

Regulation of chitin synthesis during dimorphic growth of *Candida albicans*

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***Candida albicans* has three genes encoding chitin synthase enzymes. In wild-type strains, the expression of *CHS2* and *CHS3* peaked 1–2 h after the induction of hyphal growth, whilst mRNA levels in a non-germinative strain, CA2, remained low under the same conditions. *CHS1* gene expression did not peak during germ tube formation but remained at low levels in both yeast and hyphal growth. The pattern of gene expression did not predict the changes in measured chitin synthase activities or changes in chitin content during dimorphic transition. Chitin synthase activity increased steadily, and did not peak shortly after germ tube induction, and activity profiles were similar in germ-tube-competent and germ-tube-negative strains. The phenotype of a $\Delta chs2$ null mutant suggested that *CHS2* encoded the major enzyme activity *in vitro* and was largely responsible for elevated chitin synthase activities in microsomal preparations from hyphal cells compared to yeast cells. However, CaChs3p was responsible for synthesis of most chitin in both yeast and hyphae. Three independent chitin assays gave markedly different estimates of the relative chitin content of yeast and hyphae and wild-type and *chs* mutants. Only one of the methods gave a significantly higher chitin content for hyphal compared to yeast cell walls and a lower chitin content for hyphae of the $\Delta chs2$ null mutant compared to the parental strain.**

Keywords: *Candida albicans*, dimorphism, gene expression, chitin synthase, chitin

INTRODUCTION

Chitin synthase isoenzymes of fungi catalyse the synthesis of the structural polysaccharide chitin, the (1–4)- β -homopolymer of *N*-acetylglucosamine that is important for cell shape, strength and viability (Bulawa, 1993; Shaw *et al.*, 1991; Yarden & Yanofsky, 1991). The hyphal cell walls of the dimorphic human pathogenic fungus *Candida albicans* have been reported to have a higher chitin content than yeast cell walls (Chattaway *et al.*, 1968; Elorza *et al.*, 1994; Sullivan *et al.*, 1983) and the specific chitin synthase activity of hyphae has been estimated to be twice that of yeast cells (Braun & Calderone, 1978). Therefore, chitin synthesis in *C. albicans* is regulated in a temporal and spatial manner. The study of chitin synthesis is relevant not only to our understanding of fungal growth and morphogenesis but

also in relation to its potential as a target for antifungal drugs. The enzyme chitin synthase is common to all fungi but has not been found in mammalian cells, although an enzyme that is capable of synthesizing short chitin oligosaccharides has recently been described in *Xenopus* (Semino & Robbins, 1995).

The study of chitin synthesis is most advanced in *Saccharomyces cerevisiae*, where genes for three chitin synthase isoenzymes have been characterized (reviewed by Bulawa, 1993). There is no evidence for functional redundancy of chitin synthase isoenzymes in *S. cerevisiae* – targeted gene disruption experiments suggest that each isoenzyme performs a separate role and functions at a distinct stage of the cell cycle. ScChs1p is the major activity *in vitro* yet $\Delta chs1$ mutants have only a subtle phenotype of bud lysis under certain conditions. The ScChs1p is thought to be involved in cell wall repair following chitinase-mediated release of the daughter bud (Bulawa *et al.*, 1986; Cabib *et al.*, 1989, 1992). ScChs2p synthesizes chitin of the primary septal plate (Bulawa & Osmond, 1990; Silverman *et al.*, 1988; Shaw

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Abbreviation: MMF, mixed membrane fraction.

et al., 1991) while ScChs3p is responsible for the bulk of chitin production in both the lateral cell wall and at the chitin ring that marks the site of new bud development and eventually gives rise to the bud scar (Bulawa, 1992; Shaw *et al.*, 1991; Valdivieso *et al.*, 1991). The functional role of the chitin synthase enzymes in *S. cerevisiae* is also supported by immunocytochemical studies. The ScChs-2p was found at the neck of buds at late mitosis while the ScChs3p formed a surface ring in pre- and post-budded cells (Chuang & Schekman, 1996).

C. albicans also has three chitin synthase genes, *CaCHS1*–3, numbered in the order in which they were isolated (Au-Young & Robbins, 1990; Chen-Wu *et al.*, 1992; Sudoh *et al.*, 1993). The *CaCHS1* and *CaCHS2* genes belong to classes II and I, respectively, as defined by Bowen *et al.* (1992). The two gene products are apparently zymogenic and are activated *in vitro* by proteolysis. The *CaCHS3* gene is a class IV enzyme (Din *et al.*, 1996) and until recently enzymes in this class were thought to be non-zymogenic. However, the *ScCHS3* gene product is activated by proteases if the substrate UDP-N-acetylglucosamine is used to protect the enzyme from proteolysis during its isolation (Choi *et al.*, 1994b).

Although CaChs1p has a pH optimum similar to ScChs1p (Au-Young & Robbins, 1990), it is more similar at the amino acid level to the predicted ScChs2p. Reciprocally, *CaCHS2* is more similar to *ScCHS1* at the predicted amino acid level and is shown here to encode the major chitin synthase activity when assayed *in vitro*. It is likely that the *CHS1* gene of *C. albicans* is the functional homologue of *ScCHS2* which is involved in the synthesis of the chitin plate in the primary septum (Mio *et al.*, 1996; C. A. Munro, K. Winter, C. E. Bulawa, A. J. P. Brown & N. A. R. Gow, unpublished). The activation and regulation of the chitin synthase enzymes *in vivo* is not well understood.

Preliminary reports based on very limited Northern analyses suggest that *CaCHS2* and *CaCHS3* are expressed preferentially in the hyphal phase of growth whilst *CaCHS1* appears to be expressed at a low level in both yeast and hyphae (Chen-Wu *et al.*, 1992; Sudoh *et al.*, 1993). Here we extend these studies and show that *CHS* gene expression is regulated differentially during yeast/hypha transitions induced by a wide variety of culture conditions that support filamentous growth.

