The Gβ-like protein CpcB is required for hyphal growth, conidiophore morphology and pathogenicity in Aspergillus fumigatus

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CpcB (cross pathway control B) encodes a yeast Cpc2 and mammalian RACK1 (receptor for activated protein kinase C) ortholog, which is a WD repeat protein with functional homology to the β subunit of heterotrimeric G proteins in Aspergillus fumigatus. Previous study has reported that CpcB governs growth and development in both A. fumigatus and Aspergillus nidulans. However, little is known about the functional identities of CpcB orthologs and their relationships with G protein complexes. In this study, we verified that cytoplasmic AfcpcB acts as a Gβ-like protein ortholog and plays important roles in hyphal growth, conidiophore morphology, cell wall integrity, and virulence in A. fumigatus. Furthermore, double deletion of AfcpcB and AfgpaB (Gα) causes a similar phenotype to AfgpaB mutant with abnormal multiple septa conidiophores but exhibits sparse conidiation with white and fluffy colonies. Thus, the exacerbated conidiation defect suggests that AfcpcB has its own specific function compared to the Gα subunit of AfgpaB or the G protein complex. In addition, complementation assays using AfcpcB orthologs of A. nidulans and yeasts (Saccharomyces cerevisiae, Schizosaccharomyces pombe, Candida albicans) suggest that all tested fungal AfcpcB orthologs under the A. fumigatus native promoter can largely restore hyphal growth defects in AfcpcB deletion mutant, but only the A. nidulans cpcB ortholog completely rescues the ΔAfcpcB conidiation defect, suggesting that CpcB acts as a Gβ-like protein ortholog in the Aspergillus, but may have unique and important unexplored functions that required for conidiation, which is absent in yeast.

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1. Introduction

Aspergillus fumigatus is a saprophytic filamentous fungus that ubiquitously exists in nature (Kousha et al., 2011). It is an opportunistic pathogen, causing pneumonia and invasive disseminated disease, which result in high mortality (Garcia-Vidal et al., 2008; Gibbons et al., 2012; Saracli et al., 2007). Thus, scientists and clinicians have paid increasing attention to explore A. fumigatus virulence in vitro and in vivo. Many lines of evidence have shown that virulence characteristics are partially associated with phenotypic changes of fungal cells, such as small spore size and slow hyphal growth rates, adherence abilities to host surfaces, cell surface hydrophobicity and cell wall integrity (Aimanianda et al., 2009; Chung et al., 2014; Gravelat et al., 2013). Thus, it is important to identify virulence-related genes in A. fumigatus and the phenotypes of these gene mutants. G proteins transduce signals to effectors in all eukaryotes and play important roles in controlling metabolic, neural and developmental functions (Simon et al., 1991). For example, mutations in Gα subunits cause a variety of human diseases, such as Albright hereditary osteodystrophy, McCune–Albright syndrome, and Ovarian and adrenocortical tumors (Spiegel, 1997). In addition, loss of G proteins causes diverse defects in fungal virulence, morphology and hyphal growth (Alspaugh et al., 1997; Choi et al., 1995). Canonical heterotrimeric G complex protein complexes are composed of Gα, Gβ and Gγ subunits. Among them, the Gα subunit belongs to the GTPase family, while the Gβ subunit tightly binds to the Gγ subunit, which is known to function as a Gβγ complex (Downes and Gautam, 1999). Aside from the Gβ protein, there is a class of proteins that are not Gβ- mimics (Gβ-like) but have similar structures to that of Gβ, which are termed Gβ-like proteins. Studies indicate that Gβ-like protein orthologs are associated with multiple signaling pathways. For example, mammalian RACK1 (receptor for activated protein kinase C) acts as a Gβ-like protein with important roles in assembling multiple signaling protein complexes (Chaudhuri et al., 2008;
Mathias et al., 2004; Tanaka et al., 2001). In comparison, multifunctional Asc1 (absence of growth suppressor of Cpy1) acts as a Gß-like/RACK1 ortholog in the budding yeast Saccharomyces cerevisiae, and it belongs to a group of 40S ribosomal proteins, and that represses gene expression, interacts with G protein signaling proteins and functions as a Gß subunit coupled to glucose responsiveness (Chantrel et al., 1998; Gerbasi et al., 2004; Hoffmann et al., 1999; Liu et al., 2010; Zeller et al., 2007). In A. fumigatus, CpcB (cross pathway control B) encodes a RACK1 ortholog with seven WD40 sequence repeats and is predicted to form a seven-bladed β propeller structure by SMART analysis (http://smart.embl-heidelberg.de) similar to the Gß subunit structure (Li and Roberts, 2001; Smith et al., 1999). Previous study has shown that CpcB plays a key role in Aspergillus vegetative growth and development (Kong et al., 2013). However, the relationship between AfCpcB and the G-protein complex has not been investigated in addition, there are no any reports about whether Afpcb is involved in virulence in animal model.

In this study, we used molecular techniques and phenotype analysis to verify that AfCpcB acts as a yeast Gß-like protein ortholog in A. fumigatus, and that it is required for hyphal growth, conidiophore morphology, cell wall integrity, and virulence. Moreover, the exacerbated conidiation defect in double AfCpcB and AfgpaB (Gß) deletions combined with functional ortholog complementary tests suggest that the AfgpaB CpcB has distinct functions compared to the AfGpaB Gß subunit or G-protein complex.

2. Materials and methods

2.1. Strains, primers, media, and transformation

A list of all A. fumigatus strains used in this study is given in Table 1. The total primers used in this study are shown in Table S1. The media used in the study were prepared as previously described (Jiang et al., 2014). Transformation was done according to a method described (May, 1989; Osmani et al., 1988).

2.2. Construction of A. fumigatus ΔAfpcb mutant and Afpcb-reconstituted strains

The Afpcb gene was deleted in the parental A. fumigatus A1160. For the ΔAfpcB mutant, the pyr4 selectable marker from Neurospora crassa was amplified from the plasmid pAL5 (purchased from FGSC) with the primer pair Pyr4-F/Pyr4-R, and the 5′ and 3′ flanking regions of the Afpcb gene amplified from A. fumigatus A1160 genomic DNA with the primer pairs CpcB-P1/CpcB-P3 and CpcB-P4/CpcB-P6 respectively, were fused to pyr4 selectable marker with the nested primer pair CpcB-P2/CpcB-P5. Then the resulting fusion product was transformed into the parental A. fumigatus A1160 to turn them from uracil/uridine auxotroph to autotrophy. The transformants were confirmed by diagnostic PCR using the Afpcb ORF own primer pair Cpb (S)/CpcB (A) and primer pairs CpcB-P1/R-Pyr4, CpcB-P6/F-Pyr4. For the correct transformant, the PCR product was 1478 bp with CpcB-P1/R-Pyr4, 1321 bp with CpcB-P6/F-Pyr4 and no detectable product with CpcB (S)/CpcB (A).

