Candida albicans strains deficient in CHS7, a key regulator of chitin synthase III, exhibit morphogenetic alterations and attenuated virulence

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Chitin is a structural polysaccharide present in most fungal cell walls, whose synthesis depends on a family of enzymic activities named chitin synthases (CSs). The specific role of each of them, as well as of their regulatory proteins, in cell morphogenesis and virulence is not well understood. Here, it is shown that most chitin synthesis in Candida albicans, one of the fungi most commonly isolated from opportunistic mycoses and infections, depends on CHS7. Thus, C. albicans chs7Δ null mutants showed reduced levels of chitin and CS activity, and were resistant to Calcofluor. Despite the sequence similarity and functional relationship with ScChs7p, CaChs7p was unable to restore CSIII activity in a Saccharomyces cerevisiae chs7Δ null mutant, because it was unable to direct ScChs3p export from the endoplasmic reticulum. C. albicans chs7Δ null mutants did not show any defect in growth rate, but yeast cells displayed minor morphogenetic defects affecting septum formation, and showed an increased tendency to form filaments. CaChs7p was not required for germ-tube emission, and null mutant strains underwent the dimorphic transition correctly. However, colony morphology appeared distinctively affected. chs7Δ hyphae were very curved and had irregular lateral walls, resulting in very compact colonies that seemed unable to spread out radially on the surface, unlike the wild-type. This growth pattern may be associated with the reduced virulence and high clearance rate observed when the chs7Δ strain was used in a murine model of infection. Therefore, CaChs7p is required for normal hyphal morphogenesis, suggesting that in C. albicans CSIII plays an important role in maintaining cell wall integrity, being essential when invading surrounding tissues.

INTRODUCTION

The fungal cell wall acts as an exoskeleton that provides cells with resistance to turgor pressure, while maintaining their shape. As the most external structure of fungal cells, it is in direct contact with the external medium, including the physiological defence mechanisms of the host. Accordingly, fungal cell walls play an important role in pathogenesis, and are currently under scrutiny as antifungal targets (Chaffin et al., 1998; Smits et al., 2001).

Cell wall composition varies among the different fungi, and hence in this study we focused our attention on Candida albicans, whose cell wall is basically formed by mannoproteins, glucans and chitin (Klis et al., 2001), as in Saccharomyces cerevisiae (Smits et al., 2001), but with a higher proportion of 1,6-β-glucans. C. albicans is a dimorphic organism that can grow in both the yeast and hyphal forms, and its cell wall composition varies between these states. The most significant change is in the chitin content, which appears higher in the hyphal form (Braun & Calderone, 1978), although the observed differences may vary depending on the method used to determine chitin levels (Munro et al., 1998). Structural studies on C. albicans cell walls have indicated that chitin acts as an inner structure to which 1,3-β-glucans are linked. 1,3-β-glucans form an elastic three-dimensional network maintained by hydrogen bonding to which glycosylphosphatidylinositol (GPI) proteins bind through 1,6-β-glucan chains. Additional Pir proteins are directly linked to the 1,3-β-glucan network (Klis et al., 2001). The importance of chitin in this structure is highlighted by the fact that C. albicans and S. cerevisiae cells are sensitive to nikkomycin, a specific inhibitor of chitin synthesis (Gaughran et al., 1994; Kim et al., 2002).

Abbreviations: CS, chitin synthase; ER, endoplasmic reticulum; GlcNAc, N-acetylglucosamine; HA, haemagglutinin; WGA, wheatgerm agglutinin.
C. albicans undergoes a dimorphic transition that has been traditionally related to virulence since the hyphal forms are commonly associated with infectious states (Brown & Gow, 1999). However, it appears that both the yeast and filamentous forms populate most lesions, suggesting that both could play a role in the development and progression of the infection (Calderone & Fonzi, 2001). The fact that the chitin content is higher in hyphae and that glucosamine, the metabolic precursor of chitin, is able to induce hyphal growth led to the proposal that chitin synthesis could be related to hyphal growth and virulence (Cassone, 1986). Several studies with chitin synthase (CS) -defective mutants have indicated that most chitin synthase genes (CHS genes) are not required either for the yeast-to-hypha transition, or for supporting hyphal growth (Munro & Gow, 2001). However, the specific role of chitin synthesis in virulence remains uncertain, since contradictory reports have been published. Bulawa et al. (1995) showed that chitin-deficient mutants have reduced virulence, while Mio et al. (1996) observed only minor differences in virulence in the same type of mutants. Recently, additional data on the effect of cell wall-related genes in virulence have been reviewed (Navarro-Garcia et al., 2001), but their interpretation is difficult since mutations in many of these genes could have pleiotropic effects on cell wall construction.

Our knowledge about how chitin synthesis occurs in the fungal kingdom has increased considerably in recent years (Munro & Gow, 2001; Roncero, 2002), but we know little about the enzymes/activities involved in the cross-link between chitin and glucan. Chitin synthesis occurs by means of the CS enzymes, which are encoded by a diverse family of genes, whose number varies among species: from three in S. cerevisiae to ten in Phycomyces blakesleeanus (Roncero, 2002). C. albicans has been shown to contain four such CHS genes (Munro & Gow, 2001). Three of them are relatively well characterized, and their functions have been proposed based on knowledge provided by studies on S. cerevisiae.

The class I CaChs2p enzyme seems responsible for part of the hyphal chitin (Gow et al., 1994), and the class II CaChs1p is involved in septum formation in both the yeast and hyphal forms (Munro et al., 2001). However, CaCHS1 is essential in this organism (Munro et al., 2001), in contrast to what has been shown for the S. cerevisiae homologue (see Roncero, 2002 for a review). The class IV CaChs3p, encoded by the CaCHS3 gene, is responsible for most of the chitin synthesized in this organism in both yeast and hyphal cells (Bulawa et al., 1995; Mio et al., 1996). Finally, a second class I CS, CaCHS8, has recently been described. Its function is not yet clear, although it does not appear to play any significant role in vivo (Munro et al., 2003).