Characterization of the role of *Candida* chitin synthase isoenzymes has employed the Ura-blaster technique to disrupt the *CaCHS2* (Gow *et al.*, 1994) and the *CaCHS3* (Bulawa *et al.*, 1995) genes. Neither of these genes was found to be essential and both null mutants were able to produce hyphae. The $\Delta chs3$ null mutant was characterized by an approximate 80% reduction in cell wall chitin compared to the parental strain (Bulawa *et al.*, 1995). Historically, a variety of different methods have been used to measure the chitin content of chitin synthase mutants, making it difficult to compare the data from different laboratories. One discrepancy between the different studies has been the chitin content of

the $\Delta chs2$ null mutant. Gow *et al.* (1994) reported a 40% reduction in the chitin content of the hyphal form of a $\Delta chs2$ null mutant whereas Mio *et al.* (1996) found the chitin content to be similar to that of the parental strain. We address this discrepancy and report here that chitin contents determined by different methods yield markedly different results that can account for the apparent discrepancies in the literature.

METHODS

Strains and mutants. Invasive clinical isolates of *C. albicans*, Ca94 and Ca30, and non-invasive isolates Ca88 and Ca89 were supplied by Dr G. Cole, University of Texas, Austin, TX (Cole *et al.*, 1990). *C. albicans* (Robin) Berkhout, strain 3153, was obtained from the Mycological Reference Collection (formerly at Colindale, London, now at Department of Microbiology, University of Leeds). *C. albicans* CA2 was supplied by Professor A. Cassone, Instituto Superiore di Sanita, Rome. The CA2 strain does not produce germ tubes under conditions that normally induce germ tube production *in vitro* but forms true, unstricted hyphae in vaginitis infection models (De Bernardis *et al.*, 1993). The Ura⁺ strain *C. albicans* SGY243 was a gift from R. Kelly (Kelly *et al.*, 1987). NGY2 and NGY4 are heterozygous $\Delta chs2$ null mutants with one or two disrupted copies of *CaCHS2* and NGY10 is a homozygous $\Delta chs2$ null mutant (Gow *et al.*, 1994). All were constructed from the parental Ura⁺ strain SGY243 (Table 1). The $\Delta chs3$ mutant Myco3 was supplied by C. Bulawa (Bulawa *et al.*, 1995).

Culture conditions for yeast and hyphal growth. Yeast cells of *C. albicans* were grown in the medium described by Buffo *et al.* (1984) as modified from Lee *et al.* (1975) adjusted to pH 4.5 and incubated at 25 °C. Hyphal growth occurred in the same medium at pH 6.5 and 37 °C. For cell-density-dependent dimorphic regulation, stationary phase yeast cells were grown in this medium at pH 4.5 and 25 °C and were then inoculated into fresh medium at pH 6.5 and 37 °C at an inoculum density of 2×10^7 cells ml⁻¹ (low density for hyphal growth) or $\geq 1 \times 10^8$ cells ml⁻¹ (high density for yeast growth). Hyphal growth was also induced by resuspending yeast cells grown in YPD (Sherman, 1991) in 10 vols pre-warmed 5% newborn calf serum (Gow & Goody, 1982) or in 2.5 mM N-acetylglucosamine containing 0.01 mM imidazole/HCl buffer (pH 6.6) and 0.1 mM MnSO₄ (Simonetti *et al.*, 1974) and incubating at 37 °C. In all experiments, germ tube formation was monitored using a light microscope and cell density was measured spectrophotometrically at 600 nm (LKB-Ultrospec II model 4050 spectrophotometer).

RNA extraction and Northern blot analysis. *C. albicans* cells were harvested at the various time-points by centrifugation and cell pellets were stored at -70 °C. Total RNA was extracted from the cells and Northern blots were prepared by the method of Hube *et al.* (1994). No suitable internal loading standard has been identified for Northern analysis of *C. albicans* mRNA during germ tube formation (Delbruck & Ernst, 1993; Swoboda *et al.*, 1994; Gow *et al.*, 1995). Therefore, equal loading was confirmed by examining the density of ethidium-bromide-stained rRNA bands. Sizes of *CHS* transcripts were estimated by assuming that the *C. albicans* rRNA transcripts were of similar sizes to the *S. cerevisiae* 18S and 26S rRNA bands, which are 1789 (Rubtsov *et al.*, 1980) and 3392 (Georgiev *et al.*, 1981) nucleotides, respectively.

Table 1. *C. albicans* strains

Strain	Genotype	Reference
SGY243	<i>ade2/ade2, Δura3::ADE2/Δura3::ADE2, CHS2/CHS2/CHS2</i>	Kelly <i>et al.</i> (1987)
NGY2	<i>ade2/ade2, Δura3::ADE2/Δura3::ADE2, Δchs2::hisG/CHS2/CHS2</i>	Gow <i>et al.</i> (1994)
NGY4	<i>ade2/ade2, Δura3::ADE2/Δura3::ADE2, Δchs2::hisG/Δchs2::hisG/CHS2</i>	Gow <i>et al.</i> (1994)
NGY10	<i>ade2/ade2, Δura3::ADE2/Δura3::ADE2, Δchs2::hisG/Δchs2::hisG/Δchs2::hisG</i>	Gow <i>et al.</i> (1994)
Δchs3	<i>Δura3::λimm434/Δura3::λimm434, Δchs3::hisG/Δchs3::hisG::URA3::hisG</i>	Bulawa <i>et al.</i> (1995)

The Northern blot membranes were pre-hybridized overnight in 50% (v/v) formamide, 0.5% (w/v) SDS, 5× SSC and 5× Denhardt's solution at 42 °C. Denatured salmon sperm DNA was added at a final concentration of 100 µg ml⁻¹. Probe DNA was labelled with 50 µCi (1.85 MBq) [α -³²P]dCTP by the Random Primed Labelling kit (Boehringer). After at least 16 h incubation, membranes were washed twice in a solution containing 2× SSC and 0.1% (w/v) SDS for 5 min at 20 °C followed by 20 min at 42 °C. They were then washed with 2× SSC, 0.1% (w/v) SDS for 20 min at 55 °C and finally twice with 0.2× SSC, 0.5% (w/v) SDS for 20 min at 55 °C. The expression levels of the *CHS* genes were low and membranes were normally exposed to Fuji medical X-ray film for up to 6 d. In each case, the membranes were stripped and hybridized with each of the three *CaCHS*-gene-specific probes.

