Caspofungin Exposure Alters the Core Septin AspB Interactome of *Aspergillus fumigatus*

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Abstract

Aspergillus fumigatus, the main etiological agent of invasive aspergillosis, is a leading cause of death in immunocompromised patients. Septins, a conserved family of GTP-binding proteins, serve as scaffolding proteins to recruit enzymes and key regulators to different cellular compartments. Deletion of the *A. fumigatus* septin *aspB* increases susceptibility to the echinocandin antifungal caspofungin. However, how AspB mediates this response to caspofungin is unknown. Here, we characterized the AspB interactome under basal conditions and after exposure to a clinically relevant concentration of caspofungin. While *A. fumigatus* AspB interacted with 334 proteins, including kinases, cell cycle regulators, and cell wall synthesis-related proteins under basal growth conditions, caspofungin exposure altered AspB interactions. A total of 69 of the basal interactants did not interact with AspB after exposure to caspofungin, and 54 new interactants were identified following caspofungin exposure. We generated *A. fumigatus* deletion strains for 3 proteins (ArpB, Cyp4, and PpoA) that only interacted with AspB following exposure to caspofungin that were previously annotated as induced after exposure to antifungal agents, yet only PpoA was implicated in the response to caspofungin. Taken together, we defined how the septin AspB interactome is altered in the presence of a clinically relevant antifungal.

Key words: *Aspergillus fumigatus*, septins, AspB, caspofungin, interactome

1. Introduction

Aspergillus fumigatus, the main etiological agent of invasive aspergillosis, is a leading cause of fungal mortality in immunocompromised patients [1, 2]. Although the incidence of invasive aspergillosis has increased in the last two decades due to a rise in the immunocompromised patient population, there is a lack of effective treatments and basic understanding of growth and disease [3]. The echinocandin caspofungin is guideline-recommended as a second-line therapy for invasive aspergillosis [4]. With the increasing emergence of azole resistance amongst *A. fumigatus* isolates, the echinocandins are increasingly used to treat invasive aspergillosis and it is therefore critical to understand the fungal response to these antifungal agents [5]. Caspofungin targets β -1,3-D-glucan synthase, which synthesizes a major component of the *A. fumigatus* cell wall [6, 7]. Treatment with caspofungin is fungistatic against *A. fumigatus*, yet the mechanism behind the *A. fumigatus* response to this commonly-used antifungal is not fully understood [7-9].

Septins are conserved GTPases that are involved in a myriad of cellular processes, ranging from cytokinesis to cell morphology [10-15]. Septins have been previously linked to cell wall biogenesis in *Saccharomyces cerevisiae*, where they are required for the proper localization of chitin synthase [16]. Deletion or mutation of septin genes lead to an increase in the susceptibility to caspofungin in *Candida albicans* and *A. fumigatus* [17, 18]. These fungal septins re-localize after caspofungin exposure, suggesting a role for septins in response to this echinocandin [18, 19]. However, the mechanism behind the septin-mediated response to caspofungin remains unknown. In order to further understand how AspB contributes to this caspofungin response, we analyzed the AspB protein interactome under both basal conditions and following exposure to a clinically relevant concentration of the antifungal caspofungin.

