RESEARCH ARTICLE

Pga26 mediates filamentation and biofilm formation and is required for virulence in *Candida albicans*

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Abstract

The *Candida albicans* gene *PGA26* encodes a small cell wall protein and is upregulated during *de novo* wall synthesis in protoplasts. Disruption of *PGA26* caused hypersensitivity to cell wall-perturbing compounds (Calcofluor white and Congo red) and to zymolyase, which degrades the cell wall β-1,3-glucan network. However, susceptibility to caspofungin, an inhibitor of β-1,3-glucan synthesis, was decreased. In addition, *pga26*Δ mutants show increased susceptibility to antifungals (fluconazol, posaconazol or amphotericin B) that target the plasma membrane and have altered sensitivities to environmental (heat, osmotic and oxidative) stresses. Except for a threefold increase in β-1,6-glucan and a slightly widened outer mannoprotein layer, the cell wall composition and structure was largely unaltered. Therefore, Pga26 is important for proper cell wall integrity, but does not seem to be directly involved in the synthesis of cell wall components. Deletion of *PGA26* further leads to hyperfilamentation, increased biofilm formation and reduced virulence in a mouse model of disseminated candidiasis. We propose that deletion of *PGA26* may cause an imbalance in the morphological switching ability of *Candida*, leading to attenuated dissemination and infection.

Introduction

The clinical incidence of invasive fungal infections has increased during the last decades and represents a serious threat to human health (Pfaller & Diekema, 2010). The leading cause of mycoses in humans is *Candida albicans* (Chauhan *et al.*, 2006; Pfaller & Diekema, 2010). In immunocompetent individuals, *C. albicans* behaves as a commensal that occasionally may produce superficial lesions in the skin, oral cavity, gastrointestinal tract and vagina. However, in immunocompromised patients, deep-seated, invasive infections with a high mortality rate occur (Chauhan *et al.*, 2006; Pfaller & Diekema, 2010).

The cell wall of *C. albicans* is considered to be an important virulence factor because it is the first point of contact with the host and mediates antigenicity, adhesion, dimorphism and tissue invasion (Calderone & Fonzi, 2001; Casadevall, 2007). Being made up of an extracellular matrix consisting of covalently bound polysaccharides and proteins, it provides strength to resist high internal turgor pressures and protects the cell from harmful physical, chemical and biological external threats. The organization of the fungal cell wall has been characterized in the yeast *Saccharomyces cerevisiae*, and to a lesser extent in *C. albicans* and related species (Lesage & Bussey, 2006; Ruiz-Herrera *et al.*, 2006; Klis *et al.*, 2009). The cell wall polysaccharides in these species are β-1,3-glucans, β-1,6-glucan and chitin, together accounting for about 60% of the cell wall mass. The remaining 30–40% are mannosylated cell wall proteins (CWPs) that mainly occupy the outer layer of the wall and determine the cell surface properties (Ruiz-Herrera *et al.*, 2006; Klis *et al.*, 2009). The CWPs are covalently bound to

the glucans through different kinds of linkages. The main class of CWPs are glycosylphosphatidylinositol-modified proteins (Richard & Plaine, 2007; Klis *et al.*, 2009). *Candida albicans* is predicted to encode about 115 cell wall- and/or plasma membrane-localized glycosylphosphatidylinositol proteins, many with unknown functions (Richard & Plaine, 2007). Functional characterization of some of these glycosylphosphatidylinositol proteins showed involvement in processes related to cell wall integrity and synthesis, adaptation to oxidative stress and adherence (Plaine *et al.*, 2008; Klis *et al.*, 2009; Moreno-Ruiz *et al.*, 2009).

In a previous study, we presented transcriptomics data of conditions that led to reconstruction of cell walls by *C. albicans* protoplasts (Castillo *et al.*, 2006). Among the genes upregulated was *PGA26*, encoding a predicted glycosylphosphatidylinositol-modified CWP. Here, we aimed to investigate the role of Pga26 in cell wall functioning and its importance for virulence.

Materials and methods

Strains, media and growth conditions

Strains used in this study are listed in Supporting Information, Table S1. *Candida albicans* cells were routinely grown at 28 °C in YPD medium unless otherwise specified. Cells expressing PGA26-V5 under control of the *MAL2* promoter were grown with 2% maltose instead of glucose as the carbon source. Spider medium, YE-Pro (0.1% yeast extract, 0.01% proline), Lee's medium and YPD supplemented with 10% human serum were used for morphological studies (Lee *et al.*, 1975). SCAA medium (2% casamino acids, 0.17% yeast nitrogen base without amino acids and ammonium sulphate, 0.5% ammonium sulphate) was used for experiments with reintegrant strains where *PGA26* expression is under the control of the *PCK1* promoter. Media were solidified with 2% agar. *Escherichia coli* DH5α was used for plasmid propagation using standard DNA manipulation techniques.

