Title:

Phospholipid Flippases DnfA and DnfB Exhibit Differential Dynamics within the *A. nidulans* Spitzenkörper

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Abstract:

The Spitzenkörper is a structure at the apex of growing cells in many filamentous fungi. Ultrastructural studies indicate that the Spitzenkörper is an organized mass of secretory vesicles, with different types of vesicles present in outer and inner layers. Here, we used live-cell imaging to demonstrate that the phospholipid flippases DnfA and DnfB, which preferentially localize to the outer and inner layers, respectively, exhibit different dynamics in the Spitzenkörper of *Aspergillus nidulans*. Additionally, deletion of *dnfA* partially destabilized the Spitzenkörper, while the depletion of *cdc50*, an essential β -subunit of most flippases, had dramatic effects on hyphal tip organization and morphology.

Introduction:

Filamentous fungi grow by concentrating cell expansion at a discrete region in the tip of their cells. These cells, called hyphae, can maintain a consistent shape and growth rate over large spatial and temporal scales. Coordinating the direction and rate of growth in the Ascomycete and Basidiomycete fungi is a specialized secretory structure, the Spitzenkörper (SPK). The SPK is composed of post-Golgi secretory vesicles (SV) and endocytic vesicles that are continuously released for fusion with the plasma membrane (PM) (Pinar et al., 2015; Schultzhaus et al., 2016; Schultzhaus and Shaw, 2015; Verdín et al., 2009). A large proportion of secretory traffic produced in a mycelium, therefore, transits through the SPK. Beyond the basic ultrastructure, however, there is little insight into the forces regulating SPK dynamics and organization. Interestingly, a portion of SPK vesicles are derived from endocytic vesicles (Fischer - Parton et al., 2000; Schultzhaus and Shaw, 2015; Shaw et al., 2011), and the vesicles within the SPK are organized such that larger vesicles (macrovesicles) are present around the outside while smaller vesicles (microvesicles) are concentrated within the center. These different vesicles appear to have different cargo, with glucan synthases preferentially localizing to macrovesicles and chitin synthases enriched in microvesicles (Girbardt, 1957; Grove and Bracker, 1970; Howard, 1981; López-Franco and Bracker, 1996; Riquelme et al., 2007; Sánchez-León et al., 2011; Schultzhaus et al., 2015).

Aspergillus nidulans DnfA and DnfB are segregated within the SPK. These proteins belong to a conserved group of phospholipid-translocating ATPases (“flippases”), which regulate the lipid organization of PM as well as intracellular organelles such as vacuoles and late Golgi. Their localization within the SPK suggested that the distribution of lipids on secretory vesicles could play a role in SPK structure, and provided a means to test the dynamics of different regions of the SPK (Baldrige and Graham, 2012; Schultzhaus and Shaw, 2016; Schultzhaus et al., 2015). Here, compared rates of flow of both proteins through the Spitzenkörper using Fluorescence Recovery after Photobleaching (FRAP), and examined the role of these flippases in the organization of the hyphal tip.

Results and Discussion:

A. nidulans DnfA and DnfB preferentially localize to different regions of the SPK and the PM (**Figure 1A**), with DnfA concentrated on the outside of the SPK and DnfB within the SPK core. This distribution suggests that these proteins may be present on different sets of vesicles (DnfA on macrovesicles, DnfB on microvesicles). If DnfA-GFP and DnfB-GFP are present on different vesicles, they may exhibit different FRAP recovery dynamics, as is the case for several

SPK markers in *N. crassa* (Sánchez- León et al., 2015). To test this hypothesis, we performed FRAP on DnfA-GFP and DnfB-GFP in the SPK in growing hyphae (see **Materials and Methods**) and determined the $t_{1/2}$. This quantity represents the time (s) at which one half of the final fluorescence intensity is recovered, and in this case it provides information on how rapidly new fluorophore traffic into the photobleached region. As shown in **Figure 1B**, DnfB-GFP has a significantly lower $t_{1/2}$ (15.81 $\pm$ 1.17, N=14) compared to DnfA-GFP (30.78 $\pm$ 3.17, N=13). This differential rate can also be observed in **Video 1** and **Figure 1C**, in which the concentrated SPK core labelled by DnfB-GFP is rapidly replenished when compared to the larger, more diffuse region labelled by DnfA-GFP. These quantities are similar to those calculated for some *N. crassa* proteins associated with macrovesicles (Fks-1, $t_{1/2}$ = 31.7s), and microvesicles (Ypt-1, $t_{1/2}$ = 15.5s) (Sánchez-León and Riquelme, 2015; Sánchez - León et al., 2015), but different than the *A. nidulans* Rab-GTPase Ypt31/RabE, which was found to recover with a $t_{1/2}$ of ~10s (Pantazopoulou et al., 2014). This discrepancy could be the result of the characteristics of individual proteins and their association with vesicles (i.e. membrane bound or lacking in transmembrane domains), but more work is needed to assess the full spectrum of SPK protein dynamics.