Purification of DNA probes for Northern blot analysis. Plasmid pJA16 was kindly provided by Dr J. Au-Young (Au-Young & Robbins, 1990) and contained the *CaCHS1* gene located in the vector YEp351 (Hill *et al.*, 1986). A *CaCHS1*-specific probe was prepared by digesting plasmid pJA16 with *Clal*, which released a 1 kb fragment of the *CaCHS1* ORF. The *CaCHS2*-specific probe was generated from plasmid pNG17 (Gow *et al.*, 1994) by purification of a 1.7 kb *HindIII* fragment. A partial fragment of the *CaCHS3* gene was generated by PCR. Primers 1 and 2 were constructed that were homologous to two regions at the 5' end of the ORF of *CaCHS3*. These primers were designed to include unique recognition sites for the restriction endonucleases *KpnI* and *BamHI*, respectively: primer 1 forward, 5' GTTACACCA-TGGGGACTTC 3'; primer 2 reverse, 5' TTAGATGGAT-CCCAAGCAGC 3'.

These primers were employed to PCR-amplify a 1.24 kb fragment from *C. albicans* genomic DNA. The PCR product was gel-purified using the Prep-a-Gene kit (Bio-Rad) following the manufacturer's recommendations and was then cloned into pBluescript SK(−) (Stratagene) at the *KpnI* and *BamHI* sites to give plasmid pDAS. The identity of the cloned PCR product was confirmed by sequencing and Southern analysis. Plasmid pDAS was then digested with *KpnI* and *BamHI* to release the insert, which was used as a *CaCHS3*-specific probe.

Measurement of chitin synthase activity. Yeast cells were grown on YPD at 30 °C and harvested in late exponential phase. Germ tube formation was induced by inoculation into 20% (v/v) newborn calf serum (Sigma) of <2 × 10⁷ stationary phase yeast cells ml⁻¹ or by the cell-density-dependent conditions described above. After 6 h incubation with shaking at 37 °C, cells with germ tubes were harvested. Mixed membrane fractions (MMFs) were prepared by the method of Orlean (1987). Membrane pellets were resuspended in 50 mM Tris/HCl (pH 7.5) supplemented with 30% (v/v) glycerol and stored at −20 °C. The protein concentration of each MMF preparation was determined using the Coomassie Protein Assay kit (Pierce).

Enzyme assays were performed with and without trypsin pre-treatment. Activation with trypsin was optimized for each MMF preparation but standard trypsin pre-treatment used 100 ng trypsin (µl MMF)⁻¹ incubated for 5 min at 30 °C. Longer incubations resulted in loss of chitin synthase activity. Trypsin activation was stopped by addition of 150 ng soybean trypsin inhibitor (µl MMF)⁻¹. Standard reactions for measuring chitin synthase activity were carried out in 50 µl volumes containing: 50 µg MMF protein, 25 mM *N*-acetylglucosamine, 50 mM Tris/HCl (pH 7.5), 10 mM MgCl₂ and 1 mM UDP-*N*-acetylglucosamine which included 25 nCi (0.9 kBq) UDP-[U-¹⁴C]*N*-acetylglucosamine. Reactions were carried out at 30 °C for 30 min and then were stopped by addition of 1 ml 66% (v/v) ethanol. Reaction mixtures were then filtered through GF/C filter discs which had been pre-soaked in 10% (v/v) trichloroacetic acid and reaction tubes were rinsed out with 2 × 1 ml 1% (v/v) Triton X-100. Each filter was then washed with 4 × 2 ml 66% (v/v) ethanol, dried at 80 °C and then placed in a vial for liquid scintillation counting. Results are expressed as units of chitin synthase activity. One unit represents 1 nmol UDP-*N*-acetylglucosamine incorporated into chitin min⁻¹ (mg protein)⁻¹.

Chitin measurements. Three different methods were used for chitin determinations. The method of Ride & Drysdale (1972) combines an alkaline deacetylation to convert chitin to chitosan, and a nitrous acid treatment which depolymerizes and deaminates chitosan to the aldehyde 2,5-anhydro-mannose, which is measured colorimetrically. The second method involved acid hydrolysis of whole cells to break down chitin to glucosamine (Yabe *et al.*, 1996). A third method involved the hydrolysis of chitin to *N*-acetylglucosamine by the action of chitinase (from *Streptomyces griseus*; supplied by Sigma) and β -*N*-acetylhexosaminidase (a contaminant of Sigma β -glucuronidase G-1512) and was based on the protocol of Bulawa *et al.* (1986) as modified by Mellado *et al.* (1996). *C. albicans* cells were grown in the yeast phase in YPD at 30 °C or in the hyphal phase in 20% serum at 37 °C, each for 6 h. For the first two methods, cells were freeze-dried to constant weight and the assays were performed on dried cells. For the third method (Bulawa *et al.*, 1986), wet cells were used and a sample was freeze-dried so that results could be converted to dry weights.

RESULTS

CHS expression in batch culture of the yeast form

Invasive (Ca94 and Ca30) and non-invasive (Ca88 and Ca89) clinical isolates were grown in the yeast form in YPD medium and samples were harvested at various stages of growth. The general pattern of expression of the three *CHS* genes was qualitatively similar in all four

strains although the level of transcription differed between strains (Fig. 1). Levels of expression of all three genes were highest in the mid exponential phase 5.5 h after inoculation into fresh media. *CaCHS2* and *CaCHS3* transcripts were detected at all the time-points tested but little or no *CaCHS1* message was detectable in the sample from the inoculum (0 h) and stationary phase (after 9 h) of growth.

The influence of external pH on *CHS* gene expression was examined by growing yeast cells in yeast carbon base at a pH range of 3–7. As the external pH increased, the level of expression of all three chitin synthase genes increased and expression was highest in cells grown at pH 7 (data not shown).

Levels of *CHS* mRNAs during the dimorphic transition

Yeast cells of *C. albicans* were induced to form hyphae by pH/temperature shift (Buffo *et al.*, 1984) and by inoculation into medium containing either 5% serum (Gow & Gooday, 1982) or 25 mM *N*-acetylglucosamine (Simonetti *et al.*, 1974). In pH/temperature-mediated dimorphism, growth of the yeast form is favoured at pH 4.5 and 25 °C, at pH 6.5 and 25 °C, and at pH 4.5 and 37 °C, whilst hypha formation occurs at pH 6.5 and 37 °C. *CaCHS1* appeared to be expressed constitutively throughout both yeast and hyphal phases of growth (Fig. 2). In contrast, levels of *CaCHS2* and *CaCHS3* mRNA were high in exponentially growing yeast cells (5 or 6 h time-points) but rapidly decreased and expression increased transiently during hypha formation at pH 6.5 and 37 °C. Highest levels of *CaCHS2* and *CaCHS3* mRNA were detected 1–2 h after inoculation into germ-tube-inducing media. A similar pattern of expression was also found when strain 3153 was induced to form germ tubes in the presence of serum or *N*-acetylglucosamine (data not shown). *CaCHS1* mRNA was undetectable in hyphal cells induced by *N*-acetylglucosamine.

