

PATRICK JOHN WESTFALL

The *Aspergillus nidulans* Septin AspB Plays Important Roles in Septum, Branch, and Conidiophore development.

(Under the Direction of DR. MICHELLE MOMANY)

The septin family of proteins acts as an organizational scaffold in areas of cell division and new growth. Originally discovered in *Saccharomyces cerevisiae*, septins have also been characterized in numerous other organisms such as fruit flies, mice, and humans. In *S. cerevisiae*, septins were first described as a series of 10nm filamentous rings found at the neck of a budding cell. Septins play a role in such processes as cytokinesis, bud site selection, polarity establishment, and spore formation.

Five septin homologues have been found in the filamentous fungus, *Aspergillus nidulans*. This dissertation focuses on the characterization of one of these, AspB. Null alleles of *aspB* are lethal. Localization studies using polyclonal antibodies show that AspB localizes to the site of cytokinesis in a distinctive, polar pattern as the septum develops. AspB also marks the site of branch emergence and localizes to specific interfaces as the asexual reproductive structure forms. In order to further understand the role of AspB in *A. nidulans* development, conditional mutants were generated and, based on phenotypic studies, support a role for AspB in septum formation, branch initiation, and asexual development.

INDEX WORDS: Septin, AspB, Cytokinesis, Branch development, Conidiophore development

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SEPTUM, BRANCH, AND CONIDIOPHORE DEVELOPMENT.

by

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B.S., Purdue University, 1996

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial
Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2001

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DEDICATION

This dissertation is dedicated to my wife, Jacquie W. Hilterman. Without her love and support over these last several years none of this would have been possible. I would also like to thank my parents for instilling in me at a young age a love for science and for always taking the time to answer me when I asked “why?”.

ACKNOWLEDGEMENTS

As with most things of this nature, there is never enough room to thank all those who helped me over the last 5 years. Nonetheless, I would like to say a particular thanks to my major professor, Dr. Michelle Momany, who was always willing to take the time to help me work through problems and whose infectious enthusiasm was a joy to be around. I would also like to thank my various lab mates: Gretel Guest, who was my own personal fashion consultant (who knew that plaid didn't go with stripes?) and a great friend since the beginning. Rebecca Lindsey, who kept the lab organized and made sure that I didn't monopolize too much bench space; Dr. Brian Shaw, who always had the coffee ready in the morning and made the time to drink it with me and provide insight to my scientific dilemma of the day; Xiaorong Lin, who taught me the importance of a kick-feather break and had an amazing talent to help me look at a problem from a different angle; Beth Richardson, who had the patience/tenacity to give me crash lessons in SEM or TEM when my project dictated that particular experiment. Finally, I would like to thank my graduate committee for keeping me focused and for valuable insight into my work.

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CHAPTER 1

INTRODUCTION

Septins in S. cerevisiae

Dividing cells are faced with two basic problems. They must (1) define the site of division and target the appropriate components of cytokinesis there and (2) make sure that the cellular contents are appropriately distributed. To solve these problems a complex of cellular machinery has developed that organizes the division plane. This organizational scaffold can be used to recruit specific biochemical signals that coordinate cellular activity at the site of division. Important members of this organizational complex are the septin family of proteins.

Septins have been found in all eukaryotes so far examined with the exception of plants and slime molds. They are a conserved family of proteins showing about 26% amino acid identity overall with two common motifs, a GTP-binding P-loop and a coiled-coil domain thought to be important for protein-protein interactions (Longtine *et al.*, 1996). Septins were originally discovered in the budding yeast, *Saccharomyces cerevisiae*, where they are thought to be components of a set of 10 nm filamentous rings seen just inside the plasma membrane at the mother-bud neck (Byers and Goetsch, 1976; Longtine *et al.*, 1996). Four septins were originally identified in a screen of temperature sensitive Cell Division Cycle mutants (the CDC3, CDC10, CDC11, and CDC12 gene products). When grown at non-permissive temperature, cells with temperature-sensitive mutant alleles of these genes formed elongated, multinucleate chains of cells that failed to

undergo cytokinesis (Hartwell, 1971). In addition, these cells showed mitotic delays (Carroll *et al.*, 1998; Barral *et al.*, 1999; Longtine *et al.*, 2000). More recently three other septin genes have been identified. Sep7p, like Cdc3p, Cdc10p, Cdc11p, and Cdc12p, localizes to the neck region but, unlike the other septins found at this junction, is not vital for the formation of the neck filaments (Carroll *et al.*, 1998; Mino *et al.*, 1998). The last two septins, Spr3p and Spr28p play a role in spore development (De Virgilio *et al.*, 1996; Fares *et al.*, 1996). A number of proteins have been identified that localize to the neck region in a septin-dependent manner (Table 1.1).

Table 1.1. Partial list of septin-dependent proteins in *S. cerevisiae*

Protein	Function	Reference
Cdc3p; Cdc10p; Cdc11p; Cdc12p; Sep7p	Septin protein (Neck localized)	(Longtine <i>et al.</i> , 1996) (Carroll <i>et al.</i> , 1998)
Spr3p; Spr28p	Septin protein (Sporulation specific)	(Ozsarac <i>et al.</i> , 1995) (De Virgilio <i>et al.</i> , 1996)
Gin4p; Cla4p; Nap1p	Septin organization	(Longtine <i>et al.</i> , 1998) (Longtine <i>et al.</i> , 2000)
Bud3p; Bud4p; Axl2p	Bud site selection	(Chant <i>et al.</i> , 1995) (Sanders and Herskowitz, 1996) (Roemer <i>et al.</i> , 1996)
Smt3p	Ubiquitin-like protein	(Johnson and Blobel, 1999)
Bni4p; Chs4p	Chitin ring synthesis	(DeMarini <i>et al.</i> , 1997)
Myo1p	Cytokinesis	(Bi <i>et al.</i> , 1998) (Epp and Chant, 1997) (Lippincott and Li, 1998)
Hsl1p; Hsl7p; Swe1p	Cell cycle control	(Longtine <i>et al.</i> , 2000) (Barral <i>et al.</i> , 1999) (Shulewitz <i>et al.</i> , 1999)
Kcc4p	Unknown	(Barral <i>et al.</i> , 1999)

Septin Organization at the Mother-Bud Neck

How the septins are organized at the bud neck has not yet been clearly defined, although several neck-localized proteins play a role in septin arrangement. Two models exist to explain how the septins assemble at the neck. The original model, proposed by Byers and Goetsch in 1976, was based upon their transmission electron microscopy observations of the parallel filaments found at the neck region perpendicular to the mother-bud axis. They proposed that these filaments were in fact helical and circumscribed the neck region (a in Figure 1.1) (Byers and Goetsch, 1976). However, more recently a protein kinase, Gin4p, was identified that localized to the neck region in a septin dependent manner. Knockouts of Gin4p resulted in aberrant septin localization in which the characteristic ring structure was lost and the septin localization now appeared as a series of thick bars that ran parallel to the mother-bud axis (Longtine *et al.*, 1998; Gladfelter *et al.*, 2001). This led to an alternative model in which the septins at the neck form a series of parallel filaments, with Gin4p and other proteins acting as lateral spacers between the septin filaments (b in Figure 1.1) (Longtine *et al.*, 2000; Gladfelter *et al.*, 2001).

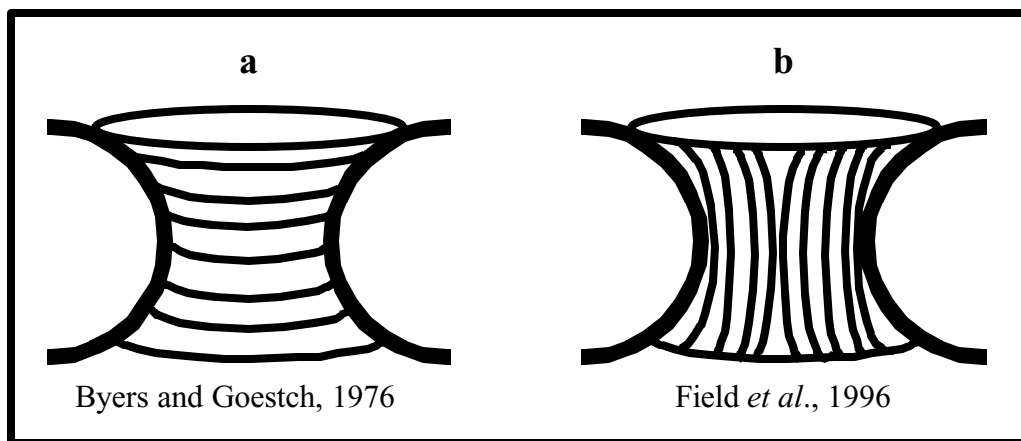


Figure 1.1 Two possible models for septin assembly in yeast.

Septin's Role in Organizing the Bud Site

Cdc3p, Cdc10p, Cdc11p, Cdc12p, and Sep7p localize to the site of bud emergence very early in the cell cycle during late G1 about 15 minutes before the bud is visible. As the bud emerges the septins stay localized to the neck region but form an hourglass like structure and that splits into two separate rings as cytokinesis takes place (Haarer and Pringle, 1987; Ford and Pringle, 1991; Kim *et al.*, 1991; Carroll *et al.*, 1998; Mino *et al.*, 1998; Cid *et al.*, 2001). At the same time that the septin ring is assembling at the presumptive bud site, a cortical actin patch localizes to the site of bud emergence. Localization of the septin ring and actin patch are independent of each other, but both depend on the Rho-related GTPase Cdc42p (Pringle *et al.*, 1995; Cid *et al.*, 2001). Cdc42p sets up the axis of formation, and in its absence cells are unable to properly polarize. Furthermore, proper Cdc42p localization is dependent on a set of already established cues, left over from the previous round of cell division (Chant, 1996). In the case of haploid cells, Bud3p, Bud4p, and Axl2p are responsible for the proper localization and activation of Cdc42 (Chant and Herskowitz, 1991; Halme *et al.*, 1996; Roemer *et al.*, 1996; Sanders and Herskowitz, 1996). All three of these proteins are localized in a septin dependent manner during the previous cell cycle. The septin ring from the previous cell cycle disassembles soon after the completion of cytokinesis. This breakdown of the septin ring is due, in part, to covalent modification by the ubiquitin-related protein, Smt3p (Johnson and Blobel, 1999).

Septins and Cytokinesis

When the bud initially emerges from the cell, it undergoes a period of apically oriented growth, with the septin ring staying localized to the neck region, and the cortical

actin patch localized to the tip of the bud. As the nucleus starts to divide, the bud switches from apical to isotropic growth and actin is relocated back to the neck region. After nuclear division is complete, cytokinesis takes place by the contraction of an actomyosin ring (Bi *et al.*, 1998; Lippincott and Li, 1998). The actomyosin ring consists of actin, type II myosin, Myo1p, and Iqg1p/Cyk1p a member of the IQGAP family of proteins (Epp and Chant, 1997; Lippincott and Li, 1998). Myo1p has been shown to co-localize with the septin ring and its proper assembly is septin dependent. Although they co-localize to the neck region, septin-dependent localization of Iqg1p/Cyk1p has not been shown. It is speculated that Iqg1p/Cyk1p may be necessary for the reassembly of actin at the neck (Lippincott and Li, 1998).

As cytokinesis takes place the newly formed crosswall, consisting of cell wall materials such as the β 1-4 glucan, chitin, begins to form. The chitin deposited in a ring at the bud neck is placed by a hierarchy of proteins that are dependent on septins for their proper localization. Chitin synthase III (ChsIII) is responsible for producing the chitin ring found at the bud neck. The ChsIII complex is activated by Chs4p, which in turn can associate with Bni4p. Two-hybrid interactions show that not only does Bni4p associate with Chs4p, but it is also capable of interacting with one of the septins, Cdc10p. The current model is that the septins at the bud neck, acting through Bni4p, can localize Chs4p, which in turn can locally activate ChsIII (DeMarini *et al.*, 1997).

Polar Structure of Septin Organization

When bud formation is disrupted in *S. cerevisiae*, a mitotic delay occurs. This delay is, in part, because the cell-cycle-inhibitory kinase Swe1p is no longer degraded (Sia *et al.*, 1998). Two regulatory proteins, Hsl1p and Hsl7p, are responsible for the

proper targeting of Swe1p for degradation and both of these regulators are localized, in a septin-dependent manner to the bud side of the neck. This localization occurs after the septin rings have formed the hourglass-like shape (McMillan *et al.*, 1998). Other proteins have also been shown to localize to the septin ring in specific domains. Like Hsl1p and Hsl7p, Kcc4p localizes exclusively to the bud side of the neck (Barral *et al.*, 1999). In contrast, Myo1p is localized exclusively in the center of the neck (Bi *et al.*, 1998). Still other proteins such as Gin4p are found co-localized with the septins throughout the neck region (Longtine *et al.*, 1998). These very specific localization patterns imply that there is an underlying polarity to the septin structure. One possible explanation is that, as the neck region matures, the septins set up a diffusion barrier, thus only allowing certain proteins access to the mother or bud side exclusively. The concept of the septins as a diffusion barrier was shown using a transmembrane protein called Ist2p, which localizes exclusively to the bud. When septin assembly was perturbed, Ist2p was found in both the mother and bud (Takizawa *et al.*, 2000). Perhaps in a similar manner, proteins such as Hsl1p or Hsl7p only have access to the bud side of the septin ring, and thus set up the apparent polarity of the structure.

Septins in Other Eukaryotes

Septins appear to be ubiquitous throughout the fungal and animal kingdoms, where their unifying characteristic seems to be their role in cytokinesis (Longtine *et al.*, 1996; Field and Kellogg, 1999; Momany *et al.*, 2001). The *Drosophila* *PNUT*, *SEP1*, *SEP2*, *SEP4*, and *SEP5* genes all encode septin homologues (Neufeld and Rubin, 1994; Fares *et al.*, 1995). Pnut, Sep1, and Sep2 localize to the cleavage furrow and intercellular bridges of cultured cells. These three *Drosophila* septins have a role in the development

of the syncytial blastoderm, which partitions approximately 6,000 nuclei into individual cells.

In mammals, septins localize not only to the cleavage furrow of dividing cells, but also to non-dividing brain cells (Trimble, 1999; Kartmann and Roth, 2001). A major difference between septins in mammals and those in fungi appears to be the identification of their interacting partners. In yeast, the septin binding partners are either bud site selection genes, involved in cytokinesis, part of a kinase cascade, or involved in ubiquitin-like modification. In contrast, all of the septin interacting partners identified so far in mammals appear to be part of the exocyst apparatus (Hsu *et al.*, 1998; Kartmann and Roth, 2001). (Hsu *et al.*, 1998). In rat brains, the exocyst complex plays an important role in the generation of synapses by collecting or targeting vesicles to sites of active growth (Hsu *et al.*, 1998; Hazuka *et al.*, 1999). Five septins from rat, KIAA0128, hCDC10, Nedd5, H5, and E-Septin were identified by co-immunoprecipitation with a component of the rat exocyst complex, Sec8. The apparent interactions of septins with the exocyst complex suggest that the septins may be playing a role in tethering vesicles to specific regions of the plasma membrane that have been targeted by the exocyst complex. As these regions become stable, septin localization is lost (Hsu *et al.*, 1998). A second example where it appears that septins may play a role in vesicle fusion is with the CDCRE-1 and Nedd5 septins that interact with a SNARE protein called syntaxin (Beites *et al.*, 1999; Kartmann and Roth, 2001). SNARE proteins have been shown to be vital for the fusion of vesicles with the plasma membrane (Sollner *et al.*, 1993). These two examples of septins playing roles in vesicle fusion could also explain their importance in

cytokinesis since new membrane (and wall material in the case of fungi) must be targeted and transported to the site of division.

Septins in Aspergillus nidulans

Aspergillus nidulans is a unique system for the study of septins because of distinct differences in the organization and regulation of cell division in filamentous fungi versus budding yeast. In yeast, each mitotic division is followed immediately by cytokinesis. In contrast, *A. nidulans* undergoes a brief period of isotropic growth, establishes polarity, and develops a germ tube that elongates by apical extension (Figure 1.1). The nucleus, meanwhile, undergoes several mitotic divisions and new nuclei move out into the germ tube. Cytokinesis takes place at the third nuclear division by placement of a crosswall, called a septum, at the basal end of the germ tube (Harris *et al.*, 1997). The mature septum in *A. nidulans* consists of a central layer of chitin flanked by layers of secondary wall material (Momany and Hamer, 1997). After subsequent mitotic divisions, septa partition the tubular hypha into regular, multinucleate compartments. Only the tip cell remains mitotically active. Basal compartments are arrested in interphase, unless the compartment forms a branch, in which case that compartment again initiates mitosis (Fiddy and Trinci, 1976; Kaminskyj and Hamer, 1998). Nearby nuclei influence the position of the septum and a mitotic event is needed for septum formation (Wolkow *et al.*, 1996). In mutants that are unable to form septa, mitosis is not affected (Harris *et al.*, 1994). Because cytokinesis in *A. nidulans* does not follow every mitosis and does not result in separate daughter cells, it is possible to distinguish the roles septins play in the organization of new growth versus partitioning.

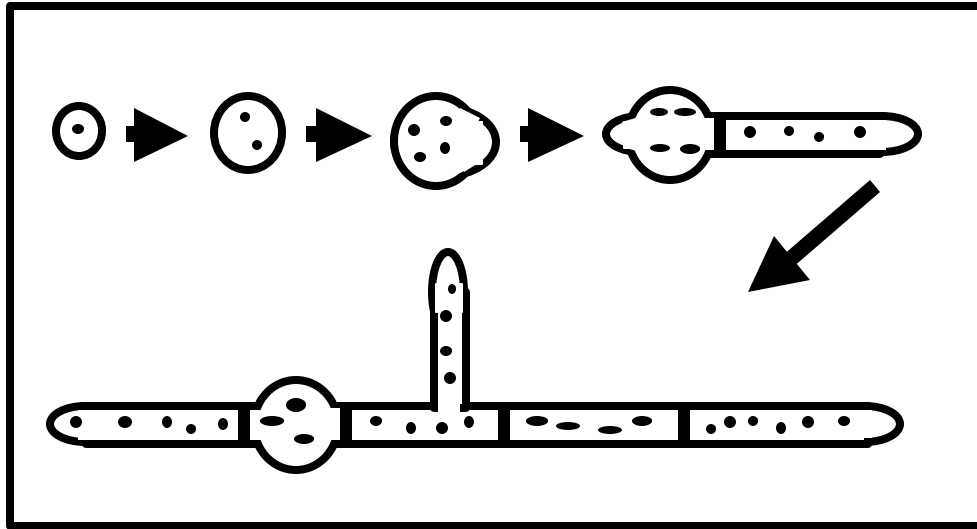


Figure 1.2 *Aspergillus nidulans* development

Five septins have been identified in *A. nidulans* and called *aspA-E* (for *Aspergillus nidulans* septin). Three of these (*aspA-C*) were identified through a PCR screen using degenerate primers (Momany and Hamer, 1996), and two others (*aspD* and *aspE*) were found in a search of the Cereon genome database (Momany *et al.*, 2001). This dissertation focuses on characterization of one of these *A. nidulans* septins, *aspB*. Through a series of immunofluorescent localization experiments with polyclonal anti-AspB antibodies and by the generation of *aspB* mutants, AspB has been shown to play an important role in cytokinesis, branch initiation, and asexual development.

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CHAPTER 2

THE *ASPERGILLUS NIDULANS* SEPTIN ASPB PLAYS PRE- AND POST-MITOTIC ROLES IN SEPTUM, BRANCH, AND CONIDIOPHORE DEVELOPMENT¹

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ABSTRACT

Members of the septin family of proteins act as organizational scaffolds in areas of cell division and new growth in a variety of organisms. Here we show that in the filamentous fungus *Aspergillus nidulans*, the septin AspB is important for cellular division, branching, and conidiation both pre- and post-mitotically. AspB localizes postmitotically to the septation site with an underlying polarity that is evident as cytokinesis progresses. This localization at the septation site is dependent on actin and occurs before the crosswall is visible. AspB localizes premitotically as a ring at sites of branching and secondary germ tube emergence. It is the only known branch site marker. In addition, AspB is found at several stages during the development of the asexual reproductive structure, the conidiophore. It localizes transiently to the vesicle/metula and metula/phialide interfaces, and persistently to the phialide/conidiospore interface. A temperature-sensitive mutant of AspB shows phenotypic abnormalities including irregular septa, high numbers of branches, and immature asexual reproductive structures.

INTRODUCTION

Actively growing cells are faced with two related problems. They must target new material to the appropriate site(s), and they must partition new material such that the resulting cells receive the full complement of cytoplasmic and genetic constituents. Both the targeting and partitioning problems can be solved by establishing organizational cues that can direct vesicle fusion or tether signals that coordinate specific biochemical reactions. Walled organisms, such as fungi and plants, have the added complication of breaking down portions of the existing rigid wall so that new growth can proceed.

Septins, a conserved family of proteins, act as organizational cues, recruiting critical proteins to areas of new growth and cell division (Longtine *et al.*, 1996; Field and Kellogg, 1999; Gladfelter *et al.*, 2001). Septins (the *CDC3*, *CDC10*, *CDC11*, and *CDC12* gene products) were originally identified as components of the 10 nm filament ring found just inside the plasma membrane at the mother-bud neck in the yeast *Saccharomyces cerevisiae* (Byers and Goetsch, 1976; Longtine *et al.*, 1996). Temperature-sensitive *cdc3*, *cdc10*, *cdc11*, and *cdc12* mutants show delays in mitosis (Barral *et al.*, 1999; Carroll *et al.*, 1998; Longtine *et al.*, 2000) and fail to undergo cytokinesis (Hartwell, 1971), resulting in elongated, multibudded, multinucleate chains of cells. Three other septin genes, *SPR3*, *SPR28*, and *SEP7*, have also been identified in *S. cerevisiae*. Spr3p and Spr28p play roles in spore development (Fares *et al.*, 1996; DeVirgilio *et al.*, 1996), whereas Sep7p is expressed in vegetative cells and localizes to the mother-bud neck (Carroll *et al.*, 1998; Mino *et al.*, 1998). Members of the septin family have two shared motifs, a GTP-binding P-loop and, with a few exceptions, a coiled-coiled domain that may function in protein-protein interactions.

Septins play several developmental roles in *S. cerevisiae*. At the bud neck, they help in organizing the septal cell wall by acting as an anchor for Bni4p, which in turn is part of a complex including the chitin synthase responsible for the formation of the bud scar (DeMarini *et al.*, 1997). During spore formation, Spr3p, Spr28p, Cdc3p, and Cdc11p are localized to the leading edge of the prospore sac, where they might influence the deposition of chitosan (Fares *et al.*, 1996; DeVirgilio *et al.*, 1996). Septins also localize several proteins responsible for marking the areas of future bud emergence (Bud3p, Bud4p, and Axl2p), and loss of proper septin function results in a randomization

of the budding pattern (Flescher *et al.*, 1993; Chant *et al.*, 1995; Pringle *et al.*, 1995; Sanders and Herskowitz, 1996; Halme *et al.*, 1996; Roemer *et al.*, 1996). Proper septin organization may also help in coordinating morphogenesis and mitosis (Lew, 2000). Several cell cycle-regulating protein kinases localize to the septin ring at the mother-bud neck, and a mitotic delay occurs if the septins fail to organize properly (Carroll *et al.*, 1998; Barral *et al.*, 1999; Shulewitz *et al.*, 1999; Longtine *et al.*, 2000).

Because of distinct differences in the organization and regulation of cell division in budding yeast versus filamentous fungi, *Aspergillus nidulans* makes a valuable system for the study of septins. In yeast, each mitotic division is followed immediately by cytokinesis. In contrast, an *A. nidulans* spore undergoes a brief period of isotropic growth, establishes polarity, and develops a germ tube that elongates by tip growth. The nucleus, meanwhile, undergoes several mitotic divisions, and new nuclei move out into the germ tube. Cytokinesis takes place after the third nuclear division by placement of a crosswall, called a septum, at the basal end of the germ tube (reviewed by Harris *et al.*, 1997; Momany and Taylor, 2000). Subsequent septa are placed such that uniform compartments, each containing 3-4 nuclei, are formed. In *A. nidulans* only the tip compartment remains mitotically active. Basal compartments are arrested in interphase unless the compartment forms a branch, in which case mitosis is again initiated (Fiddy and Trinci, 1976; Kaminskyj and Hamer, 1998).

Five septin genes have been found in *A. nidulans* (Momany *et al.*, 2000). One, *aspB* (for *Aspergillus nidulans* septin B), has been shown to be essential (Momany and Hamer, 1997a). In the present study, we show that AspB plays a role in the formation of septa, branches, and asexual reproductive structures.

MATERIALS AND METHODS

Aspergillus Strains and Growth Methods

All strains used in this study are listed in Table 1. Defined minimal medium (Käfer, 1977) with appropriate supplements was used throughout this study. For septation, branch emergence, and secondary germ tube emergence studies, conidia from appropriate strains were grown for 12 h at 37°C. For parasynchronous wave studies, strains were grown for 16 h at 37°C. For AspB localization in *sep* mutants, conidia from appropriate strains were grown for 12 h at 42°C. For conidiophore localization studies, overnight liquid cultures of *A. nidulans* strain A850 were grown at 37°C with vigorous shaking. Cultures were collected on miracloth and placed on solid medium to induce conidiation (Miller *et al.*, 1992). Conidiophores were then harvested at 5.0, 5.5, 6.0, 6.5, 7.5, and 8.0 h by scraping in water. For studies in which actin polymerization was inhibited, conidia from appropriate cultures were grown for 10 h at 37°C, transferred to medium containing 1 µg/ml Cytochalasin A (Sigma Chem. Co., St. Louis, MO) and grown for 2 h.

