Septins AspA and AspC Are Important for Normal Development and Limit the Emergence of New Growth Foci in the Multicellular Fungus Aspergillus nidulans

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Septins are cytoskeletal proteins found in fungi, animals, and microsporidia, where they form multiseptin complexes that act as scaffolds recruiting and organizing other proteins to ensure normal cell division and development. Here we characterize the septins AspA and AspC in the multicellular, filamentous fungus Aspergillus nidulans. Mutants with deletions of aspA, aspC, or both aspA and aspC show early and increased germ tube and branch emergence, abnormal septation, and disorganized conidiophores. Strains in which the native aspA has been replaced with a single copy of aspA-GFP driven by the native septin promoter or in which aspC has been replaced with a single copy of aspC-GFP driven by the native promoter show wild-type phenotypes. AspA-GFP and AspC-GFP show identical localization patterns as discrete spots or bars in dormant and expanding conidia, as rings at forming septa and at the bases of emerging germ tubes and branches, and as punctate spots and filaments in the cytoplasm and at the cell cortex. In conidiophores, AspA-GFP and AspC-GFP localize as diffuse bands or rings at the bases of emerging layers and conidial chains and as discrete spots or bars in newly formed conidia. AspA-GFP forms abnormal structures in ΔaspC strains while AspC-GFP does not localize in ΔaspC strains. Our results suggest that AspA and AspC interact with each other and are important for normal development, especially for preventing the inappropriate emergence of germ tubes and branches. This is the first report of a septin limiting the emergence of new growth foci in any organism.

Septins are novel cytoskeletal proteins first discovered in a screen for Saccharomyces cerevisiae cell cycle mutants (14). The core septin proteins, Cdc3, Cdc10, Cdc11, and Cdc12, localize to the mother/bud neck, where they assemble into heteropolymers that organize proteins necessary to complete cytokinesis and ensure proper coordination between bud formation and nuclear division (4, 16, 37, 38).

S. cerevisiae septins first appear as a cortical patch at the future bud site. They later form a ring through which the bud emerges and then develop into an hourglass-shaped complex at the base of the bud that splits into two rings to complete cytokinesis (8, 10, 23). In the dimorphic fungus Candida albicans, septins assemble during the formation of buds and pseudohyphae, localize to prebud sites, and form rings at the mother/bud neck. During hyphal growth septins localize to hyphal tips and transiently as a basal band within germ tubes (33, 39). The genome of the filamentous fungus Ashbya gossypii is 90% homologous and syntenic with the genome of S. cerevisiae, though it grows in the filamentous morphology rather than the yeast morphology (6). In A. gossypii septins localize as discrete filamentous bars at septation sites, at tips of hyphae, and at the bases of emerging branches. A. gossypii septins are not essential, though they have been shown to be involved in mitosis, sporulation, hyphal morphogenesis, and septum formation (5, 9).

In mammals, septins ensure proper growth, cell migration, vesicle trafficking, and cell division (19, 32, 35, 42). Mammalian septins form distinct filaments that colocalize with and appear to organize the actin and microtubule cytoskeletons (19, 31, 35), punctate patterns at neuron terminals and vesicles, and rings in sperm cells (2, 15, 18, 43).

Aspergillus nidulans is a multicellular filamentous fungus which has five septins, AspA, AspB, AspC, AspD, and AspE (29). All five septins are expressed during vegetative and asexual growth, with AspB having the highest expression levels (29). Immunofluorescence studies showed that AspB localizes to septa and conidiophore layers and anticipates the sites of branch emergence (40). To better understand the roles of septins in shaping the growth of multicellular organisms, we characterized A. nidulans septins AspA and AspC, orthologs of S. cerevisiae Cdc11 and Cdc12, respectively (30). Septin deletion mutants were characterized throughout vegetative and asexual development. AspA and AspC are necessary for normal development and morphogenesis as well as sporulation. AspA and AspC were found to localize as rings, caps, puncta, or filaments throughout development. Localization of AspA and that of AspC appear to be mutually dependent, as AspC was unable to localize in the ΔaspA strain and AspA localization was abnormal in the ΔaspC strain.

