Ras signaling activates glycosylphosphatidylinositol (GPI) anchor biosynthesis via the GPI–N-acetylglucosaminyltransferase (GPI–GnT) in Candida albicans

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The ability of Candida albicans to switch between yeast to hyphal form is a property that is primarily associated with the invasion and virulence of this human pathogenic fungus. Several glycosylphosphatidylinositol (GPI)-anchored proteins are expressed only during hyphal morphogenesis. One of the major pathways that controls hyphal morphogenesis is the Ras-signaling pathway. We examine the cross-talk between GPI anchor biosynthesis and Ras signaling in C. albicans. We show that the first step of GPI biosynthesis is activated by Ras in C. albicans. This is diametrically opposite to what is reported in Saccharomyces cerevisiae. Of the two C. albicans Ras proteins, CaRas1 alone activates GPI–GnT activity; activity is further stimulated by constitutively activated CaRas1. CaRas1 localized to the cytoplasm or endoplasmic reticulum (ER) is sufficient for GPI–GnT activation. Of the six subunits of the GPI–N-acetylglucosaminyltransferase (GPI–GnT) that catalyze the first step of GPI biosynthesis, CaGpi2 is the key player involved in activating Ras signaling and hyphal morphogenesis. Activation of Ras signaling is independent of the catalytic competence of GPI–GnT. This too is unlike what is observed in S. cerevisiae where multiple subunits were identified as inhibiting Ras2. Fluorescence resonance energy transfer (FRET) studies indicate a specific physical interaction between CaRas1 and CaGpi2 in the ER, which would explain the ability of CaRas1 to activate GPI–GnT. CaGpi2, in turn, promotes activation of the Ras-signaling pathway and hyphal morphogenesis. The Cagpi2 mutant is also more susceptible to macrophage-mediated killing, and macrophage cultures show better survival when co-cultured with Cagpi2.

Glycosylphosphatidylinositol (GPI)5 anchors are ubiquitously present in eukaryotes, and they anchor a wide variety of proteins to the extracellular leaflet of the plasma membrane. In yeast, as well as in fungal pathogens such as Candida albicans and Aspergillus fumigatus, GPI-anchored proteins are also incorporated into the cell wall, covalently linked to the β-1,6-glucan layer, and are important for cell wall biogenesis as well as its maintenance (1). Given that they are present in the extracellular space, a large fraction of these proteins serve as signal receptors and/or host-pathogen recognition molecules (1). Some of these surface-localized GPI-anchored proteins may even be cleaved off by cell-surface phospholipases to provide eukaryotic pathogens with an armory of enzymes or virulence factors required in colonizing/infecting the host under appropriate conditions (1, 2). In yeast and fungi, the GPI biosynthetic pathway is essential, and mutants defective in the pathway are inviable (3).

The GPI anchor, attached at the C termini of proteins carrying the appropriate GPI signal sequence, is preassembled via a sequential set of biosynthetic events in the ER (3, 4). In the first step of GPI biosynthesis, a phosphatidylinositol (PI) receives an N-acetylgalactosamine (GlcNAc) from UDP–GlcNAc. The GlcNAc–PI thus generated is then deacetylated at the sugar, triply mannosylated, and decorated with 2–3 phosphoethanolamine groups at the different mannoses. The phosphoethanolamine on the third mannose is crucial for the final step that involves attachment of the anchor to a newly translocated protein in the ER lumen to generate a complete GPI-anchored protein. In yeast and fungi, a fourth sequential mannose is obligatory, but in mammals, this requirement is not strictly upheld except in certain specific tissues (5, 6). The GPI-anchored protein is then transported via the secretory pathway to the extracellular surface of the cell membranes and/or the cell walls where they function (7).

Despite a rather conserved core, GPI biosynthesis shows species-specific differences both in terms of sequences of the reac-

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5 The abbreviations used are: GPI, glycosylphosphatidylinositol; PI, phosphatidylinositol; GPI–GnT, GPI–N-acetylglucosaminyltransferase; TRITC, tetramethylrhodamine; CFU, colony-forming unit; m.o.i., multiplicity of infection; BCA, bicinchoninic acid; G6PDH, glucose-6-phosphate dehydrogenase; ER, endoplasmic reticulum; PM, plasma membrane; PCC, Pearson’s correlation coefficient; PFA, paraformaldehyde; CFSE, carboxyfluorescein N-succinimidyl ester; PI, phosphatidylinositol; GAP, GTPase-activating protein; ROI, regions of interest; Cyt D, cytochalasin D; mRFP, monomeric red fluorescent protein.
**Figure 1. Interaction between Ras and GPI–GnT differs in *S. cerevisiae* versus *C. albicans*. Left panel, Ras2 and GPI–GnT exhibit a mutually inhibitory effect. Ras2 inhibits GPI–GnT activity; at least two GPI–GnT subunits (Gpi2 and Eri1) appear to physically interact with and inhibit Ras2 activity (11). Whether they do so directly or via another subunit is not clear. Two other GPI–GnT mutants in *S. cerevisiae* (deficient in Gpi1 and Gpi19) are reported to exhibit hyperactive Ras phenotypes (hyperfilamentation) (11, 14). Right panel, in *C. albicans* a hypofilamentous and a hyperfilamentous mutant of the GPI–GnT complex have been reported (13). Of the two subunits studied, CaGpi2 alone appears to be involved in activation of Ras signaling, and hyphal morphogenesis via CaRas1 and CaGPI19 conditional null is hyperfilamentous due to a mutually negative regulation between it and CaGPI2 (13).**

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Heterozygous mutant could control the filamentation pattern in it (13). Thus, when it came to Ras signaling and filamentation, CaGPI2 acted downstream of CaGPI19 (Fig. 1, right panel).

However, what happens at the level of the proteins? How does CaGpi2 interact with Ras signaling? Does its interaction require the formation of a functional GPI–GnT complex as was seen in *S. cerevisiae*? How do other subunits of the GPI–GnT interact with Ras signaling? Can they interact with CaRas proteins independently or do they all act via a single subunit? Is Ras an inhibitor of GPI–GnT in *C. albicans* as is reported in *S. cerevisiae*? Does the subcellular localization of Ras and its activation status matter? Is there a physical interaction between CaGpi2 and any of the two *C. albicans* Ras proteins and, if so, in which compartment does it occur? These are some of the questions we explore in this work.

We show that all the GPI–GnT subunits interact with Ras signaling via CaGpi2. Of the two *C. albicans* Ras proteins, CaRas1 is the only one that participates in this interaction. The activation of Ras signaling by CaGpi2 does not depend on the formation of a functional GPI–GnT complex. Overexpression of CaGpi2, however, alters the status of Hsp90 in the cell. CaRas1 enhances the GPI–GnT activity of the enzyme complex, and the activation is favored by its GTP-bound active state. A direct physical interaction exists between CaGpi2 and CaRas1 and the extent of this interaction is determined by the localization of CaRas1 in the ER.

**Results**

The GPI–GnT enzyme in *C. albicans* is made up of six putative subunits, based on sequence homology with *S. cerevisiae*. Of these, CaGpi3 is the putative catalytic subunit, and CaGpi1, CaGpi2, CaGpi15, CaGpi19, and CaEri1 are expected to be accessory subunits. Mutants of each of these subunits were generated as described under “Experimental procedures” and studied as described below.
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Figure 2. GPI–GnT activity is reduced in the C. albicans GPI–GnT mutants. GPI–GnT activity was measured in GPI–GnT deletion mutants relative to WT strain as explained under “Experimental procedures”. A, GPI–GnT activity in the heterozygous GPI–GnT mutants. GPI–GnT activity was measured in CaGPI1Hz, CaGPI3Hz, CaGPI19Hz, CaGPI15Hz, CaGPI19Hz, and CaERI1Hz mutants relative to BWP17. B, conditional null Cagpi1 and Cagpi3 mutants show reduced GPI–GnT activity. BWP17URA3 as well as conditional null Cagpi1 and Cagpi3 mutants were grown in both permissive (Ura+/+H11002) and repressive (Ura−/−H11001) media during secondary culture. GPI–GnT activity was estimated in these strains. The percentage of GPI–GnT activity was measured in the heterozygous GPI–GnT mutants. GPI–GnT activity was measured in all strains relative to BWP17URA3. All experiments were done three times in duplicate, and averages with standard deviation are plotted.

