The *Candida albicans* homologue of PIG-P, CaGpi19p: gene dosage and role in growth and filamentation

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Glycosylphosphatidylinositol (GPI)-anchored proteins in *Candida albicans* are responsible for a vast range of functions, and deletions in certain GPI-anchored proteins severely reduce adhesion and virulence of this organism. In addition, completely modified GPIs are necessary for virulence. GPI anchor biosynthesis is essential for viability and starts with the transfer of N-acetylglucosamine to phosphatidylinositol. This step is catalysed by a multi-subunit complex, GPI–N-acetylglucosaminyltransferase (GPI–GnT). In this, the first report to our knowledge on a subunit of the *Candida* GPI–GnT complex, we show that CaGpi19p is the functional equivalent of the *Saccharomyces cerevisiae* Gpi19p. An N-terminal truncation mutant of CaGpi19p functionally complements a conditionally lethal *S. cerevisiae* gpi19 mutant. Further, we constructed a conditional null mutant of *CaGPI19* by disrupting one allele and placing the remaining copy under the control of the MET3 promoter. Repression leads to growth defects, cell wall biogenesis aberrations, azole sensitivity and hyperfilamentation. In addition, there is a noticeable gene dosage effect, with the heterozygote also displaying intermediate degrees of most phenotypes. The mutants also displayed a reduced susceptibility to the antifungal agent amphotericin B. Collectively, the results suggest that CaGPI19 is required for normal morphology and cell wall architecture.

INTRODUCTION

Glycosylphosphatidylinositol (GPI) anchoring of proteins to the cell surface is a ubiquitous phenomenon in eukaryotes. GPI-anchored proteins span a vast range of functions, including signalling, cell wall biogenesis, virulence, adhesion and numerous enzymic activities. GPI anchor biosynthesis is essential for viability in yeast (Newman *et al.*, 2005) and for pathogenesis by various organisms such as *Trypanosoma* (Nagamune *et al.*, 2000). Though the pathway is not essential for viability in adult humans, mutations in the catalytic subunit, PIG-A, of the first complex of the biosynthetic pathway have been shown to be responsible for paroxysmal nocturnal haemoglobinuria (Kinoshita *et al.*, 1997). In addition, PIG-P, another subunit of this complex, has been implicated in the pathophysiology of Down’s syndrome patients (Choi *et al.*, 2001; Ferrando-Miguel *et al.*, 2004).

In *Candida albicans*, GPI-anchored proteins have been identified that are involved in adhesion (the ALS family of proteins and Hwp1), in cell wall biogenesis (Ecm33p) and in protection against host defences (SOD) (Richard & Plaine, 2007). Not surprisingly, therefore, disruptions in these genes, for example in Ecm33p, have been shown to cause reduced virulence (Martinez-Lopez *et al.*, 2004, 2006). Given that deletions in an anchored protein can cause such drastic effects, it is reasonable to expect that deletions in the GPI biosynthetic pathway itself will be fatal and may represent a source of fresh drug targets in an organism notorious for its multidrug resistance.

GPI biosynthesis takes place in the endoplasmic reticulum where the core structure of the GPI is assembled and proteins destined for GPI anchoring are attached to the complex GPI moiety via a transamidation reaction. GPI biosynthesis begins with the transfer of N-acetylgalactosamine (GlcNAc) from UDP–GlcNAc to phosphatidylinositol (PI). This step is catalysed in humans by a complex of seven proteins: PIG-A, PIG-P, PIG-C, PIG-H, PIG-Q, PIG-Y and DPM2 (Watanabe *et al.*, 1996, 1998, 2000; Murakami *et al.*, 2005), collectively referred to as the GPI–GnT complex. Homologues of all proteins, except DPM2, have also been identified in members of the genus *Saccharomyces* (Vossen *et al.*, 1995; Leidich *et al.*, 1995; Leidich & Orlean, 1996, 1999; Yan *et al.*, 2001). In *C. albicans*, very few studies have been carried out on the GPI anchor biosynthetic pathway itself and these have been restricted to genes further downstream in the pathway, for example...
CaSMP3 and Candida GPI7/LAS2 (Grimme et al., 2004; Richard et al., 2002). To our knowledge, no study to date has focused on the C. albicans GPI–GnT complex. The major difficulty with studying components of the first complex of the pathway in Candida is that intact microsomes from this organism appear to be inactive, unlike those from yeast and human cell lines, removing any chance of a direct assay. However, studies on CaSmp3p and Gpi7p have shown that loss of these proteins causes reduced growth (Grimme et al., 2004) and virulence, as measured by the inability of the mutants to form hyphae, and their reduced virulence in mice (Richard et al., 2002).