Construction of V5-Pga26 expressing strain

To study the localization of Pga26, a V5 epitope was introduced five amino acids after the putative Kex2 protease site. The *PGA26-V5* fusion was constructed by PCR using Phusion Hot Start High-Fidelity DNA Polymerase (Finnzymes). First, two overlapping V5-containing fragments were obtained using primer pairs LIE5/LIE6 and LIE7/LIE8, these amplicons were then fused using primers LIE5 and LIE8. For primer details, see Table S1. The resulting product was cloned into pTZ57R/T (Fermentas), and the accuracy of the insert was verified by DNA sequencing. Engineered restriction sites in LIE5 (EcoRV) and LIE8 (MluI) enabled subsequent cloning of the construct in a EcoRV/MluI-digested CIp10-MAL2p plasmid to yield pMAL-PGA13-V5. CIp10-MAL2p (Backen *et al.*, 2000) contains the inducible *C. albicans MAL2* promoter, the gene *RPS10* and *C. albicans URA3* as the selection marker. Plasmid pMAL-PGA26-V5 was digested with NcoI and integrated into the *RPS10* locus of *C. albicans* CAI4 (strain ILE9).

Disruption of the *PGA26* gene

The *PGA26* gene was disrupted by replacing the part encoding amino acids 28–102 with a *hisG::URA3::hisG* fragment using the URA-blaster method (Fonzi & Irwin, 1993). Primer sequences used to amplify the 5′ and 3′ flanking regions are given in Table S1. Correct integration of the disruption cassette in the *PGA26* locus was verified by Southern blotting and PCR, and the absence of *PGA26* expression in the homozygous mutant was checked by reverse transcriptase (RT)-PCR (detailed in Fig. S1). Transformation of *C. albicans*, DNA and RNA isolation and RT-PCR was performed as described (Pedreño *et al.*, 2004). *URA3* was restored in the *ura3* genetic backgrounds (including the CAI4 parental strain) using the CIp10 plasmid, which integrates in the *RPS10* locus (Murad *et al.*, 2000).

Construction of PGA26 reintegrant strain

An amplicon of 396 bp containing the *PGA26* ORF was generated using the primer pair PBI26-5′ and PBI26-3′ (Table S1). Both primers contain engineered BglII restriction sites through which the fragment was cloned into plasmid pTZ57R (Fermentas). One clone that was verified by sequencing was cut with BglII and the resulting fragment was cloned into the BglII site of plasmid pBI-1 (Stoldt *et al.*, 1997). pBI-1 also contains functional *C. albicans URA3* and *LEU2* genes. The resulting integration plasmid, designated pBI-PGA26, was used to transform the homozygous *pga26*Δ null mutant strain (ILE4). Plasmid pBI-PGA26 was digested with BstEII, which cuts in the *LEU2* gene, and transformed into the Ura strain ILE4. Uridine-prototroph transformants were selected for further experiments. On plasmid pBI-PGA26, *PGA26* is placed behind the *PCK1* promoter, which is repressible by glucose. Thus, growing cells in media containing or lacking glucose as the carbon source allows repression or expression of *PGA26*, respectively. Expression of *PGA26* in the reintegrant was verified by RT-PCR (data not shown). As a negative control, the same procedure was carried out using plasmid pBI-1.

Cell wall isolation and composition

Cell walls were isolated essentially as described (Pedreño *et al.*, 2004). One-minute vortexing intervals with glass beads were repeated until > 99% of the cells were broken (microscopically checked). Cell fragments were resuspended and treated with 2% sodium dodecyl sulphate (SDS), 1 mM phenylmethylsulphonyl fluoride (PMSF) solution (1 mL/

100 mg wet weight walls) at 100 °C for 10 min, and then repeatedly washed with 1 mM PMSF. If to be used for wall protein extractions, the cell wall samples were lyophilized and stored at − 20 °C.

The relative amount of different cell wall components (protein, mannan, chitin and glucans) were determined as described (Dijkgraaf *et al.*, 1996; Moreno *et al.*, 2003; Pedreño *et al.*, 2004). All assays were performed in triplicate with samples obtained from three independent biological replicates.

Transmission electron microscopy (TEM)

Exponentially growing cells were harvested by centrifugation, fixed with 2.5% glutaraldehyde and washed with distilled water. Cells were postfixed with 2% osmium tetroxide and dehydrated in increasing concentrations of ethanol. Embedding was performed with LR-White resin at 60 °C for 24–48 h. Ultrathin sections were contrasted with 2% uranyl acetate and observed in a JEM 1010 TEM (JEOL) electron microscope at 60 kV. Values of outer mannoprotein layer width are the average of five cells, each measured at two different locations in the lateral cell wall.

CWP extractions and immunoblot analysis

HF-pyridine extraction to release glycosylphosphatidylinositol-modified CWPs, and subsequent trifluoromethanesulfonic acid (TFMS) treatment for deglycosylation, was performed as described (Castillo *et al.*, 2008).

Protein separation on SDS-10% polyacrylamide gels and subsequent immunoblotting was performed using the standard methodology (Pedreño *et al.*, 2004). The Western blot was probed with anti-V5 (1 : 500) monoclonal antibody (Invitrogen), followed by incubation with rabbit anti-mouse secondary antibodies (Bio-Rad) and enhanced chemiluminescence detection.

