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Characterization of *Neurospora crassa* α-Actinin

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Abstract α-Actinin, an actin-binding protein of the spectrin superfamily, is present in most eukaryotes except plants. It is composed of three domains: N-terminal CH-domains, C-terminal calcium-binding domain (with EF-hand motifs), and a central rod domain. We have cloned and expressed *Neurospora crassa* α-actinin as GST and GFP fusion proteins for biochemical characterization and in vivo localization, respectively. The intracellular localization pattern of α-actinin suggests that this protein is intimately associated with actin filaments and plays an important role in the processes of germination, hyphal elongation, septum formation, and conidiation. These functions were confirmed by the experiments on the effect of α-actinin gene deletion in *N. crassa*.

Introduction

Cell shape, cytokinesis, intracellular motility, and tip growth in filamentous fungi are processes that involve the actin cytoskeleton [14]. Actin interacts with many associated proteins which regulate its assembly, organization, and function. The actin-binding proteins can be grouped according to their function in: (1) those that participate in the assembly of G-actin into filaments and their subsequent stability, (2) motor proteins that use F-actin as a track, and (3) those that connect actin filaments to the cell membrane or cross-link actin filaments to form different structures such as bundles, branching filaments, and three-dimensional networks [11, 15]. Examples of the latter group are all members of the spectrin superfamily which includes spectrin, α-actinin, dystrophin, and utrophin. These proteins contain three functional and structural domains: two calponin homology domains at the N-terminus, a central rod domain containing spectrin-repeats, and a C-terminal domain with EF-hand motifs [5]. The only member of the superfamily present in fungi is α-actinin [8].

α-Actinin was reported in most eukaryotic cells except plants [29–31]. In lower eukaryotes, α-actinins have been described in the protozoa *Trichomonas vaginalis* and *Dictyostelium discoideum* [1, 21], in fungi, the fission yeast *Schizosaccharomyces pombe* [34] and the filamentous fungus *Aspergillus nidulans* [33]. The *N. crassa* genome contains an open reading frame, potentially coding for an actinin-like protein with an atypical N-terminal extension preceding the conserved CH-domain. Although there is evidence for α-actinins orthologs in other filamentous fungi which share high sequence similarity with the actinin-like protein (Ainp 1) of fission yeast [7, 8, 28, 33], the biochemical and functional characteristics of these proteins in filamentous fungi has only been recently described in the study of Wang et al. [33], showing that α-actinin may act in the formation of septa in cytokinesis, septation, and conidiation [33, 34]. In *A. nidulans*, where the hyphal growth takes place exclusively at the apex, actinin may act in the maintenance of the apical dominance [33].

The goal of this study was to characterize α-actinin in *N. crassa*, analyze its spatial localization, and provide some insights into its functions.

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Materials and Methods

Strains and Culture Conditions

Wild-type *N.* crassa (FGSC 262, strain St. Lawrence STA4) and a heterokaryon α-actinin knockout strain (FGSC11835, [6]) were obtained from the Fungal Genetics Stock Center (FGSC, School of Biological Sciences, Kansas City, MO). The procedure for conidial inoculum preparation was as described earlier [7].

Construction of Recombinant Plasmids

Recombinant plasmids were constructed (A) to express and purify the fusion protein for biochemical analysis and (B) to study in vivo expression.

A. *Construction and expression of GST-α-actinin fusion protein for biochemical analysis*. The α-actinin entry in the *Neurospora* data base (NCU06429.4, Broad Institute) is a protein with two putative methionine start codons, giving two products of different predicted molecular weights (100 and 80 kDa, which we will subsequently refer to as full- and short-length α-actinin, respectively). We have used the full-length GST-fusion protein prepared earlier [8] and constructed and expressed the short-length GST-fusion protein. α-Actinin cleaved from GST was recovered and its concentration and quality was determined by measuring the A_{280} and SDS-PAGE.
B. *Construction of α-actinin-egfp gene containing plasmid and transformation*. The α-actinin-*egfp* gene fusion was constructed by PCR amplification of the sequence encoding the α-actinin gene (ncu06429.4). PCR was carried out using forward primer 5′-CGC CGCGGATCCATGGAGATGCTGGGGGTGGAG-3′ and reverse primer 5′-GTCACGTTAATTAAAT GATACCCATTCGGCTT-3′. This led to the insertion of *Bam*HI and *Pac*I restriction sites (underlined) which was used to clone the gene in the egfp gene containing plasmid pMF272 constructed by Freitag et al. [12]. Transformation of the *N. crassa his-3* mutant (FGSC 9014; rid^{RIP1} mat A his-3) and heterokaryon transformant selection was performed as described previously by Freitag et al. [12]. The correct insertion in the transformants was verified by PCR using a forward and reverse primer designed to recognize the *Neurospora* α-actinin and gfp sequence, respectively.