Generation of Antibodies and Immunofluorescence

The full-length *aspB* cDNA was PCR amplified from plasmid *paspB* (Momany and Hamer, 1997a) and fused in-frame to the glutathione *S*-transferase tag sequence in the pGEX-KG vector (Guan and Dixon, 1991) to create plasmid pMM17. pMM17 was transformed into *E. coli* strain XL1-Blue, and the GST-AspB fusion protein was induced and affinity purified using standard protocols. After SDS-PAGE, a single band of the expected 49 kD size containing approximately 200 µg of the fusion protein was excised and injected into rabbits for immunization (Harlow and Lane, 1988). Two additional

boosts containing approximately 200 μ g of fusion protein were required in order to get sufficient antibody titers as judged by western blot analysis of total *A. nidulans* protein.

The 1.5-kb *aspB* ORF was PCR amplified and fused to a six histidine tag in the Novagen (Madison, WI) pET-28b vector to create plasmid pPW17. pPW17 was transformed into *E. coli* strain BL21(DE3) and induced with 0.15 mM IPTG for 4 h at 30°C. Supernatant from lysed cells was loaded directly to a 2-ml Ni-NTA column (Amersham-Pharmacia Biotech, Uppsala, Sweden), and 1 ml fractions were collected as per standard protocols. Fractions containing a single polypeptide of the appropriate size were electrophoresed on a 10% SDS-PAGE gel and blotted to nitrocellulose. Polyclonal antibodies raised against the GST-AspB fusion were affinity purified against the His-AspB fusion as previously described (Pringle *et al.*, 1989). To test for specificity, the purified antibodies were used to probe western blots containing 5 mg/lane total protein from *A. nidulans* strain A850. A goat anti-rabbit secondary antibody conjugated to alkaline phosphatase (Sigma) was used to visualize the primary antibody. A single band of the expected size (49 kD) was recognized after 2 rounds of affinity purification (d in Figure 1). No band was visible in lanes probed with pre-immune serum (our unpublished results).

Fixation and staining of cells were as described previously (Oakley and Osmani, 1993) with an additional 2-h blocking step with 5% dry non-fat milk in buffered phosphate solution (PBS) before the addition of primary antibody. Secondary goat anti-rabbit conjugated to FITC was obtained from Jackson Immunochemical (West Grove, PA). Actin localization was done using the C4 mouse anti-actin monoclonal antibody (ICN Biomedical, Aurora, OH) and a Texas Red-labeled sheep anti-mouse secondary

(Jackson Immunochemical). For AspB localization to the conidiophore, fresh scrapings from conidiating cultures were added directly to 1 ml fixer (Oakley and Osmani, 1993). After a 30-min incubation in fixer, conidiophores were harvested by gentle centrifugation (3,000 RPM), resuspended in 1 ml of a digestion solution containing 25 mg/ml Novozyme 234 (Sigma), 50% egg white, and 1.2 M sorbitol and incubated for 2 h with gentle rocking at 37°C. Subsequent steps were as described above except that all solutions were osmotically stabilized with 1.2 M sorbitol.

Generation of Conditional aspB Alleles

A 5-kb *EcoRI/PstI* genomic fragment was subcloned from plasmid pMM1 (Momany and Hamer, 1997) into pBC KS (Stratagene, La Jolla, CA) to create plasmid pPW20, containing *aspB* and a 1-kb upstream region. pPW20 was transformed into the *E. coli* mutator strain XL1-Red (Stratagene). Plasmid DNA was isolated, linearized with *EcoRI*, and co-transformed with a marker plasmid containing the *argB* gene into *A. nidulans* strain A850 using standard protocols (Yelton *et. al.*, 1984). Approximately 4000 Arg⁺ transformants were replica plated at 30° C and 42° C. Fifty-four transformants showing a stable phenotypic defect at 42° C were crossed with *A. nidulans* strain A773 and progeny were scored. Total DNA was isolated from 12 strains with 1:1 Ts⁺:Ts⁻ segregation and blotted to nylon membranes. A PCR product containing the *aspB* ORF was used to probe blots and identified one temperature-sensitive transformant with a single copy of *aspB*. This strain, APW55, was restored to wild type growth by complementation with the original pPW20 plasmid.

Image Acquisition and Adjustment

Microscopic observations were made with a Zeiss (Thornwood, NY) Axioplan microscope. Digital images of immunofluorescence localization were acquired with an Optronics Digital Imaging System (Goleta, CA). Files were imported into Adobe PhotoShop 5.5 for adjustments to brightness and contrast. All images were rotated such that the apical tip was to the right and the basal end was to the left. For parasynchronous wave studies, images of germlings stained with Calcofluor White and Hoechst were converted to grayscale, then back to RGB color. Highlights, midtones, and shadows were then adjusted to give a false red coloring to these images.

RESULTS

AspB Localizes to Forming Septa

Septum formation in *A. nidulans* proceeds by simultaneous actin-mediated invagination of the plasma membrane and deposition of the chitinous primary septum (Momany and Hamer, 1997b). Actin appears very early in septum formation, before the chitin ring appears. To determine if AspB protein is also present during septum formation, we labeled hyphae with affinity purified anti-AspB antibodies (Figure 1). Both single and double rings of AspB were present at forming septa (Figure 2).

To determine the order of AspB intermediates in septum formation, we double labeled hyphae with antibodies against AspB and F-actin. Based on the known order of actin intermediates during septation (Momany and Hamer, 1997b), we were able to determine the progression of AspB during septum development (Figure 2). (1) A single ring of actin appears marking the site of septation, before cell wall material is deposited

(a and e in Figure 2). A single ring of AspB co-localizes with this early actin ring (i and m in Figure 2). (2) The chitinous septum becomes visible and overlaps with the actin and AspB rings (b, f, j, and n in Figure 2). (3) The invaginating actin ring forms an hourglass-like structure (g in Figure 2). The AspB ring does not invaginate, but splits into a double ring flanking the septum (o in Figure 2). (4) The actin ring continues to invaginate, eventually contracting to a very small, bright dot. The basal AspB band disappears, leaving a single ring of AspB on the apical side of the septum (d, h, l and p in Figure 2). Mature septa have no actin or AspB rings associated with them (our unpublished results).

AspB Assembly at the Septum Requires Actin and the sepA, sepG, and sepH Proteins

To determine whether actin is necessary for AspB localization, we used cytochalasin A (CA) to depolymerize F-actin (Harris *et al.*, 1994). *A. nidulans* conidia were incubated for 10 h at 37°C, then shifted to medium containing 1 µg/ml CA for 2 h. In an untreated control grown for 12 h at 37°C, rings of AspB protein were present in 15% of cells (n=100). No AspB rings were present after CA treatment (n=200), suggesting that filamentous actin is required for proper localization of the AspB ring.

To further investigate its relationship to other molecules known to be important in cytokinesis, we localized AspB protein in a group of temperature-sensitive septation (*sep*) mutants that fail to form septa when grown at restrictive temperature (Harris *et al.*, 1994). A subset of these mutants, *sepA*, *sepD*, *sepG*, and *sepH*, form septa rapidly on shift from restrictive to permissive temperature. Epistasis analysis has shown that *sepA*, *sepD*, *sepG*, and *sepH* are involved in a linear pathway, with *sepH* and *sepA* acting earlier than *sepD* and *sepG* (Morrell, 1997).

Rings of AspB protein were not seen in *sepA*, *sepG*, or *sepH* at restrictive temperature (a-b; e-h in Figure 3). However, rings of AspB were seen in *sepD* strains grown at restrictive temperature (c-d in Figure 3). These rings were present in 6% of the population, and often multiple rings were present in the same germ tube. Thus, it appears that AspB assembles downstream of *sepA*, *sepG*, and *sepH*, but upstream of *sepD*.

AspB Localization to Septa is Postmitotic

In *S. cerevisiae*, septins appear in late G1 phase to mark the point of bud emergence, and persist throughout S, G2, and mitosis until cytokinesis takes place (Longtine *et al.*, 1996). To determine whether AspB assembly at the septum occurs during or after mitosis in *A. nidulans*, we examined the nuclear state near septin rings. In 150 germlings with AspB single or double rings, all nuclei were in interphase. In the 5% of the population with mitotic nuclei, no AspB rings were present; thus, it appears that AspB assembly does not take place during mitosis.

To determine if AspB ring assembly requires exit from mitosis, AspB was localized in *A. nidulans* strain SWJ189, which contains the temperature-sensitive *bimE7* allele (James *et al.*, 1995). At restrictive temperature, *bimE7* nuclei are blocked in the pre-anaphase stage of mitosis. SWJ189 was grown 10 h at permissive temperature, then shifted to fresh medium and grown at restrictive temperature. After 120 min at restrictive temperature, approximately 80% of the tip cells examined contained mitotic nuclei, but no AspB rings were visible (n=100) (a-c in Figure 4). Forty-five minutes after shifting back to permissive temperature, AspB rings were visible in 17% of the hyphae (n=100) (d-f in Figure 4). Thus exit from mitosis appears to be needed for AspB assembly.

Septation in *A. nidulans* Occurs in a Parasynchronous Wave

Apical cells, which contain up to 30 nuclei, undergo mitosis in a wave that rapidly progresses from the tip to the base of the compartment (Rosenberger and Kessel, 1967; Clutterbuck, 1970). Although it is clear that the wave of nuclear division is followed immediately by a wave of septation, there has been disagreement on the order of septum formation in tip cells. Based on the demonstration by Wolkow *et al.* (1996) that mitosis triggers septum formation, it seemed likely that the tip-to-base wave of mitosis in the apical cell would be followed by a tip to base wave of septum formation. However, earlier work by Clutterbuck suggested that although the most apical septum formed first, there was no apparent order for formation of those septa further back (Clutterbuck, 1970).

To determine the order of septum formation in tip cells, we examined AspB and chitin rings in 46 *A. nidulans* apical compartments undergoing multiple septation events. Ninety-five percent of tip compartments showed septum progression from most to least mature moving from apex to base (i.e. septa with single AspB rings to the apical side were closer to the cell tip than septa with double AspB rings, septa with double AspB rings were closer to the cell tip than septa with single AspB rings, etc.) (Figure 5). This is the pattern that would be expected if the tip to base wave of mitosis triggers a tip to base wave of septation.

AspB Localizes Premitotically to Branch Points

After compartmentalization by septation, basal hyphal cells enter a resting state with all nuclei arrested in interphase. The nuclear number in these basal compartments averages 3 to 4 nuclei. To become mitotically active, these basal compartments must

establish a new axis of polarity by the formation of branches. No molecular markers for branching have been identified previously.

We observed AspB rings in subapical compartments where branching was occurring (a-i in Figure 6). The AspB ring was visible before any change in the profile of the hypha and persisted until the emerging branch was at least 5 μm long. To determine if AspB localization to branch points occurs before or after mitosis, we counted nuclear numbers in subapical compartments with AspB label. In 46% of subapical compartments with AspB rings, an odd number of interphase nuclei was present (n=100) (e in Figure 6). Therefore, AspB ring assembly at branch points is premitotic. Because mitosis doubles the number of nuclei, if AspB ring assembly were postmitotic, all subapical compartments with AspB rings should have an even number of nuclei. AspB localization persists at the base of the branch as nearby nuclei enter mitosis (h in Figure 6).

AspB Localizes to Secondary, but not Primary, Germ Tubes

When *A. nidulans* conidia break dormancy, they first grow isotropically, maintaining a spherical shape. By the second nuclear division, the young cells establish an axis of polarity and add new cell surface material only at the tip of the emerging germ tube. As early as the third nuclear division, some germlings send out a second germ tube from the conidium, generally 180° from the first (Momany and Taylor, 2000). Cells at the beginning stages of polarity establishment did not show AspB localization at the base of the emerging primary germ tube (n=150) (j-l in Figure 6). However, a ring structure similar to that seen at branch points was visible at sites of secondary germ tube emergence (m-o in Figure 6).

AspB Localizes to Conidiophores

In *A. nidulans*, the asexual spores are made by a specialized structure known as the conidiophore (reviewed by Adams *et al.*, 1998). Conidiophore development begins with the partitioning of a thick-walled hyphal cell, called a foot cell, that extends to form an aerial hypha. The tip of the aerial hypha swells to produce a knob-like vesicle. A layer of specialized cells, the metulae, bud from the surface of the vesicle. In turn, conidiogenous cells, the phialides, bud from the tips of the metulae. Phialides produce chains of uninucleate conidia.

To determine if AspB might have a role in organizing new growth in asexual reproduction, we induced synchronous conidiophore development, harvested developing conidiophores at several time points, and performed immunolocalization experiments. We observed AspB as a diffuse band at the base of forming metulae (d in Figure 7). This band disappeared from the vesicle/metulae interface as the metulae elongated. We also observed rings of AspB at the base of developing phialides (e in Figure 7). AspB rings disappeared from the metula/phialide interface as the phialides matured. Finally, we observed AspB at the tips of phialides producing conidiospores. The AspB band at the phialide/conidium interface was present simultaneously in all phialides (f in Figure 7).

AspB Functions in Septation, Branching, and Conidiophore Development

Previous work showed that *aspB* is an essential gene (Momany and Hamer, 1997a). To determine if AspB is needed for development of septa, branches, and conidiophores, an *A. nidulans* strain containing a temperature-sensitive allele of *aspB* was generated. When grown at restrictive temperature, this strain was able to form septa that were readily visible under DIC imaging but were only faintly visible by staining with

Calcofluor White (n=100) (c-f in Figure 8). Furthermore, the strain containing the mutant allele formed twice the number of branches when compared to wild type hyphae grown at 42°C for 10 h (n=100) (a and c in Figure 8). Finally, the *aspB-318* strain formed abnormal conidiophores that were defective in development. Most mutant conidiophores arrested at the vesicle stage of development (h and i in Figure 8); however, a small portion (16%) were able to form metulae 24 h after exposure to an air interface (n=100) (j in Figure 8). At the same time point, 100% of a wild-type population had produced metulae, phialides, and spores (n=100) (g in Figure 8).

DISCUSSION

The first clearly defined role for septins was in organization of the division plane at the septum of budding yeast (reviewed by Longtine *et al.*, 1996). Septins from a variety of organisms localize not only to cleavage planes, but also to other areas of new cell growth (reviewed by Field and Kellogg, 1999; Trimble, 1999). Our results show that the *A. nidulans* AspB protein, the first septin studied from a multicellular fungus, localizes both to sites of division and areas of new growth.

The AspB Ring at the Septation Site is Polar

Although we expected to find AspB at septation sites, the striking polarity of AspB during septum formation surprised us. Previous work showed that septation in *A. nidulans* proceeds in three distinct stages. First, a single, faint ring of actin becomes visible. Next, actin thickens into a contracting band with a ring of chitin in its furrow. Finally, actin disappears and a disk of chitin forming the primary septum remains (Momany and Hamer, 1997b). Throughout this process, actin and chitin localization

appears symmetrical -- there is no obvious difference between the apical face of the septum versus the basal face. In contrast, we found that localization of AspB is asymmetrical late in septum formation (Figures 2 and 5). The apical/basal asymmetry of AspB localization at forming septa suggests that the hypha marks polarity along its length spanning multiple cells, and that AspB either establishes or responds to these polarity cues. A similar septin asymmetry is seen in the unicellular budding yeast *S. cerevisiae* where certain proteins are localized asymmetrically in a septin dependent manner (reviewed by Gladfelter *et al.*, 2001). However, our AspB localization studies are the first demonstration of septin polarity in a multicellular organism. Although polar assembly has not been reported for animal septins, it seems likely that at least some animal septins will show a similar apical/basal polarity.

Assembly of AspB at the Septation Site Requires Actin, SepA, SepG, and SepH

Previous work has shown that actin is required for the formation of mature septa (Harris *et al.*, 1994; Momany and Hamer, 1997b). To investigate the relationship between actin and AspB, we used the actin-depolymerizing agent cytochalasin A. We observed an absence of AspB rings with drug treatment, suggesting that actin is required for either assembly or stability of the AspB ring. AspB's postmitotic localization suggests that the division site in *A. nidulans* is organized more like that of *Schizosaccharomyces pombe* than that of *S. cerevisiae*. In *S. cerevisiae*, the septins localize to the site of bud emergence early in the cell cycle and independently of actin. They act not only to organize the cytokinetic machinery, but also to localize certain kinases important for the initiation and progression of mitosis (Gladfelter *et al.*, 2001; Lew, 2000; Longtine *et al.*, 1996). In contrast, the *S. pombe* septins first localize to the

presumptive septation site late in anaphase, well after actin has marked this site, and just before the primary septum itself becomes visible (J. Pringle, personal communication). Interestingly, when grown at restrictive temperature, cells carrying the temperature-sensitive *aspB-318* allele make a septum that is readily visible by DIC imaging but stains very faintly with Calcofluor White, suggesting that the septum might not be properly organized.

Our results further suggest that the products of several of the *A. nidulans* septation (*sep*) genes are required for AspB ring formation. We performed immunolocalization experiments on temperature-sensitive septation mutants (*sepA*, *sepD*, *sepG*, and *sepH*) grown at restrictive temperature (Figure 3). We observed the AspB ring in 6% of *sepD* hyphae, and in none of the other *sep* mutants. We suspect the low percentage of the *sepD* population with the AspB ring reflects the dependence of septation upon mitosis and may indicate instability of the early septin ring. Wolkow *et al.* (1996) showed that the first mitotic division after the germling passes a critical size threshold triggers septation. Other work has shown that the initiation of septation can be separated from the progression of septation (Momany and Hamer, 1997b). Perhaps in the *sepD* mutant, mitosis triggers the initiation of septation, but the temperature-sensitive defect prevents the progression of septation. If the early AspB single ring is unstable, it might dissociate when septation does not progress. Thus, only the small portion of the population that has recently exited mitosis would be expected to show an AspB ring in *sepD* cells. Observations of AspB localization in wild-type germlings are consistent with this idea, because only about 4% show a single AspB ring with no associated chitin ring,

presumably representing the cells that have recently exited mitosis and assembled the initial septin ring.

The AspB Ring is an Early Marker for Branch Formation

Nuclei in subapical compartments are arrested in interphase until a branch is formed from the compartment. How sites for branching are chosen and how branching reactivates mitosis are not understood. However, we observed AspB localization at the nascent branch before mitosis occurred (e in Figure 6). In fact, we occasionally observed an AspB ring before any change of hyphal profile was visible. AspB localization persisted as the nuclei in the compartment entered mitosis, but was not present in any branches that were longer than 5 μm . The AspB ring is the first molecular marker identified for nascent branches. The presence of AspB at branches before resumption of nuclear division raises the possibility that the septin ring may recruit the cell cycle regulators that re-initiate mitosis in subapical compartments. Future experiments will address this possibility. In addition to this possible role in the control of nuclear division, a role for AspB in organizing new growth at branches is also consistent with our data.

The AspB Ring is Seen at Some Areas of New Growth and Not at Others

The emergence of the initial germ tube from the conidium differs from that of subsequent germ tubes in both shape and mitotic state. Primary germ tubes emerge after the first mitotic division. These germ tubes give the swollen conidiospore a pointed, pear-shaped appearance (j in Figure 6). This tapered look disappears as the germ tube lengthens. The secondary germ tubes, however, appear to be similar to branch points, with a narrower point of emergence (m in Figure 6). The fact that AspB rings localize to secondary, but not primary, germ tubes may reflect an underlying difference of

organization between these two events. One possible explanation is that a pre-existing signal is present in the conidium and directs the growth of the primary germ tube. This does not, however, mean that septins do not play a role in primary germ tube emergence, as there are at least four other septins in *A. nidulans*, and any one of these could be involved in the initial event.

AspB is Involved in the Proper Organization of the Conidiophore

In *S. cerevisiae*, the site of bud emergence is circumscribed by the septin ring late in G1 phase (Longtine *et al.*, 1996). New growth emerges exclusively within the confines of this ring as the daughter cell starts to form. Later, after mitosis, the septins act to anchor the components that will ultimately synthesize the cell wall material that separates the mother and daughter cells (DeMarini *et al.*, 1997; Gladfelter *et al.*, 2001). In *A. nidulans* conidiophores, it is possible that AspB may play an analogous role by circumscribing the site of metula emergence from the vesicle layer and phialide emergence from the metula layer. This would explain the transient AspB localization to these two interfaces and is supported by the observation that the *aspB-318* strain is unable to form the metula layer properly when grown at restrictive temperature. When conidia are formed at the tip of the phialide cells, new growth is followed immediately by cytokinesis. AspB at the tip of the phialide could be delimiting the site of conidium emergence, organizing the cytokinetic machinery, or both. It is not clear whether the simultaneous localization of AspB at the tips of the phialides reflects persistent localization or the rapid reformation of a new “bud site” as mature conidia are displaced.

Conclusion

The dynamic aspect of AspB assembly in both premitotic and postmitotic areas may in part be explained if different AspB binding partners are present at different stages of development. Our results are consistent with a model in which septins act as an organizational scaffold that recruits other proteins to specific sites.

ACKNOWLEDGEMENTS

This work was supported by NSF grant 9904629 to MM. PJW was supported by the NIH training grant in molecular and cellular mycology to UGA (AI07373). We thank Steve James (Gettysburg College) and John Hamer (Paradigm Genetics) for strains and Gretel Guest, Brian Shaw, and John Zhao for critical reading of this manuscript.

Table 2.1. *Aspergillus nidulans* strains

Strain	Genotype
A850 ^a	<i>biA1; ΔargB::trpCΔB; methG1; veA1 trpC801</i>
ASH51 ^b	<i>sepA1; pyrG</i>
AJH44 ^b	<i>sepD5; pyroA4; veA1; pyrG89; wA2</i>
AJM1 ^b	<i>sepG1; pabaA6; pyroA4; chaA1; pyrG89; veA1</i>
AJM68 ^b	<i>sepH1; wA3; veA1; pyrG89; pyroA4</i>
SWJ189 ^c	<i>bimE7; methB3; wA2</i>
A773 ^a	<i>pyrG89; wA3; pyroA4</i>
APW55 ^d	<i>aspB318; ΔargB::trpCΔB; trpC801</i>

^a Obtained from Fungal Genetics Stock Center, Department of Microbiology, University of Kansas Medical Center, Kansas City, Kansas 66160-7420.

^b Obtained from John Hamer, Department of Biological Sciences, Purdue University, West Lafayette, Indiana.

^c Obtained from Steve James, Department of Biology, Gettysburg College, Gettysburg, Pennsylvania.

^d See text.

Figure 2.1. Western analysis of affinity-purified antibodies against AspB. (a) Total protein from *A. nidulans* strain A850 stained with coomassie blue. Blots of total protein were incubated with 1:100 dilutions of (b) unpurified immune serum; (c) antibodies obtained after one round of affinity purification; (d) antibodies obtained after two rounds of affinity purification. A single 49-kD polypeptide is recognized (arrow).

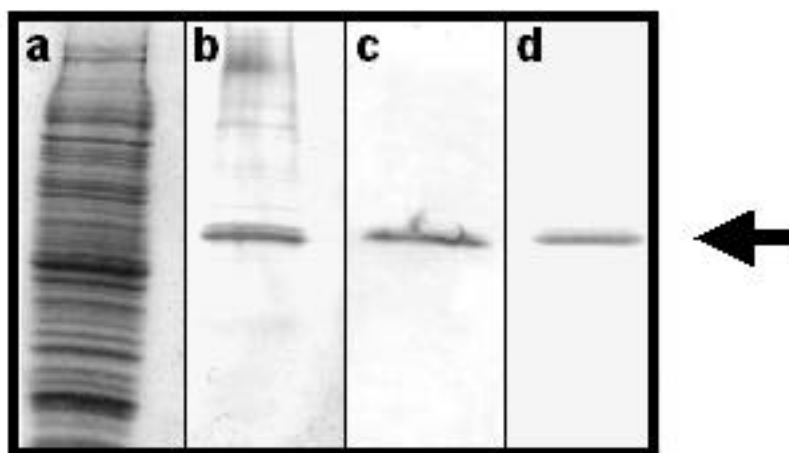


Figure 2.2. Localization of AspB and actin during septation. First row: Differential interference contrast images. Second row: Actin localization with Texas-red conjugated secondary antibody. Third row: AspB localization with FITC conjugated secondary antibody. Fourth row: Merge of rows 2 and 3. Areas of AspB and actin co-localization appear yellow. Arrows indicate location of septum. (a, e, i, and m) Early stage of septum formation. No crosswall is visible yet. (b, c, f, g, j, k, n, and o) Intermediate stages of septum formation. (d, h, l and p) Late stage of septum formation. Scale Bar = 5 μ m.