Cell wall stress and other stress-related phenotypic assays

Sensitivity to zymolyase was measured by incubating log phase cells, adjusted to a starting $OD_{600\,nm}$ of ~0.6, in 10 mM Tris-HCl, pH 7.5, containing 50 μg zymolyase 20 T mL^{-1}, and following the decrease in $OD_{600\,nm}$ in time.

Sensitivity to Calcofluor white (CFW) and Congo red (CR) was examined by spotting 3 μL of cells, (adjusted to $OD_{600\,nm} = 1$), and 10-fold serial dilutions thereof, on solid YPD containing different concentrations of the chemical tested. Growth was monitored after 3 days of incubation at 28 °C. Similarly, sensitivity to oxidative stress was determined on YPD plates containing H_2O_2, and osmotic stress on plates containing $CaCl_2$. Sensitivity to temperature upshift was assayed by keeping cells at 55 °C for different periods of time before spotting.

Minimal inhibitory concentrations (MICs) of antifungal drugs

MIC values of clinically used antifungal drugs (caspofungin, fluconazole, posaconazole, amphotericin B) were determined following CLSI guidelines (2008).

Quantification of hyphal formation

Log phase cells grown in YPD at 28 °C were inoculated at a starting $OD_{600\,nm}$ of 0.2 in prewarmed liquid YPD supplemented with 10% filter-sterilized human blood serum and incubated at 37 °C. Germ tube formation was quantified using a haemocytometer. At least 200 cells were counted for each sample. If required, cell clumps were dispersed by mild sonication (10–15 s) before counting.

Biofilm formation

Cells were grown overnight in liquid YPD, washed with phosphate-buffered saline (PBS) and resuspended in Roswell Park Memorial Institute (RPMI)-1640 medium buffered with 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid and supplemented with 0.35 mg mL^{-1} L-glutamine. Cell suspensions (100–500 μL) at a concentration of 1×10^6 cells mL^{-1} were pipetted into polystyrene flat-bottom 96-well plates to allow biofilm formation for 4, 12, 24 or 48 h at 37 °C. After incubation, biofilms were washed with PBS to remove nonadherent cells. Biofilm formation was quantified by staining with crystal violet and measuring the $OD_{570\,nm}$ (O'Toole *et al.*, 1999).

Determination of biofilm structure and thickness on Thermanox™ coverslips by confocal scanning laser microscopy (CSLM) after 24 h in RPMI medium was performed as described by Pérez *et al.* (2011) using a LSM510 META laser scanning microscope (Zeiss) with built-in software for imaging and measurements. Presented thickness values are the average of three biological replicates each measured in three different areas of the biofilm.

Virulence testing

Log-phase *C. albicans* cells were harvested, washed and resuspended in PBS. Female, 6–8-week-old immunocompetent Swiss-CD mice of about 20 g were intravenously challenged with 1.5×10^6 cells. Each group consisted of 10 mice. Viability was monitored over a period of 30 days.

Results

Deletion of *PGA26* affects cell wall integrity

The *PGA26* gene in *C. albicans* encodes a putative glycosylphosphatidylinositol-modified wall glycoprotein that is upregulated in protoplasts after enzymatic hydrolysis of the

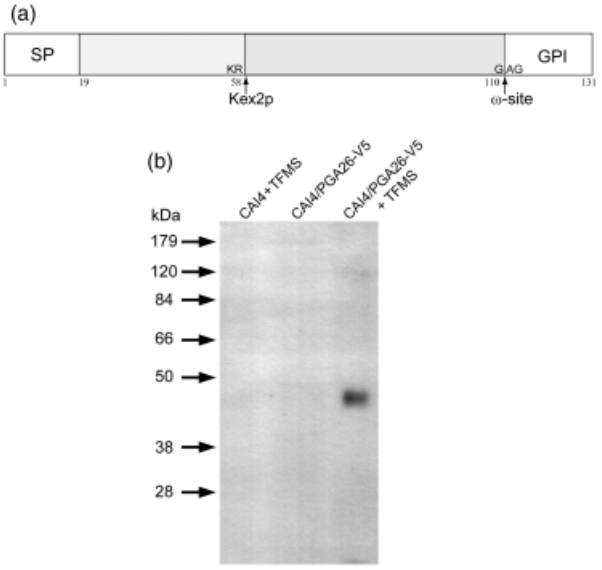


Fig. 1. Pga26 is a predicted glycosylphosphatidylinositol (GPI) protein and is localized in the cell wall. (a) The primary structure of Pga26 precursor. Post-translational processing sites are indicated. SP, signal peptide; GPI, signal peptide for GPI anchoring; ω-site, cleavage site of GPI anchor. (b) Immunoblot of HF-pyridine extracted CWP fractions from strains CAI4 (left lane) and ILE9 (CAI4, but *RPS10/rps10*::pMAL-PGA26-V5, middle and right lanes), probed with anti-V5 monoclonal antibodies. A TFMS treatment (left and right lanes) was performed to remove glycosylation. Sizes of molecular weight standards are indicated on the left.

cell wall (Castillo *et al.*, 2006). As the cell wall plays a pivotal role in primary host–pathogen interactions, we aimed to shed light on the physiological function of Pga26 through phenotypic analysis.