S. cerevisiae CSIII activity, the major activity in vivo, depends on at least another four genes: CHS4, CHS5, CHS6 and CHS7. Chs4p is involved in the activation of CSIII, but also in the anchoring of CSIII to the neck region through Bni4p and the septin ring (for a recent review, see Roncero, 2002).

CHS4 is conserved in C. albicans, and based on heterologous expression and mutant characterization it has been postulated that it would have the same function as in S. cerevisiae (Sudoh et al., 1999). However, characterization of the C. albicans Chs4p protein is far from complete. Surprisingly, C. albicans lacks any likely SHC1 gene (Roncero, 2002), recently described in S. cerevisiae as the sporulation-specific homologue of CHS4 (Sanz et al., 2002). In S. cerevisiae, Chs5p and Chs6p are Golgi proteins involved in the polarized secretion of Chs3p, although their exact function is not fully understood. It has been known for some time that C. albicans also contains a CHS5 gene, whose function has not yet been studied. According to genomic analysis, C. albicans – like all other fungi – does not have any clearly conserved CHS6 homologue (Valdivieso et al., 2004).

In S. cerevisiae, Chs7p is required for the correct export of Chs3p from the endoplasmic reticulum (Trilla et al., 1999), and several observations suggest that the amount of Chs7p regulates the overall amount of functional CSIII activity (Trilla et al., 1999). Preliminary database reports have indicated that C. albicans could contain a CHS7 homologue, and the present work is devoted to the characterization of this putative CHS7 homologue. Our results indicate that the involvement of CHS7 in chitin synthesis seems to be functionally conserved in C. albicans. In addition, we provide new insights into the role of the CSIII activity in morphogenesis and virulence.

METHODS

Strains, media and growth conditions. The strains and plasmids used in this work, and their sources, are listed in Table 1. S. cerevisiae cells were grown either in YEPD (2 % glucose, 1 % yeast extract, 2 % peptone) or in SC media (2 % glucose, 0-7 % Difco yeast nitrogen base without amino acids, BIO 101 Complete Supplement Mixture (CSM), minus the appropriate amino acid). Where indicated, galactose (2 %) was used as a carbon source instead of glucose. Agar (2 %) was added for solid media.

C. albicans yeast cells were grown routinely in YEPD supplemented with 100 µg uridine ml⁻¹ or in SC-ura at 30 °C. Hyphal cultures were obtained by growing synchronized cells at 37 °C in liquid YEPD supplemented with uridine and 20 % fetal bovine serum (FBS) (Gibco-BRL) (Gow & Gooday, 1982). Alternatively, Lee’s medium (Lee et al., 1975) was used, either alone or supplemented with 4 % FBS or with N-acetylglucosamine (GlcNAc) (Mattia et al., 1982) as carbon source. Synchronized cells were prepared as follows: cells were grown overnight at 30 °C, transferred to water for 2 h, and finally maintained without shaking in water for 24–48 h at 4 °C. Cells were then transferred to the corresponding medium and incubated at 37 °C. When required, cells were collected after 6 h of incubation.

Colony growth on solid media was observed under non-inducing conditions for filamentation growth, such as YEPD or SC containing 0-05 M potassium hydrogen phthalate buffer, pH 6-5, or 0-05 M sodium citrate buffer, pH 4-5, or under hypha-inducing conditions, such as being embedded in YEPD agar, plated on 4 % FBS-containing agar or Spider (Liu et al., 1994) medium at 37 °C. Solid media contained 2 % of pre-washed Bacto agar (Difco). Overnight cultures were resuspended in water, counted and plated at ~100 c.f.u. per plate, and incubated for 3 days.
Table 1. Strains and plasmids

<table>
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<th>Strain</th>
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<td>W303</td>
<td>MA4a can1-100 ade2-1 his3-11,15 leu2-3,12 trp1-1 ura3-1</td>
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<td>chs7A::HIS3</td>
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</tr>
<tr>
<td>Y1306</td>
<td>Y604</td>
<td>MATa ura3-52 lys2-801 ade2-101 trp1-901 his3Δ200 CHS3-3 x HA</td>
<td>Santos &amp; Snyder (1997)</td>
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<tr>
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<td><strong>C. albicans</strong></td>
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<td>CAF2-1</td>
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<td>ura3Δ::imm434/URA3</td>
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Calcofluor fluorescence was tested by plate assays in SC medium buffered with 0·05 M potassium hydrogen phthalate buffer, pH 6·2, supplemented with different Calcofluor (Blankophor BBH; Bayer) concentrations.

Uracil auxotrophs were counter-selected on SC medium containing 625 μg 5-fluoroorotic acid (5-FOA) ml⁻¹ and 100 μg uridine ml⁻¹ (Fonzi & Irwin, 1993).

The *Escherichia coli* DH5α strain was used for routine propagation of plasmids, and strain CJ236 (Bio-Rad) was used for site-directed mutagenesis. Luria–Bertani (LB) medium, supplemented with 100 μg ampicillin ml⁻¹ or 50 μg kanamycin ml⁻¹ when appropriate, was used to grow the bacteria.

