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# The PKC, HOG and $Ca^{2+}$ signalling pathways co-ordinately regulate chitin synthesis in *Candida albicans*

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## Summary

**Chitin is an essential component of the fungal cell wall and its synthesis is under tight spatial and temporal regulation. The fungal human pathogen *Candida albicans* has a four member chitin synthase gene family comprising of *CHS1* (class II), *CHS2* (class I), *CHS3* (class IV) and *CHS8* (class I). LacZ reporters were fused to each *CHS* promoter to examine the transcriptional regulation of chitin synthesis. Each *CHS* promoter had a unique regulatory profile and responded to the addition of cell wall damaging agents, to mutations in specific *CHS* genes and exogenous $Ca^{2+}$. The regulation of both *CHS* gene expression and chitin synthesis was co-ordinated by the PKC, HOG MAP kinase and $Ca^{2+}$/calcineurin signalling pathways. Activation of these pathways also resulted in increased chitin synthase activity *in vitro* and elevated cell wall chitin content. Combinations of treatments that activated multiple pathways resulted in synergistic increases in *CHS* expression and in cell wall chitin content. Therefore, at least three pathways co-ordinately regulate chitin synthesis and activation of chitin synthesis operates at both transcriptional and post-transcriptional levels.**

## Introduction

The fungal cell wall is a dynamic structure whose composition and structural organization is regulated during the cell cycle and in response to changing environmental conditions, imposed stresses and mutations in cell wall biosynthetic processes (reviewed in Klis *et al*., 2006; Ruiz-Herrera *et al*., 2006). Chitin and β(1–3)-D-glucan, represent the main structural components of the fungal cell wall. These polysaccharides oppose the positive turgor pressure within the cell and ultimately determine the morphology of the cell (Munro and Gow, 2001; Klis *et al*., 2002; Roncero, 2002). Chitin and glucan synthesis therefore play fundamental roles in maintaining fungal cell integrity during growth and morphogenesis and in adaptation to stress (Cabib, 1987; Wessels, 1990; Shaw *et al*., 1991; Sietsma and Wessels, 1994; Gooday, 1995). Because these structural polysaccharides do not occur in mammals and are essential for fungi, there is considerable potential for cell wall synthesis as a target for antifungal drugs (Munro and Gow, 1995; Munro *et al*., 2001; Odds *et al*., 2003). New generation echinocandins that target the synthesis of cell wall β(1–3)-D-glucan are proving effective agents in the treatment of opportunistic fungal pathogens such as *Candida albicans* (Denning, 2003). Chitin synthase inhibitors have not yet been discovered that have clinical use in the treatment of fungal infections (Odds *et al*., 2003).

Regulation of chitin synthesis occurs both at the transcriptional and post-translational levels and is dependent on precise targeting and activation of chitin synthases to specific locations in the plasma membrane, and the provision of adequate substrate (Munro and Gow, 1995). All fungi examined to date have multiple genes encoding chitin synthase families (Munro and Gow, 2001; Roncero, 2002; Ruiz-Herrera and San-Blas, 2003). Individual chitin synthase enzymes perform distinct functions at specific stages of the cell cycle. *Saccharomyces cerevisiae* has three chitin synthase enzymes – Chs1p (Class I), Chs2p (Class II) and Chs3p (Class IV) while *C. albicans* has four chitin synthases – two class I enzymes – *Ca*Chs2p and *Ca*Chs8p, *Ca*Chs3p (Class IV) and *Ca*Chs1p (a class II enzyme which is the orthologue of *Sc*Chs2p). Relatively little is known about the transcriptional regulation of chitin synthase genes in fungi but considerable attention has been focused on post-transcriptional regulation by Chs4–7, which influences Chs3p chitin synthase activation and localization in *S. cerevisiae* and *C. albicans*. *Sc*Chs7p controls exit of *Sc*Chs3p from the ER, *Sc*Chs5p and *Sc*Chs6p regulate its exit from the *trans*-Golgi

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network (Ziman *et al*., 1996; Santos and Snyder, 1997; Santos *et al*., 1997; Ziman *et al*., 1998; Trilla *et al*., 1999). *Sc*Chs4p tethers *Sc*Chs3p to the septins at the mother-bud neck via *Sc*Bni4p (Demarini *et al*., 1997; Trilla *et al*., 1997). Chitin synthesis is therefore influenced by endogenous and exogenous factors that directly and indirectly regulate the chitin synthase catalytic proteins.

Disruption of genes in cell wall biosynthetic pathways of *S. cerevisiae* and *C. albicans* often results in alteration and redistribution of chitin and β(1–3)-D-glucan in the cell wall, the synthesis of new cell wall proteins and changes in their cross-linking to cell wall polysaccharides (reviewed in Popolo *et al*., 2001; Klis *et al*., 2002; Klis *et al*., 2006). Defects in cell wall integrity are sensed by the transmembrane proteins of the Mid2p and the Wscp family, which signal via the Rom2p guanine nucleotide exchanger leading to activation of the Rho1p GTPase. Rho1p has many downstream targets including protein kinase C and the β(1–3)-D-glucan synthase subunits Fks1p and Fks2p (Popolo *et al*., 2001). In *S. cerevisiae* this 'cell wall salvage' or 'cell wall compensatory' pathway is activated in response to cell wall perturbing agents such as Calcofluor white (CFW), Congo Red (CR), caffeine, β-glucanases and cell wall mutations and is mediated primarily through the PKC cell integrity MAP kinase cascade and its downstream target the transcription factor Rlm1p (Lagorce *et al*., 2003; Boorsma *et al*., 2004; Garcia *et al*., 2004). In *S. cerevisiae*, elevation of chitin levels in response to activation of the salvage pathway is largely dependent upon *Sc*Chs3p (Valdivieso *et al*., 2000; Carotti *et al*., 2002). Several studies have highlighted the importance of signalling systems in co-ordinating this regulation. A higher proportion of *Sc*Chs3p localized to the plasma membrane in heat-stressed cells (Valdivia and Schekman, 2003). This mobilization of *Sc*Chs3p was dependent upon activation of Rho1p and Pkc1p, and the phosphorylation of *Sc*Chs3p by Pkc1p.

A second MAP kinase cascade, the high osmolarity glycerol response (HOG) pathway, has also been suggested to play a role in regulating cell wall architecture in *S. cerevisiae* (Garcia-Rodriguez *et al*., 2000; Kapteyn *et al*., 2001) and in *C. albicans* (Eisman *et al*., 2006). In *S. cerevisiae*, the HOG pathway is required for the response to CFW and mutants in several components of the pathway are resistant to CFW (Garcia-Rodriguez *et al*., 2000). In addition, changes in osmotic pressure have been shown to regulate chitin synthase activity in the dimorphic fungus *Benjaminiella poitrasii* suggesting the HOG pathway is involved in chitin regulation (Deshpande *et al*., 1997).

Transcript profiling studies have implicated $Ca^{2+}$ in the regulation of *ScCHS1* (Yoshimoto *et al*., 2002). In addition, sequences recognized by the $Ca^{2+}$/calcineurin-dependent transcription factor Crz1p/Tcn1p have been identified upstream of a number of genes that are upregulated in cell wall mutants that activate the cell wall salvage pathway (Lagorce *et al*., 2003; Boorsma *et al*., 2004; Garcia *et al*., 2004; Karababa *et al*., 2006). These studies directed us towards examining the role of $Ca^{2+}$ signalling in the regulation of chitin synthesis in *C. albicans*.

Each of the four *C. albicans* Chs enzymes plays a distinct role in cellular growth. *Ca*Chs1p synthesizes the septal chitin and contributes to chitin in the lateral cell wall and is essential for viability in both the yeast and hyphal forms (Munro *et al*., 2001). *Ca*Chs2p encodes the major chitin synthase activity *in vitro*, and *chs2*Δ null mutants have fractionally less chitin in hyphal cells (Gow *et al*., 1994; Munro *et al*., 1998). *Ca*Chs3p synthesizes the majority of the chitin in the lateral cell wall and the ring of chitin at the site where a new bud emerges (Bulawa *et al*., 1995; Mio *et al*., 1996). *Ca*Chs8p and *Ca*Chs2p account for almost all the measurable *in vitro* chitin synthase activity in membrane preparations but are non-essential for growth (Munro *et al*., 2003). In *C. albicans*, northern analyses suggested that *CaCHS2* and *CaCHS3* are upregulated shortly after induction of hyphal formation while *CaCHS1* is expressed at low but constant levels in both yeast and hyphae (Chen-Wu *et al*., 1992; Munro *et al*., 1998). Hyphal formation in *C. albicans* is accompanied by a three to fivefold increase in the chitin content of the cell wall (Chattaway *et al*., 1968; Sullivan *et al*., 1983; Munro *et al*., 1998).

Here we examine the regulation of chitin synthesis of *C. albicans* and describe the signalling pathways that co-ordinate this process. We used a *lacZ* reporter gene fused to the putative promoters of each of the *C. albicans CHS* genes to test hypotheses about the expression of *CHS* genes when cells are challenged with cell wall perturbing agents or subjected to environmental stresses. We show that transcriptional regulation of the *CHS* genes is stimulated via at least three pathways – the PKC and HOG MAP kinase cascades and the $Ca^{2+}$/calcineurin pathway. Each of the four chitin synthase promoters was regulated differentially, but all were activated by exogenous $Ca^{2+}$ in a calcineurin and Crz1p-dependent manner. In addition, hyper-stimulation of *CHS* gene expression was observed when multiple signalling pathways were activated simultaneously and this resulted in greatly elevated cell wall chitin levels.

## Results

### *Endogenous* CHS *promoter activity in wild-type cells and* chsΔ *mutants*

Transcriptional activity of the four chitin synthase genes of *C. albicans* was characterized using a *lacZ* reporter system. Plasmid placpoly 6 containing *URA3* and *RPS1*

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**Table 1.** *Candida albicans* strains used in this study.