Discounting signal peptides for entering the endoplasmic reticulum and glycosylphosphatidylinositol anchoring at both ends of the protein, the primary structure of Pga26 encompasses only 92 amino acids, the first 40 of which may be cleaved off by endoproteolytic Kex2 processing (Fig. 1a). Before this study, Pga26 had not been detected yet in cell wall preparations. The small protein is probably highly glycosylated; the 92 residues include two putative sites for *N*-glycosylation and 32 serines and threonines (potential *O*-glycosylation sites). The short sequence, the lack of tryptic cleavage products and predicted heavy glycosylation probably hamper the identification of the natural protein in the cell wall. However, using a fusion construct (V5-tag insertion after the predicted Kex2 site), in combination with (partial) deglycosylation of HF-pyridine-extracted CWPs by TFMS treatment, the V5-Pga26 protein was detected as a 45-kDa protein species (Fig. 1b). This strongly supports our idea that Pga26 is a glycosylphosphatidylinositol-modified CWP and is highly glycosylated. Our further studies on Pga26 focused on phenotypic analysis of deletion mutants.

First, the overall fitness of *pga26*Δ mutants was tested under standard laboratory conditions (YPD and SCAA media at 28 °C), which showed comparable growth characteristics for mutant and wild type (WT). Next, possible alterations in cell wall composition and integrity caused by deletion of *PGA26* were analysed. Spot assays with the cell wall-perturbants CFW and CR showed a clear hypersensitivity of *pga26*Δ mutants to both compounds (Fig. 2a). The exacerbation of the effects of these cell wall perturbants in the mutants suggests that Pga26 is important for maintaining wall integrity. The hypersensitivity of the homozygous *pga26*Δ mutant to CFW and CR (and other tested antifungals) was alleviated by reintegration of the *PGA26* gene (Fig. S2). Deletion of *PGA26* also increased the sensitivity to the endo-β-1,3-glucanase zymolyase (Fig. 2b). This might be caused either by structural changes in the β-1,3-glucan network or by alterations in the amount of the different cell wall polymers. Comparison of the wall composition in parental and mutant strains showed a threefold relative increase of β-1,6-glucan in the *pga26*Δ mutant strains. Taking into account possible small inaccuracies inherent to these rather difficult biochemical assays, the levels of the other measured wall components did not seem clearly altered (Fig. 2c). Analysis of cell wall ultrastructures using TEM indicated that the *pga26*Δ mutant has a fairly normal cell wall organization, but the outer mannoprotein layer is slightly thicker (46.1 ± 2.2 nm in *pga26*Δ vs. 36.6 ± 1.8 nm in WT) and seems less electron dense as compared with the WT strain (Fig. 2d).

Deletion of *PGA26* alters sensitivity to environmental stress and antifungals

Viability of the *pga26*Δ mutant upon challenges to environmental stress conditions (Fig. 3) and antifungal drugs (Table 1) was tested. *pga26*Δ cells exhibited an increased capacity to withstand heat shock (55 °C for 30 min) and osmotic stress (0.6 M $CaCl_2$). This was slightly unexpected because cell wall defects usually lead to increased vulnerability to chemical or physical insults. The *pga26*Δ mutant cells showed a slight loss of viability after exposure to severe oxidative stress (50 mM H_2O_2), whereas a lower concentration of 5 mM H_2O_2 had a negligible effect. As demonstrated in other genetic backgrounds, 5 mM H_2O_2 is a weak oxidant concentration for *C. albicans* (Alvarez-Peral *et al.*, 2002).

Disruption of several genes involved in wall architecture in *C. albicans* caused alterations in resistance to antifungal drugs (Plaine *et al.*, 2008; Moreno-Ruiz *et al.*, 2009; Moreno *et al.*, 2010). We determined MICs of clinically relevant antifungals (Table 1). For the azoles, fluconazole and posaconazole, and the polyene amphotericin B, the MICs for the *pga26*Δ mutant were lower than for the wild type, indicating that deletion of *PGA26* caused an increase in the membrane-associated antifungal drug sensitivity. The *pga26*Δ mutant showed a 20-fold increase in the MIC for