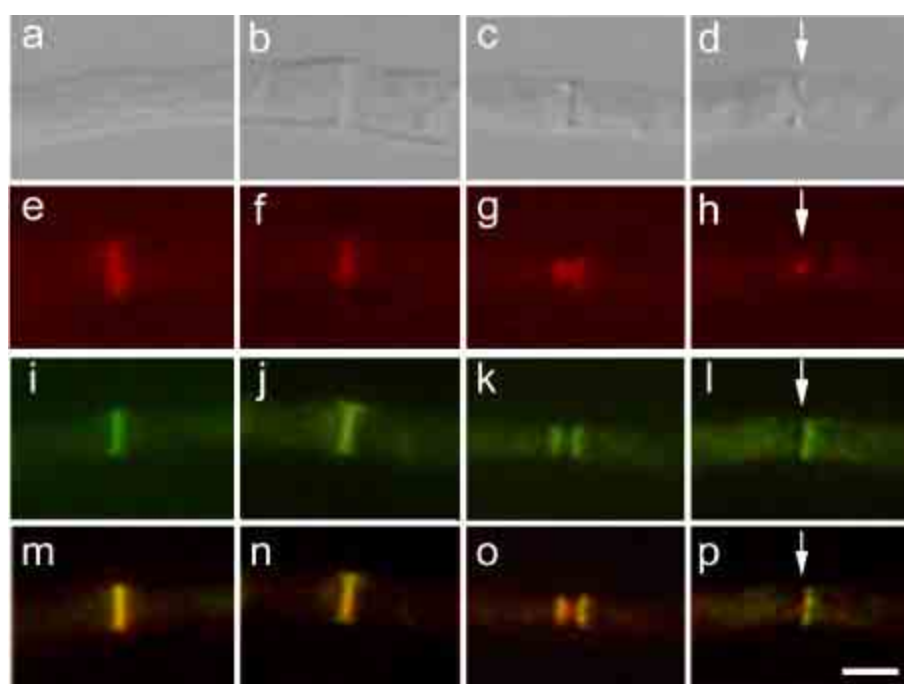


Figure 2.3. AspB localization in *sep* mutants grown at non-permissive temperature. First column: Chitin and nuclear localization with Calcofluor and Hoechst, respectively. Second column: AspB localization. (a and b) *sepA*. (c and d) *sepD*. (e and f) *sepG*. (g and h) *sepH*. Arrows mark AspB localization in *sepD*. Scale Bar = 5 μ m.

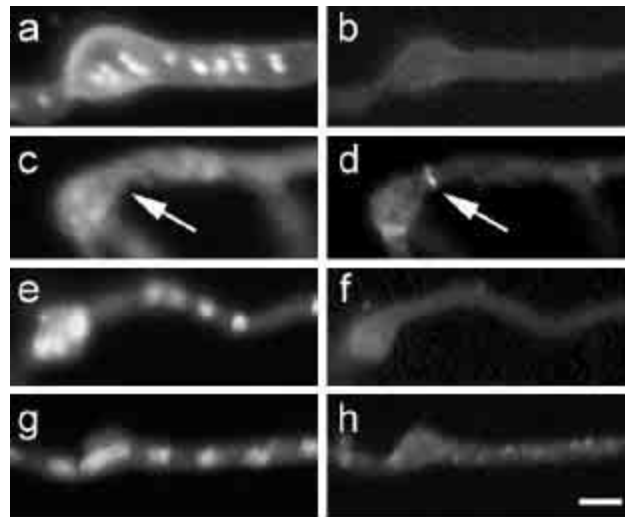


Figure 2.4. Dependence of AspB localization on mitosis. First column: DIC images. Second column: Chitin and nuclear staining with Calcofluor and Hoechst. Third column: AspB localization. (a-c) *bimE7* mutant grown at 30° C for 10 h and then shifted to 42° C for 2 h. No AspB localization was detected. (d-f) *bimE7* mutant grown at 30° C for 10 h, shifted to 42° C for 2 h, and shifted back to 30° C for 45 min. AspB localization was detected at septation sites.

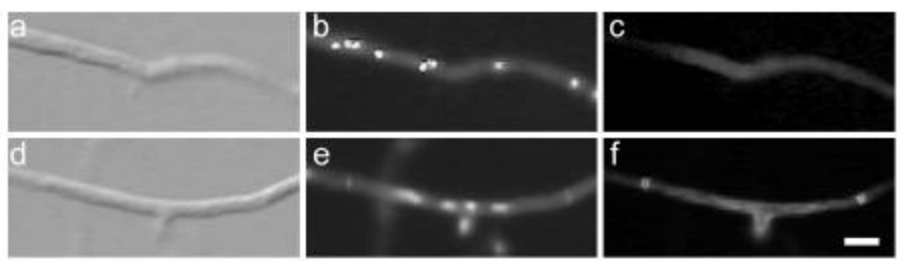


Figure 2.5. Parasynchronous formation of septa in *A. nidulans*. First column: Chitin and nuclear staining with Calcofluor and Hoechst, respectively. Images were false colored red. Second column: AspB localization. Third column: Merged images of columns 1 and 2. Areas of chitin and AspB co-localization appear yellow. Hyphae are oriented such that the basal end is to left and the apical end is to the right. Scale Bar = 5 μ m.

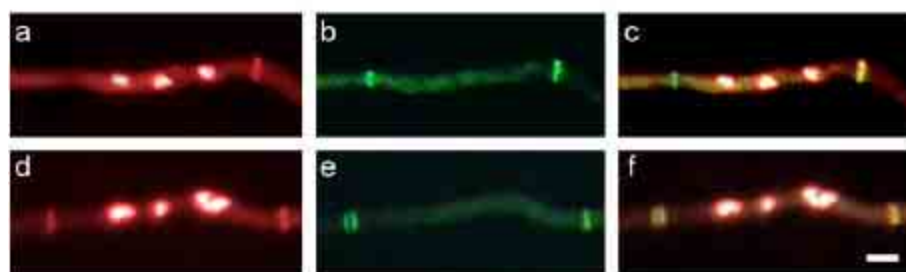


Figure 2.6. AspB localization to early branches and secondary germ tubes. First column: DIC images. Second column: Chitin and nuclear staining with Calcofluor and Hoechst. Third column: AspB localization. (a-c) AspB appears as a ring at the site of branch emergence. (d-f) Side view of emerging branch. AspB localizes as a band at the base of the branch. The odd number of interphase nuclei indicates that localization is premitotic. (g-i) AspB is localized to the branch while the compartment is in mitosis. Compact, bright nuclear staining indicates mitotic nuclei. (j-l) Emerging primary germ tube. No AspB localization is present. (m-o) Emerging secondary germ tube. AspB localizes as a ring at the site of secondary germ tube emergence. Scale Bar = 5 μ m.

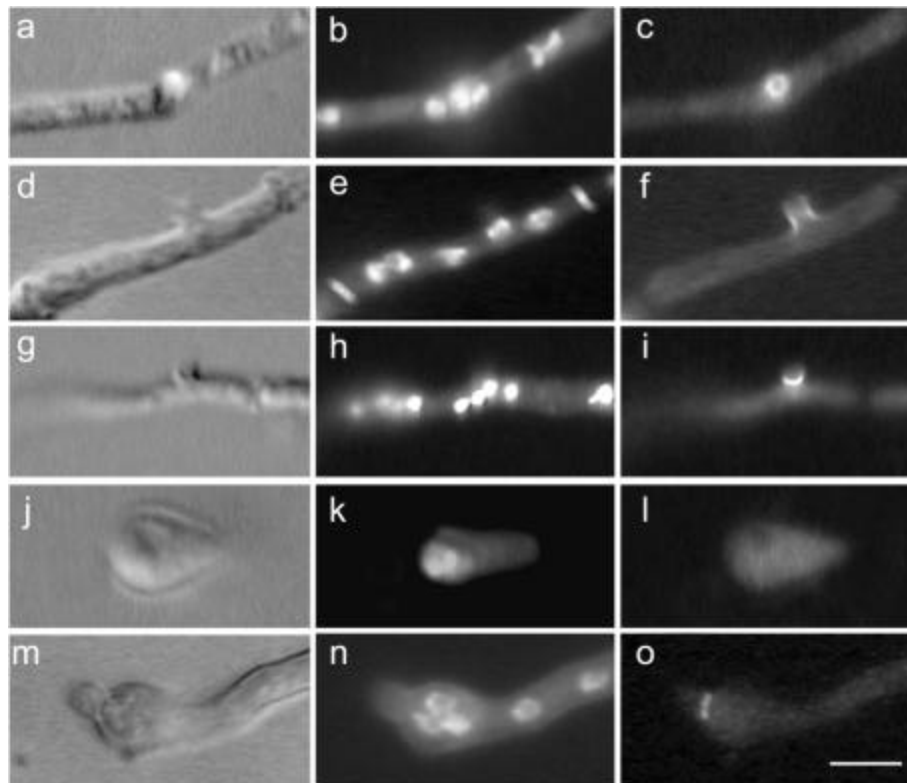


Figure 2.7. AspB localization in the conidiophore. First row: DIC images. Second row: AspB localization. (a and d) AspB localizes at the vesicle/metulae interface as metulae emerge. (b and e) AspB localizes at the metula/phialide interface. Arrow marks emerging phialide. (c and f) AspB localizes to the phialide/conidiospore interface. Arrow marks emerging conidium. Scale Bar = 5 μ m.

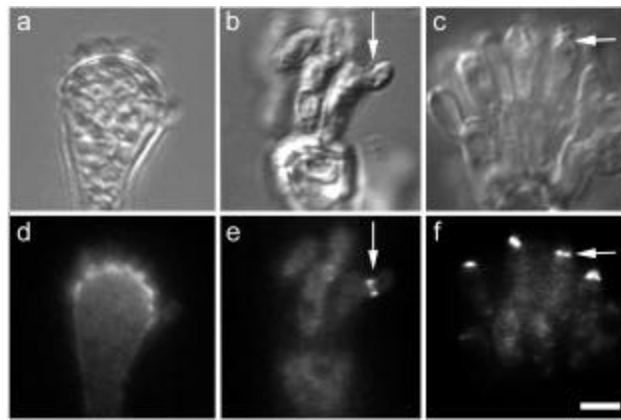
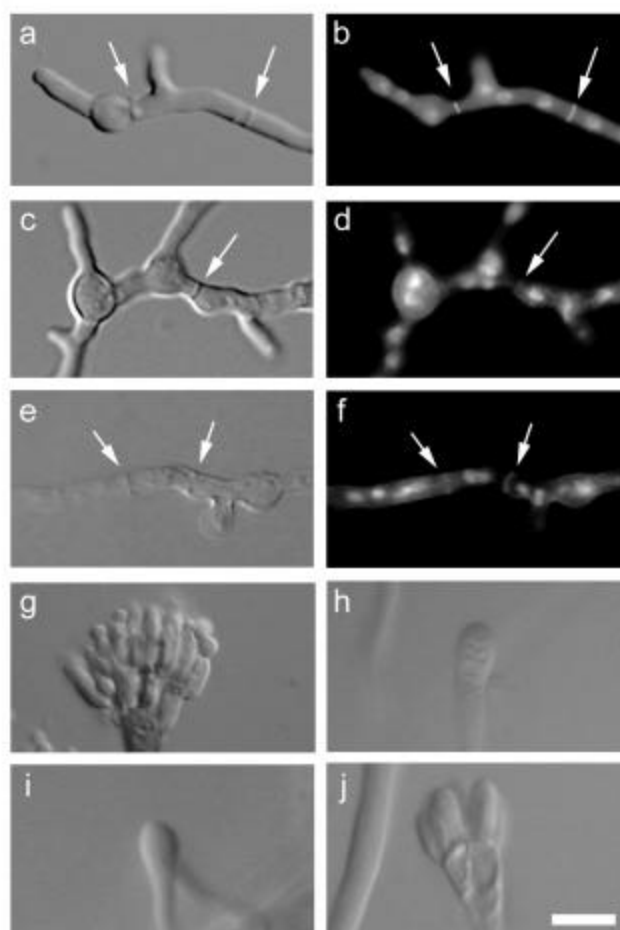


Figure 2.8. Conditional mutant of *aspB*. (a, c, and e) DIC images of vegetative hyphae. (b, d, and f) Chitin and nuclear staining of vegetative hyphae with Calcofluor and Hoechst. (a and b) Wild-type *A. nidulans* grown at 42°C for 10 h. (c-f) *A. nidulans* strain APW55 (*aspB-318*) grown at 42°C for 10 h. Note that chitin staining of septa (arrows) appears weaker than in wild type. (g-j) DIC images of conidiophores exposed to an air interface for 24 h. (g) Wild type conidiophore. (h-j) APW55 conidiophores. Scale Bar = 5µm.



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CHAPTER 3
TWO MUTANT ALLELES OF THE *ASPERGILLUS NIDULANS* SEPTIN *ASPB*
SHOW DEFECTS IN SEPTUM, BRANCH, AND CONIDIOPHORE
DEVELOPMENT¹

¹Westfall, P.J., Lindsey, R., and Michelle Momany. To be submitted to *Journal of Cell Science*.

ABSTRACT

The septin family of proteins have been found in numerous eukaryotic organisms from fungi to mammals. In areas of cellular division and growth they play an important role by acting as an organizational scaffold. In this study, two mutant alleles of the *Aspergillus nidulans* septin-encoding gene *aspB* have been identified. Strains containing either the *aspB-271* or the *aspB-318* allele make fewer septa and localization of AspB during the later stages of cytokinesis is lost. The *aspB-318* allele produces hyphae with high numbers of branches that are not able to localize AspB to the site of branch emergence. Both the *aspB-271* and the *aspB-318* alleles show delays in conidiophore development and abnormal conidiophore layering.

INTRODUCTION

Septins, by acting as cellular scaffolds, recruit proteins needed for cell division and new growth (Longtine *et al.*, 1996; Field and Kellogg, 1999; Gladfelter *et al.*, 2001). Four septins (the *CDC3*, *CDC10*, *CDC11*, and *CDC12* gene products) were originally identified in the budding yeast *Saccharomyces cerevisiae* where they were thought to be components of the 10nm filamentous rings found in the neck region of the dividing cell (Byers and Goetsch, 1976; Longtine *et al.*, 1996). Cells that contain temperature sensitive versions of *CDC3*, *CDC10*, *CDC11*, and *CDC12* are mitotically delayed (Carroll *et al.*, 1998; Barral *et al.*, 1999; Longtine *et al.*, 2000). In addition, they form elongated, multinucleate, multibudded chains of cells that are unable to undergo cytokinesis (Hartwell, 1971). Three additional septins have been more recently identified in *S. cerevisiae*. Sep7p localizes to the neck region of dividing cells, but unlike Cdc3p,

Cdc10p, Cdc11p, and Cdc12p, is not needed to proper formation of the neck filaments (Carroll *et al.*, 1998; Mino *et al.*, 1998). Spr3p and Spr28p are sporulation specific and are localized to the leading edge of the prospore sac as spores develop (De Virgilio *et al.*, 1996; Fares *et al.*, 1996).

Septins, with a few exceptions, have two motifs in common, a GTP-binding P-loop and a coiled-coil domain that may be needed for protein-protein interactions. GTP hydrolysis and filament formation of septins has been shown *in vitro*, although filament formation may not be required for proper septin function (Sanders and Field, 1994; Field *et al.*, 1996; Frazier *et al.*, 1998). A third motif, required for protein modification by the ubiquitin-related SUMO homologue, Smt3p, affects the dynamics of the septin ring. Three of the septins found at the bud neck, Cdc3p, Cdc11p, and Sep7p have Smt3p recognition sites. When these recognition sites are removed or modified, the septin ring is hyperstabilized and persists throughout several rounds of division, instead of breaking down upon exit from mitosis (Johnson and Blobel, 1999).

Numerous proteins that are important in *S. cerevisiae* development have been found to require septins for proper localization. Cdc10p interacts with Bni4p, which in turn helps localize Chitin Synthase III, the chitin synthase responsible for bud scar formation (DeMarini *et al.*, 1997). Similarly, during spore formation, the sporulation-specific septins Spr3p and Spr28p along with Cdc3p and Cdc11p, may influence the deposition of chitosan (De Virgilio *et al.*, 1996; Fares *et al.*, 1996; Longtine *et al.*, 1996). In the central region of the mother-bud neck a type II myosin, Myo1p is localized in a septin-dependent manner and is a component, along with actin and Iqg1p/Cyk1p, of the constricting actomyosin responsible for cytokinesis (Epp and Chant, 1997; Bi *et al.*,

1998). Septins also influence the position of bud emergence, by properly localizing proteins such as Bud3p, Bud4p, and Axl2p (Flescher *et al.*, 1993; Chant *et al.*, 1995; Chant and Pringle, 1995; Pringle *et al.*, 1995; Halme *et al.*, 1996; Roemer *et al.*, 1996; Sanders and Herskowitz, 1996). The septins may act as a diffusion barrier, allowing certain proteins access to either the mother or bud exclusively. In a study by Takizawa *et al.* a transmembrane protein, Ist2p, was shown to localize exclusively in the bud. However in septin mutants, this exclusive localization was lost and Ist2p was found ubiquitously (Takizawa *et al.*, 2000).

Cellular organization and regulation of cell division is markedly different in filamentous fungi versus budding yeasts. The filamentous fungus *A. nidulans* starts out as single, uninucleate spore and grows isotropically, undergoing one or two rounds of mitosis before the establishment of a germ tube (unlike yeast where each mitosis is followed by a round of cytokinesis). After the germ tube has been established nuclei move out into it. The fungus grows in an apical manner by adding new cell wall material to the tip of the growing hypha (Harris, 1999; Momany and Taylor, 2000). A mitotic event after the hypha reaches a predetermined size, results in partitioning by septa. (Wolkow *et al.*, 1996; Momany and Hamer, 1997). These compartmentalized cells contain three to four nuclei which are arrested in interphase. These nuclei only become mitotically active if a new axis of polarity is established and branches form (Kaminskyj and Hamer, 1998). During asexual development specialized branches called aerial hyphae give rise to the highly structured conidiophore, which in turn produces long chains of uninucleate spores (Adams *et al.*, 1998).

Five septin homologues (*aspA*, *aspB*, *aspC*, *aspD*, and *aspE*) have been identified in *A. nidulans* (Momany and Hamer, 1996; Momany *et al.*, 2001). Previous work showed that *aspB* is essential for development and that AspB localized to forming septa, branches, and conidiophores (Momany and Hamer, 1996; Westfall and Momany, 2002). In this study we describe two mutant alleles of the *A. nidulans* septin *aspB*. Strains containing either the *aspB-271* or *aspB-318* allele showed defects in septum formation and asexual development. In addition, strains with the *aspB-318* allele displayed a hyper-branching phenotype.

MATERIALS AND METHODS

Aspergillus Strains and Growth Methods

All strains used in this study are listed in Table 3.1. Defined minimal media with appropriate supplements were used for this study (Kafer, 1977). For all studies conidia were inoculated to coverslips in liquid medium and incubated at the appropriate temperature for times indicated below, fixed, stained, and examined as previously described (Westfall and Momany, 2002). For morphological characterization, conidia were inoculated for 10 h at 42°C. For primary and secondary germ tube studies, conidia from were inoculated at 42°C, and fixed at 6 and 10 h post-inoculation. For septation and branching localization studies, conidia were inoculated for 12 h at 42°C. For synchronized conidiophore development, 100 ml liquid cultures were inoculated overnight at 42°C with vigorous shaking. Cultures were collected miracloth and placed on solid medium at 42°C to induce conidiation (Miller *et al.*, 1992). Conidiophores were harvested at 10.0, 12.0, 13.5, 15.5, and 24 h by scraping with water. For localization

studies in conidiophores, 5 day cultures were grown at 42°C on solid medium and harvested by scraping in water. For mRNA and total protein isolation liquid cultures were inoculated overnight at 42°C with vigorous shaking.

Generation of aspB Mutant Alleles

A 5kb EcoRI/PstI genomic fragment was isolated from plasmid pMM1 to create plasmid pPW20, containing *aspB* and a 1 kb of upstream sequence (Momany and Hamer, 1996). PPW20 was transformed into the *E. coli* mutator strain XL1-Red (Stratagene, La Jolla, California). Plasmid DNA was isolated and co-transformed with a marker plasmid containing the *argB* gene into *A. nidulans* strain A850 using standard protocols (Yelton *et. al.*, 1984). The resulting 3798 Arg⁺ transformants were replica plated at 30° C and 42° C. Fifty-four transformants showing a stable phenotypic defect were crossed with *A. nidulans* strain A773 and progeny were scored. Total DNA was isolated from 12 argB- strains with 1:1 wild type:mutant segregation and blotted to nylon membranes. A PCR product containing the *aspB* ORF was used to probe DNA blots and identified two transformants with a single copy of *aspB*. These strains, APW53 and APW55, were restored to wild type growth by complementation with the original pPW20 plasmid.

Sequencing of aspB Mutant Alleles

The *aspB* open reading frame from A850, APW53, and APW55 was PCR amplified and cloned into the pGEM-T vector (Promega, Madison, Wisconsin). Isolated plasmids were transposon tagged using the GPS-1 kit (New England Biolabs, Beverly, Massachusetts) and arrayed on 96 well plates. Sequencing label was introduced using Big Dye 2.0 kit (Perkin Elmer Applied Biosystems, Boston, Massachusetts). Sequencing was performed in a 96 well format using an ABI Prism 3700 sequencer (Applied

Biosystems, Foster City, California). Analysis of sequences was performed using SeqLab 10.0 (Genetics Computer Group, University of Wisconsin, Madison, Wisconsin).

Northern and Western Blot Analysis of aspB Mutants

Northern blot analysis of strains A850, APW53, and APW55 was as per standard protocols, using 10 µg total RNA per lane. Blots were probed with a 750 base pair XhoI restriction fragment from *aspB*. Northern blots were developed and scanned using a STORM imager system (Amersham-Pharmacia Biotech, Uppsala, Sweden). Scanned images were imported into Adobe Photoshop 5.5 (Adobe Systems Inc., San Jose, California), and converted to grayscale. Percent K values were calculated for 10 pixels/lane, averaged, and used to determine the relative intensities of each signal.

Affinity purified anti-AspB antibodies were isolated as described previously (Westfall and Momany, 2002) and used to probe western blots made from SDS-PAGE gels containing 20µg total protein/lane from A850, APW53, and APW55. Western blots were developed using a goat anti-rabbit secondary coupled to horseradish peroxidase (Jackson Immunochemical, West Grove, Pennsylvania) and the ECL detection system (Amersham-Pharmacia Biotech). Developed images were scanned and relative intensities for each signal were determined as above.

For first dimension separation in 2D gel experiments, 14µg of total A850 protein was separated on 13 cm Immobiline DryStrips pH 3-10 using the IPGphor system (Amersham-Pharmacia Biotech). Second dimension separation was done using 10% SDS-PAGE gels and blotted to nitrocellulose. Detection of AspB protein on western blots was as describe above.

Antibodies and Localization

Anti-AspB antibodies were generated and affinity purified as described previously (Pringle *et al.*, 1989; Westfall and Momany, 2002). Fixation and staining of hyphae were done as described previously (Oakley and Osmani, 1993) with an additional 2 h blocking step with 5% non-fat milk in buffered phosphate solution before the addition of primary antibody. Secondary goat anti-rabbit conjugated to FITC was obtained from Jackson Immunochemical. Actin localization was done using the C4 mouse anti-actin monoclonal antibody (ICN Biomedicals, Aurora, Ohio) and a Texas Red-labeled sheep anti-mouse secondary (Jackson Immunochemical). AspB localization studies in conidiophores were done as described previously (Westfall and Momany, 2002).

Image Acquisition and Adjustment

Microscopic observations were made with a Zeiss (Thornwood, NY) Axioplan microscope. Digital images of immunofluorescence localization were acquired with an Optronics Digital Imaging System (Goleta, CA). TIFF format files were imported into Adobe PhotoShop 5.5 converted to grayscale or CMYK color and adjusted for optimal brightness and contrast.

RESULTS

Genetic Analysis of aspB Mutants

Previous attempts to delete the *Aspergillus nidulans* septin *aspB* demonstrated that the gene was essential (Momany and Hamer, 1996). In order to clarify the developmental role of the AspB, two strains containing different mutant alleles of *aspB* were generated and characterized. Strain APW53, containing the *aspB-271* allele showed

reduced septation and abnormal conidiophore development when grown at 30°C or 42°C. Strain APW55, containing the *aspB-318* allele, had a temperature sensitive defect. APW55 grew like wild type at the permissive temperature of 30° C, but showed reduced septation, hyper-branching, and abnormal conidiophore development when grown at the restrictive temperature of 42° C.

The coding region of both mutant alleles of *aspB* were cloned and sequenced. The *aspB-271* allele showed no differences within the coding region of *aspB*, but mutations were suspected within the promoter region. The *aspB-318* allele showed a single A to G change, 28 base pairs after the start of translation resulting in the substitution of a hydrophobic alanine for the wild type hydrophilic threonine.

To determine if there was any difference in the level of *aspB* mRNA expression in strains containing the *aspB-271* or *aspB-318* allele, northern blot analysis was done (a-f in Figure 1). The *aspB-271* allele showed a 20% reduction of mRNA levels compared to wild type mRNA levels of *aspB* (d and e in Figure 3.1). In contrast, the *aspB-318* allele showed no significant reduction in *aspB* mRNA levels (d and f in Figure 3.1).

Biochemical Analysis of aspB Mutants

Previous studies showed that affinity-purified polyclonal antibodies raised against AspB recognized a single 49 kD protein on western blots made from 10% SDS-PAGE gels containing total *A. nidulans* protein (Westfall and Momany, 2002). However, in western blots containing total protein from *A. nidulans* in which protein separation was allowed to go longer, the anti-AspB antibody recognized two protein bands of approximately 49 and 51 kD (a in Figure 2). Western blots containing total protein from APW53 (*aspB-271*) showed a 42% reduction of protein in the upper band, and a 20%

reduction of protein in the lower band when compared to equivalent amount of total protein from wild type *A. nidulans* (b in figure 2). Western blots containing total protein from APW55 (*aspB-318*) showed a 96% reduction of protein in the upper band, but only a 7% reduction of protein in the lower band when compared to equivalent amount of total protein from wild type *A. nidulans* (c in Figure 2).

To determine if both bands represented different forms of *aspB* or if the antibody was cross-reacting to another *A. nidulans* septin, molecular weights and isoelectric points were calculated for AspA, AspC, AspD, and AspE (Table 3.2). Western blots from two-dimensional gels containing total *A. nidulans* protein were probed with anti-AspB antibodies and recognized a single protein at pI 6.8, the expected pI of AspB (d in Figure 2).

