Candida albicans mutants in the *BNI4* gene have reduced cell-wall chitin and alterations in morphogenesis

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The *Candida albicans* *BNI4* gene was identified by homology to the *Saccharomyces cerevisiae* orthologue and encodes a predicted 1655 amino acid protein. In *S. cerevisiae* most cell-wall chitin is associated with primary septum formation and Bni4p is involved in tethering the Chs3p chitin synthase enzyme to the mother-bud neck by forming a bridge between a regulatory protein Chs4p and the septin Cdc10p. CaBni4p shows 20 % overall identity to the ScBni4p, with 73 % identity over the C-terminal 63 amino acids, which includes a putative protein phosphatase type 1 (PP1) binding domain. Northern blot analysis revealed a transcript of the expected size that was expressed in both yeast and hyphal growth forms. *C. albicans* has more chitin in its cell wall than *S. cerevisiae*, and again most chitin is synthesized by CaChs3p. The function of *CaBNI4* was investigated by performing a targeted gene disruption using the 'Ura-blast' method to delete amino acids 1120–1611 that are essential for function. The resulting *Cabni4Δ/Cabni4Δ* null mutants formed lemon-shaped yeast cells and had a 30 % reduction in cell-wall chitin, reduced hyphal formation on solid serum-containing medium and increased sensitivity to SDS and increased resistance to Calcofluor White. The *Cabni4Δ/Cabni4Δ* null mutants were unaffected in chitin ring formation, but often exhibited displaced bud sites with more obvious but flattened birth scars. Therefore, unlike in *S. cerevisiae*, the *Cabni4* mutant apparently alters chitin distribution throughout the cell wall and not exclusively at the bud-neck region.

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INTRODUCTION

Chitin is an essential polysaccharide in the fungal cell wall that is important in determining cell shape, for cytokinesis and as a possible antifungal drug target (Munro & Gow, 2001). In the human fungal pathogen *Candida albicans*, chitin is synthesized by four enzymes encoded by *CaCHS1*, *CaCHS2*, *CaCHS3* and *CaCHS8*. *CaChs3p* is responsible for the synthesis of most chitin in distinct locations of the cell wall including the lateral cell wall, chitin ring and bud scar (Bulawa *et al.*, 1995). In *Saccharomyces cerevisiae*, Bni4p is involved in localizing Chs3p to the bud-neck region where the septum is formed. The mechanism of localization of Chs3p by CaBni4p was the subject of this investigation.

In *C. albicans*, *CaChs3p* expression is increased in hyphae, which have more chitin than yeast cells (Munro *et al.*, 1998).

CaChs1p synthesizes the chitin of the primary septum, is expressed at a low level in both yeast and hyphal cells, and has a potential role in lateral cell-wall stability (Munro *et al.*, 1998, 2001, 2003). *CaChs2p* is a non-essential enzyme and its expression is increased during the yeast-to-hyphal transition (Gow *et al.*, 1994). *CaChs8p* also contributes to *in vitro* chitin synthase activity in yeast and hyphal cells and has highest identity to *CaChs2p* (Munro *et al.*, 2003). Therefore, chitin synthesis is regulated temporally and spatially at the transcriptional and post-translational levels (Roncero, 2002; Munro & Gow, 2001).

The spatial and temporal regulation of Chs3p has been studied in *S. cerevisiae* and involves a number of ancillary proteins including ScChs4p, ScChs5p, ScChs6p and ScChs7p (Trilla *et al.*, 1997, 1999; Santos *et al.*, 1997; Ziman *et al.*, 1998), septins and Bni4p (DeMarini *et al.*, 1997). In both *C. albicans* and *S. cerevisiae*, Chs4p is involved directly or indirectly in the activation of Chs3p (Sudoh *et al.*, 1999; Ono *et al.*, 2000). ScChs5p is required for targeting of ScChs3p to polarized growth sites and for cell fusion during mating (Santos *et al.*, 1997; Santos & Snyder, 2003). ScChs6p is involved in the spatial regulation and recycling of ScChs3p (Ziman *et al.*, 1998), while ScChs7p is required for the export of ScChs3p from the endoplasmic reticulum to the

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Abbreviations: PP1, protein phosphatase type 1; TEM, transmission electron microscopy.

