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Microtubule dynamics and organization during hyphal growth and branching in *Neurospora crassa*

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Abstract

By confocal microscopy, we analyzed microtubule (Mt) behavior during hyphal growth and branching in a *Neurospora crassa* strain whose Mts had been tagged with GFP. Images were assembled spatially and temporally to better understand the 3-D organization of the microtubular cytoskeleton and a clearer view of its dynamics. Cytoplasmic Mts were mainly arranged longitudinally along the hyphal tube. Straight segments were rare; most Mts showed a distinct helical curvature with a long pitch and a tendency to intertwine with one another to form a loosely braided network throughout the cytoplasm. This study revealed that the microtubular cytoskeleton of a hypha advances as a unit, i.e., as the cell elongates, it moves forward by bulk flow. Nuclei appeared trapped in the microtubular network and were carried forward in unison as the hypha elongated. During branching, one or more cortical Mts became associated with the incipient branch and were pulled into the emergence of the branch. As extension of the branch and distortion of the Mts continued, Mts soon were severed with both new Mt ends (+ and −) present in the new branch. Although the exact mechanisms for addition Mt recruitment into the branch remains an open question, the recorded evidence indicates both bulk insertion of established cortical parent-hypha Mts as well as in situ polymerization were involved. The latter conclusion was supported by FRAP studies showing evidence of Mt nucleation and polymerization assembly in the growing tip of the developing branch. Nuclei entered the branch entrapped in the advancing network of Mts.
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1. Introduction

Growth of primary hyphae occurs at the cell apex by polarized secretion of materials required for sustaining tip expansion, i.e., enzymes, membrane, and cell wall precursors (Bartnicki-García, 2002; Bartnicki-Garcia and Lippman, 1969; Harold, 1990, 1997; Riquelme et al., 2000; Trinci, 1978). As viewed with light microscopy, the cell machinery for apical growth culminates as a phase-dark globular structure or Spitzenkörper (Spk). Transmission electron microscopy (TEM) reveals the complexity of the Spitzenkörper. Whereas vesicles comprise the bulk of the Spk, there is usually an inner granular core and cytoskeletal elements (Howard, 1981; Howard and Aist, 1980; Roberson and Fuller, 1988; Vargas et al., 1993).

The emergence of a branch involves the establishment of a new site for polarized wall and plasma membrane construction, requires the assembly of a new Spk. We maintain that the same biochemical machinery that supports apical growth is also required to create branching points. Thus the first visible evidence of branching, namely an incipient lateral protrusion on the hyphal profile, is not just a localized deformation of a weakened wall under high turgor, but the orderly construction of new wall around a new growing center (Riquelme and Bartnicki-Garcia, 2004).

The cellular and molecular components involved in branch initiation are not fully understood but revolve around a fundamental question: the establishment of cell

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polarity. Recent studies have begun to identify molecular components involved in the branching process (Harris and Momany, 2004; Knechtle et al., 2003). Previously, kinetic studies suggest that branching is an expression of the intrinsic tendency of the mycelium to grow exponentially. When a hypha cannot maintain its own exponential rate of elongation, it preserves an overall exponential rate by creating a new growing point (Katz et al., 1972; Robertson, 1959; Trinci, 1969, 1970). Recent communications (Riquelme and Bartnicki-Garcia, 2004; Watters and Griffiths, 2001) suggest that branching is determined by the increasing ratio of vesicle supply over vesicle discharge at the apex. The excess in building materials reaches a critical concentration that triggers the formation of a new growing point. Accordingly, lateral branches do not use resources required to support the growth of the primary hypha (Riquelme and Bartnicki-Garcia, 2004; Trinci, 1970). Riquelme and Bartnicki-Garcia (2004) have estimated that a relatively small increase (12% increase in wall-building precursors) in precursor availability would be sufficient to trigger the origin of a new Spk and the development of a lateral branch.

