The important role of actinin-like protein (AcnA) in cytokinesis and apical dominance of hyphal cells in Aspergillus nidulans

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INTRODUCTION

Cytoskeletal proteins are known to sculpt the structural architecture of cells. They can modulate second messenger signalling pathways, providing intracellular trafficking, and organizing communication networks between inter- and intracellular components (Fischer et al., 2008; Heasman & Ridley, 2008; Sampson & Heath, 2005; Xiang & Plamann, 2003). The actin cytoskeleton is involved in many processes in eukaryotic cells, including interaction with a wide variety of actin-binding proteins such as the actin-capping proteins, the actin filament nucleators and the actin cross-linking proteins. Here, we report the identification and characterization of an actinin-like protein (AcnA) from the filamentous fungus Aspergillus nidulans. Not only did the depletion of AcnA by alcA(p) promoter repression or the deletion of AcnA result in explicit abnormalities in septation and conidiation, but also the acnA mutants induced a loss of apical dominance in cells with dichotomous branching, in which a new branch was formed by splitting the existing tip in two. Consequently, the colony showed flabellate edges. Moreover, we found that the localization of the GFP–AcnA fusion was quite dynamic. In the isotropic expansion phase of the germinated spore, GFP–AcnA was organized as cortical patches with cables lining the cell wall. Subsequently, GFP–AcnA was localized to the actively growing hyphal tips and to the sites of septation in the form of combined double contractile rings. Our data suggest that AcnA plays an important role in cytokinesis and apical dominance of hyphal cells, possibly via actin-dependent polarization maintenance and medial ring establishment in A. nidulans. This is the first report, to our knowledge, of the function of an actinin-like protein in filamentous fungi.

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Abbreviations: ABD, actin-binding domain; DIC, differential interference contrast; EFh, EF-hand; SR, spectrin repeat.
one actinin-like protein in fungi, Ain1p, which is found in Schizosaccharomyces pombe, has been identified. In this study, we identify the function of an actinin–like protein (AcnA) in the filamentous fungus Aspergillus nidulans. Our data suggest that AcnA plays important roles in cytokinesis and apical dominance of hyphal cells in A. nidulans through actin-dependent polarization maintenance and medial ring establishment.

**METHODS**

Strains, media, growth conditions and transformation. A list of A. nidulans strains used in this study is given in Table 1. The following media were used: YAG, 2% glucose, 0.5% yeast extract, trace elements of solution; YUU, YAG supplemented with 5 mM uridine and 10 mM uracil; YAGK, YAG with 0.6 M KCl; YUUK, YUU with 0.6 M KCl; MMGPR, minimal medium with 1% glycerol (v/v), nitrate salts, trace elements, 2.4 mM pyridoxine, 2.5 μg riboflavin ml⁻¹, pH 6.5, trace elements and nitrate salts were added to the media as described in the appendix to Kafer (1977); MMGTPR, MMGPR with 6.25 mM threonine. For solid media, 2% agar was added. Growth conditions, crosses and induction conditions for αc(Ap)-driven expression were as previously described (Wang et al., 2006). Transformation was done according to a method described elsewhere (May, 1989; Osmani et al., 1988). Lytic enzyme mix for transformation was composed of 0.4 mg β-d-glucanase ml⁻¹ (Interspec Products, 0439-1), 5 μl glucuronidase ml⁻¹ (Sigma) and 0.4 mg lysine enzyme L-2265 ml⁻¹ (Sigma). Media and top agar for plating transformants were made with 0.6 M KCl to stabilize the transformant protoplasts against osmotic lysis.

Tagging of AcnA with GFP in vivo. The amino acid sequence of x-actin 2 in Homo sapiens was used to search the A. nidulans protein database in GenBank. A 2073 bp sequence encoding a putative protein that was designated AcnA was found. The genomic sequence of acnA was revealed by the alignment to AcnA-5 (May, 1989; Osmani et al., 1988). Lytic enzyme mix for transformation was composed of 0.4 mg β-d-glucanase ml⁻¹ (Interspec Products, 0439-1), 5 μl glucuronidase ml⁻¹ (Sigma) and 0.4 mg lysine enzyme L-2265 ml⁻¹ (Sigma). Media and top agar for plating transformants were made with 0.6 M KCl to stabilize the transformant protoplasts against osmotic lysis.