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plasma membrane (Trilla *et al.*, 1999). ScChs3p is transported through the secretory pathway to the plasma membrane where chitin synthesis takes place, and can also be internalized again via ligand-induced endocytosis (Ziman *et al.*, 1996). ScChs3p can also be mobilized from internal stores in response to cellular stress (Valdivia & Schekman, 2003).

In *S. cerevisiae*, chitin deposition at the bud site is linked to the septin neck filaments, a family of proteins that have roles in septation and cytokinesis. The septin proteins localize to the incipient bud site and are ordered into higher structures to form a ring of neck filaments. The actin-myosin ring contracts and then the two neck filaments split, resulting in cytokinesis so that the mother and daughter cells each inherit a single septin ring (Longtine *et al.*, 1996; Lippincott *et al.*, 2001; Kinoshita, 2003). In *S. cerevisiae* there are seven septin genes: *CDC3*, *CDC10*, *CDC11*, *CDC12*, *SHS1/SEP7*, *SPR3* and *SPR28*. The latter two genes are expressed only in the sporulation phase. In *C. albicans*, homologues of all seven septin genes have been recognized. The *CaCDC3* and *CaCDC10* genes are capable of complementing defects in the respective *S. cerevisiae* genes and are thus believed to perform similar roles (Di Domenico *et al.*, 1994). Similar to the *S. cerevisiae* septins, *CaCDC3* and *CaCDC12* are essential for viability; however, the *Cacdc10Δ* and *Cacdc11Δ* mutants were viable but displayed conditional defects in cytokinesis, chitin localization and bud morphology (Warenda & Konopka, 2002). The *C. albicans* Cdc3p and Cdc11p homologues have been localized during the growth of yeast, pseudohyphae and hyphae (Sudbery, 2001). In true hyphae, an early and transient septin ring is formed that lacks Cdc3p, which does not participate in cytokinesis later in the cell cycle (Sudbery, 2001). *Cacdc11Δ* mutants were defective in ability to form hyphae on solid agar (Warenda & Konopka, 2002).

In *S. cerevisiae*, the septin Cdc10p interacts with Chs4p via Bni4p (bud neck involved). The ScBni4p protein is required for the correct targeting of ScChs3p and its activator ScChs4p to the bud-neck region. Mutants deleted

in *ScBNI4* had delocalized chitin deposition, with an aberrant cell morphology and enlarged bud necks (DeMarini *et al.*, 1997). Immunolocalization experiments confirmed that ScBni4p was normally localized to the mother-bud-neck region and that localization was dependent upon the presence of septins but not ScChs4p or ScChs3p (DeMarini *et al.*, 1997).

Yeast dihybrid analysis demonstrated that ScBni4p interacts with ScGlc7p (Uetz *et al.*, 2000). ScGlc7p is a protein phosphatase type 1 (PP1) involved in a range of physiological activities including cell polarity, cell-wall integrity and morphology (Andrews & Stark, 2000). ScBni4p is required for the localization of ScGlc7p to the incipient bud neck but not for targeting to the neck at cytokinesis (Kozubowski *et al.*, 2003). Although the precise role for this phosphatase at the bud neck is not known, it could facilitate the association of ScBni4p with the septins. ScBni4p is also believed to be necessary for the targeting of ScCrh2p, a glycosylphosphatidylinositol (GPI)-anchored mannoprotein involved in cell-wall assembly, at cytokinesis (Rodriguez-Pena *et al.*, 2002).

Chitin synthesis in *C. albicans* is significantly different from that in *S. cerevisiae* in a number of respects (Munro & Gow, 2001; Munro *et al.*, 2003), and we therefore examined the role of *CaBni4p* in growth and cellular morphogenesis in *C. albicans*. Here we describe the isolation and analysis of the *C. albicans BNI4* homologue by reverse genetics. Disruption of *CaBNI4* resulted in cells that had a phenotype that was again different, in some aspects, to that of the *bni4* mutation in *S. cerevisiae*. These were depleted in total chitin and had abnormal cell shape and bud-scar morphology and defects in hypha formation when growing on solid surfaces.