MATERIALS AND METHODS

Strains and media. Strains used in this study are listed in Table 1. Media used were previously described (13). Strain construction and growth were done by standard A. nidulans techniques (13, 17). All incubations were at 30°C and in

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Complete medium (1% glucose, 0.2% peptone, 0.1% Casamino Acids, 0.1% yeast extract, trace elements, nitrate salts, and 0.01% vitamins, pH 6.5) with amino acid supplements (17) except where noted. Green fluorescent protein (GFP) diploid strains were generated as previously reported (12) by mixing conidiospores from a wild-type strain (A773 or A850) with those from the AspA-GFP (ARL141) or AspC-GFP (ARL159) strain, respectively. Heterokaryotic germ-lings were plated into minimal agar from which only diploids could emerge. The resulting diploid conidia were streaked for isolation two times to yield strains ARL205 and ARL206, respectively. Localization was examined in four GFP bright strains for each diploid.

**Growth conditions and microscopic observations.** Preparation and growth of cells were as previously reported (28). Briefly, conidia were inoculated on sterile coverslips in liquid complete or minimal medium and incubated at 30°C in a petri dish. Cells were fixed, septa were stained with calcofluor (American Cyanamid, Wayne, NJ), and nuclei were stained with Hoechst 33258 (Sigma, St. Louis, MO). Microscopic observations were made using a Zeiss (Thornwood, NY) Axioskop microscope with appropriate filters, and digital images were acquired using an Axios (Axion Technologies) digital imaging system. For all GFP fusion observations, recipient strains that had not been transformed with GFP cassettes were viewed under identical settings to verify that there was no autofluorescence. Images were prepared using Photoshop cs version 8.0 (Adobe, Mountain View, CA). For quantitation of phenotypes, counts of 200 cells were done. All experiments were repeated at least three times with similar results. A representative data set is shown.

**Asexual structures.** Preparation of conidiophores was as previously reported (28). Briefly, conidia were inoculated on the edges of a square of complete agar medium which was sandwiched between two coverslips and placed on top of water agar to prevent the complete agar from drying out. Plates were incubated inverted at 30°C for 2 days. To observe conidiophore structures, coverslips with pores from a wild-type strain (A773 or A850) with those from the AspA-GFP (ARL141) or AspC-GFP (ARL159) strain, respectively. Heterokaryotic germ-lings were plated into minimal agar from which only diploids could emerge. The resulting diploid conidia were streaked for isolation two times to yield strains ARL205 and ARL206, respectively. Localization was examined in four GFP bright strains for each diploid.

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aerial hyphae and conidiofores attached were fixed, mounted on slides, and observed microscopically. **Fluorescent tags and gene knockouts.** The ΔaspA strain was generated by transforming *A. nidulans* A850 with plasmid pOAS15 using the protoplasting method. Approximately 800 bp upstream of the *aspA* gene (flank 1) was amplified using the Molecular Bio Products EasyStart Micro 20 kit (San Diego, CA) with primers AspAko_XbaI_1 and AspAko_BamHI_1 (Table 2), which introduced XbaI and BamHI sites, respectively. Approximately 1,000 bp downstream of the *aspA* gene (flank 2) were PCR amplified using the Molecular Bio Products EasyStart Micro 20 kit (San Diego, CA) with primers AspAko_HindIII_2 and AspAko_KpnI_2 (Table 2), which introduced HindIII and KpnI sites, respectively. The PCR products were cut with the appropriate restriction enzymes and then ligated on either side of the *argB* gene in the pArgB2 plasmid, resulting in plasmid pOAS15. Flank 1 is located at the 3' end of the *argB* gene, and flank 2 is located at the 5' end of the *argB* gene. After pOAS15 was confirmed by restriction digestion, it was used to transform *A. nidulans* A850 by the protoplasting method. The resulting transformants were checked by PCR (primers listed in Table 2) and confirmed by Southern blotting to ensure that a single homologous integration under the endogenous promoter was obtained.