| Strain | Parental strain | Genotype[a] | Source or reference |
|---|---|---|---|
| CAF2-1 | SC5314 | *URA3/ura3Δ::λimm434* | Fonzi and Irwin (1993) |
| CAI-4 | CAF2-1 | *ura3Δ::λimm434/ura3Δ::λimm434* | Fonzi and Irwin (1993) |
| NGY210 | CAI-4 | *RPS1/rps1Δ*::pCHS1plac | This study |
| NGY211 | CAI-4 | *RPS1/rps1Δ*::pCHS2plac | This study |
| NGY212 | CAI-4 | *RPS1/rps1Δ*::pCHS3plac | This study |
| NGY213 | CAI-4 | *RPS1/rps1Δ*::pCHS8plac | This study |
| C155 | C154 | *chs2Δ::hisG/chs2Δ::hisG* | Mio *et al.* (1996) |
| Myco3 | Myco4 | *chs3Δ::hisG/chs3Δ::hisG* | Bulawa *et al.* (1995) |
| NGY128 | CAI-4 | *chs8Δ::hisG/chs8Δ::hisG* | Munro *et al.* (2003) |
| C157 | C155 | *chs2Δ::hisG/chs2Δ::hisG; chs3Δ::hisG/chs3Δ::hisG* | Mio *et al.* (1996) |
| NGY138 | CAI-4 | *chs2Δ::hisG/chs2Δ::hisG; chs8Δ::hisG/chs8Δ::hisG* | Munro *et al.* (2003) |
| CM1613c | CAI-4 | *mkc1Δ::hisG/mkc1Δ::hisG* | Navarro-Garcia *et al.* (1995) |
| DSY2842 | CAI-4 | *crz1Δ::hisG/crz1Δ::hisG* | Karababa *et al.* (2006) |
| DSY2101 | CAI-4 | *cna1Δ::hisG/cna1Δ::hisG* | Sanglard *et al.* (2003) |
| CNC15 | RIM1000 | *hog1Δ::hisG/hog1Δ::hisG* | Alonso-Monge *et al.* (1999) |
| NGY258 | C155 | *chs2Δ::hisG/chs2Δ::hisG; RPS1/rps1Δ*::pCHS1plac | This study |
| NGY260 | C155 | *chs2Δ::hisG/chs2Δ::hisG, RPS1/rps1Δ*::pCHS3plac | This study |
| NGY261 | C155 | *chs2Δ::hisG/chs2Δ::hisG, RPS1/rps1Δ*::pCHS8plac | This study |
| NGY262 | Myco3 | *chs3Δ::hisG/chs3Δ::hisG, RPS1/rps1Δ*::pCHS1plac | This study |
| NGY263 | Myco3 | *chs3Δ::hisG/chs3Δ::hisG, RPS1/rps1Δ*::pCHS2plac | This study |
| NGY265 | Myco3 | *chs3Δ::hisG/chs3Δ::hisG, RPS1/rps1Δ*::pCHS8plac | This study |
| NGY290 | NGY128 | *chs8Δ::hisG/chs8Δ::hisG, RPS1/rps1Δ*::pCHS1plac | This study |
| NGY291 | NGY128 | *chs8Δ::hisG/chs8Δ::hisG, RPS1/rps1Δ*::pCHS2plac | This study |
| NGY292 | NGY128 | *chs8Δ::hisG/chs8Δ::hisG, RPS1/rps1Δ*::pCHS3plac | This study |
| NGY270 | NGY138 | *chs2Δ::hisG/chs2Δ::hisG; chs8Δ::hisG/chs8Δ::hisG; RPS1/rps1Δ*::pCHS1plac | This study |
| NGY272 | NGY138 | *chs2Δ::hisG/chs2Δ::hisG; chs8Δ::hisG/chs8Δ::hisG; RPS1/rps1Δ*::pCHS3plac | This study |
| NGY266 | C157 | *chs2Δ::hisG/chs2Δ::hisG; chs3Δ::hisG/chs3Δ::hisG; RPS1/rps1Δ*::pCHS1plac | This study |
| NGY269 | C157 | *chs2Δ::hisG/chs2Δ::hisG; chs3Δ::hisG/chs3Δ::hisG; RPS1/rps1Δ*::pCHS8plac | This study |
| NGY294 | DSY2101 | *cna1Δ::hisG/cna1Δ::hisG; RPS1/rps1Δ*::pCHS1plac | This study |
| NGY295 | DSY2101 | *cna1Δ::hisG/cna1Δ::hisG; RPS1/rps1Δ*::pCHS2plac | This study |
| NGY296 | DSY2101 | *cna1Δ::hisG/cna1Δ::hisG; RPS1/rps1Δ*::pCHS3plac | This study |
| NGY297 | DSY2101 | *cna1Δ::hisG/cna1Δ::hisG; RPS1/rps1Δ*::pCHS4plac | This study |
| NGY282 | CM1613c | *mkc1Δ::hisG/mkc1Δ::hisG*; *RPS1/rps1Δ*::pCHS1plac | This study |
| NGY283 | CM1613c | *mkc1Δ::hisG/mkc1Δ::hisG*; *RPS1/rps1Δ*::pCHS2plac | This study |
| NGY284 | CM1613c | *mkc1Δ::hisG/mkc1Δ::hisG*; *RPS1/rps1Δ*::pCHS3plac | This study |
| NGY285 | CM1613c | *mkc1Δ::hisG/mkc1Δ::hisG*; *RPS1/rps1Δ*::pCHS8plac | This study |
| NGY314 | DSY2842 | *crz1Δ::hisG/crz1Δ::hisG*; *RPS1/rps1Δ*::pCHS1plac | This study |
| NGY315 | DSY2842 | *crz1Δ::hisG/crz1Δ::hisG*; *RPS1/rps1Δ*::pCHS2plac | This study |
| NGY316 | DSY2842 | *crz1Δ::hisG/crz1Δ::hisG*; *RPS1/rps1Δ*::pCHS3plac | This study |
| NGY317 | DSY2842 | *crz1Δ::hisG/crz1Δ::hisG*; *RPS1/rps1Δ*::pCHS8plac | This study |
| NGY321 | CNC15 | *hog1Δ::hisG/hog1Δ::hisG*; *RPS1/rps1Δ*::pCHS1plac | This study |
| NGY322 | CNC15 | *hog1Δ::hisG/hog1Δ::hisG*; *RPS1/rps1Δ*::pCHS2plac | This study |
| NGY323 | CNC15 | *hog1Δ::hisG/hog1Δ::hisG*; *RPS1/rps1Δ*::pCHS3plac | This study |
| NGY324 | CNC15 | *hog1Δ::hisG/hog1Δ::hisG*; *RPS1/rps1Δ*::pCHS8plac | This study |

**a.** All strains apart from CAF2-1 are also *ura3Δ::λimm434/ura3Δ::λimm434*.

was used to create a fusion between the promoter of each *C. albicans* chitin synthase gene and the *Streptococcus thermophilus lacZ* open reading frame (ORF). A 1 kb region upstream from the ATG start codon of *CHS1*, *CHS2*, *CHS3* and *CHS8* was cloned into placpoly 6 generating, respectively, plasmids pCHS1plac, pCHS2plac, pCHS3plac and pCHS8plac. Ura$^-$ *C. albicans* cells were transformed with each linearized plasmid, homologous recombination resulted in integration of the plasmid at the *RPS1* locus and Ura$^+$ transformants were selected. Transformants were screened by Southern blot analysis and those with a single copy integration of pCHSplac (strains NGY210-NGY213, Table 1) were analysed further. The *CHS2* and *CHS3* promoters had the highest and lowest level of expression, respectively, for growth in YPD medium ($P < 0.05$) (Fig. 1). Real-time quantitative polymerase chain reaction (PCR) confirmed these results (data not shown).

The pCHSplac plasmids were transformed into isogenic mutant strains derived from CAI-4 with single or double

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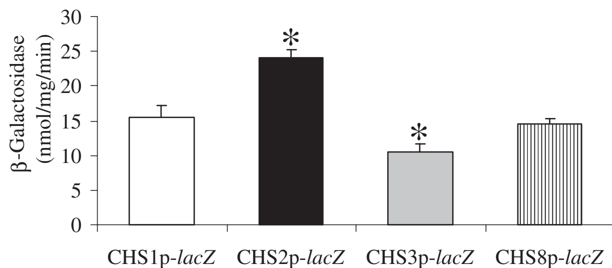


**Fig. 1.** Native *CHS* promoter activities. The β-galactosidase activity of cell-free protein extracts of parental *C. albicans* strain CAI-4 transformed with CHS1p-*lacZ*, CHS2p-*lacZ*, CHS3p-*lacZ* or CHS8p-*lacZ*, grown on YPD at 30°C. Data are from three independent experiments (average ± SD $n = 9$). Asterisks indicate significant differences from CHS1p-*lacZ* where $P \leq 0.05$.

*CHS* gene disruptions (Table 1) to test whether deletion of *CHS* genes results in a compensatory upregulation of other members of the *CHS* family. The *CHS1* promoter activity was significantly increased in the single mutants *chs3*Δ (strain Myco3) and *chs8*Δ (NGY138) and the double mutants *chs2*Δ *chs8*Δ (NGY128) and *chs2*Δ *chs3*Δ (C157) (Fig. 2). Chs1p may contribute to the maintenance of lateral wall integrity (Munro *et al.*, 2001) and play a compensatory role when *CHS3* and *CHS8* gene functions are lost. Expression from the *CHS8* promoter was stimulated when either *CHS2* or *CHS3* were deleted and was increased further in the *chs2*Δ *chs3*Δ double null mutant. *CHS3* expression was slightly elevated in *chs8*Δ and *chs2*Δ *chs8*Δ mutants while the *CHS2* promoter did not show any significant changes in any of the mutants tested (Fig. 2). Therefore, the deletion of single *CHS* genes resulted in activation of the expression of others.

## CHS *promoter activity responds to wall perturbing agents*

Transcriptional regulation of the four *CHS* genes was determined in response to various environmental changes and perturbations (Fig. 3). Growth at 37°C stimulated *CHS1*, *CHS2* and *CHS3* promoters compared with growth at 25°C (not shown) and 30°C (control conditions). The addition of SDS, which perturbs membrane integrity, or CFW that interferes with cell wall assembly, induced expression from three of the four promoters (Fig. 3). CR

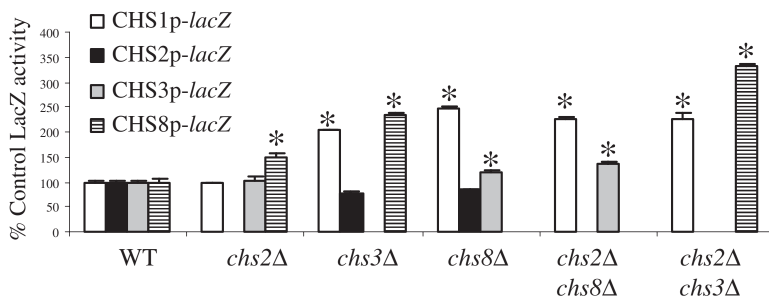


**Fig. 2.** Compensatory activation of *CHS* promoters in response to mutation in *CHS* genes. β-galactosidase activities are from cell-free protein extracts of *C. albicans* strain CAI-4 and chitin synthase mutants transformed with CHSp-*lacZ* constructs. Assays were performed on three independent YPD cultures grown at 30°C (average ± SD $n = 9$). Asterisks indicate differences where $P \leq 0.05$ compared with the wild-type parent strain transformed with the same promoter–*lacZ* fusion.

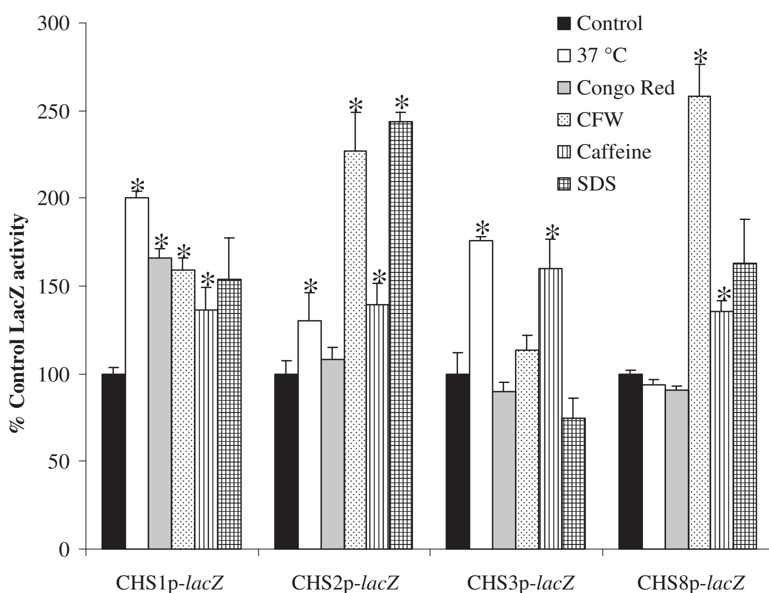


**Fig. 3.** *CHS* promoters are activated by cell wall and membrane perturbing agents. β-galactosidase activities of cell-free protein extracts of *C. albicans* strain CAI-4 transformed with the CHSp-*lacZ* constructs (strains NGY210 – NGY213, Table 2). Cells were grown on YPD supplemented with the compounds at 30°C or at 37°C and harvested at $OD_{600}$ 0.8. Assays were performed in triplicate on three independent cultures (average ± SD $n = 9$). Asterisks denote significant differences to controls that were untreated strains, NGY210 – NGY213, grown on YPD at 30°C ($P \leq 0.05$).