**Cloning and disruption of the C. albicans CHS7 gene.** In a BLAST search we identified a *C. albicans* DNA fragment of 320 bp that showed high sequence similarity to the *S. cerevisiae* CHS7 gene. This fragment was amplified by high-fidelity PCR (Roche), and used as a probe for the hybridization of a *C. albicans* genomic library in a Ycp50 vector (Navarro-Garcia et al., 1995). A positive clone was identified containing a plasmid with a 12 kb fragment. Subsequent subcloning experiments led us to select a 4·4 kb Bal–HindIII fragment hybridizing with the probe. This fragment was ligated into the Smal site of the pRS315 vector, thus creating pCRM517. After complete sequencing of the insert, the putative CaCHS7 ORF was identified in a 1·4 kb XmnI–XmnI fragment, which was used for further constructions.

The disruption cassette was constructed as follows. Using plasmid pCRM517 as a template, two PCRs were performed to synthesize two DNA fragments flanking the 5’ and 3’ ends of *CaCHS7*. The oligonucleotides for amplification of the 1·5 kb *CaCHS7*-N fragment were: *Reverses*-N1 5’-GGAAACAGCTATGACCATG-3’, complementary to the pRS315 polylinker sequence; *Ca7del2-N2* (PstI) 5’-GTGACTGGCACGAAAAGTGTGATG-3’, starting at position −7 of the ORF. The oligonucleotides for amplification of the 1·9 kb *CaCHS7*-C fragment were: *Ca7del3-N2* (PstI–BglII) 5’-TACACTGACATACAGATCTGCCGTGATGAAAGAG-3’, starting at position +895, and *Ca7del4-C2* (BamHI) 5’-CTCTAGAACTA- GTGGATC-3’, complementary to the pRS315 polylinker sequence. Both PCR products were first cloned separately into pGEM-T vectors (Promega) and then cloned in a tripartite ligation as a *hisG-URA3-hisG* cassette isolated from the pMB7 plasmid (Fonzi & Irwin, 1993), and digested with the same enzymes (see Fig. 2 for a diagram). Digestion of this
plasmid with NotI/XhoI released the disruption cassette, which was used for sequential disruption of both alleles of CaCHS7, as described by Fonzi & Irwin (1993). Correct heterozygous (CRM694 and CRM692) and homozygous (CRM695 and CRM693) disruptants were confirmed by PCR, and Southern bloting. Chromosomal DNA was digested with KpnI/EcoRI, and the probe used was the ~9 kb KpnI–XmnI fragment from pCRM517.

**Construction of other plasmids and strains.** To construct plasmid pCRM740, the CaCHS7 ORF was amplified by high-fidelity PCR using oligonucleotides CaCHS7-N (BamHI) 5'-CCAGAT-CACGGATCCGTAAATATG-3' and CaCHS7-C (HindIII) 5'-GCACTGAACTTTACCTCTCTGTG-3', and pCRM517 as a template. The result ing fragment was ligated as a BamHI–HindIII fragment into the pRS426 pGALI plasmid (Mumberg et al., 1994) digested with the same enzymes. pCRMs801 was constructed by creating a new in-frame NotI site just before the STOP codon of CaCHS7 in the pCRM740 plasmid by site-directed mutagenesis (oligonucleotide 5'-TTCTTITTTTTATTTGAAGGGGCAAACA-TACTTG-3'). NotI/StuI GFP or 3 x HA (haemagglutinin) cassettes were introduced into this newly created site. Correct constructions were confirmed by sequencing. To construct pCRM573 and pCRM758, the 1-4 kb XmnI–XmnI fragment from pCRM517, containing the CaCHS7 ORF, was inserted into the Smal site of the S. cerevisiae pRS316 and C. albicans plC14 (Zaragoza et al., 2002) vectors, respectively. To create pCRM587, the CaCHS7 fragment from pCRM573 was released by digestion with PstI/StuI at the flanking sites, and subsequent ligation into the PstI/StuI sites present in the polylinker region of the plasmid pGEMT-CaURA3, kindly provided by M. Sanchez (Universidad de Salamanca).

The revertant strain CRM776 was created by introducing the episomic pLC14-CaCHS7 (pCRM758) into the Ura3 homzygous disrupted strain. A second revertant strain was constructed by integrating the CaCHS7-containing plasmid, pCRM857, at its native locus. This was accomplished by digesting pCRM857 with Accl, a site present ~280 bp upstream from the START codon, and transforming strain CRM693 with the linearized plasmid. Transformants were recovered on selective medium, and correct homologous recombination events were verified by PCR. Strains expressing the URA3 gene at the RPS10 locus were constructed by linearizing the CIP10 plasmid (Murad et al., 2000) with SmaI, and transferring the Ura3- strains CRM692 and CRM693 in order to integrate the plasmid at the target locus. Selection was done on SC+ura plates, and correct integrations were confirmed by Southern bloting.

**Molecular biology procedures.** C. albicans was transformed by electroporation as described by Thompson et al. (1998). RNA preparations were obtained using the FastRNA Kit-Red (Q-Bio gene). For Southern and Northern blotting, Hybond-N membranes were used (Amersham Pharmacia), and blotting and hybridizations were carried out following the manufacturer’s instructions. Probes were labelled using the Rediprime II system (Amersham Pharmacia), and blots were exposed to a PhosphorImager Screen (Bass1500, Fujiilm). Western blotting was used to visualize denatured CaChs7p-3 x HA and ScChs7p-3 x HA, as described previously (Trilla et al., 1999). Blots were processed using a monoclonal anti-HA antibody (12CA5; Roche Diagnostics) and ECL chemiluminescence (Amersham Pharmacia).

