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Cell wall protection by the *Candida albicans* class I chitin synthases

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ABSTRACT

Candida albicans has four chitin synthases from three different enzyme classes which deposit chitin in the cell wall, including at the polarized tips of growing buds and hyphae, and sites of septation. The two class I enzymes, Chs2 and Chs8, are responsible for most of the measurable chitin synthase activity *in vitro*, but their precise biological functions *in vivo* remain obscure. In this work, detailed phenotypic analyses of a *chs2Δchs8Δ* mutant have shown that *C. albicans* class I chitin synthases promote cell integrity during early polarized growth in yeast and hyphal cells. This was supported by live cell imaging of YFP-tagged versions of the class I chitin synthases which revealed that Chs2-YFP was localized at sites of polarized growth. Furthermore, a unique and dynamic pattern of localization of the class I enzymes at septa of yeast and hyphae was revealed. Phosphorylation of Chs2 on the serine at position 222 was shown to regulate the amount of Chs2 that is localized to sites of polarized growth and septation. Independently from this post-translational modification, specific cell wall stresses were also shown to regulate the amount of Chs2 that localizes to specific sites in cells, and this was linked to the ability of the class I enzymes to reinforce cell wall integrity during early polarized growth in the presence of these stresses.

1. Introduction

Chitin is an essential structural polysaccharide component of cell walls and septa in fungi and is synthesized by multiple chitin synthase enzymes. Importantly, chitin is not found in human cells and therefore represents an attractive target for antifungal therapy. In *Candida albicans*, the most common serious fungal pathogen of humans, chitin is synthesized by a family of four isoenzymes which fall into three different classes of chitin synthase enzymes, Chs1 (class II), Chs3 (class IV), Chs2 and Chs8 (class I) (reviewed in Lenardon et al. (2010b)). Together, these enzymes deposit chitin at sites of growth, which includes the polarized tips of buds and hyphae, and sites of septation. Understanding how these enzymes coordinately synthesize chitin in fungal cells is a vitally important aspect of fungal cell biology and will inform strategies to exploit chitin as a target for the development of new antifungal drugs. The present study shows for the first time that the *C. albicans* class I enzymes contribute to the protection of the nascent cell wall during polarized growth and the integrity of cells experiencing cell wall stress.

Analysis of *chs* mutant phenotypes has given us clues about the individual roles of the chitin synthases during growth and cell division. For example, Chs1 is essential and is responsible for the synthesis of the primary septum (Munro et al., 2001), while Chs3 synthesizes the majority of chitin found in the cell wall as well as the chitin ring at division sites (Bulawa et al., 1995). The localization of Chs1-YFP and Chs3-YFP in live cells has provided further evidence to support these roles for Chs1 and Chs3 (Lenardon et al., 2007). The role of the two class I enzymes (Chs2 and Chs8) is less well understood, and these are revealed here.

Previous work has shown that deletion of *CHS2* and *CHS8* results in a 97–99% reduction of the chitin synthase activity that can be measured *in vitro*, with the deletion of *CHS2* alone accounting for an 80–91% reduction compared to wild-type (Munro et al., 2003), but *chs2*, *chs8* or *chs2chs8* mutants display few other obvious phenotypes under normal growth conditions (Gow et al., 1994; Mio

Abbreviations: YFP, yellow fluorescent protein; CFW, Calcofluor White; S222, serine at position 222; MIC, minimal inhibitory concentration; rpm, revolutions per minute; FCS, foetal calf serum; 5-FOA, 5-fluoroorotic acid; LB, Luria-Bertani; bp, base pairs; PBS, phosphate buffered saline; PI, propidium iodide; SEM, standard error of the mean; ANOVA, analysis of variance; SD, standard deviation.