METHODS

Strains, growth conditions. The *C. albicans* strains used in this study are listed in Table 1. Strains were grown in YPD [1 % (w/v) yeast extract, 2 % (w/v) peptone, 2 % (w/v) glucose] or SD [0.67 %

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

Strain	Relevant genotype	Parent	Reference
CAF2-1	<i>ura3Δ::imm434/URA3</i>	SC5314	Fonzi & Irwin (1993)
CAI-4	<i>ura3Δ::imm434/ura3Δ::imm434</i>	CAF2	Fonzi & Irwin (1993)
NGY81a	CAI-4 but <i>bni4Δ::hisG-URA3-hisG/BNI4</i>	CAI-4	This work
NGY81b	CAI-4 but <i>bni4Δ::hisG-URA3-hisG/BNI4</i>	CAI-4	This work
NGY82a	CAI-4 but <i>bni4Δ::hisG/BNI4</i>	NGY81a	This work
NGY82b	CAI-4 but <i>bni4Δ::hisG/BNI4</i>	NGY81b	This work
NGY83a	CAI-4 but <i>bni4Δ::hisG-URA3-hisG/bni4Δ::hisG</i>	NGY82a	This work
NGY83b	CAI-4 but <i>bni4Δ::hisG-URA3-hisG/bni4Δ::hisG</i>	NGY82b	This work
NGY84a	CAI-4 but <i>bni4Δ::hisG/bni4Δ::hisG</i>	NGY83a	This work
NGY84b	CAI-4 but <i>bni4Δ::hisG/bni4Δ::hisG</i>	NGY83b	This work

(w/v) yeast nitrogen base, 2% (w/v) glucose] at 30 °C. The hyphal form of *C. albicans* was grown in 20% (v/v) newborn calf serum or Lee's minimal medium pH 6.5 (Lee *et al.*, 1975) at 37 °C. Solid media contained 2% (w/v) agar (Oxoid). Ura⁻ strains were supplemented with 50 µg uridine ml⁻¹. Hyphal colonies were grown on 20% serum agar for 2–5 days at 37 °C.

Media for integrative transformation. Gene knockout was performed using the 'Ura-blast' protocol described by Fonzi & Irwin (1993). Ura⁺ transformants were streaked on SD plates; single colonies were picked and grown in 5 ml SD medium. Genomic DNA was extracted for Southern analysis and diagnostic PCR. Counterselection of Ura⁻ segregants was done on SD medium containing 1 mg 5-fluoro-orotic acid (5-FOA, Melford) ml⁻¹ and 50 µg uridine ml⁻¹.

Bacterial strains and plasmids. *Escherichia coli* DH5α and XL1Blue (Stratagene) were used for preparation of plasmid DNA. Plasmids pGEM-T, pBluescript and pBR322 (Promega) were used for cloning gene fragments. For sequencing and disruption cassette construction, the *CaBNI4* gene sequence was cloned as two overlapping fragments. Attempts to clone the whole gene were unsuccessful due to frequent recombination events in *E. coli*.

Plasmids p5921 and pMB7 were used for construction of Ura-blast cassettes (Fonzi & Irwin, 1993; Gow *et al.*, 1994). A 543 bp fragment from the middle of the coding region of *CaBNI4* and a 543 bp fragment covering the 3' coding and non-coding region of *CaBNI4* were obtained by PCR using restriction-site-ended primers, to facilitate the directed cloning into plasmids p5921 and pMB7 following digestion with the corresponding restriction endonucleases (Table 2). The resulting disruption plasmids, named p59BN1 and pMBN2, were derived from p5921 and pMB7 respectively, and were used to transform *C. albicans* strain CAI-4. Homologous integration resulted in the disruption of nucleotides 3630–4833 from the 4968 nucleotide ORF (corresponding to amino acids 1120–1611 out of 1655 total). This region corresponded to the putative and conserved RVXF Glc7-binding motif that associates with the hydrophobic groove of the catalytic subunit, PP1c (Egloff *et al.*, 1997), that is believed to be essential for Bni4p function.

