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Ppg1, a PP2A-Type Protein Phosphatase, Controls Filament Extension and Virulence in *Candida albicans*

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***Candida albicans*, a major human fungal pathogen, is the primary cause of invasive candidiasis in a wide array of immunocompromised patients. *C. albicans* virulence requires the ability to undergo a reversible morphological transition from yeast to filaments in response to a variety of host environmental cues. These cues are sensed by the pathogen and activate multiple signal transduction pathways to induce filamentation. Reversible phosphorylation events are critical for regulation of many of these pathways. While a variety of protein kinases are known to function as components of *C. albicans* filamentous growth signal transduction pathways, considerably little is known about the role of phosphatases. Here we demonstrate that *PPG1*, encoding a putative type 2A-related protein phosphatase, is important for *C. albicans* filament extension, invasion, and virulence in a mouse model of systemic candidiasis. *PPG1* is also important for downregulation of *NRG1*, a key transcriptional repressor of *C. albicans* filamentous growth, and is shown to affect the expression of several filament-specific target genes. An epistasis analysis suggests that *PPG1* controls *C. albicans* filamentation via the cyclic AMP-protein kinase A (cAMP-PKA) signaling pathway. We demonstrate that Ppg1 possesses phosphatase activity and that a *ppg1* catalytic mutant shows nearly equivalent filamentation, invasion, and virulence defects compared to those of a *ppg1*Δ/Δ strain. Overall, our results suggest that phosphatases, such as Ppg1, play critical roles in controlling and fine-tuning *C. albicans* filament extension and virulence as well as signal transduction pathways, transcriptional regulators, and target genes associated with these processes.**

Candida albicans, the most commonly isolated human fungal pathogen, is the leading cause of candidiasis worldwide and the fourth leading cause of hospital-acquired bloodstream infections in the United States (1–3). As a commensal, *C. albicans* is part of the normal microbiota of the oral cavity and gastrointestinal and genitourinal tracts of healthy individuals (3). Immunocompromised individuals, such as AIDS and cancer patients, neonates, organ transplant recipients, and patients with indwelling catheters, can develop disseminated candidiasis, a systemic form of the disease with an approximately 40% mortality rate (2, 4, 5). *Candida* infections have become more difficult to treat due to a limited number of antifungal therapies and increased frequency of drug-resistant isolates (6, 7).

C. albicans possesses multiple virulence properties, including the ability to undergo a reversible transition from yeast (single ovoid, budding cells) to pseudohyphal and hyphal filaments (elongated cells attached end to end) (3, 8, 9). This morphological transition allows for efficient tissue invasion, immune evasion (including macrophage lysis), and biofilm formation (3, 10–14). *C. albicans* strains that are unable to undergo a reversible yeast-filament transition are highly attenuated for virulence (12, 15, 16). The *C. albicans* morphological transition is known to be induced by a variety of cues in the host environment, including serum, temperature of 37°C, neutral pH, high CO_2, low O_2, embedded/matrix conditions, and nitrogen and carbon starvation, as well as *N*-acetylglucosamine (3, 17–19). These environmental stimuli are sensed by the pathogen and cause activation of a variety of signal transduction pathways that target specific filamentous growth transcriptional regulators (20, 21). The regulators, in turn, direct the activation of a filamentous growth transcriptional program consisting of genes specifically important for filament development as well as other virulence-related processes (22, 23). A conserved mitogen-activated protein (MAP) kinase signaling pathway responds to a variety of environmental conditions, including nitrogen starvation, and results in activation of the Cph1 transcription factor (21, 24). A Ras-cyclic AMP-protein kinase A (Ras-cAMP-PKA) pathway is also known to respond to serum, 37°C, and high CO_2 and directs activation of Efg1, a key transcriptional regulator of the filamentous growth program (21, 25, 26). Although the MAP kinase and Ras-cAMP-PKA pathways are the best characterized and appear to respond to a majority of host environmental cues, additional signaling pathways have also been identified, which direct *C. albicans* filamentation in response to neutral pH, GlcNAc, and embedded/matrix conditions (20, 21).

While a number of protein kinases in the MAP kinase and Ras-cAMP-PKA pathways function as critical filamentous growth signaling components, considerably less is known about the role of phosphatases in controlling *C. albicans* morphology. However, sequence and domain searches have indicated the presence of 28 putative phosphatases in *C. albicans*, based on comparison with *Saccharomyces cerevisiae* homologs (27). Phosphatases can regulate the activity of filamentous growth signaling pathways by dephosphorylating the kinases. As an example, a tyrosine phosphatase, Cpp1, is a repressor of filamentation and appears to function by inhibiting the MAP kinase pathway component Cek1 (28). *PTC8*, a member of the protein phosphatase M (PPM) family

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(Mg^{2+} dependent), is induced in response to growth in serum at 37°C and is important for filamentation (29) but has not been linked to any known filamentous growth signaling pathways. The Sit4 phosphatase has previously been shown to affect *C. albicans* filamentation and appears to function by controlling protein translation, cell wall biogenesis, and osmosensing, targeting the Hog1 MAP kinase pathway (30). While the *sit4*Δ/Δ mutant is highly attenuated for virulence in a mouse model of systemic candidiasis, it is unclear whether this defect can be attributed to reduced filamentation *per se* or a variety of other pleiotropic phenotypes. In addition, expression of the large majority of filament-specific genes was not affected in the *sit4*Δ/Δ mutant. Finally, another important phosphatase, calcineurin, plays a key role in controlling *C. albicans* virulence and antifungal susceptibility but does not appear to be required for the yeast-filament transition (31–33).

In order to gain more insight into the poorly understood role of phosphatases in controlling *C. albicans* morphology and filamentous growth signaling pathways, we examined morphology data collected from a previous large-scale homozygous mutant analysis (34). One particular putative phosphatase mutant identified by this analysis, *ppg1*Δ/Δ, was not defective for proliferation but appeared to have a significant defect in morphogenesis and showed reduced abundance in mouse kidneys when combined with a large pool of bar-coded mutants. *PPG1* encodes a putative type 2A serine/threonine phosphatase. Protein phosphatases in this subclass are known to regulate a variety of key cellular processes in many organisms, including cell wall integrity, actin cytoskeleton organization, auxin signaling, and polar movement in plants (35–37). In *S. cerevisiae*, the *PPG1* ortholog is required for glycogen accumulation (38). Here, we demonstrate that *C. albicans PPG1* plays an important role in controlling filament extension, invasion, and virulence. In addition, we examine the specific requirement of Ppg1 phosphatase activity for these processes as well as the relationship between *PPG1* and several known *C. albicans* filamentous growth target genes and signal transduction pathways as well as a key transcriptional regulator.