This paper focuses on one subunit of the Candida GPl–GnT complex, CaGPI19. As mentioned earlier, PIG-P in humans has been implicated in Down’s syndrome (Ferrando-Miguel et al., 2004). In yeast, this protein (Gpi19p) has been shown to be essential for viability (Newman et al., 2005). In mammalian cell lines, it is required for formation and activity of the GPI–GnT complex (Watanabe et al., 2000) where it interacts specifically with PIG-A and, less strongly, with hGPI1 (PIG-Q). Yeast Gpi19p interacts with Gpi2p, the homologue of PIG-C (Newman et al., 2005). While there is no study of interactions between subunits in Aspergillus nidulans, a recent study on a conditional pigP mutant of this organism showed subtle phenotypic variations compared with the yeast gpi19 mutant (Pilsyk & Paszewski, 2009), indicating that perhaps the arrangement of the complex may vary between organisms. The Candida homologue was of interest, as it is almost twice the size of either of its counterparts. It contains an N-terminal domain of 150 residues that is entirely missing in either PIG-P or Gpi19p and quite possibly has different roles or interactions compared with its orthologues.

We show that an N-terminal truncation mutant of CaGpi19 is able to functionally complement a conditional gpi19 mutant and describe the effect of disruption of this gene on the growth, drug resistance and virulence of C. albicans. As C. albicans undergoes a morphogenetic change to the invasive hyphal form in pathogenic mode, we also studied the effect of deletion of this protein on the morphogenetic ability of this organism. Further, we show that there is a distinct dosage-dependent effect on the phenotypes associated with its depletion.

**METHODS**

**Strains.** S. cerevisiae strain YPH500 was used to construct a conditionally lethal gpi19 strain. All C. albicans strains were derived from BWP17 (Wilson et al., 1999).

**Construction of a conditionally lethal YPH–GAL1–GPI19 strain.** We constructed a vector, pTZ-URA3-GAL1, containing the selection marker URA3 and the tightly regulated GAL1 promoter; this is tightly repressed by glucose and induced by galactose. The URA3–GAL1 fragment was amplified by using primers GPI19-GAL1 F.P and GPI19-GAL1 R.P (all primers are listed in Supplementary Table S1, available with the online version of this paper), which have 50 bp overhangs homologous to the region −250 to −200 upstream of GPI19 and to the start of the gene (+1 to +50). The resultant PCR amplicon was transformed directly into S. cerevisiae YPH500 and plated on minimal medium containing 4% galactose and 1.5% sucrose. Positive colonies were confirmed by PCR using primers that flanked the GPI19 ORF. Colonies positive for the successful integration of the GAL1 promoter at the GPI19 locus were then streaked onto glucose plates to confirm essentiality.

**Complementation of the YPH–GAL1–GPI19 strain.** Two constructs were used for complementation: YEp-Gp19, which contains the full-length CaGPI19 gene cloned (using primers FL YEPHIS F.P and FL YEPHIS R.P) between the BamHI and MluI sites of the vector YEpHIS (Figler et al., 2000); and YEp-AN-CaGpi19, which contains residues 152–298 of CaGPI19 and lacks the first 151 N-terminal residues (PCR used primers DELTA YEPHIS and FL YEPHIS R.P.). Plasmids were purified and used to transform YPH–GAL1–GPI19 using the lithium acetate method. These cells were plated on minimal media plates containing 4% galactose and 1.5% sucrose, lacking uracil and leucine. To determine whether the clones could rescue viability, colonies were streaked onto plates containing 2% glucose and incubated at 30 °C.