Disruption of the *CaBNI4* gene. Linearized p59BN1 was used to disrupt the first *CaBNI4* allele and pMBN2 was used to disrupt the second allele. pMBN2 differs from p59BN1 only in that pMBN2 contains an additional *Bam*HI site in each of the *hisG* sequences of the Ura-blast cassette. Prior to transformation, plasmids p59BN1 and pMBN2 were digested with *Sac*I and *Hind*III restriction enzymes to release the *bni4Δ*/Ura-blast disruption cassette. The digestion products were extracted with phenol/chloroform, resuspended in TE (10 mM Tris/HCl, 1 mM EDTA, pH 7.5) to a concentration of >100 ng µl⁻¹ in a volume of 20 µl. Transformation used the lithium acetate method (Bulawa *et al.*, 1995; Sanglard *et al.*, 1997). Despite numerous attempts to clone the entire *BNI4* gene both

in-frame and out-of-frame, the size of this gene and spontaneous recombination events prevented its cloning in *E. coli*. Hence reconstituted strains could not be generated as controls for aberrations due to transformation. Therefore, *Cabni4Δ/Cabni4Δ* mutants were generated from independent lineages of strains and these subjected to parallel phenotypic analysis to ensure that resulting phenotypes were a consequence of gene disruption and not a result of any unforeseen changes induced by the transformation process. This procedure has been adopted in the analysis of other large genes in *C. albicans* (Cassola *et al.*, 2004; Zaragoza *et al.*, 2000).

Analysis of nucleic acids. *C. albicans* genomic DNA was extracted using the mechanical lysis method by vortexing in Triton mix [2% (w/v) Triton X-100, 1% (w/v) SDS, 100 mM NaCl, 10 mM Tris/HCl, pH 8.0 and 1 mM EDTA] with glass beads (Sigma, 425–600 µm in diameter) followed by phenol/chloroform extraction. DNA samples were digested with restriction endonucleases, separated on a 0.8–1% (w/v) agarose gel and transferred to nylon membranes. Total RNA was extracted from 50–100 mg (wet wt) yeast and hyphal *C. albicans* cells using the Qiagen RNeasy mini extraction kit following lysis in a Hybaid Ribolyser. RNA samples were heated at 50 °C for 60 min in 55% DMSO, 1.1 M Glyoxal, separated on a 1.4% (w/v) non-denaturing agarose gel and again transferred to nylon membranes.

Southern and Northern blots were probed with α-³²P-radiolabelled fragments of *CaBNI4*. DNA sequencing was performed using the dideoxy-termination method (Sanger *et al.*, 1977) and sequences were aligned and analysed using the GCG v.10 software from HGMP (<http://www.hgmp.mrc.ac.uk>). Northern blots were re-probed with a radiolabelled *CaTEF3* fragment as loading controls (Di Domenico *et al.*, 1992). The entire gene was sequenced and submitted to GenBank: accession number AY569336.

Chitin contents. *C. albicans* cell-wall chitin was determined by the enzymic method (Bulawa *et al.*, 1986; Mellado *et al.*, 1996; Munro *et al.*, 1998) and acid hydrolysis methods described previously (Kaptein *et al.*, 2000). Triplicate samples of 50–100 mg (wet wt) yeast cells were used in the enzymic analysis and 20–30 mg (wet wt) cell-wall material were used for the acid-hydrolysis method.

Morphological observations. Yeast and hyphal cells were fixed in 10% (v/v) neutral buffered formalin (Sigma). Chitin was stained with 50 µg Calcofluor ml⁻¹ (Sigma, fluorescent brightener 28). Transmission electron microscopy (TEM) was performed on exponential-phase yeast cells after fixation with 3% glutaraldehyde (v/v) in 0.1 M sodium phosphate buffer pH 7.4. Secondary fixation was performed in 1% osmium tetroxide in distilled H₂O and cells were embedded in TAAB resin. Ultrathin silver or gold defracting sections were stained with uranyl acetate and lead citrate and examined with a Philips 301 transmission electron microscope at an accelerating voltage of 80 kV.

Table 2. Primers used in the construction of Ura-blast cassettes

Restriction sites are underlined.

