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Visualization of F-actin localization and dynamics with live cell markers in *Neurospora crassa*

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ABSTRACT

Filamentous actin (F-actin) plays essential roles in filamentous fungi, as in all other eukaryotes, in a wide variety of cellular processes including cell growth, intracellular motility, and cytokinesis. We visualized F-actin organization and dynamics in living *Neurospora crassa* cells via confocal microscopy of growing hyphae expressing GFP fusions with homologues of the actin-binding proteins fimbrin (FIM) and tropomyosin (TPM-1), a subunit of the Arp2/3 complex (ARP-3) and a recently developed live cell F-actin marker, Lifeact (ABP140 of *Saccharomyces cerevisiae*). FIM-GFP, ARP-3-GFP, and Lifeact-GFP associated with small patches in the cortical cytoplasm that were concentrated in a subapical ring, which appeared similar for all three markers but was broadest in hyphae expressing Lifeact-GFP. These cortical patches were short-lived, and a subset was mobile throughout the hypha, exhibiting both anterograde and retrograde motility. TPM-1-GFP and Lifeact-GFP co-localized within the Spitzenkörper (Spk) core at the hyphal apex, and were also observed in actin cables throughout the hypha. All GFP fusion proteins studied were also transiently localized at septa: Lifeact-GFP first appeared as a broad ring during early stages of contractile ring formation and later coalesced into a sharper ring, TPM-1-GFP was observed in maturing septa, and FIM-GFP/ARP3-GFP-labeled cortical patches formed a double ring flanking the septa. Our observations suggest that each of the *N. crassa* F-actin-binding proteins analyzed associates with a different subset of F-actin structures, presumably reflecting distinct roles in F-actin organization and dynamics. Moreover, Lifeact-GFP marked the broadest spectrum of F-actin structures; it may serve as a global live cell marker for F-actin in filamentous fungi.

1. Introduction

The importance of F-actin in tip-growing cells was recognized in fungal hyphae, in which growth and cell wall extension are focused at a single site at the apex resulting in production of a tube shaped cell (Bartnicki-Garcia and Lippman, 1977; Hasek and Bartnicki-Garcia, 1994; Heath et al., 2000; Bartnicki-Garcia, 2002). In recent years, many studies have reaffirmed the central importance of F-actin and associated proteins in growth and spatial regulation of organelles in tip-growing cells (Harris and Momany, 2004; Virag and Griffiths, 2004; Harris et al., 2005; Upadhyay and Shaw, 2008). Studies designed to disrupt F-actin function using chemical agents such as cytochalasins and latrunculin B confirmed that a polymerized actin cytoskeleton is required for normal apical growth, hyphal tip shape, and polarized enzyme secretion in different fungal organisms (McGoldrick et al., 1995; Harris et al., 1997; Torralba et al., 1998; McDaniel and Roberson, 2000; Taheri-Talesh et al., 2008).

F-actin interacts with many associated proteins that regulate its arrangement and organization. In a wide variety of species studied, F-actin can be found as patches, cables and/or actomyosin contractile rings associated with septum formation; each of these arrays is responsible for a distinct actin-dependent process (Adams et al., 1991; Roberson, 1992; Arai et al., 1998; Sandrock et al., 1999; Pruyne and Bretscher, 2000; Bretscher, 2003; Moseley and Goode, 2006; Upadhyay and Shaw, 2008; Taheri-Talesh et al., 2008). For example, tropomyosin stabilizes F-actin and regulates F-actin mechanics (Greenberg et al., 2008), Arp2/3 complex nucleates F-actin branches (Egile et al., 2005) and fimbrin cross-links F-actin into bundles and networks (de Arruda et al., 1990).

Another F-actin-dependent process important for fungal development is septum formation. The vegetative mycelia of many

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filamentous fungi are composed of multinucleated cells divided by regularly spaced septa. Septa construction requires regulation of mitotic signaling, cytokinesis and cell wall biosynthesis (Momany et al., 1995). During septum formation, a conserved set of proteins follow a similar temporal and spatial pathway in different eukaryotes to assemble F-actin-containing structures that contribute to septation (Bezanilla et al., 2000; Paoletti and Chang, 2000; Wong et al., 2002; Motegi et al., 2000, 2004). It is well established that the actin-based contractile ring functions in membrane contraction at septation, but the role of actin patches is less clear. The Arp2/3 complex is found in F-actin patches flanking the contractile ring in *Schizosaccharomyces pombe* (McCollum et al., 1996; Arai et al., 1998; Wu et al., 2006). These patches are thought to promote endocytic uptake required for cytokinesis in *S. pombe*, although the Arp2/3 complex is not required for cytokinesis in *Drosophila melanogaster* (Eggert et al., 2004).