**Preparation of microsomes and GPI–GnT assay.** Yeast strain YPH–GAL1–GPI19, transformed with either YEpHIS or YEp-AN-CaGpi19, was grown overnight in galactose, then inoculated into 250 ml cultures and grown in minimal medium containing either 4% galactose or 2% glucose at 30 °C, at 220 r.p.m. until OD500 of 2.0. Microsomes were prepared and GPI–GnT activity was assayed for each of them as described by Sobering et al. (2004) and references therein.

**RT-PCR detection of expression of CaGPI19.** Overnight cultures of BWP17 were grown in YEPD (2% glucose, 2% peptone and 1% yeast extract) to obtain yeast cells, and on 20% serum or Spider until colonies were hyphal. Hyphal cells were scraped off the plate and collected in a microcentrifuge tube; media was removed from the yeast pellet. Tri Reagent (Sigma Aldrich; 1 ml (0.3 g cells)−1) was added and glass beads were added to half the volume. Cells were lysed by breaking with glass beads for 10 cycles of vortexing (1 min with 1 min on ice). RNA was then isolated by following the manufacturer’s instructions. About 3–5 μg RNA was used for conversion to cDNA using the CaGPI19-specific reverse primer P2. CaGPI19 cDNA was then amplified by PCR using primers P1 and P2.

**Construction of CaGPI19 deletion strains.** One allele of the CaGPI19 gene was knocked out by using a PCR-mediated approach. Briefly, the HIS1 marker was amplified from SC5314, cloned into pTZ5TR/T (Fermentas) and amplified with flanking primers (HD F.P and HD R.P) containing 70 nt homologous to the start and end of the gene. BWP17 was transformed with this amplification product using the lithium acetate method; transformants were selected on synthetic defined (SD) medium [2% glucose, 0.67% yeast nitrogen base and 0.2% amino acid mix with the relevant amino acids (uracil and/or leucine) left out for the appropriate experiments] lacking histidine. The conditional null strain was constructed from the heterozygote using the pMET3-GFP-URA3 construct described by Gerami-Nejad et al. (2004).

**Growth curves.** Growth curves were obtained for the mutants compared with BWP17. For the conditional null strain, cells were grown overnight to saturation, diluted to OD600 0.1 in 50 ml SD medium either with (repressive condition) or without (permissive) 5 mM Met/Cys and grown at 30 °C. OD500 was recorded every 2 h till growth levelled off. The heterozygote growth was similarly studied in YEPD supplemented with 50 μg uridine ml−1 using three independent colonies (P1.1, P1.2 and P1.3).
Hyphal induction. For hyphal development on solid media, cells from an overnight culture were diluted to OD<sub>600</sub> 0.1 in 0.9 % saline. Dilutions were spotted onto Spider medium (1 % nutrient broth, 1 % mannitol, 0.2 % K<sub>2</sub>HPO<sub>4</sub>), or SD media containing 10 % serum and grown for 5–10 days at 37 °C. To study invasive growth, colonies were washed from the surface of the agar or gently scraped off with a sterile blade prior to washing. Invasive growth was then photographed. Liquid cultures were grown in 10 % serum for 2 h before examination under a microscope.

Effect of cell wall perturbants and antifungal drugs. Cells (1 × 10<sup>6</sup>) from an overnight saturated culture were inoculated into a fresh secondary culture and grown shaking at 220 r.p.m. at 30 °C for 5 h. A fivefold dilution series was used. Starting at OD<sub>600</sub> 0.1 (1 × 10<sup>7</sup> cells ml<sup>-1</sup>), 5 μl of each dilution was spotted onto SD Met/Cys plates containing 20 μg calcicolour white (CFW) ml<sup>-1</sup> or 0.01 % SDS. The same procedure was followed for SD Met/Cys plates containing 10 μg amphotericin B ml<sup>-1</sup>, 3 μg fluconazole ml<sup>-1</sup>, 0.4 μg miconazole ml<sup>-1</sup> and 0.04 μg ketoconazole ml<sup>-1</sup>. The three independent heterozygotes were individually screened to ensure that the behaviour was similar. Results show data for P1.1 as a representative of the heterozygotes.

