that chitin biosynthesis is a highly regulated process, with substantial translational and post-translational components; future studies will need to be designed with this in mind.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Deletion of *chsA* and *chsC* results in cell wall disorganization. (A) After 4 days, radial growth of wild type or $\Delta chsA \Delta chsC$ (5×10^3 conidia/colony spot) was similar. (B) GFP-ChsC is on the plasma membrane, especially at the hyphal tips and septa. (C) SEM of hyphal tips at 20,000 $\times$. Many of the double mutant hyphae appear shrunken and friable, consistent with structural compromise. (D) TEM of hyphae including septa at 9600 $\times$.

Fig. 2.

Growth of the chitin synthase double mutant was similar to wild type under cell wall stress. (A) Radial growth of wild type and $\Delta chsA \Delta chsC$ (2×10^3 conidia/colony spot) on media containing caspofungin was identical after four days. (B) Growth after two days of serial dilutions of wild type and $\Delta chsA \Delta chsC$ (10^5 – 10^2 conidia/spot) on media containing 10 $\mu\text{g/ml}$ caspofungin was similar.

Fig. 3.

The cell wall chitin and β -glucan contents were unchanged. (A) Total *in vitro* chitin synthase enzymatic activity is decreased in $\Delta chsA$ and $\Delta chsA \Delta chsC$. An asterisk indicates significant differences between mutant strain and wild type ($p < 0.05$). (B) Total cell wall chitin content is not changed in the chitin synthase mutants without (solid bars) or with casprofungin treatment (1 μ g/ml; striped bars). No differences were identified when comparing the mutant strains to the wild type; all strains had higher chitin content comparing casprofungin treatment to no treatment (# indicates $p < 0.05$). (C) β -1,3-glucan content is not increased in the chitin synthase mutant strains, except for $\Delta chsC$ compared to wild type (* indicates $p < 0.05$). (D) Cell wall galactomannan release increases with growth in media containing echinocandin (# indicates $p < 0.05$). Interestingly, the double mutant strain showed increased release of galactomannan as compared to wild type (* indicates $p < 0.05$) at 1 μ g/ml casprofungin, but that difference was not present after treatment with higher drug concentrations.

Fig. 4.

Transcription of the remaining chitin synthase genes is unaffected. (A) Transcriptional profiling of *A. fumigatus* chitin synthase genes in mutant strains compared to wild type under untreated growth conditions revealed no changes in chitin synthase expression. (B) Analysis of gene transcription after caspofungin treatment (4 $\mu\text{g/ml}$ for 90 min immediately prior to harvest) comparing treated to untreated samples of the same strain shows minimal changes in chitin synthase expression in the mutant strains.