To construct the Afpcb-reconstituted strain, the hygromycin resistance gene from plasmid pAN7-1 and the Afpcb gene from A. fumigatus A1160 genomic DNA individually amplified with primer pairs Hyg-F/Hyg-R and CZ-P01/CZ-P02, were fused together with primer pair CZ-P03/CZ-P04. The resulting fusion products were further transformed to ΔAfpcB mutant background. The transformants isolated were able to not only grow on medium containing 200 μg/ml hygromycin but also rescue the defect phenotypes caused by ΔAfpcB.

2.3. Construction of CpcB-GFP fusion strain

For the CpcB-GFP fusion strain, a GFP-pyr4 fragment was amplified from plasmid pFN03 (purchased from FGSC) using primer pair Gfp-pyrG (F)/Gfp-pyrG (R). A CpcB-GFP fusion tag at the C-terminal was constructed and expressed in the ΔAfCpcB mutant background. The same approach as that described previously (Nayak et al., 2006) was used to construct the CpcB-GFP fusion cassette. In brief, the long upstream including the Afpcb promoter and the downstream of Afpcb stop codon amplified from A. fumigatus A1160 genomic DNA with the primer pairs CZ-P01/CpcB-GFP-P3 and CpcB-GFP-P4/CpcB-P6, respectively, were fused with the GFP-pyr4 fragment using nested primer pair CZ-P02/CZ-P03, resulted in the CpcB-GFP fusion cassette. The resulting Cpcb-GFP fusion cassette was fused to the hygromycin resistance gene (identical to that of the AfCpcb-reconstituted strain) with the primer pair CZ-P04/CZ-P06. The final fusion product was transformed into the ΔAfCpcB mutant and the green fluorescence was observed by microscope.

2.4. Immunoblotting experiment

To extract proteins from A. fumigatus mycelia, conidial spores from the parental wild-type, Cpcb-GFP and Afpcb-GFP-reconstituted strains were inoculated into MM liquid medium and then shaken at 220 rpm on a rotary shaker at 37 °C for 20 h. Tissue was ground in liquid nitrogen with a mortar and pestle and suspended in an ice-cold extraction buffer [50 mM HEPES (pH 7.4), 137 mM KCl, 10% glycerol, 1 μg/ml peptatin A, 1 μg/ml leupeptin, 1 mM PMSF]. Equal amounts of protein (40 μg) in gel lanes were subjected to 10% SDS–PAGE and then transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon-P; Millipore) in 384 mM glycine, 50 mM Tris (pH 8.4), and 20% methanol at 250 mA for 1.5 h. The membrane was then blocked with phosphate-buffered saline (PBS) containing 5% milk and 0.1% Tween 20. Next, the membrane was probed sequentially with a 1:3000 dilution of anti-GFP antibody (Roche Applied Science) and goat anti-rabbit IgG–horseradish peroxidase diluted in PBS including

<table>
<thead>
<tr>
<th>Table 1</th>
<th>A. fumigatus strains used in this study.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain</td>
<td>Genotype</td>
</tr>
<tr>
<td>A1160</td>
<td>Δku80; pyrG; veA1</td>
</tr>
<tr>
<td>A1160C'</td>
<td>Δku80; A1160::pyrG; veA1</td>
</tr>
<tr>
<td>CZ01</td>
<td>Δku80; pyrG; AfAfpcb::pyr4; veA1</td>
</tr>
<tr>
<td>CZ02</td>
<td>Δku80; pyrG; AfAfpcb::pyr4; AfAfpcb (p)::AfAfpcb; veA1</td>
</tr>
<tr>
<td>CZ03</td>
<td>Δku80; pyrG; AfAfpcb::pyr4; AfAfpcb (p)::AfAfpcb::GFP; pyrG; veA1</td>
</tr>
<tr>
<td>CZ04</td>
<td>Δku80; pyrG; AfAfpcb::pyr4; gpdA(p)::AncpcB; veA1</td>
</tr>
<tr>
<td>CZ05</td>
<td>Δku80; pyrG; AfAfpcb::pyr4; gpdA(p)::Scasc1; veA1</td>
</tr>
<tr>
<td>CZ06</td>
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</tr>
<tr>
<td>CZ07</td>
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<tr>
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</tr>
<tr>
<td>CZ10</td>
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</tr>
<tr>
<td>CZ11</td>
<td>Δku80; pyrG; AfAfpcb::pyr4; AfAfpcb (p)::Caasc1; veA1</td>
</tr>
<tr>
<td>CZ12</td>
<td>Δku80; pyrG; AfgpaB::pyr4; veA1</td>
</tr>
<tr>
<td>CZ13</td>
<td>Δku80; pyrG; AfAfpcb::pyr4; AfAfpcb::hyg; veA1</td>
</tr>
</tbody>
</table>
5% milk and 0.1% Tween 20. The blot was developed by enhanced chemiluminescence (ECL; Amersham). X-ray films were scanned with a Silver Scanner III instrument equipped for transparency scanning (Yu et al., 2004). Other procedures in immunoblotting experiments were carried out as previously described (Shi et al., 2008).

2.5. Microscopic observation and image processing

For hyphal microscopic observations, conidia were inoculated onto pre-cleaned glass coverslips overlaid with YAG liquid media. Strains were grown on coverslips at 37 °C for the times indicated in figures prior to observation under a microscope. DNA and chitin were stained using 4,6-diamidino-2-phenylindole (DAPI) and calcofluor white (CFW) (Sigma Aldrich, St. Louis, MO), respectively, after the cells had been fixed with 4% paraformaldehyde (Polyscience, Warrington, PA) (Harris et al., 1994). Differential interference contrast (DIC) and fluorescent images of the cells were collected with a Zeiss Axio imager A1 microscope (Zeiss, Jena, Germany). For observation of conidiophore structure, the slide culture method for microscopy observation was performed as previously described with a few modifications (Lin and Momany, 2003). Conidia were inoculated on the edge of a small square of agar medium placed on top of a coverslip, which was placed in a Petri dish containing solidifiedagar to keep it moist. Another coverslip was placed on top of the agar square after inoculation. Coverslips with aerial hyphae and conidiophores attached were microscopically imaged. These images were then collected and analyzed by a SensiCam QE cooled digital camera system (Cooke Corporation, Germany) with the MetaMorph/MetaFluor combination software package (Universal Imaging, West Chester, PA).