In filamentous fungi, actin visualized by various methods has been observed in the Spk, a vesicle-rich area of the hyphal apical cytoplasm directly adjacent to the growth site (Howard, 1981; Roberson and Fuller, 1988; Bourett and Howard, 1991; Roberson, 1992; Virag and Griffiths, 2004; Harris et al., 2005; Upadhyay and Shaw, 2008; Taheri-Talesh et al., 2008). The population of F-actin in the Spk has been proposed to regulate vesicle delivery and/or fusion at the growth site (exocytosis), and may also regulate calcium channels, whose activity is important for tip growth (Bartnicki-Garcia and Lippman, 1977; Hasek and Bartnicki-Garcia, 1994; Bartnicki-Garcia, 2002; Harris and Momany, 2004; Harris et al., 2005). Cortical F-actin patches have been proposed to play a role in plasma membrane invagination during endocytosis in filamentous fungi as in yeasts (Mulholland et al., 1994; Harris and Momany, 2004; Ayscough, 2005; Rodal et al., 2005). In all fungi examined, F-actin patches are located at sites of active endocytic uptake (i.e., the subapex and septa) (Ayscough, 2005; Gachet and Hyams, 2005; Kaksonen et al., 2005; Upadhyay and Shaw, 2008; Taheri-Talesh et al., 2008).

Most studies of F-actin organization in filamentous fungi have used anti-actin antibodies or rhodamine phalloidin to label fixed cells. Filamentous actin is notoriously difficult to preserve during fixation and can often be difficult to adequately label (e.g., phalloidin is usable for only a small number of fungal species). Thus, some of the differences previously described in studies with different fungi may be caused by fixation artifacts or the source of anti-actin antibodies (Heath et al., 2000; Virag and Griffiths, 2004). Recently, live-cell imaging of F-actin has been carried out in *Aspergillus nidulans* germlings using green fluorescent protein (GFP) fused to G-actin and to different F-actin-binding proteins (ABPs) such as fimbrin and tropomyosin (Upadhyay and Shaw, 2008; Taheri-Talesh et al., 2008).

Our study was focused on visualization of the F-actin cytoskeleton in order to describe its dynamics and organization during hyphal growth and septum formation using different microscopy techniques in living mature hyphae of *Neurospora crassa*. We used ABPs, such as tropomyosin, Arp2/3 complex and fimbrin fused to GFP and a recently developed live cell F-actin reporter called Lifeact (Riedl et al., 2008).

2. Materials and methods

2.1. Strains and culture conditions

Strains used in this study are listed in Table 1A. Strains were maintained on Vogel's minimal medium (VMM) with 2% sucrose. All manipulations were according to standard techniques (Davis, 2000).

Table 1
Materials used: (A) *N. crassa* strains, (B) plasmids and (C) oligonucleotides.