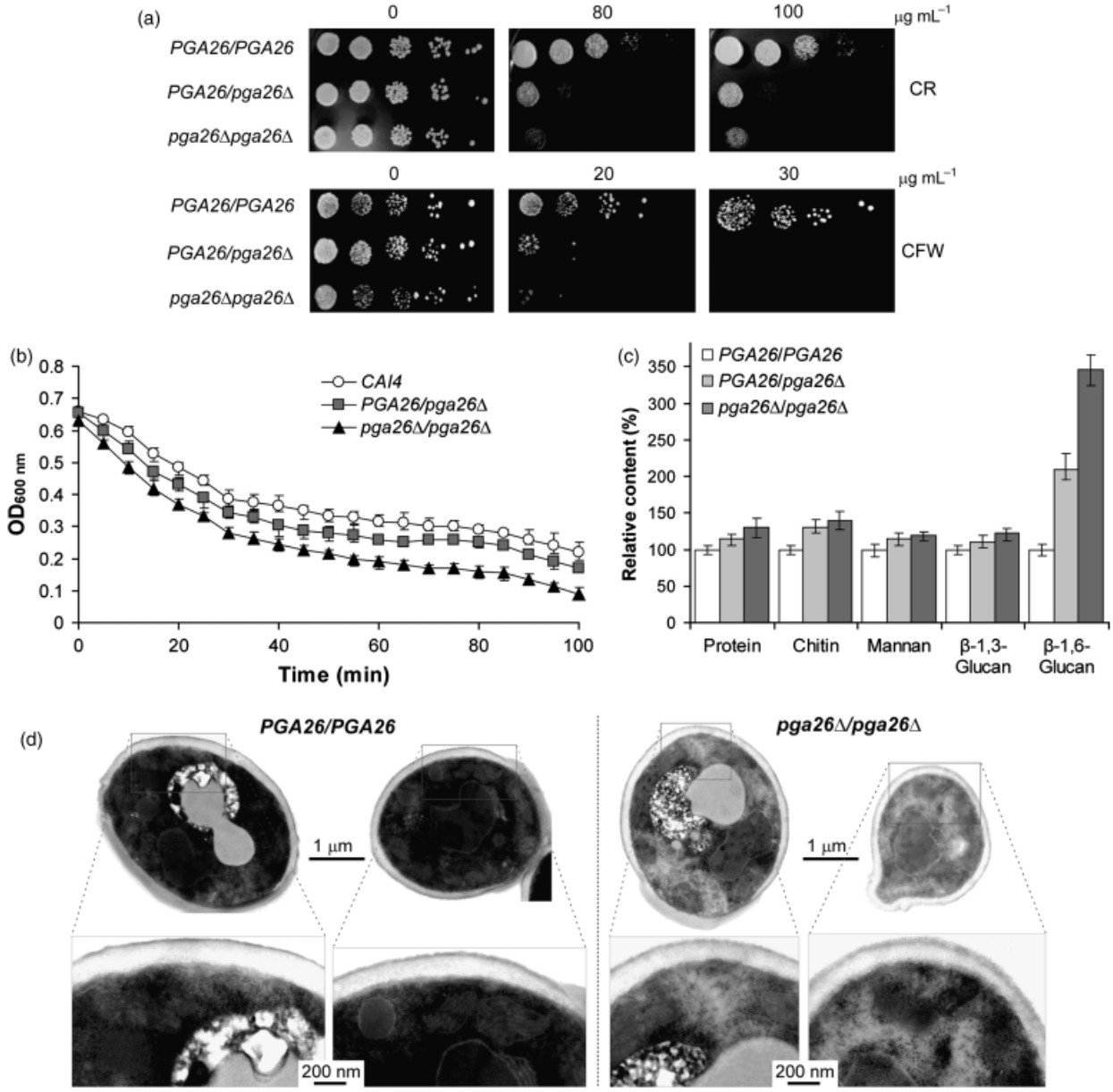


Fig. 2. Pga26 is involved in cell wall integrity. (a) Sensitivity to CR and CFW. Serial 10-fold dilutions of log phase cells were spotted on YPD containing CR or CFW at the concentrations indicated. Plates were photographed after 3 days of incubation at 28 °C. (b) Sensitivity to zymolyase. Log phase cells were incubated with 50 μg mL^{-1} zymolyase 20T and the decrease in absorbance was monitored. (c) Cell wall composition. Data represent the mean ± SD of three independent experiments. (d) TEM images showing cell wall structures in representative *pga26*Δ mutant and WT cells.

the echinocandin caspofungin. As echinocandins inhibit *de novo* β-1,3-glucan synthesis, this supports the idea that deletion of *PGA26* affects cell wall integrity and architecture.

Deletion of *PGA26* enhances filamentation and biofilm formation

Rapid switching between yeast and hyphal morphologies is generally regarded as an important virulence factor in *C. albicans* (Sudbery *et al.*, 2004). Different filamentation-inducing conditions were tested to analyse whether Pga26 influences the yeast-to-hyphae transition. Mutant colonies grown on solid Spider and YE-Pro media appeared to be enlarged and wrinkled, and showed more profound filamentation than the parental strain on higher magnification images (Fig. 4a). On Lee's medium, even at 28 °C, or 37 °C, in the absence of proline and biotin (two specific inducers), filamentation of *pga26*Δ mutants occurred readily (within 1 h), whereas the parental strain remained essentially in the yeast form (Fig. 4b). Filamentation at 37 °C was

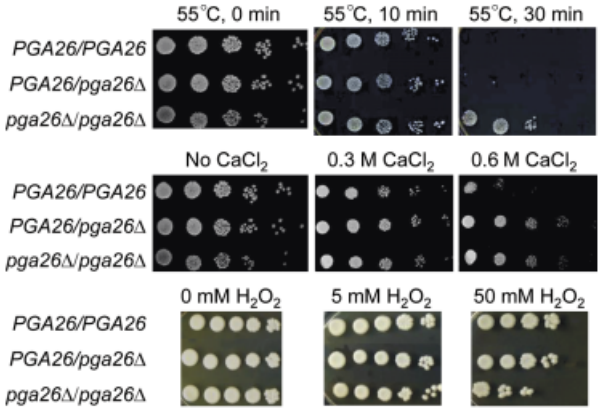


Fig. 3. Deletion of *PGA26* alters the sensitivity to environmental stresses. Sensitivity to 10 or 30 min heat shock (55 °C), and exposure to osmotic ($CaCl_2$) and oxidative (H_2O_2) stress at indicated concentrations was tested.