AspB Mutants Show Defects in Septation, Branching, and Conidiophore Development

To determine the affect of the *aspB-271* and *aspB-318* mutations on *A. nidulans* development, phenotypic characterization was performed on hyphal cultures. In order to determine if the mitotic rate of either mutant strain was affected, the number of nuclei in both wild type and mutant germlings grown for 10 h at 42° C was counted. Forty-four percent of the germlings with the *aspB-271* allele had more than 65 nuclei. In contrast, only 7% of the wild type germlings and 2% of the germlings with the *aspB-318* allele had more than 65 nuclei (a in Figure 3). Strains containing either the *aspB-271* or *aspB-318* alleles also showed reduced septation compared to the wild type strain (n=200)(a in Figure 3; a-c in Figure 4). Strains containing the *aspB-318* allele produced twice the number of branches and were ten times more likely to have a third or fourth germ tube compared to wild type strains (n=200) (b and c in Figure 3; d and f in Figure 4). Finally,

strains containing the *aspB-271* and *aspB-318* alleles showed delays in asexual development, abnormal asexual development, and reduced spore production, with the *aspB-271* strain often producing anucleate and multinucleate spores.

The aspB-318 Mutant Produces More Secondary Germ Tubes

AspB does not localize to spores undergoing initial germ tube emergence, but does localize to spores undergoing secondary germ tube emergence in a ring similar to that seen at points of branch emergence (Westfall and Momany, 2002). To investigate whether the *aspB-271* or *aspB-318* mutations affected primary germ tube emergence, A850 (wild type), APW53 (containing the *aspB-271* allele), and APW55 (containing the *aspB-318* allele) conidia were germinated for 6 h at 42° C. Forty-two percent of A850 and APW53 and 38% of APW55 conidia were able to establish polarity and had initiated primary germ tubes at this time point (n=150). To investigate whether the *aspB-271* or *aspB-318* mutations affected secondary germ tube emergence, A850, APW53, and APW55 conidia were germinated for 10 h at 42° C. Eighty-three percent of A850, 90% of APW53, and 94% of APW55 germlings showed secondary germ tubes emerging from the conidium (n=150). In addition, 62% of the germlings with the *aspB-318* allele showed three or more germ tubes emerging from the conidium versus only 6% of wild type strains and 11% of strains carrying the *aspB-271* allele (n=150) (c in figure 3).

The aspB-271 and aspB-318 Mutants are Unable to Localize AspB in Late Septation

Septation in *A. nidulans* takes place by concurrent actin-mediated invagination of the plasma membrane and deposition of cell wall materials to form a primary septum (Momany and Hamer, 1997). AspB, actin, and chitin have very distinct localization patterns throughout this process. Initially, AspB and actin localize to the site of septation

as single rings. As septation progresses the AspB ring splits into two separate rings, the actin ring invaginates and forms an hourglass like structure, and a chitin becomes visible as cell wall material starts to fill. When septation nears completion, the AspB ring to the basal side of the septum is lost and actin localizes to a single point near the center of the crosswall. Mature septa have a single central pore and no AspB or actin localization (Momany and Hamer, 1997; Westfall and Momany, 2002).

To determine if AspB localized properly in mutant strains, hyphae were grown for 12 h at 42° C and co-labeled with affinity purified anti-AspB antibodies and an anti-actin antibody (Figure 5). APW53 containing the *aspB-271* allele, was able to localize a single ring of AspB in the early stages of septation (g-i in Figure 5). However, in the later stages of septation, indicated by hourglass-like actin localization, AspB was no longer present (j-l in Figure 5). Chitin staining with Calcoflour White, appeared much fainter in the mutant strain, when compared to wild type septal staining (a, d, g, and j in Figure 5). APW55, containing the *aspB-318* allele, was also able to localize AspB at the earliest stages of septum formation, but the ring of AspB appeared much broader and less well defined when compared to wild type AspB rings at the same stage of septum development (b and n in Figure 5). In addition, AspB localization was lost at later stages of septum development and chitin rings did not appear as bright as wild type chitin rings (a and p-r in Figure 5).

The aspB-318 Mutant Does Not Localize to Branch Points

After hyphae are compartmentalized by septation, basal cells contain three to four nuclei that are arrested in interphase. Resumption of mitotic activity occurs when a

branch forms. The only identified molecular marker for branching in *A. nidulans* is AspB (Westfall and Momany, 2002).

In wild type cells, AspB localizes pre-mitotically as a ring at the base of emerging branches. This localization persists throughout mitosis, but is lost after the branch is greater than 5 μm long (Westfall and Momany, 2002). In strains containing the *aspB-271* allele, branching appeared normal and AspB was able to localize to the site of branch emergence (d-f in Figure 6). In strains containing the *aspB-318* allele no AspB localization was found at the base of emerging branches. In addition, the base of branches from strains carrying the *aspB-318* allele appeared to be much broader compared to branches in wild type cells (a and g in Figure 6).

The aspB-271 and aspB-318 Mutants Disrupt Normal Asexual Development

Asexual reproduction in *A. nidulans* takes place in a specialized structure called a conidiophore (Adams *et al.*, 1998). Thick walled basal cells produce a specialized branch called an aerial hypha. The aerial hypha swells at its tip to produce a knob-like vesicle. The vesicle then produces a layer of bud-like growths, or metulae. Metulae in turn produce a second layer of bud-like growths, or phialides. Phialides are the conidiogenous cells, producing long chains of uninucleate, asexual spores from their tips. During asexual development, AspB localizes transiently to the base of developing metulae and phialides. AspB localizes persistently to the tips of phialides as they produce spores (Westfall and Momany, 2002).

The *aspB-271* and *aspB-318* mutants cause delays in asexual development and form abnormal conidiophores. Synchronized conidiophore development at 42° C was induced and conidiophores were examined at various time points for emergence of

metulae, phialides, or conidiospores. In wild type strains metulae were evident after 12 h, phialides after 13.5 h, spores after 15.5 h, and mature conidiophores after 24 hours of exposure to an air interface (a and c in Figure 7). In strains with the *aspB-271* mutation, metulae were present after 12 h and phialides were visible after 13.5 h, but spores were not seen until 24 hours after exposure to an air interface (a-c in figure 7; a-d in Figure 8). Scanning electron microscopy of the *aspB-271* mutants showed that the metulae layer also appeared less dense and the phialides appeared to be abnormally long, often with multiple phialides forming, one on top of the other (b and c in Figure 8). Furthermore, in strains with the *aspB-271* allele, the number of conidiophores producing spores was greatly reduced. One hundred percent of conidiophores produced spores in wild type strains after 24 hours of exposure to the air interface, but only 25% of conidiophores in the *aspB-271* mutant produced spores. Those spores that were produced by the *aspB-271* mutant were often either anucleate or multinucleate and not of a uniform size (e in figure 9).

Metulae and phialides were not visible in strains expressing the *aspB-318* gene product until after 24 hours of exposure to an air interface (a-c in Figure 7). Spores from the *aspB-318* mutant, although uninucleate and normal sized, did not appear until after 48 hours of exposure to an air interface (f in figure 9).

To examine if AspB was localized in asexual reproductive structures from either mutant strain, conidiophores were isolated from plates grown at 42° C for five days. Conidiophores were incubated with affinity purified anti-AspB antibody. AspB localized to the tips of the phialides in strains carrying either the *aspB-271* allele or the *aspB-318*

allele (d-f in Figure 9). However, similar to its localization at septa, the *aspB-318* gene product appeared to be more diffuse than AspB localization in wild type conidiophores.

DISCUSSION

Originally identified as cytokinetic elements, septins have been shown to be part of a complex that not only coordinates division, but also directs new growth, serves as a morphogenetic checkpoint, and helps define cellular domains (Longtine *et al.*, 1996; Field and Kellogg, 1999; Gladfelter *et al.*, 2001). Our results support similar roles for the *A. nidulans* septin AspB. *AspB* mutants had abnormal septa, produced large numbers of branches and secondary germ tubes, and had deformed asexual reproductive structures.

Expression Levels and Modification of AspB May Affect A. nidulans Development

Total loss of *aspB* expression in *A. nidulans* is lethal, and attempts to place *aspB* behind an inducible promoter were unsuccessful, indicating that the level of *aspB* expression is critical (Momany and Hamer, 1996; our unpublished results). Sequence analysis of the *aspB-271* allele revealed no base pair changes within the coding region of the *aspB* gene. However, base pair changes in the promoter region seem likely because *aspB* mRNA levels are reduced in the APW53 strain. The reduction of expression levels in the *aspB-271* allele due to inefficient transcription of mRNA might affect the amount of AspB protein available at the sites of septation, branching, and conidiophore development. Proteins dependent upon AspB for proper localization to these sites could also be affected.

Sequence analysis of the second *aspB* mutant allele, *aspB-318*, revealed a single base pair change within the coding region causing a switch from a threonine to alanine at

the 10th amino acid residue. Although the N-terminus of septins proteins are not highly conserved, this region may be significant for the modification of AspB. Cdc3p from *S. cerevisiae* (AspB's closest homologue) contains a consensus sequence for modification by Smt3p, the *S. cerevisiae* SUMO homologue, at amino acids 8-15 (Johnson and Blobel, 1999). The region of AspB containing the threonine to alanine switch also fits the consensus sequence for SUMO recognition (Johnson and Blobel, 1999; Johnson and Gupta, 2001; Takahashi *et al.*, 2001). A SUMO homologue has been identified, but not characterized in *A. nidulans* (our unpublished results). SUMO acts to compartmentalize and stabilize modified proteins (Muller *et al.*, 2001). Loss of stabilization, because of the *aspB-318* mutation, could account for the loss of AspB localization during late septation and at branch points.

Quantification of AspB levels revealed that anti-AspB antibodies recognized two protein bands of similar size on western blots containing total protein from both wild type and mutant (a-c in Figure 1). Two dimensional gel analysis showed that neither protein band recognized by the anti-AspB antibody was one of the other *A. nidulans* septin proteins. The *aspB* gene has a 57 base pair, in-frame intron 184 base pairs from the translational start site (Momany and Hamer, 1996; Momany *et al.*, 2001). Differential splicing of this intron is not predicted to change AspB's isoelectric point. Thus both versions would appear as a single point on 2D gels. If this intron is transcribed it would explain the appearance of the second, larger molecular weight band (Table 3.2).

Differential splicing of septins has been demonstrated in mammalian septins and has been suggested to play an important role in ovarian and breast cancer (Kalikin *et al.*, 2000; Russell *et al.*, 2000; Zieger *et al.*, 2000).

Mutations in AspB Affect Septation and Mitotic Rate

Compartmentalization in filamentous fungi allows for cellular differentiation, regulates branching, prevents cytoplasmic leakage after wounding, and ensures only tip cells are mitotically active. AspB's role during septation is demonstrated by the reduced number of septa present in strains containing the *aspB-271* and *aspB-318* alleles (b in Figure 3; a-c in Figure 4). Although the products of both alleles are able to localize to the site of septation initially, this localization is lost at the later stages of septation (g-r in Figure 5). In those septa that are able to form in either of the mutant strains, chitin staining, as visualized with Calcofluor White, is reduced, suggesting that the cell wall material at the septum is not properly organized. Furthermore, the *aspB-318* gene product's initial diffuse localization is indicative of a problem with ring assembly. Alternatively, the *aspB-318* gene product may not be properly modified causing it to become unstable and disassemble prematurely.

Septation in *A. nidulans* is dependent upon an exit from mitosis (Momany and Hamer, 1997). However, loss of septation can also influence the mitotic rate. Strains carrying the *aspB-271* allele had an unusually high number of nuclei present in approximately half the population (a in Figure 3). Partitioning of nuclei by septa allows the fungus to control the mitotic rate by arresting compartmentalized nuclei in interphase. Only tip cells, which can contain up to 30 nuclei, undergo mitosis (Rosenberger and Kessel, 1967; Clutterbuck, 1970). The *aspB-271* gene product, by reducing compartmentalization, results in nuclear doubling at an exponential rate.

The aspB-318 Gene Product Affects Branching and Secondary Germ Tube Emergence

Branching in *A. nidulans* involves not only establishing a new polar axis and directing growth there, but the resumption of the mitotic cycle in nuclei previously arrested in interphase. AspB is the only marker identified in filamentous fungi for branch emergence (Westfall and Momany, 2002). Mutant strains carrying the *aspB-318* allele showed a hyperbranching phenotype with almost twice the number of branches as wild type strains grown under the same condition. Furthermore, AspB was not able to localize properly at branchpoints in strains with the *aspB-318* allele (g-i in Figure 5). This suggests either AspB or AspB dependent proteins may regulate branching, and in the absence of this regulation branching occurs more frequently.

Similar to the hyperbranching phenotype, strains with the *aspB-318* allele produced more secondary germ tubes than wild type strains grown under the same conditions. Neither of the *aspB* mutant alleles affected the rate of primary germ tube emergence. Previous studies showed that AspB did not localize to the primary germ tube but was present at the base of subsequent germ tubes that emerge from the conidium (Westfall and Momany, 2002). The results from this study further support the hypothesis that secondary germ tubes emergence is more analogous to branching than to primary germ tube emergence.

AspB Mutants Cause Delays in Asexual Development

Asexual development in *A. nidulans* results in the formation of a highly structured, multi-layered conidiophore. Since the emergence of the metulae and phialides layers is so similar to the emergence of buds in *S. cerevisiae*, it is reasonable to expect a scaffolding involving septins to mark the sites of new growth emergence. In fact,

emerging metulae and phialides are marked transiently, and spores are marked persistently by AspB (Westfall and Momany, 2002). If the septin ring failed to form properly, disassembled early, or if septin dependent proteins were not properly recruited to the site of growth emergence, multibudded, multinucleate metulae, phialides, or spores might form, similar to the phenotype seen in temperature sensitive *CDC3*, *CDC10*, *CDC11*, and *CDC12* mutants when grown at restrictive temperature (Hartwell, 1971). The *aspB* mutant phenotypes support this idea by showing delays in metulae and phialide emergence and by forming multiple layers of phialides (a-f in Figure 4; a-d in Figure 8). Furthermore, spores produced by the strains carrying the *aspB-217* allele were often either anucleate or multinucleate and of irregular size.

Conclusion

The *Aspergillus nidulans* septin, AspB, plays an important role in development by organizing the septum, marking the site of branch emergence, marking the site of secondary germ tube emergence, and organizing the asexual reproductive structure. A model, with AspB acting as a scaffold for the recruitment of proteins important in these processes, is supported by our mutant studies. *AspB* mutants were unable to properly organize septa, branches, and conidiophores. Further characterization of other *A. nidulans* septins, along with the identification of septin interacting proteins will be of importance in helping to understand how these proteins influence development.

Table 3.1. *Aspergillus nidulans* strains

Strain	Genotype
A850 ^a	<i>biA1; ΔargB::trpCΔB; methG1; veA1 trpC801</i>
A773 ^a	<i>pyrG89; wA3; pyroA4</i>
APW53 ^b	<i>aspB-271; ΔargB::trpCΔB; methG1; trpC801</i>
APW55 ^b	<i>aspB-318; ΔargB::trpCΔB; methG1; trpC801</i>

^a Obtained from Fungal Genetics Stock Center, Department of Microbiology, University of Kansas Medical Center, Kansas City, Kansas 66160-7420.

^b This study

Table 3.2. *A. nidulans* septin predicted molecular weights and isoelectric points

<i>A. nidulans</i> septin	Molecular weight (kD)	predicted pI
AspA	43.1	4.9
<u>AspB</u> ^a	48.9	6.8
<u>AspB</u> ^b	51.0	6.8
AspC	46.3	8.7
AspD	38.7	8.1
AspE	52.5	5.7

^a Predicted size without intron

^b Predicted size with intron

Figure 3.1. Northern blot analysis of mutant *aspB* alleles. 10 μ g of total RNA from a) A850 (wild type), b) APW53 (*aspB-271*), c) APW55 (*aspB-318*). Identical gel was probed with 750kb fragment from *aspB*. e) A850, f) APW53, g) APW55.

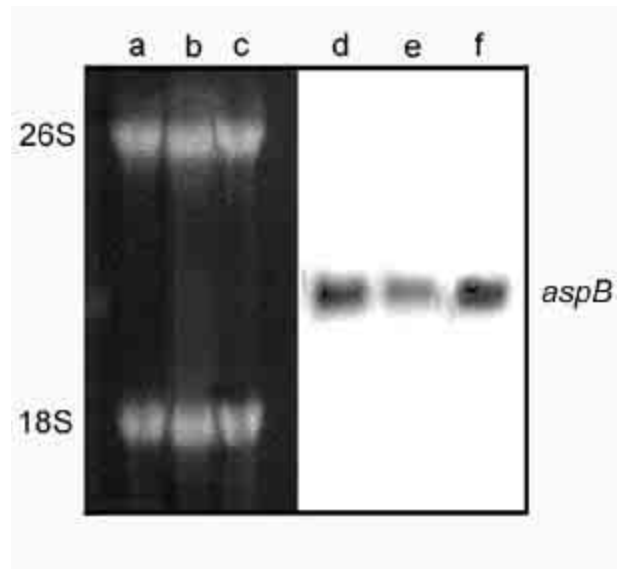


Figure 3.2. Western blot analysis of protein from wild type and mutant strains probed with affinity purified anti-AspB antibody. 20 μ g of total protein from a 20% SDS-PAGE gel was loaded per lane. a) A850 (wild type), b) APW53 (*aspB-271*), c) APW55 (*aspB-318*). d) Two dimensional gel analysis of total protein from *A. nidulans* strain A850. A protein “dot” at pI 6.8 is recognized.

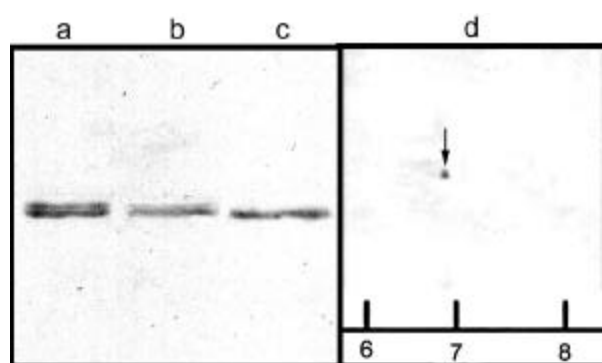


Figure 3.3. Mitotic Rate, septation, branching, and germ tube emergence in *aspB* mutants after 10 h of growth at 42° C. a). Percent of population with respect to number of nuclei. b) Number of septa with respect to number of nuclei. c) Number of branches with respect to the number of nuclei. d) Percentage of population with primary and secondary germ tubes with respect to time.

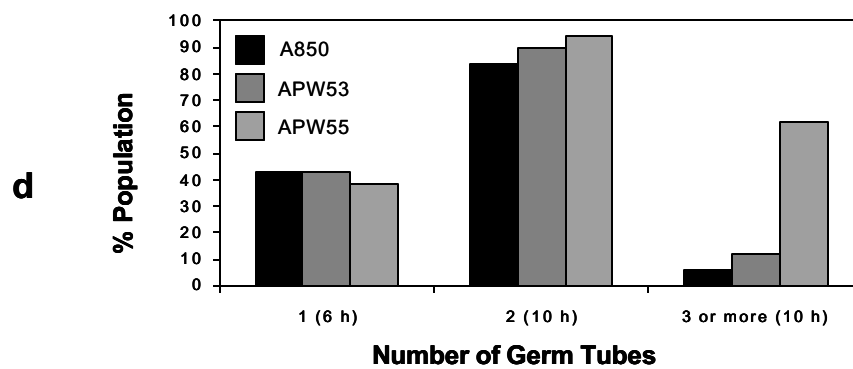
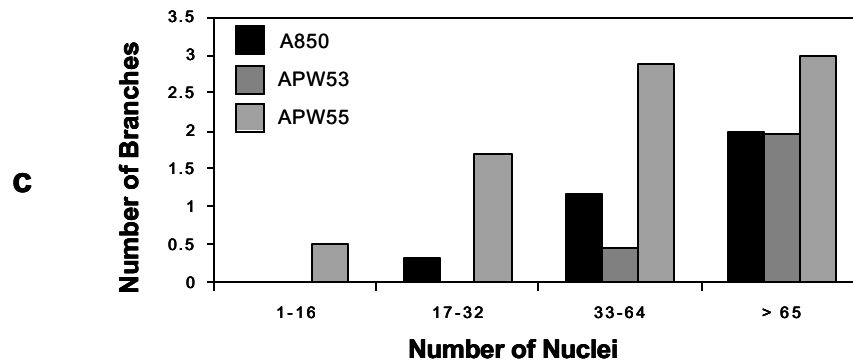
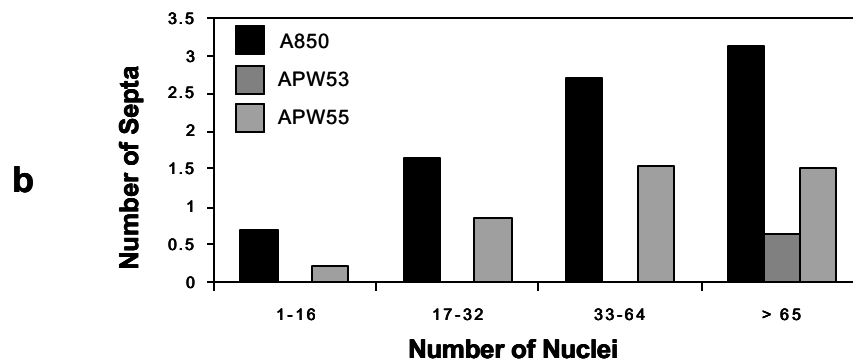
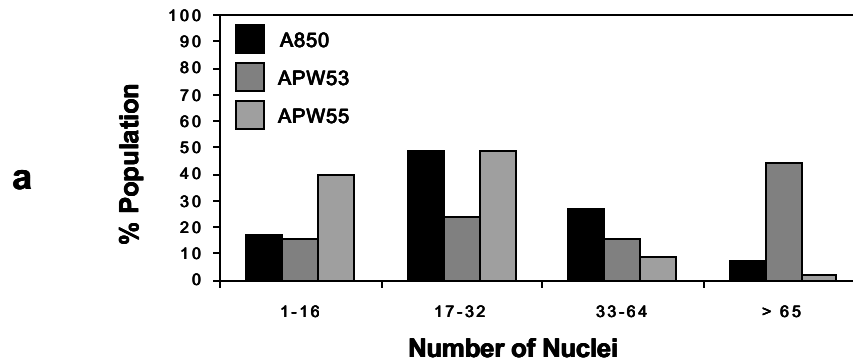


Figure 3.4. Morphology of *aspB* mutants. First column: A850 (wild type). Second column: APW53 (*aspB-271*). Third column: APW55 (*aspB-318*). a-c) Chitin and nuclear staining with Calcofluor and Hoescht of germlings grown for 12 h at 42° C. d-f) DIC image of germlings grown for 12 h at 42° C.

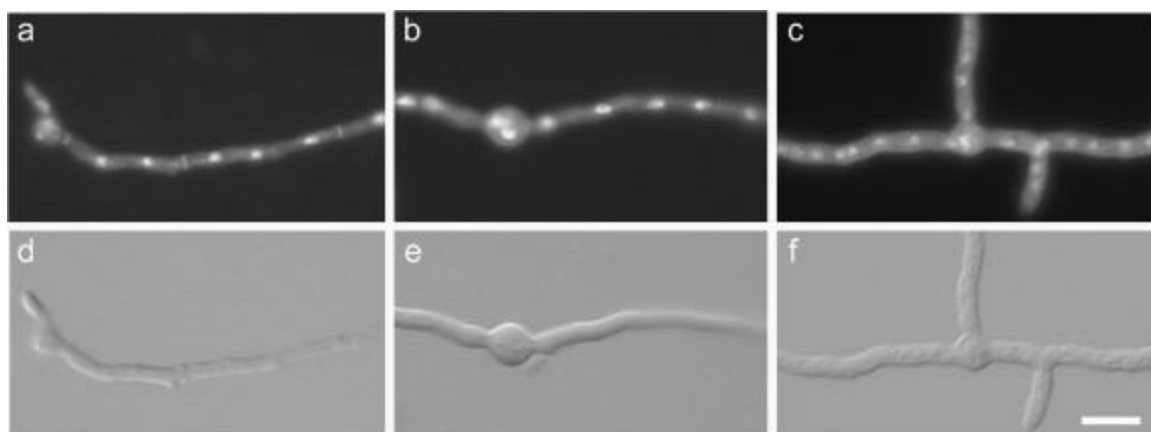


Figure 3.5. AspB localization at septa in mutants. First column: Chitin and nuclear staining with Calcofluor and Hoescht. Second column: AspB localization with FITC conjugated secondary antibody. Third column: Actin localization with Texas-red conjugated secondary antibody. a-c) Early septum formation in A850 (wild type). d-f) Late septum formation in A850. g-i) Early septum formation in APW53 (*aspB-271*). j-l) Late septum formation in APW53 (*aspB-271*). m-o) Early septum formation in APW55 (*aspB-318*). p-r) Late septum formation in APW55 (*aspB-318*).