2.6. Transmission electron microscopy

Transmission electron microscopy examination was carried out mainly as described in previous research (Horiiuchi et al., 1999). In brief, to observe cell wall melanin/pigment layer variations, the sections, cut on an ultramicrotome using a glass knife, were incubated on water agar (2% w/v) for 2 h, the spores then washed dealdehyde with 0.3 M phosphate buffer (pH 7.3) at 4 °C. After the cells had been fixed with 4% paraformaldehyde (Polyscience, Warrington, PA) (Harris et al., 1994). Differential interference contrast (DIC) and fluorescent images of the cells were analyzed by a SensiCam QE cooled digital camera system (Cooke Corporation, Germany). For observation of conidiophore structure, the slide culture method for microscopy observation was performed as previously described with a few modifications (Lin and Momany, 2003). Conidia were inoculated on the edge of a small square of agar medium placed on top of a coverslip, which was placed in a Petri dish containing solidifiedagar to keep it moist. Another coverslip was placed on top of the agar square after inoculation. Coverslips with aerial hyphae and conidiophores attached were microscopically imaged. These images were then collected and analyzed by a SensiCam QE cooled digital camera system (Cooke Corporation, Germany) with the MetaMorph/MetaFluor combination software package (Universal Imaging, West Chester, PA).

2.7. RNA isolation and RT-PCR analysis

The fresh conidia were cultured at 37 °C, 220 rpm for 18 h in MM liquid medium before mycelial aggregates were harvested, squeeze-dried, and stored at −80 °C until needed for total RNA isolation. The mycelia were then pulverized to a fine powder in the presence of liquid nitrogen. Total RNA was isolated using Trizol (Invitrogen, 15596-025) following manufacturer instruction. One hundred milligrams of mycelia per sample was used as the starting material for the determination of total RNA. Reverse transcription polymerase chain reaction (RT-PCR) was carried out using HiScript™ Q RT SuperMix for qPCR (+gDNA wiper) (VazymeTM, R123-01), and then cDNA as the template was used for testing the AfpcpB transcript in the parental wild-type, ΔAfpcpB mutant and AfpcpB-reconstituted strains with primer pair CpcB (S)/CpcB (A).

2.8. Samples preparation for RNA-seq

For induction of asexual development, conidia (10⁶/ml) of control parental wild-type and ΔAfpcpB mutant strains were inoculated in liquid MM, at 37 °C, 220 rpm for 18 h. Then the mycelia were harvested by filtering through Miracloth (Calbiochem, CA) and transferred to solid MM and incubated at 37 °C for 20 h for air exposed asexual developmental induction (See et al., 2003). RNA samples were prepared to perform digital transcriptome analyses by the RNA-seq approach.

2.9. Functional complementary test in the ΔAfpcpB mutant

To express AfpcpB orthologs of AncpcB in Aspergillus nidulans, Scasc1 in S. cerevisiae, Spcpc2 in Schizosaccharomyces pombe, and Caasc1 in Candida albicans, two promoters that one was AngpdA constitutive promoter and the other was AfpcpB native promoter were used to drive the expression of AfpcpB orthologs. For the control of the AngpdA promoter, the coding sequence of each ortholog amplified from corresponding specie cDNA with the primer pair CZ-PAN-F/CZ-PAN-R for AncpcB, CZ-PSC-F/CZ-PSC-R for Scasc1, CZ-PSP-F/CZ-PSP-R for Spcpc2 or CPC-α-F/CPC-α-R for Caasc1 was ligated with the reconstituted plasmid vector pBARGPE1 which contains the AngpdA promoter, the trpc terminator and the hygromycin resistance gene. The resulting plasmids were transformed into the ΔAfpcpB mutant to yield the ortholog expression strains of CZ04 (AncpcB), CZ05 (Scasc1), CZ06 (Spcpc2) and CZ07 (Caasc1). For the control of the AfpcpB promoter, the fragment of 5’ flank was amplified from A. fumigatus A1160 genomic DNA with the primer pair CZ-P01/CZ-PAN-P3 for AncpcB, CZ-P01/CZ-PSC-P3 for Scasc1, CZ-P01/CZ-PSP-P3 for Spcpc2 or CZ-P01/CZ-PCa-P3 for Caasc1. Similarly, the fragment of 3’ flank was created with the primer pair CZ-P05/CZ-PAN-P4 for AncpcB, CZ-P05/CZ-PSP-P4 for Scasc1, CZ-P05/CZ-PCa-P4 for Spcpc2 and CZ-P05/CZ-PCa-P4 for Caasc1. The coding sequence of each ortholog amplified from corresponding specie cDNA with the primer pair CZ-PAN-S/CZ-PAN-A for AncpcB, CZ-PS-S/CZ-PS-A for Scasc1, CZ-PS-S/CZ-PS-P for Spcpc2, or CPC-α-S/CPC-α-P for Caasc1 was fused together with the corresponding fragment of 5’ flank and the fragment of 3’ flank using the nested primer pair CZ-P02/CZ-P03, respectively. The resulting fusion products were individually fused to the fragment of hygromycin resistance gene (identical to that of the AfpcpB-reconstituted strain), resulted in final fusion products relating with Scasc1, Spcpc2 and Caasc1 with the nested primer pair CZ-P04/CZ-P06. Then, all the final fusion products were transformed into the ΔAfpcpB mutant to create ortholog expression strains of CZ08 (AncpcB), CZ09 (Scasc1), CZ10 (Spcpc2) and CZ11 (Caasc1).