All PCRs and fusion PCRs were conducted using Invitrogen AccuPrime PfX DNA polymerase as previously described (44). The ΔaspC strain was generated by a complete replacement of the *aspC* gene with the *AfpyrG* gene. This was done by amplifying three separate fragments: the first fragment was 1 kb upstream of the *aspC* start codon (A850 template), the second fragment was 1 kb downstream of the *aspC* stop codon (A850 template), and the third fragment was the *AfpyrG* gene (pFNO3 plasmid template), which included 30 bp before the *aspC* start codon and 30 bp after the stop of the *aspC* stop codon added to facilitate fusion PCR (primers listed in Table 2). Each DNA fragment was run on an 0.8% agarose gel, and the bands were excised and cleaned with the Qiagen QIAquick gel extraction kit (Maryland). Equal amounts of each fragment were used in the fusion PCR. After fusion PCR the DNA was run on a gel, excised to ensure that a single band was obtained, and gel purified with the Qiagen QIAquick gel extraction kit (Maryland). The cleaned DNA was used to transform *A. nidulans* ∆nkuA strains by protoplasting. The resulting transformants were checked by PCR, which utilized primers that flanked the entire fusion PCR product (Table 2). The colonies that were positive for the fusion cassette were analyzed by Southern blotting to confirm that only a single copy of the cassette was incorporated by homologous recombination at the desired location.

Cassettes for GFP fusions were created in the S. Osmani lab and obtained through the Fungal Genetics Stock Center (http://www.fgsc.net/) (44). GFP strains were constructed by amplifying the target gene (*aspA* or *aspC*) excluding the stop codon (A850 template), 2 kb downstream of the target gene (A850 template), and the GFP *AfpyrG* cassette (pFNO3 template) with the addition of approximately 30 bp before and after the stop codon added to the 5' and 3' ends to facilitate the fusion PCR (primers listed in Table 2). Each PCR product was run on an 0.8% agarose gel, and the band was excised and cleaned with the Qiagen QIAquick gel extraction kit (Maryland). Equal amounts of each fragment were used for the fusion PCR. After fusion PCR the DNA was run on a gel, excised to ensure that a single band was obtained, and gel purified with the Qiagen QIAquick gel extraction kit (Maryland). *A. nidulans* ∆nkuA strains were then transformed with the cleaned DNA by the protoplasting method. The resulting transformants were checked by PCR, which utilized primers that flanked the entire fusion PCR product (Table 2). The colonies that were positive for the fusion cassette were analyzed by Southern blotting to confirm that only a single copy of the cassette was incorporated by homologous recombination at the desired location. At least four independent transformants were characterized.

**RESULTS**

In ΔaspA, ΔaspC, and ΔaspA ΔaspC strains, germ tubes emerge early, germ tube and branch emergence increases, and septation and conidiation are reduced. In the filamentous fungus *A. nidulans* development begins when uninucleate asexual
Conidiospores break dormancy in the presence of a carbon source. Soon after conidia break dormancy, nuclear division begins and a single primary germ tube emerges and grows by tip extension (11, 13, 27). After germlings pass a critical size threshold, nuclear division triggers septum formation (41). This normally occurs when germlings contain 8 to 16 nuclei. At about the same time a secondary germ tube emerges from the hypha (21). To determine if the septins AspA and AspC play roles in A. nidulans development, we compared wild-type strains to strains in which individual septin genes were deleted (\(\Delta aspA\), ASH5, and \(\Delta aspC\), ARL161) or in which both septins were deleted (\(\Delta aspA\ \Delta aspC\), ARL162). After 5 h at 30°C, no germ tubes were yet visible in the wild type, while 52 to 58% of deletion strain cells had germ tubes (\(n = 200\); Fig. 1 and 2). When incubated for 8 h, 92% of wild-type cells had one germ tube, while 75 to 82% of single deletion mutants and 95% of double deletion mutants had two or more germ tubes (Fig. 1 and 2). If two germ tubes formed in the wild-type strain, they were usually separated by 180°. In the septin deletion mutants multiple germ tubes were adjacent to each other or separated by 45°, 90°, or 180° (data not shown). Branch emergence was assayed by counting the number of hyphae that had formed at least one branch. When cells were incubated for 14 h, branches emerged in 7% of wild-type cells, 58% of \(\Delta aspA\) cells, 56% of \(\Delta aspC\) cells, and 82% of the double deletion mutants (\(n = 200\); Fig. 1 and 2). In addition to branching early, the mutants also showed more branches or branch initials relative to wild type.