2-Dimensional Gel Electrophoresis and Western Blot

Procedures for the preparation of cell-free extracts from the mycelia, determination of protein concentration, analysis in 2-D gel and Western blot have been described earlier [7]. *N. crassa* α-actinin-specific antibody used for immunoblotting was the one prepared earlier in our laboratory [8].

Localization of α-Actinin In Situ

Experimental details have been described in a previous study [7]. Anti-*Neurospora* α-actinin was used at 1:20 dilution and FITC conjugated anti-rabbit secondary antibody at a dilution of 1:80.

In Vivo Expression of α-Actinin-GFP and Acquisition of Live Cell Images

Cultures were prepared in liquid medium as described above. For long-term image acquisition times (i.e., images taken over a period of 12 h), germinating spores were deposited in an 8-chambered cover glass (Lab-Tek, ref 155411) and covered with a small block of Vogel's [32] medium containing 1.5% agar to maintain the cells in the same plane. Hyphal images were observed with a Leica SP2 microscope and processed with Adobe Photoshop 8.0.

Cell Wall Staining with Calcofluor

Slides coated with Vogel's medium containing 1.5% sucrose were inoculated with conidia and grown at 25°C for 8 h in a moist chamber. The cell wall of the mycelium was stained with 10 μM calcofluor (Fluorescent Brightener 28) for 3 min and covered with a coverslip. An Orthoplan epiillumination microscope (Leica) equipped with fluotar optics and a selective filter combination was used to visualize calcofluor fluorescence patterns. Fluorescent micrographs were obtained with a Leica-DFC490 camera and processed with Adobe Photoshop 8.0.

Results

Features of *Neurospora* α-Actinin

Western blot analysis of the full-length and short-length α-actinin along with native *Neurospora* α actinin is shown in Fig. 1a. The Mr and pI of full-length (110 kDa, pI 5.9) and short-length (80 kDa, pI 5.6) proteins correspond to the theoretical values based on their sequences. The "native" *Neurospora* α-actinin has all the features of short-length α actinin (Fig. 1a). The 80-kDa protein corresponds to the conserved region containing the characteristic domains of the α-actinins (Fig. 1b).

The results also show that the GST fusion did not disrupt the expression of α-actinin.

