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

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-blast 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, 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.

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.

Glycosylphosphatidylinositol (GPIs) have important roles in cell wall biogenesis in yeast. These glycolipids, which have the conserved core structure H2N-CH2-CH2-PO42-6Manz1,2Manz1,6Manz1,4GlcNz1,6Ins 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-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; Kapeyn 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-fluoro-orotic acid; GPI, glycosylphosphatidylinositol; JBSM, jack bean a-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-glucanosyltransferases 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 GPs 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 (Grime 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, α1,2-linked Man to the third, α1,2-linked Man of the GPI precursor by the Smp3 mannosyltransferase (Grime et al., 2001).

The C. albicans genome encodes a sequence homologue of Smp3p (Grime 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 CaSM3P complements the growth and GPI mannosylation defects in S. cerevisiae and that repression of CaSM3P expression in C. albicans leads to a block in GPI assembly and the accumulation of a Manα1-GPI precursor glycolipid. CaSm3p therefore adds a fourth mannose during assembly of C. albicans GPs, and this activity is critical for growth of C. albicans because repression of CaSM3P 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 CaSm3p 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–PcAAL–GPI13 strains are described by Grime et al. (2001).YPD and SD media were prepared as described by Sherman (1991) and supplemented with 80 μg uridine ml⁻¹ (YPDU or SDU) when required for growth of Cauara3 strains. SMA medium contained 2% (w/v) maltose instead of glucose. Inositol-free medium was prepared as described by Grime 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 μg CFW ml⁻¹ (Sigma-Aldrich).

Cloning of CaSM3P and construction of expression plasmids. A candidate CaSM3P 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 CaSM3P 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 (2μ) (Christiansson et al., 1992; Sikorski & Hieter, 1989) and DNA sequencing of this fragment confirmed that the cloned CaSM3P gene was identical to the candidate CaSM3P sequence in Contig 6–2467.

Disruption of CaSM3P. The CaSM3P gene was subcloned into the BamHI site of the cloning vector pNEB193 (New England Biolabs) and a 1455 nt BsoBI–EcoRV fragment was removed from the resulting plasmid and replaced with a blunt-ended 4.1 kb HindIII–BglII containing the ‘ura-blaster’ cassette from pMB-7 (Fonzi & Irwin, 1993) to create plasmid pΔCaSM3P. The hisG-CaURA3–hisG cassette replaced 97% of the CaSM3P coding region and was flanked by 659 and 645 nt of DNA homologous to chromosomal DNA sequence immediately 5’ and 3’ to CaSM3P, respectively. A 5·5 kb linear disrupting fragment (Fig. 1a) was excised from pΔCaSM3P by digestion with PaeI and SacI, 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 CaSM3P 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 negative 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 CaSM3P locus. A 172 bp DIG-labelled hybridization probe complementary to the 3’ untranslated region of CaSM3P 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 Casmp3p::hisG–CaURA3–hisG/CaSM3P 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 CaSM3P::hisG–CaURA3–hisG locus to...
GGATCCGATAACTGATTCGT
GGATCCAAGAATGGTGAGAG

be amplified were examined by Southern blotting to verify the
were not studied further. Colonies from which this band could not
transform a Casmp3
CaSMP3
copy of CaSMP3
too great to allow product amplification. Colonies from which the
CaSMP3
::

Table 1. Oligonucleotide primers used in this study

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

Casm3:: hisG was verified by Southern blotting (Fig. 1a, c). A second
transformation was carried out using the same Pmel–SaeI disrupting
dNA fragment and two independent heterozygous Casm3:: 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
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*Engineered restriction sites are underlined. Other features are indicated in bold.

Creation of a CaSMP3-repressible strain. A Casm3:: hisG
CaSMP3
strain was constructed in which expression of the remaining
CaSMP3
allele was placed under the control of the maltose-
inducible, glucose-repressible Pmal2 promoter (Backen et al., 2000).
A 536 bp EcoRI–KpnI DNA fragment containing Pmal2 was amplified
by PCR from S. albicans genomic DNA using primers 13 and
14 and cloned into pUCl9. A 3-9 kb HindIII fragment containing
CaUra3
was next inserted in the pUCl9 polynucleot 13 to the
CaSMP3
promoter, yielding plasmid pPmal2-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 pPmal2-Ura3. The resulting plasmid, pPmal2-
Smp3-Ura3, was linearized by digestion with BstZ171, whose unique recognition site is away mid way along
the CaSMP3
homologous DNA. The resulting linear fragment was used to transform a
Casm3:: hisG/CaSMP3
strain to uracil prototrophy
and transformants were screened by whole-cell PCR for integration of
pPmal2 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
pPmal2-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).