Construction of GPI–GnT deletion mutants

The generation of CaGPI2 and CaGPI19 heterozygous and conditional null mutants in the BWP17 strain of C. albicans was described previously (13, 15, 16). The other Cagpi heterozygous and conditional null mutants were similarly generated in the BWP17 strain. Because C. albicans has two alleles of each gene, the heterozygous mutants of the GPI–GnT genes were constructed by disruption of one allele of a specific gene by one of the two selection markers, HIS1 or ARG4. Repeated attempts to make homozygous nulls were unsuccessful in all cases. Hence, conditional null mutants were generated by replacing the endogenous promoter of the surviving allele with a repressible MET3 promoter in the heterozygous strain background.

The successful generation of the heterozygous and conditional null deletion mutants was first confirmed by PCR and then by monitoring the relative transcript levels of the corresponding GPI–GnT subunit gene vis-à-vis the WT control using real time-PCR (RT-PCR). The transcript levels of CaGPI1, CaGPI2, CaGPI3, CaGPI15, CaGPI19, and CaERI1 were significantly down-regulated in their respective deletion mutants (Fig. S1). This confirmation allowed us to use these mutants for further studies.

GPI–GnT activity is reduced in the GPI–GnT deletion mutants

The percentage of GPI–GnT activity was measured in the GPI–GnT deletion mutants with respect to that of the WT strain, BWP17. Significantly lower GPI–GnT activity was observed in the heterozygous mutants CaGPI2/CaGPI2 (CaGPI2Hz), CaGPI15/CaGPI15 (CaGPI15Hz), CaGPI19/ CaGPI19 (CaGPI19Hz), and CaERI1/Caeri1 (CaERI1Hz) mutant strains as compared with BWP17, suggesting that they were required for GPI–GnT activity (Fig. 2A). The heterozygous mutants CaGPI1/Caagpi1 (CaGPI1Hz) and CaGPI3/ Caagpi3 (CaGPI3Hz) had ~80%, or greater, relative GPI–GnT activity suggesting haplosufficiency of these genes when it came to maintaining GPI–GnT activity at near normal levels. Hence, the conditional null mutants were examined. Both Cagpi1 and Cagpi3 conditional null mutants were grown in repressive growth media, and GPI–GnT activity was estimated in these mutants relative to WT control strain (Fig. 2B). The Cagpi1 conditional null mutant (~20%) and the Cagpi3 conditional null mutant (~40%) had drastically reduced GPI–GnT activity as compared with the control strain under repressive growth conditions. Thus, CaGpi1 and CaGpi3 are also essential for GPI–GnT activity in C. albicans.

Depletion of any one of CaGpi1, CaGpi2, CaGpi3, CaGpi15, CaGpi19, or CaEri1 affects Ras signaling and hyphal morphogenesis

As mentioned earlier, Cagpi2 is hypofilamentous due to decreased Ras signaling, and Cagpi19 is hyperfilamentous due to enhanced Ras signaling as compared with the WT strain (13, 15). Hyphal morphogenesis of the four remaining GPI–GnT subunit mutants of C. albicans were examined in Spider medium as well as YEPD at 37 °C. Down-regulation of CaGPI1, CaGPI3, and CaGPI15 led to decreased hyphal growth with respect to BWP17 under hypha-inducing conditions, whereas down-regulation of CaERI1 resulted in hyperfilamentation (Fig. 3A). The phenotypes of CaGPI2Hz and CaGPI19Hz are also shown for comparison (Fig. 3A). The revertant strains were restored in their hyphal morphogenesis phenotypes, indicating that the effect was specifically caused by the gene defect in each case (Fig. 3B). Similar results were obtained for hyphal growth of the strains in liquid Spider medium as well (Fig. 3, C and D).

Hyphal growth in the GPI–GnT mutants correlate with CaGpi2 levels

As seen previously in the CaGPI2Hz and CaGPI19Hz mutant strains (13), are the hyphal morphologies of the other GPI–GnT mutants also correlated with the levels of CaGPI2 in them? To test this hypothesis, we first examined mRNA transcript as well as protein expression levels of CaGPI2 in the various GPI–GnT deletion mutants (Fig. 4A, panels i and ii, and Fig. S2). The transcript and protein expression levels of CaGPI2 were down-regulated in the hypofilamentous strains, CaGPI1Hz, CaGPI2Hz, CaGPI15Hz, and CaGPI19Hz as compared with the control. In contrast, CaGPI2 was up-regulated in the hyperfilamentous CaGPI19Hz and CaERI1Hz strains. Thus, hyphal growth in the GPI–GnT mutants correlates with CaGPI2 expression levels.

Next, we overexpressed CaGPI2 in the hypofilamentous CaGPI1Hz, CaGPI3Hz, and CaGPI15Hz strains. In each case, hyphal growth was restored back to WT levels as was the Ras-dependent cAMP/PKA signaling (Fig. 4B, panels i and ii, and Fig. S3, panel i). Disrupting one allele of CaGPI2 in the CaGPI19Hz or CaERI1Hz strain, however, reversed their hyperfilamentation phenotype and reduced cAMP/PKA signaling (Fig. 4C, panels i and ii, and Fig. S3, panel ii). The converse was not true. Disruption of one allele of CaERI1 in the CaGPI2Hz strain did not induce greater filamentation in it (Fig. 4D, panel i). We have previously shown that disruption of one allele of CaGPI19 in the CaGPI2Hz strain also does not induce hyperfilamentation (13). Furthermore, overexpression of CaGPI1, CaGPI3, and CaGPI15 in the CaGPI2Hz strain also did not restore hyphal morphology (Fig. 4D, panel ii). Taken
CaGpi2 stimulates GPI–GnT activity

C. albicans has two Ras proteins. Because both RAS1 and RAS2 are nonessential genes in C. albicans, a complete null mutant, made by deleting both copies of CaRAS1 and CaRAS2, was used for this study (17). Caras1/Caras2 had lower GPI–GnT activity (~50%) as compared with WT BWP17 (Fig. 6A), suggesting that deletion of CaRAS genes causes a reduction in GPI–GnT activity. This is in complete contrast to that reported in S. cerevisiae where deletion of RAS2 (homologous to CaRAS1) leads to a significant increase in GPI–GnT activity (11).

CaRAS1 and CaRAS2 were now separately reintroduced as single copies in the Caras1/Caras2 mutant to generate strains that express only one Ras protein at a time. A strong ACT1 promoter was used to ensure sufficient CaRas1 and CaRas2 levels, given that both alleles of CaRAS1 and CaRAS2 were deleted in the Caras1/Caras2 mutant. The expression of the CaRas proteins was confirmed by transcript level analysis (Fig. S4A), and their filamentation phenotypes (Fig. S4B) were in complete agreement with what has been reported previously (17).

GPI–GnT activity was assayed in Caras1/Caras2/CaRAS1 and Caras1/Caras2/CaRAS2, compared with Caras1/Caras2/URA3 (Fig. 6A). Restoration in GPI–GnT activity was observed together, it may therefore be inferred that CaGpi2 is downstream of all GPI–GnT subunits in regulation of Ras signaling.