**Table 1. A. nidulans strains used in this study**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Reference or source</th>
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<tr>
<td>GR5</td>
<td>pyrG89::w3A3, pyroA4, veA1</td>
<td>FGSC*</td>
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<tr>
<td>R21</td>
<td>pbaA1, ya2, veA1</td>
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<tr>
<td>LB01</td>
<td>chaA1, veA1</td>
<td>This study</td>
</tr>
<tr>
<td>WJA01</td>
<td>pyroA4, nkuA:: argB2, riboB2, veA1</td>
<td>This study</td>
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<td>WJA02</td>
<td>pyrG89::αc(Ap)(p):: GFP- AcnA:: pyr4, pyroA4, nKuA:: argB2, riboB3, veA1</td>
<td>This study</td>
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<tr>
<td>WJA03</td>
<td>pyrG89::ΔacnA:: pyrG, pyroA4, nKuA:: argB2, riboB2, veA1</td>
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*FGSC, Fungal Genetics Stock Center

**Cloning of acnA cDNA and expression of AcnA protein in Escherichia coli BL21.** The full-length cDNA of acnA was cloned by PCR amplification from the UniZAP A. nidulans cDNA library (McCluskey, 2003) with primers pET-ACN-Nde-5’- (ATCGCATA-TGATTTGGACGTGAGTTGCG) and pET-ACN-BamH-3’- (GTAAGGCATCTCCACGTCGCGTTGATAGGCAGAT), and subcloned into pET-28a (Novagen) to make a His-tag fusion at the N terminal of AcnA. The recombinant expression vector was confirmed by sequencing, and was designated pET28a-Acn. E. coli BL21(DE3) cells (Novagen) that had been transformed with the pET28a-Acn construct were grown in Luria–Bertani (LB) medium in the presence of kanamycin (50 μg ml⁻¹) to OD₆₀₀ 0.6 at 30 °C. Protein expression was induced by the addition of 1 mM IPTG and incubation at 30 °C for 4–6 h. The cells were then lysed at 37 °C for 15 min in lysis buffer containing 0.02 M sodium phosphate, 0.5 M NaCl, 40 mM imidazole, 0.3 mg lysozyme ml⁻¹, pH 7.4, in the presence of one Protease Inhibitor Cocktail Tablet (Roche). The cell lysate was sonicated, and the soluble proteins were collected by centrifugation at 12,000 r.p.m. (13,400 g) for 15 min. Recombinant protein containing a His–AcnA fusion was purified on a HiTrap affinity column (Ni Sepharose 6 Fast Flow; GE). Immunoblotting experiments were done as described by Shi et al. (2008) with anti-His (N-terminal) mouse monoclonal primary antibodies (Novagen).

**Immunoblotting experiments and Southern hybridization.** To extract proteins from A. nidulans mycelia, conidial spores from WJA02 were inoculated in liquid medium and shaken at 220 r.p.m. on a rotary shaker at 37 °C for 18 h. Total proteins were extracted and separated on a 12% Tris-glycine SDS gel. Immunoblotting experiments were done as previously described (Shi et al., 2008) with anti-GFP (N-terminal) mouse monoclonal primary antibodies (Roche Applied Science). For Southern hybridization, Zeta-Probe membranes (Bio-Rad) were probed with DIG-labeled probes, and processed as described in the manufacturer’s protocol (Roche Applied Science).