MATERIALS AND METHODS

Strains and DNA constructions. The genotypes for all strains used in this study are shown in Table S1 in the supplemental material. The wild-type (WT) control strain (DK318) has been described previously (39). A fusion PCR strategy (40) was used to generate the *ppg1*Δ/Δ strain. *PPG1* 5′ and 3′ flanking fragments were generated using primers MAO7 and MAO8 for the downstream flank as well as primers MAO5 and MAO6 for the upstream flank (all primers used in this study are described in Table S2 in the supplemental material). A second round of fusion PCR was then performed using the *PPG1* 5′ and 3′ flanks, as well as *HIS1* and *LEU2* markers, generated with primers RZO37/RZO38 and plasmids pSN52 and pSN40 (40), to yield *ppg1*Δ::*HIS1* and *ppg1*Δ::*LEU2* PCR products. SN152 (40) was transformed with *ppg1*Δ::*HIS1* as well as wild-type alleles of *C. albicans ARG4* (made using primers DKO400/DKO401) and *LEU2* (made using primers DKO404/DKO405) in order to generate the *ppg1*Δ/+ strain (MAY7). SN152 was sequentially transformed with *ppg1*Δ::*HIS1*, *ppg1*Δ::*LEU2*, and *C. albicans* wild-type *ARG4* to generate the *ppg1*Δ/Δ strain (MAY34). We used an *SAT1* split-marker approach (41) to construct the *ppg1*Δ/Δ::*PPG1* add-back strain (MAY50). Briefly, a PCR product containing *PPG1* 5′ upstream sequences, open reading frame (ORF), and terminator (T) was amplified from SC5314 genomic DNA using primers MAO155/MAO157. The 3′-flanking sequences downstream of the *PPG1* terminator were also amplified with primers MAO158/MAO159. Partial, overlapping *SAT1* cassettes were generated by PCR using plasmid pSFS2 (42) as a template and primers GSO128/GSO129 for the 5′ fragment and GSO127/GSO130 for the 3′ fragment. The 5′ *PPG1* flank + ORF + T as well as the 5′ partial *SAT1* cassette were digested with KpnI and ligated together. A similar ligation reaction was used to join the 3′ *PPG1* terminator flank and the 3′ partial *SAT1* cassette following digestion with SacII. Primers MAO56 and GSO129 were used to generate an ~5-kb 5′ split-marker fragment (*PPG1* 5′-flanking region, ORF, and Terminator plus a 3.6-kb 5′ *SAT1* partial cassette). Primers MAO160 and GSO130 were used to generate an ~2-kb fragment (the *PPG1* 3′-flanking region downstream of Terminator plus a 1.4-kb 3′ *SAT1* partial cassette). Each split-marker fragment was purified with the GeneElute PCR cleanup kit (Sigma) and concentrated by ethanol precipitation. DNA pellets (0.5 to 1 μg) were resuspended in 5 μl sterile ultrapure water for transformation into strain *ppg1*Δ/Δ (MAY34).

In order to generate the *ppg1*Δ/Δ::$ppg1_{H248A\ H173A\ D90L}$ strain (MAY55), we first used site-directed mutagenesis to introduce mutations into the *PPG1* ORF as follows: the D90L mutation was generated using upstream nonmutagenic primer MAO77 and internal mutagenic primer MAO133, the H173A mutation was generated using internal nonmutagenic primer MAO150 and internal mutagenic primer MAO151, and the H248A mutation was generated using internal nonmutagenic primer MAO152 and internal mutagenic primer MAO153. These three fragments were combined with an additional PCR fragment containing the 3′ end of the ORF (generated using the internal mutagenic primer MAO154 and downstream nonmutagenic primer MAO78) in an overlap PCR to make a final fragment containing the *ppg1* ORF with all three mutations. A PCR fragment containing the *PPG1* 5′-flanking sequences, mutated ORF, and terminator was then used in the *SAT1* split-marker approach described above to generate strain *ppg1*Δ/Δ::$ppg1_{H248A\ H173A\ D90L}$ (MAY55). The *SAT1* split-marker approach was also used to construct strains with *TPK1* deleted. The *TPK1* 5′ upstream and 3′ downstream flanking regions were PCR amplified using primers MAO138/MAO139 and MAO140/MAO141, respectively. The 5′ and 3′ *TPK1* flanks were fused to the partial *SAT1* cassettes as described above. Primers MAO148 and GSO129 were used in a PCR to generate an ~4.2-kb 5′ split-marker fragment (*TPK1* 5′-flanking region + 3.6-kb 5′ *SAT1* partial cassette). Primers MAO149 and GSO130 were used to generate an ~2-kb 3′ split-marker fragment (*TPK1* 3′-flanking region + 1.4-kb 3′ *SAT1* partial cassette). Following purification and concentration as described above, these fragments were used to transform both wild-type (SN152) and *ppg1*Δ/Δ (MAY34) strains. Homozygous *tpk1*Δ/Δ and *ppg1*Δ/Δ *tpk1*Δ/Δ deletion mutations were generated using the *SAT* flipper method (42) (DK318 served as the starting strain for the *tpk1*Δ/Δ mutant) followed by retransformation with the 5′ and 3′ *TPK1-SAT1* split-marker fragments. The *ppg1*Δ/Δ *efg1*Δ/Δ mutant was generated using the *SAT* flipper method by transforming the *ppg1*Δ/Δ (MAY34) strain with a previously described *efg1*Δ::*SAT1* deletion fragment (43).

To construct MAY73 (*tetO-PPG1*), primers MAO126/MAO128 were used to generate a *PPG1* upstream fragment (positions −566 to −98 relative to the *PPG1* start codon), which was digested with KpnI and cloned into plasmid pEL9 (44). Primers MBO127/MBO129 were used to generate a second PCR product from positions −33 to +466 relative to the start ATG of *PPG1*, which was cloned into the resulting plasmid digested with SacII to generate pEL9-PPG1. This plasmid was used as a template to amplify an ~12-kb fragment using PhireTaq (Thermo Scientific), which included the *tetR* transactivator, *SAT1* marker, *tetO*, and *PPG1* flanks. This fragment was transformed into strain DK318 to generate *tetO-PPG1* strain MAY73. Correct integration of all constructs as well as absence of the ORF in homozygous deletion mutants was verified by whole-cell PCR. For recombinant bacterial expression, WT *PPG1* and $ppg1_{H248A\ H173A\ D90L}$ were cloned into the BamHI and NcoI restriction sites of expression vector pAG10H (kindly provided by P. John Hart), a modified pET19d vector with a tobacco etch virus-cleavable His-10 tag fused to the N terminus of the target protein (Ppg1) (45).