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et al., 1996; Munro et al., 2003). The expression profile of the class I genes indicates that they may be involved in providing protection to cells during cell wall stresses since *CHS2* and *CHS8* are 3–3.5-fold up-regulated at the level of transcription when cells are grown in the presence of caspofungin, an echinocandin drug which targets β(1,3)-glucan synthesis in fungal cell walls (Walker et al., 2008), and 9–12-fold up-regulated when cells are grown in the presence of $CaCl_2$ and Calcofluor White (CFW) (Munro et al., 2007). This up-regulation of transcription correlates with an overall increase in the *in vitro* chitin synthase activity in membranes prepared from yeast cells treated with caspofungin or $CaCl_2$ and CFW (Munro et al., 2007; Walker et al., 2008). More recently, it has been shown that Chs2, and Chs2 and Chs8 can form salvage septa in the absence of all other chitin synthases, including the normally essential Chs1, provided that chitin synthesis has been activated by pretreatment of cells with $CaCl_2$ and CFW (Walker et al., 2013). It is also known that the effect of the Chs1 inhibitor (RO-09-3143) on wild-type cells is fungistatic, whereas it is fungicidal in a *chs2* mutant background (Sudoh et al., 2000). These observations suggest that Chs2 and Chs8 have significant biological functions under stress conditions that are not yet fully understood.

Other studies have shown that Chs8 is involved in chitin microfibril morphogenesis. *CHS8* is required for the synthesis of long chitin microfibrils in the septa of yeast and hyphae, and Chs2-YFP and Chs8-YFP are both located at sites of septation in yeast cells immediately prior to cytokinesis (Lenardon et al., 2007). Chs8-YFP has also been observed at septation sites in hyphae (Lenardon et al., 2007). A global analysis of the *C. albicans* phosphoproteome showed that Chs2 is phosphorylated on the serine at position 222 (S222) (Beltrao et al., 2009), although the significance of the phosphorylation of class I chitin synthases has not been investigated. Ultimately, the true biological function of the class I enzymes in *C. albicans* remains to be clarified.

The objective of this work was therefore to elucidate the biological function of Chs2 and Chs8 during normal growth conditions in *C. albicans*, to investigate the biological consequence of phosphorylation of Chs2 on S222 and to investigate the mechanism(s) by which the class I enzymes provide protection to cells during cell wall stress.

2. Materials and methods

2.1. Strains, media and growth conditions

Yeast cells were grown in YPD broth (1% (w/v) yeast extract, 2% (w/v) mycological peptone, 2% (w/v) glucose supplemented with 25 μg/ml uridine (YPD+uri), or SD broth (0.67% (w/v) yeast nitrogen base without amino acids with ammonium sulphate, 2% (w/v) glucose supplemented with 25 μg/ml uridine at 30 °C with shaking at 200 rpm. In some experiments, YPD+uri broth was supplemented with a sub-MIC concentration of caspofungin (0.016 μg/ml Cancidas®, Merck and Co., Inc., USA), or 0.2 M $CaCl_2$ and 0.1 mg/ml CFW (Fluorescent Brightener 28, Sigma–Aldrich Co.). To induce hyphal growth, yeast cells that had been grown overnight in YPD+uri were inoculated into pre-warmed 20% (v/v) foetal calf serum (FCS) supplemented with 25 μg/ml uridine at a concentration of 1×10^7 cells/ml and incubated at 37 °C with shaking at 200 rpm. Transformants were selected and maintained on SD plates (SD broth with 2% (w/v) purified agar) with appropriate auxotrophic supplements, and recycling of the *URA3* marker was achieved by plating cells on SD plates containing 0.1% (w/v) 5-fluoroorotic acid (5-FOA) and uridine (25 μg/ml). *E. coli* strains containing the plasmids pBSCHS2-2, pDDB57 and pYFP-URA3 were grown on Luria-Bertani (LB) medium (0.5% (w/v) yeast extract, 1% (w/v) tryptone, and 1% (w/v) NaCl) supplemented with 100 μg/ml ampicillin. *E. coli* containing pSN52 was grown on LB medium supplemented with 30 μg/ml kanamycin.