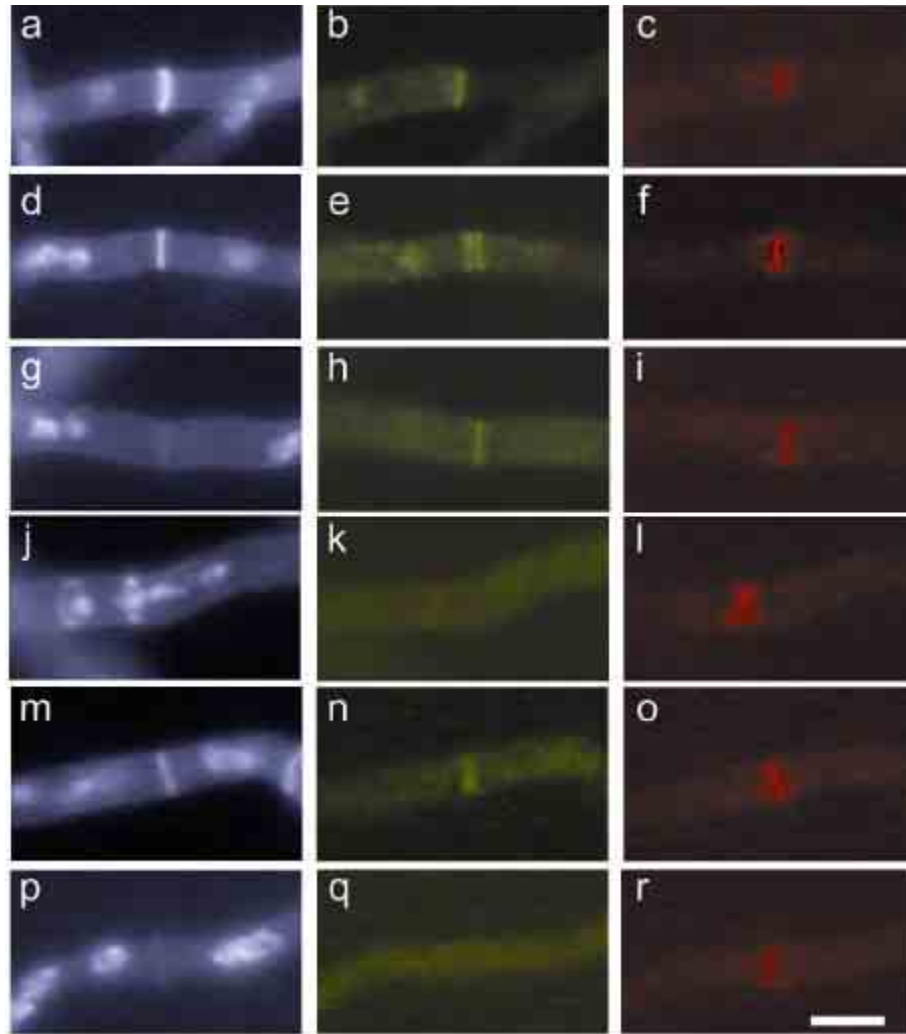


Figure 3.6. AspB localization at branches in mutants. First column: DIC images of emerging branches. Second column: AspB localization with FITC conjugated secondary antibody. Third column: Actin localization with Texas-red conjugated secondary antibody. a-c) A850 (wild type), d-f) APW53 (*aspB-271*), g-i) APW55 (*aspB-318*).

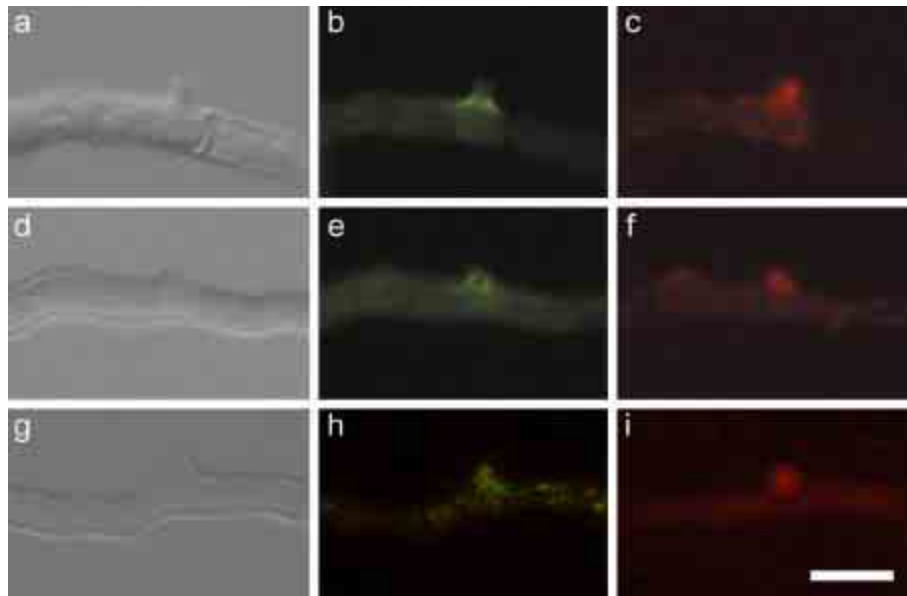


Figure 3.7. Staged conidiophore development in mutants. a) Presence of metulae with respect to time. b) Presence of phialides with respect to time. c) Presence of spores with respect to time. d) Stages of normal asexual development in *A. nidulans*.

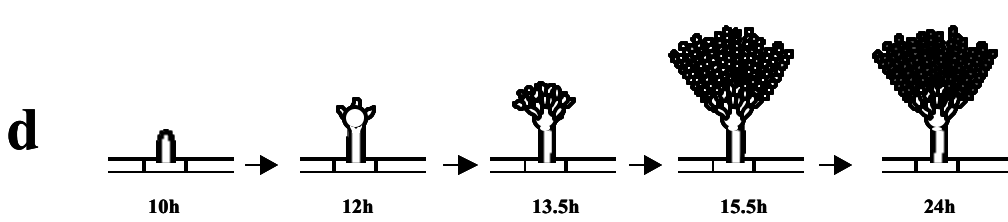
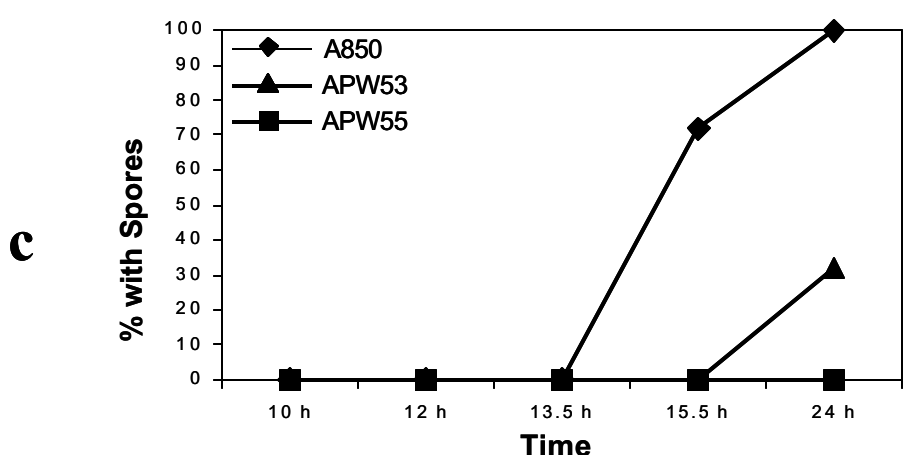
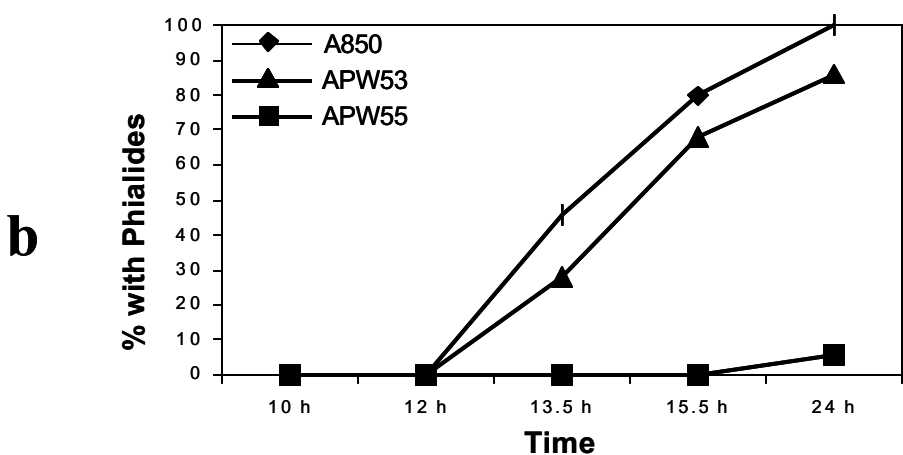
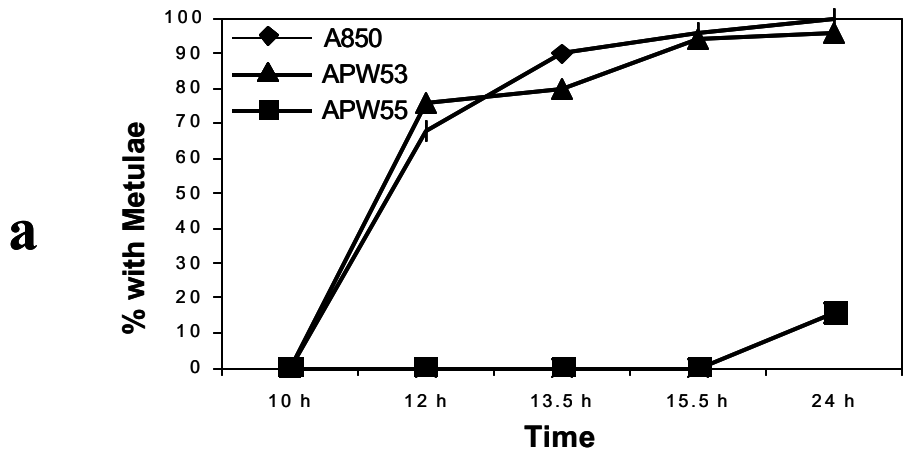


Figure 3.8. Cryofixation SEM of APW53 conidiophores. a) Vesicle formation 10 h post-introduction to an air interface. b) Metulae formation 12 h post-introduction to an air interface. c) Phialide formation 15.5 h post-introduction to an air interface. d) Spore formation 24 h post-introduction to an air interface.

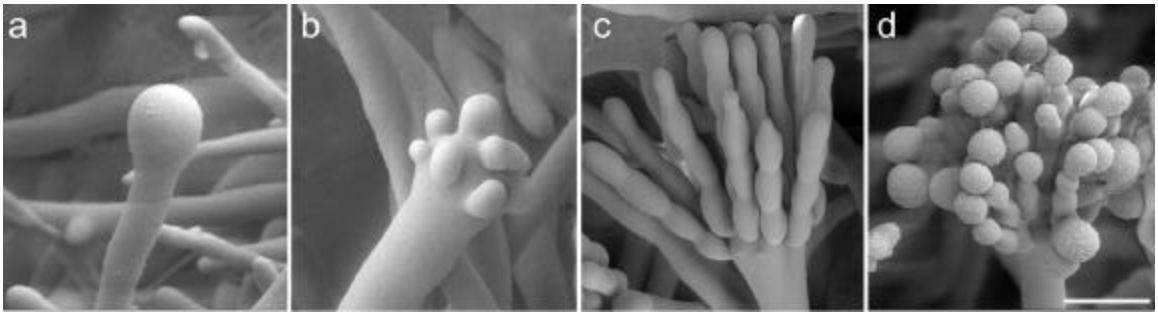
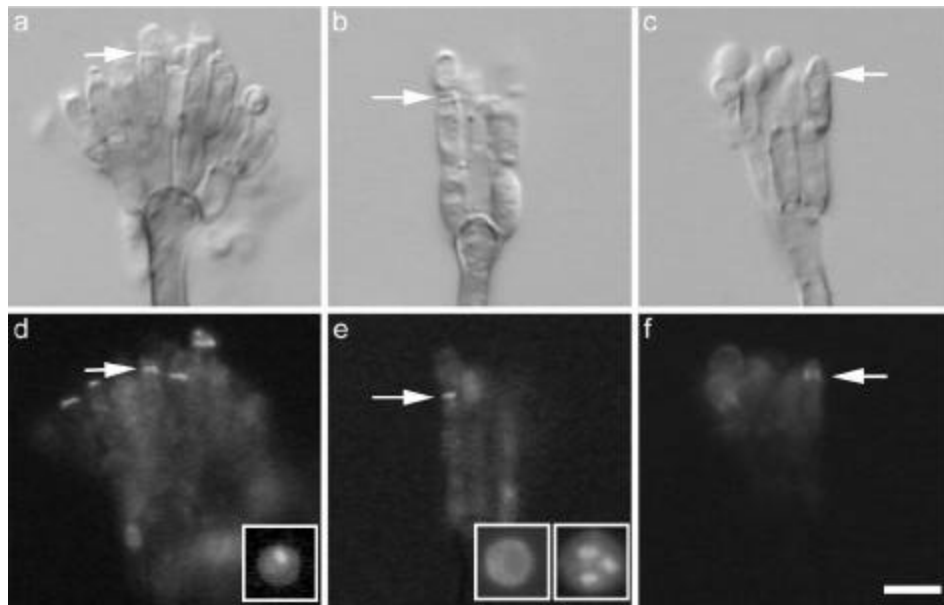


Figure 3.9. AspB localization to conidiophores in mutants. A850 (wild type). Second column: APW53 (*aspB-271*). Third column: APW55 (*aspB-318*). a-c) DIC images of conidiophores isolated from cultures grown for five days at 42° C. d-f) AspB localization in conidiophores. Arrows indicate rings of AspB at tips of phialides.



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CHAPTER 4

CONCLUSION

Septins: A Common Eukaryotic Theme

The ubiquitous presence of septins in eukaryotes, with the exception of plants and slime molds, suggests evolutionary significance. The general function of septins appears to be in cellular portioning and new growth with certain isoforms having more specialized functions. In the higher eukaryotes, they have been implicated in directing growth of synapses in brain tissue and nerve cells, and mutations in septins have been identified in certain forms of cancer (Beites *et al.*, 1999; Kalikin *et al.*, 2000; Zieger *et al.*, 2000; Kartmann and Roth, 2001). Better understanding of septins is essential not only for the characterization of this protein family, but also for an understanding of basic cell biology in higher eukaryotes.

Aspergillus nidulans is a valuable system for the study of septins. Its simple genetics, well-characterized cell biology, and quick generation time allow for ease in cytological studies and genetic manipulation. In addition, the differences in morphology and cellular biology along with its pathological significance often make it a better system than *S. cerevisiae* to use as a model for understanding certain aspects of development in higher eukaryotes. Polar growth in *A. nidulans* resembles that of nerve cells, multi-cellular differentiation is easily distinguished, and organelle structure is readily identifiable.

Septin Structure and Expression

Five septin homologues have been identified in *A. nidulans*. All five share greater than 35% amino acid identity and containing a P-loop motif. AspD is the only one that does not contain a coiled-coiled domain (Momany and Hamer, 1996; Momany *et al.*, 2001). Based upon northern blot analysis, *aspB* is the most highly expressed septin, during both vegetative and asexual development. *AspB* encodes for a 428 amino acid, 49 kD protein.

Antibodies raised against AspB identified the 49 kD protein on 10% SDS-PAGE gels. However, in experiments with 20% SDS-PAGE gels, anti-AspB antibodies resolved two bands. After determining that the second band was not another *A. nidulans* septin of similar molecular weight using 2 dimension gel analysis, we sought an alternate explanation for the second band. Comparison of the original cDNA and genomic sequence of AspB had revealed a 57 base pair, in-frame intron. Differential expression of AspB, with and without the intron, would explain the two bands seen on higher percentage SDS-PAGE western blots. Differential expression has been demonstrated for human septins and has been implicated in certain types of ovarian and breast cancer (Kalikin *et al.*, 2000; Zieger *et al.*, 2000; Kartmann and Roth, 2001). Differential expression of AspB could be important for the specificity of pre- versus post-mitotic localization or help localize proteins that are specialized for septation versus branching. The almost total absence of the higher molecular weight band in the *aspB-318* mutant could indicate its importance in branching. The *aspB-318* mutant was localized early in septation, but was totally lacking at the sites of branch emergence, suggesting that the

higher molecular weight form may have a specialized role in regulating branch emergence.

Septins Scaffolds in Cytokinesis

One of the most important aspects of septins is their ability to act as cellular scaffolds and recruit other proteins to sites of new growth. The identification of septin-interacting proteins is vital to the understanding of how septins organize and direct new growth in *A. nidulans*. Potential interacting proteins include chitin synthases, SUMO homologues, and cell cycle regulators. The polarity of septin localization during septation could help differentially localize interacting proteins to specific regions of the septum.

Mutant analysis demonstrated that chitin staining, as viewed by Calcofluor White, is not as bright in *aspB* mutants, indicating that the septum may not be properly organized. In *S. cerevisiae*, the septins help with directing the Chitin Synthase III complex, and forming the bud scar. In *A. nidulans*, the septum consists of cell wall materials, including chitin, and it is likely that AspB plays a role in directing its deposition.

Modification of septins by a SUMO-like protein has been demonstrated in *S. cerevisiae*. Three of the septins, Cdc3p, Cdc 11p, and Sep7p, contain the consensus sequence for modification by *S. cerevisiae*'s SUMO homologue, Smt3p (Johnson and Blobel, 1999; Johnson and Gupta, 2001). SUMO's usual role is to stabilize or compartmentalize modified proteins. One of the mutant alleles of *aspB* identified in this study, *aspB-318*, contains a mutation in a potential sumolation site. In addition, a SUMO-like protein has been identified, but not characterized in *A. nidulans* by searching

an available EST database. If AspB is modified by a SUMO homologue, and this modification does not take place in the *aspB-318* mutant, the result might be a highly unstable septin scaffold that is disassembled prematurely.

AspB is localized to the site of septation in an actin-dependent manner, unlike its actin-independent localization in *S. cerevisiae*. Does AspB interact directly with actin, or is some intermediate protein responsible for the recruitment of AspB to the site of septation? SepA, SepG, or SepH would be potential candidates, for such an intermediate protein,.

Septin Scaffolds in Branching and Secondary Germ Tube Emergence

The role of AspB in marking the site of branch emergence was an exciting discovery of this study. Very little is known about how branching occurs and is regulated in *A. nidulans*. Early models suggested that the septum might act as a damming mechanism, allowing for the build up of vesicles behind the crosswall and the formation of a branch (Fiddy and Trinci, 1976). This did not explain why branches sometimes occurred in the middle of the hyphal compartment or why they occasionally occurred in tip cells where septa had not yet formed. The identification of AspB as a marker of branch initiation, suggests it could recruit vesicles and promote their fusion at the site of branch emergence, similar to the role that is played by the septins found in mice and human nerve cells (Beites *et al.*, 1999; Field and Kellogg, 1999; Takizawa *et al.*, 2000; Kartmann and Roth, 2001).

The hyperbranching *aspB-318* mutant indicates that branching may be under negative regulation in wild type cells. The absence of this suppressor (perhaps dependent on AspB for its sequestration) would result in uncontrolled branching. In addition, many

of the branch bases seen in the mutant are much broader, suggesting that the area is not defined properly.

Compartmentalization of nuclei by septation results in their being arrested in interphase until a new axis of polarity is established. How this new axis of polarity is established and how nuclei become mitotically active again is not understood. A septin scaffold at the site of branch emergence could potentially explain both. Several cell-cycle-regulatory kinases have been identified in *S. cerevisiae* that localize to the septin ring at the mother bud neck. Failure of the septin ring to form does not allow for proper localization of these kinases and delays mitosis. In *A. nidulans*, proper localization of mitotic activators at branch points could signal the resumption of mitosis in nearby nuclei. Since AspB localizes pre-mitotically to the site of branch emergence, its role as part of the scaffolding used to recruit mitotic activators is a very attractive model.

AspB could play a similar role in secondary germ tube emergence from the conidium. It is becoming more and more apparent that there are both morphological and physiological differences between the initiation of polarity of the initial germ tube, and that of subsequent germ tubes and branches. Although it seems reasonable that some proteins are used for both kinds of polarity establishment events, it is also clear that AspB is only involved in the latter. Our results also show that secondary germ tube emergence from the conidium is more similar to branching from a compartmentalized segment of the hyphae. The identity of components used for initial polarity establishment has been addressed by work from our lab (Momany *et al.*, 1999). However, more work is needed to identify not only the septins and septin-dependent components of branching and

secondary germ tube emergence, but also *A. nidulans* septins that may be involved in primary germ tube emergence.

Septin Scaffold and Asexual Development

Asexual development in *A. nidulans* involves both the initiation of new growth and the compartmentalization of specialized structure. The growth that takes place during conidiophore development is morphologically similar to budding growth in *S. cerevisiae*. After vesicle formation, the site of metula emergence must be marked and those proteins responsible for the development of the structure must be recruited to these areas. Despite the morphological similarity to yeast development, this process appears to be uncoupled from nuclear development. Nuclei in the vesicle first divide, and then move out into the metula, whereas in yeast nuclear division takes place at the neck. Several components important for conidiophore development have been identified. Two of these, *brlA* and *medA* have mutant alleles that produce conidiophores with phenotypes similar to those seen in the *aspB* mutants (reviewed by Miller, 1993). It is conceivable that the septin scaffold formed at the vesicle/metulae and then the metula/phialides interface helps recruit those like BrlA and MedA to their proper location.

Unlike the transient localization of AspB to the vesicle/metula and metula/phialade interfaces, its localization at the tips of phialides as spores emerge is persistent. The persistence could be for two reasons: (1) the area is constantly undergoing new growth and AspB is acting as a scaffold for the necessary proteins needed to maintain growth this , or (2) the area is constantly undergoing rounds of cytokinesis as each new spore is separated and long chains of spores form. Considering

the phenotype of the *aspB-271* allele the second scenario seems more likely because the irregular spore size and inconsistent nuclear number suggest that a cytokinetic defect is occurring.

Future Septin Studies in A. nidulans

Although the characterization of septins in *A. nidulans* has been started, much work remains to be done. Northern blot analysis of septins from *A. nidulans* has shown them to be ubiquitously expressed, with *aspB* and *aspC* showing the highest level of expression (Momany *et al.*, 2001). *AspC*, based on its higher level of expression during vegetative growth, might have similar localization patterns to *AspB* during septation and branching. *aspA*, *aspD*, and *aspE* are more likely to be involved in asexual development, since their mRNA levels peak during this stage of the life cycle. Septin-specific antibodies, GFP-tagged septins, or His-, or HA-tagged septins would all allow for localization studies and provided a clearer understanding of which septins are involved in which developmental process.

The identification of protein-protein interactions, both between septins and with other proteins, remains one of the biggest areas to be investigated. Yeast two-hybrid systems have been developed for *A. nidulans* and would be a valuable tool for identifying these interactions. In addition, co-immunoprecipitation or “fishing” experiments using tagged septins bound to columns could identify proteins that are able to bind septins. Once these septin interacting proteins have been identified, it remains to elucidate their specific roles, either in cytokinesis, branching, or conidiophore development, or perhaps some novel role.

The study of septins has grown quickly in the last 30 years. Despite having been identified in so many organisms, their function still is not clearly defined. In yeast they define the bud site, organize the division plane, act as a morphological checkpoint, and play a role in spore formation. In some higher eukaryotes they direct vesicle fusion in nerve tissue. Localization and genetic studies of septins in *A. nidulans* show that, like yeast, they play a role in cytokinesis and spore development. However, the septins' role in marking the site of branch emergence in *A. nidulans* also suggest that, like mice and humans, they may organize areas of new growth and direct vesicle fusion. By examining septins in *A. nidulans* we are afforded the opportunity to study the potential of all of these aspects of development in one system.

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APENDIX A
ASPERGILLUS NIDULANS SWO MUTANTS SHOW DEFECTS IN POLARITY
ESTABLISHMENT, POLARITY MAINTENANCE, AND HYPHAL
MORPHOGENESIS¹

¹Momany, M., Westfall, P.J. and G. Abramowsky. 1999. *Genetics*. 151:557-567
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ABSTRACT

When the spores of filamentous fungi break dormancy, they grow isotropically, adding new cell wall material uniformly in every direction. Later they switch to polarized growth, with new cell wall material added to the tip of an emerging germ tube. In order to identify genes involved in the synthesis and localization of new cell wall growth in filamentous fungi, we screened a collection of temperature-sensitive *A. nidulans* mutants for swollen cells. Defects in either cell wall localization or synthesis can presumably disrupt morphogenesis, giving rise to misshapen, swollen hyphae. Because cell wall localization ultimately depends upon cell polarity, we expected that most, if not all, of our mutants would have polarity defects. We have isolated mutants representing eight genes involved in polarity establishment, polarity maintenance and hyphal morphogenesis. Our results suggest that polarity establishment and polarity maintenance are separate events and that a persistent signal is required for apical extension in *A. nidulans*.

INTRODUCTION

All cells must organize areas of new growth in order to maintain their shapes and function properly. Fungi employ distinctive patterns of new cell wall addition that ultimately lead to their growth as unicells, in the case of yeasts, or as hyphae, in the case of filamentous fungi. These distinctive patterns of cell wall deposition are the visible result of changes in cellular polarity. Many details of the polarity establishment that determines localization of new cell wall material are known for the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. In contrast, the processes of polarity

establishment and subsequent new cell wall synthesis and localization are relatively unexplored in filamentous fungi.

The budding yeast *S. cerevisiae* shifts between isotropic and polarized growth modes (Lew and Reed, 1995; Roemer et al., 1996). The nascent bud emerges from a specific site on the cell surface and grows by apical extension, adding new material at the tip of the bud. After DNA replication is completed, this polar growth is replaced by isotropic growth and the bud begins to expand uniformly. Eventually, a septum separates the mother cell and daughter bud. Mother and daughter each begin the process again with emergence of a new bud in a polarized manner. Throughout this process, actin is concentrated in areas of growth where it is thought to allow transport of vesicles carrying materials needed to build the new cell wall. Several gene products required for the selection of the new growth sites have been identified. These include actin, the septins and products of the BUD genes.