**Chitin and CS-activity determinations.** Chitin measurements were performed as described previously (Trilla et al., 1999), using chitinase from Serratia marcescens (Sigma-Aldrich). Total amounts of chitin were expressed as nmol of GlcNAc released per 100 mg cells (wet weight). C. albicans yeast and hyphal cultures were prepared as described above, and harvested after 6 h incubation. For CS activity assays, total cell membranes were prepared as described by Choi & Cabib (1994), and CS activities were measured as described by Choi (1998). When required, the proteolytic activation step was implemented by incubating 4 ml membrane suspension with 0.2 ml trypsin (1 mg ml−1) in a total volume of 6 ml (33 mM) Tris/HCl, pH 7.5. Proteolysis was stopped by adding 0.2 ml aprotinin (Sigma) solution (2 mg ml−1). Membranes were diluted in the appropriate buffer (32 mM), and tested at the desired pH and ion concentrations. The assay system for CS activity consisted of 50 μg membranes, 32 mM GlcNAc, 1 mM UDP-α-D-glucose, as described by Fonzi & Irwin (1993). Correct heterozygous (CRM694 and CRM693) disruptants were confirmed by PCR, and Southern blotting. Chromosomal DNA was digested with KpnI/EcoRI, and the probe used was the ~9 kb KpnI–XmnI fragment from pCRM517.

**Antifungal-drug susceptibility tests.** C. albicans cells (1 x 10⁴ cells ml⁻¹) were incubated at 30 °C for 48 h in SC medium with different concentrations of the test compounds. Measurements were made after 24 and 48 h. Stock solutions were prepared at 10 mg ml⁻¹ in DMSO, and diluted to 2 mg ml⁻¹ for use, with DMSO used as the solvent.

**Microscope techniques and image processing.** Chitin was stained with Calcofluor or wheat germ agglutinin–fluorescein isothiocyanate complex (WGA-FITC) as described previously (Roncero et al., 1998), and visualized using the appropriate UV (340–380 nm excitation and 425 nm emission wavelengths) or FITC (480–440 nm excitation and 527–530 nm emission wavelengths) filters, respectively.

CaChs7p-GFP was directly localized in S. cerevisiae cells containing plasmid pCRMs801, and growing exponentially with galactose as the carbon source. The GFP filter used was GFP blue (470–440 nm excitation and 525–550 nm emission wavelengths). To localize Chs3p-3 x HA, indirect immunofluorescence was performed as described previously (Trilla et al., 1999). Mouse HA.11 anti-HA antibody (BabCo) was used at a dilution of 1:175 as the primary antibody. Alexa Fluor 594 goat anti-mouse antibody (Molecular Probes) diluted 1:400 was used as the secondary antibody. The filter used in this case was the N21 green (515–560 nm excitation and 590 nm emission wavelengths). All microscopic techniques were performed with a Leica RX150 photomicroscope, using an epifluorescence system with a 100 W Hg lamp. Phase-contrast, Calcofluor and WGA-FITC images were obtained with a Leica DC100 digital camera. For GFP and immunofluorescence, a Sensys (Photometrics) digital camera and Leica Q540 v2.3 software were used. Colony photographs at low magnification were obtained with a Zeiss SV11 stereomicroscope equipped with an adapted Canon G3 digital camera. Digital photographs were processed using the Adobe PHOTOSHOP 5.5 software package.

**Animal studies.** Female ICR mice (Harlan) weighing 23–27 g were used. C. albicans strains were maintained as stock cultures in aliquots in no. 2 Nutrient Broth (Oxoid) supplemented with 20% (v/v) glycerol at −80 °C. Cell concentrations were estimated directly by cell counting in a Bürker chamber, and by plating diluted aliquots of the suspension on Sabouraud dextrose agar (Difco) plates. On the day of infection, an aliquot of each frozen culture was thawed and resuspended in sterile saline solution. Cells were counted in a Bürker chamber, diluted with sterile saline to yield the desired c.f.u. ml⁻¹, and a 0.5 ml volume was injected intravenously into the tail vein. In addition, the cell viability of the inoculum was determined by plating serial dilutions of each suspension on Sabouraud plates.
Two different assays were carried out: in the first one, strains CRM695 and CAF2-1 were injected at concentrations of $10^4$, $10^5$, $10^6$ and $10^7$ c.f.u. ml$^{-1}$. Eight different mice were inoculated with each strain and concentration. Mice were monitored twice daily, and deaths were recorded up to day 30 after infection. LD$_{50}$ values, determined using the Spearman–Kärber method (Finney, 1964), were estimated after 10, 20 and 30 days. The second assay was carried out with strains CAF2-1, CRM695, CRM796 and CRM797 at an inoculum size of $10^6$ c.f.u. ml$^{-1}$, using ten mice for each strain. Deaths were recorded until day 12 after injection. Mice that survived to day 12 were euthanized by CO$_2$ asphyxiation. The kidneys of the dead mice were removed aseptically, and immediately stored at $-20$ °C until processed. Kidneys were weighed, suspended in 5 ml of normal saline solution supplemented with 1% peptone (PSS, Difco) and homogenized (Stomacher 80, PBI). Colony counts were determined by plating two 0-05 ml aliquots of undiluted tissue homogenate, and serial 10-fold dilutions on Sabouraud agar plates. Colonies were counted after 2 days of incubation at 35 °C. Survival was analysed with the Mantel–Haenszel log-rank test. For comparison of quantitative counts of the kidneys between groups, an analysis of variance (ANOVA) with the Newman–Keuls multiple comparison test was performed. All data analysis was carried out using the GraphPad Prism version 4.0 computer package (GraphPad Software).

RESULTS

Cloning of the CaCHS7 gene and analysis of the sequence

Previous work in our laboratory identified the ScCHS7 gene as being directly involved in the regulation of chitin synthesis in S. cerevisiae. A BLAST search with this gene in different databases identified a C. albicans gene with significant similarity. Starting from a small fragment of 320 bp, we were able to clone the putative CaCHS7 gene (see Methods for details).