For simple staining, mid-exponential-phase cultures were resuspended in PBS, pH 7.0, at OD<sub>600</sub> 0.2 and incubated in the dark for 30 min with 100 μg CFW ml<sup>-1</sup>. Cells were washed with PBS and observed under a confocal microscope (Olympus FV1000) using a 405 nm laser.

GPI anchor levels. Overnight cultures of strains were washed in PBS and diluted to OD<sub>600</sub> 0.2 in PBS. Cells were incubated with 20 nM FLAER (Pinewood Scientific Services) for 30 min on ice, washed with PBS, resuspended in 50 % glycerol and visualized using confocal microscopy or an Olympus IX71 fluorescence microscope with an FITC filter.

Estimation of ergosterol. The percentage of ergosterol was determined as described by Arthington-Skaggs et al. (1999).

**RESULTS**

**CaGPI19 is able to functionally complement a conditionally lethal gpi19 mutant**

CaGpi19p was identified by BLASTp searches against the human PIG-P as well as the *Saccharomyces* Gpi19p. The *Candida* protein has 35 % identity to Gpi19p. Most of the conserved residues are in the predicted transmembrane regions with only a few scattered in the cytosolic C terminus. It also contains 151 extra residues at its N terminus that are absent in the yeast and human orthologues. GPI19 is an essential gene in yeast (Newman et al., 2005). To determine whether CaGPI19 was the functional equivalent of the yeast gene, we constructed the conditionally lethal YPH-GAL1-GPI19 strain in which the native promoter of GPI19 is replaced with that of GAL1 (Fig. 1a). This strain grows normally in the presence of galactose. When shifted to medium containing glucose, the GAL1 promoter is tightly repressed and the strain was unable to grow (Fig. 1b). We transformed this strain with YEp-CaGpi19 containing the full-length CaGPI19 gene and YEp-AN-CaGpi19 encoding the C-terminal portion of the protein. While CaGpi19p was unable to complement the growth defect, the deletion mutant AN-CaGpi19p was able

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**Fig. 1.** (a) Construction of the YPH-GAL1-GPI19 conditionally lethal strain by PCR amplification of the wild-type YPH500 strain with primers flanking the up- and downstream regions of the GPI19 ORF, which amplified a fragment of 1.2 kb. After integration of the GAL1 promoter, the same PCR amplified a band of 2.6 kb. (b) YPH-GAL1-GPI19 (top) is able to grow in medium containing galactose but is inviable when shifted to media containing 2 % glucose. The bottom strain is YPH500. (c) The rescue of viability by AN-CaGpi19 (i) on glucose plates. Neither the vector alone (YEpHIS; ii) or the full-length CaGPI19 clone (YEp-Gpi19; iii) were able to rescue the growth defect. (d) GPI–GnT assay to monitor the activity of the complex. Microsomes isolated from YPH-GAL1-GPI19 strain grown in galactose (red) showed maximum activity. Microsomes containing the YEpHIS vector and grown in glucose (blue) were barely able to catalyse the transfer. Membranes from YPH-GAL1-GPI19 containing AN-GPI19 and grown in glucose (black) were active, though considerably less so than those containing Gpi19p from cells grown in permissive conditions. Radioactive lipids were detected using the Bioscan AR 2000.
to rescue the growth defect on glucose plates (Fig. 1c). Growth of the complemented strains in glucose was much slower than in galactose, probably due to low sequence homology between the yeast and Candida orthologues. However, given that ΔN-CaGpi19p is able to restore growth under permissive conditions, we conclude that it is the functional homologue of Gpi19p.