Media and growth conditions. Yeast extract-peptone-dextrose (YEPD) medium (46) at 30°C was used as the standard non-filament-inducing growth conditions for all strains. Solid YEPD medium plus 10% fetal bovine serum (FBS), Spider medium, and Lee's pH 6.8 medium were prepared as previously described (24, 39, 47). Liquid serum and temperature induction experiments were performed by growing strains overnight in YEPD medium at 30°C to an optical density at 600 nm (OD_{600}) of ~4.0 and diluting 1:10 into 50 ml of prewarmed medium as described previously (39). Aliquots of cells were harvested at specific postinduction time points for RNA preparation and microscopy. For the liquid serum and temperature induction epistasis experiment, strains were grown overnight in YEPD medium at 30°C to saturated density and then diluted in prewarmed YEPD medium at 30°C or YEPD medium plus 10% serum at 37°C and harvested at the 3-h postinduction time point. The *tetO-PPG1* strain was grown in both liquid and solid YEPD media at 30°C in the presence or absence of 20 μg/ml doxycycline (Dox; Sigma-Aldrich, St. Louis, MO). Liquid cultures were grown overnight to an OD_{600} of ~1.0, and aliquots of cells were taken for microscopy and RNA extraction.

RNA preparation and Northern analysis. RNA extractions were carried out using the hot acid phenol protocol (48). Probe preparation and Northern analysis (using 3 μg of total RNA from each sample) were carried out as described previously (39). Blots were scanned using a phosphorimager and visualized as described previously (39). The primers used to generate probes for Northern analysis are indicated in Table S2 in the supplemental material.

Invasion assays. Invasion assays were performed as described previously (49) with minor modifications. Briefly, saturated overnight cultures were diluted to an OD_{600} of 1.0, and four additional 1:10 serial dilutions were performed. Three microliters from each dilution was spotted onto the specific solid agar medium (see Fig. 4), and cells were grown for 2 days. Spot images were taken before and after the plates were washed with double-distilled water (ddH_2O).

Expression of Ppg1 in *E. coli*. Expression constructs for His-tagged Ppg1 and $Ppg1_{H248A\ H173A\ D90L}$ were transformed into an *Escherichia coli* Rosetta 2(DE3) strain (Stratagene). Expression strains were induced by the addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG; Sigma-Aldrich), followed by a 16-h incubation at 19°C. Cells were harvested, and extracts were prepared using the nickel-nitrilotriacetic acid (Ni-NTA) Fast Start kit protocol (Qiagen). Recombinant proteins were purified from the supernatant of bacterial extracts using Ni-NTA agarose affinity chromatography. Eluted fractions were pooled and analyzed using SDS-PAGE (data not shown). Protein concentrations of the fractions were determined using a Bradford assay (50). Western analysis was performed as described previously (51) using 20 μg of recombinant purified protein, a 1:20,000 dilution of mouse monoclonal anti-His antibody (GenScript), and a 1:5,000 dilution of secondary goat anti-rabbit IgG antibody (Invitrogen).

Phosphatase activity assay. Phosphatase assays were performed according to the manufacturer's instructions as follows: 20 μg of recombinant Ppg1 protein samples was incubated with 100 μl of p-nitrophenyl phosphate (pNPP) substrate solution (Sigma-Aldrich, St. Louis, MO). The colorimetric pNPP substrate turns yellow upon the release of a phosphate group by the phosphatase. The pNPP reaction was stopped after 15 min by the addition of 50 μl of 3 M NaOH solution. Activity was measured by spectrophotometer as absorbance at 405 nm. All assays were performed in biological duplicate and technical quadruplicate.

Virulence and histological assays. Virulence in the mouse model of systemic candidiasis was assessed, and subsequent histological analysis of kidney sections was performed as described previously (39). All animal experimentation was conducted following the National Institutes of Health guidelines for housing and care of laboratory animals and performed in accordance with institutional regulations after pertinent review and approval by the Institutional Animal Care and Use Committee at The University of Texas at San Antonio. Briefly, overnight cultures of each strain were washed three times in sterile pyrogen-free saline, and 200 μl of cell suspension (containing the appropriate inoculum size) was used to inject individual 6- to 8-week-old female BALB/c mice by lateral tail vein. Survival was monitored after infection. Moribund mice were sacrificed, and their deaths were recorded on the next day. Survival data and differences between groups were analyzed using the Kaplan-Meier log rank test. Analyses were performed using Prism by GraphPad Prism ver. 6.04 (GraphPad Software Inc., San Diego, CA).

RESULTS

***C. albicans* Ppg1 possesses phosphatase activity that is abolished upon mutation of the putative catalytic site.** Ppg1, a putative serine/threonine protein phosphatase type 2A, was first identified in *C. albicans* as part of an amino acid sequence-based screen for orthologs of previously annotated *S. cerevisiae* protein phosphatases (the identity was 60.8%) (27). A model generated using homology detection and structure prediction HMM-HMM (where HMM stands for hidden Markov model) comparison (HHpred) software (http://toolkit.tuebingen.mpg.de/hhpred) predicted that Ppg1 possesses a conserved putative catalytically active binuclear center (Fig. 1A). In order to confirm that *C. albicans PPG1* encodes a phosphatase, recombinant His-tagged Ppg1 was expressed in bacteria and purified. As shown in Fig. 1B and C, $Ppg1_{(HIS)10}$ was stably expressed and showed significant phosphatase activity compared to vector-only and substrate-only controls. We also observed that $Ppg1_{(HIS)10}$ phosphatase activity was abolished upon treatment with okadaic acid (see Fig. S1 in the supplemental material), a specific inhibitor of serine/threonine phosphatases of types 1, 2A, and 2B (52). We observed that mutation of three highly conserved amino acid residues in the conserved binuclear center (H248, H173, D90) completely abolished $Ppg1_{(HIS)10}$ phosphatase activity (Fig. 1C). Importantly, phosphatase activity was not abolished as a consequence of reduced protein stability since the catalytic mutant was expressed at a level equivalent to that of wild-type $Ppg1_{(HIS)10}$ (Fig. 1B).