Table 1. *In vitro* susceptibility of the *pga26*Δ deletion mutant to antifungal drugs

	MICs (μg mL^{-1})	
	PGA26/PGA26	*pga26Δ/pga26Δ*
Fluconazole	0.250	0.125
Posaconazole	0.125	0.031
Amphotericin B	0.125	0.062
Caspofungin	0.016	0.250

Values are representive outcomes of at least three independent experiments using a broth microdilution procedure.

accompanied by clumping and flocculation. Quantitative measurements of germ-tube formation in liquid YPD medium supplemented with 10% human serum showed that the *pga26*Δ null mutant was almost completely filamented (*c.* 98%), already after 15 min of induction (Fig. 4c), while the amount of germ tubes in the parental strain was about 30%. The heterozygous mutant showed an intermediate level of filamentation. Even in the absence of serum, the *pga26*Δ mutant, but not parental cells, exhibited a significant degree of hyphae formation at 37 °C, reaching 85%.

The ability to form biofilms also appeared to be positively affected by disruption of the *PGA26* gene. In a crystal violet staining assay, the homozygous null mutant showed an almost twofold increase in biofilm formation after 24 h (Fig. 5a). After 48 h, the amount of biofilm measured was decreased. Apparently, ageing of a *C. albicans* biofilm is accompanied by partial dissociation or detachment of biofilm biomass, which, subsequently, is removed by washing (Pérez *et al.*, 2006). In a complementary experiment using confocal microscopy (CSLM), measurements of biofilm thickness after 24 h also indicated increased biofilm formation of the *pga26*Δ mutant (210 ± 20 μm) as compared with the parental strain (150 ± 17 μm) (Fig. S3).

