Deficiencies in the essential Smp3 mannosyltransferase block glycosylphosphatidylinositol assembly and lead to defects in growth and cell wall biogenesis in *Candida albicans*

Stephen J. Grimme,¹ Paul A. Colussi,² Christopher H. Taron² and Peter Orlean¹

Correspondence

Peter Orlean

p-orlean@uiuc.edu

¹Department of Microbiology, University of Illinois, Urbana, IL 61801, USA

²New England Biolabs, 32 Tozer Road, Beverly, MA 01915, USA

Glycosylphosphatidylinositols (GPIs) are essential for viability in yeast and have key roles in cell wall construction. Assembly of *Saccharomyces cerevisiae* GPIs includes the addition of a fourth, side-branching mannose (Man) to the third Man of the core GPI glycan by the Smp3 mannosyltransferase. The *SMP3* gene from the human pathogenic fungus *Candida albicans* has been cloned. *CaSMP3* complements the inviable *S. cerevisiae* *smp3* null mutant and, when expressed in an *S. cerevisiae* *smp3/gpi13* double mutant, it permits *in vivo* conversion of the Man₃-GPI precursor that accumulates in that mutant to a Man₄-GPI. One allele of *CaSMP3* was disrupted using the ura-blaster procedure, then the remaining allele was placed under the control of the glucose-repressible *MAL2* promoter. Repression of *CaSMP3* expression leads to accumulation of a GPI precursor glycolipid whose glycan headgroup contains three mannoses and bears a phosphodiester-linked substituent on its first Man. Under repressing conditions, cells exhibited morphological and cell wall defects and became inviable. *CaSmp3p* therefore adds a fourth, α 1,2-linked Man to trimannosyl GPI precursors in *C. albicans* and is necessary for viability. Because addition of a fourth Man to GPIs is of less relative importance in mammals, Smp3p is a potential antifungal target.

Received 19 April 2004

Revised 5 June 2004

Accepted 9 June 2004

INTRODUCTION

The fungal cell wall is a complex of cross-linked polysaccharides and glycoproteins that is critical for maintenance of the integrity and shape of the cell as it grows and divides (Orlean, 1997). The wall is also a key determinant of fungal virulence because its surface bears glycoproteins that mediate adhesion of pathogenic fungi to host tissues (Hoyer, 2001; Sundstrom, 2002). Because of its importance in fungal growth and virulence, cell wall biogenesis has long been eyed as an attractive target for new antifungal agents.

Glycosylphosphatidylinositols (GPIs) have important roles in cell wall biogenesis in yeast. These glycolipids, which have the conserved core structure H₂N-CH₂-CH₂-PO₄α6Manα1,2Manα1,6Manα1,4GlcNα1,6Ins phospholipid, are preassembled in the endoplasmic reticulum, then transferred to the COOH terminus of a subset of secretory proteins that have a consensus signal-attachment site (Nuoffer *et al.*, 1993). Surveys of the *S. cerevisiae* and *Candida albicans*

genomes have led to the prediction that 60–70 of the proteins encoded in *S. cerevisiae* and 50–100 *C. albicans* gene products may receive a GPI (Caro *et al.*, 1997; De Groot *et al.*, 2003; Hamada *et al.*, 1998; Lee *et al.*, 2003; Sundstrom, 2002). Such proteins then transit the secretory pathway, to be anchored in the external face of the plasma membrane. In some, perhaps most cases, the GPI glycan moiety can participate in a transglycosylation reaction and become cross-linked to cell wall 1,6-β-glucan, thereby serving to anchor glycoproteins to the wall (De Nobel & Lipke, 1994; De Sampaio *et al.*, 1999; Fujii *et al.*, 1999; Hamada *et al.*, 1999; Kapteyn *et al.*, 1996; Lu *et al.*, 1994).

GPIs are essential in *S. cerevisiae* and *Schizosaccharomyces pombe* (Colussi & Orlean, 1997; Leidich *et al.*, 1994). *S. cerevisiae* *gpi* mutants show aberrant cellular morphology (Leidich *et al.*, 1994, 1995; Leidich & Orlean, 1996), sensitivity to cell wall-disruptive agents (Benghezal *et al.*, 1995; Vossen *et al.*, 1995) and altered cell wall composition (Vossen *et al.*, 1997). These defects can be attributed to the collective effects of blocks in the GPI-dependent transport (Doering & Schekman, 1996; Nuoffer *et al.*, 1993) of cell wall assembly enzymes and cell wall proteins to the cell surface, and to abolition of GPI-dependent cross-linking of

Abbreviations: CFW, Calcofluor White; EthN-P, phosphoethanolamine; 5-FOA, 5-fluoroorotic acid; GPI, glycosylphosphatidylinositol; JBαM, jack bean α-mannosidase.

glycoproteins to cell wall polysaccharides (Ram *et al.*, 1994; Richard *et al.*, 2002b; Vossen *et al.*, 1997). For example, GPI-anchored 1,3- β -glucanotransferases such as *S. cerevisiae* Gas1p and its homologues in *C. albicans* and *Aspergillus fumigatus* elongate cell wall 1,3- β -glucan chains (Mouyna *et al.*, 2000), and deficiencies in these cell surface enzymes severely perturb cell wall organization in *S. cerevisiae* and *C. albicans* (Fonzi, 1999; Popolo *et al.*, 1997; Popolo & Vai, 1998; Ram *et al.*, 1998).

Glycoproteins predicted to receive a GPI anchor and which are expressed on the cell surface have important roles in the virulence and morphogenesis of the opportunistic human pathogen *C. albicans*. Among these are the agglutinin-like sequence (Als) proteins and the Hwp1p transglutaminase substrate, which have been implicated, respectively, in adhesion and attachment to mammalian cells (reviewed by Hoyer, 2001; Staab *et al.*, 1999). In *Candida glabrata*, the GPI-modified adhesin Epa1p is primarily responsible for adhesion of this pathogenic yeast to human epithelial cells (Cormack *et al.*, 1999).

The key roles of GPIs in fungal growth and virulence make this post-translational modification of protein a potential antifungal target and random screens have indeed yielded inhibitors of GPI assembly (Sütterlin *et al.*, 1997; Tsukahara *et al.*, 2003). Importantly, it may prove to be possible to target GPI assembly selectively in eukaryotic microbes because, although many of the steps and enzymes involved in GPI anchoring are conserved, differences can occur that are critical for protozoa or for yeast, but which are absent from or of diminished importance in mammals (Grimme *et al.*, 2001; Smith *et al.*, 1997). To identify such variations and evaluate their importance in a human pathogenic fungus, we have initiated a detailed biochemical investigation of GPI assembly in *C. albicans*. We are focusing on a variation that is essential in *S. cerevisiae*, but not required for GPI attachment to protein in mammals, namely, the addition of a fourth, α 1,2-linked Man to the third, α 1,2-linked Man of the GPI precursor by the Smp3 mannosyltransferase (Grimme *et al.*, 2001).

The *C. albicans* genome encodes a sequence homologue of Smp3p (Grimme *et al.*, 2001) and we report here an analysis of the biochemical role of this protein in GPI assembly in *C. albicans*. We show that *CaSMP3* complements the growth and GPI mannosylation defects in *S. cerevisiae* and that repression of *CaSMP3* expression in *C. albicans* leads to a block in GPI assembly and the accumulation of a Man₃-GPI precursor glycolipid. *CaSmp3p* therefore adds a fourth mannose during assembly of *C. albicans* GPIs, and this activity is critical for growth of *C. albicans* because repression of *CaSMP3* expression leads to loss of cell viability. Our results allow us to make predictions about the GPI assembly pathway and GPI anchor structure in *C. albicans*, and they validate GPI biosynthesis in general and *CaSmp3p* in particular as potential antifungal drug targets.

METHODS

C. albicans and *S. cerevisiae* strains, media and growth conditions.

All *C. albicans* strains were derived from CAI-4 (Fonzi & Irwin, 1993). The *S. cerevisiae* *smp3-2* and *smp3-2/gpi13::KanMX4-P_{GAL}-GPI13* strains are described by Grimme *et al.* (2001). YPD and SD media were prepared as described by Sherman (1991) and supplemented with 80 μ g uridine ml⁻¹ (YPDU or SDU) when required for growth of *Caura3* strains. SMal medium contained 2% (w/v) maltose instead of glucose. Inositol-free medium was prepared as described by Grimme *et al.* (2001) and contained 2% glucose or 2% maltose as appropriate. Counter-selection on 5-fluoroorotic acid (5-FOA) was carried out on SDU supplemented with 1 mg 5-FOA ml⁻¹ (Toronto Research Chemicals). Calcofluor White (CFW) sensitivity was tested on solid YPD medium containing 10–20 μ g CFW ml⁻¹ (Sigma-Aldrich).