Mixed membranes from YPH-GAL1-GPI19 transformed with YEp-ΔN-CaGpi19 were assayed for GPI–GnT activity. As a control, membranes from YPH-GAL1-GPI19 transformed with the YEpHS vector were tested. Membranes from the strain containing YEp-ΔN-CaGpi19 that were grown in glucose were capable of GlcNAc transfer to PI while those containing the vector alone were barely active (Fig. 1d). However, microsomes from the strain grown in galactose (in which Gpi19p was present) had significantly better activity than those containing YEp-ΔN-CaGpi19. This is consistent with slower growth on glucose plates, suggesting that while CaGpi19p is the functional equivalent of Gpi19p it has reduced functionality compared with its Saccharomyces homologue.

Deletions in CaGPI19 lead to modest growth defects

CaGPI19 is expressed in both yeast and hyphal forms of Candida, as determined by reverse transcription PCR (Fig. 2a). To investigate whether the gene was essential, we constructed a conditional null strain wherein one copy of the gene was disrupted and the second was under the control of the MET3 promoter. The MET3 promoter has been widely used and is reported to be repressed up to 85-fold by methionine and/or cysteine (Care et al., 1999). Construction of the strains was verified by using PCR (Fig. 2b, c).

The heterozygote (Cagpi19/CaGPI19) and conditional null (Cagpi19/MET3-GFP-CaGPI19) were tested for growth defects. Gene dosage effects have been reported for many Candida genes (Köhler & Fink, 1996; Martinez-Lopez et al., 2004) and haploinsufficiency in yeast is well documented (Deutschbauer et al., 2005). In repressive conditions, the conditional null showed a modest decrease in growth (doubling time of 159 min) compared with BWP17 (132 min) in liquid minimal media (Fig. 3a), indicating that the repression of the gene was the cause of the slow growth. Even under permissive conditions, however, there is a small growth defect (140 min). This is indicative of a gene dosage effect (Deutschbauer et al., 2005). Growth on solid media showed similar results (Fig. 3b). We detected expression of GFP–CaGpi19p by immunofluorescence, even with 10 mM Met/Cys, suggesting a basal level of protein expression (Supplementary Fig. S1, available with the online version of this paper). Further, the repression lifts with time; after 24 h, growth of the mutant caught up with the control, probably due to the expenditure of Met/Cys in the medium, and though the effect was still clear it was not as prominent as in the early stages of growth. We also noticed a tendency of the conditional null to clump and aggregate (Fig. 3d). The extent of clumping is less than that reported for CaSmp3p (Grimme et al., 2004).

Given the slower growth in the conditional null in permissive media (in which it must behave like a heterozygote), we expected a similar growth defect in the heterozygote, Cagpi19/CaGPI19. However, the growth defect in YEPD was minimal, with a doubling time of 120 min compared with 115 min for the wild-type (Fig. 3c). The growth curve was verified using three independent colonies to ensure that this slight growth defect was not an experimental error. The difference in growth from the parent strain may be due to differences in the strength of the natural promoter versus the weaker MET3 promoter. Alternatively, the conditional null expresses a GFP-tagged form of CaGpi19p that may be less efficient compared with the untagged CaGpi19p expressed in the heterozygote and parent.

Surface GPI anchor levels are reduced on depletion of CaGpi19p

FLAER, an inactive form of aerolysin tagged with Alexa 488, selectively binds to GPI-anchored proteins on the cell
surface (Brodsky et al., 2000). On incubating the strains with FLAER, we observed bright green clusters of GPI-anchored proteins on the surface (Fig. 4a). Similar granular appearance of FLAER-bound GPI anchors on cell surfaces has been reported in other systems as well (Vats et al., 2005). Both the heterozygote (Cagpi19/CaGPI19) and the conditional null mutant (Cagpi19/MET3-GFP-CaGPI19) showed a decrease in detectable GPI-anchored proteins when grown in minimal medium containing Met/Cys, indicating that decreasing levels of CaGPI19 had a deleterious effect on GPI anchor production (Fig. 4a, b).