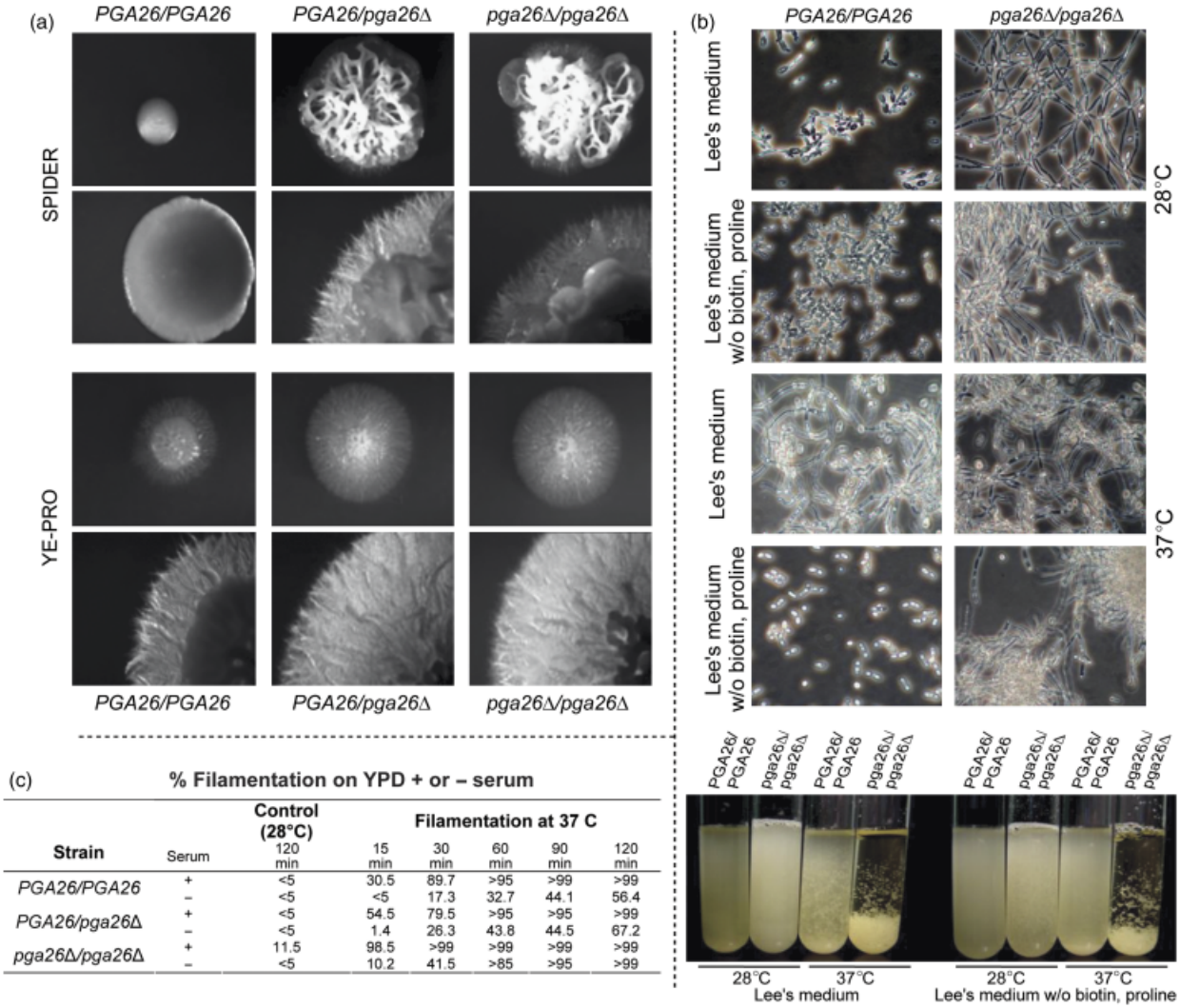
PGA26 is required for virulence

Possible involvement of *PGA26* in virulence was tested in a mouse model of systemic infections. After 27 days, all mice infected with the parental strain had died while $> 70\%$ of the mice injected with the *pga26*Δ null mutant were still alive (Fig. 5b). The heterozygous *PGA26*/*pga26*Δ mutant showed intermediate virulence.

Discussion

Fungal cell walls are essential organelles that determine cell shape and protect against physical injury and other stresses imposed by the external environment. In pathogenic fungi, the cell wall is important for the primary interactions with host tissues and thus crucial for the establishment of infections (Ruiz-Herrera *et al.*, 2006; Klis *et al.*, 2009). However, the molecular mechanisms underlying the dynamic roles of the cell wall in virulence remain mostly unresolved. In a previous study, we have identified the gene *PGA26*, encoding a small predicted glycosylphosphatidylinositol protein, as being upregulated during the *de novo* wall synthesis by *C. albicans* protoplasts (Castillo *et al.*, 2006). Orthologs of *PGA26* with unknown function are also present in other pathogenic *Candida* species such as *Candida dubliniensis* and *Candida tropicalis*.

Our results showed that Pga26 is localized in the cell wall, and that disruption of *PGA26* caused phenotypes that strongly suggest an involvement of the gene product in cell wall construction. Mutants were hypersensitive to zymolyase, CR and CFW. On the other hand, a decreased susceptibility was observed towards caspofungin, which inhibits β-1,3-glucan synthesis. Deletion of *PGA26* did not result in major structural or compositional changes of the cell wall, although TEM pictures showed a slightly widened outer mannoprotein layer and we found a relative increase in β-1,6-glucan. Taken together, and also keeping in mind its induced expression during wall recovery in protoplasts, the function of Pga26 in cell wall construction seems to reinforce newly synthesized wall structures. A similar role in ensuring cell wall stability has recently been proposed for two other glycosylphosphatidylinositol-modified CWPs in *C. albicans*, Pga59 and Pga62, which upon deletion show comparable phenotypes for caspofungin, CR and CFW (Moreno-Ruiz *et al.*, 2009). Interestingly, sequences in Pga59 and Pga62, which might be involved in cell wall reinforcement through interactions with wall polysaccharides, show weak similarity to the Pga26 sequence. To compensate for loss of skeletal strength, increased levels of chitin in the cell wall are often observed in cell wall-related mutants (Kapteyn *et al.*, 2000; Klis *et al.*, 2009; Moreno-Ruiz *et al.*, 2009). In *pga26*Δ deletion mutants, we measured only a slight increase in chitin. Also frequently observed in cell wall mutants are pleiotropic phenotypes such as altered



Strain	Serum	Control (28°C) 120 min	Filamentation at 37 C 15 min	30 min	60 min	90 min	120 min
PGA26/PGA26	+	<5	30.5	89.7	>95	>99	>99
	–	<5	<5	17.3	32.7	44.1	56.4
PGA26/pga26Δ	+	<5	54.5	79.5	>95	>95	>99
	–	<5	1.4	26.3	43.8	44.5	67.2
pga26Δ/pga26Δ	+	11.5	98.5	>99	>99	>99	>99
	–	<5	10.2	41.5	>85	>95	>99

Fig. 4. Deletion of *PGA26* leads to hyperfilamentation. (a) Exponentially growing cells were plated on Spider and YE-Pro media and incubated at 37 °C. Colony morphology was inspected microscopically after 7 days on higher magnification images of the edges of the colonies (bottom panels). (b) Filamentation in complete Lee's medium, or in the same medium without biotin and proline, at either 28 or 37 °C after 1 h of incubation. Flocculation in glass tubes (bottom panel) was monitored 1 min after stopping the agitation. (c) Germ tube formation in YPD at 37 °C, with or without addition of human blood serum, was quantified at the indicated time points. A control at 28 °C was measured at 120 min.

sensitivities to conditions that affect the biosynthesis and integrity of the plasma membrane. In line with this, *PGA26*-disrupted mutants showed increased sensitivities to membrane-targeting antifungal drugs (amphotericin B and azoles).