Next, we looked at the role of flippases in SPK structure and dynamics during growth. In *A. nidulans*, the SPK generally maintains its position within the center of the hyphal tip, resulting in the production of straight hyphae, as seen in **Figure 1D** and **Video 2** through labelling of the SPK with GsaA-mCherry, a subunit of *A. nidulans* 1, 3- β -glucan synthase. When the flippase *dnfA* is deleted, however, hyphae are wider and bulbous and exhibit a reduction in growth rate of ~70% (Schultzhaus et al., 2015). Accordingly, as shown in the lower four panels of **Figure 1D** (which comprise image sequences of two separate hyphae), the SPK moves laterally within the apical dome during growth, and occasionally diffuses and recovers. This effect could be explained if DnfA has a role in altering the lipid asymmetry of vesicles within the SPK, or the number of vesicles, with a resulting alteration to the interactions between molecular components (i.e. actin and vesicle-associated proteins) that contribute to SPK stability. Deleting *dnfB* did not result in an observable change in SPK dynamics (not shown). This was expected, because although DnfB strongly labels the SPK, previous studies showed that a *dnfB* deletion alone did not radically effect hyphal growth or morphology (Schultzhaus et al., 2015). Additionally, although DnfB (Drs2 in yeast) was previously implicated in the production of a specialized set of clathrin-associated secretory vesicles in budding yeast (Gall et al., 2002), there is no evidence of a subset of clathrin-labelled secretory vesicles in *A. nidulans*, as clathrin is generally excluded from the hyphal tip (personal observations). Alternatively, DnfB could be involved in the trafficking of specific cargo from the late Golgi, rather than the production or alteration of secretory vesicles.

Finally, we considered the additive effects of depleting DnfA and DnfB on hyphal tip organization, through manipulation of the *A. nidulans cdc50* homolog (AN5100). Cdc50 proteins are involved in proper localization of phospholipid flippases represented in *A. nidulans* as DnfA, DnfB, and DnfC (Saito et al., 2004). An *Ancdc50* Δ phenocopies a *dnfA* and *dnfB* double deletion mutant (Schultzhaus et al., 2015). We placed the native *Ancdc50* promoter with the *niiA* promoter that we have used previously (Hervás-Aguilar and Peñalva, 2010; Punt et al., 1995; Schultzhaus et al., 2015), which exhibits strong repression in the presence of ammonium and expression in the presence of nitrate. This allowed us to effectively observe the secretory vesicle marker GsaA-mCherry and the endocytic marker FimA-mCherry when both DnfA and DnfB are depleted.

Figure 1E shows GsaA-mCherry in *Ancdc50* expressing conditions (with 40mM sodium nitrate as a nitrogen source) and in repressing conditions (with 40mM ammonium tartrate as a nitrogen source). When *Ancdc50* is expressed, GsaA-mCherry labels the SPK. Repression of *Ancdc50* results in extremely aberrant growth with the absence of a SPK. In these cells, GsaA-mCherry is not concentrated within the apex, but is diffuse within the cytoplasm, suggesting that, together, *dnfA* and *dnfB* share an essential role in *A. nidulans* secretion. Additionally, since the phospholipid character of the membrane may play a role in the localization of endocytosis, we examined the endocytic marker FimA-mCherry when *Ancdc50* is repressed. Under inducing conditions, as in wild type, FimA-mCherry is strongly polarized. To quantify this, fluorescence intensity of a 2.5 μ m window was collected at the apex and compared to a similarly-sized region 10 μ m behind this region. Under expressing conditions, this ratio was 3.52 $\pm$ 0.08, while this value was reduced to 1.5 $\pm$ 0.19 under repressing conditions (N=5 hyphae for both, $p < 0.001$). The lifetimes of endocytic patches was not affected (not shown), which suggests that this difference results from a reduced ability to establish a normal zone of endocytosis when *Ancdc50* is repressed and lipid asymmetry is disrupted.

In conclusion, flippases DnfA and DnfB have differential dynamics within the SPK, suggesting that they associate with different vesicles. Additionally, in the likely absence of flippase function, when *Ancdc50* is depleted, secretion is highly disrupted, while the polarization of endocytosis is reduced, but not abolished. These results provide an insight into the origin and the maintenance of the SPK, as well as the role of flippases in *A. nidulans*, but more experiments, in particular biochemical tests on the components of SPK-specific vesicles, and deeper quantitative analysis of flow of vesicles through the SPK, are necessary next steps to understand this organelle.

