Mycologia, 108(3), 2016, pp. 528–532. DOI: 10.3852/15-218

Laser capture microdissection to identify septum-associated proteins in *Aspergillus nidulans*

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Abstract: To spatially resolve genetic differences at the cellular level, the laser-capture microdissection technique was developed. With this method cells can be cut from tissues with a laser beam and analyzed for DNA, RNA or protein composition. Here we adapted the technique to isolate septal microtubule-organizing center (MTOC)-associated proteins in *Aspergillus nidulans*. About 3000 septa were collected and subjected to peptide fingerprinting by mass-spectrometric analysis. We identified the microtubule polymerase AlpA and found it interacts with ApsB specifically at sMTOCs, suggesting that AlpA might be involved in the assembly or the functioning of this protein complex.

Key words: *Aspergillus*, microtubule, MTOC, septal MTOC, septum

The analysis of subtle genetic differences at the cellular or subcellular level is challenging. Whereas previously many approaches used entire tissues to identify differentially expressed genes, laser capture microdissection (LCM) allows for isolation and analysis of single cells from tissue. LCM was developed in 1996 (Emmert-Buck et al. 1996), then widely used for DNA, RNA and protein analysis in animal and plant tissues, and for fungi, chromosome preparations, and other applications. (Kubickova et al. 2002, Liu et al. 2010, MacDonald et al. 2012, Teichert et al. 2012). In fungi, subcellular structures such as the hyphal tips and septa are excellent candidates for analysis by LCM. However, in many fungi, hyphae are small with hyphal diameters of 2–3 μm. Therefore, the collected material contains only small amounts of the molecules to be analyzed. This may be less important if DNA or RNA are to be analyzed because the molecules can be amplified easily by PCR-based methods, but this amplification step is missing if proteins are analyzed. Here we describe a protocol we developed to isolate septa from *Aspergillus nidulans* to identify septum-associated proteins. We were especially interested in proteins of septal microtubule-organizing centers (sMTOCs), which were discovered in *A. nidulans* (Konzack et al. 2005). Previously, septa were isolated by LCM from *Rhizoctonia solani*, but the analysis was restricted to an electron microscopic analysis (van Driel et al. 2007).

LCM preparation of septa.—LCM was performed with a CellCut Plus system (MMI, Molecular Machines and Industries, Zürich, Switzerland) comprising an Olympus IX81 inverted microscope equipped with a UV laser (355 nm), microscope stage and isolation cup holder. Mycelia of FGSC A4 (Fungal Genetics Stock Center, Kansas City, Missouri) were grown on membrane slides in YAG medium (the hyphal diameter was 3–4 μm, which is thicker than the 2–3 μm in MM medium) and fixed in ethanol for 12 h. Media were prepared as described in Hill & Käfer (2001) After drying the slides, samples were covered with a microscope slide and observed with a 40× objective. Selected regions were cut with a UV laser through the microscope lens. The intensity and speed of the laser were 50–80% and 40–60%, respectively. Laser cutting was repeated 3–5 times to release each region. Normally to collect the cut regions, the cap of a collection tube is moved down to the membrane harboring the sample, the cut sample was collected in the cap, which was then raised again (Teichert et al. 2012). However, single septa of *A. nidulans* were too small to be collected in the cap. Therefore, we cut irregular shapes that included septa but also areas outside the hyphae (Fig. 1). About 3000 septa were collected in this way.

Mass spectrometry analysis.—The septa were processed for protein extraction and prepared for mass spectrometry. We used sodium deoxycholate (SDC) as a surfactant because it is compatible with trypsin and increases the solubility of hydrophobic proteins. In addition, SDC is easily removed from the acidified solution by adding a water-immiscible organic solvent (Masuda et al. 2007). Procedures were performed as follows. First, 50 μL of buffer (50 mM ammonium bicarbonate, 8 M urea, 1% SDC) were added to the tube with the collected septa. The tube was inverted

Submitted 4 Aug 2015; accepted for publication 28 Jan 2016.
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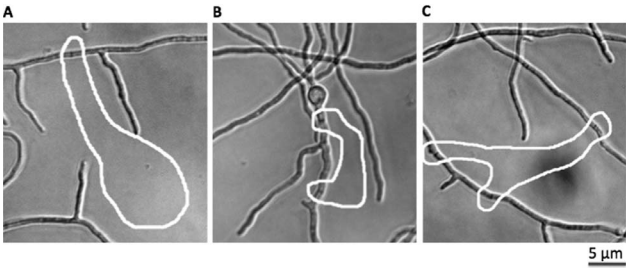