Ppg1 phosphatase activity plays an important role in controlling *C. albicans* colony morphology, filament extension, and agar invasion. As previously indicated, a large-scale systematic screen of a *C. albicans* homozygous deletion library demonstrated that the *ppg1*Δ/Δ mutant strain is defective for morphogenesis and shows reduced abundance in the kidney during infection when combined with a large pool of bar-coded mutants (34). In order to further examine and better characterize these defects, we generated both heterozygous and homozygous *ppg1* deletion mutants as well as a *ppg1*Δ/Δ::*PPG1* add-back strain. Initially, all strains were examined for colony morphology on solid filament-inducing media. As indicated in Fig. 2, the *ppg1*Δ/+ strain showed a clear reduction in fuzziness (filamentous fringes) at the edges of the colonies compared to that of the wild-type (WT) strain on both Spider (nitrogen and carbon starvation) and Lee's pH 6.8 media. However, the *ppg1*Δ/+ mutant did not appear to show a defect in colony morphology when grown on solid medium in the presence of serum at 37°C. In contrast, the homozygous *ppg1*Δ/Δ mutant showed more significant colony morphology defects than both WT and *ppg1*Δ/+ strains on Spider, Lee's pH 6.8, and particularly YEPD plus serum at 37°C media as indicated by significantly reduced wrinkling in addition to an absence of fuzziness at the edges (for Spider and Lee's pH 6.8 media). As expected, colonies of the *ppg1*Δ/Δ::*PPG1* add-back strain showed phenotypes equivalent to those observed with the *ppg1*Δ/+ strain, and all strains grew as smooth colonies under non-filament-in-

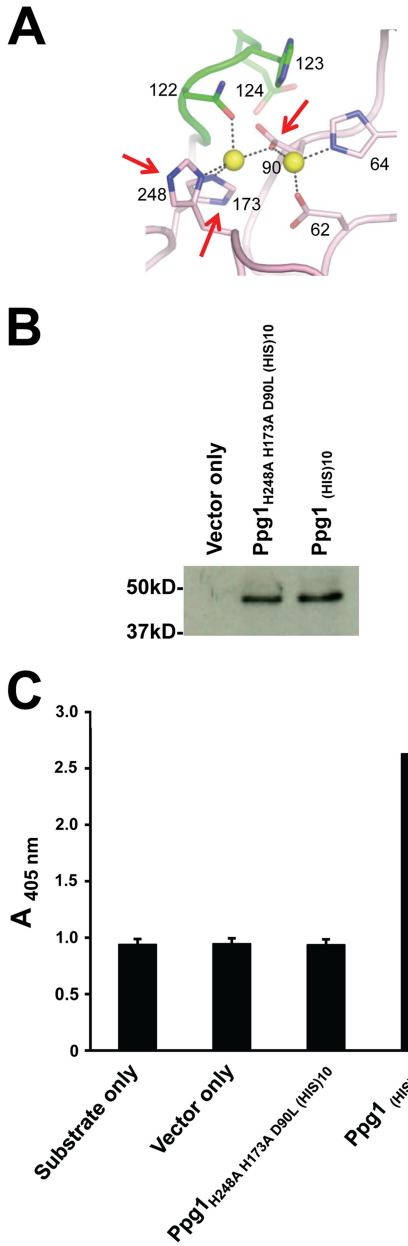

FIG 1 Mutation of the putative Ppg1 catalytic site abolishes phosphatase activity. (A) A model for the putative Ppg1 catalytically active binuclear center generated using homology detection and structure prediction HMM-HMM comparison (HHpred) software (http://toolkit.tuebingen.mpg.de/hhpred). Numbered amino acids represent highly conserved residues in the putative catalytic site. Arrows indicate mutated residues. (B) His-tagged WT and mutated Ppg1 were expressed in an *E. coli* Rosetta 2(DE3) strain and purified from the supernatant of bacterial extracts using Ni-NTA agarose (Qiagen) affinity chromatography. Western analysis was performed using 20 μg of recombinant purified proteins and an anti-His primary antibody and goat anti-rabbit IgG secondary antibody. (C) Phosphatase activity was determined using 20 μg of the indicated proteins and p-nitrophenyl phosphate (pNPP) as a substrate.

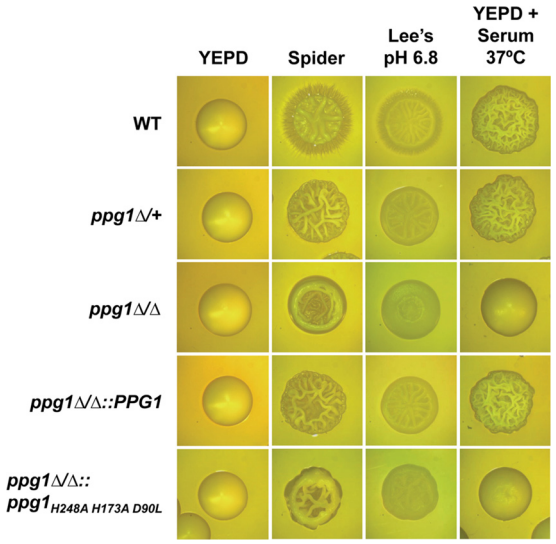

FIG 2 Ppg1 phosphatase activity controls *C. albicans* colony morphology under multiple filament-inducing conditions. Colonies of the indicated strains were grown for 2 days on solid medium under non-filament-inducing conditions (YEPD at 30°C) as well as on the indicated filament-inducing conditions (colonies on YEPD medium plus 10% serum at 37°C were grown for 2 days, and colonies on Spider medium and Lee's pH 6.8 medium were grown at 30°C for 4 days). All colonies were visualized using light microscopy.

ducing conditions (YEPD medium at 30°C). Interestingly, colonies of a *C. albicans ppg1*$_{H248A\ H173A\ D90L}$ catalytic mutant appeared nearly identical to those of the *ppg1*Δ/Δ strain but showed slightly greater wrinkling under all solid-medium filament-inducing conditions (Fig. 2). These results indicate that the *ppg1*Δ/Δ colony morphology defects can be largely attributed to a defect in Ppg1 phosphatase activity.

To determine the effect of constitutive high-level *PPG1* expression on *C. albicans* colony morphology, we generated a strain in which a single allele of *PPG1* was under the control of an *E. coli tet* operator. In the absence of doxycycline (Dox), a tetracycline derivative, a strong transactivator protein binds as a dimer to the *tet* operator and directs transcriptional activation. As shown in Fig. S2A in the supplemental material, Northern analysis confirmed that *PPG1* is expressed only in the absence of Dox and only when the *tet* operator is present. Under solid-medium non-filament-inducing conditions (YEPD medium at 30°C), constitutive high-level *PPG1* expression did not affect *C. albicans* colony morphology (see Fig. S2B in the supplemental material). Interestingly, however, *PPG1* expression resulted in a noticeable decrease in wrinkling when colonies were grown on Spider medium and YEPD + serum at 37°C. We did not observe any effect of *PPG1* expression on filamentation under a variety of liquid-medium inducing conditions (data not shown). These findings indicate that constitutive high-level *PPG1* expression results in increased colony morphology defects under certain filament-inducing conditions.