Cloning of *CaSMP3* and construction of expression plasmids.

A candidate *CaSMP3* gene, identified by TBLASTN searching (Altschul *et al.*, 1990) of the unfinished *C. albicans* genome, was found on Contig 6-2467 (encoded by nucleotides 5966–7463). A 2789 bp DNA fragment containing the putative *CaSMP3* locus was amplified from *C. albicans* genomic DNA by PCR using primers 1 and 2 (Table 1). The fragment was cloned into the plasmids pRS415 (CEN) and pRS425 (2 μ) (Christianson *et al.*, 1992; Sikorski & Hieter, 1989) and DNA sequencing of this fragment confirmed that the cloned *CaSMP3* gene was identical to the candidate *CaSMP3* sequence in Contig 6-2467.

Disruption of *CaSMP3*. The *CaSMP3* gene was subcloned into the *Bam*HI site of the cloning vector pNEB193 (New England Biolabs) and a 1455 nt *Bsa*BI–*Eco*RV fragment was removed from the resulting plasmid and replaced with a blunt-ended 4.1 kb *Hind*III–*Bgl*II containing the ‘ura-blaster’ cassette from pMB-7 (Fonzi & Irwin, 1993) to create plasmid p Δ *CaSMP3*. The *hisG*-*CaURA3*-*hisG* cassette replaced 97% of the *CaSMP3* coding region and was flanked by 659 and 645 nt of DNA homologous to chromosomal DNA sequence immediately 5' and 3' to *CaSMP3*, respectively. A 5.5 kb linear disrupting fragment (Fig. 1a) was excised from p Δ *CaSMP3* by digestion with *Pac*I and *Sac*I, introduced into strain CAI-4 by electroporation (De Backer *et al.*, 1999) and uracil prototrophs were selected on SD medium containing 1 M sorbitol. Colonies were tested for homologous recombination of the disrupting DNA fragment at the chromosomal *CaSMP3* locus by carrying out two whole-cell PCR reactions (Ling *et al.*, 1995) using either primers 3 and 6 or 4 and 5 (Table 1, Fig. 1a). Genomic DNA was prepared from representative transformants that yielded PCR products that spanned both the 5' and 3' integration junctions, and Southern blotting was performed to confirm that the disrupting DNA had been integrated at the chromosomal *CaSMP3* locus. A 172 bp DIG-labelled hybridization probe complementary to the 3' untranslated region of *CaSMP3* was amplified from genomic DNA using primers 10 and 11 (Table 1, Fig. 1) and the PCR DIG Probe Synthesis Kit (Roche Molecular Biochemicals). Amplification with Expand High Fidelity Polymerase required a 1:6 ratio of DIG-11-dUTP to dTTP. Hybridization was performed using DIG Easy Hyb Granules and the probe was detected with DIG Wash and Block Buffer Set using NBT/BCIP chromogenic detection (Roche Molecular Biochemicals).

Selection for the *CaURA3* gene was removed from *Casmp3::hisG*-*CaURA3*-*hisG*/*CaSMP3* strains and counter-selection with 5-FOA imposed for colonies that had excised *CaURA3*, leaving behind a single *hisG* sequence (Fig. 1a). Resistant colonies were transferred to YPDU medium and tested for the inability to grow on SD medium. Candidate ‘loop outs’ were screened by whole-cell PCR for the amplification of a product diagnostic for the presence of *hisG* using primers 7 and 8 (Table 1, Fig. 1a). Genomic DNA was isolated from positive colonies and the conversion of the *CaSMP3::hisG*-*CaURA3*-*hisG* locus to

Table 1. Oligonucleotide primers used in this study

Primer	Sequence (5'–3')*	Features
1	CGCGGATCCGATAACTGATTCGT	<i>Bam</i> HI
2	CGCGGATCCAAGAATGGTGAGAG	<i>Bam</i> HI
3	ATTGACCGGCAATGTAGGATCTAG	
4	CAACCACGGCTTCTTTCATCAG	
5	CGTTGGGCAATAAATCCAATAACA	
6	GGATTATTGCCAACGTGATATG	
7	GGAGGTGCGGATATGAGGTAGC	
8	AACGTGCTGGAAGAAGAGCTACTC	
9	AAGTTCTCATACCTGGCGTTGTATAAC	
10	ATCCAACCTGGTTTAGGTGTCTATCAGT	
11	CAAGTAAAGACAGGGACAGAACATGATAAAT	
12	GCCACTGAAACCACGGTTAATAAC	
13	CCGAATTCTGTCTAGTACCATCTGTAC	<i>Eco</i> RI
14	GGGGTACCTGATTATTAGTTAAACCAC	<i>Kpn</i> I
15	GGGGTACCTATGATCAAGATCAACTTC	<i>Kpn</i> I, <i>CaSMP3</i> start
16	GCTCTAGACCCAGTATAGAATAAATTC	<i>Xba</i> I, amber codon

*Engineered restriction sites are underlined. Other features are indicated in bold.

Casmp3::hisG was verified by Southern blotting (Fig. 1a, c). A second transformation was carried out using the same *PacI*–*SacI* disrupting DNA fragment and two independent heterozygous *Casmp3::hisG/CaSMP3* strains as recipients for integration of the ura-blaster cassette at the remaining *CaSMP3* locus. Uracil prototrophic transformants were screened by whole-cell PCR for the amplification of a DNA fragment diagnostic of the wild-type *CaSMP3* allele using primers 3 and 9 (Table 1 and Fig. 1a). Primer 9 anneals to a region of the *CaSMP3* coding sequence that should no longer be present if both alleles are disrupted.

Creation of a *CaSMP3*-repressible strain. A *Casmp3::hisG/CaSMP3* strain was constructed in which expression of the remaining *CaSMP3* allele was placed under the control of the maltose-inducible, glucose-repressible P_{MAL2} promoter (Backen *et al.*, 2000). A 536 bp *EcoRI*–*KpnI* DNA fragment containing P_{MAL2} was amplified by PCR from *C. albicans* genomic DNA using primers 13 and 14 and cloned into pUC19. A 3.9 kb *HindIII* fragment containing *CaURA3* was next inserted in the pUC19 polylinker 3' to the *CaMAL2* promoter, yielding plasmid p $P_{MAL2}URA3$. A 566 bp *KpnI*–*XbaI* fragment containing the *CaSMP3* start codon and the first 560 bp of the *CaSMP3* coding region was amplified by PCR using primers 15 and 16, and cloned into p $P_{MAL2}URA3$. The resulting plasmid, P_{MAL2} -*SMP3*-*URA3*, was linearized by digestion with *BstZ171*, whose unique recognition site is about midway along the *CaSMP3* homologous DNA. The resulting linear fragment was used to transform a *Casmp3::hisG/CaSMP3* strain to uracil prototrophy and transformants were screened by whole-cell PCR for integration of P_{MAL2} at the *CaSMP3* locus using primers 4 and 12 (Table 1 and Fig. 1a). PCR-positive colonies were also tested for their inability to yield a 1.9 kb amplification product diagnostic for *CaSMP3* using primers 3 and 9. Although primer 9 can anneal within the P_{MAL2} -*SMP3* fusion, the 7.2 kb distance between these primers is too great to allow product amplification. Colonies from which the diagnostic fragment could be amplified still contain a wild-type copy of *CaSMP3* controlled by its native promoter and these strains were not studied further. Colonies from which this band could not be amplified were examined by Southern blotting to verify the absence of a wild-type *CaSMP3* locus and to confirm the integration

of the *MAL2* promoter in front of an intact copy of *CaSMP3* (Fig. 1b, c).