Methods:

Aspergillus culture conditions and genetic techniques

A. nidulans strains were cultured on minimal medium (MM) with supplements following standard protocols (Kaminskyj, 2001). Strains producing DnfA-GFP (ZSS3) and DnfB-GFP (ZSS2) were constructed previously (Schultzhaus et al., 2015). *gsaA::mCherry* or *fimA::mCherry* strains were obtained through insertion of the mCherry coding sequence immediately prior to the stop codon, and *niiA::Ancdc50* was produced by insertion of the *niiA* promoter before the *Ancdc50* start codon as previously described (Schultzhaus et al., 2015). Expressing conditions used 40mM sodium nitrate as a nitrogen source, and repressing conditions used 40mM ammonium chloride as a nitrogen source, as in previous studies (Hervás-Aguilar and Peñalva, 2010; Schultzhaus and Shaw, 2016). Genetic manipulation was done using previously described protocols (Oakley et al., 2012). For each transformation, eight transformants were tested by PCR with primers flanking the transformation construct insertion site, as well as for expected fluorescence, growth, and development phenotypes.

Image Capture and Analysis

Conidia were streaked onto a plate of solid MM with supplements and incubated for 16-20 hours in 30°C, after which a block of agar containing the leading hyphae was excised and placed upside down on a 25mm x 60mm coverslip containing a 15 μ m drop of liquid MM (inverted microscope), or on a microscope slide and the cells were covered with 15 μ m of liquid

minimal medium followed by a coverslip (upright microscope). All cells were then incubated at 30°C for 30 minutes to facilitate attachment to coverslips.

FRAP for DnfA, DnfB were done on an Olympus FV1000 FluoView inverted laser scanning confocal microscope system (Olympus, Japan) as previously described (Lara-Rojas et al., 2016). FRAP included capturing an image every 5s, and bleaching a circular region encompassing the Spitzenkörper for seven complete scans with full laser intensity. FRAP analysis was completed using the ImageJ FRAP Profiler plugin (from the Hardin lab <http://worms.zoology.wisc.edu/research/4d/4d.html#frap>) for image sequences in which cell growth was unperturbed and the cell tip did not leave the plane of focus during image capture. The StackReg ImageJ plugin was used to align growing hyphal tips for automated FRAP analysis.

Imaging of GsaA-mCherry and FimA-mCherry was completed on an Olympus BX-51 microscope equipped with a Hamamatsu Orca-ER CCD camera (Hamamatsu, Japan) driven by Slidebook 5.0 Software (Intelligent Imaging Innovations, Colorado, USA) as previously described (Shaw and Upadhyay, 2005). All figures and videos were processed for publication using ImageJ and Adobe Photoshop 5.0 (Adobe, California, USA).

Figure Legends:

Figure 1. Spitzenkörper protein dynamics in *Aspergillus nidulans*. **A** Localization of flippases DnfA and DnfB within the Spitzenkörper of *A. nidulans*. **Thin Arrows** indicate plasma membrane localization of DnfA. **Thick Arrows** indicate the core of the Spitzenkörper as labeled by DnfB-GFP. **B.** $t_{1/2}$ of DnfA-GFP and DnfB-GFP fluorescence recovery profiles. The mean $t_{1/2}$ of DnfA-GFP and DnfB-GFP were found to be significantly different when compared using a Student's t-test, with a P-value of <0.001 (indicated by ***). **C.** Mean fluorescence recovery curves for DnfA-GFP (**Red**) and DnfB-GFP (**Black**). Bars on data points indicate $\pm$ standard error. **D.** SPK dynamics as visualized by GsaA-mCherry in the apex of a wild type hypha (upper two panels) and a *dnfA* deletion mutant (lower four panels). Lower two groups of panels represent two separate image sequences of separate hyphae. Arrows represent lateral SPK movement, and brackets represent SPK dissipation. Time between images = ~60 seconds. **E.** GsaA-mCherry localization when *Ancdc50* is expressed (NO₃ as nitrogen source, left panel) or repressed (NH₄ as nitrogen source, right two panels). **F.** FimA-mCherry localization when *Ancdc50* is expressed (left panel) or repressed (right panel). Asterisks in **F** represent the apex of the hyphal tip.

Video 1: Representative FRAP videos of DnfA-GFP and DnfB-GFP. This video shows a representative image sequence of DnfA-GFP fluorescence recovery in the left panel. DnfB-GFP recovery after photobleaching with similar microscope settings is shown in the right panel. In this comparison of the image sequences, DnfB-GFP can be seen recovering more quickly than DnfA-GFP which is also more diffuse in the apex, while the DnfB-GFP-labeled Spitzenkörper is highly concentrated and recovers relatively rapidly. Scale bar = 5 μ m, time in mm:ss.

Video 2: Spitzenkörper dynamics in *dnfA* Δ . In this video, the Spitzenkörper is marked by GsaA-mCherry. The upper panel shows a rapidly growing wild type hyphal cell with an SPK remaining a consistent size, brightness, and location during growth. Lower panels show GsaA-

mCherry localization in a *dnfA* deletion mutant. In this mutant, hyphae exhibit markedly slower growth than in wild type, which corresponds to a somewhat smaller and more diffuse SPK, which can be seen changing in size and moving laterally within the apex during growth. Scale bar = 10µm, time in hh:mm:ss.

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Figure 1