We next sought to determine the effect of the *ppg1*Δ/Δ mutation on *C. albicans* cellular morphology under strong liquid-medium filament-inducing conditions. Both WT and *ppg1*Δ/Δ strains were induced to form filaments by growth in liquid YEPD plus serum at 37°C, and morphology was monitored at specific postinduction time points (Fig. 3). As expected, all cells grew as yeast at the zero-hour time point, just prior to induction. In addition, as observed previously, the WT strain formed short germ tubes at the 1-hour time point and much longer extended filaments (mostly hyphae) over the remainder of the time course. Similar to the wild-type strain, the *ppg1*Δ/Δ mutant was able to

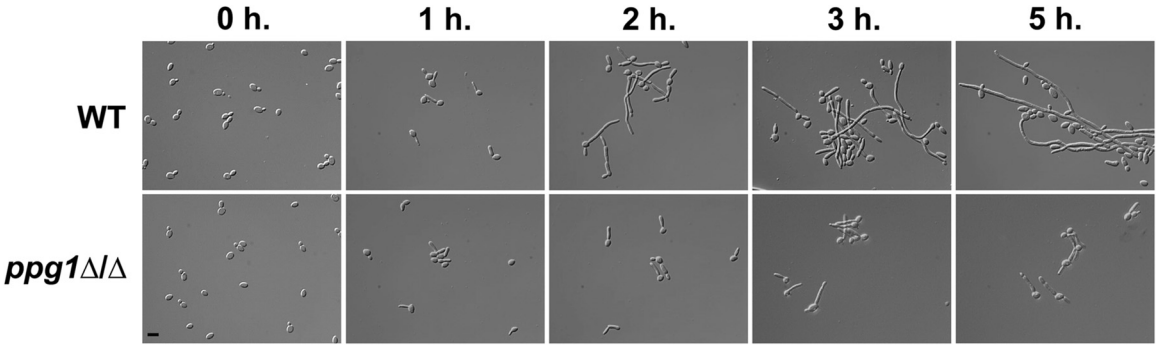

FIG 3 *PPG1* is important for filament extension in response to growth under strong filament-inducing conditions. The indicated strains were grown overnight in YEPD medium at 30°C (non-filament-inducing conditions, 0 h time point) and diluted 1:10 into prewarmed YEPD medium plus 10% serum at 37°C (strong filament-inducing conditions). Cells were harvested at the indicated time points (hours), fixed using 4.5% formaldehyde, and washed twice with 1× phosphate-buffered saline (PBS). Images were taken using differential interference contrast (DIC) microscopy. Bar, 10 μm.

form short germ tubes at the 1-hour time point. However, at the 2-, 3-, and 5-hour time points, cells of the *ppg1*Δ/Δ mutant showed significantly reduced filament lengths compared to those of the WT strain. In order to determine whether the *ppg1*Δ/Δ filament extension defect was the result of a delay in filament formation, we allowed the induction time course to proceed for additional time points past 5 h as well as overnight. However, no increase in filamentation or filament length by the *ppg1*Δ/Δ mutant was observed (data not shown). As expected, both the WT and *ppg1*Δ/Δ strains grew as yeast cells under non-filament-inducing conditions (YEPD at 30°C) (data not shown). The $ppg1_{H248A\ H173A\ D90L}$ catalytic mutant showed a filament extension defect equivalent to that of the *ppg1*Δ/Δ strain in liquid medium in response to induction by serum at 37°C (see Fig. S3 in the supplemental material). The *ppg1*Δ/Δ filament extension defect was also rescued in the *ppg1*Δ/Δ::*PPG1* add-back control strain. Altogether, our results indicate that *PPG1*, and more specifically Ppg1 phosphatase activity, plays an important role in controlling *C. albicans* filament extension under strong liquid filament-inducing conditions.

During infection, *C. albicans* filamentous growth is known to play an important role in promoting the invasion of both mucosal cell layers and a variety of host tissues. We performed a simple *in vitro* assay to determine whether the *ppg1*Δ/Δ mutation also affects this important virulence trait by examining agar invasion under both non-filament-inducing and a variety of filament-inducing conditions. As shown in Fig. 4, we observed that the *ppg1*Δ/Δ mutant is highly defective for agar invasion under multiple filament-inducing conditions. Similar to the *ppg1*Δ/Δ strain, the $ppg1_{H248A\ H173A\ D90L}$ catalytic mutant also showed a significant defect in agar invasion. However, this defect appeared to be slightly less severe than that of the *ppg1*Δ/Δ mutant on Lee's pH 6.8 medium as well as YEPD medium plus serum at 37°C. Interestingly, both the *ppg1*Δ/+ and the *ppg1*Δ/Δ::*PPG1* add-back strains did not appear to show any agar invasion defect, indicating that expression of a single *PPG1* allele is sufficient for normal invasion. As expected, none of the strains were able to invade the agar under non-filament-inducing conditions (YEPD at 30°C). Overall, these results indicate that in addition to filament extension, *PPG1* and more specifically Ppg1 phosphatase activity also play an important role in controlling the ability of *C. albicans* to invade surfaces.

***PPG1* is important for *NRG1* downregulation and affects the expression of multiple filament-specific transcripts in response to serum at 37°C.** Previous studies have shown that downregulation of *NRG1*, a key transcriptional repressor of filament-specific genes, represents a critical event important for both *C. albicans* filamentation and expression of the filamentous growth program (22, 53, 54). In order to determine whether *PPG1* plays a role in controlling this regulatory event, aliquots of cells from the liquid serum and temperature induction time course experiment described in Fig. 3 (including additional 15- and 30-min time points) were harvested for total RNA preparation, and a Northern analysis was performed to examine *NRG1* transcript levels. As shown in Fig. 5, in the WT strain the *NRG1* transcript level decreased by the 30-min postinduction time point, remained low through much of the time course, and increased again by the 5-hour time point. In contrast, very little, if any, reduction in *NRG1* transcript level was observed in the *ppg1*Δ/Δ strain, indicating that *PPG1* is important for *NRG1* downregulation in response to serum at 37°C.