2.10. Construction of ΔAfpcpB single mutant and ΔAfpcpBΔAfpcpB double mutant strains

To construct the ΔAfpcpB mutant strain, the 5’ and 3’ flanking regions of the AfpcpB gene amplified from A. fumigatus A1160 genomic DNA with the primer pairs GpaB-P1/GpaB-uu-P3 and GpaB-uu-P4/GpaB-P6, respectively, were fused with pyr4 selectable marker with the nested primer pair GpaB-P2/GpaB-P5, resulted in the AfpcpB gene deletion construct. The resulting AfpcpB gene deletion construct was transformed into the parental A. fumigatus A1160 strain to turn them from uracil/uridine auxotroph to auxotroph. The transformants of ΔAfpcpB isolated were confirmed by PCR with primer pairs GpaB (S)/GpaB (A), GpaB-P1/R-Pyr4, and GpaB-P6/F-Pyr4. As a result, the PCR products were 2283 bp for GpaB-P1/R-Pyr4, 2017 bp for GpaB-P6/F-Pyr4 and no detectable product with GpaB (S)/GpaB (A), suggesting that this gene was correctly deleted. For ΔAfpcpBΔAfpcpB double mutant,
the 5' and 3' flanking regions of the \(AfgpaB\) gene amplified from \(A. fumigatus\) A1160 genomic DNA with the primer pairs GpaB-P1/GpaB-P3 and GpaB-P4/GpaB-P6, respectively, were fused with the hygromycin resistance marker (identical to that of the \(AfgpaB\)-reconstituted strain) with the nested primer pair GpaB-P2/GpaB-P5, resulting in the double deletion construct. After being transformed into the \(AfgpaB\) mutant background, the transformants resistant to 200 \(\mu\)g/ml hygromycin were isolated and confirmed by PCR using the primer pairs of GpaB (S)/GpaB (A), GpaB-P1/R-Pyr4 and GpaB-P6/F-Pyr4. The PCR products were 2266 bp with GpaB-P1/R-Pyr4, 1979 bp with GpaB-P6/F-Pyr4 and no detectable product with GpaB (S)/GpaB (A), suggesting that the \(AfgpaB\) gene was correctly deleted in the parental \(AfgpaB\) mutant.

2.11. Virulence test

The virulence assay in the immunosuppressed murine model was performed as previously described with a few modifications (D’Enfert et al., 1996; Jiang et al., 2014; Ma et al., 2008). Considering the possible robustness, white male rather than female ICR mice (six-week-old, 20–22 g) were given intraperitoneal injection of cyclophosphamide (150 mg/kg of body weight) on day 0, mice were anesthetized with pentobarbital sodium by intraperitoneal injection, and inoculated each containing 15. On day 0, mice were anesthetized with pentobarbital sodium by intraperitoneal injection, and inoculated by endotracheal intubation with 30 \(\mu\)l saline with 1 \(\times\) 10\(^6\) conidia or without conidia as the control. The immunosuppression was maintained by injection of cyclophosphamide (75 mg/kg of body weight) on day 3 and 6. Mice were kept in sterile conditions and rendered with sterilized water containing tetracycline (1 mg/ml, Sigma) to prevent bacterial infection. The mortality was monitored daily and documented for 10 days after inoculation. Lungs were removed from the dead animal and maintained in 10% (v/v) Formalin before periodic acid–Schiff staining, according to the standard procedure (Schmalhorst et al., 2008).

3. Results

3.1. \(AFCpcB\) is a predicted \(G\beta\)-like protein ortholog in \(A. fumigatus\) based on phylogenetic analysis

We performed BLASTP analysis of the \(A. fumigatus\) genome database with the \(S. cerevisiae\) \(G\beta\)-like Asc1 as a query and identified \(AFCpcB\) (AFUB_070060) as the best hit (\(E\)-value = 1e–108, identity = 60.4%). We performed subsequent BLASTP analysis of the \(S. cerevisiae\) genome database with \(A. fumigatus\) CpcB as a query and identified ScAsc1 as the best hit (\(E\)-value = 1.7e–101, identity = 60%), suggesting that \(AFCpcB\) and ScAsc1 are potential orthologs. We performed additional BLASTP searches using the \(AFCpcB\) amino acid sequence and identified the orthologs listed in Fig. 1. \(AFCpcB\) contains 316 amino acids and shares high identity with \(G\beta\)-like orthologs ranging from 60.4% to 95.6%. In contrast, the corresponding \(G\beta\) subunits shared low identity with \(AFCpcB\), ranging from 14.4% to 19.8%. Therefore, \(AFCpcB\) shares higher identities with \(G\beta\)-like orthologs than with \(G\beta\) proteins. Furthermore, additional BLASTP searches also revealed that \(AFCpcB\) orthologs are conserved in mammals and fungi. Phylogenetic relationship analysis showed that \(AFCpcB\) is closely related to atypical \(G\beta\)-like proteins and distant from typical \(G\beta\) subunits in other species (Fig. 1).

3.2. \(AfcpcB\) deletion causes severe growth and conidiation defects

To characterize the functions of \(AFCpcB\), we generated the \(AfgpaB\) strain by replacing its ORF with the \(N. crassa\) pyr4 marker in the control parental \(A. fumigatus\) A1160. We referred to this strain as CZ01. Diagnostic PCR analysis showed that the fusion allele was located at the native gene locus and there was no \(AfcpcB\) transcript in the \(AfgpaB\) mutant by RT-PCR analysis, suggesting that the \(AfgpaB\) gene was fully deleted (Fig. S1). As shown in Fig. 2A, the \(AfgpaB\) mutant displayed reduced radial growth and conidia production compared to the parental wild-type strain (referred to as A1160) in YAG liquid medium. To further confirm \(AFCpcB\)’s function in hyphal growth, we examined the growth phenotypes of the \(AFCpcB\) mutant in YAG liquid medium where the \(AfgpaB\) mutant exhibited delayed conidial germination and

![Fig. 1. Phylogenetic analysis of \(AFCpcB\) with identified and predicted \(G\beta\) proteins and \(G\beta\)-like proteins. Amino acid sequences were aligned with a ClustalW multiple sequence alignment program (Larkin et al., 2007) and the phylogenetic tree was constructed with bootstrap values shown via MEGA 6. Organism sources and NCBI accession numbers are Drosophila melanogaster (Dm) Rack1 (NP_477249), Homo sapiens (Hs) Gnb2l1 (CAG33259), Cryptococcus neoformans (Cn) Gnb2 (AAX94564), Neurospora crassa (Nc) Cpc2 (CAA57460), A. fumigatus (Af) CpcB (EDP50066), Aspergillus nidulans (An) CpcB (XP_661767), Schizosaccharomyces pombe (Sp) Cpc2 (CAB11079), S. cerevisiae (Sc) Asc1/Cpc2 (NP_013834), Candida albicans (Ca) Asc1 (P83774.2), S. pombe Git5 (AAD09020), S. cerevisiae Ste4 (AAAD3114), C. albicans Ste4 (XP_721045), Homo sapiens Gjb1 (NP_002065), C. neoflavus Gph1 (AAD03598), N. crassa Gb subunit Gnb-1 (AF941286.1), A. fumigatus Sfasd (EAL91392) and A. nidulans Sfasd (AAC3436).