[3 H]Inositol labelling of lipids and GPI glycan headgroup analysis. Radiolabelling of *S. cerevisiae* strains with [3 H]inositol was carried out as described previously (Grimme *et al.*, 2001). For [3 H]inositol labelling of *C. albicans* strains, exponentially growing cells cultured in SMal were harvested and resuspended at 0.01 OD₆₀₀ units ml^{−1} in inositol-free medium containing either 2% (w/v) maltose or 2% (w/v) glucose and incubated at 30 °C for 12–16 h until the cultures had reached an OD₆₀₀ of approximately 1.0. Ten OD₆₀₀ units ml^{−1} of these cultures was harvested and resuspended in 1 ml fresh medium containing the same carbon source in which the cells had previously been grown. Cells were radiolabelled for 3 h with 15 μ Ci (555 kBq) [3 H]inositol, after which radiolabelling was terminated by addition of NaN₃ to a final concentration of 10 mM. Lipids were then extracted as described by Taron *et al.* (2000), separated by TLC on Silica Gel 60 plates (Alltech) using chloroform/methanol/water (4:4:1 or 5:5:1, by vol.) as solvent, and 3 H-labelled lipids were detected by fluorography using Biomax MS film and a Biomax TransScreen-LE intensifying screen (Eastman-Kodak). Treatments of 3 H-labelled lipids with phosphatidylinositol-specific phospholipase C (PI-PLC) and methanolic NH₃ were performed as described by Taron *et al.* (2000). For GPI headgroup glycan analysis, 200 OD₆₀₀ units of *Casmp3::hisG/P_{MAL2}-CaSMP3* cells that had been cultured in inositol-free medium containing 2% (w/v) glucose for 16 h were radiolabelled with 1.4 mCi (51.8 MBq) [3 H]inositol at 30 °C for 3 h. Isolation of GPI intermediates by preparative TLC, delipidation with methanolic NH₃, re-*N*-acetylation with acetic anhydride, HF dephosphorylation, exo-glycosidase treatment and high performance TLC were performed as described by Grimme *et al.* (2001).

Staining and microscopy. *C. albicans* strains were continuously cultured at 30 °C in medium containing Glc or Mal for 20 or 36 h by diluting exponentially growing cells into fresh medium every 8 h. Approximately 5.0×10^6 cells were harvested, washed once with 1 ml ice-cold PBS, resuspended in 100 μ l PBS and stained with propidium iodide (5 μ g ml^{−1}) and Hoechst 33342 (25 μ g ml^{−1})

supplied by Molecular Probes. Cells were visualized by epifluorescence, bright field or phase-contrast microscopy using a Nikon Eclipse TE300 inverted microscope. Images were acquired using a Princeton Instruments MicroMAX-800PB cooled CCD camera and IPLab imaging software (v3.2.3).

Materials and reagents. Exoglycosidases were obtained from Prozyme. [2-³H]myo-Inositol (specific activity 555–740 GBq mmol⁻¹) was obtained from American Radiolabelled Chemicals. Oligonucleotides were synthesized by Integrated DNA Technologies and DNA sequencing was performed at the University of Illinois Genetic Engineering and Sequencing Facility.

RESULTS

A *C. albicans* protein functionally equivalent to *S. cerevisiae* Smp3p

The *C. albicans* genome contains an ORF that encodes a protein with 35 % identity and 54 % similarity to ScSmp3p and which has the amino acid motif HQEXRF characteristic of the subgroup of dolichol phosphate mannose-utilizing mannosyltransferases defined by ScSmp3p (Grimme *et al.*, 2001). The predicted CaSmp3 protein contains 498 aa and

hydropathy analysis (Kyte & Doolittle, 1982) indicates the presence of eight to nine potential membrane-spanning segments.

To show whether the putative *CaSMP3* gene encodes the functional equivalent of ScSmp3p, we tested whether it complements *S. cerevisiae* strains with conditional and null mutations in *SMP3*. Expression of *CaSMP3* under the control of its own promoter on centromeric and 2 μ plasmids in *S. cerevisiae* *smp3* cells restored the ability of the conditional mutant to grow at 37 °C (Fig. 2a) and corrected the hypersensitivity of the *smp3* mutant to CFW (Fig. 2b). Furthermore, plasmids expressing *CaSMP3* complemented the lethal null mutation in *S. cerevisiae* *SMP3* (data not shown).

We next investigated whether *CaSMP3* expression corrects the biochemical defect in *S. cerevisiae* *smp3* cells. The *smp3* mutant accumulates trimannosyl GPI precursors (Man₃-GPIs) that can be detected by radiolabelling with [³H]inositol (Grimme *et al.*, 2001). Accumulation of this lipid is greatly reduced in *smp3* cells expressing *CaSMP3* (Fig. 3a, lanes 5 and 6), consistent with almost complete alleviation of the biosynthetic defect in *smp3*. To show that *CaSMP3* encodes a protein that is capable of mannosylating Man₃-GPIs, we conducted an *in vivo* mannosyltransferase assay using the *S. cerevisiae* *smp3/gpi13* double mutant as illustrated in Fig. 3(b). When expression of *GPI13* is repressed in this strain at 25 °C, it accumulates a Man₄-GPI (Fig. 3c, lane 1), but at 37 °C, when the *smp3* block is superimposed in the double mutant, only the upstream Man₃-GPI is formed (Fig. 3c, lane 2). We therefore tested whether expression of *CaSMP3* from a plasmid in the *smp3/gpi13* double mutant would overcome the *smp3* block at 37 °C and restore Man₄-GPI formation. This was indeed the case: *smp3/gpi13* cells harbouring a 2 μ plasmid containing *CaSMP3* now accumulated a [³H]inositol-labelled lipid that had the same TLC mobility as the Man₄-GPI bearing one phosphoethanolamine (EthN-P) moiety that accumulates in the Gpi13p-depleted strain (Fig. 3c, lanes 3 and 4). Formation of this GPI is strong evidence that *CaSMP3* encodes a protein capable of mannosylating a trimannosyl

GPI *in vivo*. Taken together, these results show that CaSmp3p is the functional equivalent of ScSmp3p.

Disruption of *CaSMP3* and phenotypic consequences of repressing its expression

To investigate whether *CaSMP3*, like its *S. cerevisiae* counterpart, is an essential gene and whether deficiencies in CaSmp3p affect GPI biosynthesis and growth, we attempted to isolate homozygous *CaSMP3* null mutants using the ura-blaster strategy (Fonzi & Irwin, 1993). A 5.5 kb linear disrupting fragment (Fig. 1a) was created in which 97 % of the *CaSMP3* coding region was replaced by the *hisG*-*CaURA3*-*hisG* cassette which remained flanked by chromosomal DNA sequence immediately 5' and 3' to *CaSMP3*. This fragment was introduced into strain CAI-4 by electroporation and uracil prototrophs were selected. Three distinct colony morphologies were observed, large round colonies, slightly smaller wrinkled colonies and small round colonies. Sixty independent transformants obtained in three separate transformation experiments were tested for integration of the disruption cassette at a *CaSMP3* locus by screening their DNA for the ability to serve as template for PCR amplification of DNA fragments across both junctions of the predicted integration event. Eighteen colonies, all of which were originally large and round, yielded PCR products. Genomic DNA was isolated from eight positive colonies and analysed by Southern blotting. Of these, seven showed the hybridization pattern predicted if integration of *hisG*-*CaURA3*-*hisG* had occurred at one of the *CaSMP3* alleles. To 'loop out' the ura-blaster cassette, one Ura⁺ heterozygote from each of two independent primary transformations was grown first without selection for *CaURA3*, then in the presence of 5-FOA to select for colonies that had excised the *CaURA3* gene. Candidate *Casmp3::hisG*/*CaSMP3* loop-outs (48 from each of the Ura⁺ heterozygotes) were screened by whole-cell PCR for the presence of *hisG* sequences and by Southern blotting to verify the conversion of the *CaSMP3::hisG*-*CaURA3*-*hisG* locus to *Casmp3::hisG* (Fig. 1c, lanes 2 and 7).

Attempts were next made to create a homozygous Δ *CaSMP3*

Fig. 1. Modifications introduced into the *CaSMP3* chromosomal locus. (a) Strategy for disruption of *CaSMP3*. Shown diagrammatically, from top to bottom, are the chromosomal *CaSMP3* locus, the predicted organization of the *CaSMP3* locus after integration of the disrupting ura-blaster cassette and the *hisG*-disrupted *CaSMP3* locus remaining after recombination between the *hisG* repeats and excision of *CaURA3*. (b) Strategy for placing *CaSMP3* under the control of the *CaMAL2* promoter (M2p). Shown diagrammatically, from top to bottom, are plasmid P_{MAL2}-*SMP3*-*URA3* linearized by digestion with *Bst*Z17I, the chromosomal *CaSMP3* locus and the predicted organization of the *CaSMP3* locus after integration of P_{MAL2} following homologous recombination. The approximate sites of annealing of oligonucleotide primers 1–10 (Table 1) are indicated. The approximate region of DNA complementary to the hybridization probe is indicated by the grey vertical bars. H, *Hind*III site; B, *Bam*HI site. (c) Southern blot analysis of *C. albicans* genomic DNA digested with either *Hind*III (lanes 1–4) or *Bam*HI (lanes 6–9). The resulting digestion fragments and sites of hybridization to the DIG-labelled probe are indicated in panels (a) and (b). Samples are as follows: DIG-labelled DNA MW marker VII (lane 5), CAI-4 (lanes 1 and 6), heterozygous *Casmp3::hisG*/*CaSMP3* diploid (lanes 2 and 7), *Casmp3::hisG*/P_{MAL2}-*CaSMP3* (lanes 3 and 8) and a triploid *Casmp3::hisG*/P_{MAL2}-*CaSMP3*/*CaSMP3* strain (lanes 4 and 9). Digestion with *Hind*III yields diagnostic fragments of 6.1 (*CaSMP3*), 1.4 (*Casmp3::hisG*) and 4.8 kb (P_{MAL2}-*CaSMP3*), and digestion with *Bam*HI yields diagnostic fragments of 5.2 (*CaSMP3*), 3.9 (*Casmp3::hisG*) and 10.3 kb (P_{MAL2}-*CaSMP3*).