Most manifestations of candidiasis appear to be associated with the establishment of biofilms. In clinical settings, *C. albicans,* often in conjunction with other species of the human microbial community, readily forms biofilms on tissues or medical devices and shows morphologies ranging from blastospores to filaments. Notably, filamentation seems to play an important role in the development of *C. albicans* biofilms (Garcia-Sanchez *et al.*, 2004). In *pga26*Δ mutants, we observed a twofold increase in biofilm mass formed after 24 h of incubation under biofilm-inducing conditions. Furthermore, *pga26*Δ mutants showed rapid and strongly increased myceliation. Even under some non-inducing conditions, *pga26*Δ cells quickly developed germ tubes. These results suggest that a lack of Pga26 introduces cell wall defects that shift the morphological balance towards the formation of hyphae. Of note, analysis of *PGA26* expression in wild-type strains during the transition from yeast to hyphae showed that its expression seems morphology-independent (results not shown), in agreement with genome-wide profiling studies (Nantel *et al.*, 2002). As β-glucan is a component of the extracellular biofilm matrix, alterations in wall incorporation or secretion of β-glucans might also influence the biofilm-forming capacity (Nett *et al.*, 2007). In *pga26*Δ cell walls, the content of the major

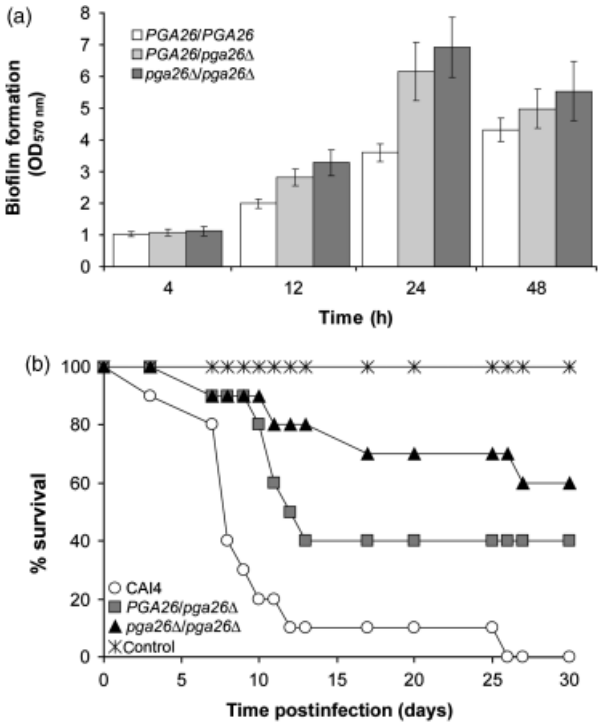


Fig. 5. Absence of Pga26 increases the biofilm-forming capacity, but reduces virulence in mice. (a) Biofilm formation on polystyrene was monitored at different time intervals. The amount of biofilm formed was measured using crystal violet staining and measuring the $OD_{570\,nm}$. Presented values are averages ± SD of three independent experiments. (b) Pga26 is required for full virulence. Systemic mouse tail vein-infection model using groups of 10 SWISS CD-1 mice infected with inocula containing 1.0×10^6 *Candida albicans* cells. The control is injection with buffer without *C. albicans* cells.

component β-1,3-glucan appears normal, whereas the less abundant β-1,6-glucan shows a threefold relative increase in mass.

The *pga26*Δ mutant displayed a profound reduction in virulence in a mouse model of disseminated candidiasis (Fig. 5b). Such attenuated infectivity resembles that of knockout mutants of other CWPs, such as Pga29 (De Boer *et al.*, 2010), Als proteins (Hoyer *et al.*, 2008) and Pga31 and Pga13 (S. Gelis, unpublished data). As morphological switching is considered an important virulence factor, the observed hyperfilamentation in combination with attenuated virulence is interesting. In invasive *C. albicans* infections, the hyphal growth form stimulates biofilm formation and is crucial for tissue invasion and escape from phagocytic immune cells. On the other hand, the yeast form is more suited and important for dissemination through blood vessels. Indeed, strains that are locked in either of the two growth forms are avirulent (Lo *et al.*, 1997; Murad *et al.*, 2001). We propose that the cell wall defect caused by deletion of *PGA26* strongly stimulates hyphal growth, thereby affecting the vital morphological balance between yeast and hyphal forms that is needed for the establishment of invasive infections.

In conclusion, our results point to an important role for Pga26 in cell wall construction and integrity, with large implications for morphological development and fungal virulence.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Construction and verification of the *pga*26Δ null mutant.

Fig. S2. Reintegration of *PGA*26 restores drug sensitivity phenotypes.

Fig. S3. Deletion of *PGA*26 leads to increased thickness of biofilms.

Table S1. *Candida albicans* strains and oligonucleotide primers used in this study.

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