The dimorphic transition was also controlled by inoculum density in a manner similar to that described by Chen-Wu *et al.* (1992). A high inoculum density ($\sim 1 \times 10^8$ cells ml⁻¹) supported yeast growth with only 15% germ tubes present after 4 h. In contrast, at a low inoculum density of 2×10^7 cells ml⁻¹, 81% hypha formation occurred by 4 h. *CaCHS1* mRNA was detected at low levels in the yeast phase and highest levels of expression of *CaCHS1* occurred when there were 81% germ tubes present (Fig. 3). *CaCHS2* and *CaCHS3* were expressed in both yeast and hyphal phases of growth but these peaked at a sustained high level during hypha formation (Fig. 3). The overall pattern of expression of the three genes was similar in all conditions supporting hypha development and differed significantly from control cultures grown at either the same external pH (Fig. 2) or temperature (Fig. 3), suggesting that changes in external pH and/or temperature were unlikely to explain the observed patterns of transcriptional regulation.

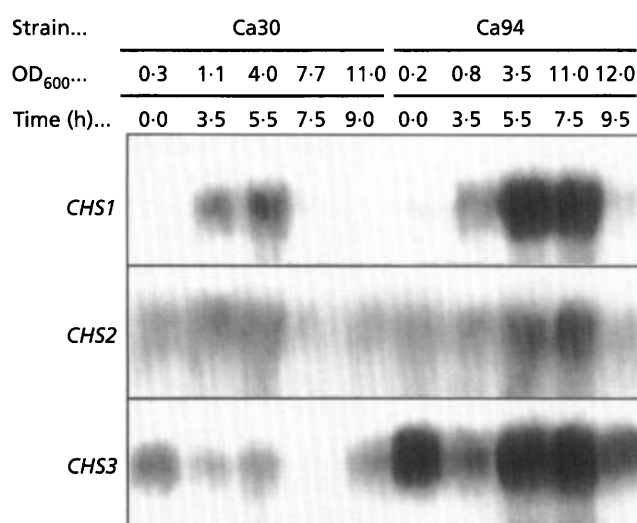


Fig. 1. Chitin synthase gene expression during growth in the yeast form of two invasive clinical isolates of *C. albicans*. Stationary phase yeast cells of strains Ca30 and Ca94 were diluted 50-fold with fresh YPD medium, grown at 30 °C and samples were removed at various time-points. RNA was extracted, subjected to Northern blotting and hybridized to probes specific for *CHS1*, *CHS2* and *CHS3*.

The pattern of *CHS* gene expression of strain 3153 was also compared to that of strain CA2 during hypha development. Strain CA2 is impaired in germ tube formation *in vitro*, but not *in vivo* (De Bernardis *et al.*, 1993). When grown at low inoculation density under conditions that normally induce germ tube formation (Buffo *et al.*, 1984), CA2 formed fewer than 5% germ tubes. Northern analysis indicated that *CaCHS1* mRNA levels in CA2 were comparable to those of strain 3153 under conditions that would normally stimulate germ tube formation (Fig. 4). However, *CaCHS2* and *CaCHS3* expression increased more slowly in CA2 in these media and peaked 4 h after induction compared to 1–2 h after induction for germ-tube-positive strains. This transcriptional pattern differed from that in germ-tube-positive strains grown under hypha-inducing or -repressive conditions. Therefore, under all conditions tested a transient increase in the transcription of *CaCHS2* and *CaCHS3* was correlated with germ tube development.

Chitin synthase activity during the dimorphic transition

Chitin synthase activity, with and without trypsin pre-treatment, was measured for germ-tube-positive (3153) and germ-tube-negative (CA2) strains (Fig. 5). Cells were grown in YPD for yeast growth and serum-containing medium for hyphal cultures and under conditions employed previously for culture-density-regulated dimorphism. The pattern of chitin synthase activities of the two strains was similar but not predicted by the patterns of *CHS* gene expression. For example, at

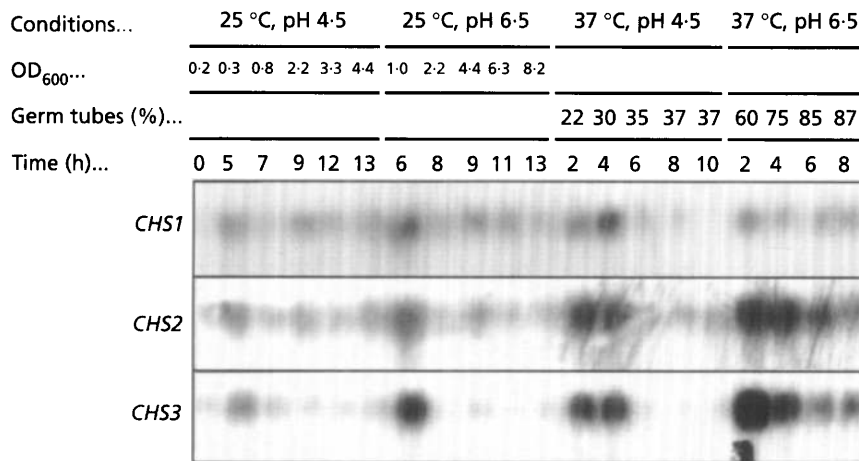


Fig. 2. Expression of chitin synthase genes during pH/temperature-stimulated dimorphism. *C. albicans* strain 3153 was grown to stationary phase in the medium of Buffo *et al.* (1984), pH 4.5, at 25 °C. Washed inocula of yeast cells were resuspended in fresh medium at: pH 4.5, 25 °C; pH 6.5, 25 °C; pH 4.5, 37 °C; or pH 6.5, 37 °C. RNA was extracted and hybridized with probes specific for *CHS1*, *CHS2* and *CHS3*.