Fig. 2. *CaSMP3* corrects temperature and CFW sensitivity of the *S. cerevisiae smp3* mutant. *S. cerevisiae smp3-2* was transformed with centromeric and 2µ plasmids containing *CaSMP3* behind its native promoter or with the empty vectors and transformants were tested for growth on YPD medium at 37 °C (a) and for growth on YPD medium supplemented with 15 µg CFW ml⁻¹ (b).

strain by disruption of the second *CaSMP3* allele in *Casmp3::hisG/CaSMP3* heterozygotes by a second round of transformation with the ura-blaster cassette used to disrupt the first allele. Three independent *Casmp3::hisG/CaSMP3* strains arising from two different primary transformations were used as recipients. Uracil prototrophs were screened by whole-cell PCR for amplification of a DNA fragment diagnostic of the wild-type *CaSMP3* allele. Because one of the PCR primers was designed to anneal in a region of the *CaSMP3* coding sequence that should not be present if both *CaSMP3* alleles have been disrupted, homozygous Δ *CaSMP3* strains would yield no PCR product. However, all 80 uracil prototrophs tested PCR-positive, indicating that they harboured a wild-type copy of *CaSMP3* and suggesting that isolation of a homozygous Δ *CaSMP3* strain is not possible because *CaSMP3* is an essential gene in the CAI-4 background. Whole-cell PCR tests designed to amplify DNA fragments spanning the *CaSMP3* locus and so determine whether the *CaURA3* marker had integrated into a *CaSMP3* allele were also performed. This analysis revealed that in

some transformants the second integration event had occurred at the same site as the first, that some transformants were likely to be triploid for *CaSMP3* and that some transformants had ectopically integrated the *hisG-CaURA3-hisG* cassette. Because attempts to disrupt the second copy of *CaSMP3* did not yield any homozygous null mutants, we conclude that *CaSMP3* is an essential gene in strain CAI-4.

To confirm that loss of *CaSMP3* leads to loss of viability and interruption of GPI assembly, we constructed a *Casmp3::hisG/CaSMP3* strain in which expression of the remaining *CaSMP3* allele was placed under the control of the maltose-inducible, glucose-repressible *CaMAL2* promoter (*P_{MAL2}*) (Backen *et al.*, 2000) (Fig. 1b). First, a plasmid was assembled whose DNA insert consisted, 5' to 3', of a 536 bp DNA fragment containing *P_{MAL2}*, a DNA fragment consisting of the *CaSMP3* start codon and the first 560 bp of *CaSMP3* coding sequence, and the *CaURA3* gene. This plasmid was linearized by digestion with *Bst*Z17I,

Fig. 3. *CaSMP3* complements the GPI biosynthetic defect in *S. cerevisiae smp3* mutants. (a) Suppression of the [³H]inositol lipid labelling defect in *smp3*. Cells were labelled with [³H]inositol, radiolabelled lipids extracted, separated by TLC in chloroform/methanol/water (4 : 4 : 1, by vol.) and detected by fluorography. Lanes: 1 and 2, lipids from wild-type *S. cerevisiae* cells harbouring the empty 2μ plasmid (vector) or the 2μ plasmid containing *CaSMP3* (2μ *CaSMP3*), labelled at 25 and 37 °C respectively; 3 and 4, lipids from *smp3*-2 cells containing vector alone labelled at 25 and 37 °C; 5 and 6, *smp3* cells containing 2μ *CaSMP3* labelled at 25 and 37 °C. (b) Scheme illustrating the test for conversion of the trimannosyl GPI that accumulates in the *S. cerevisiae smp3/gpi13* mutant to a Man₄-GPI upon expression of *CaSMP3*. The structures of GPI headgroups are shown, with side-branching EthN-PS omitted for simplicity. Ins represents inositol; M, mannose; G, glucosamine; P, phosphate; E, ethanolamine. (c) Expression of *CaSMP3* in the *S. cerevisiae smp3/gpi13* mutant bypasses the *smp3* block and allows formation of a Man₄-GPI. The *smp3-2/gpi13::KanMX4-pGAL-GPI13* strain harbouring 2μ *CaSMP3* was incubated for 16 h at 25 °C in SD to repress *GPI13* expression and portions of these cultures were then shifted to 37 °C for 30 min or maintained at 25 °C. [³H]inositol-labelling was then carried out for 3 h at 37 or 25 °C, radiolabelled lipids were extracted, separated by TLC using chloroform/methanol/water (4 : 4 : 1, by vol.) and detected by fluorography (lanes 3 and 4). A control *smp3/gpi13* strain harbouring the empty 2μ plasmid was grown, temperature-shifted and radiolabelled in parallel (lanes 1 and 2). The GPI headgroup structures indicated were determined previously (Grimme *et al.*, 2001; Taron *et al.*, 2000) (side-branching EthN-PS are omitted).

whose single recognition site lies about halfway along the *CaSMP3* fragment. The linearized plasmid was used to transform a *Casmp3::hisG/CaSMP3* heterozygote to uracil prototrophy. Such transformants were predicted to arise upon homologous recombination between the *CaSMP3* sequences at either end of the linear fragment and the remaining intact chromosomal *CaSMP3* locus. Integration of the linear fragment results in a truncation of the *CaSMP3* allele under control of its native promoter and reconstitution of a complete *CaSMP3* fused to P_{MAL2}. Transformants containing the P_{MAL2}-*CaSMP3* fusion were initially identified by whole-cell PCR tests, and Southern blot analysis confirmed the correct integration of the P_{MAL2}-*CaSMP3*

fusion fragment and the absence of a wild-type *CaSMP3* allele (Fig. 1c, lanes 3 and 8). A representative triploid *Casmp3::hisG/P_{MAL2}-CaSMP3/CaSMP3* strain is also shown (Fig. 1c, lanes 4 and 9).

Repression of *CaSMP3* expression leads to a block in GPI precursor assembly

We next tested whether a shift of *Casmp3::hisG/P_{MAL2}-CaSMP3* to glucose-containing medium leads to interruption of the *C. albicans* GPI precursor assembly and, if so, which step is blocked. Exponential-phase cultures of *Casmp3::hisG/P_{MAL2}-CaSMP3* grown in synthetic medium

containing 2% (w/v) maltose were harvested and diluted to $0.01 \text{ OD}_{600} \text{ units ml}^{-1}$ in inositol-free medium containing 2% (w/v) glucose or 2% maltose and incubated for a further 12–16 h before being radiolabelled with $15 \mu\text{Ci}$ (555 kBq) [^3H]inositol for 3 h. Cultures of strain CAI-4 and of the *Casmp3::hisG/CaSMP3* heterozygote were radiolabelled in parallel in medium containing glucose or maltose supplemented with $80 \mu\text{g}$ uridine ml^{-1} . Radiolabelled lipids were extracted, separated by TLC and detected by fluorography.

Cultures that had been shifted to glucose-containing medium accumulated a major [^3H]inositol-labelled lipid (Fig. 4, lane 7) that had a TLC mobility similar to that of the trimannosyl GPI bearing a single EthN-P that accumulates in the *S. cerevisiae smp3* mutant (Fig. 4, lane 6). The control *Casmp3::hisG/P_{MAL2}-CaSMP3* cells that had been radiolabelled in maltose-containing medium did not show accumulation of any [^3H]inositol-labelled GPI lipids (Fig. 4, lane 8). Neither the wild-type CAI-4 strain, nor the *smp3::hisG/SMP3* heterozygote accumulated any aberrant lipids (lanes 1–4). The accumulation of a [^3H]inositol-labelled lipid after a shift of *Casmp3::hisG/P_{MAL2}-CaSMP3* cells to glucose-containing medium is strong evidence that expression of the *P_{MAL2}*-regulated gene was repressed, leading to depletion of CaSmp3p and interruption of GPI biosynthesis.