We also examined the expression of several filament-specific target genes (*ALS3*, *HWP1*, *ECE1*) in WT versus *ppg1*Δ/Δ strains in response to induction by serum at 37°C (Fig. 5). Surprisingly, all of these genes showed somewhat increased expression during the early serum and temperature induction time points (up until 2

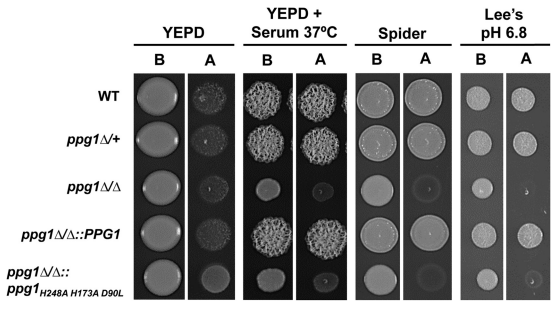

FIG 4 Ppg1 phosphatase activity is important for agar invasion under a variety of filament-inducing conditions. The indicated strains were grown in YEPD at 30°C overnight. A total of 6×10^3 cells of each strain were spotted onto the indicated solid media. Following 2 days of growth at 30°C (or 37°C for the YEPD + serum condition), images were taken before (B) and after (A) each plate was washed with ddH_2O.

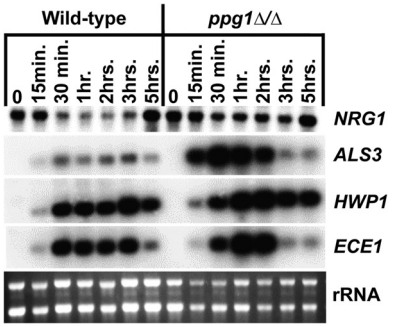

FIG 5 *PPG1* is important for *NRG1* downregulation and controls filament-specific gene expression in response to serum at 37°C. Cells were harvested at the indicated time points from the strains grown as described for Fig. 3 and used to prepare total RNA for Northern analysis. Three micrograms of total RNA was loaded in each lane, and Northern blots were probed for the indicated transcripts. rRNA is included as a loading control.

h). In addition, two filament-specific transcripts (*ALS3* and *ECE1*) also showed a sharp reduction at the later time points (3 and 5 hours postinduction). These results indicate that *PPG1* affects both the transcript level and induction kinetics of multiple filament-specific genes under strong filament-inducing conditions.

Functional relationship between *PPG1* and the cAMP-PKA filamentous growth signaling pathway. Previous studies have shown that the cAMP-PKA filamentous growth signaling pathway is required for transient removal of the Nrg1 protein from the promoters of filament-specific genes and that a downstream target transcription factor of this pathway, Efg1, is required for downregulation of the *NRG1* transcript (54, 55). Our finding that *PPG1* is also important for *NRG1* downregulation suggested a possible functional relationship between *PPG1* and the cAMP-PKA pathway. In order to test this hypothesis, we generated *ppg1*Δ/Δ *tpk1*Δ/Δ and *ppg1*Δ/Δ *efg1*Δ/Δ double mutants. *TPK1* encodes a catalytic subunit of cAMP-dependent protein kinase (56). *TPK1* rather than *TPK2*, which encodes the other cAMP-dependent protein kinase catalytic subunit, was chosen for epistasis analysis because the *tpk1*Δ/Δ mutant shows the greatest phenotypic difference compared to the *ppg1*Δ/Δ mutant under liquid-medium filament-inducing conditions. The epistasis analysis was performed using *ppg1*Δ/Δ, *tpk1*Δ/Δ, *efg1*Δ/Δ, *ppg1*Δ/Δ *tpk1*Δ/Δ, and *ppg1*Δ/Δ *efg1*Δ/Δ mutants. All of these strains, in addition to a wild-type control strain, were induced to form filaments by growth in liquid YEPD medium plus serum at 37°C, and aliquots of cells were fixed for microscopy (Fig. 6). As observed previously, the *ppg1*Δ/Δ mutant showed a significant defect in filament extension. Also consistent with previous observations (56), the *tpk1*Δ/Δ mutant showed a level of filamentation similar to that of a WT strain. Interestingly, the *ppg1*Δ/Δ *tpk1*Δ/Δ double mutant showed significant filamentation with a phenotype identical to that of the *tpk1*Δ/Δ single mutant. In addition, both the *efg1*Δ/Δ and *ppg1*Δ/Δ *efg1*Δ/Δ mutants showed identical phenotypes and grew entirely as yeast cells (consistent with a previous report for the *efg1*Δ/Δ mutant [12]). These results indicate that both *efg1*Δ/Δ and *tpk1*Δ/Δ mutations are epistatic to the *ppg1*Δ/Δ mutation during liquid-medium filament induction in response to serum at 37°C and suggest that *PPG1* functions upstream of *TPK1* and *EFG1* to affect the *C. albicans* cAMP-PKA signaling pathway. Similar epistatic relationships were observed when colony morphologies were examined on solid Spider medium (see Fig. S4 in the supplemental material). However, the *tpk1*Δ/Δ and *ppg1*Δ/Δ *tpk1*Δ/Δ mutants showed reduced fuzziness (filamentation) at the colony fringes compared to the WT strain (as previously reported for the *tpk1*Δ/Δ mutant [56]) and the *ppg1*Δ/Δ *efg1*Δ/Δ mutant showed mild colony wrinkling, suggesting that Efg1 and Ppg1 may perform partially independent functions under this condition. A similar epistasis analysis has suggested that *PPG1* does not function via the *C. albicans* MAP kinase signaling pathway (data not shown).