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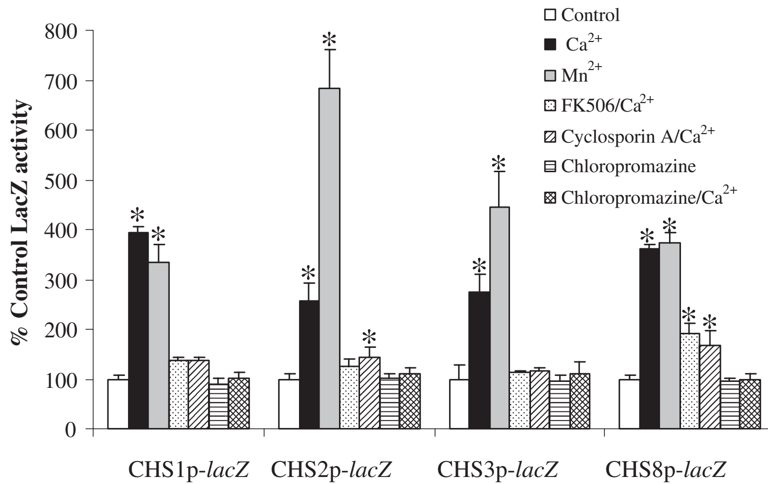


**Fig. 4.** *CHS* promoters response to $Ca^{2+}$ and $Mn^{2+}$. β-galactosidase activities were measured in cell-free protein extracts of *C. albicans* strain CAI-4 transformed with the CHSp-*lacZ* constructs (strains NGY210 – NGY213, Table 2). Cells were grown at 30°C on YPD supplemented with $Ca^{2+}$ and $Mn^{2+}$ ± FK506, cyclosporin A or chloropromazine. Assays were performed in triplicate on three independent cultures (average ± SD $n = 9$). Asterisks indicate that there are significant differences to untreated control samples prepared in the same experiment from strains NGY210 – NGY213 ($P \leq 0.05$).

stimulated only *CHS1* expression. Caffeine, an inhibitor of cAMP phosphodiesterase, stimulates dual phosphorylation of *Sc*Slt2, the MAP kinase component of the PKC cell wall integrity signal transduction pathway (Martin *et al*., 2000). The addition of 12 mM caffeine to the growth medium resulted in significantly elevated expression from all four *CHS* promoters.

The *CHS* transcriptional response to cations and salts – 200 mM $Ca^{2+}$, $Mn^{2+}$, $K^{2+}$, $Li^{2+}$, $Mg^{2+}$ or 800 mM NaCl was tested. Addition of $K^{2+}$, $Li^{2+}$, $Mg^{2+}$ or $Na^{2+}$ had no detectable effects (data not shown), however, exogenous $Ca^{2+}$ and $Mn^{2+}$ activated all four *CHS* promoters (Fig. 4). Some response was observed even with 5 mM $Ca^{2+}$ (data not shown). Exogenously applied $Ca^{2+}$ leads to activation of the calcineurin pathway, which induces dephosphorylation of the Crz1p transcription factor (Cyert, 2003). The calcineurin specific inhibitors FK506 and Cyclosporin A inhibited $Ca^{2+}$ activation of *CHS* transcription (Fig. 4). Activation of *CHS* expression with $Mn^{2+}$ was also reduced, but not totally blocked, by simultaneous addition of FK506 (data not shown) suggesting the $Mn^{2+}$-specific activation occurred in part through the calcineurin signalling pathway but also involved a calcineurin-independent mechanism. These results suggest that $Ca^{2+}$ and $Mn^{2+}$ activated *CHS* expression via both calcineurin/Crz1-dependent and independent mechanisms. The calmodulin inhibitor chloropromazine had no effect on *CHS* transcriptional activity but chloropromazine with 200 mM $Ca^{2+}$ completely inhibited the $Ca^{2+}$-activation response. The calcium ionophore A23187 also inhibited the $Ca^{2+}$-activation response (data not shown). Inhibition by chloropromazine suggested that the observed $Ca^{2+}$ stimulation involved the classical $Ca^{2+}$ signalling pathway acting through calmodulin and calcineurin.

To corroborate these findings the four *CHS*-reporter constructs were transformed into null mutant strains lacking genes involved in the calcineurin pathway. The Crz1p transcription factor is dephosphorylated when the phosphatase calcineurin is activated by $Ca^{2+}$/calmodulin. It then enters the nucleus and induces expression of a number of genes, many of which encode proteins with cell wall-related functions (Yoshimoto *et al*., 2002; Lagorce *et al*., 2003; Garcia *et al*., 2004; Karababa *et al*., 2006; Pardini *et al*., 2006). Putative calcium-dependent response element (CDRE) motifs that are recognized by the Crz1p transcription factor were found in the promoter region of the *CHS* genes in *C. albicans* (Table 2). In the *cna1*Δ strain, which is mutated in one of the calcineurin subunits, the *CHS2* and *CHS8* promoters still responded to $Ca^{2+}$, but the response of the *CHS2* promoter was reduced (Fig. 5). The $Ca^{2+}$ responses of the *CHS1* and *CHS3* promoters were not significantly different to the untreated *cna1*Δ control. Stimulation with $Ca^{2+}$ was abrogated by simultaneous addition of FK506. In the *crz1*Δ mutant background, expression from the *CHS1* promoter was elevated when cells were grown in YPD, and addition of $Ca^{2+}$ did not further stimulate *CHS1* expression (Fig. 5). The response of the *CHS2* and *CHS3* promoters to exogenous $Ca^{2+}$was significantly different in the *crz1*Δ mutant compared with wild-type cells. Therefore, *CHS2* and *CHS3* were activated in part via the $Ca^{2+}$/calcineurin/Crz1 pathway but Crz1p repressed the expression of *CHS1*. Transformation of the CHSp-*lacZ* constructs into the double calcium channel mutant *mid1*Δ *cch1*Δ had no significant effect on $Ca^{2+}$-stimulated activation of expression (data not shown).

### *Synergistic stimulation of* CHS *promoters by combined* $Ca^{2+}$ *and CFW treatment*

Addition of $Ca^{2+}$ or CFW stimulated the *CHS* promoters – therefore we tested the effects of combinations of $Ca^{2+}$ and CFW treatments. All four promoters were hyper-stimulated by combined treatment with $Ca^{2+}$ and CFW

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**Table 2.** Putative transcription factor binding sites.

| Gene | Position[a]/strand | CDRE[b] | Position[a]/strand | Sko1 binding site[c] |
|---|---|---|---|---|
| CHS1 | –351/+ | GGGCTTC | –121/+ | TACGT |
| | –380/– | TGGCTTG | –851/– | TACGT |
| | –744/+ | AGGCTCC | | |
| | –810/+ | TGGCTCT | | |
| CHS2 | –539/– | TGGCTTT | | |
| | –879/+ | GGGCGTG | | |
| | –918/+ | AGGCTGA | | |
| CHS3 | –498/– | AGGCGGG | –130/+ | TACGT |
| | –904/– | AGGCTCA | –665/– | TACGT |
| CHS8 | –847/– | TGGCTTC | –782/+ | TACGT |
| | –893/+ | AGGCTTA | | |

**a.** Start codon A taken as position 1.
**b.** Crz1p consensus NGGC(G/T)CA.
**c.** Sko1p binding site consensus TACGT.

(Table 3). The pCHSplac plasmids were transformed into mutant strains lacking MAP kinase genes of the PKC and HOG signal transduction pathways (Table 1). The first mutant tested had a disrupted *MKC1* gene that encodes the MAP kinase of the PKC pathway (Navarro-Garcia *et al*., 1998). In the *mkc1*Δ strain background the *CHS8* promoter, and to a lesser extent, the *CHS2* promoter had reduced activity. All four *CHS* promoters were still stimulated by $Ca^{2+}$ in the *mkc1*Δ mutant (Table 3), therefore the $Ca^{2+}$-induced upregulation of *CHS* promoters can be independent of the PKC pathway. However, the ability of the *CHS2* and *CHS8* promoters to respond to CFW was impaired in the *mkc1*Δ mutant. All four promoters were stimulated by the combined $Ca^{2+}$/CFW treatment but the level of stimulation of *CHS2* and *CHS8* promoters was significantly less in *mkc1*Δ cells compared with wild-type cells. The response of the *CHS* promoters to CFW and combined $Ca^{2+}$/CFW was also examined in the *crz1*Δ mutant (Table 3). Again the response of *CHS2* and *CHS8* promoters to CFW was significantly reduced in the *crz1*Δ mutant and the $Ca^{2+}$/CFW induction of all four promoters was dramatically reduced (three- to five-fold).

Three of the four *CHS* promoter sequences contained ATF/CREB elements – potential binding sites for the Sko1p transcription factor that is regulated by Hog1p (Table 2) (Proft *et al*., 2005). Expression from the *CHS1*, *CHS2* and *CHS8* promoters was reduced in the *hog1*Δ mutant compared with wild-type cells in YPD and in the presence of $Ca^{2+}$ or CFW (Table 3). In contrast, expression from the *CHS3* promoter was increased in the *hog1*Δ mutant, suggesting Hog1p normally repressed *CHS3* transcription. In the *hog1*Δ mutant the *CHS3* promoter still responded to exogenous $Ca^{2+}$, but not to CFW and combinations of $Ca^{2+}$ and CFW stimulated the *CHS3* promoter in both the *hog1*Δ and wild-type backgrounds. The $Ca^{2+}$/CFW-induced stimulation of the *CHS1* and *CHS2* promoters was not significantly altered in the *hog1*Δ strain but the level of expression from the *CHS8* promoters was significantly less than in wild-type cells. Together these data suggest that Mkc1p, Crz1p and Hog1p play significant roles in the $Ca^{2+}$/CFW hyper-stimulation of *CHS* promoters.

### *Exogenous $Ca^{2+}$ stimulates chitin synthase activity*

We tested whether the $Ca^{2+}$-activated *CHS* gene expression translated into measurably higher chitin synthase enzyme activity. *C. albicans y*east cells were cultured in YPD or YPD plus 100 mM $Ca^{2+}$ for 5 h and membrane fractions of wild type (CAI-4), *chs*Δ and signalling mutant strains were prepared and assayed for chitin synthase activity. The specific chitin synthase activity of wild-type mixed membrane fractions (MMF) increased slightly when exogenous $Ca^{2+}$ was added to the growth medium (Fig. 6). In the *mkc1*Δ mutant, Chs activity was comparable to the control strain and did not increase upon addition of $Ca^{2+}$. The *hog1*Δ mutant had markedly elevated Chs activity compared with the control and addition of $Ca^{2+}$ did not further stimulate chitin synthase activity. Chitin synthase activity of the *crz1*Δ mutant was comparable to wild type and decreased in $Ca^{2+}$-treated cells. As shown previously (Munro *et al*., 2003), the Chs activity of the *chs2*Δ *chs8*Δ double mutant was only around 5% of wild-type levels and no further stimulation was observed when cells were grown in the presence of $Ca^{2+}$. The *chs3*Δ mutant had reduced Chs activity but this was elevated in response to $Ca^{2+}$. Therefore, Chs2p and Chs8p are mainly responsible for the elevated Chs activity in response to $Ca^{2+}$ and this was mediated via Crz1p, Mkc1p and Hog1p. Attempts were made to measure chitin synthase activity from membranes prepared from cells grown in the presence of CFW and $Ca^{2+}$/CFW. No detectable Chs activity was found (not shown). CFW inhibition of *in vitro* chitin synthase activity has been reported previously (Roncero and Duran, 1985) and was shown to be dependent upon pH of the growth medium (Roncero *et al*., 1988).