We next performed various chemical and enzymic treatments to confirm that the lipid accumulated by *Casmp3::hisG/P_{MAL2}-CaSMP3* under repressing conditions is indeed a GPI, and to determine the step in GPI assembly that is blocked upon CaSmp3p depletion. The accumulated lipid is sensitive to mild base hydrolysis (Fig. 5a, lanes 1 and 2), indicating that it has ester-linked fatty acyl chains, but the lipid is resistant to cleavage by phosphatidylinositol-specific phospholipase C (Fig. 5a, lanes 3 and 4), a property consistent with the presence of an acyl chain esterified to inositol.

We next analysed the glycan headgroup of the accumulating lipid to determine the number of mannose residues present and the presence and location of phosphodiester-linked substituents on these residues. The [^3H]inositol-labelled lipid was isolated by preparative TLC and its headgroup glycan released by deacylation with mild base. The aqueous-soluble material was converted to a neutral glycan by re-*N*-acetylation of the GlcN followed by dephosphorylation with 50% aqueous HF. Portions of the ^3H -labelled neutral glycan headgroups were treated with non-specific jack bean α -mannosidase (JB α M) or α 1,2-specific mannosidase. The sizes of the undigested and α -mannosidase-treated glycans were determined by separating using high performance TLC and comparing their mobilities to those of [^3H]glucose oligosaccharide standards (Grimme *et al.*, 2001; Taron *et al.*, 2000). The untreated neutral glycan had a chromatographic mobility corresponding to that of Man₃-GlcNAc-Ins (Fig. 5b, lanes 3 and 5). Treatment with JB α M changed its mobility to that of

Fig. 4. Shift of *Casmp3::hisG/P_{MAL2}-CaSMP3* cells to glucose leads to accumulation of [^3H]inositol-lipids. CAI-4, *Casmp3::hisG/CaSMP3* and *Casmp3::hisG/P_{MAL2}-CaSMP3* cells were labelled with [^3H]inositol in maltose- or glucose-containing medium and radiolabelled lipids were extracted, separated by TLC using chloroform/methanol/water (5:5:1, by vol.) and detected by fluorography. Lanes: 1 and 2, CAI-4 cells grown and labelled in medium containing glucose and maltose, respectively; 3 and 4, *Casmp3::hisG/CaSMP3* cells grown and labelled in glucose and maltose, respectively; 7 and 8, *Casmp3::hisG/P_{MAL2}-CaSMP3* cells grown and labelled in glucose and maltose, respectively; 5 and 6, samples of [^3H]inositol-labelled lipids from *S. cerevisiae gpi13* and *smp3* strains labelled under non-permissive conditions. The ^3H -labelled lipid about half way up lane 6 corresponds to lipid 3-2 (Grimme *et al.*, 2001), which was proposed to be a Man₃-GPI lacking the EthN-P side-branch. The GPI headgroup structures for the lipids that accumulate in the *S. cerevisiae smp3* and *gpi13* mutants are shown (Grimme *et al.*, 2001; Taron *et al.*, 2000). Abbreviations are defined in the legend to Fig. 3(b).

GlcNAc-Ins (lane 4), whereas 1,2- α -mannosidase digestion generated Man₂-GlcNAc-Ins (lane 6). These results indicate that the accumulated GPI contains three mannoses with the outermost Man in α 1,2 linkage.

Yeast GPI precursors can receive side-branching EthN-Ps on Man-1 or Man-2 during their assembly (Canivenc-Gansel *et al.*, 1998; Grimme *et al.*, 2001; Taron *et al.*, 2000). *S. cerevisiae smp3* mutants accumulate two isoforms of Man₃-GPIs with EthN-P on either Man-1 or Man-2.

Therefore, we investigated the presence of phosphodiester-linked substituents on the accumulating *C. albicans* Man₃-GPI. To do this, a sample of deacylated neutral glycan was incubated first with JBαM, then with HF. This order of treatments yielded Man-GlcNAc-Ins (Fig. 5b, lane 7), indicating that the glycan bore an HF-labile substituent on Man-1 that blocked cleavage of this residue by JBαM. Only trace amounts of Man₂-GlcNAc-Ins (Fig. 5b, lane 7) were observed, suggesting that the HF-labile substituent on the isolated lipid is present almost exclusively on the first mannose.

Growth defects of *Casmp3::hisG/P_{MAL2}-CaSMP3* strains

Casmp3::hisG/P_{MAL2}-CaSMP3 strains showed slow growth on solid glucose-containing, repressing medium, but formed smaller colonies than cells of the *Casmp3::hisG/CaSMP3* heterozygote. This slow growth may reflect slight leakiness

Fig. 5. Structural characterization of the lipid that accumulates in *Casmp3::hisG/P_{MAL2}-CaSMP3* cells after a shift to glucose-containing medium. (a) Susceptibility of the accumulated lipid to mild base and PI-PLC treatment. Lipids isolated from radio-labelled *Casmp3::hisG/P_{MAL2}-CaSMP3* cells were incubated with methanol/water or methanolic ammonia (mild base) (lanes 1 and 2) or without or with PI-PLC (lanes 3 and 4). Reaction mixtures were then butanol-extracted and separated by TLC in chloroform/methanol/water (4:4:1, by vol.), after which radio-labelled lipids were detected by fluorography. (b). Analysis of the GPI headgroup glycan. Lipid from 200 OD₆₀₀ units of [³H]inositol-labelled *Casmp3::hisG/P_{MAL2}-CaSMP3* cells that had been cultured in medium containing 2% glucose was isolated as detailed in Methods, then deacylated and re-*N*-acetylated. Samples of this ³H-labelled material were then submitted to the following treatments, then separated by high performance TLC and detected by fluorography. Lanes: 3 and 5, dephosphorylation with 50% aqueous HF; 4, dephosphorylation followed by incubation with JBαM; 6, dephosphorylation followed by incubation with *Aspergillus saitoi* 1,2- α -mannosidase; 7, incubation with JBαM, followed by treatment with 50% aqueous HF. Lane 1 displays a series of NaB[³H]₄-reduced glucose oligomers (G1-G6). Lane 2, [³H]Ins. Ins, M0, M1, M2, M3 and M4 indicate the positions of inositol, GlcNAc-Ins, Man-GlcNAc-Ins, Man₂-GlcNAc-Ins, Man₃-GlcNAc-Ins and Man₄-GlcNAc-Ins, respectively (Grimme *et al.*, 2001; Taron *et al.*, 2000).

of P_{MAL2} and the possibility that only low levels of CaSmp3 protein are needed to sustain viability. Leaky expression of *S. cerevisiae* Smp3p was also observed when its expression from the *GAL10* promoter was repressed (Grimme *et al.*, 2001). Growth under repressing conditions was also observed for the essential *CaCHS1* gene when the only functional copy was expressed behind the *MRP1* promoter (Munro *et al.*, 2001). Strikingly, however, *Casmp3::hisG/P_{MAL2}-CaSMP3* cells formed large aggregates of irregularly shaped cells under repressing conditions in glucose-containing medium (Fig. 6a, panel 1), in contrast to the *Casmp3::hisG/P_{MAL2}-CaSMP3* cells on maltose or the heterozygote on glucose (Fig. 6a, panels 2 and 3). Moreover, the aggregates of *Casmp3::hisG/P_{MAL2}-CaSMP3* cells included cells that had become permeable to propidium iodide and had therefore lost cell integrity (Fig. 6a, panel 4) (Zaragoza *et al.*, 2002). When grown in Glc, many *Casmp3::hisG/P_{MAL2}-CaSMP3* aggregates included enlarged cells, and most of these enlarged cells also contained enlarged vacuoles as visualized using bright field optics (Fig. 6b, panel 1). Some of these cells (indicated by the arrows in Fig. 6b, panel 1) contained multiple nuclear structures that could be stained with the dye Hoechst 33342 (Fig. 6b, panel 2). Interestingly, these enlarged cells were still viable as they were not stained with propidium iodide; however, a portion of the cells in the aggregate were stained with propidium iodide and were therefore necrotic (Fig. 6b, panel 3). The aberrant morphologies of *Casmp3::hisG/P_{MAL2}-CaSMP3* cells on glucose-containing medium resemble those of *S. cerevisiae* *gpi1* and *gpi2*

Fig. 6. Shift of *Casmp3::hisG/P_{MAL2}-CaSMP3* cells to glucose leads to growth defects. (a) Aggregation and propidium iodide staining of *Casmp3::hisG/P_{MAL2}-CaSMP3* cells cultured in repressing SD medium (panels 1 and 4) or in inducing SMal medium (panels 2 and 5) for 20 h. Parental *Casmp3::hisG/CaSMP3* cells incubated in SD medium are shown in panels 3 and 6. Cells were stained with propidium iodide and photographed using phase-contrast optics (panels 1–3) or epifluorescence (panels 4–6). Bar, 100 μ m. (b) Depletion of CaSmp3p results in morphological defects. Repressed *Casmp3::hisG/P_{MAL2}-CaSMP3* cells were cultured in YPD medium for 36 h and stained with Hoechst 33342 and propidium iodide. Cells were photographed using bright field optics (panels 1 and 4) or epifluorescence to detect nuclear staining (panels 2 and 5) and necrotic cells (panels 3 and 6). The bar represents 10 μ m.