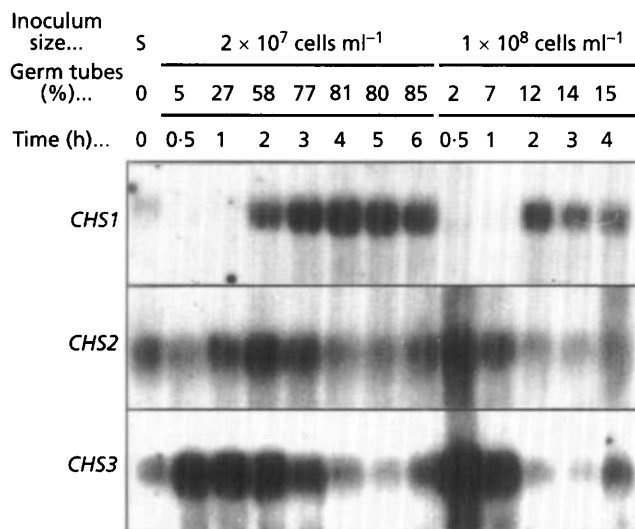


Fig. 3. Expression of chitin synthase genes during inoculum-density-regulated dimorphism. *C. albicans* strain 3153 was grown in the medium of Buffo *et al.* (1984), pH 4.5, at 25 °C to stationary phase. Washed inocula of yeast cells were incubated in fresh medium at pH 6.5 at 2×10^7 cells ml⁻¹ or 1×10^8 cells ml⁻¹ and incubated at 37 °C. Low inoculum density supported hypha formation whilst high inoculum density maintained growth mainly in the yeast form. RNA was extracted from cells harvested at different time-points up to 4 h and analysed by Northern blotting with probes specific for the three *CHS* genes. S, Stationary phase.

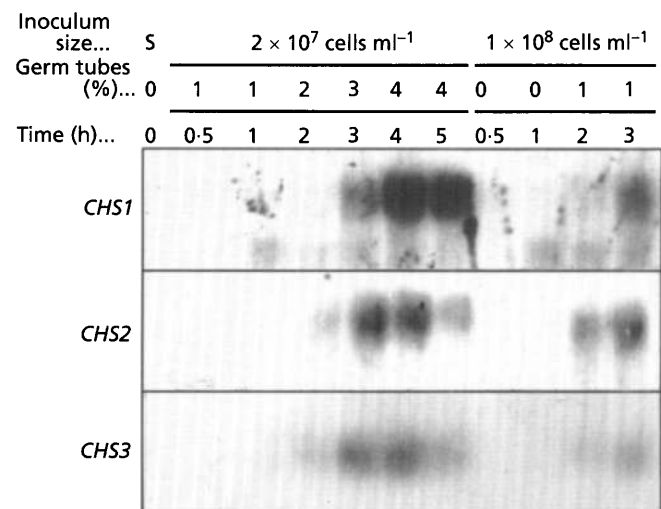


Fig. 4. Expression of *CHS* genes during growth of a non-germinative *C. albicans* strain, CA2, under conditions which stimulate germ tube induction in wild-type strains. A culture was grown to stationary phase in the medium of Buffo *et al.* (1984), pH 4.5, at 25 °C and then subcultured into fresh medium at pH 6.5 and 37 °C at either 2×10^7 cells ml⁻¹ (conditions that would normally support germ tube formation) or 1×10^8 cells ml⁻¹ (which maintained growth in the yeast form). RNA was extracted and subjected to Northern blot analysis with probes specific for the three *CHS* genes of *C. albicans*. S, Stationary phase.

2 h post hypha induction, the levels of *CaCHS2* and *CaCHS3* mRNA were markedly higher in strain 3153 compared to CA2. However, at this time-point the only difference in the chitin synthase activities was a slight decrease in the activity of CA2 prior to trypsin pre-treatment. Cells grown in serum and YPD also had similar changes in chitin synthase activity in the two strains but activities were higher in these two media compared to the cells isolated from pH/temperature-regulated cultures (data not shown). Therefore, chitin synthase production must also be regulated at a post-transcriptional and/or post-translational level.

Chitin synthase activity of $\Delta chs2$ mutants

The chitin synthase activity of the $\Delta chs2$ null mutant was reduced three- to fivefold compared to the SGY243 parent in both yeast and hyphal cells (Fig. 6). Strain SGY243 has three copies of the *CaCHS2* gene (Gow *et al.*, 1994). Progressive decreases in total chitin synthase activity were observed in strains harbouring one, two or three disrupted copies of the *CaCHS2* gene. The residual 20% chitin synthase activity in the $\Delta chs2$ null mutant presumably corresponds to CaChs1p and CaChs3p activities. Chitin synthase activities were compared

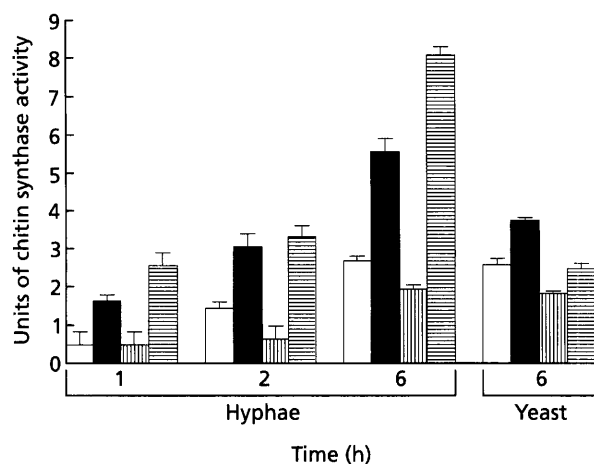


Fig. 5. Measurement of chitin synthase activity in strain 3153 and the non-germinative strain CA2. Stationary phase yeast cells grown on the medium of Buffo *et al.* (1984), pH 4.5, at 25 °C were inoculated into fresh medium at pH 6.5 and 37 °C at an inoculum density of 2×10^7 cells ml⁻¹ (low density for hyphal growth) or $\geq 1 \times 10^8$ cells ml⁻¹ (high density for yeast growth). Cells were harvested at 1, 2 and 6 h time-points and MMFs were prepared. The total chitin synthase activity of each membrane fraction was measured as described in Methods. Errors bars are SD ($n = 3$). □, 3153; ■, 3153 plus trypsin; ▨, CA2; ▩, CA2 plus trypsin.