2. Materials and Methods

2.1. Strain, media, and culture conditions

Cultures were grown on glucose minimal media (GMM) at 37°C, except where otherwise specified. *Escherichia coli* DH5α competent cells were used for cloning. The *aspB-egfp nimX-rfp* strain was generated by cloning 1 kb of *nimX* and the terminator sequence into the pJW24-RFP-NS vector. The resulting plasmid was digested with NotI and SalI and transformed into the *akuB*^{KU80, pyrG⁻} *aspB-egfp* strain [20]. Deletion of the *arp2* ortholog, which we named *arpB* due to a gene involved in melanin biosynthesis sharing the *arp2* name [21], was attained by replacing the 1.6 kb *arpB* (Afu1g13330) with the 3.0 kb *Aspergillus parasiticus* *pyrG* cassette. Approximately 1 kb of upstream and downstream flanking regions of *arpB* were PCR-amplified from AF293 genomic DNA and cloned into the pJW24 plasmid. The resulting plasmid was digested with NotI and SalI and transformed into the *akuB*^{KU80} *pyrG⁻* strain. Deletion of *cyp4* was performed by similarly replacing the 0.8 kb *cyp4* (Afu2g03720) with the 3.0 kb *A. parasiticus* *pyrG* cassette. The resulting plasmid was digested with NotI and SalI and transformed also into the *akuB*^{KU80} *pyrG⁻* strain. The Δ *ppoA* strain has been previously described [22] and the *OE::ppoA* strain was constructed in the AF293.1 background [23] through promoter replacement with a fusion PCR product [24] consisting of ~ 1 kb flanking regions around the *A. parasiticus* *pyrG::A. nidulans gpdA* promoter cassette from pJMP9 [25]. Strains generated in this study were confirmed by PCR (data not shown) and Southern blotting (Fig. S1). The *aspB-egfp ppoA-rfp* strain was generated by cloning 1 kb of *ppoA* and terminator sequence into the pJW24-RFP-NS vector. The resulting plasmid was digested with NotI and SalI and transformed into the *akuB*^{KU80, pyrG⁻} *aspB-egfp* strain (Table S1).

2.2. Protein extraction, AspB-EGFP fusion protein purification and LC-MS/MS analysis

The *A. fumigatus* strain expressing the *aspB-egfp* fusion construct under the control of the *aspB* native promoter was grown in GMM liquid media and GMM liquid media supplemented with 1 µg/mL of caspofungin for 24 h at 37°C. Total cell lysate was obtained by homogenizing the fungal mycelia (1 g wet weight) as previously described [26]. Total protein from the crude extract was quantified by the Bradford method and purified using the GFP-Trap[®] affinity purification (Chromotek) as previously described [26].

Resin-bound proteins were then reduced with 5 mM dithiothreitol for 30 min at 37°C and alkylated with 12 mM iodoacetamide for 45 min at room temperature. Trypsin digestion was allowed to proceed on-resin for 18 h at 37°C. Samples were then subjected to a 90 min chromatographic separation on a Waters NanoAquity UPLC equipped with a 1.7 µm BEH130 C₁₈ 75 µm I.D. X 250 mm reversed-phase column as previously described [26]. The analytical column was coupled to a Thermo QExactive Plus high-resolution mass spectrometer through an electrospray ionization source. The instrument was operated in a data-dependent mode of acquisition with a precursor MS scan from m/z 375-1600 at r=70,000 followed by ten MS/MS spectra at r=15,000 using a 26% CID energy setting.

Mass spectra were processed with Mascot Distiller (Matrix Science) and were then submitted to Mascot searches (Matrix Science) against an NCBI_RefSeq_Aspergillus database at 5 ppm precursor and 0.02 Da product ion mass tolerances. Static mass modifications corresponding to carbamidomethylation on Cys residues and dynamic mass modifications corresponding to oxidation on Met residues and deamidation on Asn and Gln residues were included. Searched spectra were imported into Scaffold v4.3 (Proteome Software) and scoring thresholds were set to

yield a 0.1% protein false discovery rate. A minimum of two unique peptides from each protein was required for identification.

2.3. Antifungal susceptibility testing

To determine the effect of antifungal agents on the three AspB interactant deletion strains ($\Delta ppoA$, $\Delta cyp4$ and $\Delta arpB$) as well as the *ppoA* overexpression strain (*OE::ppoA*), 10^4 conidia from these strains and the AF293 control (wild-type) strain were inoculated on GMM agar supplemented with either caspofungin (1 $\mu\text{g/mL}$) or nikkomycin Z (2 $\mu\text{g/mL}$) and growth visualized after 3 days of incubation at 37°C. Antifungal susceptibility testing was also performed following standard CLSI criteria [27] and the minimal effective concentration (MEC) of the anti-cell wall agents determined for each strain [28].