Fig. 7. Shift of *Casmp3::hisG/P_{MAL2}-CaSMP3* cells to glucose leads to CFW sensitivity. WT *CaSMP3* cells (lane 1), *Casmp3::hisG/CaSMP3* cells (lane 2) and *Casmp3::hisG/P_{MAL2}-CaSMP3* cells (lanes 3 and 4) were cultured in YPD (lanes 1–3) or YPMal (lane 4) for 36 h prior to 10-fold serial dilution and plating onto (a) YPD or (b) YPD containing 20 µg CFW ml⁻¹. Plates were incubated 36–60 h at 37 °C.

mutants, which also form aggregates containing enlarged, sometimes irregularly shaped cells that have failed to separate (Leidich & Orlean, 1996).

The morphological defects of *Casmp3::hisG/P_{MAL2}-CaSMP3* cells indicated a severe impairment in normal cell wall biogenesis and we therefore tested their sensitivity to the fluorescent brightener CFW, an empirical criterion for cell wall defects (Ram *et al.*, 1994). *Casmp3::hisG/P_{MAL2}-CaSMP3* cells grown in Glc-containing, repressing medium prior to serial dilution and plating on solid media were clearly hypersensitive to 20 µg CFW ml⁻¹ (row 3 in Fig. 7a and b). In contrast, *Casmp3::hisG/P_{MAL2}-CaSMP3* cells pre-grown in medium containing Mal, which induces expression of *CaSMP3*, grew at least as well as wild-type and heterozygous cells on CFW (Fig. 7b, lanes 1, 2 and 4). We conclude that depletion of the *CaSmp3* protein perturbs cell wall biogenesis and leads to morphological aberrations, and ultimately to loss of cell integrity.

DISCUSSION

Our results allow us to assign a biochemical function to *CaSmp3p* and to make predictions about GPI anchor structure and the GPI assembly pathway in the human opportunistic fungal pathogen *C. albicans*. *CaSMP3* corrects

the growth and GPI assembly defects of *S. cerevisiae smp3* mutants, which are defective in the addition of a side-branching α 1,2-linked Man during GPI precursor assembly, and repression of *CaSMP3* expression in *C. albicans* leads to the accumulation of a GPI with the glycan structure Man α 1,2Man α Man-GlcN-Ins modified with an HF-labile substituent on Man-1. The accumulation of a Man₃-GPI demonstrates that *C. albicans* makes GPIs and is strong evidence that *CaSmp3p* adds a fourth Man to trimannosyl GPI precursors in *C. albicans*. The HF-labile substituent is most likely to be EthN-P, for this substituent has been detected on Man-1 of *S. cerevisiae* and mammalian GPI precursors (Canivenc-Gansel *et al.*, 1998; Grimme *et al.*, 2001; Hirose *et al.*, 1992; Kamitani *et al.*, 1992; Taron *et al.*, 2000).

Our findings demonstrate the importance of GPIs for growth of *C. albicans*. Our inability to isolate homozygous *CaSMP3* null mutants suggests that *CaSMP3* is an essential gene and this notion receives strong support from our finding that repression of *CaSMP3* expression leads to loss of viability and to defective cell wall assembly. Consistent with an essential role for GPIs in *C. albicans*, it has not proven possible to isolate homozygous null mutants in *CaGPI3*, the catalytic subunit of the first enzyme in GPI assembly (E. Robinson, P. A. Colussi & P. Orlean, unpublished). The requirement for *CaSmp3p*-dependent addition of a fourth Man to complete GPI anchoring in *C. albicans* leads us to predict that protein-bound GPIs in *C. albicans* contain at least four mannoses, as is the case in *S. cerevisiae* (Fankhauser *et al.*, 1993) and *Aspergillus fumigatus* (Fontaine *et al.*, 2003).

The only other *C. albicans* gene involved in GPI biosynthesis that has been studied to date is *CaGPI7*, which encodes a protein whose *S. cerevisiae* counterpart is proposed to add an EthN-P side branch to the second GPI Man (Benachour *et al.*, 1999). Disruption of this gene is not lethal (Richard *et al.*, 2002b), nor does it abolish the presumably GPI-dependent membrane anchoring of protein; however, proteins predicted normally to be cross-linked to the cell wall in a GPI-dependent manner are released into the growth medium (Richard *et al.*, 2002a). Our findings lead to the prediction that *CaSmp3p* depletion should, by blocking GPI attachment, affect both plasma membrane and cell wall localization of proteins that receive a GPI.

One explanation for the effect of *CaGPI7* deletion on cell wall anchorage of protein is that *CaGpi7p*-dependent addition of EthN-P to Man-2 of GPIs is important for subsequent cross-linkage to cell wall constituents (Richard *et al.*, 2002a). The result of our EthN-P-positioning experiment on the Man₃-GPI that accumulates upon *CaSMP3* repression raises questions about the addition of EthN-P to Man-2 in *C. albicans*. Thus, the GPI that accumulates in the *S. cerevisiae smp3* mutant consists of a mixture of approximately equal amounts of a Man₃-GPI isoform bearing EthN-P on Man-1, and a Man₃-GPI so modified on Man-2 (Grimme *et al.*, 2001), indicating that EthN-P can

be added to Man-2 at the trimannosyl stage in *S. cerevisiae*. In contrast, *C. albicans* SMP3-repressed strains accumulate a Man₃-GPI modified predominantly on Man-1. Explanations for this are that EthN-P addition to Man-2 of *C. albicans* GPIs is a very minor modification during GPI precursor assembly or that most EthN-P is added to Man-2 very late in the GPI-anchoring pathway to free or protein-bound Man₄-GPIs.

Fungi and mammals differ with respect to the importance of the fourth mannosylation step for GPI anchoring in each type of organism. Addition of the fourth Man during GPI precursor assembly is mandatory in fungi. This is clear from the facts that protein-bound GPIs in *S. cerevisiae* all bear at least four mannoses (Fankhauser *et al.*, 1993), that *S. cerevisiae* GPI transamidase mutants accumulate Man₄-GPIs (Benghezal *et al.*, 1995) and that deletion of the fourth GPI mannosyltransferase gene, *SMP3*, is lethal in *S. cerevisiae* and *C. albicans*. Smp3p-dependent addition of a fourth Man is necessary for subsequent addition of the 'bridging' EthN-P moiety on Man-3 through which the GPI becomes linked to protein (Grimme *et al.*, 2001). In contrast, addition of a fourth Man does not appear to be an obligatory step in all mammalian cells. Thus, protein-bound GPIs on individual GPI-anchored proteins in animals can bear GPIs with three or with four mannoses (Homans *et al.*, 1988; McConville & Ferguson, 1993; Roberts *et al.*, 1988; Stahl *et al.*, 1992). However, the capacity of mammalian cells to attach Man₄-GPIs to protein has so far not been obvious from studies of GPI precursor assembly in cultured mammalian cell lines: such analyses readily detect Man₃-GPIs, but formation of Man₄-GPIs is at best rare (Hirose *et al.*, 1992; Hong *et al.*, 2000). Tellingly too, mutant mammalian cell lines defective in transfer of GPIs to protein accumulate Man₃-GPIs, not Man₄-GPIs (Mohny *et al.*, 1994; Ohishi *et al.*, 2000), and, moreover, these Man₃-GPIs bear EthN-P on Man-3. These findings indicate that in mammals, in contrast to fungi, a fourth Man is not a prerequisite for addition of the bridging EthN-P and that transfer of GPIs to proteins can occur irrespective of the presence or absence of Man-4 on GPI precursors.

Although the GPI anchoring pathway in mammalian cells does not require a fourth mannosylation step, the occurrence of Man₄-GPIs suggests that a fourth GPI mannosyltransferase may nonetheless occur in mammals, and a functional human Smp3 homologue has been identified (Taron *et al.*, 2004). This gene is expressed at highest relative levels in the brain and colon, raising the possibility that a fourth mannose can be added to a subset of human GPIs in a tissue-specific manner.