**Construction of the acnA deletion strains.** A strain containing an acnA-null mutation was created by PCR using plasmid pKS-pyr4, which carries the Neurospora crassa pyr4 gene as a selectable nutritional marker for fungal transformation as a template. A sequence of about 500 bp that corresponded to the regions immediately upstream of the acnA start codon was amplified with the primers AcnA-pre-Not-5’ (GAATTCGGCGCCGCCATTCGAGAATGCAAGCAACCG; NotI site underlined) and AcnA-pre-Xba-3’ (CCGTCTAGAGTGGCAGGATATGCGAAGCTTC; XbaI site underlined).
Table 2. Primers used in this study

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</tr>
<tr>
<td>AcnA-3’</td>
<td>GAATGTCAAAGCCGAAGCTGACATGTAAGG</td>
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<td>ACN-self-5’</td>
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<td>ACN-post-3’</td>
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<tr>
<td>pET-ACN-BamH-3’</td>
<td>GCAAGGTAGGTAGGTAGGTACCTTGTACCTTTCGG</td>
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<tr>
<td>GFP-5’</td>
<td>GACACCTCTGGTCACAGAGATG</td>
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<tr>
<td>Diag-pyr4-3’</td>
<td>GTGCGGAGGCTCAGCGATATATATTG</td>
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<tr>
<td>AcnA-pre-Not-5’</td>
<td>GAATGCGGGCCCGCGCATTCGAATGCGAAGCCCG</td>
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<td>AcnA-pre-Xba-3’</td>
<td>CCGCTCTAGATTGAGCGATATGCGGTTTC</td>
</tr>
<tr>
<td>AcnA-post-Pst-5’</td>
<td>GTCTCGAGCCGAGCTATGCTACTTTTCGG</td>
</tr>
<tr>
<td>AcnA-post-Kpn-3’</td>
<td>GCCCAGGATACCTTGTACGATTGAAAGCCCG</td>
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Microscopy and imaging processing. For microscopic observations, conidia were inoculated onto pre-cleaned glass coverslips on the appropriate agarose media, and grown at 37 °C. The cells had been fixed with 4 % formaldehyde (Polysciences), and fluorescent detection of actin was done essentially as described in earlier studies (Prigozhina et al., 2001), using anti-actin rabbit monoclonal (Epitomics;1 : 50) and Cy3-conjugated anti-rabbit IgG (Invitrogen, 1 : 1000) as primary and secondary antibodies, respectively. Differential interference contrast (DIC) and fluorescent images of the live cells were collected with a Zeiss Axio imager A1 microscope and the GFP signal was observed using a Chroma GFP filter set (catalogue no. 41017). Images were acquired and analysed using a Sensicam QE cooled digital camera system (Cooke) together with control and MetaMorph/MetaFluor combination package analysis software (Universal Imaging). The images were assembled in Adobe Photoshop 7.0.

RESULTS

AcnA is a putative α-actinin homologue

The amino acid sequence of α-actinin 2 in Homo sapiens was used to search the A. nidulans protein database in GenBank. A putative 690 aa protein was found and designated AcnA. Because the structure of the α-actinin homologue comprised three domains, an actin-binding domain (ABD), a spectrin repeat (SR) domain and an EF-hand (EH) superfamily domain, we aligned the three domains between AcnA and human α-actinin 2. AcnA was 27.1 % identical to α-actinin 2 of Homo sapiens over its entire length and 44.13 % identical to this protein over its N-terminal 213 aa, which includes a conserved actin-binding motif. Amino acids 292–414 of AcnA were 23.4 % identical to the first spectrin-like repeat (amino acids 282–391) of α-actinin 2 of Homo sapiens, and amino acids 415–523 of AcnA were 23.2 % identical to the fourth spectrin-like repeat (amino acids 640–739) of the Homo sapiens protein. Thus, it seems that AcnA has only two spectrin-like repeats, which is consistent with its shorter overall length compared with that of the other known α-actinins. Amino acids 524–675 of AcnA were 13.85 % identical to the two EFh motifs (amino acids 757–785 and 793–821) of α-actinin 2 of Homo sapiens, and this region of AcnA likely contains two functional Ca$^{2+}$-binding sites.

