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Tools and techniques

Experimental measurement of endocytosis in fungal hyphae

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ABSTRACT

The present study examines the notion that polarized exocytosis in the tips of growing hyphae creates an excess of plasma membrane and thus the need for its removal by endocytosis. To measure endocytosis experimentally, we developed a photobleaching (FRAP) procedure to count endocytic events in hyphae of *Neurospora crassa* carrying a fluorescent tag on the actin-binding protein fimbrin (FIM-1-GFP). Given 40 nm as the average diameter of endocytic vesicles, we calculated that about 12.5% of the plasma membrane discharged in the apex becomes endocytosed in the subapex. According to our calculations, the GFP-tagged hyphae of *N. crassa,* measured under the constrained conditions of confocal microscopic examination, needed about 8800 vesicles/min to extend their plasma membrane or about 9800/min, if we include predicted demands for cell wall growth and extracellular secretion. Our findings support the notion that exocytosis and endocytosis operate in tandem with the latter serving as a compensatory process to remove any excess of plasma membrane generated by the intense exocytosis in the hyphal tips. Presumably, this tandem arrangement evolved to support the hallmark features of fungi namely rapid cell extension and abundant secretion of hydrolytic enzymes.

1. Introduction

Whereas exocytosis is a well-documented process in fungal hyphae (Grove and Bracker, 1970; Howard, 1981; Mollenhauer and Morre, 1966; Roberson et al., 2010), the reverse process namely endocytosis has had a controversial history. In Girbardt's classic study (1969), he found it necessary to consider the possibility that the abundance of vesicles he had discovered in hyphal tips of fungi were poised to be secretory vesicles and not the result of endocytosis. Girbardt ruled out an endocytic origin by showing that growing hyphae in a medium containing K_2TeO_3, which stained the plasma membrane, failed to stain the vesicles in the cytoplasm (Girbardt, 1969). Similarly, Torralba and Heath (2002) found evidence against endocytosis in the mycelium of *Neurospora crassa* (Torralba and Heath, 2002). In contrast, the existence of endocytosis in yeast cells had been known much earlier (Riezman, 1985), and was later confirmed by characterizing a series of several proteins that participate in this process (Engqvist-Goldstein and Drubin, 2003; Kaksonen, 2008; Lu et al., 2016; Munn, 2001; Riezman, 1985; Smith et al., 2001). In mycelial fungi, the existence of endocytosis has now been convincingly demonstrated by live imaging of fungal transformants displaying endocytic proteins tagged with GFP. The largely confined region where most exocytosis and endocytosis take place in fungal hyphae, namely the hyphal apex and a subapical collar respectively (Araujo-Bazan et al., 2008; Atkinson et al., 2002; Berepiki et al., 2010; Delgado-Alvarez et al., 2010; Echauri-Espinosa et al., 2012; Lara-Rojas et al., 2016; Read and Kalkman, 2003; Taheri-Talesh et al., 2008; Upadhyay and Shaw, 2008), makes these tubular cells excellent subjects for studying both processes and their relationship. The present work extends a preliminary study (Bartnicki-Garcia et al., 2015) that suggested that endocytosis was needed to recycle excess of membrane produced by exocytosis. A key purpose of this study was to measure endocytosis in growing hyphae and estimate its relevance viz a viz exocytosis. We followed two tracks: 1) we developed a technique to measure endocytosis experimentally by monitoring the appearance of endocytic patches after photobleaching the endocytic collar of hyphae of *N. crassa* genetically modified by tagging specific proteins with green fluorescent protein (GFP); and 2) we used a spreadsheet to calculate vesicle contribution to plasma membrane as well as cell wall growth. This is the first attempt to collect quantitative data on the endocytic recycling of excess of membrane deposited by exocytosis. We concluded that fungi satisfy the demands for cell wall construction and extracellular enzyme secretion by exocytic discharge of the required materials in many more vesicles than are needed for plasma membrane extension thus creating a need to recycle excess membrane via endocytosis.

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2. Methods

2.1. Lifeact-GFP and FIM-1-GFP strains and culture conditions

Strain TRM49-OC30 (mat a *his-3*$^{+}$::P*ccg-1-lifeact-egfp*$^{+}$) and TRM08-DD02 (mat a *his-3*$^{+}$::P*ccg-1-fim-sgfp*$^{+}$) were used in this study (Delgado-Alvarez et al., 2010). The strains were maintained on Vogel's minimal medium (VMM) with 1.5% sucrose. All manipulations were according to standard techniques (Davis, 2000).