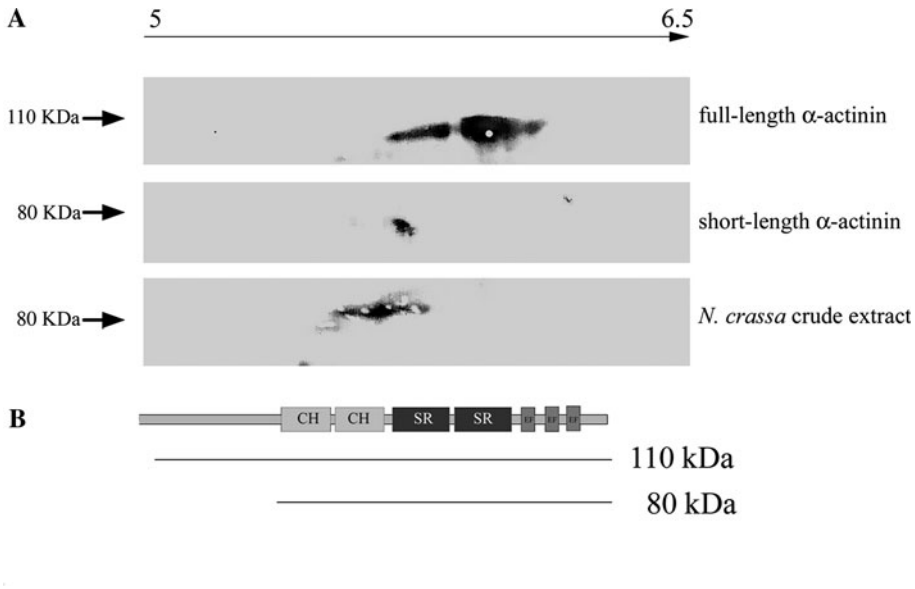

Fig. 1 2D-PAGE (10%) analysis of the *N. crassa* α-actinin and the GST-α-actinin constructs. **a** *N. crassa* crude extract and purified full (110 kDa)- and short (80 kDa)-length α-actinin were immunoblotted with anti-*Neurospora* α-actinin antibody. **b** Diagrammatic representation of α-actinin construction, *bars* underline the region of the protein reacting with the anti-*Neurospora* α-actinin antibody shown in (**a**) (*CH* calponin homology domain, *SR* spectrin repeat, *EF* EF-hand motif)

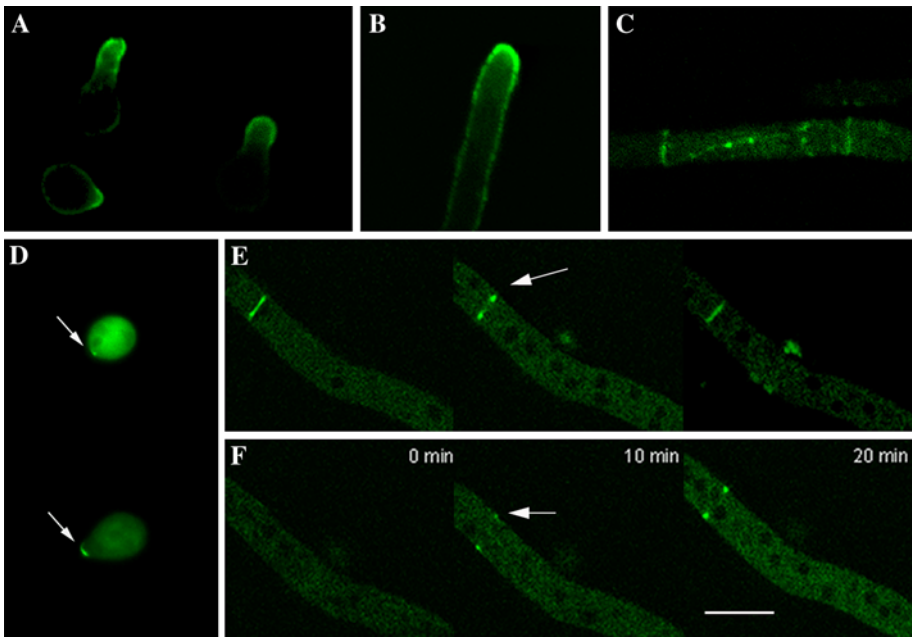

Fig. 2 Localization of α-actinin in *N. crassa*. **a**–**c** Immunolocalization of *N. crassa* α-actinin during different stages of growth. Germinating conidia, emerging germ tubes, and growing hyphae corresponding to 1 h (**a**) and 12 h (**b**) growth. The α-actinin was accumulated in the tip of the hypha (**b**). Localization of α-actinin in the septum (**c**). **d**–**f** Laser scanning confocal microscopy images of *Neurospora* hyphae expressing α-actinin-GFP. Localization in the tip region of the emerging tube (**d**) and at septa (**e**, **f**). **e** Stack series of the hyphae at 30 min, the septal pore was visualized in the medial acquisition image. **f** Time series corresponding to the first step of septum formation. *Bar* 10 μm

α-Actinin Localization

Immunofluorescence microscopy of α-actinin in the germ tubes and growing hyphae showed that the protein was localized in the tip region of the emerging germ tubes (Fig. 2a), apices of growing hyphae (Fig. 2b), and at septa in the distal parts of the hyphae (Fig. 2c). The results were further analyzed by monitoring α-actinin-GFP fluorescence. Intense GFP fluorescence was observed at the presumptive sites of germ tube initiation and the tips of emerging germ tubes (Fig. 2d). In the growing hyphae α-actinin-GFP was also located at septa (Fig. 2e, f) but did not persist after completion of these structures.