Because septin deletion mutants appeared to develop more rapidly than wild type and because nuclear division is known to trigger septation (41), we scored septation based on nuclear number rather than time of incubation. After 11 h of incubation, nuclei and septa were labeled and only hyphae with 16 nuclei were scored. All wild-type cells, 12% of \(\Delta aspA\) cells, and 3% of \(\Delta aspC\) cells with 16 nuclei had septa (data not shown). To determine whether septation in the septin deletion mutants was reduced or simply delayed, we examined cells after 15 h of incubation, a time when all hyphae had >32 nuclei. All wild-type cells and 95% of \(\Delta aspA\) cells had at least one septum. In contrast, only 28% of \(\Delta aspC\) and 38% of \(\Delta aspA\ \Delta aspC\) cells had at least one septum (\(n = 200\); Fig. 2). The intensity of septum staining with calcofluor was reduced and difficult to see and photograph in \(\Delta aspC\) and \(\Delta aspA\ \Delta aspC\) cells compared to wild-type and \(\Delta aspA\) cells (data not shown). In plate assays of growth, we saw no obvious defect of septin deletion mutants with addition of the cell wall-perturbing agent calcofluor and no obvious difference in radial growth rate at restrictive temperature (data not shown).

During asexual reproduction (conidiation), an aerial hypha emerges from the main hypha and swells at its tip to form a vesicle. From the vesicle two ordered layers of cells emerge sequentially (metulae and phialides), ultimately giving rise to chains of conidiospores.

We compared conidiophores (asexual structures) of wild type to septin-deleted strains (Fig. 3). Wild type had regular layers and a chain of conidiospores (Fig. 3A). \(\Delta aspA\), \(\Delta aspC\), and \(\Delta aspA\ \Delta aspC\) mutants had irregular and fused layers in the conidiophore and produced fewer spores (Fig. 3B to H).
possible, fusions were integrated at the *aspA* or *aspC* locus behind the native *asp* promoter, replacing the wild-type gene. To determine whether the GFP tag might interfere with septin function, we compared the phenotypes of Asp-GFP fusion strains (AspA-GFP, ARL141, and AspC-GFP, ARL161) with those of wild-type and septin-deleted strains. Germ tube and branch emergence, septation, and conidiation were all wild type in the septin-GFP strains, indicating that the GFP tag does not interfere with function (Fig. 2 and data not shown).

AspA-GFP was visible as spots or short rods in dormant conidia. There was generally a single very bright spot either alone or with dimmer spots (Fig. 4A). As the conidium expanded, AspA-GFP localization became more punctate and cortical (Fig. 4A and B), and as polarization occurred, AspA-GFP was found at the base and growing tip of the germ tube (Fig. 4B). In hyphae, AspA-GFP showed cytoplasmic localization that was often punctate at the cortex and brighter at hyphal tips and emerging branches (Fig. 4C, D, E, and G). AspA-GFP was also visible as a ring or cap at forming septa and emerging branches (Fig. 4E and F). In conidiophores, AspA-GFP localized transiently to each individual layer as it emerged, persistently to the phialide-conidiospore interface at the base of the forming chain of spores and generally as a single bright spot in each conidium, either alone or with dimmer spots (Fig. 5). Localization of AspA-GFP was also examined in a heterozygous diploid strain containing one copy of *aspA-GFP* and one of native *aspA* (ARL205). Localization of AspA-GFP in the heterozygous diploid strains was identical to localization in the haploid strain (data not shown). Localization of AspC-GFP was virtually indistinguishable from AspA-GFP at all stages examined in both haploid and heterozygous diploid strains (data not shown).