2.2. Laser Scanning Confocal Microscopy (LSCM)

Agar plugs (6 mm diameter) of the GFP-tagged strain *of N. crassa* were grown on VMM for 18 h at 30°C. The "inverted agar block" method (Hickey et al., 2002) was used for live-cell imaging and FRAP studies. An inverted laser scanning microscope FV1000 FluoView™ (Olympus, Japan) equipped with a Multi-line Ar laser was used for excitation of GFP at 488 nm. An UPlanFLN 60x, (NA 1.42) oil immersion and an UPlanSapo 20x/0.75 objectives were employed. Time-lapse images were recorded simultaneously by fluorescence and DIC (Differential Interference Contrast) channels with FluoView™ software (Olympus, Japan).

2.3. Fluorescence Recovery After Photobleaching (FRAP)

FRAP experiments were performed on growing tips of mature hyphae. Images were recorded with the focal plane set closest to the cell surrface. The time series, recorded one frame every 2–3 sec, began with control frames recorded to show the hyphae were actively growing before photobleaching the endocytic collar region. A 0.609 mW laser pulse intesity was applied to the specimens in a region of interest (ROI) for ~4 sec. To quantitate endocytosis, we counted the number of endocytic patches appearing in an area of 5 × 5 μm (25 μm^2) on the single frame after photobleaching Each patch was considered an endocytic event and the total number of events/sec calculated for the entire collar area. Before and after the photobleaching pulse, laser intensity was kept at 5% to avoid phototoxic effects. The time series was recorded at one frame every 2–3 sec. DIC images were simultaneously recorded to determine hyphal diameter and elongation rates, parameters used to calculate the area of exocytosed plasma membrane. Final figures were created with Adobe Photoshop CC 2017 (Adobe Systems Inc., San Jose, CA).

3. Results

3.1. Confocal microscopy of endocytosis and exocytosis in fungal hyphae

By following actin dynamics of hyphae tagged with Lifeact-GFP, the regions where endocytosis and exocytosis take place are clearly revealed (Fig. 1). This shows the unique advantage of fungal hyphae to study endocytosis with most endocytic events concentrated in a subapical region forming a distinct collar. The collar starts at ~5 μm from the apical region and extends for about 9 μm, as previously reported by Delgado-Alvarez et al. (2010) (Fig 2). The intensity of the endocytosis process can be readily appreciated in low magnification videos of growing hyphae tagged with Lifeact-GFP (Supplementary Movie 1). In the apical region, the intense labeling of the Spitzenkörper signals a strong interaction of exocytic vesicles with actin filaments. Note that fimbrin is a clear component of the endocytic collar but not of the Spitzenkörper.

3.2. Experimental measurement of endocytic events

To quantify the frequency of endocytic events, FRAP experiments were done on hyphae of *N. crassa* tagged with FIM-1-GFP. Individual hyphae were selected for evidence of normal growth, Spitzenkörper presence, regular morphology and stable fluorescence. Hyphal tips were examined by focusing on the upper cortex close to the plasma membrane where actin cables and actin patches are localized (Delgado-Alvarez et al., 2010). The entire process was recorded, analyzing the frames before, during and after photobleaching (Fig. 3). Following bleaching of the collar, the new fluorescent patches were counted, they