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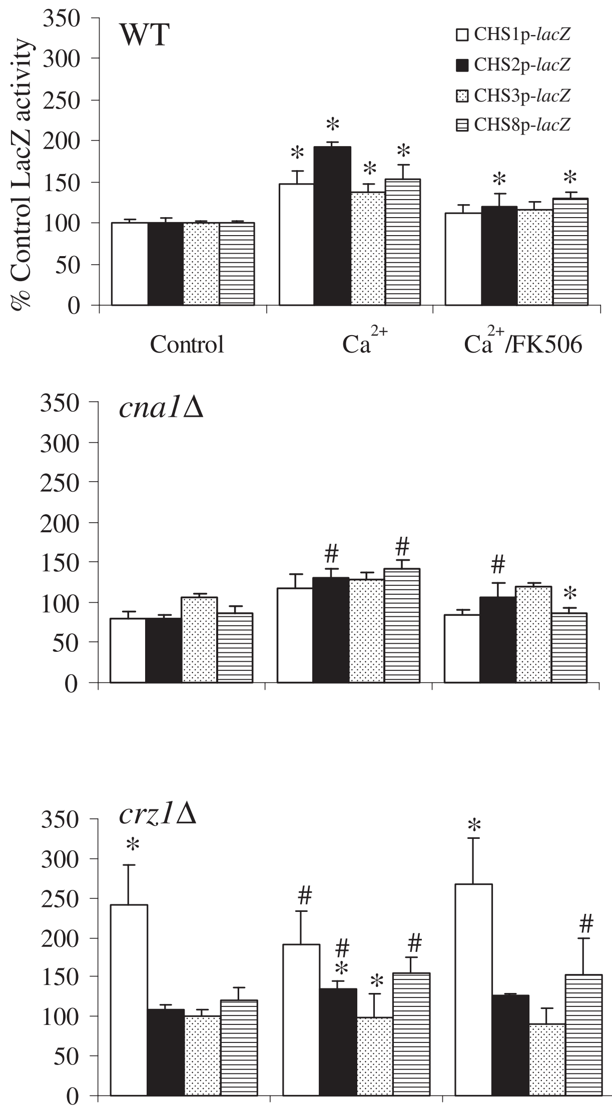


**Fig. 5.** Stimulation of the *CHS* promoters by $Ca^{2+}$ is dependent upon calcineurin and Crz1p. β-galactosidase activities of protein extracts of CAI-4 (control), *cna1*Δ and *crz1*Δ transformed with the CHSp-*lacZ* constructs were measured. Cells were grown at 30°C on YPD, YPD supplemented with $Ca^{2+}$ ± FK506. Triplicate assays were performed on three independent cultures (average ± SD, $n = 9$). In WT asterisks indicate significantly different to untreated control of that promoter under the same conditions ($P \leq 0.05$). For mutants, an asterisk indicates a significant difference relative to wild type under the same conditions, a number sign indicates a significant difference to the untreated control in the same mutant background ($P \leq 0.05$).

### *Treatment with $Ca^{2+}$ and CFW elevates cell wall chitin levels*

The chitin content of cells was measured under conditions where $Ca^{2+}$ stimulated *CHS* gene expression and *in vitro* Chs activity. Addition of $Ca^{2+}$, CFW and $Ca^{2+}$/CFW resulted in elevated cell wall chitin levels in wild-type CAI-4 cells (Fig. 7) with the combination of $Ca^{2+}$/CFW giving the greatest stimulation. Chs3p is responsible for the synthesis of the majority of the chitin in the *C. albicans* cell wall (Bulawa *et al.*, 1995; Mio *et al.*, 1996). In the *chs3*Δ mutant, chitin levels were dramatically reduced and $Ca^{2+}$-treatment had only a slight effect on chitin content. The *chs2*Δ *chs8*Δ mutant behaved similarly to wild type. In the *mkc1*Δ mutant, chitin levels were lower than in parental controls, but were elevated after combined $Ca^{2+}$/CFW treatment. However, addition of $Ca^{2+}$ or CFW alone had little effect on chitin content in the *mkc1*Δ mutant. Chitin levels of untreated *crz1*Δ cells were significantly higher than wild-type cells again suggesting that under some conditions Crz1p represses chitin synthesis. Chitin levels of *crz1*Δ were elevated by CFW or $Ca^{2+}$/CFW treatments but did not respond, or were repressed, when treated with $Ca^{2+}$ alone. Untreated *hog1*Δ cells had wild-type chitin levels that were increased marginally when cells were grown with $Ca^{2+}$ but the activation with CFW or $Ca^{2+}$ plus CFW was significantly reduced compared with wild-type cells. These results suggest that elevated cell wall chitin content in response to combined treatments with $Ca^{2+}$ and CFW is due mainly to Chs3p and that the PKC and HOG and to a lesser extent the $Ca^{2+}$/Crz1 pathways are involved in this Chs3p-dependent stimulation of chitin synthesis.

### *$Ca^{2+}$- and CFW-dependent phosphorylation of Mkc1p and Cek1p*

The phosphorylation status of the Mkc1p and Cek1p MAP kinases was examined in order to assess the status of the PKC and SVG (STE vegetative growth) pathways in the strains and treatments described above (Navarro-Garcia

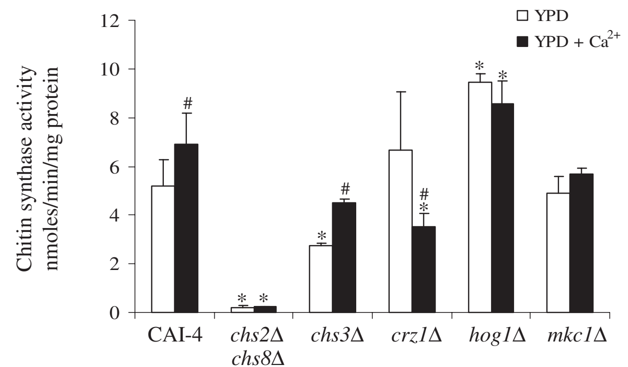


**Fig. 6.** Exogenous $Ca^{2+}$ elevates *in vitro* chitin synthase activity of yeasts cells of *C. albicans* strains. Activities are from mixed membrane fractions after trypsin-treatment isolated from mid-exponential cells grown at 30°C in YPD (open bars) or YPD + 100 mM $Ca^{2+}$ (black bars). Triplicate assays were performed (average ± SD, $n = 3$). Asterisks indicate significant differences from CAI-4 under the same conditions, a number sign indicates significant differences to untreated samples in the same strain background ($P \leq 0.05$).

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**Table 3.** The *CHS* promoters are hyper-stimulated by combined $Ca^{2+}$/CFW treatment.

| Strain | Treatment | Mean % *lacZ* activity | Fold change[a] | *P*-value | Fold change[b] | *P*-value |
|---|---|---|---|---|---|---|
| CHS1p-*lacZ* | | | | | | |
| wt | Control | 100 ± 7 | | | | |
| | +$Ca^{2+}$ | 174 ± 38 | **1.74** | 1.60E-05 | | |
| | +CFW | 190 ± 34 | **1.90** | 3.71E-02 | | |
| | +Ca/CFW | 888 ± 59 | **8.88** | 1.30E-06 | | |
| *mkc1Δ* | Control | 91 ± 9 | | | 0.91 | 7.06E-01 |
| | +$Ca^{2+}$ | 189 ± 25 | **2.09** | 4.00E-04 | 1.20 | 1.00E+00 |
| | +CFW | 141 ± 41 | 1.55 | 2.27E-01 | 0.82 | 7.08E-01 |
| | +$Ca^{2+}$/CFW | 735 ± 40 | **8.11** | 4.40E-10 | 0.91 | 3.08E-01 |
| *crz1Δ* | Control | 209 ± 11 | | | **2.09** | 3.10E-10 |
| | +$Ca^{2+}$ | 202 ± 22 | 0.97 | 1.00E+00 | 0.55 | 7.81E-01 |
| | +CFW | 154 ± 19 | **0.74** | 3.70E-04 | 0.39 | 7.91E-01 |
| | +$Ca^{2+}$/CFW | 411 ± 174 | 1.96 | 2.78E-01 | **0.22** | 1.43E-03 |
| *hog1Δ* | Control | 39 ± 12 | | | **0.39** | 2.00E-06 |
| | +$Ca^{2+}$ | 48 ± 9 | 1.22 | 9.94E-01 | **0.70** | 1.44E-09 |
| | +CFW | 47 ± 13 | 1.18 | 1.00E+00 | **0.62** | 2.80E-03 |
| | +$Ca^{2+}$/CFW | 592 ± 219 | **15.00** | 8.79E-03 | 1.69 | 3.85E-01 |
| CHS2p-*lacZ* | | | | | | |
| wt | Control | 100 ± 3 | | | | |
| | +$Ca^{2+}$ | 226 ± 43 | **2.26** | 1.52E-07 | | |
| | +CFW | 221 ± 23 | **2.21** | 7.67E-08 | | |
| | +$Ca^{2+}$/CFW | 889 ± 121 | **8.89** | 3.05E-06 | | |
| *mkc1Δ* | Control | 68 ± 3 | | | **0.68** | 9.98E-11 |
| | +$Ca^{2+}$ | 196 ± 13 | **2.88** | 1.77E-08 | 1.27 | 6.02E-01 |
| | +CFW | 76 ± 3 | **1.12** | 7.00E-03 | **0.51** | 1.08E-08 |
| | +$Ca^{2+}$/CFW | 379 ± 18 | **5.56** | 5.96E-10 | **0.63** | 5.20E-11 |
| *crz1Δ* | Control | 142 ± 10 | | | **1.42** | 3.80E-05 |
| | +$Ca^{2+}$ | 153 ± 12 | 1.08 | 8.91E-01 | **0.48** | 2.13E-04 |
| | +CFW | 147 ± 6 | 1.04 | 1.00E+00 | **0.47** | 9.56E-06 |
| | +$Ca^{2+}$/CFW | 402 ± 43 | **2.84** | 2.13E-06 | **0.32** | 1.06E-05 |
| *hog1Δ* | Control | 43 ± 3 | | | **0.43** | 2.30E-14 |
| | +$Ca^{2+}$ | 54 ± 2 | **1.24** | 8.95E-06 | **0.55** | 1.58E-09 |
| | +CFW | 38 ± 1 | **0.88** | 8.63E-03 | **0.40** | 1.40E-09 |
| | +$Ca^{2+}$/CFW | 354 ± 113 | **8.15** | 2.00E-03 | 0.92 | 2.67E-01 |
| CHS3p-*lacZ* | | | | | | |
| wt | Control | 100 ± 7 | | | | |
| | +$Ca^{2+}$ | 154 ± 26 | **1.54** | 1.69E-04 | | |
| | +CFW | 118 ± 10 | **1.18** | 1.36E-02 | | |
| | +$Ca^{2+}$/CFW | 693 ± 33 | **6.93** | 2.94E-10 | | |
| *mkc1Δ* | Control | 101 ± 8 | | | 1.01 | 1.00E+00 |
| | +$Ca^{2+}$ | 184 ± 23 | **1.82** | 1.20E-04 | 1.18 | 4.27E-01 |
| | +CFW | 104 ± 25 | 1.03 | 1.00E+00 | 0.87 | 9.94E-01 |
| | +$Ca^{2+}$/CFW | 732 ± 93 | **7.23** | 2.08E-06 | 1.04 | 1.00E+00 |
| *crz1Δ* | Control | 110 ± 8 | | | 1.10 | 4.45E-01 |
| | +$Ca^{2+}$ | 89 ± 6 | 0.81 | 8.87E-02 | **0.52** | 1.20E-04 |
| | +CFW | 91 ± 4 | 0.83 | 2.32E-01 | 0.70 | 3.00E-01 |
| | +$Ca^{2+}$/CFW | 288 ± 41 | **2.61** | 1.66E-04 | **0.38** | 6.10E-10 |
| *hog1Δ* | Control | 156 ± 14 | | | **1.56** | 1.29E-05 |
| | +$Ca^{2+}$ | 251 ± 21 | **1.61** | 2.06E-07 | **1.04** | 6.24E-07 |
| | +CFW | 135 ± 9 | **0.86** | 2.12E-05 | 0.73 | 5.15E-02 |
| | +$Ca^{2+}$/CFW | 988 ± 237 | 6.32 | 2.16E-04 | 0.91 | 2.12E-01 |
| CHS8p-*lacZ* | | | | | | |
| wt | Control | 100 ± 9 | | | | |
| | +$Ca^{2+}$ | 158 ± 16 | 1.58 | 2.28E-11 | | |
| | +CFW | 263 ± 14 | 2.63 | 8.27E-07 | | |
| | +$Ca^{2+}$/CFW | 1237 ± 421 | 12.37 | 9.01E-09 | | |
| *mkc1Δ* | Control | 27 ± 5 | | | **0.27** | 1.75E-09 |
| | +$Ca^{2+}$ | 76 ± 17 | **2.86** | 7.52E-04 | 1.81 | 1.61E-01 |
| | +CFW | 16 ± 2 | **0.59** | 6.41E-03 | **0.22** | 4.38E-08 |
| | +$Ca^{2+}$/CFW | 378 ± 47 | **14.12** | 8.50E-07 | **1.14** | 3.81E-06 |
| *crz1Δ* | Control | 141 ± 8 | | | **1.41** | 1.99E-06 |
| | +$Ca^{2+}$ | 160 ± 12 | 1.13 | 1.13E-01 | **0.72** | 3.95E-07 |
| | +CFW | 233 ± 16 | **1.65** | 4.08E-07 | **0.63** | 4.13E-09 |
| | +$Ca^{2+}$/CFW | 529 ± 94 | **3.74** | 9.74E-05 | **0.30** | 4.42E-05 |
| *hog1Δ* | Control | 43 ± 8 | | | **0.43** | 2.03E-08 |
| | +$Ca^{2+}$ | 48 ± 5 | 1.11 | 9.99E-01 | **0.70** | 1.09E-07 |
| | +CFW | 34 ± 6 | 0.79 | 6.00E-01 | **0.30** | 3.70E-09 |
| | +$Ca^{2+}$/CFW | 315 ± 69 | **7.29** | 1.23E-04 | **0.59** | 7.10E-04 |