[^H]inositol labelling of lipids and GPI glycan headgroup analysis. Radiolabelling of S. cerevisiae
strains with [^H]inositol was carried out as described previously (Grimme et al., 2001). For
[^H]inositol labelling of C. albicans strains, exponentially growing
cells cultured in SMal were harvested and resuspended at 0:01
OD500 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 OD500 of approximately
1-0. Ten OD500 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 µCi (555 kBq) [^H]inositol, after which
radiolabelling was terminated by addition of NaN3 to a final concen-
tration of 10 mM. Lipids were then extracted as described by Taron
et al. (2000), separated by TLC on Silica Gel 60 plates (Alltech)
using chlorform/methanol/water (4:4:1 or 5:5:1, by vol.) as
solvent, and ^H-labelled lipids were detected by fluorography
using Biomax MS film and a Biomax TransScreen-LE intensifying
screen (Eastman-Kodak). Treatments of 3H-labelled lipids with
exo-glycosidase treatment and high performance TLC were performed
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using Biomax MS film and a Biomax TransScreen-LE intensifying
screen (Eastman-Kodak).

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 5×10^8 cells were harvested, washed once with
1 ml ice-cold PBS, resuspended in 100 µl PBS and stained with
propidium iodide (5 µg ml−1) and Hoechst 33342 (25 µg 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.

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

We next investigated whether \textit{CaSMP3} expression corrects the biochemical defect in \textit{S. cerevisiae} \textit{smp3} cells. The \textit{smp3} mutant accumulates trimannosyl GPI precursors (Man\textsubscript{3}-GPIs) that can be detected by radiolabelling with \textsuperscript{[3]H}\textit{H}inositol (Grimme et al., 2001). Accumulation of this lipid is greatly reduced in \textit{smp3} cells expressing \textit{CaSMP3} (Fig. 3a, lanes 5 and 6), consistent with almost complete alleviation of the biosynthetic defect in \textit{smp3}. To show that \textit{CaSMP3} encodes a protein that is capable of mannosylating Man\textsubscript{4}-GPIs, we conducted an \textit{in vivo} mannosyltransferase assay using the \textit{S. cerevisiae} \textit{smp3}/\textit{gpi13} double mutant as illustrated in Fig. 3(b). When expression of GPI13 is repressed in this strain at 25°C, it accumulates a Man\textsubscript{4}-GPI (Fig. 3c, lane 1), but at 37°C, when the \textit{smp3} block is superimposed in the double mutant, only the upstream Man\textsubscript{3}-GPI is formed (Fig. 3c, lane 2). We therefore tested whether expression of \textit{CaSMP3} from a plasmid in the \textit{smp3}/\textit{gpi13} double mutant would overcome the \textit{smp3} block at 37°C and restore Man\textsubscript{4}-GPI formation. This was indeed the case: \textit{smp3}/\textit{gpi13} cells harbouring a 2\mu plasmid containing \textit{CaSMP3} now accumulated a \textsuperscript{[3]H}\textit{H}inositol-labelled lipid that had the same TLC mobility as the Man\textsubscript{4}-GPI bearing one phosphoethanolamine (EthN-P) moiety that accumulates in the \textit{gpi13p}-depleted strain (Fig. 3c, lanes 3 and 4). Formation of this GPI is strong evidence that \textit{CaSMP3} encodes a protein capable of mannosylating a trimannosyl GPI \textit{in vivo}. Taken together, these results show that CaSmp3p is the functional equivalent of ScSmp3p.