Our demonstration that formation of Man₄-GPIs is mandatory in fungi but not an obligatory step in mammals highlights fungal Smp3 mannosyltransferases, as well as subsequent enzymes in GPI precursor assembly that require the presence of a fourth Man, as potential selective targets for antifungal agents. The availability of the

CaSMP3 gene will permit a detailed biochemical characterization of the CaSmp3 protein and facilitate development of high-throughput assays for inhibitors. Our ability to create *C. albicans* strains with conditional defects in GPI assembly will permit us to identify further GPI assembly intermediates and chart the *C. albicans* GPI assembly pathway, as well as probe the phenotypic consequences of blocking GPI anchoring in this human pathogenic fungus.

ACKNOWLEDGEMENTS

Supported in part by Grant GM46220 from the National Institutes of Health to P.O. P.O. is a Burroughs Wellcome Scholar in Molecular Pathogenic Mycology. C.H.T. thanks Dr Donald Comb of New England Biolabs for financial support. We thank M. Glaser for use of a Nikon Eclipse TE300 inverted microscope and IPLab imaging Suite.

REFERENCES

- Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990). Basic local alignment search tool. *J Mol Biol* **215**, 403–410.
- Backen, A. C., Broadbent, I. D., Fetherston, R. W., Rosamond, J. D., Schnell, N. F. & Stark, M. J. (2000). Evaluation of the *CaMAL2* promoter for regulated expression of genes in *Candida albicans*. *Yeast* **16**, 1121–1129.
- Benachour, A., Sipos, G., Flury, I., Reggiori, F., Canivenc-Gansel, E., Vionnet, C., Conzelmann, A. & Benghezal, M. (1999). Deletion of *GPI7*, a yeast gene required for addition of a side chain to the glycosylphosphatidylinositol (GPI) core structure, affects GPI protein transport, remodeling, and cell wall integrity. *J Biol Chem* **274**, 15251–15261.
- Benghezal, M., Lipke, P. N. & Conzelmann, A. (1995). Identification of six complementation classes involved in the biosynthesis of glycosylphosphatidylinositol anchors in *Saccharomyces cerevisiae*. *J Cell Biol* **130**, 1333–1344.
- Canivenc-Gansel, E., Imhof, I., Reggiori, F., Burda, P., Conzelmann, A. & Benachour, A. (1998). GPI anchor biosynthesis in yeast: phosphoethanolamine is attached to the α 1,4-linked mannose of the complete precursor glycopospholipid. *Glycobiology* **8**, 761–770.
- Caro, L. H., Tettelin, H., Vossen, J. H., Ram, A. F., van den Ende, H. & Klis, F. M. (1997). In silico identification of glycosylphosphatidylinositol-anchored plasma-membrane and cell wall proteins of *Saccharomyces cerevisiae*. *Yeast* **13**, 1477–1489.
- Christianson, T. W., Sikorski, R. S., Dante, M., Shero, J. H. & Hieter, P. (1992). Multifunctional yeast high-copy-number shuttle vectors. *Gene* **110**, 119–122.
- Colussi, P. A. & Orlean, P. (1997). The essential *Schizosaccharomyces pombe* *gpi1*⁺ gene complements a bakers' yeast GPI anchoring mutant and is required for efficient cell separation. *Yeast* **13**, 139–150.
- Cormack, B. P., Ghori, N. & Falkow, S. (1999). An adhesin of the yeast pathogen *Candida glabrata* mediating adherence to human epithelial cells. *Science* **285**, 578–582.
- De Backer, M. D., Maes, D., Vandoninck, S., Logghe, M., Contreras, R. & Luyten, W. H. (1999). Transformation of *Candida albicans* by electroporation. *Yeast* **15**, 1609–1618.
- De Groot, P. W., Hellingwerf, K. J. & Klis, F. M. (2003). Genome-wide identification of fungal GPI proteins. *Yeast* **20**, 781–796.
- De Nobel, H. & Lipke, P. N. (1994). Is there a role for GPIs in yeast cell-wall assembly? *Trends Cell Biol* **4**, 42–45.

- De Sampaio, G., Bourdineaud, J. P. & Lauquin, G. J. (1999). A constitutive role for GPI anchors in *Saccharomyces cerevisiae*: cell wall targeting. *Mol Microbiol* **34**, 247–256.
- Doering, T. L. & Schekman, R. (1996). GPI anchor attachment is required for Gas1p transport from the endoplasmic reticulum in COP II vesicles. *EMBO J* **15**, 182–191.
- Fankhauser, C., Homans, S. W., Thomas-Oates, J. E., McConville, M. J., Desponds, C., Conzelmann, A. & Ferguson, M. A. (1993). Structures of glycosylphosphatidylinositol membrane anchors from *Saccharomyces cerevisiae*. *J Biol Chem* **268**, 26365–26374.
- Fontaine, T., Magnin, T., Melhert, A., Lamont, D., Latge, J. P. & Ferguson, M. A. (2003). Structures of the glycosylphosphatidylinositol membrane anchors from *Aspergillus fumigatus* membrane proteins. *Glycobiology* **13**, 169–177.
- Fonzi, W. A. (1999). *PHR1* and *PHR2* of *Candida albicans* encode putative glycosidases required for proper cross-linking of β -1,3- and β -1,6-glucans. *J Bacteriol* **181**, 7070–7079.
- Fonzi, W. A. & Irwin, M. Y. (1993). Isogenic strain construction and gene mapping in *Candida albicans*. *Genetics* **134**, 717–728.
- Fujii, T., Shimoi, H. & Iimura, Y. (1999). Structure of the glucan-binding sugar chain of Tiplp, a cell wall protein of *Saccharomyces cerevisiae*. *Biochim Biophys Acta* **1427**, 133–144.
- Grimme, S. J., Westfall, B. A., Wiedman, J. M., Taron, C. H. & Orlean, P. (2001). The essential Smp3 protein is required for addition of the side-branching fourth mannose during assembly of yeast glycosylphosphatidylinositols. *J Biol Chem* **276**, 27731–27739.
- Hamada, K., Fukuchi, S., Arisawa, M., Baba, M. & Kitada, K. (1998). Screening for glycosylphosphatidylinositol (GPI)-dependent cell wall proteins in *Saccharomyces cerevisiae*. *Mol Gen Genet* **258**, 53–59.
- Hamada, K., Terashima, H., Arisawa, M., Yabuki, N. & Kitada, K. (1999). Amino acid residues in the omega-minus region participate in cellular localization of yeast glycosylphosphatidylinositol-attached proteins. *J Bacteriol* **181**, 3886–3889.
- Hirose, S., Prince, G. M., Seveler, D., Ravi, L., Rosenberry, T. L., Ueda, E. & Medof, M. E. (1992). Characterization of putative glycoinositol phospholipid anchor precursors in mammalian cells. Localization of phosphoethanolamine. *J Biol Chem* **267**, 16968–16974.
- Homans, S. W., Ferguson, M. A., Dwek, R. A., Rademacher, T. W., Anand, R. & Williams, A. F. (1988). Complete structure of the glycosyl phosphatidylinositol membrane anchor of rat brain Thy-1 glycoprotein. *Nature* **333**, 269–272.
- Hong, Y., Maeda, Y., Watanabe, R., Inoue, N., Ohishi, K. & Kinoshita, T. (2000). Requirement of PIG-F and PIG-O for transferring phosphoethanolamine to the third mannose in glycosylphosphatidylinositol. *J Biol Chem* **275**, 20911–20919.
- Hoyer, L. L. (2001). The ALS gene family of *Candida albicans*. *Trends Microbiol* **9**, 176–180.
- Kamitani, T., Menon, A. K., Hallaq, Y., Warren, C. D. & Yeh, E. T. (1992). Complexity of ethanolamine phosphate addition in the biosynthesis of glycosylphosphatidylinositol anchors in mammalian cells. *J Biol Chem* **267**, 24611–24619.
- Kapteyn, J. C., Montijn, R. C., Vink, E., de la Cruz, J., Llobell, A., Douwes, J. E., Shimoi, H., Lipke, P. N. & Klis, F. M. (1996). Retention of *Saccharomyces cerevisiae* cell wall proteins through a phosphodiester-linked β -1,3- β -1,6-glucan heteropolymer. *Glycobiology* **6**, 337–345.
- Kyte, J. & Doolittle, R. F. (1982). A simple method for displaying the hydropathic character of a protein. *J Mol Biol* **157**, 105–132.
- Lee, S. A., Wormsley, S., Kamoun, S., Lee, A. F., Joiner, K. & Wong, B. (2003). An analysis of the *Candida albicans* genome database for soluble secreted proteins using computer-based prediction algorithms. *Yeast* **20**, 595–610.
- Leidich, S. D. & Orlean, P. (1996). *Gpi1*, a *Saccharomyces cerevisiae* protein that participates in the first step in glycosylphosphatidylinositol anchor synthesis. *J Biol Chem* **271**, 27829–27837.
- Leidich, S. D., Drapp, D. A. & Orlean, P. (1994). A conditionally lethal yeast mutant blocked at the first step in glycosyl phosphatidylinositol anchor synthesis. *J Biol Chem* **269**, 10193–10196.
- Leidich, S. D., Kostova, Z., Latek, R. R., Costello, L. C., Drapp, D. A., Gray, W., Fassler, J. S. & Orlean, P. (1995). Temperature-sensitive yeast GPI anchoring mutants *gpi2* and *gpi3* are defective in the synthesis of N-acetylglucosaminyl phosphatidylinositol. Cloning of the *GPI2* gene. *J Biol Chem* **270**, 13029–13035.
- Ling, M., Merante, F. & Robinson, B. H. (1995). A rapid and reliable DNA preparation method for screening a large number of yeast clones by polymerase chain reaction. *Nucleic Acids Res* **23**, 4924–4925.
- Lu, C. F., Kurjan, J. & Lipke, P. N. (1994). A pathway for cell wall anchorage of *Saccharomyces cerevisiae* alpha-agglutinin. *Mol Cell Biol* **14**, 4825–4833.
- McConville, M. J. & Ferguson, M. A. (1993). The structure, biosynthesis and function of glycosylated phosphatidylinositols in the parasitic protozoa and higher eukaryotes. *Biochem J* **294**, 305–324.
- Mohney, R. P., Knez, J. J., Ravi, L., Seveler, D., Rosenberry, T. L., Hirose, S. & Medof, M. E. (1994). Glycoinositol phospholipid anchor-defective K562 mutants with biochemical lesions distinct from those in Thy-1-murine lymphoma mutants. *J Biol Chem* **269**, 6536–6542.
- Mouyna, I., Fontaine, T., Vai, M., Monod, M., Fonzi, W. A., Diaquin, M., Popolo, L., Hartland, R. P. & Latge, J. P. (2000). Glycosylphosphatidylinositol-anchored glucanoyltransferases play an active role in the biosynthesis of the fungal cell wall. *J Biol Chem* **275**, 14882–14889.
- Munro, C. A., Winter, K., Buchan, A., Henry, K., Becker, J. M., Brown, A. J., Bulawa, C. E. & Gow, N. A. (2001). Chs1 of *Candida albicans* is an essential chitin synthase required for synthesis of the septum and for cell integrity. *Mol Microbiol* **39**, 1414–1426.
- Nuoffer, C., Horvath, A. & Riezman, H. (1993). Analysis of the sequence requirements for glycosylphosphatidylinositol anchoring of *Saccharomyces cerevisiae* Gas1 protein. *J Biol Chem* **268**, 10558–10563.
- Ohishi, K., Inoue, N., Maeda, Y., Takeda, J., Riezman, H. & Kinoshita, T. (2000). Ggalp and *gpi8p* are components of a glycosylphosphatidylinositol (GPI) transamidase that mediates attachment of GPI to proteins. *Mol Biol Cell* **11**, 1523–1533.
- Orlean, P. (1997). Biogenesis of yeast wall and surface components. In *The Molecular and Cellular Biology of the Yeast Saccharomyces: Cell Cycle and Cell Biology*, pp. 229–362. Edited by J. R. Pringle, J. R. Broach & E. W. Jones. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory.
- Popolo, L. & Vai, M. (1998). Defects in assembly of the extracellular matrix are responsible for altered morphogenesis of a *Candida albicans* *phr1* mutant. *J Bacteriol* **180**, 163–166.
- Popolo, L., Gilardelli, D., Bonfante, P. & Vai, M. (1997). Increase in chitin as an essential response to defects in assembly of cell wall polymers in the *ggp1Δ* mutant of *Saccharomyces cerevisiae*. *J Bacteriol* **179**, 463–469.
- Ram, A. F., Wolters, A., Ten Hoopen, R. & Klis, F. M. (1994). A new approach for isolating cell wall mutants in *Saccharomyces cerevisiae* by screening for hypersensitivity to calcofluor white. *Yeast* **10**, 1019–1030.