Activation of Ras signaling by CaGpi2 is independent of GPI–GnT activity

GPI–GnT activity is restored in the revertant strain expressing CaGPI2 as compared with the CaGPI2Hz strain (Fig. 5A). However, in all the other hypofilamentous GPI–GnT mutant strains, GPI–GnT activity decreases upon overexpressing CaGPI2 (Fig. 5A). Likewise, disruption of CaGPI2 in the hyperfilamentous GPI–GnT mutant strains does not result in restoration of GPI–GnT activity (Fig. 5B), even though it reverses the hyperfilamentation phenotype. A transcript level analysis in the WT strain overexpressing CaGPI2 suggests that the levels of the different GPI–GnT subunits are significantly altered (Fig. 5C, panel i). Although the levels of CaGPI1, CaGPI3, and CaGPI15 are up-regulated upon overexpression of CaGPI2, the levels of CaGPI19 and CaERI1 are down-regulated, thereby hampering formation of a functional enzyme complex. Likewise, the expression levels of all the GPI–GnT genes are up-regulated in the CaGPI2Hz strain, whereas that of CaGPI2 is reduced (Fig. 5C, panel ii). Thus, it appears that a certain optimal stoichiometry of the subunits is required for constitution of an active GPI–GnT enzyme complex.

Figure 3. Defects in GPI anchor biosynthesis affects hyphal morphogenesis in C. albicans. A, depletion of any one of the GPI–GnT subunits affects hyphal morphogenesis on solid media. Filamentation of the GPI–GnT heterozygous strain relative to BWP17 is shown. A representative image on the 7th day of growth is shown for all strains. B, hyphal growth phenotype was restored in each GPI–GnT revertant on solid medium. A representative image on 10th day of growth is shown for all strains except CaERI1Hz/pACT1-CaERI1 for which the growth on the 7th day is shown. The experiments were done three times again in duplicate using independently grown cultures for confirmation of the result in A and B. C, depletion of any one of the GPI–GnT subunits affects hyphal morphogenesis in liquid media. GPI–GnT heterozygous strains as well as BWP17 control were grown in liquid Spider medium for 2 h. The number of yeast and pseudohyphal and hyphal cells were counted, and the data were plotted as a fraction of total. D, hyphal growth phenotype was restored in each GPI–GnT revertant in liquid media. The GPI–GnT revertant strains displayed normal hyphal growth relative to BWP17/URA3 in liquid Spider media after 1 h of growth. Averages of three sets of independent experiments done in duplicate along with standard deviations are shown in C and D.
in the former but not in the latter strain. Thus, CaRAS1 alone exerts control on the first step of the GPI anchor biosynthetic pathway. It has been previously shown that in the CaGPI2Hz strain overexpression of CaRas1, but not CaRas2, restores hyphal morphogenesis (13). Thus, CaRas1 exerts control over GPI–GnT as well as hyphal morphogenesis in C. albicans.

CaRas1G13V is a better activator of the GPI–GnT complex than CaRas1

CaRas1 cycles between an inactive GDP-bound form and an active GTP-bound form. To ascertain whether stimulation of the GPI–GnT activity requires the GTP-bound active form of CaRas1, we introduced a G13V mutation in CaRas1. CaRas1G13V is constitutively activated and cannot interact with the GTPase-activating protein (GAP), Ira2, to return back to its inactive GDP-bound state (18, 19). Expression of CaRas1G13V was extremely low as compared with that of WT CaRas1 (Fig. 6B, panel i). This is in keeping with what has been previously reported for this mutant protein in C. albicans (20). Interestingly, despite its low expression, CaRas1G13V was able to enhance GPI–GnT activity by roughly 1.5-fold as compared with WT CaRas1 as well as BWP17URA3 (Fig. 6B, panel ii), suggesting that GTP-bound CaRas1 is a better activator of the GPI–GnT enzyme complex than WT CaRas1.

CaRas1 can stimulate GPI–GnT at the ER

How does post-translational modification and localization of CaRas1 affect GPI–GnT activity in C. albicans? Nascent Ras proteins synthesized in the cytosol are prenylated, allowing them to transiently associate with the ER membrane where they are further palmitoylated before being finally transported to the inner leaflet of the plasma membrane (PM) (21, 22). Hyphal morphogenesis in C. albicans involves the PM-localized CaRas1 preferentially (20). CaRas1 has two cysteine residues at positions 287 and 288 that serve as sites for palmitoylation and prenylation, respectively. CaRas1C288S cannot be prenylated and is known to be localized in cytosol, whereas CaRas1C287S cannot be palmitoylated and remains associated with the ER (20). Neither CaRas1 variant efficiently supports hyphal growth, but CaRas1C288S is the more severely affected of the two. To test which of these variants best interact with the GPI anchor biosynthesis pathway, we studied GPI–GnT activity in the Caras1/Caras2 null strain, wherein these protein variants were exclusively
expressed using the CaRAS1 endogenous promoter. Because these are not overexpression strains, they are expected to behave as heterozygous rather than as overexpression strains.

The expression levels and the specific localizations of the CaRas1 variants in the strains Caras1/Caras2/pRAS1-CaRas1, Caras1/Caras2/pRAS1-CaRas1C287S, and Caras1/Caras2/pRAS1-CaRas1C288S were confirmed by immunofluorescence using confocal microscopy (Fig. S5). The expressed CaRas1 variants were also quantified using Western blots (Fig. 6C, panel i). Expression of CaRas1C287S and CaRas1C288S is higher than that of WT CaRas1 and yields single bands of ~46 kDa (although CaRas1C288S had slightly faster migration) as they do not undergo proteolytic cleavage that CaRas1 experiences when localized to the plasma membrane (23).

Fluorescence resonance energy transfer (FRET) was employed to assess the actual likelihood of interaction. It must be pointed out that FRET depends inversely on the sixth power of the distance between two fluorophores and hence drops rapidly with small increases in distance. Thus, a positive FRET is seen as an indication of a physical interaction between the donor and the acceptor. CaGpi2–His6 was detected using an anti-His antibody and a TRITC-labeled secondary antibody. CaRas1 was detected using an anti-Ras antibody and a secondary antibody that was FITC-labeled to provide us with a fluorescence donor–acceptor pair. Once again, we saw that CaGpi2 was predominantly localized to the ER (acceptor–red channel), whereas CaRas1–FITC was localized to PM as well as ER (donor–green channel) in the prebleach image (Fig. 7B). After photobleaching...
of the acceptor fluorophore (CaGpi2–His6–TRITC), an increased fluorescence of donor molecules (CaRas1–FITC) was detected, indicating that the two proteins were in close proximity (Fig. 7B). More than 10 cells were analyzed and consistently produced a positive FRET between the two proteins (6.3 ± 0.2 nm) and likely to physically interact. No FRET was observed when regions of interest (ROIs) exclusively at the PM were selected and where only CaRas1 localization was clearly visible.

To ensure that the FRET observed is specific to the two proteins being studied, we tagged CaErg11 (another ER-localized enzyme, lanosterol 14α-demethylase, involved in ergosterol biosynthesis) with His6 and repeated the FRET experiments. CaRas1 was probed with anti-Ras antibody and a secondary antibody tagged with FITC, whereas CaErg11–His6 was detected with an anti-His antibody and a TRITC-labeled secondary antibody in BWP17–CaErg11–His6 cells. No FRET was observed between the donor acceptor pair, suggesting that CaRas1 and CaErg11 are not in close proximity and do not interact (Fig. 7C).

CaGpi2 overexpression phenocopies a hsp90 mutant

We had previously reported that the strain overexpressing CaGpi2 is hyperfilamentous (13). We discovered that it was also sensitive to heat shock (Fig. 8A), a phenotype typical of hyperactive Ras mutants (18). A transcript level analysis suggested that CaHSP90 was down-regulated in this strain (Fig. 8B). For further confirmation of the role of Hsp90, we disrupted one allele of CaHSP90 in the CaGPI2Hz background (CaGPI2Hz/CaHSP90Hz) and examined its filamentation phenotype at 30 °C. The CaGPI2Hz/CaHSP90Hz strain was hyperfilamentous as compared with the parental strain even at 30 °C, clearly indicat-
Hsp90 depletion overcomes the filamentation block in the CaGPI2H strain (Fig. 8C).