CaGPI19 deletion leads to alterations in the cell wall

In Candida, as well as in yeast, cell wall biogenesis and architecture depend on a number of GPI-anchored proteins (Martinez-Lopez et al., 2006). Hence, we tested the effect of disruption of CaGPI19 on the cell wall integrity. A gene dosage effect was noticeable. Cell wall architecture was affected; the heterozygote and conditional null showed resistance to SDS (Fig. 5a). This could be due to either an increase in chitin, as reported for other GPI and cell wall mutants (Grimme et al., 2004), or a change in composition of the membrane. For the heterozygote, three independent colonies were tested. Staining of exponential phase cells with CFW showed that the distribution of chitin appears to have changed in Cagpi19/CaGPI19 as well as in Cagpi19/MET3-GFP-CaGPI19 (Fig. 5b). The parent strain shows a tendency to concentrate chitin at the septa, whereas the mutants show a uniform distribution over the cell surface. The conditional null displayed this difference even in media lacking Met/Cys. Mean fluorescence intensities suggest a modest increase in CFW for the mutants (Fig. 5c). Unexpectedly, however, we observed no difference in behaviour of the mutants in comparison with the control on plates containing CFW (Fig. 5d). Cell wall defects usually cause hypersensitivity to CFW (Ram et al., 1994). Nonetheless, taking the staining data in conjunction with the behaviour of the mutants on SDS plates, we conclude that disruption of CaGPI19 leads to aberrations in cell wall biogenesis.
Repression of CaGPI19 leads to increased filamentation

The conditional null showed a dramatic increase in hyphae formation and displayed hyperfilamentation on 10% serum as well as Spider medium (Fig. 6). The heterozygote displayed increased filamentation and invasiveness to an intermediate degree between the parent strain and the null mutant.

Equal numbers of cells from all three strains were spotted on 10% serum in either SD medium containing Met/Cys or YEPD and grown for up to a week. The conditional null displayed an early change of morphology, responding faster to serum (hyphae appeared in 3 days) than the heterozygote, which in turn developed hyphae faster (~5 days) and showed more hyphae and invasiveness than the control, BWP17 (~6 days). Similar results were observed following growth on Spider medium. Faster development of hyphae occurred even in liquid medium (data not shown) with both mutants developing hyphae after just 1 h, while BWP17 took longer (~2.5 h).

Deletions in CaGPI19 affect drug resistance

We tested the disruptants for sensitivity to several common antifungals used in treatment of candidiasis to determine how far-reaching the effects of depleting CaGPI19p were. With the exception of amphotericin B, we found no appreciable difference in the response of the heterozygote to any of the other drugs compared with the wild-type strain on solid media (Fig. 7). When grown in the presence of amphotericin B, we noticed a definite increase in resistance of the heterozygote compared with BWP17. The Cagpi19/MET3-GFP-CaGPI19 strain, however, displayed greater sensitivity to azoles in addition to resistance to amphotericin B (Fig. 7).

DISCUSSION

Our results indicate that CaGPI19 is the functional equivalent of GPI19. The deletion mutant of the CaGPI19 gene restores viability of the conditionally lethal gpi19 strain and partially restores GPI–GnT activity in membranes depleted of Gpi19p. The inability of the full-length protein to rescue growth suggests that the extra 151 N-terminal residues interfere with formation of the yeast GPI–GnT complex. Sequencing of the amplified CaGPI19 gene confirms that this sequence is in-frame with the C-terminal half. Growth of the complemented strains containing D\(_N\)-CaGpi19p on glucose plates was slow, probably due to the reduced GPI–GnT activity of membranes containing D\(_N\)-CaGpi19p in comparison with those containing Gpi19p. This is most likely due to the low homology between the two. The C-terminal cytosolic domain is, presumably, where interactions take place, since the first 60 residues of Saccharomyces Gpi19p are reported to be non-essential for GPI–GnT activity (Newman et al., 2005). Sequence divergence at residues involved in mediating interactions between proteins in the complex may cause reduced functionality. It may also reflect differences in architecture of the GPI–GnT complex in C. albicans compared with yeast.