CaCHS7 encodes a protein of 310 amino acids, showing 50% identity (67% similarity) to its S. cerevisiae counterpart. In addition, Chs7p appears to be conserved among fungi (data not shown). Moreover, these proteins maintained the overall secondary structure, with the previously described seven putative transmembrane domains (Trilla et al., 1999), and the hydropathy profiles of CaChs7p and ScChs7p were virtually identical (Fig. 1a). These similarities suggest functional conservation between the different Chs7p homologues.

Following publication of the complete sequence of the C. albicans genome (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=genomes), we realized that our cloned gene differed significantly from the published sequence in the promoter region. The absence of a 28 bp region in our clone was the most significant observation, although other minor changes were also detected (Fig. 1b). In order to confirm this, we reamplified the promoter region and several independent clones were sequenced. Surprisingly, both types of sequence were isolated, showing that both are represented in the C. albicans genome. Both types of sequence were already present in the SC5314 strain (Gillum et al., 1984), the original clinical isolate from which strains CAF2-1 and CAI4 were derived (data not shown). Therefore, C. albicans apparently contains an allelic heterogeneity at the promoter region of the CaCHS7 locus.

![Fig. 1. Analysis of the CaCHS7 gene. (a) Hydrophobicity profile of CaChs7p (solid line) and ScChs7p (dotted line). (b) Alignment of the promoter region of both genomic alleles of the CaCHS7 gene. Contig refers to the sequence identical to that deposited in databases, while alternative refers to the second sequence identified in this work. Note the 28 bp deletion detected in one of the alleles.](http://mic.sgmjournals.org)
**C. albicans chs7Δ null mutants show reduced chitin and CS-activity levels**

In order to establish the function of CaCHS7 in *C. albicans*, a null chs7Δ/chs7Δ mutant was created in the CAI4 strain by serial disruptions using the URA3-blaster technique (Fonzi & Irwin, 1993), and following the strategy outlined in Fig. 2(a) (see Methods for specific details). The absence of the 2.3 kb band characteristic of the wild-type strain indicated that both wild-type alleles of the CaCHS7 had been deleted. These results prove that chs7Δ/chs7Δ homozygous mutants are viable.

To test whether CaChs7p was involved in chitin synthesis, we carried out several tests. chs7Δ/chs7Δ mutants were resistant to 2 mg Calcofluor ml⁻¹, as observed for the chs3Δ/chs3Δ mutant (Fig. 2c), and after Calcofluor staining chs7Δ cells showed reduced fluorescence (Fig. 2d), clear indications of low chitin levels (Roncero et al., 1988), and pointing to diminished levels of CSIII activity. Minor amounts of chitin were present in the septa, probably due to unaffected levels of CSII activity. The heterozygous CHS7/chs7Δ strain behaved in essentially the same way as the control strain (data not shown). Taken together, these results strongly support the direct involvement of CaCHS7 in the regulation of chitin synthesis.

A more precise chitin localization was achieved after cellular staining with WGA-FITC, a chitin-specific stain (Roncero et al., 1988). chs7Δ/chs7Δ mutants showed very reduced levels of WGA-FITC staining, and chitin appeared localized exclusively to the septa in both yeast and hyphal cells (Fig. 2e), although some staining was also observed at some hyphal tips. By contrast, fluorescence in the wild-type strain was intense and fairly uniform (Fig. 2e), as described for *S. cerevisiae*. Again, the heterozygous strains behaved similarly to the control (data not shown).

According to these results, the amount of chitin in the chs7Δ null mutant fell to 25% of the wild-type levels, and the reduction was similar in both yeast and hyphal cells (Table 2). The absolute values obtained were very similar to those of the chs3Δ mutant (Table 2). As compared to the corresponding control, chitin levels in the different chs7Δ strains did not change as a function of the levels of the URA3 gene. However, the absolute levels of chitin were significantly lower in all uracil-auxotroph strains. Interestingly, chitin levels in the yeast heterozygous strains were modestly, but reproducibly, reduced as compared to the wild-type.

Reintroduction of a wild-type copy of the CaCHS7, either in an episomal plasmid in the chs7Δ mutant (CRM776), or by reintegration of the gene at its native locus in the chromosome (CRM870), restored Calcofluor staining and sensitivity (Fig. 2c, d), as well as normal levels and distribution of chitin, as assessed by WGA-FITC staining (Fig. 2e) or by quantitative determinations (Table 2). These results demonstrate that the reduction in chitin levels was directly due to the absence of the CaCHS7 gene.

Following the strategy used in *S. cerevisiae* (Choi & Cabib, 1994), which was recently revised for *C. albicans* (Choi, 1998), we measured CS activity in wild-type and chs7Δ strains at different pHs, and in the presence of different cations. CS activity was unaffected in the mutant when Mg²⁺ was used as activator, suggesting normal CSII activity. In contrast, the chs7Δ null mutant was deficient in the CS activity measured at alkaline pH and in the presence of Co²⁺ (data not shown), conditions compatible with those described for CSIII activity in *S. cerevisiae* (Choi & Cabib, 1994).

The chs7Δ/chs7Δ mutant did not show altered sensitivity to several antifungal agents, except for a 15-fold decrease in the MIC of nikkomycin (Table 3), in agreement with a defect in chitin synthesis (see Discussion).

**Heterologous expression of CaCHS7 in S. cerevisiae**

Owing to the high similarity between CaChs7p and ScChs7p proteins, and the apparent functional conservation in chitin synthesis, we attempted heterologous complementation in the *S. cerevisiae* chs7Δ mutant previously obtained in our laboratory. CaCHS7 expressed under its own promoter failed to complement the *S. cerevisiae* chs7Δ mutation with either monocopy or multicopy vectors (data not shown). We also expressed the CaCHS7 gene under the GAL1
Chitin synthesis and morphogenesis

(a) Diagram of chitin synthesis and morphogenesis pathways.