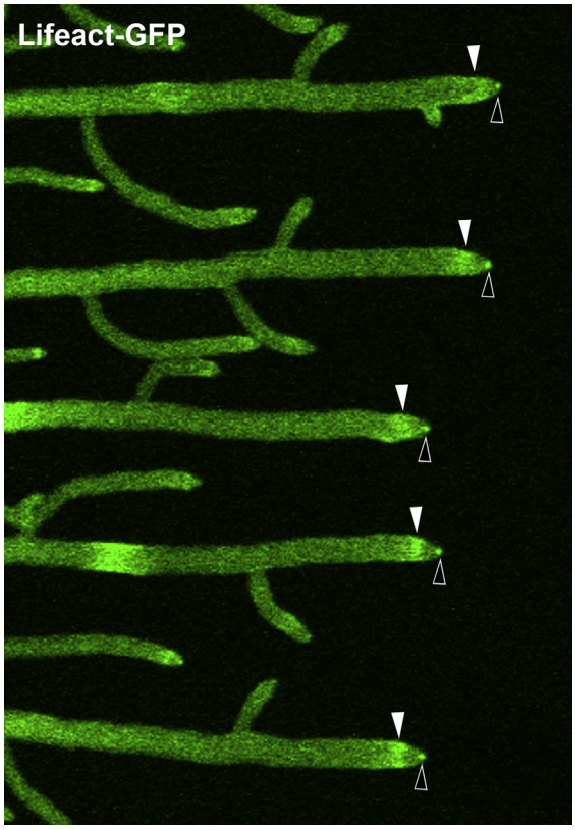

Fig. 1. Scene from Supplementary Movie 1 showing growing hyphae of *Neurospora crassa* labeled with Lifeact-GFP. The green fluorescence shows actin accumulation in the Spitzenkörper (empty arrowhead) and the endocytic collar (filled arrowhead). Bar = 10 μm.

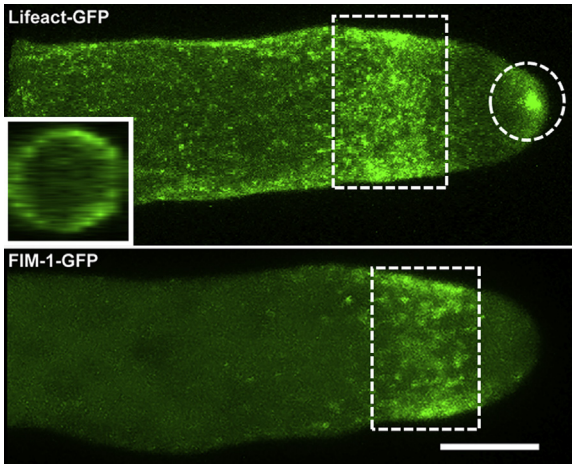

Fig. 2. 3D reconstruction of two hyphal tips of *Neurospora crassa* whose actin (top) or fimbrin (bottom) were tagged with Lifeact GFP or FIM-1-GFP respectively. Lifeact-GFP shows actin present in the Spitzenkörper (circle) and the endocytic subapical collar (rectangle). The insert is a transverse view of the middle of the collar showing that endocytic patches are distributed uniformly around the circumference of the collar. Fimbrin is only present in the endocytic region (rectangle). Bar = 5 μm.

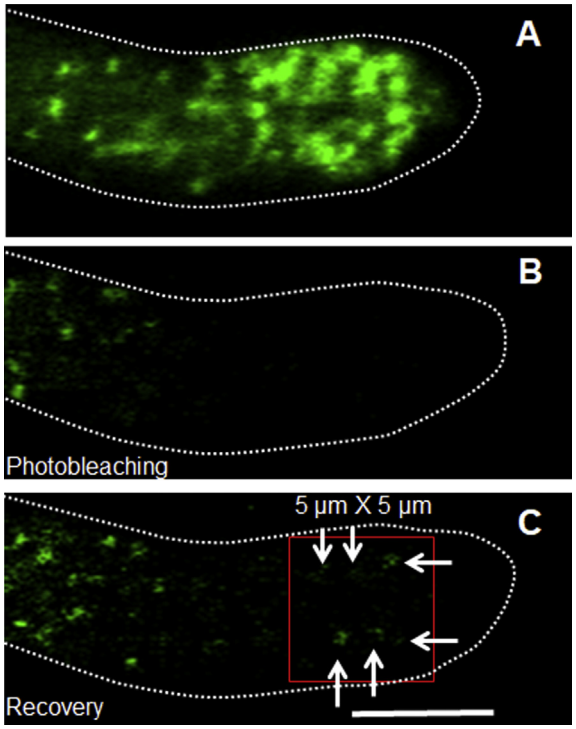

Fig. 3. Stages in a FRAP experiment to count endocytic events in a mature hypha of *Neurospora crassa* labeled with FIM-1-GFP. A. Single plane confocal image of the endocytic collar of a hypha immediately prior to photobleaching. B. 6 sec later C. Recovery after 2 sec (square shows area of new endocytic events counted). Bar = 5 µm.