\(G\beta\)-like proteins: DmRACK (66.4%), HsGnb2l1 (72.2%), NcCpc2 (79.2%), AFCpcB (96.6%), ScAsc1/Cpc2 (59.0%), SpCpc2 (66.9%), CaAsc1 (63.1%), SgGh5 (18.8%), ScSt4 (17.1%), HaGbl1 (18.4%), CaGph1 (17.7%), NcG nb1 (17.3%), and AnSfasd (16.5%).
shortened hyphal growth compared to the parental wild-type strain under the same culture conditions. To confirm that these phenotypes were specifically caused by the \( \text{AfcpcB} \) deletion, we constructed an \( \text{AfcpcB} \)-reconstituted strain by introducing the parental wild-type \( \text{AfcpcB} \) allele driven by the endogenous \( \text{AfcpcB} \) promoter and linked with the hygromycin resistance gene into the \( \text{D}\text{AfcpcB} \) mutant, referred to as CZ02. As a result, the \( \text{AfcpcB} \)-reconstituted strain exhibited almost normal conidiation and growth compared to the parental wild-type strain under both solid and liquid culture conditions, suggesting that the \( \text{D}\text{AfcpcB} \) mutant phenotype can be rescued by introducing the \( \text{AfcpcB} \) gene. Taken together, these results suggest that \( \text{AfCpcB} \) is required for hyphal growth and conidiation development in \( \text{A. fumigatus} \).

In order to identify genes that were regulated by \( \text{AfcpcB} \) during asexual conidiation development, we analyzed the full genome transcriptomes of \( \text{A. fumigatus} \) parental wild-type strain and \( \text{D}\text{AfpcB} \) mutant. For this analysis, samples were prepared as described in materials and methods. Comparison of both transcriptomes revealed that 241 genes were downregulated and 332 genes were upregulated in the \( \text{D}\text{AfpcB} \) mutant compared to its wild-type progenitor by digital gene expression analysis. Gene Ontology (GO) classification analysis showed that the differentially expressed genes were involved in 376 GO groups. In addition, many of the differentially expressed genes identified in GO groups were focused on metabolic process, oxidoreductase activity, catalytic activity and so on. As a result shown in Table 2, comparison of both transcriptomes under the same condition revealed that some of conidiation associated genes such as \( \text{wetA} \), \( \text{abaA} \) and \( \text{rodA}/\text{rodB} \) were shown to be down-regulated to some extent (fold change was about 0.4) in the \( \text{D}\text{AfpcB} \) mutant compared to its wild-type progenitor (>2-fold change, \( P \)-value <0.05), suggesting that \( \text{D}\text{AfpcB} \) may affect these genes transcriptional profiling. In comparison, the expression of the regulator of conidiation, \( \text{brlA} \), did not be changed significantly, suggesting that \( \text{D}\text{AfpcB} \) may have no obvious effect on the \( \text{brlA} \) transcriptional profiling at this selected time point.

Previous studies have shown that conidiation is induced not only after hyphae exposure to the atmosphere, but also by...
environments that are unsuitable for growth, such as nutritional starvation, osmotic stress or salt stress (Etchebeste et al., 2008, 2009, 2010). Therefore, we aimed to determine whether the defective ΔAfCpcB phenotypes were restored when cultured in the presence of salt or osmotic stresses. To test this hypothesis, we inoculated the ΔAfCpcB mutant spores on solid minimal medium (MM) without or with diverse salt stresses (KCl, NaCl, CaCl₂ or MgCl₂) or osmotic stress (sorbitol), as shown in Fig. 2B. Unexpectedly, although the ΔAfCpcB mutant showed slightly increased conidia production compared to the parental wild-type strain, with the exception of CaCl₂ which showed no restoration, neither osmotic stress nor salt stress conditions were able to restore the ΔAfCpcB mutant growth defects, suggesting that salt and osmotic stresses cannot bypass the requirement of AfCpcB during hyphal growth and conidiation.

3.3. AfCpcB localizes to the cytoplasm with the predicted molecular weight

To further ascertain AfCpcB’s function and localization during growth and development, we transformed a CpcB-GFP fusion construct into the ΔAfCpcB mutant strain, generating the CZ03 strain with the GFP tag fused at the C-terminus of AfCpcB under the native promoter. We further tested whether transformed CpcB-GFP was functional by examining whether the defective ΔAfCpcB deletion phenotypes observed in the CZ01 background strain could be rescued. Expectedly, CpcB-GFP fully complemented the hyphal growth and conidiation defects associated with loss of AfCpcB, suggesting that the CpcB-GFP fusion protein was functional (Fig. 3A). The CpcB-GFP signal was strong by fluorescent light microscopy, indicating that CpcB-GFP was abundantly expressed. Moreover, we used the specific nuclear stain DAPI (4,6-diamidino-2-phenylindole) to visualize the distribution of nuclei, by which we confirmed that AfCpcB was cytoplasmic (Fig. 3B). Moreover, Western blotting analysis with an anti-GFP antibody detected AfCpcB as a polypeptide between 50 and 75 kDa, which is consistent with the predicted mass of approximate 62 kDa (35.0 kDa of AfCpcB plus 26.9 kDa of GFP). Therefore, we defined the molecular weight of AfCpcB protein as about 35.0 kDa (Fig. 3C).

3.4. Functional complementation of fungal AfCpcB orthologs

Based on the highly conserved amino acid sequences of fungal orthologs, we investigated whether AfCpcB orthologs were functionally interchangeable. Thus, we carried out functional complementation experiments using selected AfCpcB orthologs in the ΔAfCpcB mutant. As shown in Fig. 4A, we individually amplified and constructed full-length cDNA of AfCpcB orthologs under the control of the constitutive AngpdA promoter. After being transformed into the ΔAfCpcB mutant strain, all colonies expressing AfCpcB orthologs of S. cerevisiae, S. pombe and C. albicans, referred to as CZ05, CZ06, CZ07, respectively, did not remarkably restore conidiation and hyphal growth, although their colony size was slightly larger than the parental recipient ΔAfAgpB mutant strain. In comparison, colonies expressing the A. nidulans AfCpcB ortholog almost completely rescued the defective ΔAfCpcB phenotypes in regards to colony size and conidiation. We referred to this strain as CZ04 (Fig. 4B and C). To rule out the possibility that the gpdA promoter was unable to properly drive the expression of yeast orthologs, we constructed the orthologs mentioned above under the control of the native A. fumigatus cpcB promoter (Fig. 4A). The transformed strains expressing AfCpcB orthologs from A. nidulans, S. cerevisiae, S. pombe and C. albicans were referred to as CZ08, CZ09, CZ10, and CZ11, respectively. As a result, all of the yeast orthologs exhibited significant restoration of growth and conidiation with larger colony sizes and increased amounts of conidia compared to those under the control of the gpdA promoter. Upon growth on YAG media for 2 days, the conidia production per cm² was 33.2% in the ΔAfCpcB mutant, but approximately 50% in yeast ortholog strains (CZ09–CZ11). In particular, conidiation production was 93.2% in the A. nidulans ortholog strain (CZ08) versus the parental wild-type strain. Furthermore, the radial growth diameter was 55.8% in the ΔAfCpcB mutant, but increased to 80.8% in the transformed S. pombe ortholog strain (CZ10), 86.5% in the transformed S. cerevisiae ortholog strain (CZ09), 90.5% in the transformed C. albicans ortholog strain (CZ11), and 98.1% in the transformed A. nidulans ortholog strain (CZ08) versus the parental wild-type strain (Fig. 4B and C). Notably, under the control of the AfCpcB promoter, hyphal growth was largely restored by yeast AfCpcB orthologs in the AfCpcB mutant compared to the restoration of conidiation, and A. nidulans ortholog expression almost fully restored the defect in both growth and conidiation. In addition, we found that the native AfCpcB promoter drove yeast AfCpcB orthologs better than the AngpdA constitutive promoter in the A. fumigatus system. Finally, these data suggest that all of the AfCpcB orthologs tested can significantly rescue the hyphal growth defect in the ΔAfCpcB mutant, and only Ancbpc but not other tested yeast AfCpcB orthologs can almost functionally substitute for AfCpcB in conidiation.