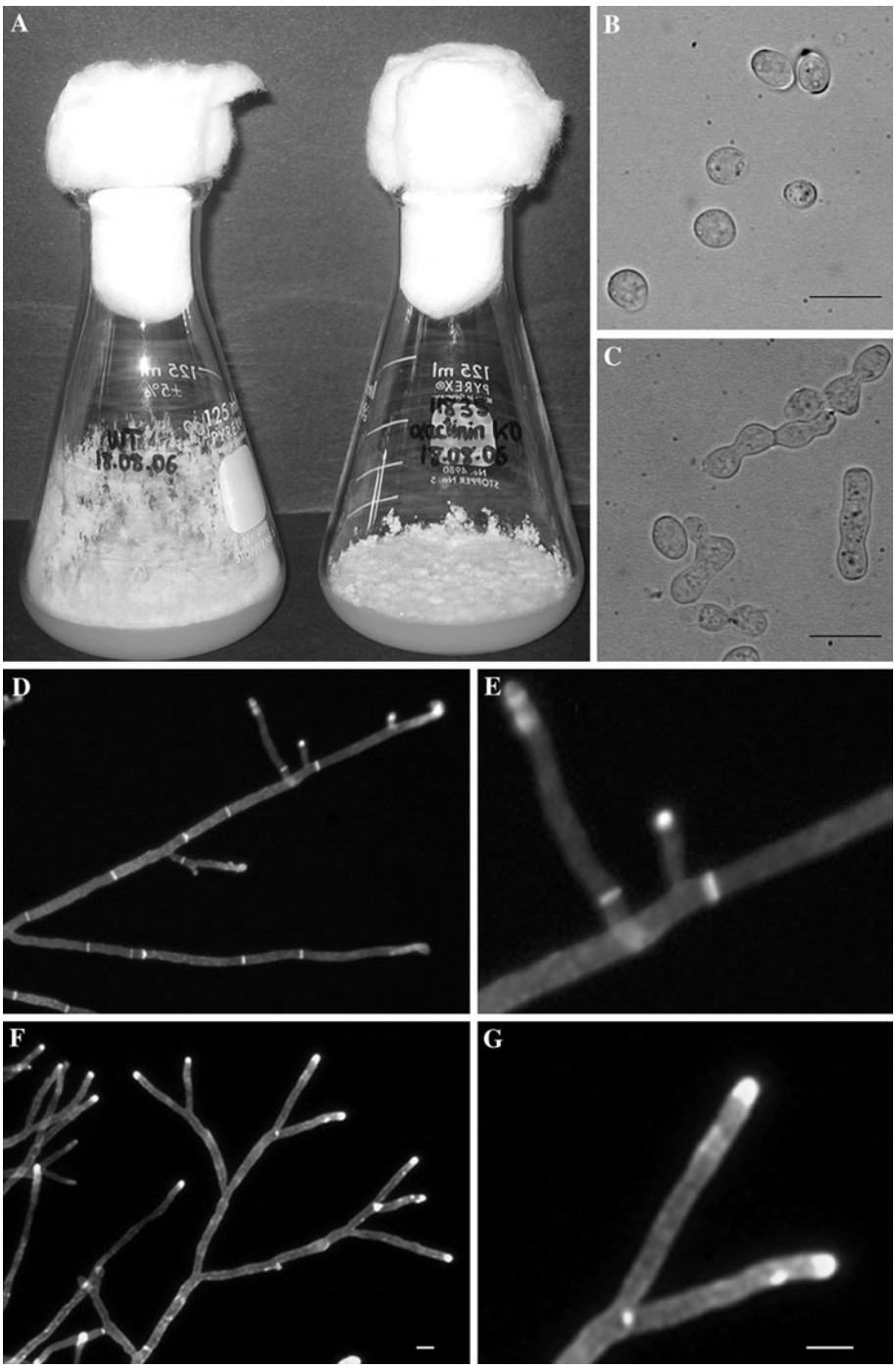

Fig. 3 Phenotype of the α-actinin heterokaryon knock-out strain. **a** Wild type (wt) (*left*) and α-actinin heterokaryon knock-out strain (*right*). Optical microscopy images of conidial suspension from wild type (**b**) and α-actinin heterokaryon knockout strain (**c**). Calcofluor-stained hyphae of *N. crassa* wt (**d**, **e**) and α-actinin heterokaryon knock-out strain (**f**, **g**). **e**, **g** are images at higher magnification of (**d**, **f**), respectively. *Bar* 10 μm