Hsp90 down-regulation affects the transcription of several proteins, including Hog1 MAPK, as well as their phosphorylation (24). Hence, we monitored the levels of phosphorylated Hog1 as a measure of Hsp90 activity and found that the strain overexpressing CaGpi2 had reduced Hsp90 activity (Fig. 8D). Thus, CaRas1 may be activated upon overexpression of CaGpi2 due to a down-regulation of Hsp90 in the cell. Activating Hsp90 in these cells with the help of tamoxifen (25) results in enhanced Hsp90 activity and reversal of the hyperfilamentation phenotype (Fig. 8, D and E), indicating that Hsp90 is indeed affected upon CaGpi2 overexpression.

**Cagpi2 heterozygous strain is more susceptible to killing by MH-S macrophage cells and is less able to kill the macrophage cell line**

Given the effect of CaGpi2 on GPI anchor biosynthesis, we expected it to have reduced levels of GPI-anchored proteins that are involved in adhesion/virulence in *C. albicans*. To confirm this, Western blottings were performed using a polyclonal anti-Als5 antibody. As expected, levels of Als5, a heavily glycosylated adhesin belonging to the agglutinin-like sequence family of proteins, were reduced to $\sim 65 \pm 12\%$ in the CaGPI2H strain (Fig. 9A). In addition, we have already shown above that Ras signaling as well as hyphal growth are also affected in this strain. Hence, we expected it to be attenuated in virulence. To examine whether this was indeed the case, we co-cultured cells from a murine alveolar macrophage cell line (MH-S) with BWP17 and CaGPI2H strain for 18 h. As can be seen from Fig. 9, B and C, the internalization of *C. albicans* cells was found to be higher for the CaGPI2H strain than for BWP17 and CaGPI2H strain for 18 h. As can be seen from Fig. 9, B and C, the internalization of *C. albicans* cells was found to be higher for the CaGPI2H strain than for BWP17 strain; 25.83 $\pm 2.8\%$ MH-S was able to phagocytose BWP17, whereas 53.01 $\pm 1.9\%$ MH-S was able to phagocytose CaGPI2H strain. This was found to be the case even when phagocytosis was inhibited with the help of cytochalasin D (Cyt D); 14.4 $\pm 2.5\%$ MH-S was able to phagocytose BWP17, and 36.7 $\pm 2.1\%$ MH-S was able to phagocytose CaGPI2H cells after pretreatment with Cyt D (Fig. 9D). Thus, down-regulation of CaGPI2 resulted in enhanced phagocytosis of *C. albicans* cells by cytoskeleton-dependent and -independent pathways.

GPI biosynthesis and Ras signaling in *C. albicans*
To study the survivability of the *C. albicans* cells, the macrophage cells were then lysed, and the fungal cells obtained were plated on YEPD. Colony-forming units (CFU) were counted in each case. CFU of BWP17 alone after incubation at 30 °C for 24 h were $0.5 \times 10^6$. This was reduced by roughly 20% after it was co-cultured with MH-S cells (Fig. 9E). CFU of *CaGPI2Hz* strain, however, were reduced by 54% after co-culturing with MH-S as compared with the same strain in the absence of MH-S ($0.5 \times 10^6$) as shown in Fig. 9E. The significantly greater reduction in CFU of the *CaGPI2Hz* strain compared with that of BWP17 co-cultured with MH-S suggests that *CaGPI2Hz* strain is more susceptible to killing by macrophages.

We also examined the live cell recovery of MH-S after co-culturing these cells with *C. albicans* strains (Fig. 9F). Live-cell recovery of MH-S was ~67% when co-cultured with BWP17 at a 1:1 multiplicity of infection (m.o.i.) and ~53% when co-cultured with BWP17 at 1:5 m.o.i. Cell recovery of MH-S was ~80% and ~86% when co-cultured with *CaGPI2Hz* strain at m.o.i. 1:1 and 1:5, respectively. Thus, the survival rates for MH-S cells co-cultured with *CaGPI2Hz* cells are higher, suggesting that the *CaGPI2Hz* strain is likely to be less virulent than the WT strain.

**Discussion**

Unlike *S. cerevisiae*, *C. albicans* is a true filamentous fungus and a pathogen. The ability to switch between yeast and hyphal forms is a major virulence trait of the organism and is regulated, among others, by the Ras-signaling pathway (26). The organism is also adept at forming drug-resistant biofilms on catheters or medical implants, another trait that depends quite considerably on the Ras-signaling pathway (27, 28). These attributes of the organism are also closely regulated with a transcriptional program that induces expression of virulence factors like Als3, Ecm33, and Hwp1, a large majority of which are GPI-anchored proteins (1, 29). Mutants defective in GPI-anchored proteins at their cell surface are attenuated in virulence as well (30, 31).

Hence, the idea that the first step of GPI anchor biosynthesis cross-talks with Ras signaling in *C. albicans* is interesting specifically from the point of view of the virulence of the organism.

In *S. cerevisiae*, an organism often chosen as the model organism for studying different fungal pathways, Ras2 is shown to...
be an inhibitor of GPI–GnT activity, and the GPI–GnT complex, in turn, is shown to be an inhibitor of Ras signaling (10, 11). The results presented here show that this model does not hold true in C. albicans, and generalizations based on model organisms alone can sometimes be misleading.

In C. albicans, Ras signaling enhances GPI–GnT activity. Of the two Ras proteins in C. albicans, only CaRas1 (a close homolog of S. cerevisiae Ras2) enhances GPI–GnT activity. The interaction of CaRas1 with the GPI–GnT complex is favored by the GTP-bound active form of CaRas1 and can be achieved by CaRas1 that is either cytoplasmic or ER-localized. Thus, it appears that the pool of CaRas1 present in the endomembranes, during its transit to the PM via the ER, is able to activate GPI–GnT activity (Fig. 10). A positive correlation between CaRas1 and GPI–GnT activities would ensure that not only the virulence factors but also the biosynthesis of GPI anchors necessary for their localization and function are coordinated with hyphal morphogenesis. Thus, C. albicans is programmed for optimizing its chances for successful infections.

CaRas1 activity also is in turn stimulated by the CaGpi2 subunit of the GPI–GnT complex resulting in hyphal morphogenesis. No other GPI–GnT subunit appears to participate in this, and the activation of CaRas1 does not require the formation of a functional GPI–GnT complex. This too is in contrast with what has been reported in S. cerevisiae where two subunits, Gpi2 and Eri1, were both shown to interact with Ras2 in co-immunoprecipitation assays (11). However, it remains unclear whether both subunits independently interact with Ras2 or whether one of the subunits acts as a bridge for the other. It is also unclear whether other subunits of the GPI–GnT may be involved in the interaction, nor is it clear whether this interaction occurs with Ras2 localized at the PM or with Ras2 that associates with the ER during its transit to the PM. In C. albicans, using co-localization studies along with FRET analysis, we show that CaRas1 physically interacts with CaGpi2 in the ER (Fig. 10). This is also in keeping with our observation that CaRas1 localized to endomembranes is able to stimulate GPI–GnT activity.