FIG. 1. Sampling of septa by laser microdissection. One, two or three septa were encircled in A, B and C, respectively. The area should be irregular and at least 100 μm^2. Bar = 5 µm.

so that the lid was covered with buffer, and incubated 30 min at 42 C. The sample was then sonicated on ice for 3 min (10 s interval every 5 s sonication). After 20 min incubation at RT, the sample was vortexed and transferred to a new cup and centrifuged at 13 000 rpm at 4 C for 1 min. Afterwards, dithiothreitol (DTT) was added to the supernatant to a final concentration of 10 mM and the sample was incubated at 50 C for 30 min. Iodoacetamide was added to a final concentration of 55 mM and the sample was incubated in the dark for 30 min at RT. After four-fold dilution of the sample with 50 mM ammonium bicarbonate solution, trypsin was added (Excision Grade, Bovine Pancreas, Calbiochem) to 1 µg/µL and the sample was incubated at 37 C overnight. Afterward, 100 µL ethyl acetate was added to 100 µL of the digested sample and the mixture was acidified with 0.5% trifluor-acetic acid to about pH 2. After centrifugation at 15 700 *g* for 2 min the aqueous phase containing the digested peptides was collected.

The peptide sample was desalted with StageTips C18 (Thermo Scientific) according to the manufacturer's instructions and eluted with 20 µL 80% acetonitrile containing 5% formic acid. The eluate was concentrated in the SpeedVac to a volume of approximately 5 µL. Afterward separation was performed on a Nano-LC Ultra system (Eksigent) using the following columns and chromatographic conditions: Peptides were loaded on a RPC trap column (ReproSil C18, 0.3 × 10 mm) and subsequently separated with an analytical column (C18 PepMap 100, 3 µm bead size, 75 µm i.d.; 15 cm long, LC Packings) with a linear gradient (A: 0.1% formic acid in water, B: 0.1% formic acid in 84% ACN) at a flow rate of 280 nl/min. The gradient used was: 1–60% B in 30 min, 100% B for 15 min. Mass spectrometry was performed on a linear ion trap mass spectrometer (Thermo LTQ Orbitrap XL, Thermo Electron) online coupled to the nano-LC system. For electrospray ionization, a distal coated SilicaTip (FS-360-50-15-D-20) and a needle voltage of 1.4 kV was used. The LTQ Orbitrap was operated in parallel mode performing precursor mass scanning in the Orbitrap (60.000 FWHM resolution at m/z 400) and isochronous acquisition of five data-dependent CID MS/MS scans in the LTQ ion trap. Afterward the LC-ESI-MS/MS data were used for a database search with the software Mascot (Matrix Science) with the *A. nidulans* protein sequences. Peptide mass tolerance was set to 50 ppm, a significance threshold $p < 0.05$ with an ion-score cut-off of 20. The mass-spectrometric analysis was done commercially by TopLab GmbH (Munich, Germany).

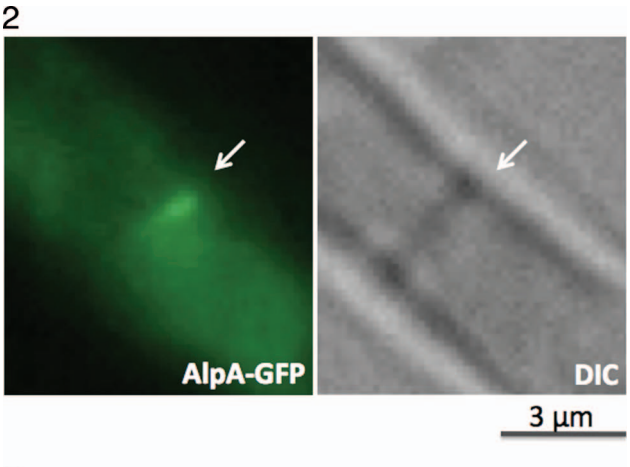
Mass Spec analysis of septa of *A. nidulans* collected by LCM resulted in the identification of seven proteins, one of which was ANID_05521 (TABLE I). 14 peptides from the N-terminal part of ANID_05521 were detected, giving a sequence coverage of 21% and a protein score of 643. Six additional proteins were identified with lower scores by the presence of only one or two peptides, including ANID_04189. Orthologs of ANID_04189 are involved in septum formation in *S. pombe* and in *S. cerevisiae* (TABLE I). Because of the low coverage of these peptides, we did not analyze them further.