Name	Sequence	Position and orientation
SACIDIS	5'-GAAGAGCTCCAACACTGGTAACAATGG-3'	Nucleotides 3087–3106 of <i>BNI4</i> ORF →
BGLIIDIS	5'-GAAAGATCTGAGTCGATTGATCAACAATC-3'	Nucleotides 3630–3611 of <i>BNI4</i> ORF ←
SALIDIS	5'-GAAGTCGACGATAGACATCCTGATACTGC-3'	Nucleotides 4833–4852 of <i>BNI4</i> ORF →
HINDIIDIS	5'-GAAAAGCTTACACCAGATTGCTACAACA-3'	382–500 nucleotides downstream of <i>BNI4</i> stop codon ←

The *CaBNI4* gene was originally identified by homology searches to the Stanford *C. albicans* genome database using

ScBNI4 nucleotide and protein sequences. A contig was identified that contained a 202 bp sequence which showed 73 % identity to *ScBNI4* at the amino acid level and 68 % identity at the nucleotide level and corresponded to the 3' region of the gene. The complete ORF for *CaBNI4* of 4.968 kb (orf 6.2835 of contig 6.2168) shares 20 % overall

(b)

N terminal region: 31.9% identity over 141 aa

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ScBNI4 1 MSDSISDSKSELLNSTFYSS-TSIN-TLDHARTFRNSLILKE-ISDQSLNSSIK--PCESVLDRDVESSVLQ 68
      ||| | | |::: |||: |||: :::: |||: :::: |||: | |::: : : | |
CaBNI4 1 MADSTFD---EFFDNDFYSAPTSINNSKNHVKNVRNSIQIKHSMSSRSLNEFTKDIPPSATTSTTKSNTQ 69
```

ScBNI4 69 RSPGESNARDSE--VQTVNMTTSPSLALADILNERSK-YADQKTRKAQNIESSIIEEEEEEAEQNNNSINYHE 138
: : |:| :: :| : |||||:| :|||:| | :| :::| | :| : :| :| :|

CaBNI4 70 TNPSSSKAGNTSGSTRTSIIKASPSLKALESILNEKNKYNTANTNIIIEVEEETEETEKVGKAKNHSQGLHS 142

Middle region: 70% identity over 37aa

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ScBNI4 478 -----AFSKHDT---GEVLFPKFLDNHEVDSIVSLELTRS---TKSNKRSSM----- 518
      : :| :| ||: ||| |: |||: |||||:| | :| |||||:|
CaBNI4 957 TNNNVHVHTEDKPNTQLLGEALFPKSLNAHEVESIVSLERSRSMKSVKSNKRSSFVNVDGS 1016
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C terminal region: 73% identity over 63aa

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ScBNI4 830 GVRFSSQIILYDTYGEFYDRHPEISTCNQLTPQLAQMIKLELNLKSAMEVHDDSRCYTHFY 892
      |||||:| |||||:| |||||: : ||||| |||:| ||| :| |:| :| |||||:
CaBNI4 1593 GVRFSSRIILYDTYNEDEYDRHPDTATCNQLTPLLAQIKDELNSFKSEMEIHVESKCYTHFF 1655
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Fig. 1. (a) Alignment of the predicted protein sequences of ScBni4p and CaBni4p based on CLUSTALW analysis at the EBI web site (<http://www.ebi.ac.uk>). The three regions of homology are outlined, with the corresponding percentage identities. The coiled-coil regions were predicted using the COILSCAN program in GCG version 10 and are shown by the hatched box. The region disrupted in *C. albicans bni4Δ/bni4Δ* is shown by the shaded box. (b) Local alignment of ScBni4p and CaBni4p with the highest identity at the C-terminal region. The grey box shows the conserved PP1 binding domain and the shaded bar below the sequence shows the 19 amino acids included in the corresponding region deleted in *Cabni4Δ/Cabni4Δ*.

identity to ScBni4p, and alignment of ScBni4p and CaBni4p sequences revealed three main regions of homology, at the N-terminal, mid-protein and C-terminal regions (Fig. 1a). The highest identity is at the C-terminal 63 amino acids; this region also contains the putative Glc7p binding domain, GVRF (Fig. 1b). Attempts to clone full-length *CaBNI4* in pBluescript were unsuccessful; therefore, *CaBNI4* was cloned as two overlapping fragments of 3.7 kb and 2.7 kb. A 5.5 kb fragment containing the full-length *CaBNI4* gene was successfully cloned into the low-copy plasmid pBR322, but this was susceptible to recombination in *E. coli*. No evidence was found for another *BNI4*-like sequence in the *C. albicans* genome database and no homologous sequence was identified by low-stringency Southern hybridization using a probe specific to the 3' region of the *CaBNI4* gene (not shown).