One issue, however, continued to puzzle us. CaRas1 needs to be localized at the plasma membrane for sensing external cues and inducing hyphal morphogenesis (20, 26). How then does CaGpi2, acting at the ER, activate CaRas1 signaling and hyphal morphogenesis? One possible explanation is that the physical interaction of CaRas1 with CaGpi2 stabilizes the association of the former with the ER and promotes its palmitoylation, thereby increasing its chances of transport and localization at the PM. Thus, depletion of CaGpi2 could perhaps reduce the levels of CaRas1 available at the PM for signaling in the cAMP/PKA pathway for hyphal morphogenesis. Overexpression of CaGpi2, however, could perhaps increase the available pool of GPI biosynthesis and Ras signaling in C. albicans

Figure 9. Comparative virulence study of BWP17 and CaGPI2Hz. A, levels of GPI-anchored proteins are reduced in the CaGPI2Hz. Panel i, Western blotting using polyclonal anti-Als5 antibody shows a decrease in the levels of Als5 in the CaGPI2Hz relative to the WT. Panel ii, bar graph showing the quantification of Als5 protein levels. B and C, phagocytosis of yeast form of BWP17 and CaGPI2Hz, respectively, by MH-S cells. MH-S cells were cultured on coverslips for 12 h, then co-cultured with CFSE-labeled BWP17 or Als5 protein levels. F, plots of the difference between control and treated cells. The significance of any difference was calculated by using one-tailed distribution in a two-sample equal variance test. *p < 0.05; **p < 0.005; and ***p < 0.0005 represent statistically significant difference between control and treated cells. The significance of any difference was calculated by using one-tailed distribution in a two-sample equal variance test. Student’s t-test.
CaRas1 at the PM and promote filamentation (Fig. 10). This might be one possible explanation for why the WT BWP17 strain overexpressing CaGpi2 is hyperfilamentous (13). The strain overexpressing CaGpi2 is also heat-shock-sensitive, a phenotype typical of hyperactive Ras mutants (18). A more detailed analysis suggested that CaGpi2 overexpression also results in Hsp90 down-regulation, which is reflected in a decrease in transcript levels as well as in Hsp90 activity. Activating Hsp90 in these cells with the help of tamoxifen (25) results in reversal of the hyperfilamentation phenotype, indicating that Hsp90 is indeed affected upon CaGpi2 overexpression. Furthermore, disrupting one allele of Hsp90 in CaGPI2Hz could reverse its filamentation defect and promote filamentation even at 30 °C. It is well known that Hsp90 along with its co-chaperone, Sgt1, modulates the Ras-signaling pathway by inhibiting the interaction of GTP-bound CaRas1 with Cyr1, the adenyl cyclase, and activate cAMP production. However, this interaction is inhibited by Hsp90 along with its co-chaperone, Sgt1 (32). As a consequence of this modulation, CaRas1-dependent cAMP signaling is maintained at basal levels. When Hsp90 levels are down-regulated, due to CaGpi2 overexpression as shown in this work or by CaGpi19 down-regulation (33), the interaction of CaRas1 with Cyr1 is promoted at the cost of its interaction with Ira2. Thus, the filamentation pathway remains turned on even at 30 °C, resulting in a hyperfilamentous phenotype.

GPI biosynthesis and Ras signaling in C. albicans

Figure 10. Model depicting the possible interaction between Ras signaling and GPI–GnT in C. albicans. Depending on their filamentation phenotypes, GPI–GnT mutants of C. albicans can be classified into two sets, hyperfilamentous and hypofilamentous. In each mutant, CaGpi2 expression levels alone dictate the filamentation phenotype. CaRas1 physically interacts with CaGpi2 to stimulate GPI–GnT activity. GTP-bound CaRas1 is a better activator. CaGpi2, in turn, helps activate CaRas1-dependent cAMP/PKA activity and promote filamentation. This does not require formation of a functional GPI–GnT. It is possible that physical interaction of CaRas1 with CaGpi2 assists ER association of CaRas1 and thereby promotes its palmitoylation as well as transport/localization to the PM. This perhaps increases the pool of CaRas1 available for hyphal signaling at the PM. At the PM, CaRas1 cycles between an active GTP-bound and an inactive GDP-bound form by the action of Ira2, a GAP, and Cdc25, a guanine nucleotide exchange factor (20, 49). For cAMP-dependent signaling, CaRas1-GTP needs to associate with Cyr1, the adenyl cyclase, and activate cAMP production. However, this interaction is inhibited by Hsp90 along with its co-chaperone, Sgt1 (32). As a consequence of this modulation, CaRas1-dependent cAMP signaling is maintained at basal levels. When Hsp90 levels are down-regulated, due to CaGpi2 overexpression as shown in this work or by CaGpi19 down-regulation (33), the interaction of CaRas1 with Cyr1 is promoted at the cost of its interaction with Ira2. Thus, the filamentation pathway remains turned on even at 30 °C, resulting in a hyperfilamentous phenotype.

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These results join the growing trend of reports in the literature suggesting multiple roles for the same protein. In the GPI biosynthetic pathway alone, two such proteins, Arv1 and PIGN, have been identified. Human and yeast Arv1 play a role not only in the delivery of the early GPI anchor intermediate to the first mannosyltransferase during GPI biosynthesis but also work independently to control lipid homeostasis (34, 35). Similarly, PIGN protein transfers phosphoethanolamine to the first mannose in the GPI biosynthetic pathway and also independently works to prevent protein aggregation in the ER (36, 37). We propose that CaGpi2 also performs more than one function: one within the GPI–GnT to form a functional enzyme complex as well as to coordinate the activation of the enzyme by CaRas1 and another at the level of Hsp90 to regulate Ras signaling and hyphal morphogenesis.

Unlike Ras proteins that tend to be highly conserved and therefore difficult to specifically target, CaGpi2 in C. albicans shares barely 30% identity with the human homolog Pig-C. Targeting it specifically could result in not only inhibiting GPI biosynthesis but also work independently to control lipid homeostasis. PIGN protein transfers phosphoethanolamine to the first mannosyltransferase during GPI biosynthesis. In the GPI biosynthetic pathway alone, two such proteins, Arv1 and PIGN, have been identified. Human and yeast Arv1 play a role not only in the delivery of the early GPI anchor intermediate to the first mannosyltransferase during GPI biosynthesis but also work independently to control lipid homeostasis (34, 35). Similarly, PIGN protein transfers phosphoethanolamine to the first mannosyltransferase during GPI biosynthesis. In the GPI biosynthetic pathway alone, two such proteins, Arv1 and PIGN, have been identified. Human and yeast Arv1 play a role not only in the delivery of the early GPI anchor intermediate to the first mannosyltransferase during GPI biosynthesis but also work independently to control lipid homeostasis (34, 35). Similarly, PIGN protein transfers phosphoethanolamine to the first mannosyltransferase during GPI biosynthesis. In the GPI biosynthetic pathway alone, two such proteins, Arv1 and PIGN, have been identified. Human and yeast Arv1 play a role not only in the delivery of the early GPI anchor intermediate to the first mannosyltransferase during GPI biosynthesis but also work independently to control lipid homeostasis (34, 35). Similarly, PIGN protein transfers phosphoethanolamine to the first mannosyltransferase during GPI biosynthesis. In the GPI biosynthetic pathway alone, two such proteins, Arv1 and PIGN, have been identified. Human and yeast Arv1 play a role not only in the delivery of the early GPI anchor intermediate to the first mannosyltransferase during GPI biosynthesis but also work independently to control lipid homeostasis (34, 35). Similarly, PIGN protein transfers phosphoethanolamine to the first mannosyltransferase during GPI biosynthesis. In the GPI biosynthetic pathway alone, two such proteins, Arv1 and PIGN, have been identified. Human and yeast Arv1 play a role not only in the delivery of the early GPI anchor intermediate to the first mannosyltransferase during GPI biosynthesis but also work independently to control lipid homeostasis (34, 35). Similarly, PIGN protein transfers phosphoethanolamine to the first mannosyltransferase during GPI biosynthesis. In the GPI biosynthetic pathway alone, two such proteins, Arv1 and PIGN, have been identified. Human and yeast Arv1 play a role not only in the delivery of the early GPI anchor intermediate to the first mannosyltransferase during GPI biosynthesis but also work independently to control lipid homeostasis (34, 35). Similarly, PIGN protein transfers phosphoethanolamine to the first mannosyltransferase during GPI biosynthesis. In the GPI biosynthetic pathway alone, two such proteins, Arv1 and PIGN, have been identified. Human and yeast Arv1 play a role not only in the delivery of the early GPI anchor intermediate to the first mannosyltransferase during GPI biosynthesis but also work independently to control lipid homeostasis (34, 35). Similarly, PIGN protein transfers phosphoethanolamine to the first mannosyltransferase during GPI biosynthesis.