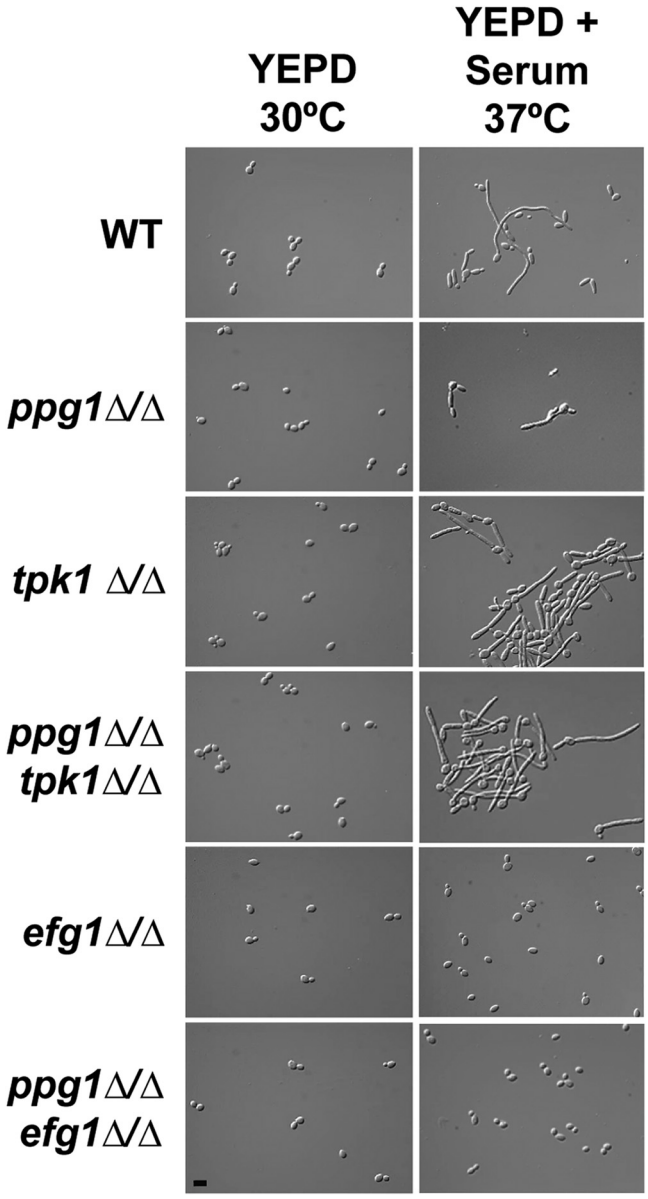

FIG 6 The *tpk1*Δ/Δ and *efg1*Δ/Δ mutations are epistatic to the *ppg1*Δ/Δ mutation with respect to cellular morphology under strong liquid-medium filament-inducing conditions. The indicated strains were grown overnight in YEPD at 30°C, saturated cultures were diluted under the indicated filament-inducing (YEPD + serum at 37°C) and -noninducing (YEPD at 30°C) conditions, and cells were harvested at the 3-h postinduction time point. After fixing in 4.5% formaldehyde, cells were washed twice with 1× PBS and visualized using DIC microscopy. Bar, 10 μm.

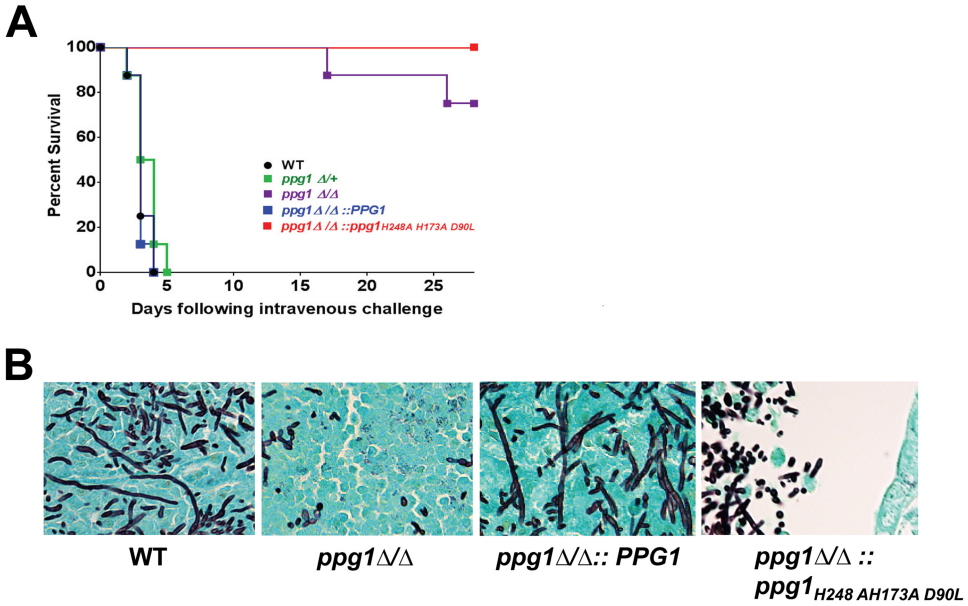

FIG 7 Ppg1 phosphatase activity is important for *C. albicans* virulence and filament extension during infection *in vivo*. Eight ($n = 8$) female BALB/c mice (6 to 8 weeks old) were inoculated by tail vein with 3.5×10^5 CFU of the indicated strains, and survival was monitored for 28 days. A Kaplan-Meier test indicated that *ppg1*Δ/Δ and *ppg1*Δ/Δ::*ppg1*$_{H248A\ H173A\ D90L}$ strains showed a statistically significant difference in virulence compared to the wild-type strain ($P < 0.0001$). (B) Kidneys from mice infected with the indicated strains were fixed, paraffin embedded, sectioned, and stained with Grocott-Gomori methenamine silver (GMS).

Ppg1 phosphatase activity is important for *C. albicans* filament extension and virulence in a mouse model of systemic candidiasis. In order to determine the requirement of *PPG1* and Ppg1 phosphatase activity for virulence in a mouse model of systemic candidiasis, mice were inoculated by tail vein with WT, *ppg1*Δ/+, *ppg1*Δ/Δ, *ppg1*Δ/Δ::*PPG1* add-back, and *ppg1*Δ/Δ::*ppg1*$_{H248A\ H173A\ D90L}$ mutant strains. None of these strains showed a significant growth defect at 37°C as determined by a growth curve. As indicated in Fig. 7A, all mice inoculated with wild-type, *ppg1*Δ/+, and *ppg1*Δ/Δ::*PPG1* strains died within 5 days. In contrast, all mice inoculated with the *ppg1*Δ/Δ::*ppg1*$_{H248A\ H173A\ D90L}$ catalytic mutant and 75% of mice inoculated with the *ppg1*Δ/Δ strain were alive at 28 days postinfection. These results indicate that Ppg1, and more specifically Ppg1 phosphatase activity, is important for virulence in a mouse model of systemic candidiasis. Interestingly, strains bearing a single allele of *PPG1* were not defective for virulence. A histological analysis indicated, as expected, that long extended filaments were present in tissues infected with WT and *ppg1*Δ/Δ::*PPG1* add-back strains (Fig. 7B). In contrast, both *ppg1*Δ/Δ and *ppg1*Δ/Δ::*ppg1*$_{H248A\ H173A\ D90L}$ catalytic mutant strains generally showed a mixture of yeast and short filaments in the kidneys during infection *in vivo*, also associated with reduced tissue penetration and lack of invasive growth. Overall, these results indicate that Ppg1 and Ppg1 phosphatase activity are important for filament extension, tissue invasion, and virulence during infection *in vivo* and are consistent with our previous *in vitro* findings.

DISCUSSION

Protein phosphatases are known to function as key regulatory molecules in controlling a wide variety of biological processes. Previous studies in yeast and higher eukaryotes have shown that PP2A-type phosphatases, in particular, control many cellular processes, including actin cytoskeleton organization, mitosis, DNA replication, cell wall integrity, cellular proliferation, apoptosis, gene expression, and signal transduction (35, 37, 57–59). In the major human fungal pathogen *Candida albicans*, protein phosphatases are known to regulate cell cycle progression, cation homeostasis, cell wall integrity, macrophage killing, antifungal drug resistance (e.g., as mediated by calcineurin), osmotolerance, and the response to oxidative stress and DNA-damaging agents (33, 60–66). However, considerably less is known about the ability of phosphatases to control morphology, one of the most important *C. albicans* virulence traits.