2.4. Fluorescence microscopy

Conidia (10^4) of the AspB-EGFP NimX-RFP or AspB-EGFP PpoA-RFP strain were cultured on coverslips immersed in 10 mL of GMM+UU broth and incubated for 20 h at 37°C, as previously described [29]. To examine co-localization after exposure to anti-cell wall agents, caspofungin (1 $\mu\text{g/mL}$) was added after 18 h of incubation and samples imaged after 2 h following drug co-incubation at 37°C. Localization patterns were visualized using an Axioskop 2 plus microscope (Zeiss) equipped with AxioVision 4.6 imaging software.

3. Results

3.1. AspB interacts with the septin complex, cytoskeleton, and cell-cycle regulatory proteins

Previously, the septin AspB has been shown to interact with other members of the septin family in *Aspergillus fumigatus* and *Aspergillus nidulans* [18, 30]. AspB was purified from a

strain expressing the *aspB-egfp* construct under the control of its native promoter using a GFP-Trap® affinity matrix and the AspB bound protein complex subjected to LC-MS/MS analysis for the identification of interactants [18]. A total of 334 proteins co-purified with AspB, including all of the other four *A. fumigatus* septins (Table 1) (Fig. 1A). In addition to the septin complex, AspB co-purified with cytoskeletal proteins: actin, tubulin (alpha-1, beta subunit, and TubB subunit), and Arp 2/3 complex components. Previously identified septin-interacting proteins from *S. cerevisiae* and *C. albicans*, such as Cla4 and NimX^{Cdc28}, were also co-purified with AspB in *A. fumigatus*, indicating that septin interactions are somewhat conserved across different fungal species [31-33]. A strain co-expressing *nimX-rfp* and the *aspB-egfp* construct under the control of their respective native promoters showed that NimX^{Cdc28} colocalized with 65% of the AspB double bars observed (Fig. S2), validating its interaction with AspB.

3.2. *AspB* protein interactions are altered following caspofungin exposure

Previously, septins have been implicated in response to cell wall stress in different fungal species [18, 19]. In *A. fumigatus*, the AspB localization pattern is altered after exposure to anti-cell wall agents nikkomycin Z and caspofungin [18]. Furthermore, deletion of *aspB* increases the susceptibility of *A. fumigatus* to these anti-cell wall agents. However, how AspB contributes to the response to anti-cell wall agents remains elusive. We grew the *aspB-egfp* expressing strain in the presence and absence of caspofungin to define this differential protein interactome. Following caspofungin exposure, 265 proteins continued to be pulled down with AspB-GFP and 69 proteins no longer co-purified under this condition (Fig. 1B). Proteins involved in hyphal growth and polarity, such as Cla4, MobB, and RasB, no longer co-purified with AspB. An additional 54 proteins co-purified with AspB only following exposure to caspofungin. Most of

these proteins are involved in metabolism and secondary metabolism (two proteins involved in pseurotin A production and one uncharacterized synthase) (Table 2)[34].