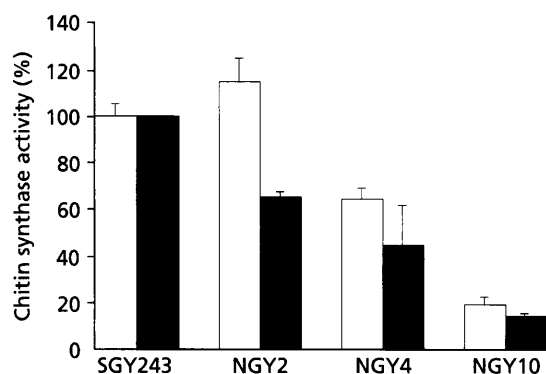


Fig. 6. Measurement of chitin synthase activity of $\Delta chs2$ mutants compared to their parental strain SGY243. Strains NGY2, NGY4 and NGY10 have one, two and three copies of the *CHS2* gene disrupted, respectively. The activities were measured after trypsin pre-treatment and the activities of SGY243 membrane preparations from yeast and hyphal cells were taken as 100%. For strain SGY243, hyphal membranes had approximately twofold higher activity than yeast membranes. Representative data from one of three independent experiments are presented. Mean values from independent treatments were not significantly different ($P < 0.05$). Error bars are SD ($n = 3$). □, Yeast; ■, hyphae.

when Co^{2+} replaced Mg^{2+} in the assay (Table 2). There was little difference in the level of the total activity in wild-type cells, although the trypsinized membranes gave slightly higher activity with Mg^{2+} whereas the untreated membranes had higher activity in the presence of Co^{2+} . Trypsinized membranes from the $\Delta chs2$ null

mutant also had marginally increased activity in the presence of Co^{2+} , suggesting that CaChs1p and CaChs3p may be activated preferentially by Co^{2+} while CaChs2p is most efficiently activated by Mg^{2+} . The differences in activities with Mg^{2+} and those with Co^{2+} were not statistically significant ($P < 0.05$). The inhibition of the chitin synthase activity by a mixture of nikkomycins X and Z was compared for membranes from SGY243 and the $\Delta chs2$ null mutant (Table 2). In wild-type membranes, chitin synthase activity was decreased by 70% in the presence of 2.5 μM nikkomycin whereas 50 μM nikkomycin was required to reduce the chitin synthase activity of the $\Delta chs2$ null mutant by 70%.

Measurement of chitin contents

The chitin content of the cell wall of yeast and hyphal forms of strain SGY243 and the $\Delta chs2$ null strain NGY10 was determined using three chitin content assays (Fig. 7). The nitrous acid method (Ride & Drysdale, 1972) gave similar values for the chitin content of yeast cells of both strains with only marginally elevated values for $\Delta chs2$ yeast cells. The value for the chitin content of the hyphal form of SGY243 was double that of the chitin content of the yeast form whereas hyphae of the $\Delta chs2$ null mutant contained about the same amount of chitin as the yeast form. As reported previously, the hyphal form of the $\Delta chs2$ null mutant had a 44% reduction in chitin content compared to the wild-type strain (Gow *et al.*, 1994). However, when chitin content was determined by measuring glucosamine released by acid hydrolysis (Yabe *et al.*, 1996) the $\Delta chs2$ null mutant showed elevated chitin levels in the yeast form, and there was no significant difference in the chitin content of hyphae in parental and $\Delta chs2$ null strains. A third chitin content assay in which the *N*-acetylglucosamine released from chitin by the action of chitinase and β -*N*-acetylhexosaminidase was measured (Bulawa *et al.*, 1995) gave similar results to those obtained by acid hydrolysis but yielded much lower chitin contents than the previous two methods (Fig. 7). The parental and $\Delta chs2$ mutant strains had similar chitin contents and there was no appreciable increase in the amount of chitin in the hyphal forms. Pre-treating cells with lyticase (Sigma) or Zymolyase-20T (ICN Biomedicals) to digest β -glucans that may have been covalently attached to the chitin did not increase the amount of *N*-acetylglucosamine released by this method.

The chitin content of the $\Delta chs3$ null mutant of *C. albicans* was also measured using the three chitin assays. The Ride & Drysdale (1972) and the acid hydrolysis protocols both showed that the yeast and hyphae of the $\Delta chs3$ null mutant had 32–39% of the chitin content of the parental strain CAI4, comparable to the 60% reduction in chitin content reported by Mio *et al.* (1996), measured using the acid hydrolysis method. Chitin determination by the enzymic method gave an 85% reduction in both yeast and hyphae which is again

Table 2. Chitin synthase activities measured in the presence of Mg^{2+} , Co^{2+} and nikkomycins X and Z

Results are expressed as units of chitin synthase activity. One unit represents 1 nmol UDP-N-acetylglucosamine incorporated into chitin min^{-1} (mg protein^{-1}).

Strain	Trypsin	Chitin synthase activity in the presence of:		
		Mg^{2+} *	Co^{2+} *	Nikkomycin (μM)†
SGY243 (wild-type)	—	3.0 ± 1.0	4.0 ± 0.7	7.2 ± 1.4 (0.0)
	+	7.6 ± 0.7	5.8 ± 1.6	2.1 ± 0.6 (2.5)
NGY10 (Δchs2)	—	1.0 ± 0.5	1.4 ± 0.5	1.4 ± 0.4 (10.0)
	+	1.4 ± 0.3	1.7 ± 0.8	0.9 ± 0.1 (0.0)
				0.7 ± 0.1 (10.0)
				0.3 ± 0.1 (50.0)

* Each value represents the mean \pm SD of triplicate samples measured from three independent experiments.

† Trypsin-activated yeast MMFs were used for measurement of chitin synthase activity and the assay was performed in the presence of magnesium. Values represent the mean \pm SD of triplicate samples.

similar to previous examination of yeast cells of the *Candida* Δchs3 mutant using this method (Bulawa *et al.*, 1995). Therefore we conclude that the apparent disparities in the published literature on chitin contents of various *CHS* null mutants reflect differences in the methods used for chitin determinations.

One notable difference between the three assays was their ability to break down ultrapure crystalline chitin (Katakura Chikkarin). The Ride & Drysdale (1972) method detected only 28% and the acid hydrolysis method 60%, while the enzymic method was unable to break down crystalline chitin at all.