The fission yeast *Schizosaccharomyces pombe* also switches between two distinct growth patterns (Mitchison and Nurse, 1985). Immediately after dividing by fission, new cells show unipolar growth, extending only from one end of the cell. Early in G2, the cell switches to bipolar growth, extending from both ends. After cytokinesis, mother and daughter begin the cycle again with unipolar growth at one end. As is true for *S. cerevisiae*, actin is concentrated in the regions of new cell wall (Marks and Hyams, 1985). Recently two genes important for establishing polarity in *S. pombe* have been identified. The *tea1* and *pom1* gene products are found at the ends of the fission yeast at various times in the cell cycle and are thought to play a role in localization of the growth machinery (Bahler and Pringle, 1998; Mata and Nurse, 1997).

Like yeast, filamentous fungi employ two distinct growth modes. When spores of filamentous fungi break dormancy, they first grow isotropically, adding new cell wall material uniformly in every direction. Later they switch to polarized growth, with new cell wall material forming an emerging germ tube (Bartnicki-Garcia and Lippman, 1969; Harris, 1997). This polarized growth continues with the addition of cell wall material exclusively at the tip of the germ tube, a process known as apical extension. Apical extension ultimately shapes cells into the elongated filaments, or hyphae, typical of many fungi. As is true for budding and fission yeast, actin is concentrated in the areas of new cell wall growth (Heath, 1990). The Spitzenkörper, a distinctive collection of vesicles, is also seen in the growing tip where it is thought to function as a "vesicle supply center," organizing the materials needed to build the new cell wall (Reynaga-Pena et al., 1997). As pointed out by Harris (1997), the critical difference in polarity establishment between yeasts and filamentous fungi is that the axis of polarity in yeasts re-orient, but once established, the axis of polarity in filamentous fungi remains fixed.

Though the signal(s) responsible for polarity establishment in filamentous fungi are still unknown, several molecules implicated in subsequent hyphal extension and morphogenesis have recently been identified. In *Neurospora crassa*, deletion of the microtubule dependent motor protein kinesin results in cells that are shorter and fatter than wild-type (Seiler et al., 1997) and a mutant with defects in the regulatory subunit of cAMP-dependent protein kinase shows hyphal swelling (Bruno et al., 1996). In *Aspergillus nidulans*, loss of type I myosin function leads to enlarged cells that cannot grow by apical extension (McGoldrick et al., 1995) and mutation of *sepA*, which encodes an FH 1/2 protein, results in cells with abnormally wide hyphae and no septa (Harris et

al., 1997). Normal patterns of hyphal development can also be disrupted by mutations in *samB*, which encodes a novel Zn-finger containing protein (Kruger and Fischer, 1998).

In order to identify genes involved in the synthesis and localization of new cell wall material in filamentous fungi, we screened a collection of temperature-sensitive *A. nidulans* mutants for swollen cells. Defects in either cell wall localization or synthesis can presumably disrupt morphogenesis, giving rise to misshapen, swollen hyphae. Because cell wall localization ultimately depends upon cell polarity, we expected that most, if not all, of our mutants would have polarity defects. We have isolated mutants representing eight genes involved in polarity establishment, polarity maintenance and hyphal morphogenesis. Our results suggest that polarity establishment and polarity maintenance are separate events and that a persistent signal is required for apical extension.

MATERIAL AND METHODS

Aspergillus Strains and Growth Methods

Strains used in this study are listed in Table A.1. Generation of the temperature-sensitive mutant collection has been previously described (Harris et al., 1994). Media used were complete medium (1% glucose, 0.2% peptone, 0.1% yeast extract, 0.1% casamino acids, nitrate salts, trace elements, and 0.01% vitamins, pH 6.5), or minimal medium (1% glucose, nitrate salts, trace elements, pH 6.5). Trace elements, vitamins, nitrate salts, and amino acid supplements are described in the appendix to Kafer (1977). For solid media 1.8% agar was added.

Strain construction and genetic analysis were by standard *A. nidulans* techniques (Harris et al., 1994; Kafer, 1977). In addition to strains listed in Table A.1, swo mutants with pabaA6 or argB markers were generated by crossing the original temperature-sensitive swo isolates listed in Table A.1 (swo, pabaA6; biA1) to AH12 (argB; chaA-1). Swo strains with reciprocal argB and pabaA6 markers were used for diploid construction and crosses.

Staining and Microscopy

Examination of phenotypes in liquid medium has been previously described by Harris et al.(1994). Ten ml complete liquid medium was inoculated with $1-5 \times 10^4$ conidia/ml, poured into a Petri dish containing a glass coverslip, and incubated at 42°C for 12-16 hours. Coverslips with adhering germlings were fixed in 3.7% formaldehyde, 50mM phosphate buffer (pH7.0) and 0.2% triton for 30-60 minutes. Coverslips were then washed with water, incubated 5 minutes with 10 µg/ml Calcofluor (American Cyanamid) and 100ng/ml Hoechst 33258 (Sigma), washed again and mounted on a microscope slide for viewing. Germlings were photographed using a Zeiss Axioplan microscope and Zeiss MC100 microscope camera system with Kodak Tmax 100 film.

Localization of Cell Wall Addition

We initially attempted to label cell walls with the lectin wheat germ agglutinin coupled to fluorescein isothiocyanate (FITC; Sigma). In multiple experiments, the lectin did not label a band at the basal end of the germ tube. Therefore, we raised antibodies against total cell walls for use in labeling experiments. *A. nidulans* A28 was grown for 48 hours in complete liquid medium. Hyphae were homogenized and walls were isolated as previously described (Bull, 1970). Antibodies were produced by the Monoclonal

Antibody Production Facility at the University of Georgia as follows. Mice were inoculated with 50 μ g of cell walls in incomplete Freund adjuvant and boosted four times as previously described (Ste-Marie et al., 1990). Serum was collected and shown to contain antibodies against *A. nidulans* cell walls by an ELISA assay using cell walls as antigen.

For data presented in Figure A.3, conidia were inoculated to coverslips in 10ml complete liquid medium and incubated at 30°C for 4, 5, 6, or 7 hours. Coverslips with adhering germlings were removed from liquid medium and incubated with anti-cell wall antibody (diluted 1:1,000 in PBS) at room temperature for 30 minutes. Coverslips with adhering hyphae were then washed three times in phosphate buffered saline, transferred to fresh complete liquid medium, and incubated for an additional hour at 30°C.

Coverslips with adhering germlings were then fixed, stained with FITC-coupled anti-mouse mouse antibody (Sigma), washed three times in phosphate buffered saline, stained with Calcofluor and Hoescht 33258, and placed on a microscope slide for viewing. In control experiments omitting the antibodies against cell walls (primary antibody), no staining was seen. In controls omitting the final hour of incubation, the entire germling was labeled. In controls delaying treatment with antibodies against cell walls until after the final hour of incubation, the entire germ tube labeled. For data presented in Figure A.3, fifty germlings were counted for each time point. Experiments were repeated three times with essentially identical results. A typical data set is shown.

Temperature Shift Experiments

Temperature shift experiments were carried out as described above (staining and microscopy) except for changes in incubation times and temperatures. For downshift

experiments (Figure A.4), conidia were inoculated to complete medium at 42°C, incubated for 10 hours, and transferred to 30°C for 2 hours prior to fixing and staining. For upshift experiments (Figure A.5) conidia were inoculated to complete medium at 30°C, incubated for 10 hours, and transferred to 42°C for 2 hours prior to fixing and staining. Experiments were repeated 3 times with essentially identical results.

Upshift experiments with localization of new growth (Figure A.6) were carried out as described above with the following modifications. Conidia were incubated for 11.5 hours at 30°C, treated with antibodies against cell walls for 30 minutes, transferred to fresh media prewarmed to 42°C and incubated for 4.5 hours at 42°C prior to processing as described above (see Localization of cell wall addition).

RESULTS

Identification of swo Mutants

To identify genes important in cell wall synthesis and localization, we screened a pre-existing collection of 1,200 temperature-sensitive *A. nidulans* mutants (Harris et al., 1994) for strains which showed a swollen cell phenotype on solid media at restrictive temperature. Fifty-five strains were identified as putative swollen cell (swo) mutants based on an initial stereoscopic examination. The putative swo mutant phenotypes ranged from large, round cells with small germ tubes to hyphae with distended, bulging regions (data not shown).

Genetic Characterization

Because *A. nidulans* is haploid, single gene mutations are expected to segregate 1:1 in crosses with wild-type. The fifty-five putative swo mutants were crossed to wild-

type and progeny were scored for temperature-sensitivity and swelling. Nine mutants showed both 1:1 segregation and linkage of temperature-sensitivity to the swollen cell phenotype (Table A.2).

To determine whether the swo mutations are dominant or recessive, stable diploids were constructed with wild-type strains. All diploids were wild-type at restrictive temperature indicating that the swo mutations are recessive (Table A.2).

To determine if any of the nine swo mutants are allelic or tightly linked, crosses were made for every pairwise mutant combination using swo mutant strains containing reciprocal auxotrophic markers. The recovery of wild-type progeny from a cross was interpreted to mean that each parent strain had a lesion in a different swo gene. Wild-type progeny were recovered from every cross except those derived from ts6-90 by ts7-91 (data not shown). Stable diploid strains were constructed from fusion of ts6-90 and ts7-91 derivatives to determine whether they represented alleles or tightly linked genes. All ts6-90/ts7-91 diploids were temperature-sensitive indicating that the respective haploids are allelic. Ts6-90 and ts7-91 were designated swoA-1 and swoA-2, respectively. The other seven swo mutants were designated swoB - swoH (Tables A.1 and A.2).

Phenotypic Characterization

The phenotypes of the swo mutants on solid media were observed by inoculating to complete solid medium and incubating for two days at restrictive temperature. All mutants were able to send out germ tubes, though both alleles of swo A made only a few. Most of the swo mutants did not make the asexual spores (conidia) typically seen in wild-type strains after 2 days growth, though sparse conidiation was visible in cultures of

swoB, swoD, and swoF. Many morphological mutants show wild-type growth when extra osmoticum is added to the medium. This restoration to the wild-type phenotype, known as osmotic remediality, is especially likely in the case of mutants where a weakened wall cannot resist internal turgor pressure. The swo mutants were tested for osmotic remediality on solid medium containing high levels of sucrose, sorbitol, or potassium chloride. Sucrose was the best osmotic stabilizer and so only those results are reported (Figure A.1, Table A.2, and data not shown). Four of the mutants (swoA, B, F, and G) were osmotically remedial at the restrictive temperature. Three (swoC, D, and E) were osmotically remedial at the semi-permissive temperature. Only one mutant showed no osmotic remediality (swoH).

The dye Calcofluor binds to β -linked glucans and chitin (Maeda and Ishida, 1967). Some classes of cell wall mutants are resistant to the dye (Elorza et al., 1983; Roncero et al., 1988). By growing *A. nidulans* on media containing various concentrations of Calcofluor, we determined that wild-type is resistant to Calcofluor at 0.1mg/ml, but is sensitive to Calcofluor at 0.25mg/ml (data not shown). Two of the swo mutants could not grow well in the presence of 0.1mg/ml Calcofluor and thus are Calcofluor hypersensitive (swo A and B; Figure A.1, Table A.2). None of the mutants were able to grow in the presence of 0.25mg/ml Calcofluor (data not shown). Thus, none of the swo mutants are Calcofluor resistant.

The phenotypes of the swo mutants in liquid were observed by inoculating on coverslips in complete medium and incubating at permissive or restrictive temperature (Figure A.2). After 12 - 16 hours incubation, germlings were fixed and stained with Calcofluor and Hoescht 33258 to stain chitin and nuclei, respectively. At permissive

temperature, *swoB* and *swoG* showed subtle phenotypes, while all other mutants were indistinguishable from wild-type. Only 26% of *swoB* germlings had septa after overnight growth at permissive temperature, compared to 100% in wild-type control cultures. The *swoG* mutant showed a high level of dichotomous branching, a phenotype rarely seen in wild-type. At restrictive temperature, four of the *swo* mutants did not generally form germ tubes, instead showing isotropic or nonpolarized growth (*swoA*, *C*, *D*, and *F*; Figure A.2, Table A.3). The *swoA-1* allele occasionally formed germ tubes, while *swoA-2* never did. We postulate that *swoA-1* is a leaky allele. The other mutants grew in a polar manner, but exhibited hyphal swelling or other morphological abnormalities (*swoB*, *E*, *G*, and *H*; Figure A.2, Table A.3).

The switch from isotropic to polar growth in wild-type: When wild-type *A. nidulans* conidia germinate, they first expand uniformly in every direction and later grow by addition of new cell wall material at the tip of the germ tube. To define the timing of this switch from isotropic to apical growth relative to nuclear divisions, conidia of wild-type *A. nidulans* were inoculated onto coverslips in complete medium, grown for 4, 5, 6, or 7 hours, and incubated with polyclonal antibodies against cell walls for 30 minutes. Germlings were then washed, transferred to fresh medium without antibodies, and incubated for another hour. They were then fixed and stained with a fluorescently labeled secondary antibody (to label all growth prior to the final hour of incubation), Calcofluor (to label chitin), and Hoescht 33258 (to label nuclei). Cells that showed uniform antibody staining were scored as growing in a nonpolarized manner during the final hour of incubation. Cells that showed a localized unlabeled zone were scored as growing in a polarized manner during the final hour of incubation. Nuclear number and

growth polarity were scored for each time point. As shown in Figure A.3, cells grew isotropically for the first 6 hours of incubation. At 7 hours, when most of the cells had 4 nuclei, the switch to polar growth occurred. At this time cells had a slightly pointed end, rather than being perfectly round. During the eighth hour of incubation, when most of the cells had 8 nuclei, the germ tube grew by apical extension and the first septa were seen (data not shown). All of the *swo* mutants have at least four nuclei at restrictive temperature (Figure A.2) and so should be able to grow in a polar manner.

Temperature Shift Experiments

To determine the effect of the *swo* mutations on polarity, conidia were inoculated onto coverslips in liquid medium, incubated at restrictive temperature for 10 hours, shifted to permissive temperature and incubated for another 2 hours. We thought that the defect in polarity establishment would also prevent germ tube extension and so expected that the mutants that do not show polarized growth at restrictive temperature (*swoA*, C, D, and F) would not be able to make germ tubes upon release from the temperature block. Indeed, *swoC*, D, and F behaved as expected: they did not make germ tubes within two hours of the shift to permissive temperature. The *swoA* mutant, however, did make germ tubes after the shift from restrictive to permissive temperature (Table A.3, Figure A.4). In fact, each cell made 3-4 normal looking germ tubes. Interestingly, these germ tubes were almost exclusively on one half of the cell, rather than being equally spaced. Ninety-five percent of hyphae formed in *swoA*-1 and 100 percent of the hyphae formed in *swoA*-2 after the downshift emerged from only one half of the cell (Figure A.4 and data not shown). As expected, the four *swo* mutants that showed polarized growth at restrictive

temperature (swoB, E, G, and H) showed normal apical growth of hyphae after the shift from restrictive to permissive temperature (Table A.3, Figure A.4).

To determine the effect of the swo mutations on hyphal elongation, conidia were inoculated onto coverslips in complete medium, incubated at permissive temperature for 10 hours, shifted to restrictive temperature and incubated for 2 hours. We expected that only the region of the hypha that grew after the shift to restrictive temperature (i.e., the apical region) would swell. Surprisingly, none of the swo mutants showed exclusively apical swelling after the shift to restrictive temperature (Table A.3 and Figure A.5). Both swoA-1 and A-2 showed pronounced swelling along the whole hypha. swoC and D showed slight basal swelling and apparently normal apical growth. SwoB and E both showed basal swelling with normal apical growth, while swoG and H looked normal in both basal and apical regions.

To better distinguish growth that occurred at permissive temperature from growth that occurred after the shift to restrictive temperature, conidia from the nonpolar swo mutants were inoculated onto coverslips in complete medium, grown at permissive temperature for 11.5 hours, and incubated with polyclonal antibody against *A. nidulans* cell walls for 30 minutes. The germlings were then washed and transferred to fresh medium without antibodies, incubated for 4.5 hours at restrictive temperature, fixed and stained with fluorescently labeled secondary antibody, Calcofluor and Hoescht 33258 (Figure A.6). Swo A-1, swoA-2 and swoF clearly showed swelling in the region of the hypha that had grown at permissive temperature (basal). In addition, swoA-1 (the presumed leaky allele) and swoF showed swollen growth in the region of the hypha that formed at restrictive temperature (apical). The swoA-2 allele showed no new apical

extension after the shift to restrictive temperature. Swo C and D both showed normal apical extension after the shift to restrictive temperature.

DISCUSSION

Phenotypes of the swo mutants: In order to identify genes involved in the synthesis and localization of new cell wall growth, we screened a collection of temperature-sensitive *A. nidulans* mutants for a swollen cell phenotype. Because the ultimate expression of polarity decisions is the localized deposition of new cell wall material, we expected that at least some of our swo mutants would have defects in polarity. We identified nine swo mutants representing eight genes. With the exception of swoH, the mutants show restoration of the wild-type phenotype by the addition of high osmoticum to the medium, hypersensitivity to the cell wall-binding dye Calcofluor, or both (Figure A.1 and Table A.2). These phenotypes are common among wall-related fungal mutants (Borgia et al., 1996; Borgia and Dodge, 1992; Livingston, 1969). The morphologies of the swo mutants fall into two broad categories: those that exhibit nonpolar growth in liquid medium at restrictive temperature (swoA, C, D, and F) and those that exhibit polar growth in liquid medium at restrictive temperature (swoB, E, G, and H; Table A.3 and Figure A.2).

Nonpolar swo mutants show defects in polarity establishment and maintenance: It seemed likely that the nonpolar swo mutants were defective in the transition from isotropic to polar growth. In time course experiments that used antibodies against cell walls to distinguish old and new growth in wild-type *A. nidulans*, we found that the isotropic to polar switch occurred at or near the second nuclear division (Figure A.3).

This is consistent with the previous observation that the germ tube emerges concomitant with the second nuclear division in *A. nidulans* (Harris et al., 1994). All of the *swo* mutants have at least four nuclei at restrictive temperature and therefore should be capable of growing in a polar manner.

When incubated at restrictive temperature for ten hours and then shifted to permissive temperature, *swoA* made germ tubes within 2 hours, while *swoC*, *D* and *F* did not (Figure A.4). We interpret this to mean that *swoC*, *D*, and *F* are unable to establish polarity at restrictive temperature, while *swoA* is able to establish polarity, but is blocked in the maintenance of polarity required for germ tube extension. This result was surprising because there has been no previous evidence that polarity establishment can be separated from polarity maintenance. We had assumed that the same signal would be responsible for both events and so expected that none of the nonpolar *swo* mutants would be able to make germ tubes after release of the temperature block. The pattern of germ tube emergence after release of the *swoA* temperature block was also a surprise.

In wild-type *A. nidulans*, after the first germ tube emerges and grows before a second germ tube emerges. This second germ tube is made 180° opposite the first (M.M., unpublished observations) in a pattern analogous to the bipolar budding pattern of diploid *S. cerevisiae* (reviewed by (Roemer et al., 1996). The 3-4 germ tubes made by *swoA* after release of the temperature block all emerged from one half of the cell in a pattern reminiscent of axial budding in haploid *S. cerevisiae* (Figure A.4). Though the spatial pattern is similar to axial budding in yeast, the temporal pattern is very different. In contrast to emergence of a single bud in yeast cells, it appears that several of the germ tubes emerged at the same time, since they were all the same length. There are at least

three possible explanations for the synchronous emergence of multiple germ tubes. It could mean that the signal that makes the cell surface competent for germ tube emergence is overproduced or able to diffuse in *swoA* and thus a larger area of the cell surface is "competent" for germ tube emergence. Or, perhaps a large portion of the cell surface is competent to send out germ tubes in wild-type, but there is only enough of a "germ tube inducer" to cause a single germ tube to be made. In this scenario, it is the germ tube inducer that is overproduced in *swoA*. Alternatively, the *swoA* product may have a role in establishing the bipolar pattern or in suppressing the axial pattern in addition to its role in germ tube emergence.

When incubated at permissive temperature for ten hours and shifted to restrictive temperature for two hours, *swoA*, C, D, and F showed varying levels of swelling in the basal region of the hypha (Figure A.5, Table A.3). Once more this result was a surprise. We expected that the region of the hypha that formed at restrictive temperature (the apical region) would swell. Only *swoF* showed obvious swelling at the apex. Using antibodies against cell walls and longer incubation times, we were able to distinguish growth that occurred before and after the temperature shift more clearly (Figure A.6). Our results showed that both *swoA-1* (the leaky allele) and *swoF* swelled in apical regions, while *swoA-2* ceased apical extension completely at restrictive temperature. Both *swoC* and D showed only slight swelling at the basal end and made normal germ tubes after the shift to restrictive temperature. We interpret these results to mean that a persistent signal is required for polarity maintenance in *A. nidulans* and that the *swoA-2* mutant lacks this signal at restrictive temperature. The *swoA-1* and *swoF* mutants appear to be partially defective in polarity maintenance, since they are capable of some apical

extension at restrictive temperature. The *swoC* and *swoD* mutants on the other hand, appear to have no defect in apical extension once polarity has been established.

Polar *swo* mutants show defects in hyphal morphogenesis: The remaining *swo* mutants (*swoB*, *swoE*, *swoG*, and *swoH*) appear to be able to establish and maintain polarity at restrictive temperature despite defects in hyphal morphogenesis (Figure A.2). The *swoB* mutant swells irregularly along the hypha and often ruptures, leaking cellular contents. This phenotype might indicate a defect in cell wall synthesis or assembly. The idea that *swoB* has a wall synthesis defect is consistent with its osmotic remediality and Calcofluor hypersensitivity (Figure A.1, Table A.2). The phenotype of the *swoE* mutant suggests a defect in branching and/or general hyphal patterning. Indeed, *swoE* has been isolated independently in screens for mutants with defects in cell patterning (*hypA*; (Kaminskyj and Hamer, 1998) and in polarity (*podA*; Steven D. Harris, personal communication). The *swoG* mutant shows dichotomous branching at restrictive temperature, once more suggesting a defect in hyphal patterning. The *swoH* mutant shows frequent Calcofluor bright areas along the hypha, possibly indicating overproduction or mislocalization of wall material disrupting the normal hyphal patterning.

When incubated at restrictive temperature for ten hours and then shifted to permissive temperature, all of the polar *swo* mutants showed swelling of the basal region and normal growth of the apical region as expected if polarity establishment and maintenance are functioning (Table A.3, Figure A.4). When incubated at permissive temperature for ten hours and then shifted to restrictive temperature, the effects on the nonpolar *swo* mutants were more subtle than those seen with the polar *swo* mutants (Table A.3; Figure A.5). *SwoB* gave the most dramatic result, with slight swelling and

an abnormally high number of branches. This may indicate that *swoB* plays a role in branch formation. *SwoE* showed slight basal and apical swelling while neither *swoG* nor *swoH* showed obvious abnormalities. The less pronounced effect of the shift from permissive to restrictive temperature may indicate that the events that require *swoG* and *H* have been successfully completed before the shift to restrictive temperature. That is they act early in hyphal morphogenesis. In view of their phenotypes at restrictive temperature and their behavior in downshift experiments, it seems more likely that the effects of *swoG* and *H* are simply not seen within the two hour time period of growth at restrictive temperature.

Model for the roles of the *swo* genes: We propose the following model for polarity establishment, polarity maintenance, and hyphal morphogenesis in *A. nidulans* (Figure A.7):

(1) At or near the second nuclear division, the expanding conidium switches from isotropic to polar growth (Figure A.3). This switch requires establishment of an axis of polarity that anticipates the location of germ tube emergence. The products of *swoC*, *D*, and *F* are all required to establish polarity based on the inability of these mutants to make germ tubes when released from the restrictive temperature in downshift experiments (Figure A.4, Table A.3). The *swoC* and *D* gene products are not required for germ tube emergence or apical extension based on the normal hyphae made by these mutants at restrictive temperature in upshift experiments (Figures A.5 and A.6, Table A.3).

(2) After polarity is established, a separate gene product is needed to maintain the axis of polarity so that the germ tube emerges. The product of the *swoA* gene is required for polarity maintenance, but not its establishment, based on the ability of the *swoA*

mutant to make germ tubes rapidly upon release from restrictive temperature in downshift experiments (Figure A.4, Table A.3). Because of their association with apically extending tip cells, we speculate that the *swoA* gene product may interact with actin and/or the Spitzenkörper. The *swoA* protein may also show similarities to the *tea1* or *pom1* proteins that appear to maintain polarity by marking the ends of *S. pombe* cells (Bahler and Pringle, 1998; Mata and Nurse, 1997). Future work will address these issues.

(3) The polarity maintenance gene product required for germ tube emergence is also required for apical extension to continue. Even well after polarity has been established, the *swoA* mutant is not able to extend apically at restrictive temperature (Figures A.6, Table A.3). The *swoF* product also appears to play some role in polarity maintenance, in addition to its role in polarity establishment, based on impaired apical extension in upshift experiments (Figures A.5 and A.6, Table A.3).

(4) Eventually the elongating hypha forms the branches and septa seen in mature fungal colonies. The products of *swoB*, *E*, *G* and *H* all appear to be involved in establishing proper hyphal morphogenesis with postulated roles including cell wall synthesis (*swoB*) and branch formation (*swoG*). Future work will examine these later events in hyphal morphogenesis more closely.