3.3. Deletion of *ppoA* results in increased susceptibility to caspofungin in liquid media

In order to understand how AspB might regulate the response to caspofungin, we sought to analyze proteins that only co-purified with AspB after caspofungin exposure and that have been curated to be involved in response to antifungal exposure according to the *Aspergillus* Genome database (<http://www.aspergillusgenome.org/>). From the list of interactants, we selected ArpB (an Arp2 ortholog and member of the putative Arp 2/3 complex), Cyp4 (a putative cyclophilin), and PpoA (a characterized fatty acid oxygenase [22, 35]) as prototypical interactants worthy of further exploration (Table 2). Deletion of *cyp4* resulted in a reduction in radial growth under basal conditions compared to the wild-type strain (Fig. S3A); however, there was no significant difference in radial growth response to either caspofungin or nikkomycin Z exposure (Fig. S3B). Deletion of *ppoA* resulted in an increased susceptibility to nikkomycin Z, but not caspofungin, when screened on GMM agar (Fig 2). Nonetheless, the caspofungin minimum effective concentration (MEC) of the Δ *ppoA* strain was 0.25 μ g/mL, compared to the wild-type strain MEC of 1 μ g/mL (Table 3). Using a *ppoA-rfp aspB-egfp* co-expressing strain, we did not observe any AspB and PpoA co-localization under basal conditions or after exposure to anti-cell wall drugs (data not shown). Unfortunately, we were unable to assess ArpB's role in the caspofungin response as we only were able to obtain an *arpB* heterokaryon that produced inviable spores, as validated via both germination assays and viability staining with Bis-(1,3-dibutylbarbituric acid) trimethine oxonol (DiBAC) fluorescent dye (data not shown).

4. Discussion

Septins are cytoskeletal components that participate in key cellular processes, including cytokinesis, hyphal morphology, diffusion barriers, and cell wall integrity [10-13, 15, 36, 37]. In *Cryptococcus neoformans*, deletion of septin genes leads to an increased susceptibility to cell wall damaging agents such as SDS and caffeine [11]. In *C. albicans* and *A. fumigatus*, the core-septin mutants exhibit increased susceptibility to caspofungin [17, 18, 38]. The *A. fumigatus* $\Delta aspB$ strain has similar distribution of β -glucan and chitin when compared to the wild-type strain, and its β -glucan content was also similar to that of the wild-type strain [18]. However, when the $\Delta aspB$ strain is exposed to caspofungin, the amount of β -glucan released increases relative to wild-type strain, indicating aberrant cell wall assembly occurring due to the deletion of *aspB* [18]. In *C. albicans*, exposure to caspofungin leads to a rapid redistribution of septins that co-localize with chitin patches [19]. *A. fumigatus* AspB also redistributes following exposure to caspofungin, unifying the link between septins and cell wall stress in fungi [18].

Here, we explored the AspB interactome under basal conditions as well as after caspofungin exposure to further our understanding of how septins contribute to cell wall integrity in the setting of antifungal exposure with a guideline-recommended clinically approved agent. Echinocandins are increasingly used to treat invasive aspergillosis due to the emergence of azole resistant *A. fumigatus* isolates [5]. However, there is potential of *A. fumigatus* isolates to develop resistance to caspofungin [39-41]. Similar to other fungal septins, AspB interacts with the other septins, as well with the actin and microtubule networks [18, 26, 30, 42]. A significant overlap of septin-interacting proteins is observed between the proteins are pulled-down by *A. fumigatus* AspB and those from the *S. cerevisiae*, suggesting that there is some conservation in protein interaction with septin complexes across fungal species [43]. However, the septin associated kinase Gin4, which associates with the septin complex in *S. cerevisiae*, did not co-purify with

AspB [43]. It is possible that Gin4 interacts with the *A. fumigatus* septin complex at a particular stage of growth or cellular process that we failed to capture in our analyses. *A. nidulans* and *S. cerevisiae* septins' interaction is dependent on the stage of growth, providing further support to this notion [30, 43].

The AspB interactome changes following caspofungin exposure, as 69 proteins are no longer co-purified with AspB while 54 new proteins co-purified with AspB under caspofungin exposure. Proteins involved with cell polarity (Cla4, RasB, MobB) no longer interact with AspB, and new metabolically-related proteins interact with AspB after caspofungin exposure, especially those involved in secondary metabolism. In particular, we found two proteins involved in pseurotin A metabolism associated with AspB in these conditions. Pseurotin A is reported to inhibit IgE production [44], and its synthesis is enhanced during hypoxia [45]. However, the role of these metabolism-related proteins in cell wall integrity remains an open question. Of the caspofungin-mediated interactants evaluated, only PpoA is involved in response to the chitin synthase inhibitor nikkomycin Z when tested on GMM agar. However, we observed an increase in susceptibility of the $\Delta ppoA$ strain to caspofungin when tested via approved antifungal susceptibility methodology. This may suggest that PpoA is involved in some cell wall remodeling process. It is possible that more than one interacting protein contributes to cell wall integrity and they might have overlapping roles; thus, a single gene deletion analysis may not be sufficient to fully define this.