Homologues and putative homologues of α-actinins have been identified in some vertebrates, invertebrates, fungi and unicellular organisms (Fig. 1a). According to the number of SRs present in the rod domain, α-actinins can be divided into three groups; all vertebrate and invertebrate α-actinins belong to the first group, which has four repeats. Fungal actinin-like proteins belong to the second group, which has two repeats (with the exception of Cryptococcus neoformans, which has one SR repeat). The third group includes α-actinins of some unicellular organisms such as Entamoeba histolytica, which only has a single repeat. Altogether, this suggests a possible evolutionary pathway (Fig. 1b, c). Among the vertebrates, all α-actinins share a high homology; 99 % of the amino acid residues of α-actinin 2 are identical in humans and mice. Sequences of α-actinin homologues in fungi also show a high degree of identity, albeit lower than that in vertebrates. Among the selected fungal genomes, AcnA protein in A. nidulans shared the highest identity (89.1 %) with its homologue in

lined), and ligated into pLB01 at the corresponding site to generate pLB01-AcnA-pre. A sequence of about 500 bp that corresponded to the regions immediately downstream of the acnA stop codon was amplified with primers AcnA-post-Pst-5 (GGTATGTACTTTCGG; PsI site underlined) and AcnA-post-Kpn-3 (CTGGCCGGTGAATATCTAAGAG; Kpn site underlined), and ligated into pBluescript at the corresponding site to generate pKS-AcnA-post. The fragment that contained acnA-pre and pyr4 was released by digesting pLB01-acnA-pre with NotI and PstI; it was then ligated into pKS-ACnA-post that had been digested with NotI and PstI to generate pKS-AcnA-pre-pyr4-acnA-post. The linearized DNA fragment, which included a 500 bp left border of acnA, pyr4 and a 500 bp right border of acnA, was obtained by PCR using a high-fidelity DNA polymerase (Takara Biotechnology). The TN02A7 strain, which greatly reduces the frequency of non-homologous integration (Nayak et al., 2006), was transformed with the above PCR products to create the acnA-null mutation strain. A diagnostic PCR assay was performed to detect the deletion of the acnA gene. The screening medium for the acnA-null mutation strain was made as described previously (Shi et al., 2008).
Aspergillus fumigatus and 81.8% identity with the homologue in Coccidioides immitis. In comparison, the identity between A. nidulans and C. neoformans was 43.9%. Meanwhile, we found that AcnA shared 47.8% identity with its homologue from S. pombe, Ain1p, which is the only characterized actinin-like protein in fungi to date (Fig. 1b). This result implies that α-actinins are highly conserved proteins but still have some diversity in different organisms.

Characterization of AcnA protein and tagging of AcnA with GFP

From the cDNA library of A. nidulans, a 2073 bp sequence that encoded this putative protein was isolated; it matched perfectly with the sequence for the annotated AN7077 that we denoted acnA. The structure of the acnA gene has three exons and two introns (Fig. 2a). AcnA contains a putative N-terminal actin-binding surface domain and has a C-terminal EFh domain with calcium binding sites. Based on the obtained sequence, primers were designed for PCR amplification and cloning in the pET-28a expression vector. Transformation of E. coli BL21 (DE3) and subsequent induction of protein expression produced a soluble His-tagged fusion protein at the N-terminus of AcnA. Affinity purification resulted in a pure protein of the expected size (~78 kDa), with protein identity confirmed by anti-His-tag antibody (Fig. 2b). Because the molecular size of the His-tag was about 1.8 kDa, AcnA was about 76 kDa, which is consistent with its predicted size.

To study the function of AcnA in live cells of A. nidulans, a conditional alcA(p)::GFP–acnA strain, known as WJA02, was created by homologous integration according to the strategy illustrated in Fig. 2(c). PCR analysis showed that the acnA gene was fused to the GFP coding sequence under the control of alcA(p), which is regulated by the carbon source: repression by glucose on YAG, derepression by...
glycerol on MMGPR, and induction by threonine on MMGTPR. Southern blot analysis confirmed this homologous integration of only one copy at the acnA locus (Fig. 2c). To confirm the expression of the GFP–AcnA fusion protein, we carried out total protein extractions from the integrated WJA02 on MMGPR and YAG, respectively. In denaturing lysates, GFP–AcnA was detected as a band of about 103 kDa by the anti-GFP antibody. Because GFP is a 27 kDa protein, this suggests that the molecular size of AcnA is about 76 kDa, which is consistent with its predicted size. This band was completely absent from the repressive medium (Fig. 2b). The above data collectively suggest that AcnA was tagged with GFP at the predicted site in the conditional alcA(p):.GFP–AcnA strain WJA02, and that alcA(p) can be turned off completely by glucose.