Here, we report the characterization of *PPG1*, which encodes a PP2A-type serine/threonine phosphatase that plays a specific important role in controlling *C. albicans* morphology, invasion, and virulence. The *ppg1*Δ/Δ mutant was highly defective for colony morphology under a variety of solid-medium filament-inducing conditions, suggesting that *PPG1* is important for the ability of *C. albicans* to respond to multiple environmental cues. Interestingly, this mutant was capable of forming germ tubes during liquid serum and temperature filament induction but showed a significant defect in filament extension. Epistasis analysis has suggested that *PPG1* does not appear to function in the *UME6-HGC1* pathway, which has previously been shown to control hyphal filament extension (49, 67) (data not shown); *PPG1* is also unlikely to function through *EED1*, another component of this pathway (68). Consistent with the observed defect in filament extension, the *ppg1*Δ/Δ mutant was also defective for both agar invasion *in vitro* and tissue invasion during infection *in vivo*, which may at least partially account for the significantly reduced virulence of this strain. However, Ppg1 may also control additional virulence-related properties.

Our demonstration that the *ppg1*$_{H248A\ H173A\ D90L}$ catalytic mutant shows nearly equivalent defects in colony morphology, filamentation, invasion, and virulence to those of the *ppg1*Δ/Δ strain strongly suggests that these defects can be attributed

primarily to a loss in Ppg1 phosphatase activity. Interestingly, however, we did observe several minor differences in the phenotypes of *ppg1*$_{H248A\ H173A\ D90L}$ and *ppg1*Δ/Δ strains. On solid media, the *ppg1*$_{H248A\ H173A\ D90L}$ catalytic mutant showed slightly greater colony wrinkling and invasion than the *ppg1*Δ/Δ strain. In addition, while all mice inoculated with the *ppg1*$_{H248A\ H173A\ D90L}$ catalytic mutant survived within 28 days postinfection, one-quarter of the mice inoculated with the *ppg1*Δ/Δ strain succumbed to infection. These minor differences suggest that in addition to phosphatase activity, Ppg1 may possess other activities or perform other functions that make a very small contribution to *C. albicans* virulence and virulence-related properties.

Does *PPG1* function to promote *C. albicans* filament extension and virulence by targeting known filamentous growth signaling pathways and transcriptional regulators? Multiple lines of evidence suggest that Ppg1 specifically targets the cAMP-PKA filamentation pathway. We have shown that mutations in Tpk1, a catalytic subunit of the cAMP-dependent protein kinase, and Efg1, the downstream transcription factor target of the cAMP-PKA pathway, are epistatic to the *ppg1*Δ/Δ mutation with respect to filamentation in liquid YEPD plus serum at 37°C, conditions that are known to activate the cAMP-PKA pathway. These results suggest that Ppg1 functions upstream of both Tpk1 and Efg1. Consistent with this finding, we have also demonstrated that *PPG1* is specifically important for downregulation of *NRG1*, a key transcriptional repressor of filament-specific genes, in response to growth in serum at 37°C. Downregulation of *NRG1* requires Efg1, and recent work has shown that the cAMP-PKA pathway also directs a transient displacement of the Nrg1 protein from promoters of filament-specific genes (54, 55). Because Efg1 is known to functionally and/or physically interact with other transcriptional regulators that respond to different environmental cues, including Flo8, Sfl1, Sfl2, and Czf1 (69–71), Ppg1 may also modulate the function of these factors as well.

Our demonstration that *PPG1* is important for downregulation of *NRG1* may to some extent explain the observed *ppg1*Δ/Δ mutant defects in morphology and virulence. According to this hypothesis, in the absence of *NRG1* downregulation in the *ppg1*Δ/Δ mutant, filament-specific genes would not be induced (or would show reduced expression), leading to yeast phase growth. Surprisingly, we observed increased, rather than decreased, expression of three filament-specific transcripts during the early time points of serum and temperature induction in the *ppg1*Δ/Δ versus wild-type strains. However, in the case of two of these genes, there was a reduction in transcript level at the later time points. These results suggest a more complex relationship between *PPG1* and filament-specific gene expression. During the early stages of filamentation, filament-specific gene expression may show a transient increase in the *ppg1*Δ/Δ strain as a result of loss of inhibitory dephosphorylation of filamentous growth signaling pathway components that respond to environmental cues. However, in later stages of filament development Ppg1 may play a more important role in maintaining filament-specific gene expression and hyphal growth. Consistent with this hypothesis, *ppg1* mutants can form normal germ tubes but are specifically defective for filament extension in the later time points of a serum and temperature induction time course. In addition, a previous study has suggested that filament-specific genes are differentially regulated in a temporal manner during hyphal initiation versus maintenance phases (55).

How exactly is the Ppg1 phosphatase controlled in response to filament-inducing conditions? *PPG1* is not a filament-induced gene and appears to be expressed at very low constitutive levels (our unpublished observations). In addition, we have observed that high-level *PPG1* expression causes a slight reduction in colony wrinkling only in the presence of solid medium filament-inducing conditions. These results suggest that Ppg1, similar to other phosphatases, may function to modulate and carefully fine-tune the activity of major phosphorylation pathways (such as cAMP-PKA). While an epistasis analysis has suggested that Ppg1 functions independently of the MAP kinase pathway (data not shown), we cannot exclude the possibility that Ppg1 targets additional filamentous growth signaling pathways. Most likely, Ppg1 itself is regulated at the posttranslational level, possibly by phosphorylation. Our observation that the *ppg1*Δ/Δ mutant is defective for filamentation during infection *in vivo* and in response to a wide variety of environmental conditions *in vitro* suggests that Ppg1 phosphatase activity is controlled by multiple filament-inducing signals. Consistent with this hypothesis, a recent study in *S. cerevisiae* has indicated that phosphatases in the same PP2A subfamily as Ppg1 are activated by glucose at the posttranslational level via the cAMP-PKA pathway (72). At this point, the exact mechanism(s) by which *C. albicans* Ppg1 phosphatase activity is controlled by environmental cues that induce filamentation and promote virulence in the host remains unclear. It is hoped that future studies will shed more light in this area and also identify Ppg1-interacting partners and substrates. Ultimately, gaining a better understanding of how phosphatases, such as Ppg1, function to regulate and fine-tune the activity of *C. albicans* filamentous growth signaling pathways should improve our knowledge of the complex regulatory circuits that control morphology and virulence in fungal pathogens and could provide information leading to the development of more effective antifungal strategies.

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