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Characterization of the major Woronin body protein HexA of the human pathogenic mold *Aspergillus fumigatus*

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ABSTRACT

In filamentous fungi, the septal pore controls the exchange between neighbouring hyphal compartments. Woronin bodies are fungal-specific organelles that plug the pore in case of physical damage. The Hex protein is their major and essential component. Hex proteins of different size are predicted in the data base for pathogenic and non-pathogenic *Aspergillus* species. However, using specific monoclonal antibodies, we identified 2 dominant HexA protein species of 20 and 25 kDa in *A. fumigatus*, *A. terreus*, *A. nidulans*, and *A. oryzae*. HexA and Woronin bodies were found in *A. fumigatus* hyphae, but also in resting conidia. Using monoclonal antibodies, a GFP-HexA fusion protein, and an RFP protein fused to the putative peroxisomal targeting sequence of HexA, we analyzed the spatial localization and dynamics of Woronin bodies in *A. fumigatus* as well as their formation from peroxisomes. In intact hyphae, some Woronin bodies were found in close proximity to the septal pore, while the majority was distributed in the cytoplasm. Septum-associated Woronin bodies show a minimal lateral movement, while the cytosolic Woronin bodies are highly dynamic. The distribution of Woronin bodies and their co-localization pattern with peroxisomes revealed no evidence that Woronin bodies arise predominantly at the apical tip of *A. fumigatus* hyphae. We found that Woronin bodies are able to plug septal pores of *A. fumigatus* in case of damage. Woronin bodies therefore contribute to the stress resistance and potentially also to the virulence of *A. fumigatus*, which renders them a potential target for future anti-fungal strategies.

Introduction

The mycelium of filamentous fungi is a three-dimensional network of interconnected multi-nucleated hyphae. Individual cells or compartments are separated by septa, but in the absence of stress, their cytoplasm remains connected via the septal pores. This organisation allows an efficient exchange and transport of molecules and even organelles; it enables filamentous fungi to respond to external cues as a functional entity and to exploit environmental resources in a very efficient manner. However, the presence of a continuous cytoplasm also poses a problem, as local injuries represent a potential threat for the whole hyphal network. *Pezizomycotina* have developed an organelle to plug septal pores in case of damage, and these so-called Woronin bodies were first described by Michail Stepanowitsch Woronin in 1864 (Woronin, 1864). In many fungi, Woronin bodies are found in proximity to the septal pore. They contain a dense core that assembles at the peroxisomal membrane (Jedd and Chua, 2000). Its major component, the Hex-1 protein, forms a crystal lattice that is essential for Woronin body formation and function (Yuan et al., 2003). In damaged cells, Woronin bodies rapidly plug septal pores (Reichle and Alexander, 1965; Collinge and Markham, 1985), and mutants lacking *hex-1* or homologous genes show extensive cytoplasmic bleeding after cellular wounding and several secondary defects such as impaired conidiation (Jedd and Chua, 2000; Soundararajana et al., 2004; Maruyama et al., 2005). Our current knowledge on the organization and function of Woronin bodies has been established using *Neurospora crassa* as a model organism for filamentous fungi. However, *N. crassa* has some unique features, such as an extremely rapid growth and a continuous and fast cytoplasmic flow, thus distinguishing it from most other filamentous fungi, e.g. important fungal pathogens, such as *Aspergillus fumigatus*.

A. fumigatus is the major cause of invasive aspergillosis, a severe and systemic infection in immunocompromised patients. The high mortality is largely due to the suboptimal diagnostic and therapeutic options and the unspecific symptoms of this disease (McCormick et al., 2010). The identification of new therapeutic target structures is therefore an urgent need. For 2 reasons, Woronin bodies are particularly interesting in this context; they are fungal-specific organelles, and their loss has a high probability to cause an impaired stress resistance and virulence. Here, we have started to characterize the HexA protein of *A. fumigatus*. Using monoclonal antibodies and fluorescent fusion proteins, we demonstrate that Woronin bodies plug the septal pore in damaged *A. fumigatus* hyphae. Using live

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cell imaging, we furthermore provide first insights in their spatial and temporal distribution and dynamics.

Materials and methods

Strains and media

The *A. fumigatus* strain AfS35 is a derivative of strain D141 lacking the homologous end-joining component AkuA (Krappmann et al., 2006). Aspergillus minimal medium (AMM) and yeast glucose medium (YG) medium were prepared as described (Kotz et al., 2010), but in this study AMM was supplemented with 0.2 M ammonium chloride as nitrogen source.

Sequence analysis and data base searches

Sequences from *Aspergillus* Hex-1 homologous sequences were obtained from the Central *Aspergillus* Resource (CADRE; http://www.cadre-genomes.org.uk/). Homology searches were performed using BlastP at Fungal Genomes Central – NCBI – NIH (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi?organism=fungi). Sequence alignments were generated using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). cDNA of *A. fumigatus* strain D141 was kindly provided by Sweta Samantaray and Johannes Wagener. Chromosomal and cDNA was analyzed using oligonucleotides HexA-5′ (ATG GGT TAC TAC GAC GAC GAC) and HexA-3′ (TTA CAG ACG GGA ACC GTG GAT GAC CTT). The large and the smaller amplicons obtained from cDNA were sequenced using oligonucleotide sGFP-300bp-rev (GAA GAA GAT GGT GCG CTC) (GATC AG, Constance, Germany).