AspA localizes abnormally in ΔaspC cells, and AspC fails to localize in ΔaspA cells. In most cases where septins have been studied, different septins interact to form heteropolymers. To investigate whether AspA and AspC require each other for localization, we made strains in which one septin was fused to GFP and the other septin was deleted. From crosses of AspC-GFP and ΔaspA strains, we examined four progeny that contained both the deletion and the GFP fusion based on PCR analysis (ARL182 to ARL185). In all cases we no longer observed specific localization of AspC in the ΔaspA background in vegetative or asexual growth (Fig. 6A). From crosses of AspA-GFP and ΔaspC strains, we examined two progeny that contained both the deletion and the GFP fusion based on PCR analysis (ARL198 and ARL201). AspA-GFP in ΔaspC cells localized as a tiny bright dot in conidia (Fig. 6B). AspA-GFP
continued to localize as a single very bright dot or a short bar at different locations during the remaining developmental stages (Fig. 6B), except in the conidiophore where multiple dots were seen (Fig. 6B).

**DISCUSSION**

AspA and AspC interact genetically and have similar functions in germ tube and branch emergence, septation, and conidiation. Our finding that \(/H9004\) aspA, \(/H9004\) aspC, and \(/H9004\) aspA/\(H9004\) aspC strains are viable is in sharp contrast to findings in *S. cerevisiae*, where the orthologous genes, \(CDC11\) (aspA ortholog) and \(CDC12\) (aspC ortholog), are essential (7). Phe-notypes of \(/H9004\) aspA and \(/H9004\) aspC strains were almost identical in *A. nidulans*, with both strains showing early and increased germ tube emergence, increased branch emergence, delayed or reduced septation, and disorganized conidiophores. The only phenotype that differed between the deletion strains was seen after 15 h of incubation, at which time \(/H9004\) aspA cells made normal levels of septa after an initial delay, while \(/H9004\) aspC cells showed a reduction in the total number of septa.

The very similar phenotypes in \(/H9004\) aspA and \(/H9004\) aspC strains suggest that AspA and AspC play very similar roles in germ tube and branch emergence, septation, and conidiation. The identical localization patterns of AspA-GFP and AspC-GFP are consistent with the view that these septins have similar functions. However, the increased severity of all phenotypes in the \(\Delta aspA\) \(\Delta aspC\) strains suggests that the \(Cdc11\) ortholog \(AspA\) might be the central septin in the *A. nidulans* septin complex and/or that AspA might be needed for the retention of AspC in complexes.

AspA and AspC localize as spots, rings, collars, and fila-
ments. Consistent with the idea that AspA and AspC play similar roles, AspA and AspC show virtually identical localization patterns. Typically, fungal septins localize as rings or collars through which new growth emerges, cross walls delineating compartments, or caps at hyphal tips (22). AspA and AspC show these typical fungal localization patterns, forming rings or collars through which germ tubes, branches, and conidiophore layers emerge; septa delineating hyphal compartments; and diffuse caps at tips of actively growing hyphae (Fig. 4 and 5). In addition to these typical fungal localization patterns, AspA and AspC localize as dots or short bars, puncta, or elongated filaments, patterns previously described mainly for animal septins (Fig. 4) (22).