The depletion of CaGpi19p leads to slowing of growth. Though the growth defect is not very pronounced in the heterozygote, it can be considered to be significant. Similar results have been reported by Deutschbauer et al. (2005) for heterozygous deletions in yeast. In the conditional null, the persistence of growth under repressive conditions may be attributed to the fact that the low levels of CaGpi19p are enough to keep the cell alive. This has been reported for CaSmp3p as well (Grimme et al., 2004). Similar to CaSmp3p, the CaGpi19p conditional null displays a tendency to clump. We detected no significant increase in propidium iodide staining (data not shown), suggesting that the levels of CaGpi19p observed under repression are enough to maintain viability. Thus, while depletion of CaGpi19p does affect...
growth and morphology, the extent of the defect does not allow us to conclusively state that it is essential.

There is a definite drop in levels of GPI anchors in the heterozygote as well as in the CaGpi19p-depleted Cagpi19/MET3-GFP-CaGPI19 strain, but production is not completely abolished. The spots appear to be membrane-localized and may represent clusters of GPI-anchored proteins in the plasma membrane (Supplementary Fig. S2, available with the online version of this paper). Thus,
depletion of CaGpi19p may either destabilize the GPI–GnT complex and/or cause a reduction in the extent of complex formation; either situation would lead to diminishment of the transferase activity. In the absence of a direct assay, though, it is unclear if CaGpi19p is essential for the formation of the complex itself in C. albicans, although such a model has been proposed in yeast and mammalian systems (Newman et al., 2005; Watanabe et al., 2000).

There is a perceptible gene dosage effect on depletion of the protein. Heterozygous strains show noticeable changes in the cell wall and morphogenetic ability. This dosage-dependency appears to be significant; expression of PIG-P has also been shown to be upregulated twofold in the fetal cortex brain of individuals with Down’s syndrome (Ferrando-Miguel et al., 2004). It is the only Down’s syndrome critical region gene shown to be expressed in developing mouse tongues, prompting the speculation that its overexpression causes malformation (Choi et al., 2001). A recent paper on involvement of PIG-P in embryonic signalling and development reported that overexpression as well as depletion of this protein caused the same defects (Shao et al., 2009). Therefore, any disturbances in gene copy number of this protein appear to be detrimental to normal functioning of the cell. Given the subtle variations in phenotypes, the role of PIG-P in various organisms and perhaps even its stoichiometry in the GPI–GnT complex may differ and could be important for normal growth and functioning of the organism.

CFW staining clearly hints at a difference in cell wall biogenesis. The pattern of deposition as well as the amount of chitin in the cell wall has changed. Surprisingly, on solid medium, we were unable to detect any sensitivity to CFW at all the concentrations tested (up to lethal concentrations). This is quite unlike the deletion of CasMP3 and GP17, which, though further downstream in the GPI biosynthetic pathway, show a marked sensitivity to CFW (Grimme et al., 2004; Richard et al., 2002). The reason for this difference is not clear, though it may be noted that deletions of different GPI-anchored proteins did not have the same effect on cell wall chitin (Plaine et al., 2008). In the A. nidulans pigP conditional null, there was also no sensitivity to any of the cell wall perturbing agents used (Pilsyk & Paszewski, 2009). The complexity of interactions and the different types of GPI-anchored proteins involved in cell wall architecture may lead to different rescue pathways.

The differences in behaviour of Casmp3p and CaGpi17p may also be due to CaGpi19p being involved in additional regulatory contacts. The human GPI–GnT complex is regulated by DPM2, a subunit of the dolichol phosphate mannos synthase enzyme. This subunit does not appear to be present in C. albicans (Oswal et al., 2008) although DPM1 and DPM3 are. The possibility exists that one of these proteins may have taken over the function of DPM2. Thus, we cannot rule out the prospect of regulatory interactions with other glycosylation pathways. That GPI–GnT proteins are involved in regulating other signalling events has been established. For example, PIG-P is necessary for correct localization of Wnt signalling receptors during mammalian embryogenesis (Shao et al., 2009).