TABLE I. *A. nidulans* proteins indentified with nano-LC-ESI-MS/MS

Protein	Description	Score	Queries matched
ANID_05521	AlpA, MT polymerase, spindle function	643	26
ANID_06750	Domain(s) with predicted oxidoreductase activity and role in metabolic process	36	1
ANID_02138	DEAD/DEAH box helicase	32	2
ANID_00944	ATP-dependent RNA helicase Rok1	31	1
ANID_03024	Nuclear protein	26	1
ANID_04189	MkkA, essential mitogen-activated protein kinase kinase (MAP2K), mutants arrest as branched germlings; ortholog in *S. pombe* is a component of septum; component of bud neck and bud tip in *S. cerevisiae*	24	1
ANID_11793	Ortholog(s) have roles in cellular response to biotic stimuli and filamentous growth of a population of unicellular organisms in response to biotic stimuli	22	1

AlpA (ANID_05521) is a highly conserved protein in eukaryotes, whose orthologs include *Saccharomyces cerevisiae* Stu2, *Schizosaccharomyces pombe* Alp14, *Xenopus laevis* XMAP215 and human TOG. They are known to be involved in microtubule and spindle functions including microtubule polymerization, spindle formation, kinetochore function and cell morphogenesis (Garcia et al. 2001, Ohkura et al. 2001, Brouhard et al. 2008, Takeshita et al. 2013). In *A. nidulans*, AlpA localizes at the plus end and the lattice of MTs and acts as an MT polymerase (Enke et al. 2007, Brouhard et al. 2008, Takeshita et al. 2013). By interaction with the cell-end marker TeaA at the hyphal tip cortex, AlpA also plays a role in microtubule guidance and polarity maintenance (Takeshita et al. 2013). During anaphase, AlpA migrates to kinetochores and moves to the poles with them. The role for AlpA could promote anaphase depolymerization of the spindle microtubules (Herrero et al. 2010).

Localization of AlpA.—We re-investigated the localization of AlpA and found GFP tagged AlpA at septa (FIG. 2) using strain SCE05 (Δ*argB*::*trpC*Δ*B*; *pyroA4*; *alcA(p)*::*GFP*::*alpA*) from Enke et al. (2007). The tagged protein was associated only temporarily with septa, suggesting loading of the protein to the septal MTOCs. The resolution of the pictures did not allow us to determine whether AlpA was part of the sMTOC or just loaded onto the growing MT plus end after emergence from the sMTOC. Therefore, we tested whether AlpA would interact with some sMTOC-associated proteins. To this end AlpA was fused to the C-terminal half of YFP and ApsB or gamma tubulin (MipA) to the N-terminal half. The interaction of the proteins was tested in the bimolecular fluorescence complementation assay. Plasmids pYH12 (*alcA(p)*::*YFP*^{C}::*AlpA* (N-1.0 kb), *pyr4*; Takeshita et al. 2013) and pYZ59 (*alcA(p)*::*YFP*^{N}::*apsB* (full length), *pyroA*) were co-transformed into TN02A3 (Nayak et al. 2006), yielding strain SYZ76 (Δ*nkuA*::*argB*; *alcA(p)*::*YFP*^{C}::*alpA*::*pyr4*; *alcA(p)*::*YFP*^{N}::*apsB*::*pyroA*), which was analyzed here. YFP^{C} indicates the fluorescent protein was attached at the C terminus; YFP^{N} indicates the fluorescent protein was inserted at the N terminus of the protein. About 100 hyphae were observed. The combination of ApsB with AlpA resulted in bright yellow spots in 92.4% of the septa examined. In 47.7% of the septa single spots were observed, whereas in 44.7% of the septa, a spot on either side of the septum was visible (FIG. 3). The combination of AlpA with MipA did not result in any fluorescent signal. Strain SYZ77 (Δ*nkuA*::*argB*; *alcA(p)*::*YFP*^{C}::*alpA*::*pyr4*; *alcA(p)*::*YFP*^{N}::*mipA*::*pyroA*) was constructed by co-transformation of plasmids pYZ12 and pYZ38 (*alcA(p)*::*YFP*^{N}::*mipA* (N-1.0 kb), *pyroA*). These results suggest loading of AlpA to the sMTOCs at the initiation of MT formation or a function in sMTOC formation. Of interest, at spindle-pole bodies (SPBs), the nuclear MTOCs, no interaction was observed.