**a.** Fold change with respect to the control in the same genetic background.
**b.** Ratio of fold change in the mutant compared with fold change in wild-type cells under the same conditions.
Statistically significant changes are highlighted in bold.

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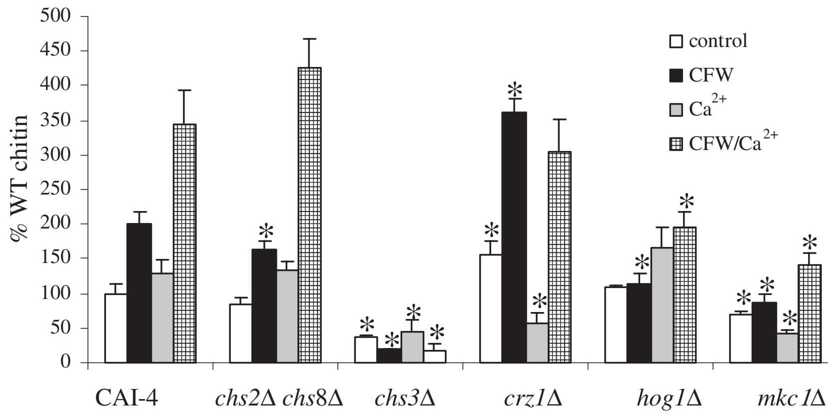


**Fig. 7.** Hyper-stimulation of cell wall chitin in response to $Ca^{2+}$/CFW. *C. albicans* strains were grown at 30°C on YPD and YPD supplemented with $Ca^{2+}$, CFW or both $Ca^{2+}$ and CFW. Cell wall chitin assays were performed five times on three biologically independent samples (average ± SD $n = 15$). Asterisks indicate significant differences from CAI-4 cells grown under the same conditions.

*et al*., 2005; Eisman *et al*., 2006). Cek1p is the *C. albicans* orthologue of *Sc*Kss1p, which is the *S. cerevisiae* MAP kinase component of pathways that regulate filamentous growth, the pheromone response and promote vegetative growth (the latter via the SVG pathway) (Lee and Elion, 1999; Eisman *et al*., 2006). The SVG pathway is constitutively activated in the *och1*Δ *N*-glycosylation mutants of *S. cerevisiae* and *C. albicans* (Lee and Elion, 1999; Bates *et al*., 2006) and in the *hog1*Δ mutant and has been implicated in the response to cell wall perturbing agents (Eisman *et al*., 2006). Phospho-Mkc1p and phospho-Cek1p were identified by western analysis as 59 kDa and 48 kDa bands, respectively, using phospho-specific antibodies (Fig. 8). Phosphorylation status was assessed 10, 30, 60 and 120 min after treatment addition, however, only 10 and 120 min time points are presented here. In non-stressed conditions, no phospho-Mkc1p was detected in wild type or *hog1*Δ yeast cells however, Mkc1p was phosphorylated in the *crz1*Δ mutant.

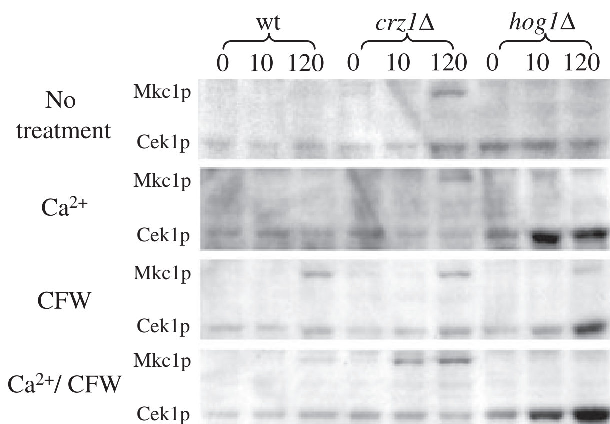


**Fig. 8.** Western blot analysis of Mkc1 phosphorylation status. *C. albicans* strains were grown at 30°C on YPD and YPD supplemented with $Ca^{2+}$, CFW or both $Ca^{2+}$ and CFW. Total protein was extracted and phosphorylated Mkc1 and Cek1 detected using phospho-specific anti-p44/42 MAP kinase antibody.

Activation of Mkc1p was observed in the *crz1*Δ strain after treatment with $Ca^{2+}$. CFW stimulated strong activation of Mkc1p in the wild type, weaker activation in *hog1*Δ and activation comparable to untreated controls in *crz1*Δ. CFW-stimulated phosphorylation of Mkc1p was observed at 2 h in wild type and *hog1*Δ and after 1 h in *crz1*Δ. Combined treatment with $Ca^{2+}$ and CFW had a synergistic effect on activation of Mkc1p in the *crz1*Δ strain where phospho-Mkc1p was detected after 10 min. In wild-type cells, $Ca^{2+}$/CFW did not give as strong a response in terms of Mkc1p phosphorylation as CFW alone. In agreement with Navarro-Garcia *et al*. (2005); Roman *et al*. (2005) and Eisman *et al*. (2006), phospho-Mkc1p was only detected in extracts prepared from *hog1*Δ cells when cells were treated with CFW. Under these conditions Cek1p appeared to be constitutively activated in the *hog1*Δ mutant. Treatment of the *hog1*Δ strain with $Ca^{2+}$, CFW and $Ca^{2+}$/CFW increased the level of phospho-Cek1p significantly. We conclude that the PKC pathway is activated when cells are treated with CFW and $Ca^{2+}$/CFW but $Ca^{2+}$ alone could not stimulate phosphorylation of Mkc1p in a wild-type background.

## Discussion

This study has shown that at least three signalling systems are involved in chitin synthesis regulation: (i) $Ca^{2+}$/calcineurin/Crz1p, (ii) PKC-Mkc1p and (iii) HOG pathways. At the transcriptional level *CHS* expression was monitored using a *lacZ* reporter gene fused to each of the four *C. albicans CHS* promoters. Each promoter was regulated differentially – the *CHS2* promoter was the most active under control conditions (YPD at 30°C) and the *CHS3* promoter was the least active. Real-time quantitative PCR confirmed these observations (data not shown). The *CHS* promoters responded to deletion of other *CHS* genes with a twofold increase in expression levels from *CHS1, CHS3* and *CHS8* promoters in several *chs*Δ mutants. In *Wangiella dermatitidis*, a melanized fungal

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pathogen of humans, a compensatory increase in *WdCHS* expression has also been described in response to chitin synthase gene disruptions (Wang *et al*., 2002). Although there is no evidence of true functional redundancy within the chitin synthases examined to date, fungi appear to upregulate certain *CHS* in compensation for loss of others perhaps to maintain a robust cell wall.

The *C. albicans CHS* promoters were found to respond to a number of environmental stimuli notably when cells were treated with cell wall perturbing drugs and when growth medium was supplemented with $Ca^{2+}$. Addition of exogenous $Ca^{2+}$, stimulated *CHS* gene expression; stimulated *in vitro* chitin synthase activity, and resulted in increased cell wall chitin mediated through Chs3p. In addition, simultaneous treatment of cells with CFW and $Ca^{2+}$ resulted in synergistically enhanced expression from all four *CHS* promoters and a threefold increase in the amount of chitin in the cell wall.

The *CHS* promoters were activated by exogenous $Ca^{2+}$ and $Mn^{2+}$ but not by equivalent concentrations of $Mg^{2+}$ or $Na^{+}$. In *S. cerevisiae*, $Ca^{2+}$ activates calcineurin via calmodulin, which induces gene expression by regulating the Crz1p/Tcn1p transcription factor. This plays a role in regulating cell wall structure including the induction of *ScCHS1* in response to $Ca^{2+}$ (Yoshimoto *et al*., 2002) and tolerance of fungi to a wide range of antifungal agents (Edlind *et al*., 2002; Sanglard *et al*., 2003; Onyewu *et al*., 2004; Karababa *et al*., 2006). $Ca^{2+}$-activation of *CaCHS* expression was blocked by inhibitors of both calmodulin and calcineurin confirming the role of this pathway in the regulation of *CHS* genes. In addition, transcription from all *CHS* promoters was reduced in the *cna1*Δ mutant in response to exogenous $Ca^{2+}$. In the *crz1*Δ mutant, basal activity of the *CHS2*, *CHS3* and *CHS8* promoters was not altered but the *CHS1* promoter was de-repressed. In addition, *crz1*Δ cells were attenuated, but not completely blocked, in their ability to activate *CHS* expression in response to exogenous $Ca^{2+}$ and the hyper-stimulation of *CHS* expression caused by cross-activation with $Ca^{2+}$ and CFW was reduced dramatically in the *crz1*Δ mutant background. *In silico* analysis of the *CHS* promoter sequences also identified potential CDREs, motifs recognized by Crz1p. Therefore, activation of the *CHS* promoters due to $Ca^{2+}$ was mainly regulated by the classical Cna1/Crz1 pathway; however, some of the $Ca^{2+}$ stimulation was Cna1p and/or Crz1p-independent indicating that calcineurin and Crz1p may have roles that are distinct from their role in this signalling pathway. Our results suggest that in *C. albicans* the $Ca^{2+}$ signalling pathway plays a major role in regulating chitin synthesis. This pathway may be vital to the co-ordination of responses to a variety of conditions that compromise cell wall integrity because it also regulates the expression of genes encoding cell wall proteins Utr2p and Crh11p (Pardini *et al*., 2006) and the glucan synthase catalytic subunit Fks1p/Gsc1p (Sanglard *et al*., 2003).