To determine whether overexpression of GFP–AcnA causes mislocalization of GFP–AcnA in the hyphae, we assessed the localization of GFP–AcnA under the different conditions under which the alcA(p) promoter activity was moderate because of the alcA promoter. This showed that the GFP–AcnA localization patterns under derepressed-conditions (MMGPR) were similar to those under induced
conditions. At the same time, we compared the GFP–AcnA localization patterns of WJA02 grown on a solid MMGPR agar with those in liquid medium. Although the GFP–AcnA signal in the liquid medium was weaker than that in the solid medium, the GFP–AcnA localization pattern was consistent under different culture conditions (data not shown). This suggests that expression of alcA(p) would not cause mislocalization of GFP–AcnA in strain WJA02. Thus, in subsequent experiments, we used glycerol as the sole carbon source in MMGPR to detect the localization of AcnA.

**AcnA is required for apical dominance of the hyphal cells**

To test whether the GFP–AcnA fusion was functional, WJA02 was inoculated under derepressing and repressing conditions. On the derepression medium MMGPR, WJA02 demonstrated normal hyphal polarity growth and the conidiation pattern was similar to that of the wild-type WJA01 strain. This indicated that the GFP–AcnA fusion was functional on the MMGPR media. The slightly slower colony growth of WJA02 may be due to a delay in the expression of AcnA in MMGPR. In order to characterize the effects of AcnA repression, a microscopy study was carried out. On MMGPR medium, WJA02 hyphae extended at their tips and grew predominantly after germination. However, on the repressing medium YAG, WJA02 clearly showed a loss of apical dominance of the cells with a dichotomous branching, in which a new branch was formed by splitting the existing tip in two, resulting in flabellate edges to the colony (Fig. 3a, c). This phenotype was a rare occurrence in wild-type WJA01 under the same culture conditions. Of wild-type germlings in YAG at 37 °C cultured for 18 h (n=130), 6.25% branched dichotomously. In contrast, 73.8% branched dichotomously (n=179) under the same conditions in the acnA-depleted strain WJA02. The switch in the branching pattern suggests that AcnA plays a role at the hyphal tip. To further examine the function of AcnA in case there was leaky expression of GFP–AcnA, an acnA-deleted mutant, WJA03, was successfully constructed. The acnA gene was deleted by replacing it with a PCR fragment in which pyr4 was flanked by about 500 bp of the sequence that flanked acnA in the genome (Fig. 3b). The tagging experiment was selected and examined by PCR assay. The acnA gene was clearly replaced by pyr4, and there was no detectable acnA gene in this strain, which was now referred to as WJA03. In the YAG and MMGPR media, WJA03 showed a loss of apical dominance in the cells with dichotomous branching (Fig. 3c), which was a similar phenotype to that observed with WJA02 in the repressing medium. Furthermore, the global deletion strain WJA03 showed more growth defects, with completely abolished conidiation, than that of the conditional knocked-down strain WJA02. These results strongly support the hypothesis that AcnA is essential for apical dominance of hyphal cells and conidiation.

**AcnA is involved in cytokinesis and conidiation**

The colonies of the depleted strain WJA02 and the deletion strain WJA03 showed abolished conidiation, indicating that the asexual cycle was disrupted. To further investigate the function of AcnA, the nuclei and cell walls were stained using DAPI and Calcofluor white, respectively. Surprisingly, the Calcofluor white staining of the acnA mutants showed no stained septa, suggesting that the hyphal cells displayed profound abnormalities in cytokinesis and septation (Fig. 4). In particular, DAPI staining revealed that cells completed mitosis without forming septa. In addition, the mycelia in WJA02 had an aberrant nuclear distribution such that the mean distance between nuclei was 4.78 ± 2.39 μm instead of 7.47 ± 3.21 μm as in the wild-type under the same conditions in YAG (Fig. 4). There was a statistically significant difference between these measurements, which suggests that there was a defect affecting septation and nuclear positioning but not nuclear division. This abnormal nuclear segregation might be the direct effect of the defect in AcnA or the result of a secondary effect, not directly linked to AcnA. To further characterize the phenotype of the acnA mutants, the hyphae of WJA02 on YAG were stained with anti-actin antibody to determine whether the block in septum formation occurred before or after the accumulation of actin associated with septum formation. Immunofluorescence analysis indicated that there was no detectable actin ring in the AcnA depletion strain WJA02 compared with the wild type, in which actin was localized to the septa (data not shown). This suggests that AcnA aids actin in localizing the presumptive division sites in A. nidulans.