The increased ability to make yeast–hyphal transitions in our mutants seems to confirm a general role for Gpi19 in morphogenesis. The A. nidulans mutant for the same gene also displays abnormal hyphae (Pilsyk & Paszewski, 2009). Temperature-sensitive gpi19 mutants show increased ability

**Fig. 7.** Effect of CaGPI19 deletion on drug sensitivities. Fresh cultures from overnight saturated cultures were spotted on SD Met/Cys plates (as described in Methods) containing 10 μg amphotericin B ml⁻¹, 3 μg fluconazole ml⁻¹, 0.4 μg miconazole ml⁻¹ and 0.04 μg ketoconazole ml⁻¹. The single allele deletion of CaGPI19 results in resistance to amphotericin B. Further repression of the expression of CaGpi19p results in sensitivity to azoles.
to invade agar and show filamentous growth (Newman et al., 2005). Malformation of the tongue in individuals with Down’s syndrome is attributed to PIG-P.

Also noteworthy is the interaction of Ras with the GPI biosynthetic pathway. In yeast, interaction of Ras2p with the GPI–GnT complex leads to a mutual negative regulation of GPI biosynthesis and Ras signalling (Sobering et al., 2003, 2004). Mutants in GPI biosynthesis often display phenotypes similar to those of hyperactive Ras. Our results show that, unlike CaGPI7, perturbation of CaGPI19 significantly increases hyphal transition and invasive behaviour in Candida. Since Ras signalling is also required for serum-induced hyphal differentiation in Candida (Feng et al., 1999), this suggests that the hyper-filamentous phenotype seen following CaGPI19 disruption may be due to disruption of interaction between Candida Ras and the GPI–GnT complex in a similar fashion to that of yeast. Many proteins have been suggested to negatively regulate the GPI–GnT complex in a similar fashion to that of yeast. Further work is in progress to determine whether the hyperfilamentation is due to a specific physical interaction of CaGpi19p with Ras1p.

Interestingly, CaGpi19p-depleted Cagpi19/MET3-GFP-CaGPI19 cells show an increased resistance to amphotericin B. This polyene antibiotic is used clinically for treatment of candidiasis and reports of resistant strains are rare. Ergosterol levels are reported to be the main cause for resistance to this drug, with both increased as well as decreased levels of ergosterol being correlated with amphotericin B sensitivity (Pasrija et al., 2005; Sanglard et al., 2003). In many reports of fungal resistance to this drug, there have also been increases in chitin or glucon levels (Bahmed et al., 2002; Seo et al., 1999). However, in Candida there is at least one report that correlates amphotericin B resistance with decreased chitin levels (Bahmed et al., 2003). From our results, however, it appears that chitin levels have increased. A preliminary analysis suggests that ergosterol levels dropped in the conditional null (0.00016 %) compared with the wild-type (0.00032 %), while there is no significant decrease in the heterozygote (0.00029 %). While ergosterol levels alone may not be enough to explain the polyene resistance in the heterozygote, it may be a cumulative effect of cell wall changes and ergosterol levels. In the CaGpi19p-depleted cells, taking into account the azole sensitivity, the ergosterol pathway does seem to be affected. In yeast, Gpi19p interacts with Gpi2p, another GPI-GnT subunit, which in turn was shown to interact with Erg11p encoding lanosterol demethylase, the enzyme targeted by the azoles (Miller et al., 2005; Davierwala et al., 2005). A similar interaction in Candida cannot be ruled out.

There was no difference in the heterozygote’s response to azoles. Our results with CaGPI19 suggest that single-allele disruption of CaGPI19 is not enough to affect azole susceptibilities and it is only when levels are greatly depleted that sensitivity to azoles is noticed, thus confirming a dosage-dependency of this gene. CaGPI7 mutants do not show any difference in response to either amphotericin B or azoles (Richard et al., 2002), though it should be noted that this gene is not essential and its product is responsible for a GPI-modifying, rather than synthetic, activity. Thus, while the data suggest a specific interaction of GPI–GnT with ergosterol biosynthesis, we do not discount the possibility that perturbation of other GPI biosynthetic enzymes may also affect azole sensitivities.

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