Generation of monoclonal antibodies

The last 1065 bp of AFUA_5G08830 (corresponding to exon 4) were amplified using the oligonucleotides HexA-5′-XhoI (GCG**CTCGAG**TACCGTGAATCCACTTCTCGC) and HexA-3′-XhoI (CGC**CTCGAG**CAGACGGGAACCGTGGATGAC). The fragment obtained after digestion with XhoI was cloned into the XhoI site of the expression vector pET21b(+) (Novagen, Darmstadt, Germany). After transformation into *E. coli* strain BL21, a clone over-expressing a His-tagged protein of approximately 40 kDa was selected, and the fragment was sequenced. The recombinant protein was purified using Protino Ni-IDA 100 columns according to the instructions of the manufacturer (Macherey-Nagel, Düren, Germany). Balb/c mice were immunized, and hybridomas were generated and cloned according to standard procedures (Celis et al., 1994). The CatA-specific monoclonal antibody D40-3 was raised against a recombinant protein representing the N-terminal half of CatA (Schwienbacher, 2005).

Immunofluorescence

Hyphae or germlings grown on glass cover slips in AMM at 30 °C were fixed with 3.7% formaldehyde/PBS and washed twice with PBS. For cell wall digestion, samples were incubated in 300 μl PBS containing 60 mg bovine serum albumin and 6 mg lysing enzymes from *Trichoderma harzianum* (Sigma) for 1 h at 37 °C. Treated cells were then permeabilized using 0.5 ml 0.2% Triton X-100 in PBS (1 min at RT) and subsequently blocked with 2% goat serum/PBS for 1 h. After 3 washing steps with PBS, samples were incubated with the primary antibody in a humid chamber for 30 min and washed 3 times with PBS. Bound antibodies were stained using Cy3-labelled anti-mouse immunoglobulin (Dianova, Hamburg, Germany). After another 3 washes with PBS, samples were mounted with Vecta Shield (Vector Laboratories, Burlingame, California, USA) and analyzed using a Leica SP5 confocal scanning microscope (Leica Microsystems, Wetzlar, Germany).

Protein extraction and western blot

For protein extractions from resting conidia, 75-cm^2 flasks containing YG agar were inoculated with AfS35 conidia and grown at 37 °C for 3 days. Conidia were harvested in sterile water, and the pellet was frozen overnight at −80 °C and lyophilized. The resulting dry material was ground with a mortar and pestle in liquid nitrogen. The ground powder was resuspended in sample buffer (2% [w/v] SDS, 5% [v/v] mercaptoethanol, 60 mM Tris/Cl pH 6.8, 10% [v/v] glycerol, 0.02 [w/v] bromophenol blue), heated at 94 °C and immediately extracted twice using a Fast Prep 24 device (M.P. Biomedical, Irvine, CA) (speed: 6.5 m/s for 35 s), followed by a final heat denaturation at 94 °C for 5 min. Samples were separated on 12% SDS gel, blotted onto nitrocellulose membranes, and finally stained with the appropriate primary and alkaline phosphatase-labelled secondary antibodies.

For protein extractions from hyphae, 4×10^7 resting conidia were inoculated in 20 ml AMM and incubated over night at 37 °C. Proteins were extracted and analyzed as described above. The histidine hepta-peptide motif in the non-spliced HexA polypeptide was detected using the His-Tag antibody #2365 from CellSignaling Technology (Danvers, MA 01923, USA).

Construction of strains expressing fluorescent fusion proteins

In order to fuse the PTS1 signal from HexA to the C terminus of the red fluorescent protein, the *rfp* gene was amplified using gpdA-5′-NsiI and RFP-3′-PTS1. The core *hexA* gene was amplified using HexA-short-5′-Bsp1407I (GCG TGT ACA ATG GGT TAC TAC GAC GAC) and HexA-3′ (TTA CAG ACG GGA ACC GTG GAT GAC CTT). The PCR product was digested, cloned into the pSK379 backbone, and introduced into *A. fumigatus* AfS35 using protoplast transformation. The strain expressing cytosolic RFP was generated using a derivative of pSK379 containing the *rfp* gene under the control of the *gpdA* promoter.

Live-cell imaging

To visualize the effects of fludioxonil on germ tubes, resting conidia of a derivate of strain Afs35 expressing cytoplasmic RFP were inoculated in 8-well ibidi-chambers (ibidi GmbH, Martinsried, Germany) containing 300 μl AMM. Germ tubes were generated by overnight incubation at 30 °C. Fungal cells of the appropriate length were treated with 1 μg/ml fludioxonil for 4 h as described previously (McCormick et al., 2012). Samples were subsequently analyzed at 37 °C using a Leica SP-5 microscope equipped with an environmental chamber adjusted to 37 °C (Leica Microsystems).

Results

The hexA locus of A. fumigatus

The data base entries for the Hex-1 protein of *N. crassa* (acc. no. P87252) and the HexA protein of *A. fumigatus* AFUA_5G08830 show a substantial difference in their molecular weights, being 19.1 kDa for Hex-1 and 61 kDa for HexA. Searching for homologues in other *Aspergillus* species also revealed substantial differences: the predicted HexA proteins of the pathogenic species *A. terreus* (ATET_06581) and *A. fumigatus* are substantially larger (∼60 kDa) than their homologues in *A. oryzae* (AO090020000450) and *A. nidulans* (ANIA_04695) (∼20 kDa). Larger HexA homologues are also predicted in other pathogenic fungi, e.g. *Penicillium marneffei* and *Coccidioides posadasii*. An alignment of the HexA protein sequences