2.2. Construction of chs mutant strains

A heterozygous *CHS8*/*chs8* mutant was constructed using a PCR based method adapted from Noble and Johnson (2005). Primers MDL24 and MDL25 (Table S1) with 100 bp homology to the sequence immediately upstream of the start codon and 100 bp immediately downstream of the stop codon of *CHS8* were designed to anneal to sequences immediately adjacent to the *Candida dubliniensis HIS1* marker in pSN52 (Noble and Johnson, 2005). The resulting PCR product was transformed into *C. albicans* strain BWP17 (Table 1). His^+ colonies were screened by PCR using primers MDL28 and MDL29 (Table S1) to confirm that one copy of *CHS8* had been replaced by the *CdHIS1* marker. The resulting strain was designated *CHS8*/*chs8Δ0* (NGY609, Table 1).

The second *CHS2* and *CHS8* allele of the *CHS2*/*chs2Δ0* and *CHS8*/*chs8Δ0* heterozygous mutants (NGY479 and NGY609; Table 1) was disrupted using the mini ura-blaster method (Wilson et al., 2000). The disruption cassette containing *dpl200-URA3-dpl200* flanked by 100 bp of sequence homologous to the region of *CHS2* or *CHS8* immediately 5′ to the start codon and 100 bp of sequence homologous to the region of *CHS2* or *CHS8* immediately 3′ of the stop codon was PCR-amplified from pDDB57 (Wilson et al., 2000) using primers KP08, KP09 or KP17, KP18 (Table S1). The PCR product was transformed into the *CHS2*/*chs2Δ0* or *CHS8*/*chs8Δ0* heterozygous mutant. Ura^+ colonies were screened by PCR using primers KP10 and KP11 or KP19 and KP11 (Table S1). After 5-FOA selection to recycle *URA3*, the correct construction of the strains was confirmed by PCR using primers KP10 and KP12 or KP19 and KP20 (Table S1) and by Southern analysis using a probe generated by PCR using the primers KP13 and KP14 or KP21 and KP22 (Table S1) which amplifies the sequence from −511 to −29 relative to ATG^{CHS2} or −808 to −220 relative to ATG^{CHS8}. These strains were designated *chs2Δ0* and *chs8Δ0* (NGY603 and NGY608; Table 1).

C. albicans strains that express mutant forms of Chs2 were constructed by re-introducing one copy of the mutated *chs2* alleles at the native chromosomal locus of the *chs2Δ0* null mutant strain (NGY603; Table 1). The promoter, ORF and terminator of the *CHS2* gene (−664 to +3177 relative to ATG^{CHS2}) were amplified by PCR using the primers CHS2Ia and CHS2IIb (Table S1) which introduced an *Apa*I and *Sal*I site at each end. Similarly, the *CHS2* terminator (+3031 to +3177 relative to ATG^{CHS2}) was amplified using the primers CHS2IIIa and Chs2IIIb (Table S1) which introduced an *Xba*I and *Not*I site at each end. The two PCR products were then ligated between the *Apa*I and *Sal*I sites, and the *Xba*I and *Not*I sites respectively of pBS-URA3 (Lenardon et al., 2010a) to generate the plasmid pBSCHS2-2. pBSCHS2-2 contains the *CHS2* promoter, *CHS2* ORF, *CHS2* terminator, *URA3* and a second copy of the *CHS2* terminator between the *Not*I and *Apa*I sites in pBluescript SK+ (Stratagene) which facilitates the integration of the *Not*I-*Apa*I cassette at the *CHS2* locus by homologous recombination and recycling of the *URA3* selective marker.

The QuickChange®II XL Site-Directed Mutagenesis kit (Stratagene) was used to generate site-specific mutations in the *CHS2* ORF in the plasmid pBSCHS2-2. An S222A mutation was generated by changing the TCA at position +664 in the *CHS2* ORF to GCA using the mutagenic oligonucleotide primers KPS222A1 and KPS222A2 (Table S1). This point mutation created a version of Chs2 that cannot be phosphorylated on S222. To generate a form of Chs2 that mimics constitutive phosphorylation on S222, an S222E mutation was introduced by changing the TCA to GAA using the mutagenic oligonucleotide primers KPS222E1 and KPS222E2 (Table S1). Point mutations were confirmed by DNA sequencing