3.5. The genetic relationship between Gα-AfGpaB and Gβ-like AfCpcB

The interaction between Gβ-like protein and Gz subunit protein has been verified in yeast. For example, the Gβ-like/AfCpcB protein ortholog Gib2 interacts with the Gz subunit Gap1 in Cryptococcus neoformans (Palmer et al., 2006); the Gβ-like AfCpcB protein ortholog Asc1 also can interact with the Gz subunit Gap2 in S. cerevisiae (Zeller et al., 2007). However, the genetic interaction between Gβ-like protein orthologs and Gz subunit proteins remains unknown in A. fumigatus. BLASTP analysis shows that ScGpa2 and CnGpa1 share identities of 55.4%, 67.2% with AfGpaB, respectively in the A. fumigatus genome database, suggesting that AfGpaB is a potential ScGpa2 or CnGpa1 ortholog in A. fumigatus. To determine the genetic relationship between AfGpaB and AfCpcB, we performed single AfAgpB knockout by replacing its ORF with the N. crassa pyr4 marker in the control parental strain A1160, referred to as CZ12. Meanwhile, we constructed a double AfAgpB and AfCpcB deletion through a secondary knockout by replacing its ORF with the hygromycin resistance gene in the ΔAfAgpB mutant, referred to as CZ13. Diagnostic PCR analysis showed that the fusion allele was located at the native gene locus, and there was no PCR product with the AfAgpB ORF primers in CZ12 and CZ13, suggesting that the AfAgpB gene was fully deleted in both ΔAfAgpB and ΔAfCpcBΔAfAgpB mutants. We next inoculated equal number of conidia from the parental wild-type, ΔAfCpcB mutant, ΔAfAgpB mutant and ΔAfCpcBΔAfAgpB mutant strains onto YAG agar and maintained them at 37 °C for 3 days. As shown in Fig. 5A, ΔAfAgpB mutant conidiation was slightly reduced compared to the parental wild-type strain. Interestingly, double deletion of

<table>
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<th>Table 2</th>
<th>The mRNA fold changes of conidiation related genes in the ΔAfCpcB mutant compared to parental wild-type strain.</th>
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<td>brlA: Key C2H2 Zn finger developmental activator</td>
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<tr>
<td>AFUB_070140</td>
<td>wetA: Developmental regulator for conidial maturation</td>
</tr>
<tr>
<td>AFUB_005170</td>
<td>abd: Developmental activator with ATTS domain</td>
</tr>
<tr>
<td>AFUB_057130</td>
<td>rodA/rodB: Hydrophobin</td>
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ΔAfCpcBΔAfGpaB yielded a nearly white and fluffy colony, which was notably devoid of conidia. Quantitative analyses of conidia and hyphal growth suggest that ΔAfCpcBΔAfGpaB significantly exacerbated the conidiation defect, and partially rescued the hyphal growth defect caused by ΔAfCpcB deletion (Fig. S3).

The low conidiation rate in the ΔAfCpcBΔAfGpaB mutant may be due to a defect in conidiophore development. To dissect the possible basis of the conidiation defect, we imaged conidiophores from the mutants and parental wild-type strain using a previously described sandwich coverslip protocol (Lin and Momany, 2003). As shown in Fig. 5B, vegetative mycelia of the control parental wild-type strain developed into complete conidiophores with visible phialides connected with chains of numerous conidia. For all ΔAfCpcB, ΔAfGpaB and ΔAfCpcBΔAfGpaB mutants, we observed conidiophore structure with numerous conidia to some extent, but the ΔAfCpcB and ΔAfCpcBΔAfGpaB mutants exhibited significantly reduced numbers of conidiation structures compared to their parental wild-type strain in the same microscopic visual field. Moreover, conidiophore production was delayed by approximately 12 h in the ΔAfCpcB mutant and 24 h in the ΔAfCpcBΔAfGpaB mutant compared to the parental wild-type strain. Notably, the septa at the foot cell stalk were present in all mutant strains ΔAfCpcB, ΔAfGpaB and ΔAfCpcBΔAfGpaB, but not in the parental wild-type strain, which did not show any detectable septa at the stalks of the foot cell under the same conditions. In particular, the ΔAfCpcBΔAfGpaB double mutant exhibited more septa than the ΔAfCpcB or ΔAfGpaB single mutants by repeated observation.

Abnormal conidiophore morphology may due to abnormal septation during cell division (Zhong et al., 2014). We speculated that the sparse conidiation phenotype in the ΔAfCpcBΔAfGpaB mutant was due to abnormal septation during cell division. To test this hypothesis, we observed the cell division and septation phenotypes in the parental wild-type strain, ΔAfCpcB, ΔAfGpaB and ΔAfCpcBΔAfGpaB mutants by inoculating conidial spores into YAG liquid medium at 37°C, and the hyphae were stained with DAPI and CFW. Unexpectedly, there were no obvious detectable differences in polarized growth or septum formation between the ΔAfCpcBΔAfGpaB mutant and the control parental wild-type strain. Likewise, nuclei and septa were distributed along hyphal cells in ΔAfCpcB and ΔAfGpaB mutants similar to the parental wild-type strain. (Fig. 5C and D) Although we cannot conclude whether septa in ΔAfCpcB, ΔAfGpaB or ΔAfCpcBΔAfGpaB cells were completely normal, the similarity in their appearance and position as well as the fact that conidia can form suggest that a large part of the septation process can occur independently of AfCpcB and AfGpaB. Taken together, these data suggest that it is possible that abnormal septa distribution at the stalk and decreased conidiation structures are associated with the conidiation defects in mutant strains.