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for the hyphal induction of the fungal strains at 37 °C. The bacterial strains were grown in Luria-Bertani (LB) broth at 37 °C.

Generation of deletion mutants

Gene disruption in C. albicans is based on PCR-mediated disruption and homologous recombination between target gene and selection marker disruption cassette (39). Lithium acetate method was used for C. albicans transformations (40). The CaGPII2Hz and CaGPII1Hz mutants were previously reported (13, 15). Using the same strategy, CaGPII1Hz, CaGPII3Hz, CaGPII15Hz, and CaERI1Hz mutants were made in BWP17 using the PCR-mediated disruption approach (39). For the generation of CaGPII1Hz mutant, the ARG4 marker was used (Table 2 and Table S1). CaGPII3Hz, CaGPII15Hz, and CaERI1Hz mutants were made using the HISI marker (Table 2 and Table S1). The colonies were confirmed by PCR using gene-flanking primers (Table S1) in all cases.

Generation of conditional null mutants

Conditional null mutants, Cagpi1 and Cagpi3, were also made using PCR-mediated promoter replacement approach as described previously for other GPI–GnT mutants (13, 15). The pMET3–URA3–GFP plasmid was used to replace the endogenous gene promoter with MET3 (41). Gene expression from the MET3 promoter can be repressed by controlling concentrations of Met/Cys in the growth medium (42).

Generation of revertant strains

Revertant strains were generated as reported previously (13). Revertant strains for all GPI–GnT subunits (with the exception of CaGPI3) were created in their respective heterozygous strains. For generating CaRAS1 and CaRAS2 revertant strains, pACT1–GFP vector carrying either CaRAS1 or CaRAS2 was used to transform Caras1/Caras2 mutant. The CaGPI3 revertant was generated by transforming the CaGPII3Hz mutant with the pADH1–CaGPI3 plasmid that had the target gene cloned under the control of the ADH1 promoter. Gene-specific as well as locus-specific primers were used to confirm the integration in all cases by PCR.

The plasmid pRAS1–CaRas1 with appropriate site-specific mutations and an endogenous RAS1 promoter (20) was used to create CaRas1 variant strains Caras1/Caras2/pRAS1–CaRas1, Caras1/Caras2/pRAS1–CaRas1C287S, and Caras1/Caras2/pRAS1–CaRas1C287S, Caras1/Caras2/pADH1–CaRas1G13V was generated by cloning Caras1G13V downstream of the basal pADH1 promoter. The plasmids were digested with PacI (except the pADH1–CaRas1G13V; plasmid that was digested with Stul) and used to transform the Caras1/Caras2 mutant. Integration of these plasmids occurred at the endogenous Caras1 locus (pADH1–CaRas1G13V; plasmid integrated at the RPS1 locus) and were confirmed by PCR.

Ura3 gene produces significant effects on cellular proteome and virulence of C. albicans (43). Thus, to rule out the effect of URA3 on other genes in revertant strains, appropriate control strains were generated by reintegration of one copy of URA3 at RPS1 locus in BWP17 and all mutant strains as necessary. Integration at the RPS1 locus in the positive clones was confirmed by PCR using GFP forward primer, FPGFP-BamHI, and RPS1 reverse internal primer, RPCaRPS1 (Table S1).

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**Table 1**

List of different plasmids used in the study

| Serial no. | Plasmids                     | Refs.          |
|------------|------------------------------|----------------|
| 1.         | pACT1–CaGPI                  | A. Brown, Aberdeen, UK |
| 2.         | pACT1–CaRAS1                 | This study     |
| 3.         | pACT1–CaRAS2                 | This study     |
| 4.         | pAP14 (pRAS1–CaRas1)         | 20             |
| 5.         | pAP22 (pRAS1–CaRas1C287S)    | 20             |
| 6.         | pAP23 (pRAS1–CaRas1C287S)    | 20             |
| 7.         | pADH1–CaRas1G13V            | 20             |
| 8.         | pACT1–CaGPII                 | This study     |
| 9.         | pACT1–CaGIPI2               | 13             |
| 10.        | pADH1–CaGIPI3               | This study     |
| 11.        | pACT1–CaGPI15               | This study     |
| 12.        | pACT1–CaGIPI19              | This study     |
| 13.        | pACT1–CaERI1                | This study     |
| 14.        | pRS–ARG4                    | 39             |
| 15.        | pMET3–URA3–GFP              | 41             |
| 16.        | pTZ–HIS1                    | 15             |

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Experimental procedures

**Materials**

All chemicals were purchased from Sigma, SRL, Merck, or Qualigen. Growth media were from HiMedia. Primers used in this study were synthesized by Xcelris or Sigma. DNA ladders and enzymes were from Bangalore Genei (India), Fermentas, or New England Biolabs. UDP-[6-3H]GlcNAc was from American Radiochemicals. Rabbit anti-His polyclonal antibody was purchased from Santa Cruz Biotechnology, goat anti-rabbit TRITC IgG, goat anti-mouse TRITC IgG, and goat anti-mouse FITC IgG were from Bangalore Genei; mouse anti-Ras mAb was from Merck Millipore; and anti-glucose-6-phosphate dehydrogenase (G6PDH) antibody was from Sigma, anti-phospho-p38 MAPK (Thr-180/Tyr-182) was from New England Biolabs. The production of polyclonal anti-A5s antibody in rabbit was outsourced to Merck India Ltd. (38). ER tracker BODIPY FL GL was purchased from ThermoFisher Scientific.

**Plasmids and strains**

The C. albicans parental strain used in this study is BWP17, and all mutants were made in this background (39). Plasmids and strains used in this study are listed in Tables 1 and 2. The primers used are listed in Table S1. WY-ZY4 strain (BWP17-CaRas1/CaRas2 mutant) was a kind gift from Prof. Yue Wang (Institute of Molecular and Cell Biology, National University of Singapore) (17). Plasmids expressing different mutant forms of CaRas1 were a kind gift from Prof. Deborah A. Hogan (Dept. of Microbiology and Immunology, Geisel School of Medicine, Dartmouth College, Hanover, NH) (20). The pACT1–GFP vector was a kind gift from Prof. Alistair Brown (Aberdeen Fungal Group, University of Aberdeen, Aberdeen, UK). The pADH1–GFP vector was made in the laboratory.

**Growth conditions of strains**

All fungal strains were grown at 30 °C in yeast extract/peptone/dextrose (YPD) media or synthetic defined dextrose minimal media (SD media) supplemented with specific amino acids based on the auxotrophy status of the strain. MET3-regulatable conditional mutants were grown in permissive (Met− Cys−) or repressive (5–10 mM Met/Cys) minimal media growth conditions for regulating gene expression from the MET3 promoter. Spider medium or YEPD medium was used for the hyphal induction of the fungal strains at 37 °C. The bacterial strains were grown in Luria-Bertani (LB) broth at 37 °C.
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**Table 2**

| Serial no. | Strains | Genotype | Refs. |
|-----------|---------|----------|-------|
| 1. | BWPI7 | CaGPI17::mRFP | This study |
| 2. | BWPI7–URA3 | CaGPI17::mRFP | This study |
| 3. | BWPI7–Caras1/Cara2 | CaGPI17::mRFP/URA3 | This study |
| 4. | BWPI7–Caras1/Cara2–URA3 | CaGPI17::mRFP/URA3 | This study |
| 5. | BWPI7–Caras1/Cara2 acting as a reporter under the constitutive ADH1 promoter | CaGPI17::mRFP/URA3 | This study |
| 6. | BWPI7–Caras1/Cara2–URA3 | CaGPI17::mRFP/URA3 | This study |
| 7. | BWPI7–Caras1/Cara2–URA3 | CaGPI17::mRFP/URA3 | This study |
| 8. | BWPI7–Caras1/Cara2–URA3 | CaGPI17::mRFP/URA3 | This study |
| 9. | BWPI7–Caras1/Cara2–URA3 | CaGPI17::mRFP/URA3 | This study |
| 10. | BWPI7–Caras1/Cara2–URA3 | CaGPI17::mRFP/URA3 | This study |

**Generation of overexpression strains**

Overexpression strains were generated as reported previously (13). CaGPI2 was overexpressed in each GPI–GnT heterozygous mutant using the plasmid pACT1–CaGPI2. Integration at the RPS1 locus in the positive clones was confirmed using CaGPI2 forward primer, FPCAPl–HindIII, and RPS1 reverse internal primer, RPCaRPS1 (Table S1).