DISCUSSION

We have characterized the expression pattern of the three genes encoding chitin synthase isoenzymes in *C. albicans*, and the relative contribution of the isoenzymes to enzyme activity *in vitro* and chitin formation through an analysis of strains with specific null mutations. Northern analyses of the three chitin synthase genes of *C. albicans* described here and previously (Chen-Wu *et al.*, 1992; Sudoh *et al.*, 1993) showed that they were regulated differentially at the transcriptional level. Previous reports of *CHS* gene expression in *C. albicans* found elevated levels of *CaCHS2* and *CaCHS3* mRNA in hyphal cells and a preference for *CaCHS1* expression in yeast cells (Chen-Wu *et al.*, 1992; Sudoh *et al.*, 1993). However, in this more expansive study we found *CaCHS1* was expressed equally in yeast and hyphal cells. *CaCHS1* expression exhibited a different pattern to that of *CaCHS2* and *CaCHS3*. Highest levels of *CaCHS1* mRNA occurred in the late exponential phase of yeast phase growth. *CaCHS2* and *CaCHS3* were co-expressed and exhibited a transient peak of expression 1–2 h after induction of the hyphal growth phase under all the conditions tested. Levels then subsided by 4 h of hyphal induction. The elevated level of *CaCHS2* and

CaCHS3 mRNA in hyphal cells is commensurate with hyphal cells having a higher chitin content and chitin synthase activity than yeast cells. However, both the Δchs2 and Δchs3 null mutants formed hyphae efficiently and in wild-type cells the elevated chitin content of hyphal cells was maintained even when *CaCHS2* and *CaCHS3* mRNA levels declined. Therefore these genes are not required for hypha formation and the temporal pattern of transcription does not tightly parallel the extent of chitin synthesis. Under conditions that normally induced filamentous growth, the non-germinative strain CA2 did not show the initial stimulation of *CaCHS2* and *CaCHS3* expression observed in germ-tube-competent strains. However, total chitin synthase activity (mostly due to *CaChs2p* activity) increased in a similar way to that seen in the wild-type strain 3153. This again underlines the general conclusion that there was no overt relationship between up-regulated *CHS* gene expression and changes in chitin synthase activity, chitin content and cell shape.

Measurement of chitin synthase activity of the Δchs2 null mutant showed that *CaChs2p* is the major activity when assayed *in vitro*. Previous reports of zymogenic chitin synthase activity measured in wild-type cells can therefore be contributed in the main to *CaChs2p* activity since this dominant activity will normally mask the other enzymes. In *S. cerevisiae*, the *Chs1p* activity was responsible for most activity *in vitro* (Bulawa *et al.*, 1986). The chitin synthase activity remaining in the *Candida* Δchs2 null mutant had a preference for Co^{2+} over Mg^{2+} in both untreated and trypsin-treated membranes, whereas the native zymogenic activity encoded by *CaCHS2* was maximal in the presence of Mg^{2+} . The *Candida* *Chs2p* and the *Saccharomyces* *Chs1p* share the properties of having the highest total *in vitro* activity and similar metal ion requirements. In addition, the activity remaining in the *Candida* Δchs2 null mutant was

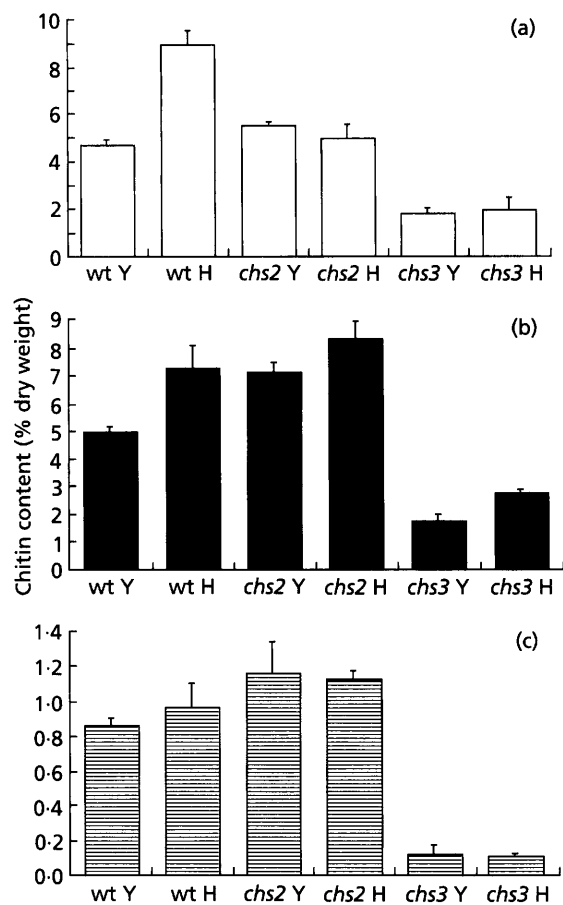


Fig. 7. Chitin content of *C. albicans* strains SGY243 and the Δ chs2 null mutant NGY10 were measured following three protocols: (a) the nitrous acid method of Ride & Drysdale (1972); (b) the acid hydrolysis method (Yabe *et al.*, 1996); and (c) the chitinase extraction method (Bulawa *et al.*, 1986). The chitin contents of at least three independent cultures were measured using each method. Assays were performed a minimum of three times and each sample was measured in triplicate. The means from independent cultures were not significantly different ($P < 0.05$). Error bars represent \pm SE. Y, Yeast; H, hyphae; wt, wild-type.

much more resistant to nikkomycin compared to the CaChs2p activity. In *S. cerevisiae*, ScChs1p is the most sensitive to nikkomycin while ScChs2p is least sensitive (Gaughran *et al.*, 1994). These observations coupled with evidence from deduced protein sequences (Chen-Wu *et al.*, 1992) again suggest that the CaCHS2 gene product is more closely related to the ScCHS1 gene product than to the *Saccharomyces* Chs2p.

In wild-type strains, chitin synthase activity increased steadily up to 6 h after germ tube induction. The activity measurements did not parallel the mRNA levels, which were highest for CaCHS2 and CaCHS3 1–2 h after the switch to hyphal growth. This may indicate regulation at the post-transcriptional level and may reflect in part the relative stability of the chitin synthase isoenzymes (Chuang & Schekman, 1996; Ziman *et al.*, 1996). In *S. cerevisiae*, Choi *et al.* (1994a) showed that post-trans-

lational regulation of ScCHS1 and ScCHS3 appeared to be predominant over translational regulation, whereas ScCHS2 was apparently regulated both at the transcriptional level and by synthesis and degradation of the gene product. There are many possible post-transcriptional regulatory processes that can modulate chitin synthesis (Gooday, 1995; Gooday & Schofield, 1995; Munro & Gow, 1995). These include provision of substrate, allosteric activation of chitin synthase, phosphorylation, dephosphorylation, modulation of the assembly of the chitin chains in the cell, activation of zymogenicity and in the case of Chs3p regulation by other proteins of a multienzyme complex including homologues of ScChs4p (Csd4p/Cal2p) and ScChs5p (Cal3p) (Bulawa, 1993; Santos *et al.*, 1997; Trilla *et al.*, 1997).