	Genotype, description, or sequence	Reference
(A) Strains		
Wild type 2489	*mat a*	FGSC2489
9717	*mat A his-3*$^{-}$; *Δmus-51::bar*$^{+}$	FGSC9717
dRFP-TPM	*mat a his-3*$^{+}$*::Pccg-1-drfp-tpm-1*	This study
TRM47-OC28	*mat a his-3*$^{+}$*::Pccg-1-tpm-1-sgfp*$^{+}$	This study
TRM08-DD02	*mat a his-3*$^{+}$*::Pccg-1-fim-1-sgfp*$^{+}$	This study
TRM10-DD04	*mat a his-3*$^{+}$*::Pccg-1-fim-1 (abd1)-sgfp*$^{+}$	This study
TRM11-DD05	*mat a his-3*$^{+}$*::Pccg-1-fim-1 (abd2)-sgfp*$^{+}$	This study
TLS-NG01	*mat a his-3*$^{+}$*::Parp2-arp-2-sgfp*$^{+}$	This study
TLS-NG02	*mat a his-3*$^{+}$*::Pccg-1-arp-3-sgfp*$^{+}$	This study
TRM49-OC30	*mat a his-3*$^{+}$*::Pccg-1-lifeact-egfp*$^{+}$	This study
(B) Oligonucleotides		
FimF	GC**TCTAGA**ATGAATGTCCTCAAGATCCAG	This study
FimR	CC**TTAATTAA**CTGCATCTTGTCATAGGTAGCCAT	This study
Abd1F	C**TCTAGA**ATGTTCTTGAAGGCCACCCAGGTC	This study
Abd1R	CC**TTAATTAA**GGCCAGTTGGCGGCCTTG	This study
Abd2F	C**TCTAGA**ATGGAGAAGCTTGAGGTCGAG	This study
Abd2R	CC**TTAATTAA**CTACTGCATCTTGTCATAGGT	This study
TropF	GC**TCTAGA**ATGGACCGCATCAAGGAG	This study
TropR	CC**TTAATTAA**GATGTTGGCAATATCAGCCT	This study
Arp2F	**ACTAGT**TGATTGGTTCTTGCTGGGCG	This study
Arp2R	**GGATCC**CAGCCCTAGGACCCAACTTCTCCAACAC	This study
Arp3F	**TCTAGA**ACTAGTCAGCACACCACCGCAACAAT	This study
Arp3R	**GGATCC**CAGCAGATCCACCAGGTCCTCCG	This study
LifactF	GGG**TCTAGA**ATGGGTGTCGCAGATTTGAT	This study
LifactR	CAC**GGGCCC**TTACTTGTACAGCTCGTCC	This study
(C) Plasmids		
pMF272	*Pccg-1-sgfp*$^{+}$	AY598428
pRM47-OC28	*Pccg-1-tpm-1-sgfp*$^{+}$	This study
pRM08-DD02	*Pccg-1-fim-1-sgfp*$^{+}$	This study
pRM10-DD04	*Pccg-1-fim-1 (abd1)-sgfp*$^{+}$	This study
pRM11-DD05	*Pccg-1-fim(abd2)-sgfp*$^{+}$	This study
pRM49-OC30	*Pccg-1-lifeact-egfp*$^{+}$	This study
pLS-NG01	*Pccg-1-arp-2-sgfp*$^{+}$	This study
pLS-NG02	*Parp-2-arp-2-sgfp*$^{+}$	This study
pLS-NG03	*Pccg-1-arp-3-sgfp*$^{+}$	This study

Restriction enzymes sequence in bold.

2.2. Construction of GFP-containing plasmids

Standard PCR and cloning procedures (Sambrook et al., 1989) were used to fuse the *sgfp* gene to the carboxyl terminus of *fim*, abd1, abd2, (truncated version of fimbrin), *arp-2*, *arp-3* and *tpm-1*. We constructed a fimbrin full-length GFP fusion and two truncated versions, each containing one actin-binding domain (abd1 or abd2) (Fig. 1A) (Wang et al., 2004b). All the genes and truncated versions of fimbrin were amplified by PCR from *N. crassa* (FGSC 2489) genomic DNA. Primers used are listed in Table 1B. PCR was performed in a Bio-Rad Thermal Cycler with Platinum Hi-fi *Taq* polymerase (Invitrogen, Carlsbald, CA) according to the manufacturer's instructions. The amplified and gel-purified PCR products were digested with *XbaI* and *PacI* and ligated into *XbaI*- and *PacI*-digested plasmid pMF272 (GenBank Accession No. AY598428). This yielded pRM08-DD01 (*fim*), pRM11-DD02 (abd1), pRM10-DD03 (abd2) and pRM47-OC28 (*tpm-1*) (Table 1C). Only those without changes to the amino acid sequence were used in this study. *N. crassa* transformed with the Abd1-GFP construct showed no specific localization of GFP and was not examined further.

The *arp-3* gene amplified was digested with *XbaI* and *BamHI* and cloned into *XbaI*- and *BamHI*-digested pMF272 giving rise to the pLS-NG02 (*arp3*) (Table 1C). The *arp-2* coding region and 943 nucleotides of sequence upstream of the ATG were amplified, digested with *SpeI* and *BamHI* and ligated into a *SpeI*- and *BamHI*-digested pMF272 lacking the *ccg-1* promoter producing the pLS-NG01 (*arp-2*) (Table 1C). To create this derivative, pMF272 was digested with *NotI* and *XbaI*, ends were filled in with Klenow, and the larger fragment was self-ligated. In *arp-2* and *arp-3* constructs,