Northern analysis of *CaBNI4* revealed a transcript of the

expected size in both exponential-phase yeast cells and 4 h after induction of hyphal growth (Fig. 2).

Disruption of the *CaBNI4* gene

To determine the role of *CaBNI4* we disrupted both chromosomal copies of the gene, in parallel lineages of strains generated using the Ura-blaster protocol. Disruption was confirmed by Southern analysis (Fig. 2a) and the absence of transcript in the null mutants was confirmed by Northern analysis (Fig. 2b). Growth rates of the yeast form in YPD and hypha formation in Lee's medium were the same for CAI-4 and the *Cabni4Δ/Cabni4Δ* (NGY83a, NGY83b, NGY84a, NGY84b) mutants (not shown). Growth rates were measured in liquid culture in YPD at 30 °C for yeast and germ-tube formation was measured in Lee's medium at 37 °C for both Ura⁺ and Ura[−] strains. No effect was seen on hypha formation in the Ura⁺ and

Fig. 2. Southern and Northern analysis of *Cabni4Δ*. (a) Disruption of *CaBNI4* by Southern analysis. Genomic DNA was digested with *Cla*I (C) and probed with an 865 bp radiolabelled probe (hatched box) of the 3' region of *CaBNI4* (from nucleotide 4585 of *BNI4* ORF to 482 nucleotides downstream of the stop codon). Lanes: 1, CAI-4; 2, NGY82a (*bni4Δ::hisG/BNI4*); 3, NGY84a (*bni4Δ::hisG/bni4Δ::hisG*). (b) Northern analysis of *CaBNI4* showing loss of the *CaBNI4* transcript in the null mutant. RNA was extracted from exponential-phase yeast cells and from hyphae 4 h after germ-tube induction in 20% serum. Loading of RNA was estimated by ethidium bromide staining of the rRNA species and by reprob-ing with *CaTEF3* (probes corresponding to nucleotides 2970–4942 are shown as hatched boxes). Lanes: 1, CAI-4 yeast; 2, CAI-4 hyphae; 3, NGY84a (*bni4Δ/bni4Δ*) yeast; 4, NGY84a (*bni4Δ/bni4Δ*) hyphae.

Fig. 3. Reduced hypha formation of *Cabni4Δ/Cabni4Δ* mutant (NGY83a) compared to CAF2-1 parent and *CachS3Δ/CachS3Δ* mutant on 20% serum agar. Scale bars, 1 mm.

Ura⁻ *Cabni4Δ/Cabni4Δ* mutants induced in Lee's medium at pH 6.5 and 37 °C. However, the *Cabni4Δ/Cabni4Δ* mutants displayed a reduced ability to form hyphae when grown on solid 20 % serum (Fig. 3). In contrast, *CachS3Δ/CachS3Δ* mutants were not affected in hypha formation on serum agar (Fig. 3).

Phenotype of the *bni4* mutant

The *Cabni4Δ/Cabni4Δ* mutants were screened against SDS (0.01–0.05 %), Calcofluor White (100–400 µg ml⁻¹) (Fig. 4), NaCl (0.2–1 M), and Hygromycin B (100 µg ml⁻¹) (not shown) to determine their sensitivity to these plasmolysing or cell-wall-damaging agents. SDS sensitivity

was slightly increased for the *Cabni4Δ/Cabni4Δ* mutants compared to the CAF2-1 parent, whilst an increased resistance to Calcofluor White was observed, indicative of a reduction in cell-wall chitin (Fig. 4).

The chitin contents of yeast cells and cell walls of yeast and hyphae were determined using chitinase-digestion and acid-digestion methods (acid-hydrolysis data shown in Fig. 5). In both cases the *Cabni4Δ/Cabni4Δ* mutants contained significantly (*P* > 0.01) less chitin compared to the wild-type strain (Fig. 5). Overall, in *Cabni4Δ/Cabni4Δ* mutant strains, yeast cells had 30 % less chitin and hyphal cells 40 % less chitin compared to the parental strain.

Fig. 4. Increased sensitivity of *Cabni4Δ/Cabni4Δ* mutant to SDS and increased resistance to Calcofluor White compared to CAF2-1. *C. albicans* strains were grown as a starter culture of yeast cells in YPD overnight. Serial dilutions corresponding to 5 × 10¹–5 × 10⁴ yeast cells were spotted across solid medium containing (a) SDS or (b) Calcofluor White and plates were incubated at 30 °C for 2–5 days.