Many of the conditions that stimulated the *CHS* promoters including growth at 37°C, treatment with cell wall perturbing agents CFW, CR and SDS and the cAMP-phosphodiesterase inhibitor caffeine lead to hyper-phosphorylation of Slt2p/Mkc1p (De Nobel *et al*., 2000; Martin *et al*., 2000; Navarro-Garcia *et al*., 2005). We used the *mkc1*Δ MAP kinase null mutant to test the role of the PKC pathway in chitin synthesis regulation. In the *mkc1*Δ mutant background the *CHS2* and *CHS8* promoters were less responsive to CFW but were still stimulated by $Ca^{2+}$ and compared with wild-type cells only the *CHS2* promoter had a significantly reduced response to $Ca^{2+}$/CFW. However, there was a dramatic decrease in chitin levels in the *mkc1*Δ mutant under all conditions tested suggesting post-transcriptional regulation of Chs3p occurs via the PKC pathway. This pathway has been shown to regulate Chs3p in *S. cerevisiae* (Valdivia and Schekman, 2003).

The HOG signalling pathway was the third pathway implicated in *CHS* transcriptional regulation. Promoter sequences recognized by the HOG-regulated transcription factor Sko1p were identified in the *CHS1*, *CHS3* and *CHS8* promoters. Loss of the HOG pathway in non-stressed conditions resulted in reduced expression of *CHS1*, *CHS2* and *CHS8* but increased expression of *CHS3*. Therefore, as with Crz1p, blocking a particular signalling pathway had both positive and negative effects on *CHS* expression. Although the *CHS1*, *CHS2* and *CHS8* promoters had attenuated responses to either CFW or $Ca^{2+}$ in the *hog1*Δ mutant, only the *CHS8* promoter had markedly reduced activity in response to the combined $Ca^{2+}$/CFW treatment compared with wild-type cells. Despite the apparently low basal level of expression of *CHS2* and *CHS8* in the *hog1*Δ mutant, the levels of chitin synthase enzyme activity were greater than in wild-type cells. This suggests the presence of a compensatory mechanism that is activated in response to loss of Hog1p that acts post-transcriptionally and results in enhanced Chs enzyme activity. Nevertheless, the amount of chitin in the wall of the *hog1*Δ mutant synthesized in response to co-stimulation with $Ca^{2+}$ and CFW was significantly (40%) lower than wild type implying that the HOG pathway is involved in activation of chitin synthesis via Chs3p.

The $Ca^{2+}$/calcineurin, PKC and HOG pathways contribute to the hyper-stimulation of chitin synthesis in response to $Ca^{2+}$/CFW treatment. The role of the $Ca^{2+}$-signalling pathway appears to be mainly in regulating *CHS* transcription, whereas the PKC and HOG pathways also contribute to regulation of chitin synthase enzyme activity and total cell wall chitin content. The Mkc1 pathway positively regulates *CHS* expression, chitin synthase activity and chitin levels in the cell wall while the $Ca^{2+}$/Crz1 and HOG pathways have both positive and negative regulatory

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effects on different *CHS* genes and Chs isoenzymes (Fig. 9).

Interpretation of experiments studying *CHS* expression is complicated by cross-talk between signalling pathways and by compensatory mechanisms that are triggered in mutants defective in single signalling pathways. For example, mutants blocked in the HOG pathway have a constitutively active Cek1 MAP kinase, which contributes to a CR resistance phenotype (Roman *et al*., 2005; Eisman *et al*., 2006). We examined the phosphorylation status of Mkc1p and Cek1p in cells treated with $Ca^{2+}$, CFW and $Ca^{2+}$/CFW. We confirmed phosphorylation of Cek1p in the *hog1*Δ mutant and enhanced phosphorylation of Cek1p when *hog1*Δ was treated with $Ca^{2+}$, CFW and $Ca^{2+}$/CFW. Despite activation of Cek1p, chitin levels are reduced in the *hog1*Δ mutant suggesting Cek1p may not make a major contribution to chitin regulation. Our findings also corroborate the observations of Navarro-Garcia *et al*. (2005) that Hog1p was required for phosphorylation of Mkc1p under a variety of conditions, but not with CFW treatment. In addition, our Western analyses suggested that Mkc1p was phosphorylated in the *crz1*Δ mutant. These data suggest that these pathways do not operate in isolation and that mutations in one pathway results in activation of others as in the case of Cek1p activation in the *hog1*Δ mutant and Mkc1p phosphorylation in the *crz1*Δmutant. Hence, the activation of Mkc1p is not solely responsible for the elevated chitin synthesis under the conditions tested. Instead, the PKC, HOG and $Ca^{2+}$ signalling pathways all contribute to the regulation of chitin synthesis.

In conclusion, the $Ca^{2+}$/Crz1p, PKC-Mkc1p and HOG signalling pathways co-ordinate the regulation of chitin synthesis in *C. albicans*. The use of multiple pathways may enable the fungus to fine-tune the co-ordinated assembly of cell wall chitin to exogenous stresses by modulating chitin synthesis. *CHS* gene expression responded to a wide range of environmental conditions and individual *CHS* genes and Chs enzymes responded differently to these stresses. This regulation is vital for the maintenance of a robust cell wall during growth and morphogenesis but also under conditions where the integrity of the cell wall is compromised by treatments with antifungal drugs that target fungal cell wall synthesis.

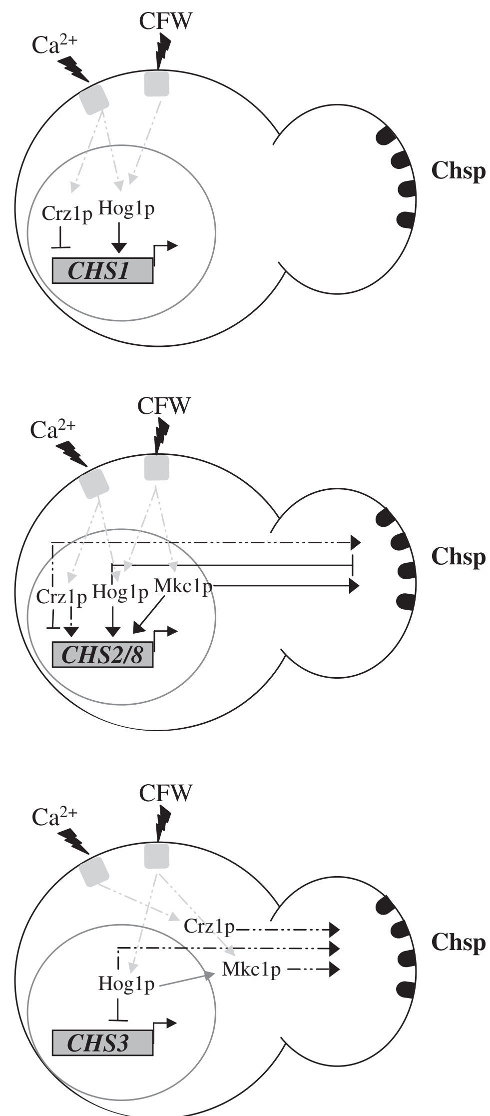


**Fig. 9.** Summary model of the co-ordinated regulation of chitin synthesis. Solid lines indicate regulation under non-stress conditions and dotted lines indicate regulation in response to various stresses. Under normal growth conditions Hog1 is required for basal levels of *CHS1* transcription and Crz1p acts as a repressor. $CaCl_2$ or CFW-stimulated *CHS1* expression is dependent upon Crz1p and Hog1p, while the PKC pathway does not play a major role. The contribution of these three pathways to post-transcriptional regulation of Chs1p cannot be determined by measurement of *in vitro* chitin synthase activity and chitin content because Chs1p is only a minor contributor to these. *CHS2* and *CHS8* are regulated by the Crz1p, Hog1p and Mkc1p pathways under normal growth conditions. Although Crz1p represses *CHS2* expression it is required for $CaCl_2$ stimulation. Hog1p and Mkc1p are both required for normal control levels of *CHS2* and *CHS8* mRNA and CFW-activated transcription is dependent upon both pathways. In addition, Hog1p is required for $CaCl_2$ activation of *CHS2* and *CHS8* expression. In Chs activity assays, Hog1p negatively regulates Chs2p and Chs8p and Crz1p is responsible for their activation due to the presence of exogenous $CaCl_2$. Hog1p had a significant negative contribution to *CHS3* promoter activity but all three pathways were involved in elevation of chitin levels due to treatment with $CaCl_2$ and CFW – a measure of Chs3p post-transcriptional regulation.

## Experimental procedures

### *Strains, media and growth conditions*

*Candida albicans* strains used in this study are listed in Table 1. *C. albicans* cultures were maintained on solid YPD medium comprising 1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose, 2% (w/v) agar. Yeast cells of *C. albicans* were grown at 30°C in YPD with shaking at 200 r.p.m.

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### *Transformation of* C. albicans

Ura$^{-}$ *C. albicans* strains were cultured in 10 ml of YPD supplemented with 25 μg ml$^{-1}$ uridine at 30°C for 36–72 h. After centrifugation, the cell pellets from 200 μl of cells were resuspended in 100 μl of OSB (200 mM LiAc pH 7.5, 100 mM DTT, 50% v/w PEG 6000, 10 mg ml$^{-1}$ Clontech herring testis carrier DNA) and then transforming DNA was added. Samples were incubated at 43.5°C for 60 min and spread over SD agar plates (2% (w/v) D-glucose, 0.67% (w/v) yeast nitrogen base (YNB) (Bio 101, Carlsbad), 1.5% (w/v) purified agar, Oxoid) and incubated at 30°C. Single colonies were picked and grown in 5 ml of SD medium, and genomic DNA was extracted for Southern analysis.

### *Construction of plasmids and* C. albicans *strains*

The placpoly-6 vector was used for the promoter-fusion reporter system and was based on a plasmid previously described by Uhl and Johnson (2001). This contains the *CaURA*3 and the *CaRPS1* genes and was used to create fusion between the promoter of each *CaCHS* gene and the *S. thermophilus lacZ* ORF. A 1 kb upstream region from the ATG start codon of each *CHS1*, *CHS2*, *CHS3* and *CHS8* ORF was cloned into the *Pst*I–*Xho*I sites of placpoly-6 generating pCHS1plac, pCHS2plac, pCHS3plac, pCHS8plac respectively. Ura$^{-}$ *C. albicans* cells were transformed with these plasmids previously cut within the *RPS1* gene with StuI to target homologous recombination at the neutral chromosomal *RPS1* locus and the *URA*3 gene was the selectable marker (Murad *et al*., 2000). Southern analysis was used to screen transformants and only those with single integrations of each pCHSplac plasmid were selected. Genomic DNA from each transformant was digested with *Xho*I/*Bam*HI and hybridized to 693 bp *RPS1* specific probe.

### *Measurement of β-galactosidase activity*

The expression of each *CHS* gene in *lacZ* promoter fusions was measured using a modified version of the assays described previously (Rose and Botstein, 1983). *C. albicans* cells were grown with shaking at 200 r.p.m. at the chosen growth condition and harvested at $OD_{600} < 1$. Yeast cells were centrifuged at 3000 *g* for 5 min at 4°C and the pellet was resuspended in 0.5 ml of ice–cold water and transferred to microcentrifuge tubes. The cells were then centrifuged at 13 000 *g* for 5 min and resuspended in 0.5 ml of breaking buffer [100 mM TRIS-HCl pH 7.5, 0.01% (w/v) SDS, 1 mM dithiothreitol (DTT), 10% (v/v) glycerol, pepstatin 4 μg ml$^{-1}$, 1 × proteinase cocktail tablets EDTA-free (Roche)]. Approximately equal volumes of glass beads (Sigma, Poole, UK G9268) and cell pellet were used and the cells were disrupted using a Fastprep cell breakage machine (Thermo Savant, Middlesex, UK) using six cycles of 30 s with chilling on ice for 1 min in between each cycle. The extract was centrifuged at 13 000 r.p.m. for 10 min and the protein concentration of the supernatant was measured using Coomassie® Protein Assay Reagent Kit (Pierce Biotechnology, Perbio, Rockford, UK). Varying quantities of protein extract, 30–300 μl, were added to Z-buffer (60 mM $NaH_2PO_4$, 40 mM $Na_2HPO_4$, 10 mM KCl, 1 mM $MgSO_4$), in a total volume of 800 μl. The reaction was initiated by adding 200 μl of *o*-nitrophenyl-*β*-D-galactopyranoside (ONPG) stock solution (4 mg ml$^{-1}$ in phosphate buffer) and incubated until the yellow *o*-nitrophenol product was produced. The reaction was stopped by addition of 400 μl of 1 M $Na_2CO_3$. The specific *β*-galactosidase activity was measured in terms of the yield of product *o*-nitrophenol at the absorbance of 420 nm.