**Intracellular distribution of GFP–AcnA**

According to the phenotypes of WJA02 on the derepression medium MMGPR, the GFP–AcnA fusion protein was functional. WJA02, which was expressed as a GFP–AcnA fusion protein under the control of the alcA promoter, was used to examine AcnA localization. In this strain, the GFP–AcnA fusion protein was biologically active under this derepression condition. During the period of isotropic expansion, GFP–AcnA was organized as cables and cortical patches close to the cell wall. Moreover, when the germinating spore established a polarity axis by an apical extension, the cortical cable of GFP–AcnA localized to the emergence of a germtube at the hyphal tip. Subsequently, GFP–AcnA, which formed double parallel cables for the majority of the time, further extended to align with the cell wall through the whole cell, and predominantly localized to the actively growing hyphal tips (Fig. 5a). The occasional weakness of the fluorescence of the double parallel cables of GFP–AcnA in the middle of hyphae may be attributable to the fact that the GFP–AcnA localized at different focal planes. This localization pattern suggests that to function, AcnA in A. nidulans forms an anti-parallel dimer, which might be consistent with the typical characterization of α-actinins in mammalian cells. Furthermore, at the sites of
septation, a dense GFP–AcnA accumulation in the form of double rings embracing the septum was present at times (Fig. 5b, arrowed). Interestingly, two different shapes of double rings of GFP–AcnA were found (Fig. 5b, left and right), while no single ring was observed during our experiment. Furthermore, at the septum sites of the mature hyphae, these double rings of GFP–AcnA could no longer be seen. These data suggest that GFP–AcnA is highly expressed and dynamic during germination, hyphal growth and cytokinesis in A. nidulans. No detectable GFP
fluorescence was found when the alcA(p):GFP–AcnA strain WJA02 was cultured on the repressing medium YAG, which was tested as a control (data not shown).

Normal actin distribution is AcnA-dependent

To further study the relation of AcnA function with actin in A. nidulans, we determined the distribution of actin during cytokinesis and septation in acnA depletion cells. The normal actin localization was not detectable in WJA02 cells under repression conditions when indirect immunofluorescence with anti-actin mAbs was used to visualize the actin cytoskeleton. As shown in the bottom panel of Fig. 6, none of the germlings had detectable actin rings or actin caps. In addition, we were unable to find any clear subapical patches in WJA02 cells (Fig. 6). In contrast, under the same conditions, wild-type TN02A7 cells displayed normal actin organization; actin predominated at the hyphal tip and further localized, in addition to septa (data not shown), to cortical actin patches (Fig. 6, top panel). Thus, AcnA appears to be involved in the normal localization of the actin cap and of the actin patches at the hyphal tip, and in the establishment of the actin ring at the septum. Apparently, the loss of apical dominance in AcnA depletion is reflected by aberrant organization of the actin cytoskeleton.

DISCUSSION

Evolution of α-actinins

The sequence alignment suggests that α-actinin is a conserved protein during evolution; the degree of identity is very high, particularly in the N terminus and C terminus. The most significant differences in the sequence are located