Similarly, plasmids pACT1–CaGPI1, pADH1–CaGPI3, and pACT1–CaGPI15 were used to transform CaGPI2 heterozygous mutants to generate CaGPI2Hz/pACT1–CaGPI1, CaGPI2Hz/pADH1–CaGPI3, and CaGPI2Hz/pACT1–CaGPI15 strains. Positive colonies were confirmed by PCR using appropriate gene-specific and locus-specific primers (Table S1).

For tagging of CaGPI2 with His6 at its C terminus for immunofluorescence and FRET studies, primers FP CaGPI2–His and RP CaGPI2–mRFP were used (Table S1). CaGPI2 was tagged at its C terminus with mRFP for protein expression studies by a PCR-mediated approach. Primers FP CaGPI2–mRFP and RP CaGPI2–ARG4 (Table S1) were used to amplify mRFP–ARG4 cassette from pJET–mRFP–ARG4 plasmid, and the amplicon was used to transform relevant strains to produce BWPI7–CaGPI2–mRFP, CaGPI15Hz/CaGPI2–mRFP, and CaER11Hz/CaGPI2–mRFP, respectively.

**Generation of double heterozygous strains**

Double heterozygous mutant strains were generated as reported earlier (13). Briefly, appropriate selection markers (ARG4 or HIS1) were amplified by PCR using primers that included sequences of the gene to be disrupted as well as the nutritional marker and a heterozygous strain transformed with CaGPI2.
the amplicon (39). Selection was done on S.D. His<sup>-</sup>Arg<sup>-</sup> plates, and colonies were confirmed by PCR using gene-flanking primers (Table S1).

**Quantification of transcript levels through RT-PCR**

RNA was extracted as described previously (13). The cDNA was prepared from total RNA (3 μg) using cDNA preparation kit (Bio-Rad). Transcript levels of different genes were quantified using SYBR Green PCR Master Mix (Applied Biosystems) by comparative C<sub>T</sub> method using RT primers (Table S1) (44). GAPDH was taken as an internal control for all the experiments.

**Preparation of microsomes from C. albicans**

Microsomes from C. albicans were prepared using a protocol previously standardized in our lab (13) with minor modifications. Tris-EDTA (TE) buffer (50 mM Tris-HCl, pH 7.5, 10 mM EDTA) was used in place of TM buffer (Tris-MgCl<sub>2</sub>) to improve GPI–GnT activity. Briefly, cells were pelleted down from secondary cultures (200 ml) of each strain grown overnight at 30 °C, washed with ice-cold TE buffer, resuspended in 4 ml of same buffer, and lysed by vortexing with glass beads. Cell lysates were centrifuged at 1000 × g for 10 min at 4 °C, and the supernatant was centrifuged at 12,000 × g for 15 min at 4 °C. Ice-cold 8.0 mM CaCl<sub>2</sub> was added dropwise to the supernatant with constant stirring at 4 °C. The sample was centrifuged at 8000 × g and 4 °C for 10 min to pellet out microsomal membranes. The pellet was resuspended in 300 μl of ice-cold Tris-EDTA buffer (50 mM, pH 7.5) containing 10% glycerol, aliquoted in microcentrifuge tubes, and flash-frozen in liquid nitrogen for storage at −80 °C.

**GPI–GnT assay**

GPI–GnT assay was performed using microsomes (~1500 μg of total protein) as described previously (45). Briefly, the microsomes were incubated at 30 °C for 1 h with UDP-[6-<sup>3</sup>H]GlcNAc (1–2 μCi; 60 Ci/mmol) in presence of tunicamycin (0.25 mg/ml) in a 120-μl reaction volume. Lipids were extracted in 10:10:3 chloroform/methanol/water, dried under nitrogen, resuspended in water-saturated n-butanol, and partitioned against water. The butanol phase was dried, and glycolipids were dissolved in 10–20 μl of water-saturated butanol, spotted on HPTLC plates, and resolved in 65:25:4 chloroform/methanol/water. Radiolabeled glycolipids were detected and quantified by Bioscan AR-2000 TLC scanner using Winscan software. Because endogenous Gpi12 present in the microsomes deacetylate the product of the GPI–GnT reaction, both [6-<sup>3</sup>H]GlcNAc–PI and [6-<sup>3</sup>H]GlcN–PI are detected in our assays. The sum of the areas under the peaks for the two species obtained by integration is used to quantify the activity of the microsomal fraction. The total radioactive counts obtained in the control strain was taken as 100%, and activity of all other strains was calculated relative to the control strain. Heat-killed microsomes were used as the negative control.

**Western blottings**

All strains were grown overnight in YEPD media, and the secondary culture of all strains was grown in fresh media (100 ml) at 30 °C for 10–12 h. Lysates were prepared in ice-cold lysis buffer (150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, protease inhibitor mixture) using acid-washed glass beads. The cell lysate was centrifuged at 3000 rpm (for CaRas1 and Als5) or at 15,000 rpm (for p38 MAPK) for 10–15 min at 4 °C, and the supernatant was used for further analysis. Protein concentration of lysates was estimated using the BCA kit (BCA1; Sigma). Protein samples were separated by 12% SDS-PAGE (for CaRas1 and p38 MAPK) and 6% SDS-PAGE (for Als5). Protein was transferred to a polyvinylidene difluoride membrane at 50 V overnight followed by blocking with 5% skimmed milk in PBS with 0.05% Tween 20 or 5% BSA in PBS with 0.05% Tween 20 for 1 h. The blot was then probed with primary antibody (1:3000 for anti-Ras1 (05-516; Merck-Millipore), 1:500 for anti-phospho-p38 MAPK (Thr-180/Tyr-182; catalog no. 9211; CST), 1:1000 for anti-Als5 (GeNei), and 1:2500 for anti-G6PDH (A9521; Sigma)) at 4 °C overnight followed by washing three times with PBST (PBS + 0.05% Tween 20). Appropriate secondary antibodies (goat anti-rabbit IgG-HRP, sc-2004, Santa Cruz Biotechnology; goat anti-mouse IgG-HRP, 114068001A, GeNei) were then added at a dilution of 1:5000 for 2 h followed by three washes with PBST. The blot was developed on an X-ray film using ECL reagent (Luminata Forte, WBLUF0100, Merck-Millipore).

**Hyphal morphology**

Hyphal morphology was monitored in different strains as described previously (13). Equal numbers of cells (0.1 OD<sub>600</sub> mm) of each strain were spotted on YEPD medium plates and Spider medium plates and incubated at 37 °C. For hyphal growth in the presence of tamoxifen (52 μM), the plates were incubated at 30 °C for 10–15 days. Hyphal morphology was observed in Nikon SMZ1500 microscope. Images at different time intervals (7–10 days) were captured. Hyphal morphology in liquid media was also monitored in different strains as described previously (13).