Fig. 5. Chitin content of *Cabni4Δ* mutants NGY83a, NGY83b, NGY84a and NGY84b and their respective parent strains. Ura⁻ and Ura⁺ strains were included to see whether *URA3* status affected chitin content. The chitin content as measured after acid hydrolysis of triplicate yeast (Y) and hyphal (H) cell-wall samples. Cell walls were pretreated with SDS and Zymolyase 20-T to remove unbound carbohydrate and glycoprotein. Error bars represent mean standard deviation; *n* = 3.

Morphological alterations

The effect of the chitin reduction for the *Cabni4Δ/Cabni4Δ* null mutants was visualized using Calcofluor White staining (Fig. 6). Mutant hyphal cells stained less brightly with Calcofluor. Only minor differences were noted between the

Calcofluor staining of yeast cells in the mutant. A slightly aberrant bud-scar pattern was observed in the *Cabni4Δ/Cabni4Δ* mutant, in which bud scars appeared to be more randomly positioned over the yeast cell surface. Chitin rings in dividing cells were difficult to detect using Calcofluor White in both wild-type and *Cabni4Δ/Cabni4Δ* mutants. Yeast cells were more elongated, and 'lemon-shaped' cells were frequently observed (Fig. 6k).

Analysis of *Cabni4Δ/Cabni4Δ* by TEM showed that the primary septum was intact and normal and again suggested there were minor differences in the budding pattern (Fig. 7f) and the formation of lemon-shaped cells (Fig. 7d).

DISCUSSION

We isolated and characterized a *C. albicans* gene that is the only homologue of the *BNI4* of *S. cerevisiae* in the *C. albicans* genome sequence. The gene product of *ScBNI4* has been implicated in the spatial deposition of chitin to the bud-neck region by forming a complex by which Chs3p is linked to the septin bud-neck filaments via Chs4p and Bni4p. Immunolocalization studies confirmed the position of ScBni4p at the bud-neck region (DeMarini *et al.*, 1997). The similarity between ScBni4p and CaBni4p is mostly in the C-terminal region and deletion of 492 amino acids in the C-terminal region caused loss of the *BNI4* transcript and influenced subtly the phenotype of both yeast and hyphal cells. In the *C. albicans* mutants, bud scars and primary septa were formed but only minor changes in the shape and distribution of the bud scars was found.

In *S. cerevisiae* it has been shown that Bni4p interacts with

Fig. 6. Wild-type (CAI-4) cells (a–d), *Cabni4Δ/Cabni4Δ* (NGY84a) null mutant cells (e–k) stained with Calcofluor White. Bud scars (thin arrows) were clearly visible on *Cabni4Δ/Cabni4Δ* yeast cells that had undergone several budding events (h, i). Mutant strains had bud scars at non-polar locations (thick arrow, j). Thicker arrows indicate the elongated, 'lemon-shaped' cells that were frequently observed in *Cabni4Δ/Cabni4Δ* mutants (k). Bars, 5 μm (a, b, e, f, h–k) and 13.5 μm (c, d, g).

Fig. 7. TEM of parent CAI-4 (a, c, e) *Cabni4Δ/Cabni4Δ* null mutant (NGY84a) (b, d, f) showing protuberant and flattened bud scars, birth scars and lemon-shaped cells (d). Vacuoles (v) are indicated; bars, 0.5 μm.

Chs4p and the septin Cdc10p whilst Chs4p interacts with Cdc12p (DeMarini *et al.*, 1997). Therefore, if the same is true in *C. albicans*, then the Chs4p–Cdc12p interaction may be sufficient for targeting of Chs3p to the bud site since the bud neck and primary septum were not markedly affected in the mutant. To date no other *ScBNI4* homologue has been identified in the *C. albicans* genome sequencing project and no homologues were identified here by low-stringency hybridization; therefore, no evidence exists for the formal possibility that function of Bni4p can be achieved by an alternative homologue protein.