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Fig. 4. Comparison of septation and nucleus distribution in hyphal cells between the wild-type and the conditional mutant WJA02. Left panels, the hyphae of the acnA mutant WJA02 cultured on YAG medium were stained with Calcofluor white to visualize the septa; the nuclei were visualized with DAPI. Middle panels, DAPI and Calcofluor double staining of TN02A7. Bars, 10 μm. Right panel, quantification analysis of mean value of distances between nuclei in WJA02 and the wild-type strain TN02A7 on YAG. For WJA02, n=137; for wild-type TN02A7 strain, n=179. The differences in the median values between the treatment groups are greater than would be expected by chance; there is a statistically significant difference by t test (P<0.001).
in the rod domain (Broderick & Winder, 2005; Virel & Backman, 2007). It seems possible that modern \( \alpha \)-actinins evolved from an ancestral \( \alpha \)-actinin protein by duplicating its two repeats. The first intragenic duplication produced the second repeat, which became the fourth repeat (SR4) in modern \( \alpha \)-actinins. A subsequent second intragenic duplication added two more repeats (SR2 and SR3) (Dixson et al., 2003; Virel & Backman, 2004).

Interestingly, a conserved-domain BLAST from NCBI predicted that \( \alpha \)-actinin proteins from fungi such as \( S. \) pombe and \( A. \) nidulans have no conserved SR in the domain, which results in a monomeric protein with two tandem \( \alpha \)-actinin-type actin-binding motifs without spectrin-like repeats. In contrast, using the conserved-domain BLAST tool, the retrieved predicted SRs of fungal actinin sequences from the SwissProt, TIGR and Sanger Institute databases showed two SRs, which implies that two conserved SR domains exist in the actinin-like or predicted actinin proteins of fungi. Our protein sequence alignment analysis indicated that amino acids 292–414 of AcnA are 23.4% identical to the first spectrin-like repeat (amino acids 282–391) of \( \alpha \)-actinin 2 of \( Homo \) sapiens, and amino acids 415–523 of AcnA are 23.2% identical to the fourth spectrin-like repeat (amino acids 640–739) of \( \alpha \)-actinin 2 of \( Homo \) sapiens. Thus, as in some other fungal species, AcnA has only two spectrin-like repeats (Fig. 1b).

**Possible regulation of AcnA in cytokinesis**

In the past few years, an accumulating body of literature has revealed that \( \alpha \)-actinin plays multiple important roles in mammalian cells. Its functions include linking the cytoskeleton to many different transmembrane proteins at many junctions, regulating the activity of a variety of receptors, and serving as a scaffold for the connection of the cytoskeleton to diverse signalling pathways (Cabello et al., 2007; Chi et al., 2008; Choi et al., 2008; Sjoblom et al., 2008). However, evidence for the function of \( \alpha \)-actinins in cytokinesis currently remains inconclusive. Early studies indicated that \( \alpha \)-actinins might be involved in cytokinesis (Fujiwara et al., 1978; Mabuchi et al., 1985; Sanger et al., 1987). Recently, it was found that although endogenous \( \alpha \)-actinin primarily accumulates at the equatorial region in normal rat kidney (NRK) epithelial cells and colocalizes along actin filaments during cytokinesis, overexpression of \( \alpha \)-actinin causes the failure of cytokinesis (Mukhina et al., 2009).
Moreover, in the fission yeast *S. pombe*, an α-actinin-like protein (Ain1p) is localized to the actomyosin-containing medial ring in an F-actin-dependent manner during cytokinesis. However, *ain1* deletion cells have no obvious defects under normal growth conditions, except for displaying severe cytokinesis defects under certain stress conditions (Wu et al., 2001). Little is known about the precise function of α-actinin during cytokinesis. Our results demonstrate that *acnA* depletion or deletion mutants have severe cytokinesis defects under normal culture conditions in *A. nidulans* (Fig. 4). Some published evidence indicates that septum formation is dependent on mitosis and requires the recruitment of actin to the site of septum formation in *A. nidulans* (Boyce et al., 2003; Harispe et al., 2008; Harris, 2001; Sharpless & Harris, 2002; Upadhyay & Shaw, 2008; Westfall & Momany, 2002). The formation of an actin ring in *A. nidulans* predicts the site of septum formation and provides a visual marker of the progress of cytokinesis. Meanwhile, septation is observed when the first signs of chitin deposition become visible through Calcofluor white staining during the formation of septa. Therefore, by staining hyphae for actin and chitin, an approximate stage at which a mutant is blocked during cytokinesis and septation can be determined. We report here that *acnA* mutants exhibited defects in septa and conidiation. This indicates that AcnA is required for actin accumulation during the formation of septa. Furthermore, from the localization pattern of GFP–AcnA as a set of double rings embracing the septum site, AcnA may function as an adaptor or an anchor protein for the actin ring. Thus, the defect of AcnA in mutant cells results in their failure to recruit actin to presumptive division sites, resulting in the lack of development of the septum. These observations introduce the possibility that modulation of actin by AcnA regulates actomyosin contraction rings during cytokinesis.