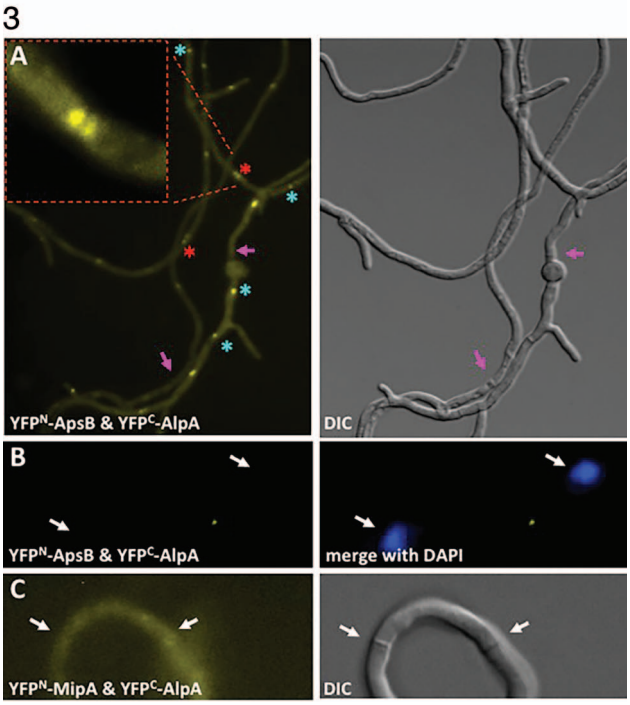


FIG. 2. Localization of GFP tagged AlpA at septum (arrows).

FIG. 3. Bimolecular fluorescence complementation assay between (A, B) AlpA and ApsB or (C) gamma tubulin (MipA). A. Interaction of ApsB and AlpA at septa. Strain SYZ76 (*alcA(p)*::*YFP*^{C}::*alpA, alcA(p)*::*YFP*^{N}::*apsB*) was incubated in MM (2% glycerol) overnight at 28 C. Single spots at septa (cyan asterisk). One spot on each side of the septum (red asterisk). Septa lacking signal (pink arrow). B. Interaction of ApsB and AlpA not at spindle pole bodies. Strain SYZ76 stained with DAPI (to label nuclei) and observed in the YFP channel. Arrows indicate the nuclei. C. Lack of interaction between AlpA and MipA. Strain SYZ77 prepared as in A. No significant YFP signal was visualized at septa (arrows) or spindle pole bodies. Hyphae are 3 µm diam.

These results document structural and functional differences between SPBs and sMTOCs. The observations of localization suggest that AlpA comprises a subunit of the sMTOC or is required for its assembly or functioning. Supporting this suggestion is the observation that in *S. cerevisiae* the AlpA ortholog, Stu2, interacts with Spc72 (the ortholog of ApsB) and is required for the anchorage of astral microtubules to the SPBs (Chen et al. 1998, Usui et al. 2003). We do not have any evidence for a similar function in *A. nidulans* because we did not observe interactions between AlpA and ApsB at the SPBs, only at sMTOCs. This is an interesting finding, and further analyses are required to determine if AlpA plays a role in MTOC assembly, structure or function.

Other than AlpA, no other septum-associated proteins (aside from ANID_04189, which had few recovered peptides; TABLE I) were identified, probably due to few proteins in the preparation. This raises doubts as to the abundance of AlpA at septa, as the protein is also found at the microtubule plus ends in the cytoplasm. However, we did not find other highly abundant cytoplasmic proteins such as tubulin or ribosomal proteins in the analysis of septa. This shows that the LCM method we describe here is suitable for the isolation of septum-associated proteins.

Conclusions.—To facilitate the collection of septa, the LCM process should be automated. This could be achieved by fluorescently labeling the structures of interest. In the case of septa, sMTOC-associated proteins such as ApsB or GcpC or a septum-specific kinase, KfsA, could be fluorescently tagged and used as localization markers (Takeshita et al. 2007, Zekert et al. 2010). However, the system currently does not allow sampling of individual septa in *A. nidulans* because of their small size. The use of fungi with larger hyphae, such as *Sordaria macrospora* or *Neurospora crassa,* would provide opportunities to automate sampling collection until the collection processes are more refined.

ACKNOWLEDGMENTS

This work was financed by the research unit FOR1334 of the German Science foundation (DFG) and the Mexican Consejo Nacional de Ciencia y Tecnología (CONACYT). YZ was a fellow of the Chinese Scholarship Council (CSC).

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