(b) Southern blot analysis showing the presence of CaCHS7 and its deletion mutants.

(c) Overview of mutant strains used:
- CAF2 (WT)
- CRM894 (CHS7/cha7Δ)
- CRM895 (CHS7Δ/cha7Δ)
- CRM742 (chs3Δ/cha3Δ)
- CRM777 (WT+plC14)
- CRM776 (cha7Δ+plC14-CaCHS7)
- CRM870 (cha7Δ::CaCHS7)

(d) Fluorescence microscopy images of mutant strains:
- CAF2-1
- CRM895
- CRM870
- CRM742

(e) Additional images of mutant strains:
- CAF2-1
- CRM895
- CRM870
promoter in a multicopy vector (pCRM740) (Mumberg et al., 1994). This construct provided high levels of CaCHS7 transcription in S. cerevisiae, which led to high amounts of protein after 90 min of growth in galactose medium, as determined by Western blotting (data not shown). Under these conditions, CaCHS7 also failed to complement the S. cerevisiae chs7Δ mutation, as determined by Calcofluor staining (Fig. 3a) and Calcofluor resistance assays (data not shown). In addition, the levels of chitin in this strain were similar to those observed in the original null mutant. By using a CaChs7p-GFP chimera (pCRM801) expressed in S. cerevisiae, the protein appeared to be correctly expressed and localized at the endoplasmic reticulum (ER) (Fig. 3b), as expected. The fact that CaCHS7 lacks the CUG codon, and that the protein is highly expressed in S. cerevisiae argues against an expression problem, and points directly to the absence of functional activity.

ScChs7p is involved in the correct export of ScChs3p from the ER (Trilla et al., 1999). We therefore tested the exit of ScChs3p-3×HA from the ER only in the presence of CaChs7p. In a wild-type strain, ScChs3p-3×HA localized to the neck region (Fig. 3c, left), while in the chs7Δ mutant ScChs3p-3×HA remained retained in the ER (Fig. 3c, centre). In cells expressing CaCHS7, ScChs3p-3×HA showed a perinuclear location (Fig. 3c, right) identical to that observed in the original chs7Δ mutant, suggesting that it is not exported from the ER. These results are consistent with the absence of complementation of the chs7Δ mutation.

**Effect of chs7Δ deletion on morphogenesis**

chs7Δ mutant strains showed normal growth rates at all temperatures tested; however, they exhibited some minor morphogenetic defects. Mutant cells showed prominent bud scars, and a significant percentage of cells displayed aberrant septa (Fig. 2d). When mutant strains were plated on non-inducing media for filamentation they showed an increased tendency to grow as filamentous forms. On SC medium buffered to pH 6.2 (Fig. 4a), but also on YEPD medium (data not shown), colonies of the chs7Δ strain had a more wrinkled appearance in comparison with the control strain. This was accompanied by a significant increase in the presence of filamentous cells (data not shown). In liquid media at 28°C, yeast cells tended to form small clumps that disappeared readily after gentle sonication (Fig. 4b), but at 37°C mutant cultures were mostly composed of filamentous forms, resulting in highly flocculated cultures (Fig. 4c). The phenotypes of chs7Δ and chs3Δ were identical under all conditions tested (Fig. 4). The reintroduction of the

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### Table 3. MIC (μg ml⁻¹) of antifungal agents for C. albicans strains CRM695 and CAF2-1

<table>
<thead>
<tr>
<th>Antifungal</th>
<th>CRM695</th>
<th>CAF2-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nikkomycin</td>
<td>3-2</td>
<td>50</td>
</tr>
<tr>
<td>Amphotericin B</td>
<td>1-6</td>
<td>1-6</td>
</tr>
<tr>
<td>Caspofungin</td>
<td>0-4</td>
<td>0-4</td>
</tr>
<tr>
<td>Amnyosymin</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Miconazole</td>
<td>0-8</td>
<td>1-6</td>
</tr>
<tr>
<td>Papulacandin</td>
<td>0-8</td>
<td>0-8</td>
</tr>
<tr>
<td>Tunicamycin</td>
<td>3-1</td>
<td>6-2</td>
</tr>
<tr>
<td>FK506</td>
<td>&gt;100</td>
<td>&gt;100</td>
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</tbody>
</table>

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*Chitin amounts are expressed as nmol GlcNAc per 100 mg (wet weight) of cells. Relative data compared to the corresponding wild-type are indicated in parentheses. See Methods for details on protocol. ND, Not determined; WT, wild-type.

†Cells were collected growing in yeast or hyphal form, as described in Methods.
wild-type CaCHS7 gene restored normal growth characteristics in liquid media, but colonies on solid media were more wrinkled than the wild-type, although very similar to the heterozygous strain.

CaChs7p did not seem to be required for the normal yeast-to-hyphae transition in liquid media in either of the different conditions tested (YEPD or Lee’s medium supplemented with FBS or GlcNAc at 37 °C). Hyphal growth and germ-tube emissions in this mutant took place with the same kinetics as in the controls, and no evident differences were observed in the microscopic aspect of the hyphal extensions (data not shown). Previous work on C. albicans mutants with reduced levels of chitin (chs3Δ) also failed to identify any morphogenetic problems in hyphal extension. However, the absence of lateral cell wall chitin in the chs7Δ mutant prompted us to search for different phenotypes.