ACKNOWLEDGEMENTS

We express our thanks to John Hamer for generously providing the temperature-sensitive mutant collection, to Jered Brown for technical assistance, and to Debbie Haas for critical reading of the manuscript. This work was supported by a grant to M.M. from the Division of Energy Biosciences, U.S. Department of Energy. P.J.W. was supported

by a pre-doctoral training grant in molecular and cellular mycology from the National Institutes of Health.

Table A.1

A. nidulans strains

Strain	Genotype
A28a	pabaA6; biA1
AH12b	argB; chaA-1
AGA20	swoD
AGA24	swoC
AJB11	swoF
AJB15	swoB
AJB16	swoA-2
AJB17	swoE
APW14	swoH
APW18	swoA-1
APW22	swoG
ts1-67c	swoB; pabaA6; biA1
ts1-168c	swoG; pabaA6; biA1
ts6-90c	swoA-1; pabaA6; biA1
ts7-20c	swoH; pabaA6; biA1
ts7-28c	swoD; pabaA6; biA1
ts7-91c	swoA-2; pabaA6; biA1
ts7-99c	swoF; pabaA6; biA1
ts8-160c,d	swoE; pabaA6; biA1
ts10-4c	swoC; pabaA6; biA1

a Available from Fungal Genetics Stock Center, Dept. of Microbiology, University of Kansas Medical Center, Kansas City, KS.

b Obtained from John Hamer, Dept. of Biological Sciences, Purdue University, West Lafayette, IN.

c Original isolate from temperature-sensitive collection (Harris et al., 1993); Collection obtained from John Hamer, Dept. of Biological Sciences, Purdue University, West Lafayette, IN.

d Isolated independently in screens for hypercellular mutants (hypA, Kaminskyj and Hamer, 1998) and polarity defective mutants (podA, Steve Harris, personal communication).

Table A.2
swo Mutant Characteristics

Allele Designation	Ts+:Ts-a	Dominant/ Recessive ^b	Osmotic Remedial ^c	Calcofluor Hypersensitivity ^d
swoA-1	50:50	R	42°C	yes
swoA-2	50:50	R	42°C	yes
swoB	50:50	R	42°C	yes
swoC	49:50	R	37°C	no
swoD	53:47	R	37°C	no
swoEe	44:52	R	37°C	no
swoF	53:47	R	42°C	no
swoG	47:53	R	42°C	no
swoH	45:55	R	no	no

a Temperature-sensitivity of progeny from mutant by AH12 cross.

b D = dominant, R = recessive; Based on phenotype of swo/wildtype diploid.

c Based on ability of 1M sucrose to restore wild type growth on solid medium at semi-permissive (37°C) or restrictive temperature (42°C) relative to growth at the same temperature without sucrose; see Figure A.1.

d Based on ability of 0.1mg/ml Calcofluor to inhibit growth relative to wild type at permissive temperature (30°C); None of the swo mutants were resistant to Calcofluor at 0.25mg/ml (data not shown); see Figure A.1.

e Isolated independently in screens for hypercellular mutants (*hypA*, Kaminskyj and Hamer, 1998) and polarity defective mutants (*podA*, Steve Harris, personal communication).

Table A.3
Polarization of swo mutants

G.T. at 42°C? ^a	Mutant	Downshift ^b (42°C → 30°C)	Upshift ^{c,d} (30°C → 42°C)
no	swoA-1	Multiple hyphae, axial	Basal swells, new apical growth swells
no	swoA-2	Multiple hyphae, axial	Basal swells, no new apical growth
no	swoC	No hyphae	Basal swells slightly, new apical growth normal
no	swoD	No hyphae	Basal swells slightly, new apical growth normal
no	swoF	No hyphae	Basal swells slightly, new apical growth swells
yes	swoB	Basal swells slightly, apical normal	Basal swells, apical swells slightly, abnormal branching
yes	swoE	Basal swells, apical normal	Basal swells, apical swells at tip
yes	swoG	Basal swells, apical normal, hyperbranching	Basal normal, apical normal
yes	swoH	Basal swells, apical normal	Basal normal, apical normal

a G.T.= Germ tube; see Figure A.1.

b Basal = the older end of the germling, the conidium end; Apical = the newer end of the germling, the hyphal tip end; Conidia were inoculated onto coverslips in liquid medium, incubated at restrictive temperature for 10 hours, shifted to permissive temperature and incubated for another 2 hours; see Figure A.4.

c Conidia were inoculated onto coverslips in liquid medium, incubated at permissive temperature for 10 hours, shifted to restrictive temperature and incubated for another 2 hours; see Figure A.5.

d For the swo mutants that do not make germ tubes at 42°C, antibodies against total *A. nidulans* cell walls were used to distinguish growth that occurred before and after the temperature shift ; see Figure A.6.

Figure A.1. Phenotypes of swo mutants on solid medium. The swo mutants were inoculated to complete medium, complete medium with 1M sucrose, or complete medium with 0.1mg/ml Calcofluor and incubated for two days at permissive temperature (30°C), semi-permissive temperature (37°C), or restrictive temperature (42°C), as indicated. Top row (from left to right): APW18 (swoA-1), AJB16 (swoA-2), AJB15 (swoB), AGA24 (swoC), AGA20 (swoD), AJB17 (swoE); second row (from left to right): AJB11 (swoF), APW22 (swoG), APW14 (swoH), A28 (wild-type).

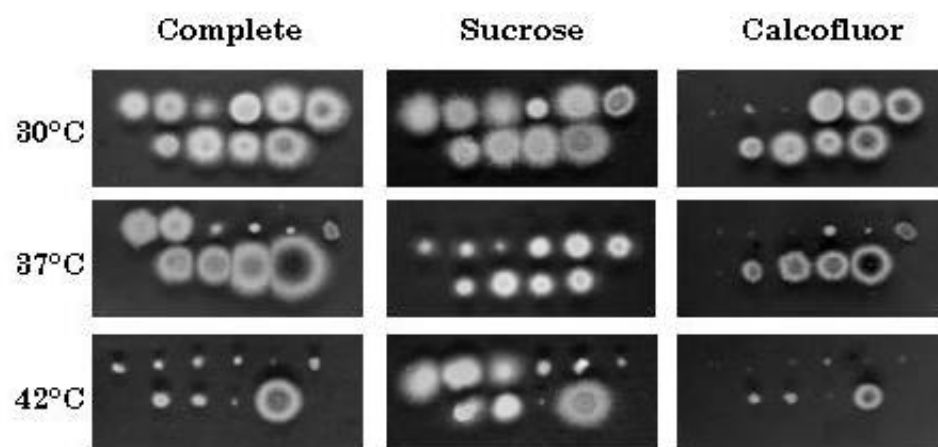


Figure A.2. Phenotypes of swo mutants in liquid medium. The swo mutants were inoculated onto coverslips in complete liquid medium and incubated at restrictive temperature for 12 - 16 hours. Germlings were fixed and stained with Calcofluor and Hoescht 33258 to stain chitin and nuclei, respectively. All strains are as listed in Figure 1. All micrographs are at the same magnification. Scale bar = 10 μ m.

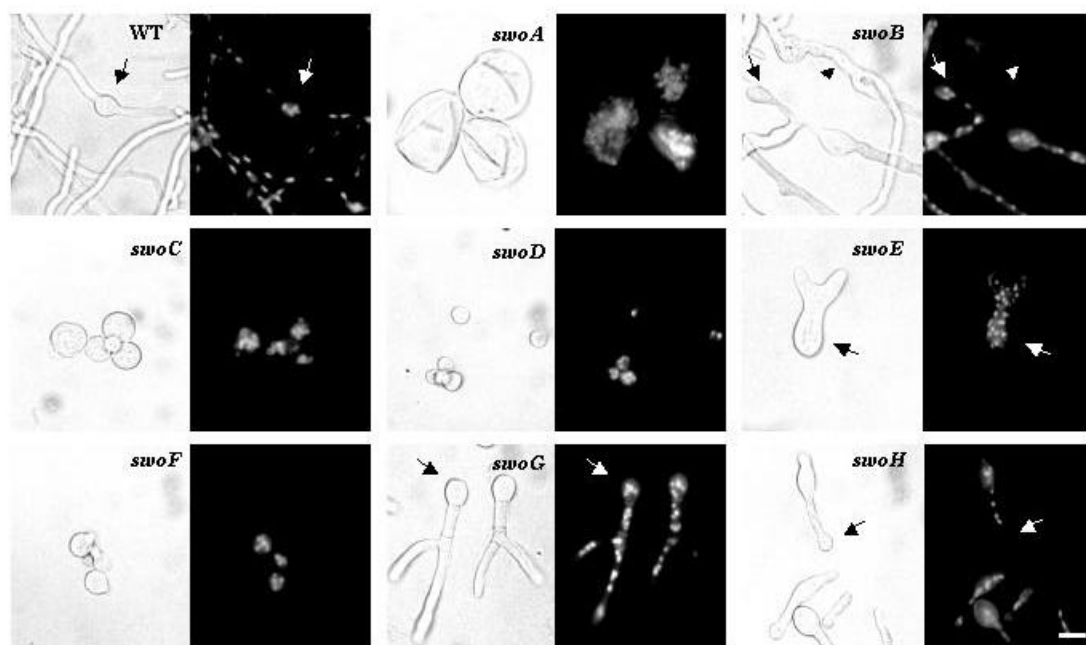
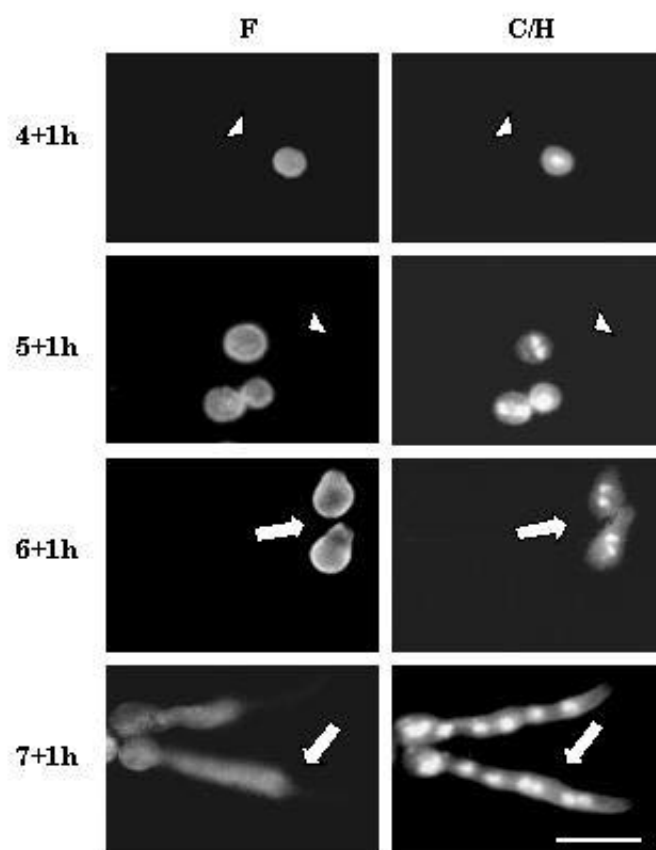


Figure A.3. The switch from isotropic to polar growth. Conidia of wild-type *A. nidulans* (A28) were inoculated onto coverslips in complete medium, grown for 4, 5, 6, or 7 hours, incubated with polyclonal antibodies against cell walls, washed, transferred to fresh medium without antibodies, and incubated for another hour. They were then fixed and stained with an FITC-labeled secondary antibody, Calcofluor, and Hoescht 33258. (A) Left column shows FITC staining (labels growth that occurred prior to the last hour of incubation). Right column shows Calcofluor and Hoescht 33258 staining (labels entire hypha and nuclei). Arrowheads denote examples of cells that grew in a nonpolar manner during the final hour of incubation. Arrows denote examples of germlings that grew in a polar manner during the final hour of incubation. All micrographs are at the same magnification. Scale bar = 10 μm . (B) Nuclear number and growth polarity were scored for 100 germlings for each time point. Cells that showed uniform antibody staining were scored as nonpolar. Cells that showed a localized unlabeled zone were scored as polar.

A



B

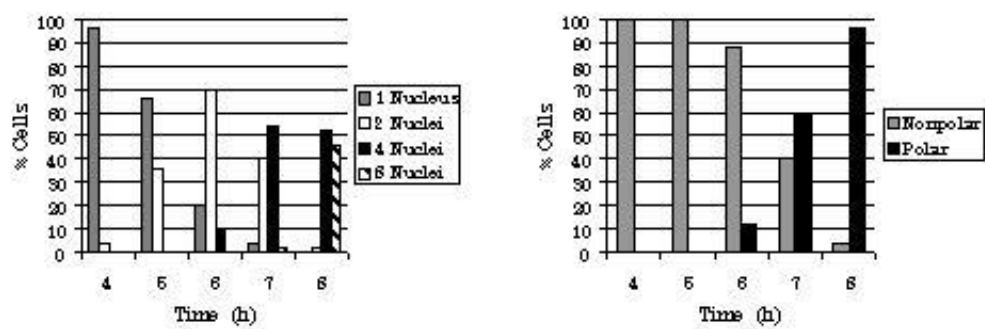


Figure A.4. Phenotypes of two mutants shifted from restrictive to permissive temperature. Conidia were inoculated onto coverslips in liquid medium, incubated at restrictive temperature for 10 hours, shifted to permissive temperature and incubated for another 2 hours, fixed and stained with Calcofluor and Hoescht 33258. Note that many regions of the hyphae are so Calcofluor-bright that nuclei are not visible. All micrographs are at the same magnification. Scale bar = 10 μ m.

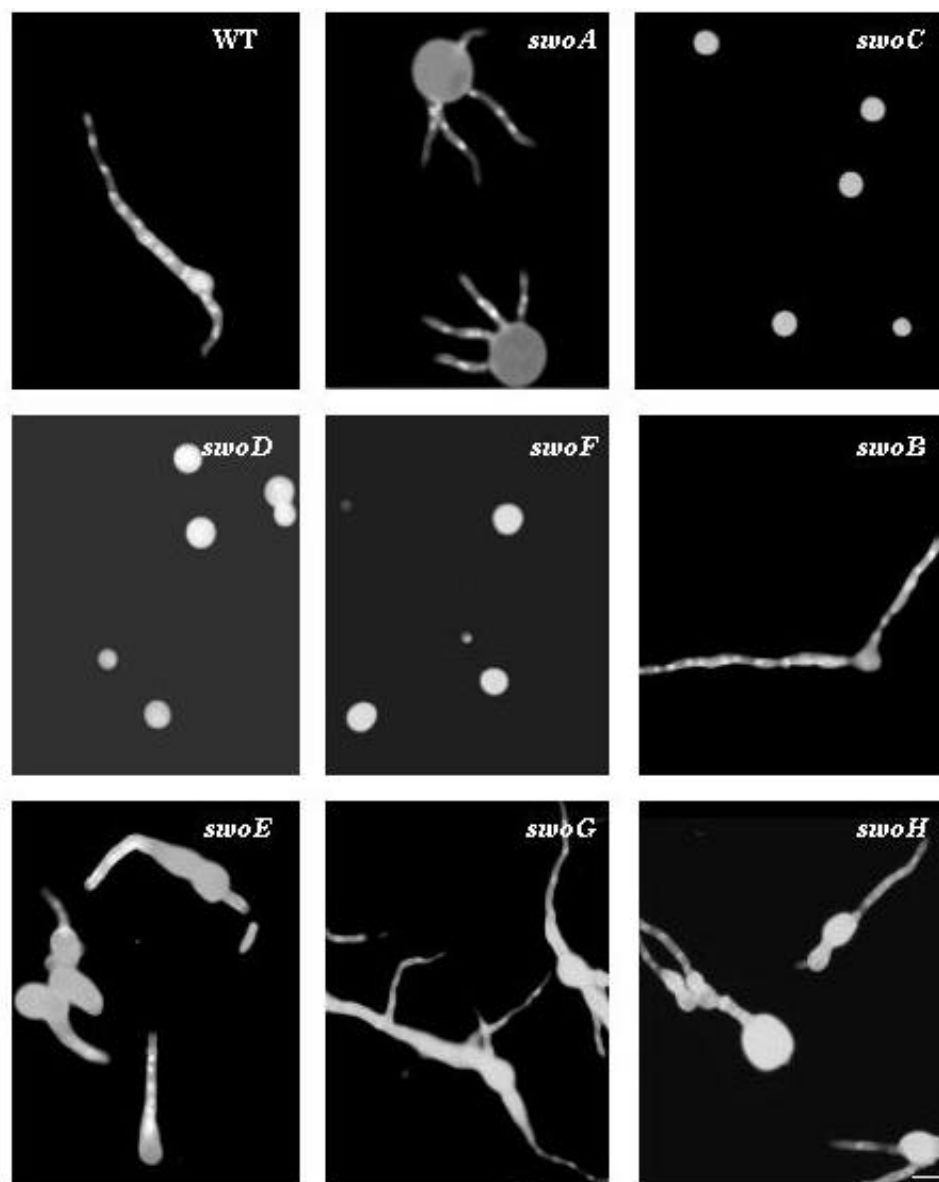


Figure A.5. Phenotypes of two mutants shifted from permissive to restrictive temperature. Conidia were inoculated onto coverslips in liquid medium, incubated at permissive temperature for 10 hours, shifted to restrictive temperature and incubated for another 2 hours, fixed and stained with Calcofluor and Hoescht 33258. Note that many regions of the hyphae are so Calcofluor-bright that nuclei are not visible. All micrographs are at the same magnification. Scale bar = 10 μ m.

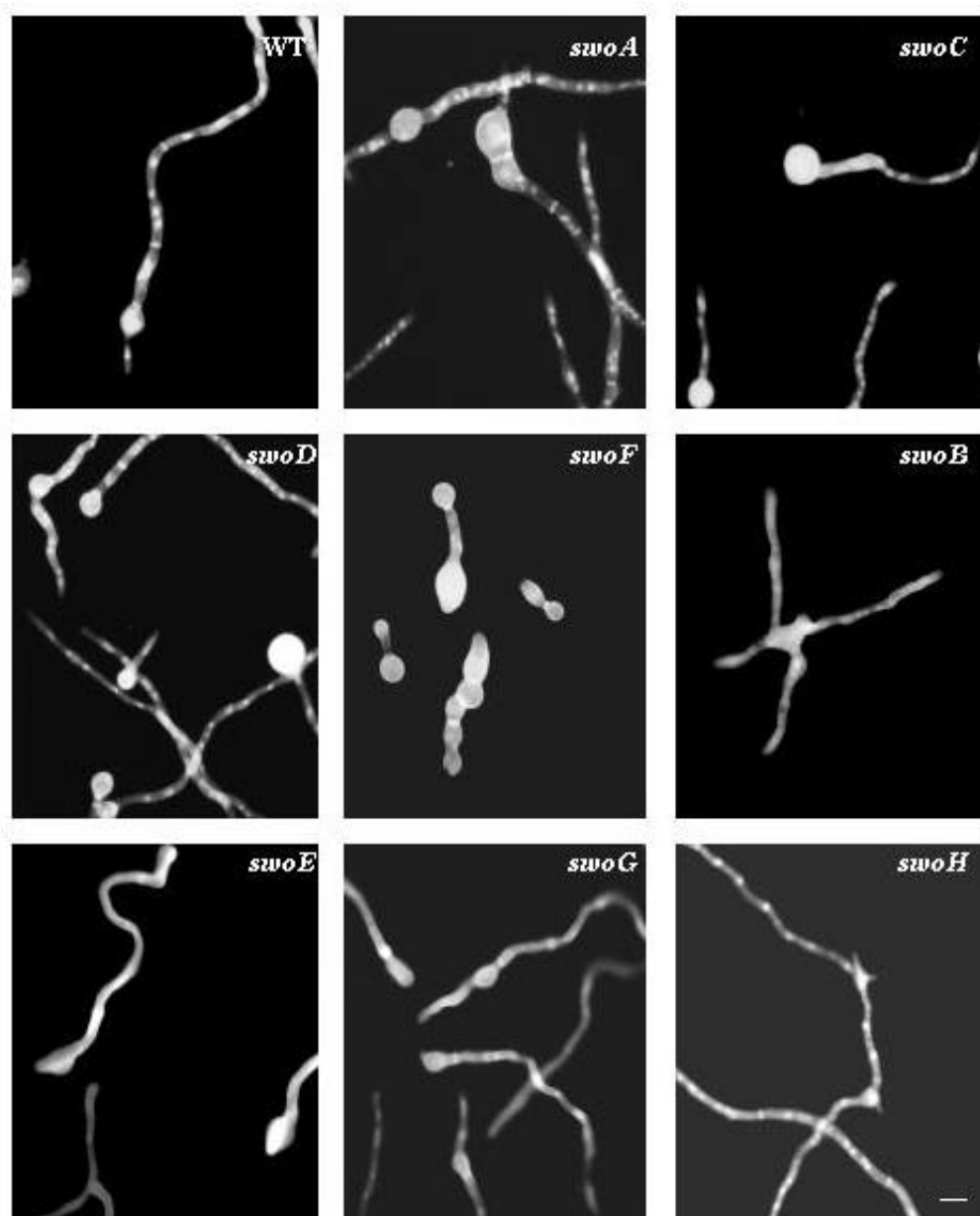


Figure A.6. Localization of growth occurring at permissive and restrictive temperature in the nonpolar *swo* mutants. Conidia from the nonpolar *swo* mutants were inoculated onto coverslips in complete medium, grown at permissive temperature for 11.5 hours, incubated with polyclonal antibodies against cell walls, transferred to fresh medium without antibodies and incubated for another 4.5 hours at restrictive temperature. Germlings were then fixed and stained with fluorescently labeled secondary antibody, Calcofluor and Hoescht 33258. Left column shows FITC staining (labels growth that occurred at permissive temperature). Right column shows Calcofluor and Hoescht 33258 staining (labels entire hypha and nuclei). Arrows mark examples of regions that grew after the switch to restrictive temperature. All micrographs are at the same magnification. Scale bar = 10 μm .

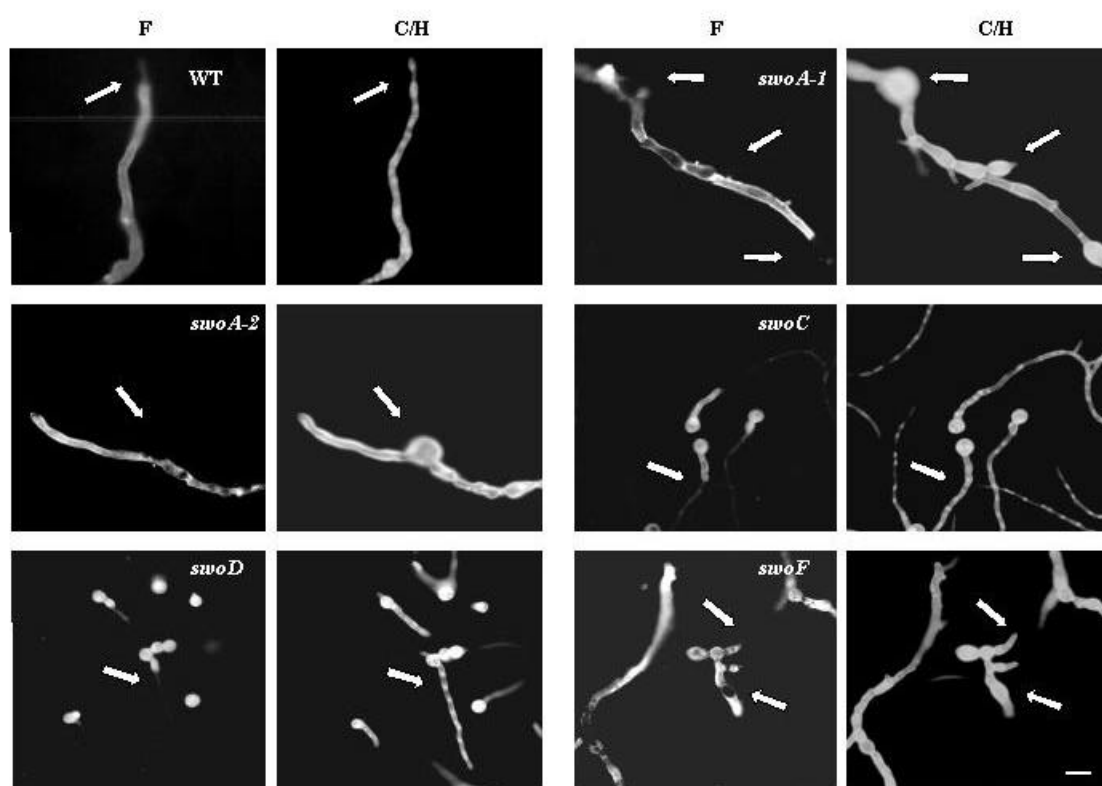
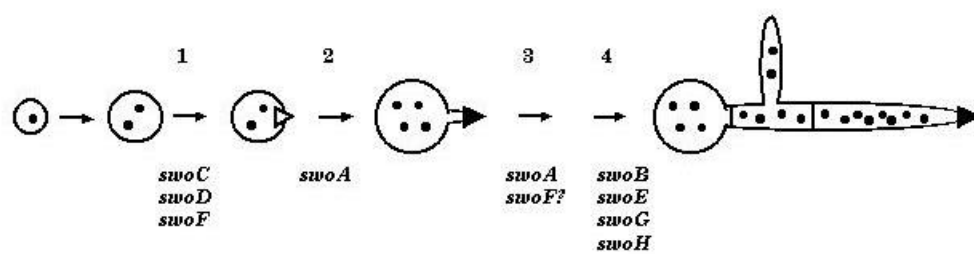


Figure A.7. Model of polarity establishment, polarity maintenance, and hyphal morphogenesis. At or near the second nuclear division the conidium switches from isotropic to polar growth. Presumably, a molecular marker tags the site from which the germ tube will emerge (open triangle). A separate signal, or perhaps a modification of the polarity establishment marker, is sent to initiate germ tube emergence and apical extension (closed triangle). As the hypha matures, it forms septa and branches. See text for postulated roles of the *swo* gene products.