**Fig. 6.** Dependence of actin localization on the function of AcnA in *A. nidulans*. Bottom panel, localization of actin in the absence of AcnA in WJA02 in YAG medium by indirect immunofluorescence staining with anti-actin mAbs. There were no detectable actin patches and caps on the hyphal tips. Top panel, localization of actin in wild-type TN02A7 under the same conditions as above. Actin was predominantly localized at the hyphal tips and further localized to the cortical actin patches. The correlated position of GFP–AcnA in germlings is shown using DIC at the left. Bars, 10 μm.
AcnA is involved in the apical dominance of hyphal growth

Filamentous fungi are ideal systems for studying the process of polarized growth, because the growing hyphae are among the most polarized cells in nature; their life cycles are dominated by hyphal growth exclusively at the cell apex (for reviews, see Harris, 2006; Momany, 2005; Takeshita et al., 2008). At the sites of tip growth, multiple protein complexes assemble and coordinate to ensure that incoming building materials reach the appropriate destination sites such that polarized growth is maintained (Fischer et al., 2008; Taheri-Talesh et al., 2008). In A. nidulans, cortical actin patches are highly mobile throughout the hypha, and are clustered near the apical hyphae and scattered behind the tips. Thus, the actin cap at the tip is often seen as a subapical ring that embraces the hyphal tube in living hyphae (Araujo-Bazan et al., 2008; Harispe et al., 2008; Upadhyay & Shaw, 2008). Earlier studies have verified that the mislocalization of actin results in the disruption of the polarized growth machinery. Thus, the actin cytoskeleton must play an important role in tip growth (Harris et al., 1994; Torralba et al., 1998). In this study, acnA mutants clearly showed a loss of apical dominance in cells with dichotomous branching; a new branch was almost always formed by splitting an existing tip in two. In comparison, published data suggest that mutants in sepA, which encodes formin, and spa or bemA, which encode the polarisome, display dichotomous branching (split tips), similar to acnA mutants in A. nidulans (Leeder & Turner, 2008; Sharpless & Harris, 2002;Virag & Harris, 2006; Virag et al., 2007). In fact, SepA, which is localized at the hyphal tip constantly and at the septum transiently, participates in two actin-mediated processes (i.e. septum formation and polarized growth), but the localization pattern of SepA is different from that of AcnA. In addition, the presence of the polarisome, identified in Saccharomyces cerevisiae as a 12S multiprotein complex, indicates important roles in bud site selection. Moreover, the deletion of spa2 in Saccharomyces cerevisiae results in the mislocalization of actin and defects in polarization. SpaA, a homologue of Spaz in A. nidulans, is localized exclusively at the hyphal tip, which indicates that it plays a role in the stabilization of the hyphal polarity axes (Shih et al., 2005; van Drogen & Peter, 2002). It is not clear whether the function of AcnA is related to those of SepA and SpaA in regulating correct actin localization. Current data suggest that depletion of AcnA induces a defect in the subapical actin ring that embraces the hyphal tube. It seems that the absence of the normal subapical rings in apolar cells may result in apical, dichotomous branching. This dichotomous branching (not just random branching) phenotype suggests that AcnA does not affect on-site selection of germ tube emergence during polarity growth. The dichotomous branching of acnA mutants and the dense accumulation of GFP–AcnA at the tip suggest that AcnA acts on the apical dominance of hyphal growth. The increase in apical branching in acnA deletion and deletion mutants implies a role for AcnA in the stabilization of hyphal polarity axes. In summary, although our current results may not fully address the precise mechanism of regulation by AcnA of cytokinesis and apical dominance of hyphal growth, our results do provide informative insights into the function of the α-actinin homologue in A. nidulans.

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