Table 1
FRAP experiment to estimate membrane internalized by endocytosis. A total of 60 hyphae of *N. crassa* tagged with FIM-1-GFP were analyzed.

	AVG	MED	SE
Exocytosis (by geometry)			
Growth rate (µm/min)	6.7	6.2	0.5
Hyphal diameter (µm)	8.4	8.3	0.2
Exocytic vesicle diameter (µm)*	0.08		
Exocytic vesicle surface (µm^2)	0.02011		
Exocytosed membrane (µm^2/min)	179.9	169.2	9.7
Endocytosis			
Endocytic collar length (µm)*	9.0		
Endocytic collar area (µm^2)	236.7	234.2	3.7
Endocytic vesicle diameter (µm)*	0.04		
Endocytic vesicle surface (µm^2)	0.00503		
FRAP experiments			
Endocytic events/min/µm^2	21.4	20.3	1.2
Total endocytic events in the collar/min	5129	4799	602
Endocytosed membrane (µm^2/min)	25.8	24.1	1.5
Percentage of endocytosed membrane %	12.5	14.2	1.8

* Endocytic collar length and vesicle diameter were considered constant throughout.

varied in intensity but were all counted. Simultaneous calculations were made of the amount of plasma membrane generated from measurements of hyphal elongation. The amount of membrane endocytosed was calculated by assuming that each fluorescent patch internalized a vesicle of 0.04 µm in diameter. An examination of 60 hyphae revealed an average of 5129 endocytic events in the collar per min (Table 1). This corresponds to 25.8 µm^2 min^{-1} of plasma membrane internalized in hyphae growing at 6.7 ± 0.05 µm min^{-1}. We calculated that the amount of endocytosed membrane was 12.5% of the total of plasma membrane generated by exocytosis.

Table 2
Geometric calculations of exocytosis, plasma membrane and cell wall growth extension in an average hypha of *Neurospora crassa* tagged with FIM-1-GFP

A	B	C	D	E Formulas
2	*Exocytosis and plasma membrane extension*			
3	Growth rate	6.7	µm/min	
4	Outer wall diameter	8.40	µm	
5	Inner wall (PM) diameter	8.32	µm	C4 − (2 * C6)
6	Wall thickness	0.04	µm	
7	Plasma membrane generated in 1 µm of growth	26.1	µm^2	PI() * C5
8	Plasma membrane surface/min	175.1	µm^2	C7 * C3
9	Exocytic vesicle diameter (µm)	0.08	µm	
10	Exocytic Vesicle surface	0.02011	µm^2	PI() * C9^2
11	PM vesicles discharged/min for PM extension only	8710		C8/C10
12	FRAP measurement of internalized PM (Table 1)	12.5%		
13	CW vesicles discharged/min	9799		C11 + (C12 * C11)
14				
15	*Exocytosis including cell wall growth*			
16	Cell volume (measured at inner wall)/1 µm hyphal length	54.37	µm^3	PI() * (C5/2)^2
17	Cell volume (measured at outer wall)/1 µm hyphal length	55.42	µm^3	PI() * (C4/2)^2
18	Cell wall volume/1 µm hyphal length	1.05	µm^3	C17 − C16
19	Exocytic vesicle volume	0.00027	µm^3	(PI() * C9^3)/6
20	PM Vesicles discharged per 1 µm of hyphal extension	1300		C7/C10
21	CW vesicles discharged per 1 µm of hyphal extension	1463		C13/C3
22	Cell wall volume generated by PM vesicles/1 µm of hyphal extension	0.35	µm^3	C19 * C20
23	Cell wall volume generated by CW vesicles/1 µm of hyphal extension	0.39	µm^3	C21 * C19
24	Cell wall volume derived from the volume of CW vesicles	37%		C23/C18

Calculations made on an Excel Spreadsheet (xlsx file included in supplementary section) with formulae shown on column E. PI() = 3.1416
PM vesicles refer to vesicles that contribute solely to plasma membrane growth
CW vesicles refer to vesicles that contribute to both plasma membrane and cell wall growth.