**Heat-shock sensitivity assay**

Sensitivity to heat shock was monitored in different strains as described previously (16). Briefly, equal numbers of cells before and after heat treatment at 48 °C for 8 min were spotted on YEPD plates and incubated at 30 °C. Growth was monitored for 2–3 days, and images were captured at different time intervals.

**Immunofluorescence**

Primary cultures (10 ml) for each strain were grown until late log phase. Secondary culture cells were grown to early log phase and fixed at room temperature in 3.5% formaldehyde for 30 min. The cells were washed with 1× PBS, resuspended in lyticase buffer (Tris-Cl, pH 7.2, 0.1 M MgCl<sub>2</sub>, sorbitol), and incubated with 2 μl of lyticase (Sigma) for 20–30 min to digest cell walls. The cells were permeabilized with 1% Triton X-100 in 1× PBS for 15–20 min at room temperature. The cells were incubated in blocking solution (1% BSA in 1× PBS) for 30 min at room temperature. Primary antibody (anti-Ras1; 05-516; Merck-Millipore) was added to cells and incubated overnight at 4 °C. The cells were washed twice with 1× PBS and incubated with secondary antibody (goat anti-mouse IgG–TRITC; J. Biol. Chem. (2018) 293(31) 12222–12238 12235)
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11503801A; GeNei) for 2 h at room temperature. After washing twice with 1× PBS, the cells were resuspended in 80% glycerol. The cells were transferred onto polylysine-coated coverslips and mounted onto glass slides. Images of immunostained cells were recorded using a confocal microscope (Olympus Fluoview FV1000).

To visualize CaGpi2–mRFP expression levels in the C. albicans strains, because protein expression levels for GPI–GnT subunits are low (15, 46), immunofluorescence experiments with rabbit anti-mRFP antibodies (Abcam) and TRITC-labeled goat anti-rabbit IgG antibodies (GeNei) were performed. The fluorescence intensity in the cells was quantified using Olympus Fluoview software.

FRET analysis

For FRET, BWP17–CaGpi2–His6 was used as experimental strain, and BWP17–CaErg11–His6 was used as negative control strain. In experimental strain BWP17–CaGpi2–His6, CaRas1–FITC was the fluorescence donor, and CaGpi2–His6–TRITC was the fluorescence acceptor. In negative control strain BWP17–CaErg11–His6, CaRas1–FITC was the donor and CaErg11–His6–TRITC was used as the acceptor. The tagging did not affect the functioning of the protein, and the strains behave like the WT in each case. The primary antibody His probe sc-803 (Santa Cruz Biotechnology) was used to detect His6-tagged proteins. Samples were prepared as described under “Immunofluorescence.” Acceptor photobleaching method was used for estimating FRET (47). This method measures the increase in donor emission upon acceptor photobleaching. Secondary antibodies conjugated to the FITC (goat anti-mouse IgG–FITC, 112038001A, GeNei) and TRITC (goat anti-mouse IgG–TRITC, 11503801A, and/or goat anti-rabbit IgG–TRITC, 115028001A, GeNei) were used to generate the donor–acceptor pairs. An Olympus Fluoview FV1000 confocal microscope with a ×100 oil immersion plan–apochromat lens with a numerical aperture of 1.4 with ×3 optical zoom was used for image acquisition.

FITC was excited with a 488-nm HeNe laser at 8% power, and emission was detected after passage through a 500–530-nm bandpass filter. TRITC was excited with a 543-nm HeNe laser at 10% power, and emission was detected after passage through a 550–590-nm long-pass filter. Photobleaching of TRITC, the acceptor dye, was performed with a 543-nm HeNe laser at 100% power. A control neighboring cell was also monitored before and after photobleaching to ensure that the photobleaching was specific. Images were acquired in a specific order, i.e. an image of FITC alone (pre-bleach donor) and an image of TRITC alone (pre-bleach acceptor), followed by photobleaching of TRITC, another image of FITC alone (post-bleach donor), and an image of TRITC (post-bleach acceptor) to show complete photobleaching of the acceptor molecule. FRET intensity was expressed as the increase in fluorescence (post-bleach donor minus pre-bleach donor) in arbitrary units. FRET efficiency was obtained by the increase in fluorescence normalized to the fluorescence intensity of the pre-bleach donor. As a negative control, CaRas1 was detected using anti-Ras antibody and a FITC-labeled secondary antibody, whereas CaErg11–His6 was detected with anti-His antibody and a secondary antibody tagged with TRITC in BWP17–CaErg11–His6 strain and analyzed for FRET. Background corrections were done using an ROI outside the cell. Bleed-through corrections were done by monitoring fluorescence in the red channel in control samples that were stained with the FITC-labeled antibody alone.

Statistical significance of data

Unless otherwise stated, statistical significance of the data (p value) was calculated in SigmaPlot 8.0 using Student’s t test. The p value ≤0.05 is considered not significant and is depicted by n.s.; p value ≤0.01 is depicted by *; p value ≤0.001 is depicted by **; and p value ≤0.0001 is depicted by ***.

Macrophage-mediated killing and phagocytic assays

Maintenance of cell line—Murine alveolar macrophage (MH-S) cell line was grown in RPMI 1640 medium (Sigma) supplemented with 10% fetal bovine serum (Gibco, Life Technologies, Inc.), 50 µg/ml gentamicin sulfate, in a humidified atmosphere containing 5% CO2 at 37 °C. Exponentially growing cells were used in all experiments. Cell line was maintained as adherent cultures and subcultured by trypsinization. Live cell recoveries were counted using trypan blue exclusion method using hemocytometer.

CFSE labeling of C. albicans—C. albicans cells (100 million) were labeled with 10 µM CFSE (Sigma) for 30 min at room temperature followed by two washes with phosphate-buffered saline at 2000 × g for 5 min at 4 °C. Flow cytometric analysis by a FACSCalibur flow cytometer (BD Biosciences) at FL1 channel using CellQuest software indicated that by using this protocol more than 95% of the C. albicans was labeled with CFSE, and labeling was stable up to 18 h. For all FACS experiments, relative fluorescence intensity of 10,000 cells was recorded as single parameter histograms (log-scale 1024 channels, 4 decades).

Confocal microscopy—For visualization of uptake of C. albicans by MH-S cells, 0.3 million cells were cultured on glass coverslips overnight. Cells were then co-cultured with CFSE-labeled BWP17 and CaGPI2Hz cells at m.o.i. (1:5) for 3 h at 37 °C. Cells were then washed, fixed with 2% paraformaldehyde (PFA), followed by washing twice with quencher (ammonium chloride), and examined using a confocal laser-scan microscope (Olympus Fluoview FV1000). Five images each were captured having Z-sections (depths of 0.1 µm).

Co-culture assay in vitro—MH-S cells (0.3 million) cultured in 24-well plates were incubated with CFSE-labeled BWP17 and CaGPI2Hz for 3 h at different m.o.i. (1:5) for indicated time periods at 37 °C in a CO2 incubator. The cells were then harvested with PBS and fixed in 2% PFA. Uptake of stained BWP17 and CaGPI2Hz by MH-S cells was assessed by flow cytometry. For monitoring phagocytosis-independent uptake of C. albicans, MH-S cells were incubated for 1 h with Cyt D (Sigma) (2.5 µg/ml) to inhibit actin polymerization and cytoskeletal rearrangement (48). These cells were then washed and incubated with stained BWP17 and CaGPI2Hz strains for 3 h following similar conditions and procedures as mentioned above.

Macrophage-mediated killing of BWP17 and CaGPI2H—To determine the extent of killing and/or phagocytosis, 0.1 million MH-S cells were seeded in 48-well cell culture plates and kept for adherence followed by addition of C. albicans at m.o.i. 1:5
for 18 h. The mammalian cells were lysed using 0.2% Triton X-100 (Sigma), and the fungal cells were plated on YEPD plates and kept at 30 °C for 24 h. The number of C. albicans was determined as CFU/ml = number of colonies × dilution factor/volume of culture plate.

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