### *Measurement of chitin synthase activity*

Mixed membrane fractions were prepared from exponential phase yeast cells and their chitin synthase activities measured as described previously (Munro *et al*., 1998). MMF proteins were activated by limited incubation with 100 ng trypsin μl$^{-1}$ MMF at 30°C and the reactions were stopped by addition of 150 ng soybean trypsin inhibitor μl$^{-1}$ MMF. Briefly, standard reactions for measuring chitin synthase activity were carried out in a 50 μl volume and were composed of; 50 μg MMF protein, 25 mM *N*-acetylglucosamine, 1 mM UDP-*N*-acetylglucosamine which included 25 nCi UDP-[U-$^{14}$C] *N*-acetylglucosamine, 50 mM Tris-HCl pH 7.5 and 10 mM $MgCl_2$. Incubations were carried out at 30°C for 30 min and the reaction was stopped by addition of 1 ml of 66% (v/v) ethanol. The reaction mixture was then filtered through GF/C filter discs (Whatman), which had been presoaked in 10% (v/v) trichloroacetic acid. The reactions tubes were rinsed out with 2 × 1 ml of 1% (v/v) Triton X-100 and each filter was then washed with 4 × 2 ml of 66% (v/v) ethanol. The radiolabelled chitin synthesized in the reaction was trapped on the filters and unincorporated substrate was removed by washing. Filters were dried at 80°C and their radioactivity counted in a scintillation counter.

### *Measurement of cell wall chitin content*

Cell walls were prepared from 10 ml of *C. albicans* stationary phase yeast cultures grown in YPD and the chitin content was measured as described previously (Munro *et al*., 2003). Cells were disrupted with glass beads (Sigma, G9268) using a Fastprep cell breakage machine (Thermo Savant, Middlesex, UK) until at least 95% of cells were disrupted. They were then washed five times with 1 M NaCl and extracted in SDS-MerOH buffer (50 mM Tris, 2% sodium dodecyl sulphate (SDS), 0.3 M β-mercaptoethanol, 1 mM EDTA; pH 8.0) at 100°C for 10 min, then washed in $dH_2O$. Cell wall pellets were resuspended in sterile $dH_2O$, freeze dried, and the dry weight of recovered cell walls was measured. Chitin contents were determined by measuring the glucosamine released by acid hydrolysis of purified cell walls (Kapteyn *et al*., 2000).

### *Culture conditions*

Cells were grown in normal laboratory media and under conditions of various environmental stresses. Cells were grown overnight in YPD then transferred to YPD supplemented with different agents: 1 M sorbitol, 0.8 M NaCl, 0.2 M $CaCl_2$, 100 μg ml$^{-1}$ CFW, 200 μg ml$^{-1}$ CR, 0.05% SDS, 12 mM caffeine, 25 mM DTT, 23 mM glucosamine, 50 μg ml$^{-1}$

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cyclosporin A, 1 μg ml$^{-1}$ FK506, 1 mM chloropromazine, 4 μM A23187. Cells were harvested at $OD_{600}$ 0.8.

### *Western analysis*

Western analysis was performed using the method of Millar *et al*. (1995) with some modifications. Overnight cultures of the wild type, *crz1*Δ and *hog1*Δ strains were diluted 1:50 into 25 ml of YPD supplemented with uridine and incubated shaking for 4 h at 30°C. The mid-log phase cultures were then treated with a final concentration of 100 mM $CaCl_2$, 100 μg ml$^{-1}$ CFW, or both for 0, 10, 30, 60 or 120 min. No-treatment controls were also performed. After treatment, cells were harvested by centrifugation (1500 *g*, 2 min, 4°C) and washed in 1 ml of cold lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5% NP40, 2 μg ml$^{-1}$ Leupeptin, 2 μg ml$^{-1}$ Pepstatin, 1 mM PMSF, 2 mM $Na_3VO_4$, 50 mM NaF). Cells were collected by centrifugation (800 *g*, 5 min, 4°C) and resuspended in 250 μl of cold lysis buffer. Cells were broken using a FastPrep machine in the presence of acid-washed glass beads (4 × 15 s bursts at speed 6.5 with 1 min on ice between bursts). The extracts were clarified by centrifugation (16 000 *g*, 5 min, 4°C). Protein concentration in the cleared lysate was estimated using the method described by Bradford (1976) with BSA as a standard.

Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using the XCell *SureLock*™ Mini-Cell system (Invitrogen) with NuPAGE®Novex Bis-Tris 4–12% precast gels (Invitrogen) in NuPAGE® MOPS-SDS Running Buffer (Invitrogen) containing NuPAGE® Antioxidant (Invitrogen) as per the manufacturer's instructions. Approximately 15 μg of protein was loaded in each lane. The proteins were transferred to Invitrolon™ PVDF Membranes (Invitrogen) in NuPAGE® Transfer Buffer containing methanol using the XCell II™ Blot Module (Invitrogen) following the manufacturer's instructions.

Following transfer, the membranes were rinsed in PBS and blocked in PBS-T + 10% BSA (PBS, 0.1% Tween-20, 10% (w/v) BSA, 50 mM (NaF) for 30 min at room temperature. The membranes were then incubated overnight at 4°C in PBS-T + 5% BSA (PBS, 0.1% Tween-20, 5% (w/v) BSA, 50 mM (NaF) containing a 1:1000 dilution of Phospho-p44/42 Map Kinase (Thr202/Tyr204) Antibody (Cell Signaling Technology). The membranes were washed five times for 5 min in PBS-T (PBS, 0.1% Tween-20) and then incubated for 1 h at room temperature in PBS-T + 5% BSA containing a 1:2000 dilution of Anti-rabbit IgG, HRP-linked Antibody (Cell Signaling Technology). The membranes were washed three times for 5 min in PBS-T and the signal was detected using LumiGLO™ Reagent and Peroxide (Cell Signaling Technology) as per the manufacturer's instructions.

### *Statistical analyses*

Statistical significant differences in the assay results were determined with SPSS software using ANOVA and Post Hoc Dunnett's *T*-test, $P < 0.05$. When the results displayed unequal variance the Kruskal–Wallis non-parametric test or Dunnett's T3 test were applied.

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## References

Alonso-Monge, R., Navarro-Garcia, F., Molero, G., Diez-Orejas, R., Gustin, M., Pla, J., *et al.* (1999) Role of the mitogen-activated protein kinase Hog1p in morphogenesis and virulence of *Candida albicans*. *J Bacteriol* **181:** 3058–3068.

Bates, S., Hughes, H.B., Munro, C.A., Thomas, W.P., MacCallum, D.M., Bertram, G., *et al.* (2006) Outer chain *N*-glycans are required for cell wall integrity and virulence of *Candida albicans*. *J Biol Chem* **281:** 90–98.

Boorsma, A., de Nobel, H., ter Riet, B., Bargmann, B., Brul, S., Hellingwerf, K.J., and Klis, F.M. (2004) Characterization of the transcriptional response to cell wall stress in *Saccharomyces cerevisiae*. *Yeast* **21:** 413–427.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72:** 248–254.

Bulawa, C.E., Miller, D.W., Henry, L.K., and Becker, J.M. (1995) Attenuated virulence of chitin-deficient mutants of *Candida albicans*. *Proc Natl Acad Sci USA* **92:** 10570–10574.

Cabib, E. (1987) The synthesis and degradation of chitin. *Adv Enzymol* **59:** 59–101.

Carotti, C., Ferrario, L., Roncero, C., Valdivieso, M.H., Duran, A., and Popolo, L. (2002) Maintenance of cell integrity in the *gas1* mutant of *Saccharomyces cerevisiae* requires the Chs3p-targeting and activation pathway and involves an unusual Chs3p localization. *Yeast* **19:** 1113–1124.

Chattaway, F.W., Holmes, M.R., and Barlow, A.J.E. (1968) Cell wall composition of the mycelial and blastospore forms of *Candida albicans*. *J Gen Microbiol* **51:** 367–376.

Chen-Wu, J.L., Zwicker, J., Bowen, A.R., and Robbins, P.W. (1992) Expression of chitin synthase genes during yeast and hyphal growth phases of *Candida albicans*. *Mol Microbiol* **6:** 497–502.

Cyert, M.S. (2003) Calcineurin signaling in *Saccharomyces cerevisiae*: how yeast go crazy in response to stress. *Biochem Biophys Res Commun* **311:** 1143–1150.

De Nobel, H., Ruiz, C., Martin, H., Morris, W., Brul, S., Molina, M., and Klis, F.M. (2000) Cell wall perturbation in yeast results in dual phosphorylation of the Slt2/Mpk1 MAP kinase and in an Slt2-mediated increase in FKS2-*lacZ* expression, glucanase resistance and thermotolerance. *Microbiology* **146:** 2121–2132.

Demarini, D.J., Adams, A.E.M., Fares, H., De Virgilio, C., Valle, G., Chuang, J.S., and Pringle, J.R. (1997) A septin-based hierarchy of proteins required for localized deposition of chitin in the *Saccharomyces cerevisiae* cell wall. *J Cell Biol* **139:** 75–93.

© 2007 The Authors

Denning, D.W. (2003) Echinocandin antifungal drugs. *Lancet* **362:** 1142–1151.

Deshpande, M.V., O'Donnell, R., and Gooday, G.W. (1997) Regulation of chitin synthase activity in the dimorphic fungus *Benjaminiella poitrasii* by external osmotic pressure. *FEMS Microbiol Lett* **152:** 327–332.

Edlind, T., Smith, L., Henry, K., Katiyar, S., and Nickels, J. (2002) Antifungal activity in *Saccharomyces cerevisiae* is modulated by calcium signalling. *Mol Microbiol* **46:** 257–268.

Eisman, B., Alonso-Monge, R., Roman, E., Arana, D., Nombela, C., and Pla, J. (2006) The Cek1 and Hog1 mitogen-activated protein kinases play complementary roles in cell wall biogenesis and chlamydospore formation in the fungal pathogen *Candida albicans*. *Eukaryot Cell* **5:** 347–358.

Fonzi, W.A., and Irwin, M.Y. (1993) Isogenic strain construction and gene mapping in *Candida albicans*. *Genetics* **134:** 717–728.

Garcia, R., Bermejo, C., Grau, C., Perez, R., Rodriguez-Pena, J.M., Francois, J., *et al.* (2004) The global transcriptional response to transient cell wall damage in *Saccharomyces cerevisiae* and its regulation by the cell integrity signaling pathway. *J Biol Chem* **279:** 15183–15195.

Garcia-Rodriguez, L.J., Duran, A., and Roncero, C. (2000) Calcofluor antifungal action depends on chitin and a functional high-osmolarity glycerol response (HOG) pathway: evidence for a physiological role of the *Saccharomyces cerevisiae* HOG pathway under noninducing conditions. *J Bacteriol* **182:** 2428–2437.

Gooday, G.W. (1995) The dynamics of hyphal growth. *Mycol Res* **99:** 385–394.

Gow, N.A.R., Robbins, P.W., Lester, J.W., Brown, A.J.P., Fonzi, W.A., Chapman, T., and Kinsman, O. (1994) A hyphal-specific chitin synthase gene (*CHS2*) is not essential for growth, dimorphism, or virulence of *Candida albicans*. *Proc Natl Acad Sci USA* **91:** 6216–6220.