Heterokaryon α-Actinin Knockout Strain Phenotype

The α-actinin knockout strain was deposited in the FGSC as a heterokaryon [6], considering that the homokaryon has a lethal phenotype because of the poor or non-existent germination of the first generation ascospores. Compared to the wild type, the heterokaryon shows shortened aerial hyphae (Fig. 3a) in Davis and De Serres [9] medium. In addition, conidial counting revealed that the number of conidia was reduced by half in the heterokaryon knock-out strain as compared to the wild type (data not shown).

Further, the conidia remained attached in chains due to a defect in the separation (Fig. 3c). They also lacked the ovoid shape, characteristic of the wild type (Fig. 3b). In addition, the morphology of the hyphae was different with respect to their branching pattern. The mutant showed a predominantly dichotomous branching phenotype (Fig. 3f,

g) compared to the sympodial branching in the wild type (Fig. 3d, e).

Discussion

Neurospora α-actinin annotation in the MIPS database (NCU20705) is a predicted protein of about 80 kDa with a pI of 5.27. These characteristics correspond to the immunoreacting peptide of the crude extract in Western blots revealed with the antibody, prepared against α-actinin from *N. crassa*. The *Neurospora* α-actinin has been annotated in the MIPS database as an α-actinin-related protein based on the initiator methionine located immediately upstream of the coding sequence for the first CH-domain. All close homologs in other fungi confirm that this methionine is the initiator and the resulting protein contains all the domains that define α-actinins. However, there are two start codons present in the ncu06429.4 gene annotation of the Broad Institute database and we cloned the gene from cDNA which included the first start codon indicating that this N-terminal extension is present in α-actinin mRNA and could be an UTR region or pro-domain of the protein. An alternative interpretation could be that the translation of mRNA is initiated at both methionine codons located in the ORF.

The heterokaryon mutant of α-actinin apparently affects the pattern of cell growth caused by the loss of apical dominance. The dichotomous branching phenotype of the α-actinin heterokaryon knockout strain is probably a consequence of splitting at the tips. This phenomenon has also been observed in the *A. nidulans* α-actinin mutant [33] as well as in other *A. nidulans* mutants affecting other actin-binding proteins [13, 25, 27, 33]. The dichotomous branching phenotype observed in cytochalasin A-treated *Neurospora* [20] and *Neurospora* actin mutants [26] suggest that α-actinin along with actin take part in the establishment of the branching patterns. The localization of *Neurospora* α-actinin at the germ tube initiation sites and in the tips of the emerging germ tubes suggests that α-actinin plays a role in the germ tube polarization during spore germination and hyphal growth. A similar localization has been observed for actin, which was shown to be essential for germ tube initiation [3]. Recent experiments on hyphal tip growth have shown that endocytic pathways, particularly actin and actin-binding proteins, are involved in tip growth [2, 25].

Despite the fact that the functional complementation of the mutant by the gene encoding the fusion protein is not yet available, a similar localization of α-actinin at septa was observed with specific antibody (in situ) and monitored with GFP fusion protein (in vivo). The disappearance of α-actinin from the septa once its formation is complete has also been described for other proteins required for septation in filamentous fungi, such as SepA (formin), MyoA (myosin I), tropomyosin, fimbrin, and actin [4, 10, 17, 19, 22, 24, 25, 33, 35]. The α-actinin localization at septa was also observed in *S. pombe* and plays a role in cytokinesis [34].

The list of newly identified proteins and molecules that interact with α-actinin has increased recently [16, 23] demonstrating that α-actinin not only acts as an actin-binding protein but also as a scaffold for other protein–protein interactions and as a linker to connect the cytoskeleton to diverse signalling pathways [18].

Although the role of α-actinin in *N. crassa* has not yet been completely elucidated, its localization and effects on the phenotype of knock-out heterokaryon strain strongly suggest that the protein participates, together with actin, in the coordination of cellular activities such as germination, septum formation, and branching.

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