Changes in the structural integrity of the *Candida* cell wall are expressed as changes in the sensitivity of cells to various inhibitors that stress the wall in various ways. Sensitivity to Hygromycin B, SDS, Calcofluor and NaCl have been shown to depend on the permeability, porosity or chitin content of the cell wall, and several glycosylation or chitin synthase mutants have increased sensitivity to these compounds (Timpel *et al.*, 1998, 2000; Warit *et al.*, 2000; Binley *et al.*,

1999; Navarro-Garcia *et al.*, 1995). The *Cabni4Δ/Cabni4Δ* mutant had decreased sensitivity to Calcofluor White, reflecting the reduced chitin content, and increased sensitivity to SDS that may reflect reduced integrity of the wall.

We have demonstrated a reduction in cell-wall chitin in the *Cabni4Δ/Cabni4Δ* null mutant, indicating that in *C. albicans*, Bni4p is involved in targeting of Chs3p to the cell surface. However, disruption of *CaBNI4* caused loss of chitin over the whole cell wall as visualized by Calcofluor staining. We propose that *CaBni4p* may have a global, septin/bud-neck-independent role in the deposition of chitin, by either the targeting or activation of the Chs3p chitin synthase enzyme.

The lemon shape of the *bni4Δ/bni4Δ* mutants suggests that Chs3p targeting and chitin content are vital for normal yeast morphology. The *C. albicans bni4Δ/bni4Δ* mutant was also reduced in hyphal formation on 20 % serum agar. Germ-tube formation was, however, normal in both Lee's

and 20 % serum liquid media. A number of other cell-wall mutants of *C. albicans* such as *int1Δ* (Gale *et al.*, 1998), *hwp1Δ* (Tsuchimori *et al.*, 2000) and *cdc10Δ* and *cdc11Δ* septin mutants (Warenda *et al.*, 2003) have been found to respond differently to hypha-inducing signals in liquid and solid media. For example, mutants of the non-essential septins *cdc10Δ* and *cdc11Δ* were only mildly affected for hyphal growth in liquid culture, whereas hyphal growth was profoundly affected on solid media (Warenda *et al.*, 2003). The *Cabni4Δ/Cabni4Δ* mutant described here was more severely defective in hyphal growth on solid media even in comparison with the *cdc10Δ* and *cdc11Δ* mutants. The zinc finger transcription factor *Czf1* has also been shown to be involved in the regulation of hyphal formation only when the cell was in contact with solid medium (Brown *et al.*, 1999). These observations suggest that different or additional regulatory processes are involved in the formation of hyphal cells on solid and in liquid media and that Bni4p may be important in the deposition of chitin that maintains the filamentous cell shape when hyphal cells are growing on surfaces.

It is not known what proteins are involved in the targeting of Chs enzymes, other than Chs3p, to specific cellular locations. Analysis of disrupted *CHS* genes in *C. albicans* suggests that Chs3p, Chs2p and Chs1p may all participate in the formation of chitin in the cell wall of hyphae (Gow *et al.*, 1994; Bulawa *et al.*, 1995; Munro *et al.*, 2001). The activity of these enzymes is not thought to be dependent on Chs4p. No association of Bni4p or Chs4p with other Chs proteins was identified by dihybrid analysis in *S. cerevisiae* (DeMarini *et al.*, 1997). However, the possibility that Bni4p is involved in the targeting of other chitin synthase enzymes to the surface has yet to be fully explored. Although the phenotype of the *bni4Δ/bni4Δ* mutant of *C. albicans* differs significantly in some respects from the equivalent *S. cerevisiae* mutant, our data support the evidence that disruption of the chitin synthase cell-targeting machinery influences the composition, architecture and function of the fungal cell wall.

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