Kapteyn, J.C., Hoyer, L.L., Hecht, J.E., Muller, W.H., Andel, A., Verkleij, A.J., *et al.* (2000) The cell wall architecture of *Candida albicans* wild-type cells and cell wall-defective mutants. *Mol Microbiol* **35:** 601–611.

Kapteyn, J.C., ter Riet, B., Vink, E., Blad, S., de Nobel, H., Van den Ende, H., and Klis, F.M. (2001) Low external pH induces *HOG1*-dependent changes in the organization of the *Saccharomyces cerevisiae* cell wall. *Mol Microbiol* **39:** 469–480.

Karababa, M., Valentino, E., Pardini, G., Coste, A.T., Bille, J., and Sanglard, D. (2006) *CRZ1*, a target of the calcineurin pathway in *Candida albicans*. *Mol Microbiol* **59:** 1429–1451.

Klis, F.M., Mol, P., Hellingwerf, K., and Brul, S. (2002) Dynamics of cell wall structure in *Saccharomyces cerevisiae*. *FEMS Microbiol Rev* **26:** 239–256.

Klis, F.M., Boorsma, A., and De Groot, P.W. (2006) Cell wall construction in *Saccharomyces cerevisiae*. *Yeast* **23:** 185–202.

Lagorce, A., Hauser, C.N., Labourdette, D., Martin-Yken, H., Arroyo, J., Hoheisel, J.D., and François, J. (2003) Genome-wide analysis of the response to cell wall mutations in the yeast *Saccharomyces cerevisiae*. *J Biol Chem* **278:** 20345–20357.

Lee, B.N., and Elion, E.A. (1999) The MAPKKK Ste11 regulates vegetative growth through a kinase cascade of shared signaling components. *Proc Natl Acad Sci USA* **96:** 12679–12684.

Martin, H., Rodriguez-Pachon, J.M., Ruiz, C., Nombela, C., and Molina, M. (2000) Regulatory mechanism for modulation of signalling through the cell integrity Slt2-mediated pathway in *Saccharomyces cerevisiae*. *J Biol Chem* **275:** 1511–1519.

Millar, J.B., Buck, V., and Wilkinson, M.G. (1995) Pyp1 and Pyp2 PTPases dephosphorylate an osmosensing MAP kinase controlling cell size at division in fission yeast. *Genes Dev* **9:** 2117–2130.

Mio, T., Yabe, T., Sudoh, M., Satoh, Y., Nakajima, T., Arisawa, M., and Yamada-Okabe, H. (1996) Role of three chitin synthase genes in the growth of *Candida albicans*. *J Bacteriol* **178:** 2416–2419.

Munro, C.A., and Gow, N.A.R. (1995) Chitin biosynthesis as a target for antifungals. In *Antifungal Agents: Discovery and Mode of Action.* Dixon, G.K., Copping, L.G., and Hollomon, D.W. (eds). Oxford: Bios Scientific Publishers, pp. 161–171.

Munro, C.A., and Gow, N.A.R. (2001) Chitin synthesis in human pathogenic fungi. *Med Mycol* **39S:** 41–53.

Munro, C.A., Schofield, D.A., Gooday, G.W., and Gow, N.A.R. (1998) Regulation of chitin synthesis during dimorphic growth of *Candida albicans*. *Microbiology* **144:** 391–401.

Munro, C.A., Winter, K., Buchan, A., Henry, K., Becker, J.M., Brown, A.J., *et al.* (2001) Chs1 of *Candida albicans* is an essential chitin synthase required for synthesis of the septum and for cell integrity. *Mol Microbiol* **39:** 1414–1426.

Munro, C.A., Whitton, R.K., Hughes, B., Rella, M., Selvaggini, S., and Gow, N.A.R. (2003) *CHS8*-a fourth chitin synthase gene in *Candida albicans* contributes to *in vitro* chitin synthase activity, but is dispensable for growth. *Fung Genet Biol* **40:** 146–158.

Murad, A.M., Lee, P.R., Broadbent, I.D., Barelle, C.J., and Brown, A.J.P. (2000) CIp10, an efficient and convenient integrating vector for *Candida albicans*. *Yeast* **16:** 325–327.

Navarro-Garcia, F., Sanchez, M., Pla, J., and Nombela, C. (1995) Functional characterization of the *MKC1* gene of *Candida albicans*, which encodes a mitogen-activated protein kinase homolog related to cell integrity. *Mol Cell Biol* **15:** 2197–2206.

Navarro-Garcia, F., Alonso-Monge, R., Rico, H., Pla, J., Sentandreu, R., and Nombela, C. (1998) A role for the MAP kinase gene *MKC1* in cell wall construction and morphological transitions in *Candida albicans*. *Microbiology* **144:** 411–424.

Navarro-Garcia, F., Eisman, B., Fiuza, S.M., Nombela, C., and Pla, J. (2005) The MAP kinase Mkc1p is activated under different stress conditions in *Candida albicans*. *Microbiology* **151:** 2737–2749.

Odds, F.C., Brown, A.J.P., and Gow, N.A.R. (2003) Antifungal agents: mechanisms of action. *Trends Microbiol* **11:** 272–279.

Onyewu, C., Wormley, F.L., Perfect, J.R., and Heitman, J. (2004) The calcineurin target, Crz1, functions in azole tolerance but is not required for virulence of *Candida albicans*. *Infect Immun* **72:** 7330–7333.

© 2007 The Authors

Pardini, G., De Groot, P.W., Coste, A.T., Karababa, M., Klis, F.M., De Koster, C.G., and Sanglard, D. (2006) The *CRH* family coding for cell wall GPI proteins with a predicted transglycosidase domain affects cell wall organization and virulence of *Candida albicans*. *J Biol Chem* **281:** 40399–40411. M606361200 (Epub ahead of print).

Popolo, L., Gualtieri, T., and Ragni, E. (2001) The yeast cell wall salvage pathway. *Med Mycol* **39S:** 111–121.

Proft, M., Gibbons, F.D., Copeland, M., Roth, F.P., and Struhl, K. (2005) Genomewide identification of Sko1 target promoters reveals a regulatory network that operates in response to osmotic stress in *Saccharomyces cerevisiae*. *Eukaryot Cell* **4:** 1343–1352.

Roman, E., Nombela, C., and Pla, J. (2005) The Sho1 adaptor protein links oxidative stress to morphogenesis and cell wall biosynthesis in the fungal pathogen *Candida albicans*. *Mol Biol Cell* **25:** 10611–10627.

Roncero, C. (2002) The genetic complexity of chitin synthesis in fungi. *Curr Genet* **41:** 367–378.

Roncero, C., and Duran, A. (1985) Effect of Calcofluor white and Congo red on fungal cell wall morphogenesis: in vivo activation of chitin polymerization. *J Bacteriol* **163:** 1180–1185.

Roncero, C., Valdivieso, M.H., Ribas, J.C., and Duran, A. (1988) Effect of Calcofluor white on chitin synthases from *Saccharomyces cerevisiae*. *J Bacteriol* **170:** 1945–1949.

Rose, M., and Botstein, D. (1983) Construction and use of gene fusions to *LACZ* (Beta-galactosidase) that are expressed in yeast. *Methods Enzymol* **101:** 167–180.

Ruiz-Herrera, J., and San-Blas, G. (2003) Chitin synthesis as target for antifungal drugs. *Curr Drug Targets Infect Disord* **3:** 77–91.

Ruiz-Herrera, J., Elorza, M.V., Valentin, E., and Sentandreu, R. (2006) Molecular organization of the cell wall of *Candida albicans* and its relation to pathogenicity. *FEMS Yeast Res* **6:** 14–29.

Sanglard, D., Ischer, F., Marchetti, O., Entenza, J., and Bille, J. (2003) Calcineurin A of *Candida albicans*: involvement in antifungal tolerance, cell morphogenesis and virulence. *Mol Microbiol* **48:** 959–976.

Santos, B., and Snyder, M. (1997) Targeting of chitin synthase 3 to polarized growth sites in yeast requires Chs5p and Myo2p. *J Cell Biol* **136:** 95–110.

Santos, B., Duran, A., and Valdivieso, M.H. (1997) *CHS5*, a gene involved in chitin synthesis and mating in *Saccharomyces cerevisiae*. *Mol Cell Biol* **17:** 2485–2496.

Shaw, A.J., Mol, P.C., Bowers, B., Silverman, S.J., Valdivieso, M.H., Durán, A., and Cabib, E. (1991) The function of chitin synthase 2 and 3 in the *Saccharomyces cerevisiae* cell cycle. *J Cell Biol* **114:** 111–123.

Sietsma, J.H., and Wessels, J.G.H. (1994) Apical wall biogenesis. In *The Mycota I.* Wessels, J.G.H., and Meinhardt, H. (eds). Berlin: Springer-Verlag, pp. 125–141.

Sullivan, P.A., Yin, C.Y., Molloy, C., Templeton, M.D., and Shepherd, M.G. (1983) An analysis of the metabolism and cell wall composition of *Candida albicans* during germ tube formation. *Can J Microbiol* **29:** 1514–1525.

Trilla, J.A., Cos, T., Duran, A., and Roncero, C. (1997) Characterization of *CHS4* (*CAL2*), a gene of *Saccharomyces cerevisiae* involved in chitin biosynthesis and allelic to *SKT5* and *CSD4 Yeast* **13:** 795–807.

Trilla, J.A., Duran, A., and Roncero, C. (1999) Chs7p, a new protein involved in the control of protein export from the endoplasmic reticulum that is specifically engaged in the regulation of chitin synthesis in *Saccharomyces cerevisiae*. *J Cell Biol* **145:** 1153–1163.

Uhl, M.A., and Johnson, A.D. (2001) Development of *Streptococcus thermophilus lacZ* as a reporter gene for *Candida albicans*. *Microbiology* **14:** 1189–1195.

Valdivia, R.H., and Schekman, R. (2003) The yeasts Rho1p and Pkc1p regulate the transport of chitin synthase III (Chs3p) from internal stores to the plasma membrane. *Proc Natl Acad Sci USA* **100:** 10287–10292.

Valdivieso, M.H., Ferrario, L., Vai, M., Duran, A., and Popolo, L. (2000) Chitin synthesis in a *gas1* mutant of *Saccharomyces cerevisiae*. *J Bacteriol* **182:** 4752–4757.

Wang, Q., Liu, H., and Szaniszlo, P.J. (2002) Compensatory expression of five chitin synthase genes, a response to stress stimuli, in *Wangiella (Exophiala) dermatitidis*, a melanized fungal pathogen of humans. *Microbiology* **148:** 2811–2817.

Wessels, J.G.H. (1990) Role of cell wall architecture in fungal tip growth generation. In *Tip Growth in Plant and Fungal Cells.* Heath, I.B. (ed.). San Diego, CA: Academic Press, pp. 1–28.

Yoshimoto, H., Saltsman, K., Gasch, A.P., Li, H.X., Ogawa, N., Botstein, D., *et al.* (2002) Genome-wide analysis of gene expression regulated by the calcineurin/Crz1p signaling pathway in *Saccharomyces cerevisiae*. *J Biol Chem* **277:** 31079–31088.

Ziman, M., Tsung, M., and Schekman, R.W. (1996) Role of *CHS6*/*CSD3* in the membrane trafficking of Chs3p. A *Saccharomyces cerevisiae* chitin synthase whose localization is temporally and spatially regulated. *Mol Biol Cell* **7:** 1893.

Ziman, M., Chuang, J.S., Tsung, M., Hamamoto, S., and Schekman, R. (1998) Chs6p-dependent anterograde transport of Chs3p from the chitosome to the plasma membrane in *Saccharomyces cerevisiae*. *Mol Biol Cell* **9:** 1565–1576.

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