Based on the white and fluffy colony phenotype of the double mutant, we speculated that the cell surface pigment layer of the double mutant was reduced. To test our hypothesis, we examined the cell wall surface construction in all four strains by transmission electron microscopy (TEM). The samples were processed and observed as described in previous research (Horiuchi et al.,...
Fig. 4. Functional complementary test in the ΔAfpcB mutant. (A) Illustration of the orthologs' expression constructs. The A. nidulans gpd promoter 'AngpdA(p)' and the AfpcB promoter 'AfpcB(p)' were used to express AfpcB orthologs respectively; 'CDS' indicates a coding sequence of each ortholog; 'AngpdA(t)' and 'AfpcB(t)' indicate the putative transcriptional termination regions, respectively. (B) The colony morphology of the AfpcB orthologs expressed in the ΔAfpcB mutant under the control of 'Angpd(p)' and 'AfpcB(p)'. Equal number of conidia (2 x 10⁴) were inoculated in each plate, and cultured at 37 °C for 2 days. (C) Quantitative data for the diameters of the colonies and numbers of conidia in related strains cultured at 37 °C for 3 days. Error bars represent standard deviations from three replicates. Letters represent significant differences among values for the strains (Duncan's multiple range test, P < 0.05).
Fig. 5. Phenotypic comparison of \textit{AfcpcB} and \textit{AfgpaB} single and double mutants. (A) Colony morphology of the parental wild-type, \textit{AfcpcB} mutant, \textit{AfgpaB} mutant, and \textit{AfcpcB:Afgpab} mutant strains grown on YAG medium at 37°C for 3 days. (B) Conidiophores morphology of the parental wild-type strain and the \textit{AfgpaB} mutant strain grown for 2 days, \textit{AfcpcB} mutant strain grown for 2.5 days, and the \textit{AfcpcB:Afgpab} mutant strain grown for 3 days on YAG medium at 37°C. Bars, 10 μm. (C and D) Hyphal morphology of parental wild-type strain and the \textit{AfgpaB} mutant strain grown for 8 h, the \textit{AfcpcB} mutant strain grown for 12 h, and the \textit{AfcpcB:Afgpab} mutant strain grown for 12 h in YAG liquid medium at 37°C. Bars, 10 μm. (E) Transmission electron microscopic observations of the conidia of the parental wild-type, \textit{AfcpcB} mutant, \textit{AfgpaB} mutant, and \textit{AfcpcB:Afgpab} mutant strains.
As shown in Fig. 5E, the electron-dense pigment layer of the cell wall surface in both ΔAfCpcB and ΔAfGpaB mutants appeared disrupted. Moreover, the dense staining layer of the cell surface in the double mutant ΔAfCpcBΔAfGpaB was severely disrupted and almost absent. Therefore, we conclude that double deletions of AfCpcB and AfGpaB cause the exacerbated defect of cell wall integrity and both AfCpcB and AfGpaB may be associated with cell wall integrity.

3.6 AfCpcB deletion mutant virulence is attenuated in an immunosuppressed mice model

To evaluate the virulence of the ΔAfCpcB mutant, we compared the differences in virulence of the parental wild-type, ΔAfCpcB mutant, and AfCpcB-reconstituted strains via a cyclophosphamide and hydrocortisone-treated mouse model of invasive aspergillosis. For each group of fifteen mice, 1 × 10⁶ conidia in 0.3 ml physiological saline was inoculated into the immunocompromised mice by endotracheal intubation, while the control group was inoculated with 0.3 ml saline alone. The mice were kept in sterilized environments and monitored daily. As shown in the survival curve (Fig. 6A), the survival rate of mice infected with the ΔAfCpcB mutant was significantly high compared to those infected with the parental wild-type strain or the AfCpcB-reconstituted strain. Specifically in the day 5 post-inoculation analyses, the survival percentage of the ΔAfCpcB mutant, parental wild-type and AfCpcB-reconstituted strains was 60%, 27% and 7%, respectively, and there was a significant difference between the survival rates of the parental wild-type strain and the ΔAfCpcB mutant strain. We next examined the reasons for the survival difference by using lung tissue collected from mice inoculated with each strain that had died on the same day. As shown in Fig. 6B, histopathologic examinations of lungs sections from the parental wild-type strain and the AfCpcB-reconstituted strain revealed a high degree of invasion around lung airways, with hyphae spreading throughout the lung parenchyma and angioinvasion. However, ΔAfCpcB mutant-infected lung tissue yielded fewer colonies and shorter hyphae than the parental wild-type strain or AfCpcB-reconstituted strain. These observations may explain the decreased mortality in ΔAfCpcB mutant-infected mice compared to controls. In summary, histopathological analysis of lungs was consistent with survival data and the relevance of AfCpcB in A. fumigatus virulence.

4. Discussion

Functional analysis has shown that both AfCpcB and G protein signaling proteins, such as GpaA, GpaB, SfaD and PkaC1, play important roles in vegetative growth and asexual sporulation development in the Aspergilli (Lafon et al., 2006; Liebmann et al., 2003, 2004; Shin et al., 2009). In addition, the interaction between Gli-like protein orthologs and the G protein complex has been verified in yeast. The possible relationships between the Gli-like protein AfCpcB and G protein complex will provide new insights into G protein complex function in the Aspergilli. In this study, we generated an AfCpcB and AfGpaB double mutant, which exhibited synthetic conidiation defects compared to the two parental mutants, suggesting that AfCpcB may synthetically function with AfGpaB during conidiation. In addition, comparison of transcriptomes between AfCpcB deletion and its parental strain under the same condition revealed that the expression of G protein signaling associated genes such as gpaB, acyA and pkaC1 have no significant differences (Table 3). These results suggest that AfCpcB deletion may have no detectable effect on the mRNA expression for the other G protein signaling associated genes at our selected tested time point under the conidiation developmental induction. Interestingly, the growth diameter of the double mutant is increased compared to the parental ΔAfCpcB mutant, suggesting that ΔAfGpaB partially suppresses the growth defect caused by ΔAfCpcB, and hence, AfGpaB may counteract AfCpcB during hyphal growth. The abnormal multiple septa phenotype in ΔAfCpcB, ΔAfGpaB and ΔAfCpcBΔAfGpaB strains is also found in the mutation strains of protein kinases, such as A. nidulans LAMMER Kinase (known to conserve the motif “EHLAMMERILG”) LkbA plays important roles in the vegetative growth and asexual and sexual development andALKbA caused abnormal conidiophore morphogenesis including multiple septa in conidiophore stalk. However, over-expression of cell division nmnX (a mitotic cyclin-dependent kinase) in the ALKBa mutant resulted in conidiophore stalk septa disappeared (Kang et al., 2013). This result suggests that conidiophore morphogenesis and cell division are closely connected. For cell division, septa are required for filamentous fungi to produce multicellular hyphae and septum formation is essential to assemble