After induction of filamentation on solid medium (FBS-containing agar), wild-type cells formed extensive hyphae. However, chs7Δ colonies appeared much more compact and their diameters were reduced (Fig. 5a). Similar differences in the morphology of the colonies were also observed after growing cells on Spider medium or embedded in YEPD medium (data not shown). Under the

**Fig. 3.** Complementation of the *S. cerevisiae* chs7Δ mutant by the CaCHS7 gene. (a) Chitin staining after Calcofluor treatment (see Methods) of *S. cerevisiae* chs7Δ cells transformed with the indicated plasmids. Cells were grown in galactose medium. Note the absence of staining in all strains except that transformed with the wild-type ScCHS7 gene. (b) Intracellular localization of ScChs7p-GFP and CaChs7p-GFP expressed in *S. cerevisiae*. Note the perinuclear staining indicative of ER localization. (c) ScChs3p-3×HA localization in different strains. Localization at the neck in the control strain (left panel) is lost in the Δchs7 mutant (centre panel). Expression of CaCHS7 (right panel) does not correct the ER localization to that observed in the original mutant.

**Fig. 4.** Cell growth under non-filamentation conditions. Diluted cultures of the indicated strains were grown in SC–ura, diluted, and plated or inoculated in different media. Colony morphology on SC medium, pH 6–7, was recorded after 2 days at 28 °C (a). Exponential-phase cultures of the indicated strains grown in Lee’s medium at 28 (b) or 37 °C (c). Cell morphology of the indicated cultures was assessed by phase-contrast microscopy.
microscope, the control hyphae exhibited long, linear apexes that tracked straight forwards across the surface, in contrast to the mutant hyphae that showed shorter apexes. The colonies of heterozygous CHS7/chs7Δ and revertant strains appeared to be identical, and very similar to the control strain (Fig. 5a).

Early colonies of the chs7Δ mutant showed a high proportion of curved hyphal extensions (Fig. 5b). Instead of growing straight, as occurred in the wild-type or heterozygous strains, these hyphae were curved and many of the mutant hyphae extended parallel to the colony border, or even inside it. Closer inspection of the hyphae indicated that the curved morphology in the mutant was linked to irregular lateral walls that displayed numerous swollen portions (Fig. 5c, right). By contrast, wild-type (Fig. 5c, left) as well as heterozygous and revertant hyphae (Fig. 5b and data not shown) were rather regular and uniform.

Considering that chs7Δ hyphae did not show any significant defects in liquid media it may be assumed that chs7Δ phenotypes are exacerbated by growth on solid media.

Reduced virulence of the C. albicans chs7Δ/chs7Δ mutant

Since the above characteristics suggested that scavenging functions could be compromised in the chs7Δ null mutant, we tested the effect of the defect in chitin synthesis on a mouse systemic-infection model. This issue has been previously addressed using different chs3Δ mutants, although with contradictory results (Bulawa et al., 1995; Mio et al., 1996). Immunocompetent mice were inoculated with different concentrations of micro-organisms, and mouse survival was monitored after 10, 20 and 30 days. Since all mutants were prototrophs for uracil, CAF2-1 was used as a control strain. In comparison with the control strain, the chs7Δ/chs7Δ mutant displayed reduced virulence at a 10⁶ c.f.u. ml⁻¹ inoculum size (Fig. 6a). At the highest inoculum (10⁷ c.f.u. ml⁻¹) there was still a small but measurable delay in mortality (Fig. 6a). The chs7Δ mutant was essentially avirulent at low (10⁴–10⁵ c.f.u. ml⁻¹) inoculum sizes (data not shown).

In order to test the possible influence of URA3 expression in virulence (Brand et al., 2004), we used chs7Δ/chs7Δ

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**Fig. 5.** Colony morphology under filamentation conditions. Colony morphology, at low magnification through a stereomicroscope, of different strains grown in 4% serum-supplemented agar for 3 days at 37 °C (a). Colony morphology at a different magnification after 24 h of growth (b). Hyphal morphology of wild-type and chs7Δ strains (c). Bars, 15 μm.
strains expressing the URA3 gene inserted at the high-expression RPS10 locus (CRM797). This homozygous mutant also showed reduced virulence as compared to the control strain and its virulence was similar to that of the original chs7Δ/chs7Δ Ura+ strain (CRM695) (Fig. 6b). In agreement with the in vitro phenotypes, the heterozygous mutant was virulent, similarly to the control (Fig. 6b). In this experiment, we also determined the rate of C. albicans survival in mice by analysing the number of cells present in mouse kidneys (Fig. 6c). The number of c.f.u. recovered from both homozygous mutants was significantly lower than that obtained from the control and heterozygous strains. This result is consistent with the reduced virulence of the chs7Δ null mutant.

DISCUSSION

The regulation of CSIII has been assumed to be conserved across the fungal kingdom based on genomic studies, although experimental support for this is lacking. Only the C. albicans homologue of CHS4 has been cloned and partially characterized (Sudoh et al., 1999), and the results obtained confirm its participation in the regulation of CSIII. A key element in the regulation of S. cerevisiae CSIII is Chs7p (Trilla et al., 1999), which is conserved among fungi (C. Roncero, unpublished observations). Nevertheless, CHS7 is not present in Schizosaccharomyces pombe or in Caenorhabditis elegans, two organisms that contain chitin, but not specific homologues of CSIII activity. These results support the idea of the evolutionary conservation of Chs7p function with CSIII activity, and here we attempted to confirm this hypothesis experimentally. Unfortunately, an experimental approach is not possible in filamentous fungi since there is no a clear way of testing CSIII activity either in vivo or in vitro. Therefore, our work focused on the characterization of the Chs7 protein from the dimorphic fungi C. albicans, which contains traceable CSIII activity.

