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

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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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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 (Santella *et al.*, 2008; Schafer & Schroer, 1999; Schroer *et al.*, 1994; Sjoblom *et al.*, 2008). Among the actin cross-linking proteins, α -actinin is one of the best characterized. It was first isolated from rabbit skeletal muscle (Ebashi & Ebashi, 1965) and subsequently has been verified in both muscle and non-muscle cells from many animals (Flood *et al.*, 1995; Furukawa & Fechheimer, 1994; Furukawa *et al.*, 2003; Simon *et al.*, 1988). Furthermore, its presence in fungi and unicellular organisms has been reported or predicted from the relevant sequencing

databases (Dixon *et al.*, 2003; Virel & Backman, 2004, 2007).

In the past few years, a growing body of literature has revealed that α -actinin plays multiple important roles in the cell: it links the cytoskeleton to many different transmembrane proteins at numerous junctions, regulates the activity of a variety of receptors, and functions as a scaffold for the connection of the cytoskeleton to diverse signalling pathways (Sadeghi *et al.*, 2002; Schulz *et al.*, 2004; Sjoblom *et al.*, 2008; Weins *et al.*, 2007). In addition, the localization of α -actinin to the cleavage furrows of chick embryos (Fujiwara *et al.*, 1978), sea urchin eggs (Mabuchi *et al.*, 1985) and cultured mammalian cells (Sanger *et al.*, 1987) suggests a role in cytokinesis. In mammalian cells, there are four α -actinin-encoding genes, and they produce at least six different protein products; each has been found within a specific tissue type and has a specific expression profile (Sjoblom *et al.*, 2008). However, the nature of these roles is not clear, and the genetic studies provide only limited insight into other aspects of α -actinin function *in vivo*. Thus, a simple and genetically tractable model system, such as fungi, provides a unique approach to study the function of the actin cytoskeleton in cellular morphogenesis and cytokinesis. However, currently, only

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 M 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 *alcA*(p)-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 α-actinin 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 *A. nidulans* genome project at the AN7707 locus. The conditional *acnA* allele, *alcA*(p)::GFP-*acnA* was constructed in plasmid pLB01 (Liu & Morris, 2000). A fragment starting from the predicted initiation codon of *acnA* was amplified from the wild-type genome GR5 with primers AcnA-5' (ATATAGCGGCGCTGTTGACGGTTGACGAG; *NotI* site underlined) and AcnA-3' (GAATG TCTAGACGTCGTTCCAACCTCGTAG; *XbaI* site underlined) (Table 2). The 651 bp amplified DNA fragment was digested with *NotI* and *XbaI*, and cloned into pLB01 at corresponding sites. This plasmid was transformed into the TN02A7 strain. Homologous integration of the *acnA* sequence generated two copies of *acnA*: a truncated *acnA* gene with its own

promoter and a *gfp-acnA* fusion gene under the control of the *alcA* promoter. Candidate transformants were subjected to PCR analysis using a forward primer designed to recognize the *gfp* sequence and a reverse primer designed to recognize the *acnA* sequence.

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-TGATGTTGACGGTTGACGAGTCGTGGG) and pET-ACN-BamH-3'-(GTAAGGGATCCTCACACGCTGCCGTTGATAGGCGCATTG), 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-labelled 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' (GAATTGCGGCCGCGCATTTCAG-AATGCGCAACCG; *NotI* site underlined) and AcnA-pre-Xba-3' (CCGTTCTAGAGTTGAGCGATATGCAGGTTTC; *XbaI* site under-

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

Strain	Genotype	Reference or source
TN02A7	<i>pyrG89;pyroA4,nkuA::argB2;riboB2;veA1</i>	Nayak <i>et al.</i> (2006)
GR5	<i>pyrG89;wA3;pyroA4;veA1</i>	FGSC*
R21	<i>pabaA1,yA2;veA1</i>	FGSC*
LB01	<i>chaA1;veA1</i>	This study
WJA01	<i>pyroA4,nkuA::argB2;riboB2;veA1</i>	This study
WJA02	<i>pyrG89;alcA</i> (p)::GFP- <i>AcnA::pyr4,pyroA4,nkuA::argB2;riboB2;veA1</i>	This study
WJA03	<i>pyrG89;ΔacnA::pyrG,pyroA4,nkuA::argB2;riboB2;veA1</i>	This study