Table 3

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Fig. 6. Virulence of ΔAfCpcB in the immunosuppressed mice model of invasive pulmonary aspergillosis. (A) ICR mice were immunocompromised with cyclophosphamide and hydrocortisone acetate, and inoculated with 1 × 10⁶ conidia by endotracheal intubation. Compared to the parental wild-type and the AfCpcB-reconstituted strains, the ΔAfCpcB mutant strain showed a significant attenuation in virulence as measured by murine survival. (B) Histopathological analyses were conducted using lung tissue collected from mice inoculated with each strain post-inoculated at the same day. Periodic acid–Schiff (PAS) stain was utilized to visualize fungal growth. The lungs from mice inoculated with the parental wild-type strain or the reconstituted strain showed obvious invasive fungal growth compared to the ΔAfCpcB mutant.
the septal band at the end of mitosis in A. nidulans, which is a dynamic structure containing actin, septin and formin proteins. Assembly relies on a conserved protein kinase cascade termed the Septation Initiation Network (SIN) in S. pombe or the Mitotic Exit Network (MEN) in S. cerevisiae (McCollum and Gould, 2001). In A. nidulans, protein kinases such as NimX likely regulate septum formation via SIN/MEN (Harris, 2001; Harris and Kraus, 1998; Osmani et al., 1994), and cyclin-dependent kinases (CDKs), Aurora kinases, Polo-like kinases (PLks), and NIMA (never-in-mitosis A)-related kinases (NEKs) are associated with mitosis regulation in A. nidulans (Bachewich et al., 2005; Fry et al., 2012; Govindaraghavan et al., 2014; Mogilevsky et al., 2012; Shen et al., 2014). Therefore, protein kinases are required for cell division and septa formation. Aside from protein kinases, protein phosphatases are also involved in maintaining conidiophore morphogenesis, such as calcineurin and two protein phosphatase regulatory subunits (ParA and PabA) (Juvvadi et al., 2008; Zhong et al., 2014). Combined with the fact that the mammalian ortholog of CpcB, RACK1, functions as a receptor for activated protein kinase C, we deduce the abnormal conidiophore morphology with septa distribution in the stalk of foot cell may be related to loss of protein kinase function, and both CpcB and GapB may regulate protein kinases during asexual conidiation.

In addition, ScCpc2, SpCpc2 and RACK1 are functionally interchangeable during different growth conditions in budding and fission yeast systems (Hoffmann et al., 1999; McLeod et al., 2000). In this study, as shown in Fig. 4, yeast orthologs (ScCpc2, SpCpc2 and CaasC1) driven by the AfCpcB native promoter can largely restore the hyphal growth defect in the ΔAfCpcB mutant, suggesting that AfCpcB orthologs possess well-conserved functions in growth. Hence, ScCpc2, SpCpc2 and CaasC1 are functional AfCpcB orthologs. In comparison, even when driven by the AfCpcB native promoter, the conidiation defect was only partially restored by yeast orthologs when compared to the parental wild-type strain, suggesting that the conidiation defect cannot be restored by yeast orthologs. As mentioned above, the conidiation defect is likely associated with conidiophore development. We investigated whether yeast orthologs could rescue the abnormal conidiophore morphology of the ΔAfCpcB mutant. As we expected, the number of septa in the stalk was reduced at a certain extent by the expression of yeast orthologs. In comparison, AncpcB almost completely restored hyphal growth and conidiation to the normal levels, suggesting that the Aspergilli CpcB possesses common functions in hyphal growth, but distinct functions in conidiation compared to yeast orthologs. To further dissect conserved domains for the AfCpcB orthologs, we performed protein structure predicted analysis by SMART (http://smart.embl-heidelberg.de) in CpcB orthologs from A. fumigatus, A. nidulans and orthologs from yeasts (S. cerevisiae, S. pombe, C. albicans). As a result, all tested CpcB orthologs belong to a WD40 protein family and each member of this family contains seven repeated WD domains. Generally, one WD domain is composed of about 40–60 amino acids in length that begins with glycine-histidine (GH) and ends with tryptophan–aspartic acid (WD) (Neer et al., 1994; Yu et al., 2000). BLASTP analysis further indicates all orthologs contain numbers of amino acids from number 310 to 320. Moreover, we found that AnCpcB shares an identity of 95.6% with AfCpcB, whilst yeast homologs only share 60–70% identities with AfCpcB (Fig. 52). Therefore, it suggests that low identities of amino acids between A. nidulans and yeasts CpcB homologs may be the major reason for causing functional differences in conidiation complementary tests.

In summary, we verified that AfCpcB plays important roles in hyphal growth, conidiophore morphology, cell wall integrity, and virulence. The AfCpcB and AfGapB double mutant phenotypes suggest that AfCpcB associates with the G protein complex but also has its own specific function compared to the Gα subunit AfGapB or G-protein complex. In addition, complementation assays indicated that the Aspergilli CpcB might have unique functions that are required for conidiation, but which are absent in yeast Gα-like protein orthologs. Moreover, we speculate that the unique functions of AfCpcB are distinct from nonpathogenic yeasts (S. cerevisiae, S. pombe) and may be associated with the attenuated virulence of the ΔAfCpcB mutant. Therefore, further study is required to determine AfCpcB’s important unexplored functions.

Acknowledgments

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fgb.2015.04.007.

References

Chantrel, Y. et al., 1998. The transcriptional regulator Hap1p (Cyp1p) is essential for anaerobic or heme-deficient growth of Saccharomyces cerevisiae: genetic and molecular characterization of an extragenic suppressor that encodes a WD repeat protein. Genetics 148, 559–569.
Etetebest, O. et al., 2009. The bZIP-type transcription factor FIBB regulates distinct morphogenetic stages of colony formation in Aspergillus nidulans. Mol. Microbiol. 73, 775–789.


Aspergillus nidulans