We cloned the CaCHS7 gene, and in the course of the work we realized that the sequence in the promotor region of our clone differed significantly from the published one (Fig. 1b). This discrepancy is due to an allelic heterogeneity in the locus that results in the co-existence of both alleles, at least in the SC5314 background. In order to confirm that both alleles were functional, we constructed both heterozygous mutants (data not shown). They showed minor phenotypic defects as compared to the wild-type and behaved similarly in terms of chitin deposition and Calcofluor resistance, suggesting that both alleles are functional and contribute redundantly to chitin synthesis. However, it remains to be seen whether the expression of either allele is regulated differently, as has recently been shown for the SAP2 locus (Staib et al., 2002).

Characterization of C. albicans chs7Δ strains by several methods (Figs 2 and 3, Table 2) revealed that this mutant was deficient in the major CS activity in vivo, which was measurable at alkaline pH, and in the presence of Co2+. However, the use of different cations or pH values did not allow us to discriminate between a defect in CSI or CSIII, because the correspondence between C. albicans and S. cerevisiae CS enzymes is limited in terms of pH and cation requirements (Choi, 1998).

These results indicate not only that CaChs7p is very similar to the ScChs7p protein (Fig. 1a), but also that both proteins have been functionally conserved during evolution. Despite that, we observed a lack of complementation of the S. cerevisiae chs7Δ mutant by the CaCHS7 gene (Fig. 3). To rule out that this might be an exceptional case, we also cloned the Aspergillus fumigatus AfCHS7 gene (unpublished results) and expressed it in S. cerevisiae, obtaining similar results. The direct reason for this absence of complementation is that neither CaChs7p nor AfChs7p efficiently promotes the export of ScChs3p from the ER (Fig. 3c, data not shown). It is possible that the different sequence of
Overall hyphal growth in liquid media was not compromised in the chs7Δ mutant, and hyphal formation occurred normally. However, when grown in embedded, serum-supplemented or Spider solid medium, the chs7Δ mutants gave rise to more compact colonies as compared to the wild-type (Fig. 5a). On serum-supplemented plates, colonies were characterized by irregular and curved hyphae that seemed unable to spread out onto the surface, as the wild-type did (Fig. 5). The molecular reason for such a growth pattern is presently unclear, but a likely explanation could be that the lack of chitin might compromise the integrity of the lateral cell walls. It could be speculated that the absence of chitin would not be a problem when cells are growing as yeast or hyphae in liquid media; however, these defects do become apparent when hyphal cells have to colonize solid substrates, probably when cell wall strength is more necessary.

Interestingly, the morphogenetic alterations observed during hyphal formation in the chs7Δ strain were very similar to those reported for the septin mutants (Warenda & Konopka, 2002), and lead to reduced virulence in both types of mutant (Warenda et al., 2003). Defective septin rings lead to irregular chitin deposition in lateral hyphal walls (Warenda & Konopka, 2002), suggesting that the correct assembly of chitin, but not the total amount that appears unaffected in septin mutants, is a requirement for successful colonization. Moreover, these similarities suggest a direct relationship between septins and CSIII activity in the same process, although the levels of molecular interaction have not yet been elucidated. However, the phenotypes of both types of mutants differed significantly during yeast growth at 37 °C, suggesting that the defect in chitin ring assembly would trigger a specific morphogenetic response.

A possible extrapolation from these results would be that chs7Δ mutants could be impaired for foraging in nature, thereby compromising their virulence. This indeed seems to be the case, since chs7Δ/achs7Δ mutants, although virulent, showed attenuated virulence at all inoculum sizes (Fig. 6a, b). Application of the Spearman–Kärber method indicated that the LD₅₀ (c.f.u. ml⁻¹) determined at 30 days was almost two orders of magnitude higher in the chs7Δ/achs7Δmutant strain (1 x 10⁶) as compared to the CAF2-1 strain (1.8 x 10⁴). This could be explained in terms of the reduced ability of the mutant to invade surrounding tissues. Data on heterozygous strains support this interpretation, since the almost normal virulence correlated with their colony growth on solid hyphae-inducing media. This lower invasiveness of the chs7Δ/achs7Δ mutants is in accordance with the significantly higher clearance rates observed in vivo (Fig. 6c).

A general overview of the results presented indicates that all the phenotypes observed are independent of the URA3 expression levels. However, the absolute levels of chitin vary depending on the expression of this gene, in clear agreement with a recent report linking URA3 expression and metabolic regulation (Brand et al., 2004).

Since the evidence accumulated in S. cerevisiae links the function of Chs7p exclusively to the regulation of chitin synthesis (Roncero, 2002), it may be assumed that the lack of Chs7p would be equivalent to the lack of CSIII activity. If this were the case, the phenotypes of chs7Δ and chs3Δ null mutants should be similar. The defects in chitin synthesis and localization appeared to be similar between both mutants, and our results showing reduced virulence are in clear agreement with those reported for chs3Δ (Bulawa et al., 1995), thus rebutting another report on chs3Δ characterization (Mio et al., 1996). Comparison of chs3Δ and chs7Δ colony morphologies proved to be difficult since our chs3Δ strain (Mio et al., 1996) was unable to grow on Spider medium, and grew poorly on serum-supplemented solid medium or when embedded in agar. However, under these conditions chs3Δ colonies appeared very compact and resembled those observed for chs7Δ. Therefore, it may be concluded that chs7Δ and chs3Δ strains behave similarly.

From all the above results it may be concluded that the function of Chs7p is maintained among fungi, suggesting that the chitin synthesis machinery is evolutionarily conserved. Finally, our observations reveal that the major in vivo CS of C. albicans is not required for overall growth or morphogenesis, but does appear to be essential for the control of certain morphogenetic processes required for hyphal extension and full virulence.

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