The insolubility and chemical resistance of chitin make quantitative determinations of chitin content difficult. Measurement of chitin in *C. albicans* has given a wide range of values in the past. Chattaway *et al.* (1968) measured chitin contents of around 3% for the alkali-insoluble fraction of yeast cell walls and 10% for the same fraction isolated from hyphae, whereas Sullivan *et al.* (1983) estimated the chitin content of isolated cell walls on a dry weight basis to be 0.6% for yeast and 2.7% for hyphae. Elorza *et al.* (1994) determined that chitin constituted 10% of wall polysaccharides in yeast cells and 21.6% in hyphae. These figures are difficult to compare with the results presented here, which were measured in extractions of whole cells. Chitin is in the innermost layer of the fungal cell wall and is masked by and covalently linked to other components of the wall, including mannoproteins and (1–3)- β - and (1–6)- β -glucan (Klis, 1994). Methods used to extract and assay chitin rely on chemical or enzymic treatments to strip off these outer layers, exposing the chitin fibrils. The method of Ride & Drysdale (1972) uses strong alkali to deacetylate chitin to chitosan. The total glucosamine method relies on acid hydrolysis to depolymerize chitin to glucosamine (Yabe *et al.*, 1996). Acid hydrolysis also liberates glucosamine from glycoproteins, which could lead to overestimation of chitin content. Prolonged hydrolysis can also result in loss of hexosamines. Chitinase extraction methods (e.g. Bulawa *et al.*, 1986) have the virtue of specificity for chitin but we found that this method gave around a threefold lower chitin content than the other two methods we investigated. However, the results from the first two methods were corrected by factors which gave a 100% yield of crystalline chitin and the chitinase method was not corrected in this fashion. This enzymic method consistently failed to demonstrate an increase in the chitin content of hyphal cells compared to yeasts as has been observed using chemical extraction methods (Chattaway *et al.*, 1968; Sullivan *et al.*, 1983; Gow *et al.*, 1994).

Chitin present in the different regions of the fungal cell wall may also vary in its crystallinity and the degree of cross-linking to other components of the cell wall, e.g. (1–3)- β -glucans (Wessels, 1986). Crystalline chitin is more resistant to degradation by chitinases (Vermeulen

& Wessels, 1984). Indeed the enzymes used here were unable to break down crystalline chitin whereas the Ride & Drysdale (1972) method detected 28% and the acid hydrolysis method 60% of crystalline chitin. Assante *et al.* (1996) also obtained only a 20% yield of purified chitin when using a modified version of the Ride & Drysdale (1972) method in which chitin was deacetylated with 4 M KOH at 130 °C. However, acid hydrolysis followed by deamination with nitrous acid achieved an almost quantitative yield and this method is probably worth considering for future experiments. The failure of chitinase to extract all the chitin in yeast and hyphal cell walls may therefore reflect the extent of chitin crystallinity and cross-linking with other polysaccharides and proteins, although we found that pretreatment of *Candida* cell walls with lyticase and Zymolyase did not increase the extraction of N-acetylglucosamine by chitinase.

The measurement of chitin may also be complicated by the presence of significant amounts of chitosan in the cell wall. Chitosan would be broken down by the Ride & Drysdale (1972) method but not by the enzymic assay. Preliminary examinations revealed only small quantities of chitosan in *C. albicans* (0.2% of dry cell weight) and equal amounts were detected in yeast and hyphae (data not shown). Therefore, the presence of chitosan is unlikely to be the cause of the anomalous chitin contents.

Previously the chitin content of the $\Delta chs2$ null mutant had been reported to be reduced by 40% in the hyphal form (Gow *et al.*, 1994). More recently, Mio *et al.* (1996) reported a much smaller decrease in hyphal chitin content in this mutant. This anomaly can be explained by the differences in the two methods used to measure chitin. This study showed that the hyphal chitin content of $\Delta chs2$ was reduced by ~44% compared to the parental strains when chitin contents were determined by the Ride & Drysdale (1972) method employed by Gow *et al.* (1994). However, little difference was seen when measured by the acid hydrolysis method used by Mio *et al.* (1996). Interestingly, all three methods gave elevated chitin levels in the yeast form of the $\Delta chs2$ null mutant compared to the parental strain. This could be explained by an increase in activity of one or both of the other isoenzymes to compensate for loss of CaChs2p; however, such an increase in activity was not detected (data not shown). Each of the methods also gave a consistently lower chitin content for the $\Delta chs3$ null mutant. Our results underline the point that chitin determinations are highly dependent on the method employed and that the apparent chitin contents inferred from extractions of cell wall material isolated from *chs* null mutant strains must be interpreted cautiously.

Measurement of total chitin synthase activity as reported here has also shown that CaCHS2 encodes the major chitin synthase activity when assayed *in vitro* and the two- to threefold increase in chitin synthase activity seen in the hyphal form was also due mainly to the CaChs2p enzyme. By analogy with *S. cerevisiae*, CaChs2p may act

in a similar way to ScChs1p, functioning as a repair enzyme during the separation of the mother and bud cell (Cabib *et al.*, 1992). However, we have shown that CaCHS2 is expressed preferentially in the hyphal form where cell division is not accompanied by cell separation, and that $\Delta chs2$ mutants may have a reduced chitin content in the hyphal form. Therefore, CaCHS2 may have an additional role to play in the hyphal form of *C. albicans*, e.g. it may be involved in maintaining cell wall integrity during branching, which may involve wall-softening by chitinases (Gooday *et al.*, 1992). However, there was no evidence of abnormal branching in the $\Delta chs2$ mutant. We did not observe the bud lysis phenotype described in $\Delta chs1$ mutants in *S. cerevisiae* (Bulawa *et al.*, 1986), even under conditions where the pH of the medium was adjusted to maximize the potentially damaging activities of chitinases which, in *S. cerevisiae*, play a role in bud separation and require subsequent chitin repair by ScChs1p (Cabib *et al.*, 1992).

Our results indicate that CaChs3p is the dominant chitin-synthesizing enzyme in both yeasts and hyphae of *C. albicans*, but that CaChs2p plays a minor role in chitin formation in the lateral cell walls of hyphae.

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