3.3. Mathematical calculations

The interactive Excel spreadsheet in Table 2 calculates the number of vesicles needed to generate the amount of plasma membrane made per unit of hyphal growth using standard geometric formulae for spheres and cylinders. The calculations are based on parameters for which actual measurements are available namely elongation rate, hyphal diameter and average vesicle diameter. This is what Collinge and Trinci (1974) did but we took the analysis one-step further and asked whether the cargo contained in these vesicles would deliver sufficient materials needed for cell wall formation or whether a greater number of vesicles would be required. According to the parameters employed for Table 2, these FIM-1-GFP tagged hyphae discharge ca. 8800 vesicles/min to extend its plasma membrane. This value increases to ca. 9800 if we include the 12.5% excess membrane calculated from the FRAP experiments (Table 1).

4. Discussion

We believe this is the first successful attempt to quantitate the endocytic process in fungi. Given uncertainties as to the actual size of an endocytic vesicle, the exact boundaries of the endocytic collar dimension, and the accuracy of the photobleaching procedure, the calculated value for endocytosed membrane, namely 12.5%, should be considered as a first approximation of the exocytosis/endocytosis ratio. In the absence of measurements of the endocytic vesicles of *N. crassa*, we relied

on images of "filasomes" (~40 nm) of *Sclerotium rolfsii* published by Roberson (1992). Filasomes are likely to be bona fide endocytic vesicles, as evidenced by their microvesicular size, their peripheral location in the cell, and the network of actin filaments surrounding them (Roberson, 1992; Takagi et al., 2003). Accurate vesicle size is essential to obtain realistic measurement of membrane dynamics not only for endocytic but also exocytic vesicles. Thus the more reliable measurements of exocytic vesicles obtained by cryofixation of *N. crassa* (Roberson et al., 2010) namely 0.08 μm in diameter are substantially smaller than the 0.10 μm values for chemically fixed hyphae used by Collinge & Trinci (1974). Accordingly, we have revised upward the frequently cited figure for vesicle discharge in a hypha of *N. crassa* growing at full speed from 38,000 to 59,000 vesicles per min (Collinge and Trinci, 1974), and this itself may be an underestimate that does not include the additional number of vesicles calculated from the amount of membrane internalized by endocytosis.

The spreadsheet calculations on vesicle contents (lumen volume) indicate that the number of vesicles required solely for plasma membrane extension deliver a mere 36% of the wall volume (Table 2). Interestingly, electron micrographs of sections of hyphal apices processed by cryofixation, for instance Fig. 3 in Roberson et al (2010), show profiles of vesicle discharging contents which becomes wall substance of equal appearance, a similarity that suggests that much of the vesicle cargo becomes cell wall. However we must also consider that a significant portion of the cell wall volume is comprised of polymers made *in situ* and also that a substantial amount of vesicle contents may be released to the medium. Clearly there is a need for quantitative information on the conversion of vesicle substance to cell wall substance.

For the present work, we did not include the possible contribution of microvesicles to plasma membrane growth. We assumed that their quantitative role would be small coupled to the possibility that there is some question about the microvesicle membrane being compatible in thickness with the plasma membrane as was found in an earlier study (Bracker et al., 1976). No such doubt exists for the membrane of macrovesicles becoming plasma membrane (Girbardt, 1969; Grove and Bracker, 1970; Riquelme and Sanchez-Leon, 2014; Roberson et al., 2010).

4.1. The recycling role of endocytosis

The finding that exocytosis generates a significant excess of plasma membrane supports our conclusion that the primary role endocytosis is to dispose of this membrane excess. The positioning of the endocytic collar a few micrometers behind the apex creates an efficient tandem operation between membrane insertion at the apex of a hypha and membrane internalization in the subapex; the possibility that some physical coordination may exist between these processes could be a fruitful topic future investigation. Besides membrane recycling, the endocytic process is being actively investigated by other researchers as a mechanism for recycling proteins of importance in apical growth (Hernandez-Gonzalez et al., 2018; Higuchi et al., 2009; Penalva, 2010; Schultzhaus and Shaw, 2015; Steinberg, 2007).

4.2. Origin of endocytosis

The internalization of membrane during endocytosis must be an energy demanding process needed to force the membrane against the high turgor of the fungal cytosol. We can speculate that such expensive process evolved to support the intense exocytosis that allows fungal hyphae to exert their superior qualities, rapid colonization of the environment by fast growth rate and effective substrate utilization by abundant secretion of substrate degrading enzymes. Endocytosis became an efficient solution to recycle the excess of membrane generated by exocytosis.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.fgb.2018.07.001.

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