*FGSC, Fungal Genetics Stock Center

Table 2. Primers used in this study

Primer	DNA sequence (5'–3')
AcnA-5'	ATATAGCGGCCGCTGTTGACGGTTGACGAG
AcnA-3'	GAATGTCTAGACGTCGTTCCAACCTCGTAG
ACN-self-5'	TTCCTGAATGTCCTCGAACCTG
ACN-post-3'	CTGGCCGGTGAATATCTAAGAG
pET-ACN-Nde-5'	ATCGCATATGATGTTGACGGTTGACGAGTCGTGGG
pET-ACN-BamH-3'	GTAAGGGATCCTCACACGCTGCCGTTGATAGGCGCATTG
GFP-5'	GACACCCTCGTCAACAGGATCG
Diag-pyr4-3'	GGTCGCAGGCCATGGATATATTGTGG
AcnA-pre-Not-5'	GAATTGCGGCCGCGCATTGAGAATGCAGCAACCG
AcnA-pre-Xba-3'	CCGTTCTAGAGTTGAGCGATATGCAGGTTTC
AcnA-post-Pst-5'	GGTCCTGCAGGGCAAGGTATGTACTTTCGG
AcnA-post-Kpn-3'	GCCCAGGTACCATTGTACATTGAAGCCCG

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' (GGTCCTGCAGGGCAA-GGTATGTACTTTCGG; *Pst*I site underlined) and AcnA-post-Kpn-3' (GCCCAGGTACCATTGTACATTGAAGCCCG; *Kpn*I 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 *Not*I and *Pst*I; it was then ligated into pKS-acnA-post that had been digested with *Not*I and *Pst*I 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 3.5 kb 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).

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. After the cells had been fixed with 4% formaldehyde (Polyscience), immunofluorescent 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). DNA and chitin were stained using 4',6-diamidino-2-phenylindole (DAPI) and Calcofluor white (both from Sigma Aldrich), respectively. 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 (EFh) 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

Fig. 1. Phylogenetic and identity analysis of α -actinin homologues in selected organisms. (a) Phylogenetic tree representing the evolutionary relationships between α -actinin homologues. The tree was constructed by DNASTAR software using the neighbour-joining method from amino acid sequences of the following actinin homologues: *A. nidulans* (XP_680976), *A. fumigatus* (XP_753806), *Coccidioides immitis* (XP_001242919), *Sclerotinia sclerotiorum* (XP_001598180), *N. crassa* (XP_957320), *Magnaporthe grisea* (XP_369960), *S. pombe* (NP_594295), *Cryptococcus neoformans* (XP_569979), *Ustilago maydis* (XP_760459), *Entamoeba histolytica* (XP_653283), *Dictyostelium discoideum* (XP_646979), *Rattus norvegicus* (AAH74001), *Mus musculus* (NP_150371) and *Homo sapiens* (NP_001094). (b) Comparison of amino acid sequence identities between AcnA of *A. nidulans* and actinin homologues from selected fungal species. Red, area belongs to the ABD, which contains two calponin homology (CH) domains; yellow, SR; blue, calcium-binding EFh. The data were analysed using conserved-domain searching in NCBI and Swiss-Prot. The sequences were obtained from NCBI (<http://www.ncbi.nlm.nih.gov/>) and Swiss-Prot (<http://www.expasy.ch/sprot/>). All actinins have one ABD on each polypeptide. Each ABD contains two calponin homology domains and one EFh, whereas the largest sequence differences between different isoforms are located in the rod domain. (c) Schematic structure of α -actinin and the possible phylogenies of the SR from unicellular organisms through fungi to vertebrates. *Entamoeba histolytica*, with the earliest diverging α -actinin, has one SR in the rod domain. *S. pombe*, with the first isolated α -actinin-like protein in fungi, includes two predicted SRs, while four SRs are present in organisms such as *Homo sapiens* and may be the result of two consecutive intragenic duplications from the single SR of α -actinin.