In conclusion, AspB interacts with a robust number of proteins that could contribute to the different roles carried out by this septin in both basal growth as well as growth following antifungal stress. After exposure to a clinically relevant concentration of caspofungin, AspB interacts with 54 new proteins, suggesting the importance of AspB in mediating this stress

response. We also observed that in both conditions, members of the canonical cell wall integrity pathway (Rho1, Mkk2, Mpk1) and AspB could serve as a scaffold for signal transduction of the cell wall integrity pathway. The number of spectral counts for Mpk1 doubles compared to the spectral counts of Rho1 and Mkk2 that remain constant. To verify this, further experiments are needed where we can quantitatively compare AspB interaction in the presence and absence of caspofungin, and the role AspB might play in signal transduction of the canonical cell wall integrity pathway.

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Figure Legends

Fig. 1 AspB interactome changes after exposure to a clinically relevant concentration of Caspofungin (A) Biological process gene ontology enrichment of the basal conditions AspB interactome **(B)** AspB interacts with 54 new proteins after caspofungin exposure.

Fig 2. Deletion of *ppoA* increased susceptibility to nikkomycin Z, but not caspofungin on agar plates. Conidia (10^4) from each strain were inoculated into GMM agar, GMM agar

containing Caspofungin (1µg/mL) or Nikkomycin Z (2 µg/mL), and incubated for 3 days at 37°C.

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397 **Table 1. Summary of AspB interacting proteins as identified by LC-MS/MS**

AspGD ID	Protein	Spectral Count
<i>Septins</i>		
AFUA_5G08540	AspA	329
AFUA_5G03080	AspC	415
AFUA_1G08850	AspD	394
AFUA_3G07015	AspE	40
<i>Cytoskeleton</i>		
AFUA_6G04740	Act1	74
AFUA_2G14990	TubB	28
AFUA_1G02550	Tubulin Alpha subunit	65
AFUA_1G10910	Tubulin Beta subunit	77
<i>Phosphorylation/dephosphorylation</i>		
AFUA_5G05900	Cla4	4
AFUA_6G07980	NimX	7
AFUA_5G09360	CnaA	3
AFUA_4G13720	MpkA	3
AFUA_1G05800	MkkA	9
AFUA_1G09170	MobB	5
<i>Cell Wall Synthesis /Hyphal Growth/Polarity</i>		
AFUA_3G14420	ChsG	4
AFUA_6G06900	Rho1	20
AFUA_2G07770	RasB	14

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404 **Table 2. Summary of AspB interacting proteins after caspofungin exposure**

AspGD ID	Protein	Spectral Count
<i>Secondary Metabolism</i>		
AFUA_8G00540	PsoA PKS-NRPS hybrid synthase	41
AFUA_5G10120	NRPS-Like	3
AFUA_8G00440	PsoF Mono-oxygenase	7
<i>Induced after exposure to antifungal agents</i>		
AFUA_1G13330	ArpB	4
AFUA_2G03720	Cyp4	3
AFUA_4G10770	PpoA	9

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406 **Table 3 Minimum effective concentration of caspofungin and nikkomycin Z on *Aspergillus***

407 *fumigatus* strains

Strain	Caspofungin (µg/mL)	Nikkomycin Z (µg/mL)
AF293	1	2
<i>ΔppoA</i>	0.25	1
<i>OE::ppoA</i>	2	4

408

Figure

Figure 1

A

B

Figure 2