AspA and AspC are involved in, but not required for, septa-
tion. While *S. cerevisiae* strains with mutations in septin core complex members make no septa and cannot complete cytokinesis (16), *Schizosaccharomyces pombe* septin mutants form septa and divide, though the process is delayed in some mutants (24, 34). Thus, it was not too surprising that *A. nidulans* \(\Delta aspA\), \(\Delta aspC\), and \(\Delta aspA\ \Delta aspC\) strains made at least some septa. After an initial delay, \(\Delta aspA\) strains made near-wild-type

![FIG. 4. AspA localizes to dormant conidia, emerging germ tubes, emerging branches, and septa. The AspA-GFP strain was incubated, and live cells were photographed using fluorescence microscopy. Panels show composites of multiple cells for each developmental stage. (A) Dormant conidia; (B) newly emerging germ tubes; (C) germlings; (D) hyphae; (E) septating hyphae; (F) branching hyphae. Scale bar, 5 μm.](http://ec.asm.org/)
levels of apparently normal septa. This is very close to the situation in *S. pombe*, where a mutant lacking Spn3, the AspA ortholog, shows normal cytokinesis (1). In contrast, ΔaspC strains made only one-third of the number of septa that the wild type did and these septa appeared to be abnormal based on staining with the chitin-binding dye calcofluor, suggesting that AspC has a unique role in septation and that this role cannot be filled by other septins. This is somewhat similar to the case in *S. pombe*, where a mutant in Spn4, the AspC ortholog, shows delayed cytokinesis, a more severe effect than loss of Spn3 (1). These differences in septin defects are consistent with recent literature showing that though different septins interact for proper function, septin dynamics within a complex can vary (5).

**AspA and AspC influence the number of new growth foci and their patterns.** During vegetative growth ΔaspA, ΔaspC, and ΔaspA ΔaspC mutants showed increased emergence of new growth foci, making extra germ tubes and branches (Fig. 1). In addition to having too many germ tubes, their spatial pattern is disrupted in ΔaspA, ΔaspC, and ΔaspA ΔaspC mutants. In wild-type *A. nidulans*, a single germ tube emerges from the conidium and is generally followed later by emergence of a second germ tube 180° relative to the first (27). In contrast, second germ tubes in the ΔaspA, ΔaspC, and ΔaspA ΔaspC mutants emerge axial or at 45° or 90° relative to the first germ tube, indicating a disruption of spatial pattern. In wild-type hyphae generally a single branch emerges from each compartment delineated by septa (21). Thus, we cannot predict the normal position for a second branch within a compartment and so cannot determine whether the multiple, closely spaced branches that emerge from compartments of ΔaspA, ΔaspC, and ΔaspA ΔaspC mutants indicate a disruption of spatial pattern or simply an increase in the number of new growth foci.

During asexual reproduction in *A. nidulans*, a regular, organized, multilayered conidiophore that bears chains of spores (conidia) is made by a process that closely resembles budding in yeast (36). During conidiation in ΔaspA, ΔaspC, and ΔaspA ΔaspC mutants, the multiple layers of the conidiophore are disorganized and the number of conidia is reduced (Fig. 3). This disorganization appears to affect different layers of the multilayered structure in individual conidiophores within a population. In some conidiophores the deletion phenotype appears to be a patterning defect, and in others it appears to be a cell division or separation defect.

In *S. cerevisiae*, septin mutants show disruption of normal axial and bipolar budding patterns (24, 26), and so the disruption of germ tube emergence patterns in ΔaspA, ΔaspC, and ΔaspA ΔaspC mutants was not too surprising. However, we were very surprised to see the increased germ tube and branch emergence in the *A. nidulans* septin mutants. Though *S. cerevisiae* septin mutants form cells with multiple buds, this multibud phenotype is thought to result from failure to complete cytokinesis, which normally separates buds from the mother cell, not from simultaneous emergence of new buds (25). During vegetative growth of filamentous fungi like *A. nidulans*, there is no separation of germ tubes and branches from the hypha comparable to the separation of buds from the mother cell at cytokinesis in yeast. Cytokinesis in filamentous fungi results in partitioning of hyphal compartments by septa, a process which still takes place in the ΔaspA, ΔaspC, and ΔaspA ΔaspC mutants, although it is delayed or reduced. This is, to our knowledge, the first report of an increase in the number of new growth foci associated with loss of septin function in any or-
ganism and raises the intriguing possibility that septins might limit new growth foci in other multicellular organisms.

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