**Fig. 1.** Modifications introduced into the \textit{CaSMP3} chromosomal locus. (a) Strategy for disruption of \textit{CaSMP3}. Shown diagrammatically, from top to bottom, are the chromosomal \textit{CaSMP3} locus, the predicted organization of the \textit{CaSMP3} locus after integration of the disrupting \textit{ura-blast} cassett and the \textit{hisG}-disrupted \textit{CaSMP3} locus remaining after recombination between the \textit{hisG} repeats and excision of \textit{Casmp3}. (b) Strategy for placing \textit{CaSMP3} under the control of the \textit{Casmp3} promoter (M2p). Shown diagrammatically, from top to bottom, are plasmid P\textsubscript{MAL2}/UR13-linearized by digestion with \textit{Bst}I, the chromosomal \textit{CaSMP3} locus and the predicted organization of the \textit{CaSMP3} locus after integration of P\textsubscript{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, HindIII site; B, BamHI site. (c) Southern blot analysis of \textit{C. albicans} genomic DNA digested with either HindIII (lanes 1–4) or BamHI (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), \textit{PI} (lanes 1 and 6), heterozygous \textit{Casmp3}::\textit{hisG} (lanes 2 and 7), \textit{Casmp3}::\textit{hisG}P\textsubscript{MAL2}::\textit{CaSMP3} (lanes 3 and 8) and a triploid \textit{Casmp3}::\textit{hisG}P\textsubscript{MAL2}::\textit{CaSMP3}/\textit{CaSMP3} strain (lanes 4 and 9). Digestion with HindIII yields diagnostic fragments of 6·1 (\textit{CaSMP3}), 1·4 (\textit{Casmp3}::\textit{hisG}) and 4·8 kb (P\textsubscript{MAL2}::\textit{CaSMP3}), and digestion with BamHI yields diagnostic fragments of 5·2 (\textit{CaSMP3}), 3·9 (\textit{Casmp3}::\textit{hisG}) and 10·3 kb (P\textsubscript{MAL2}::\textit{CaSMP3}).
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 ΔCaSMP3 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 ΔCaSMP3 strain is not possible because 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 BstZ17I,
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 PMAL2. Transformants containing the PMAL2-CaSMP3 fusion were initially identified by whole-cell PCR tests, and Southern blot analysis confirmed the correct integration of the PMAL2-CaSMP3 fusion fragment and the absence of a wild-type CaSMP3 allele (Fig. 1c, lanes 3 and 8). A representative triploid Casmp3::hisG/PMAL2-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/PMAL2-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/PMAL2-CaSMP3 grown in synthetic medium
containing 2% (w/v) maltose were harvested and diluted to 0.01 OD$_{600}$ 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 μCi (555 kBq) $[^3]$H]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 μg 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 $[^3]$H]inositol-labelled lipid (Fig. 4, lane 7) that had a TLC mobility similar to that of the trimannosyl lipid 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 $[^3]$H]inositol-labelled GPI lipids (Fig. 4, lane 8). Neither the wild-type CAI-4 strain, nor the smp3::hisGI/SMP3 heterozygote accumulated any aberrant lipids (lanes 1–4). The accumulation of a $[^3]$H]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 $[^3]$H]inositol-labelled lipid was isolated by preparative TLC and its 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 $[^3]$H-labelled neutral glycan headgroups were treated with non-specific jack bean α-mannosidase (1BzM) or α,1,2-specific mannosidase. The sizes of the undigested and α-mannosidase-treated glycans were determined by separation using high performance TLC and comparing their mobilities to those of GlcNAc-Ins (Fig. 5b, lanes 3 and 5). Treatment with 1BzM changed its mobility to that of GlcNAc-Ins (lane 4), whereas 1,2-α-mannosidase digestion generated Man$_2$-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 Man$_2$-GPIs with EthN-P on either Man-1 or Man-2.
Therefore, we investigated the presence of phosphodiester-linked substituents on the accumulating \textit{C. albicans} Man$_3$-GPI. To do this, a sample of deacylated neutral glycan was incubated first with JBzM, 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 JBzM. Only trace amounts of Man$_2$-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 of P$_{MAL2}$ and the possibility that only low levels of CaSmp3 protein are needed to sustain viability. Leaky expression of \textit{S. cerevisiae} Sm3p was also observed when its expression from the \textit{GAL10} promoter was repressed (Grimme et al., 2001). Growth under repressing conditions was also observed for the essential \textit{CaCHS1} gene when the only functional copy was expressed behind the \textit{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 \textit{S. cerevisiae} gpi1 and gpi2...
Fig. 6. Shift of Casmp3::hisG/P\textsubscript{MAL2}-CaSMP3 cells to glucose leads to growth defects. (a) Aggregation and propidium iodide staining of Casmp3::hisG/P\textsubscript{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 \(\mu\)m. (b) Depletion of CaSmp3p results in morphological defects. Repressed Casmp3::hisG/P\textsubscript{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 \(\mu\)m.
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\textsubscript{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\textsubscript{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\textsuperscript{-1} (row 3 in Fig. 7a and b). In contrast, Casmp3::hisG/P\textsubscript{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 α1,2-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 Manα1,2Manα2Man-GlcN-Ins modified with an HF-labile substituent on Man-1. The accumulation of a Man\textsubscript{4}-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 CaSMP3, 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 GPs is important for subsequent cross-linkage to cell wall constituents (Richard et al., 2002a). The result of our EthN-P-positioning experiment on the Man\textsubscript{4}-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 Man\textsubscript{4}-GPI isoform bearing EthN-P on Man-1, and a Man\textsubscript{4}-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 Man$_3$-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 Man$_4$-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 Man$_3$-GPIs (Benghezal et al., 1995) and that deletion of the fourth GPI mannosyltransferase gene, SMP3, is lethal in S. cerevisiae and C. albicans. Sm3p-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 Man$_4$-GPIs to protein has so far not been obvious from studies of GPI precursor assembly in cultured mammalian cell lines: such analyses readily detect Man$_3$-GPIs, but formation of Man$_4$-GPIs is at best rare (Hirote et al., 1992; Hong et al., 2000). Tellingly too, mutant mammalian cell lines defective in transfer of GPIs to protein accumulate Man$_4$-GPIs, not Man$_3$-GPIs (Mohney et al., 1994; Ohishi et al., 2000), and, moreover, these Man$_4$-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 Man$_4$-GPIs suggests that a fourth GPI mannosyltransferase may nonetheless occur in mammals, and a functional human Sm3p 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 Man$_4$-GPIs is mandatory in fungi but not an obligatory step in mammals highlights fungal Sm3 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 to chart the C. albicans GPI assembly pathway, as well as probe the phenotypic consequences of blocking GPI anchoring in this human pathogenic fungus.

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