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

Fig. 2. Characterization of AcnA protein and tagging of AcnA with GFP. (a) Exon-intron structure of *acnA* and domain organization of AcnA. The three exons are indicated by roman numerals. (b) Western blotting showed the expression of GFP-AcnA fusion protein in WJA02 grown in MMGPR (GFP-AcnA lane) and in repressing medium YAG (untagged lane) detected by anti-GFP antibodies. The cloning of *acnA* full-length cDNA in-frame constructed in plasmid pET28a and the expression of AcnA protein in *E. coli* BL21 (DE3) was shown by Western blotting using anti-His antibodies (His-AcnA lane); the untagged lane shows the expression of control vector pET28a under the same conditions. (c) Diagram showing the strategy used for creation of the conditional *alcA(p)::GFP-acnA* strain WJA02. The result was confirmed by PCR and Southern blotting. PCR analysis showed that WJA02 had pLB01-*acnA* integrated into the genome at the original *acnA* locus. For lanes 1 and 3, PCR primers were GFP-5' and ACN-post-3'; for lanes 2 and 4, PCR primers were ACN-self-5' and ACN-post-3'. For lanes 1 and 2, genomic DNA of wild-type TN02A7 was used as the PCR template; for lanes 3 and 4, the template was WJA02 genomic DNA. Southern blot analysis confirmed the homologous single-copy integration at the *acnA* locus of WJA02.

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 the 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 $4.78 \pm 2.39 \mu\text{m}$ instead of $7.47 \pm 3.21 \mu\text{m}$ 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 germ tube 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

Fig. 3. Phenotypic comparison of wild type and *acnA* mutants. (a) Colony phenotypic comparison of wild-type, conditional mutant WJA02 and global mutant WJA03 grown at 37 °C on MMGPR and YAG media, separately. (b) Creation strategy for *acnA* deletion strain WJA03. The result was confirmed by PCR. PCR analysis showed that *acnA* was replaced by *pyr4* at the original *acnA* locus in WJA03. For lanes 1 and 3, PCR primers were ACN-deletion-pre-Not-5' and Diag-pyr4-3'; for lanes 2 and 4, PCR primers were ACN-deletion-pre-Not-5' and ACN-post-3'. For lanes 1 and 2, genomic DNA of wild-type TN02A7 was used as the PCR template; for lanes 3 and 4, the template was WJA03 genomic DNA. (c) *acnA* mutants showed a loss of apical dominance of the cells with dichotomous branching, as revealed by DIC microscopy. Top panels show the hyphae branching laterally in wild-type and conditional mutant WJA02 on MMGPR medium. Middle panels show the hyphae branching dichotomously in the global mutant WJA03 and the conditional mutant WJA02 on the repression medium YAG. Lower panels show enlarged views of indicated areas of the middle panels.

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

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 ($P \leq 0.001$).

Fig. 5. Localization pattern of GFP-AcnA in *A. nidulans*. (a) Dynamic localization of GFP-AcnA during germination and hyphal growth in WJA02 on MMGPR medium. GFP-AcnA was organized as cables and cortical patches close to the cell wall during the period of isotropic expansion. Subsequently, the cortical cable of GFP-AcnA extended to the emergence of a germ tube at the hyphal tip. In the growing hyphal cells, GFP-AcnA cables further extended, aligned to the cell wall and predominantly localized to the actively growing hyphal tip. The correlated position of GFP-AcnA in germlings is shown using DIC. (b) GFP-AcnA was highly accumulated at the septation sites as double-twist rings (indicated with arrows). Enlarged views of the septation sites are shown; the middle panels show the enlarged areas. Bars, 10 μ m.

in the rod domain (Broderick & Winder, 2005; Virel & Backman, 2007). It seems possible that modern α -actinins evolved from an ancestral α -actinin protein by duplicating its two repeats. The first intragenic duplication produced the second repeat, which became the fourth repeat (SR4) in modern α -actinins. A subsequent second intragenic duplication added two more repeats (SR2 and SR3) (Dixon *et al.*, 2003; Virel & Backman, 2004). Interestingly, a conserved-domain BLAST from NCBI predicted that α -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 α -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 α -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 α -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 α -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 α -actinins in cytokinesis currently remains inconclusive. Early studies indicated that α -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 α -actinin primarily accumulates at the equatorial region in normal rat kidney (NRK) epithelial cells and colocalizes along actin filaments during cytokinesis, overexpression of α -actinin causes the failure of cytokinesis (Mukhina *et al.*,

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 μ m.

2007). 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.

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 hyphal apices 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 *Spa2* 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* depletion 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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