The cytoskeleton plays an important role in hyphal growth and morphogenesis (Bartnicki-García, 2002; Heath and Kaminskyj, 1989; Heath et al., 1982). The spatial arrangement of cytoskeletal elements during growth is consistent with their proposed involvement in cell morphogenesis. Mts are considered to be primarily responsible for the long distance transport of secretory vesicles to the Spk, while actin microfilaments are principally involved in their short distance transport from the Spk to the plasma membrane, as well as maintaining and controlling vesicle organization and dynamics within the Spk (Gooday, 1983; Gow, 1989; Harris et al., 2005; Hasek and Bartnicki-Garcia, 1994; Heath, 1994; Howard, 1981; Howard and Aist, 1977, 1980; McDaniel and Roberson, 1998; Roberson and Fuller, 1988). Accordingly, the Spk may be viewed as a switching station from Mt to microfilament-based vesicle transport. (Bartnicki-García, 2002; Harris et al., 2005).

Indeed, TEM images have illustrated that in the apical dome, Mts can traverse the Spk; some terminating at the plasma membrane at the extreme hyphal apex (Barja et al., 1991; Howard and Aist, 1980; Roberson and Fuller, 1988; Roberson and Vargas, 1994; Roos and Turian, 1977; Vargas et al., 1993) and that actin microfilaments are abundant in and around the Spk. Furthermore, secretory vesicles associated with the Spk are often in close association with both Mts and microfilaments (Howard, 1981; Howard and Aist, 1980; Roberson and Fuller, 1988). These cytoskeletal features and associated secretory machinery are likely involved in establishing the point of branching behind the tip (Bartnicki-García, 2002; Riquelme and Bartnicki-Garcia, 2004).

Although circumstantial evidence indicates that the cytoskeleton plays a key role in hyphal growth the exact function is still unsolved. Actin inhibition causes a drastic loss of polarized growth, but the effect does not usually persist, either because the hypha stops growing completely or because it ceases to respond to the inhibitor. Microtubule inhibition tends to produce a less severe but more persistent disruption or disorientation of tip growth. The different effects are probably a reflection of the different localization and different roles that actin and Mts play in vesicle traffic. Heath and Geitmann (2000) concluded that there was an obligatory role for F-actin in hyphal polarization and tip morphogenesis but only an indirect role for Mts. The latter conclusion was challenged by Riquelme et al. (2003) and Horio and Oakley (2005) who showed that Mt function was essential for normal hyphal morphogenesis.

To understand the complex process of tip growth, it is essential to identify and analyze the function of the structural elements potentially involved in polar elongation of branches. The main goal of this study was to examine the role of microtubular cytoskeleton during growth and branch generation using a living strain of *Neurospora crassa* whose Mts have been tagged with GFP. Previously, other authors have utilized GFP to study Mt dynamics in other fungal systems (Czymmek et al., 2005; Freitag et al., 2004; Horio and Oakley, 2005; Konzack et al., 2005; Sampson and Heath, 2005; Steinberg et al., 2001; Takano et al., 2001).

2. Materials and methods

2.1. Organisms and culture

A strain of *N. crassa* Tub N2524 whose Mts are labeled with GFP (*mat a rid*^{RIP4} *his-3*$^{+}$ *::Pccg-1Bml*$^{+}$*-sgfp*$^{+}$ β-tubulin-GFP) was kindly donated by Dr. Michael Freitag (Institute of Molecular Biology and Department of Biology, University of Oregon, Eugene 97430). The strain was maintained on Vogel's complete medium amended with 2% sucrose at room temperature (25 °C) (Freitag et al., 2004).

2.2. Confocal live-cell imaging

Neurospora crassa N2524 was grown on water agar (WA) to minimize background fluorescence. An inverted agar block method (Hickey and Read, 2003; Hickey et al., 2002) was used for live-cell imaging with an inverted laser scanning microscope (LSM-510, Carl Zeiss, Göttingen, Germany) and an argon ion laser for excitation at 488 nm wavelength and GFP filters for emission at 515–530 nm. Two oil immersion objectives were used: 63× (DIC), 1.4 N.A. planapochromatic and 100× (PH3), 1.3 N. planneofluar. Laser intensity was kept to a minimum (1.5%) to reduce photobleaching and phototoxic effects. The imaging parameters used produced no detectable background signal from any source other than from GFP. Time-lapse imaging was performed at scan intervals of 1.5–4.5 s for periods up to 40 min. Image resolution was 512 × 512 pixels and 300 dpi. The laser scan rate was of 0.996 s. Confocal images were captured using LSM-510

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