- Ram, A. F., Kapteyn, J. C., Montijn, R. C., Caro, L. H., Douwes, J. E., Baginsky, W., Mazur, P., van den Ende, H. & Klis, F. M. (1998). Loss of the plasma membrane-bound protein Gas1p in *Saccharomyces cerevisiae* results in the release of β 1,3-glucan into the medium and induces a compensation mechanism to ensure cell wall integrity. *J Bacteriol* **180**, 1418–1424.
- Richard, M., De Groot, P., Courtin, O., Poulain, D., Klis, F. & Gaillardin, C. (2002a). GPI7 affects cell-wall protein anchorage in *Saccharomyces cerevisiae* and *Candida albicans*. *Microbiology* **148**, 2125–2133.
- Richard, M., Ibata-Ombetta, S., Dromer, F., Bordon-Pallier, F., Jouault, T. & Gaillardin, C. (2002b). Complete glycosylphosphatidylinositol anchors are required in *Candida albicans* for full morphogenesis, virulence and resistance to macrophages. *Mol Microbiol* **44**, 841–853.
- Roberts, W. L., Santikarn, S., Reinhold, V. N. & Rosenberry, T. L. (1988). Structural characterization of the glycoinositol phospholipid membrane anchor of human erythrocyte acetylcholinesterase by fast atom bombardment mass spectrometry. *J Biol Chem* **263**, 18776–18784.
- Sherman, F. (1991). Getting started with yeast. *Methods Enzymol* **194**, 3–21.
- Sikorski, R. S. & Hieter, P. (1989). A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics* **122**, 19–27.
- Smith, T. K., Sharma, D. K., Crossman, A., Dix, A., Brimacombe, J. S. & Ferguson, M. A. (1997). Parasite and mammalian GPI biosynthetic pathways can be distinguished using synthetic substrate analogues. *EMBO J* **16**, 6667–6675.
- Staab, J. F., Bradway, S. D., Fidel, P. L. & Sundstrom, P. (1999). Adhesive and mammalian transglutaminase substrate properties of *Candida albicans* Hwp1. *Science* **283**, 1535–1538.
- Stahl, N., Baldwin, M. A., Hecker, R., Pan, K. M., Burlingame, A. L. & Prusiner, S. B. (1992). Glycosylinositol phospholipid anchors of the scrapie and cellular prion proteins contain sialic acid. *Biochemistry* **31**, 5043–5053.
- Sundstrom, P. (2002). Adhesion in *Candida* spp. *Cell Microbiol* **4**, 461–469.
- Sütterlin, C., Horvath, A., Gerold, P., Schwarz, R. T., Wang, Y., Dreyfuss, M. & Riezman, H. (1997). Identification of a species-specific inhibitor of glycosylphosphatidylinositol synthesis. *EMBO J* **16**, 6374–6383.
- Taron, C. H., Wiedman, J. M., Grimme, S. J. & Orlean, P. (2000). Glycosylphosphatidylinositol biosynthesis defects in Gpi11p- and Gpi13p-deficient yeast suggest a branched pathway and implicate gpi13p in phosphoethanolamine transfer to the third mannose. *Mol Biol Cell* **11**, 1611–1630.
- Taron, B. W., Colussi, P. A., Wiedman, J. M., Orlean, P. & Taron, C. H. (2004). Human Smp3p adds a fourth mannose to yeast and human glycosylphosphatidylinositol precursors *in vivo*. *J Biol Chem* doi: 10.1074/jbc.M405081200.
- Tsukahara, K., Hata, K., Nakamoto, K. & 9 other authors (2003). Medicinal genetics approach towards identifying the molecular target of a novel inhibitor of fungal cell wall assembly. *Mol Microbiol* **48**, 1029–1042.
- Vossen, J. H., Ram, A. F. & Klis, F. M. (1995). Identification of SPT14/CWH6 as the yeast homologue of hPIG-A, a gene involved in the biosynthesis of GPI anchors. *Biochim Biophys Acta* **1243**, 549–551.
- Vossen, J. H., Muller, W. H., Lipke, P. N. & Klis, F. M. (1997). Restrictive glycosylphosphatidylinositol anchor synthesis in *cwh6/gpi3* yeast cells causes aberrant biogenesis of cell wall proteins. *J Bacteriol* **179**, 2202–2209.
- Zaragoza, O., de Virgilio, C., Ponton, J. & Gancedo, C. (2002). Disruption in *Candida albicans* of the TPS2 gene encoding trehalose-6-phosphate phosphatase affects cell integrity and decreases infectivity. *Microbiology* **148**, 1281–1290.