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APENDIX B
CHARACTERIZATION OF THE *ASPERGILLUS NIDULANS* SEPTIN (*ASP*) GENE
FAMILY¹

¹Momany, M., Zhao, J., Lindsey, R., and P.J. Westfall. 2001. *Genetics*. 157:969-977
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ABSTRACT

Members of the septin gene family are involved in cytokinesis and the organization of new growth in organisms as diverse as yeast, fruit fly, worm, mouse, and man. Five septin genes have been cloned and sequenced from the model filamentous fungus *A. nidulans*. As expected, the *A. nidulans* septins contain the highly conserved GTP binding and coiled-coil domains seen in other septins. Based on hybridization of clones to a chromosome-specific library and correlation with an *A. nidulans* physical map, the septins are not clustered but are scattered throughout the genome. In phylogenetic analysis most fungal septins could be grouped with one of the prototypical *S. cerevisiae* septins, Cdc3, Cdc10, Cdc11, and Cdc12. Intron-exon structure was conserved within septin classes. The results of this study suggest that most fungal septins belong to one of four orthologous classes.

INTRODUCTION

Septins are key players in cellular organization processes ranging from cytokinesis to surface growth. Members of the septin gene family have been found in budding and fission yeast, fruit fly, worm, mouse, and man (reviewed by Field and Kellog 1999; Longtine *et al.* 1996; Trimble, 1999). In *Saccharomyces cerevisiae* there are seven septins. Multiple septins are also found in other fungi and animals, though the total number in most of these organisms is not yet known because of incomplete genome information. At least some of the septins are specific to a particular life cycle stage or tissue type.

Septin proteins are highly homologous, and all contain a P-loop nucleotide binding motif (reviewed by Longtine *et al.* 1996). Septin complexes isolated from *Drosophila* possess nucleotide binding and GTPase activity and can polymerize *in vitro* (Field *et al.* 1996). It has been suggested that the septins may represent a novel cytoskeletal element and that, like actin and tubulin, they may be regulated by nucleotide hydrolysis (Sanders and Field 1994). Most septins also contain coiled-coil domains thought to be involved in protein-protein interactions.

Septins were first described as temperature-sensitive mutations in the cell division cycle genes *CDC3*, *CDC10*, *CDC11*, and *CDC12* of *S. cerevisiae* (reviewed by Longtine *et al.* 1996). At restrictive temperature these mutants make elongated buds, do not complete cytokinesis, and lack the 10nm filament ring normally seen just inside the plasma membrane at the neck (Byers and Goetsch 1976). It now seems clear that the major components of the 10nm filament ring are Cdc3p, Cdc10p, Cdc11p, and Cdc12p. In addition to their location at the neck, Cdc3p, Cdc10p, Cdc11p, and Cdc12p are found at the site of bud emergence and at the base of the mating projection, or shmoo (Haarer and Pringle 1987; Kim *et al.* 1991). A mutation in any one of these septins results in loss of neck localization of the other three septins. A fifth septin, Sep7p, is found at the bud neck and site of bud emergence. Deletion of *SEP7* gives only a mild cytokinesis and budding defect (Carroll *et al.* 1998). The remaining septins, *SPR3* and *SPR28*, are only expressed during sporulation. The proteins Spr3p and Spr28p are found at the leading edge of developing spore walls (Devirgilio *et al.* 1996; Fares *et al.* 1996). Strains deleted in *SPR3* are less efficient in sporulation, while those deleted in *SPR28* show no phenotype. Evidence suggests that Cdc3p, Cdc10p, and Cdc11p play some role in spore

formation along with their roles in vegetative growth (Kim *et al.* 1991; Haarer and Pringle 1987).

In addition to their interactions with each other, *S. cerevisiae* septins interact with a wide variety of other proteins. Many important cytokinetic proteins localize to the neck region in a septin-dependent manner. The myosin responsible for actin ring contraction, Myo1p, is found only in the middle of the septin ring at the neck (Bi *et al.* 1998; Lippincott and Li 1998). Proteins Bni4p and Chs4p, which are involved in the deposition of the bud scar on the mother cell, are found specifically on the mother side of the neck (Demarini *et al.* 1997). The cell cycle regulators Hsl1p and Hsl7p are found on the daughter side of the neck where they may form part of the morphogenesis checkpoint delaying nuclear division when budding is delayed (Barral *et al.* 1999; Lew 2000; Shulewitz *et al.* 1999).

There are major differences in the septin-associated processes of cytokinesis and deposition of new growth in budding yeast versus filamentous fungi. In yeast the mother cell grows isotropically, adding new cell wall in every direction. Late in G1, growth shifts to a spot on the surface previously defined by a ring of septins, actin, and chitin. The polar growth of the bud continues through S phase until the middle of G2, when the bud switches to isotropic growth. As M phase ends, septation occurs, separating the mother and daughter cells, each containing a single nucleus (reviewed by Lew *et al.* 1997; reviewed by Pringle and Hartwell 1981).

In *A. nidulans*, a brief period of isotropic growth is followed by apical extension of the germ tube (reviewed by Harris 1999; Momany and Taylor 2000). Once these tip growing germ tubes reach a predetermined size, they are partitioned by septa. Only the

apical cell remains mitotically active. Basal compartments, which contain three to four nuclei, arrest in interphase until a new branch is formed (Kaminskyj and Hamer 1997). In addition to this partitioning during vegetative growth, *A. nidulans* uses budding to form its asexual reproductive spores known as conidia (reviewed by Adams *et al.* 1999). During conidiation, specialized basal cells give rise to aerial hyphae. The tips of these aerial hyphae swell to form a vesicle, and layers of cells bud off of the vesicle. Finally, uninucleate conidia bud from this multi-layered structure.

In previous work *aspB*, a septin gene cloned from *A. nidulans*, was shown to be essential (Momany and Hamer 1997). The *aspB* gene product localizes to forming septa, emerging branches, and layers of the conidiophore (Westfall and Momay, unpublished). Described here are the identification and characterization of four new septins in *A. nidulans*, *aspA*, *aspC*, *aspD*, and *aspE*. Comparison of septin sequences and intron-exon structure from a variety of fungi suggest that there are at least four classes of orthologous fungal septins.

MATERIALS AND METHODS

A. nidulans Strains and Growth Methods

A. nidulans strain A28 (*pabaA6*; *biA1*) was purchased from the Fungal Genetics Stock Center, Dept of Microbiology, University of Kansas Medical Center, Kansas City, KS, USA. All incubations were in complete medium except where noted (1% glucose, 0.2% peptone, 0.1% yeast extract, 0.1% casamino acids, nitrate salts, trace elements, and 0.01% vitamins, pH 6.5). Trace elements, vitamins, nitrate salts, and amino acid supplements are described in the appendix to Kafer (Kafer 1977).

DNA and RNA Isolation

Standard molecular biology procedures were used (Sambrook *et al.* 1989). DNA was isolated from *A. nidulans* using previously described methods (Hamer and Givan 1990). Total RNA from *A. nidulans* was extracted by using Trizo[®] Reagent (Gibco BRL). Isolation of RNA from synchronous cultures undergoing conidiation was as described by Miller *et al.* (1992). Briefly synchronous development was induced by exposing liquid cultures to an air interface. Cultures were examined microscopically to verify appropriate developmental stage at harvest times. At 0 hr vegetative hyphae were observed, at 2.5 hr aerial hyphae, at 5.0 hr vesicles and metulae initials, at 7.5 hr metulae and phialides, at 10.0 hr immature conidia, and at 13.0 hr mature pigmented conidia. Poly(A) RNA was isolated from total RNA using Gibco BRL message maker reagent assembly kit with Ambion oligo (dT), following the manufacturer's recommendations.

First-strand cDNA Synthesis

First-strand cDNA for RT-PCR was synthesized from 1.0 µg RNA using an RT-PCR cDNA synthesis kit as described by the manufacturer (Boehringer Mannheim). Reverse transcriptions were carried out in 20-µl reaction mixtures containing 20 U of AMV reverse transcriptase (Boehringer Mannheim), 15 pmol of oligo (dT)₁₅, and 50 U of Rnase inhibitor (Boehringer Mannheim). Thirty cycles were performed under the following conditions: 25^o for 10 min, at 45^o for 60 min, and at 99^o for 5 min.

Cloning of Septin Genes

The PCR amplification of a highly conserved 300 bp region of *aspA*, *aspB*, and *aspC* and the cloning of *aspB* have been previously described (Momany and Hamer 1997). cDNA clones for *aspA* and *aspC* were isolated from an *A. nidulans* library as

previously described (Momany and Hamer 1997). Genomic clones containing *aspA* and *aspC* were identified by hybridizing the corresponding cDNA clones to an *A. nidulans* chromosome-specific library (Brody *et al.* 1991). The *aspA* probe hybridized with cosmid SW18A08, on contig IIIA. The *aspC* probe hybridized with cosmids SL32D02, SW08F07 and SW24G01, which are all on contig IID. Partial sequences of *aspD* and *aspE* septin homologues were identified through a BLAST search of the Cereon Genomics *A. nidulans* sequence database using *S. cerevisiae* *CDC3*, *CDC10*, *CDC11*, and *CDC12* sequences as queries. Partial sequence information was used to design PCR primers to amplify fragments of *aspD* and *aspE* from *A. nidulans* genomic DNA. The resulting PCR products were used to probe an *A. nidulans* chromosome-specific library (Brody *et al.* 1991). The *aspD* PCR product hybridized with cosmids SW08H01 and SW11B09, which are mapped to contig VIII G. The *aspE* PCR product hybridized to cosmids SW10A02, SW04B03 and SW08E11 on contig III B and SL04C11 and SL06G03 on contig III F.

DNA Sequencing and Analysis

Both strands of *aspA*, *aspC*, *aspD*, and *aspE* genomic clones were completely sequenced using Taq polymerase cycle sequencing and an automated DNA sequencer (Model ABI 310, Perkin-Elmer Corp.) (Momany and Hamer 1997). Primer walking was performed beginning with the PCR primers used in gene identification as described above. Both strands of *aspA* and *aspC* cDNA clones were sequenced using primer walking from highly conserved regions and from flanking vector regions. To identify introns in *aspA* and *aspC*, cDNA and genomic sequences were compared. To identify introns in *aspD* and *aspE*, RT-PCR from *A. nidulans* RNA was used. Primers for RT-

PCR were based on regions in which the genomic sequence disagreed with the conserved septin consensus sequence or in which the open reading frame ended prematurely.

Consensus fungal sequences were identified for all introns (Ballance 1986). Analysis of sequences was performed using SeqLab 10.0 of the Genetics Computer Group (GCG) package of the University of Wisconsin (Devereux *et al.* 1984).

Northern Hybridization

Six identical denaturing gels containing 3 µg of poly(A) RNA for each time point were transferred to nitrocellulose membranes by standard methods (Sambrook *et al.* 1989). Probes were made by incorporation of ³²P by random priming of PCR products from *aspA*, *aspB*, *aspC*, *aspD*, *aspE* or the housekeeping gene *argB*. After hybridization and washes, the blot was exposed to a phosphor screen (Kodak) overnight at room temperature. The hybridization signals were read three times on a Storm Imager (Molecular Dynamics). Data reported is relative to the average *argB* signal.

Genomic Data Acquisition and Processing

Septin homologues were acquired through searches of GenBank using septins from *A. nidulans* as the query. All EST data were acquired from the *A. nidulans* EST database consisting of approximately 13,400 sequences from a 24h mixed vegetative and asexual culture (<http://www.genome.ou.edu/fungal.html>). Yeast microarray data is described by Spellman *et al.* (1998). Nucleotide and amino acid sequences were aligned by using ClustalX 1.8 (Thompson *et al.* 1994). Phylogenetic trees were constructed using the Neighbor-Joining method from ClustalX 1.8 with default settings (SAITOU and NEI 1987). Bootstrap values are based on one thousand replicates.

Nucleotide Sequence Accession Number

Nucleotide sequences for *aspA*, *aspC*, *aspD* and *aspE* were assigned GenBank Accession No. AF299320, AF299321, AF299322 and AF299323, respectively.

RESULTS

Septin Family Alignment

The *asp* (for *Aspergillus septin*) family consists of at least five genes: *aspA*, *aspB*, *aspC*, *aspD*, and *aspE*. Predicted products of the *asp* genes range from 343 (*aspD*) to 469 (*aspE*) amino acids in length with an estimated mass range of 38-52 kDa. The *A. nidulans* septin genes display more than 35% amino acid identity over their entire lengths. Alignment of the deduced amino acid sequences of *A. nidulans* and *S. cerevisiae* septins is shown in Figure B.1. As is true for the entire septin family, the similarity among Asp proteins is greatest in their central regions, while the amino-terminal and carboxyl-terminal regions are divergent in both length and sequence. All septin genes, including those from *A. nidulans*, have three predicted P loop GTPase domains, G1 (GXXXXGKT), G3 (DTPG), and G4 (XKXD) (Saraste *et al.* 1990). Most septins, including all *asp* products except that of *aspD*, contain predicted coiled-coil domains at or near the carboxyl termini (Lupas *et al.* 1991). These domains are thought to be involved in homotypic or heterotypic interactions among the septins themselves or in interactions between the septins and other proteins (Longtine *et al.* 1996).

Phylogenetic Analysis

Currently available fungal septin sequences were analyzed for similarities. This analysis established four main septin classes with a nearly one-to-one correspondence

among proteins from *A. nidulans*, *Schizosaccharomyces pombe*, and *S. cerevisiae* (Figure B.2). Each class contained one of the prototypical *S. cerevisiae* septins, Cdc3, Cdc10, Cdc11, or Cdc12. The Cdc3 class also contains AspB from *A. nidulans*, Cacdc3 from *Candida albicans*, Abs2 from the basidiomycetous *Agaricus bisporus* and Spn1 from *S. pombe*. The Cdc10 class contains AspD, Nccdc10 from *Neurospora crassa*, *C. albicans* Cacdc10, Spn2, and Ums1 from the basidiomycete *Ustilago maydis*. The Cdc11 class contains AspA, Spn3, Abs1, and Pbs1 from the ascomycete *Pyrenopeziza brassicae*. The Cdc12 class contains AspC, Spn4, and the *Mucor circinelloides* septin Mcs1. The predicted products of the *aspE*, *SPN5*, *SPPC584.09*, and *SPR3* genes did not cluster with septins from other fungi. Except for the *S. cerevisiae* protein Spr28, the septins from within a single fungal species were dispersed among the classes.

Intron-Exon Structure

The locations of introns in the *A. nidulans* septins were determined by comparing sequences of genomic and cDNA (Figure B.3). The number of introns varied for the *asp* genes, with one in *aspB* and *aspE*, two in *aspA*, and five in *aspC* and *aspD*. Intron-exon junctions of *asp* genes contained the consensus-splice sequences seen in other filamentous fungi, specifically, PuPy (usually GT) at the 5' end and AG at the 3' end (Balance 1986). Intron sizes varied from 51 bp (*aspD* intron III) to 180 bp (*aspA* intron I). All five *asp* genes contained an intron upstream of the G1 GTP binding domain. In *aspB*, *aspC*, *aspD*, and *aspE* the first intron is within 15 nucleotides of the codon of the G1 domain. In *aspA* the first intron is 51 nucleotides upstream of the codon for the first G1 glycine. There was no sequence conservation detected in the intron upstream of the

G1 domain. Neither the positions nor the sequences of the other *asp* introns appeared to be conserved.

The conservation of the intron position upstream of G1 within the *A. nidulans* septin family prompted us to examine intron positions in the other fungal septins for which genomic sequence was available (Figure B.3). None of the *S. cerevisiae* septins contain introns, but this is not surprising as introns are unusual in budding yeast (Fink 1987). Within the Cdc11 class of fungal septins, the sister sequences encoding *aspA* and *pbs1* both have an intron in the same position relative to the G1 domain. The more diverged *spn3* lacks this intron. Genomic sequence is not available for the *A. bisporus* septins. Within the Cdc3 class of septins, *S. pombe spn1* and *C. albicans cacdc3* have no introns, and *aspB* contains a single intron. The divergent *spn5*, which does not fall within one of the four septin classes, lacks the intron upstream of G1. Within the Cdc12 class, the sister sequences *aspC* and *mcs1* both have an intron immediately upstream of the G1 domain, while the more distant *spn4* lacks introns. The Cdc10 class contains the most conserved intron positions. *AspD* and *nccdc10* have two introns in identical positions relative to the conserved G1 and G3 domains, while *spn2* shares three identical intron positions with *aspD*. The single intron in *aspE*, which does not fall into one of the main septin classes, does not share its position with any of the other septins.

Genomic Organization

To determine the genomic organization of the *A. nidulans* septin family, a chromosome-specific cosmid library (Brody *et al.* 1991) was probed with either cDNA clones (*aspA*, *aspB* and *aspC*) or PCR products (*aspD* and *aspE*). The hybridizing clones were then located on the *A. nidulans* physical map (Prade 2000; Prade *et al.* 1997) as

shown in Figure B.4. Localization of *aspB* to chromosome I has been previously described (Momany and Hamer 1997). The *aspA* and *aspC* cDNA clones hybridized with cosmids mapped to contig IIIA and IID, respectively. The *aspD* PCR product hybridized with cosmids mapped to contig VIII G. The location of *aspE* is somewhat ambiguous as its PCR product hybridized with 4 cosmids mapped to contig IIIB and 2 cosmids mapped to contig IIIF.

Expression of asp Genes

To determine expression during asexual development, mRNA isolated from synchronized cultures undergoing conidiation was probed with the *asp* genes (Figure B.5). The *aspB* gene was the most highly expressed of the *A. nidulans* septins, ranging from 10.2- to 27.1-fold above the levels of a housekeeping gene (*argB*). The *aspD* gene was the most poorly expressed with mRNA levels ranging from 0.7- to 2.3-fold that of *argB*.

As the culture progressed from vegetative growth through conidiation, the levels of *aspB* fell, recovering slightly as conidia matured. Both *aspA* and *aspD* showed a similar declining expression pattern, though at a much lower level. The expression of *aspC* peaked as metulae budded from the vesicle layer. The expression of *aspE* was lowest during phialide development (1.1-fold above *argB*) and then increased in mature conidia (3.1-fold above *argB*). The *aspE* gene also showed the highest ratio of minimum/maximum expression (5.8), which may indicate that it is highly regulated.

To verify our RNA hybridization results we searched the *A. nidulans* EST database (<http://www.genome.ou.edu/fungal.html>) for the *asp* genes and the *argB* control.

aspB was found twice and *aspC* was found four times. None of the other *asp* genes were detected. The *argB* control was found once.

DISCUSSION

Members of the septin gene family are highly homologous and have been found in several species of fungi and animals where they play critical roles in cytokinesis and cell surface organization (reviewed by Field and Kellogg 1999). Interestingly, septins have not been reported in algae or land plants. It seems most likely that septins arose in a common ancestor of fungi and animals after divergence of the green plants (Wainright *et al.* 1993). It is also possible that septins arose in an earlier common ancestor shared by green plants, fungi, and animals and were later lost in the green plant lineage by non-orthologous gene displacement or lineage-specific gene loss. Regardless of the evolutionary path, it is not surprising that septins are present in animals and fungi and absent in plants in view of the major differences in division mechanisms. Animal and fungal cells divide centripetally, that is the division furrow progresses from the cortex to the interior. Plant cells, however, divide centrifugally, and the cell plate proceeds from the interior outward to the cortex (reviewed by Field *et al.* 1999).

The septins occur as multigene families in both fungi and animals (reviewed by Field and Kellogg 1999; Trimble 1999). In fungi, septins have been found in the three of the four phyla (for fungal phylogeny see Bruns *et al.* 1991, 1993). Multigene septin families are found in each major branch of the phylum Ascomycota. In the archaeascomycete *S. pombe* there are at least seven septins (http://www.sanger.ac.uk/Projects/s_pombe). In the hemiascomycetes *S. cerevisiae* and

C. albicans there are seven and at least two septins, respectively (<http://sequence-www.stanford.edu/group/candida/search.html>). In the euascomycete *A. nidulans* there are at least five septins. Within the phylum Basidiomycota, a *U. maydis* septin has been identified through a cytokinesis mutant (Michael Bolker, University of Marburg, unpublished results), and two *Agaricus bisporus* septins have been identified in an EST project (Ospina-Giraldo *et al.* 2000). Within the less derived phylum Zygomycota, a single septin was found in GenBank from *M. circinelloides*. The absence of septins from the fourth fungal phylum, the Chytridiomycota, and the relative paucity of septins outside the Ascomycota are undoubtedly because there are fewer genomic sequencing efforts underway in these organisms.

Even with incomplete genome data, it seems clear that most fungal septins fall into one of four classes, each containing one of the prototypical *S. cerevisiae* septins, Cdc3, Cdc10, Cdc11, or Cdc12 (Figure B.2). It seems likely that septins in each group arose by vertical descent from a common ancestor. In other words, members of each of these four septin classes appear to be orthologs. Our finding of conserved intron positions between the most similar members within septin classes also supports the concept of orthology (Figure B.3). The apparent lack of Cdc3 class septins with shared intron positions is probably because so few sequences from this class were analyzed. Spn1 and *C. albicans* Cdc3 lack introns. While none of the *S. cerevisiae* septins contain introns, this is not surprising as there are very few introns in budding yeast. A mechanism for intron loss involving reverse transcription of processed mRNA followed by homologous integration has been proposed to explain the general rarity of yeast introns (Derr *et al.*

1991; Fink 1987). Such a mechanism might also explain the apparent loss of the conserved introns from the *S. pombe* septins Spn3 and Spn4.

There is evidence for both the gain and loss of introns during evolution (Frugoli *et al.* 1998; Venkatesh *et al.* 1999). It is not possible to predict whether individual septin introns are the products of gain or loss during evolution without more sequence data, especially from the basal Chytridiomycota and Zygomycota. Nor can we yet make an informed prediction about how many septins were present in the putative common ancestor of fungi and animals. As more sequence data become available, clustering analysis and intron position in septins from lower fungi and animals should tell us more about the evolution of this important group of proteins. Indeed, intron-exon structure has been used to ascertain evolutionary relationships among angiosperm catalase genes (Frugoli *et al.* 1998) and to define clades in vertebrate evolution (Venkatesh *et al.* 1999).

Most of the *Aspergillus* septin genes are found on separate chromosomes rather than being clustered. Only *aspC* and *aspE* are on the same chromosome (III), though they are not closely linked. The *S. cerevisiae* septins are also on different chromosomes, except for *SPR28* and *SEP7*, which are 300kb apart on chromosome IV. Because duplicated genes are often clustered (Prade *et al.* 1997; Stewart and Cullen 1999), such genomic organization is consistent with the idea that fungal septins are related by vertical descent rather than duplication.

Cdc3, 10, 11, and 12 interact to form the yeast 10 nm filament neck ring. Mutation in any one of these genes abolishes neck localization of the other three. We speculate that the ancestors of the Cdc3, 10, 11, and 12 classes also formed complexes thus interacting domains have been highly conserved in different fungal species

throughout evolution. Perhaps septins that participated in more specialized tasks such as spore formation did not have to interact with each other and so were free to diverge. Consistent with this notion, the *S. cerevisiae* nonessential septin (Sep7) and the sporulation specific septins (Spr3 and Spr28) do not fall within one of the four main septin classes. Also of interest, the Mde8 septin of *S. pombe*, which groups close to Sep7 and Spr28, appears to be a most unusual septin, lacking most of the G1 consensus domain.

Based on the idea that individual members of the four septin classes participate in complexes together, it is predicted that AspA, B, C, and D proteins will interact in a complex that is critical in development, while the AspE protein will probably participate in a more specialized, nonessential process. To begin to address function, septin gene expression levels during vegetative growth and asexual development were investigated in *A. nidulans* (Figure B.5). In *S. cerevisiae*, the septins *SPR3* and *SPR28* are only expressed during sporulation. However, all five *A. nidulans* septins showed expression during vegetative growth as well as asexual sporulation, though mRNA levels did fall during sporulation. It seems likely that sporulation-specific septin orthologs are present in *A. nidulans* but have not yet been uncovered by genome projects. Comparison of the expression profiles of septins from *A. nidulans* with orthologous septins from *S. cerevisiae* revealed an interesting similarity. Based on Northern analysis and frequency in the EST database, *aspB* and *aspC* are the most highly expressed *A. nidulans* septins in vegetative and conidiating cultures. Based on publicly available microarray data (Spellman *et al.* 1998), *CDC3* and *CDC12*, the *S. cerevisiae* orthologs of *aspB* and *aspC*, respectively, are also the most highly expressed septins. Further experiments are needed

to clarify how much functional conservation exists among fungal septin orthologs. Newly available *A. nidulans* microarrays (Prade *et al.*, this issue) should allow a more thorough investigation of septin expression. As originally suggested by Longtine *et al.* (1996), it does seem likely that conserved sequence domains among the septins are important for their ability to assemble into complexes, whereas the nonconserved sequence domains are involved in interactions with other proteins.

To identify putative *A. nidulans* septin-interacting proteins, we have exploited the recently developed genomic concept of “interlogs” (reviewed by Galperin and Koonin 2000). Interlogs are interacting proteins from one species whose orthologous proteins from another species also interact. In *S. cerevisiae*, several septin-interacting proteins have been identified through genetic and biochemical methods (reviewed by Longtine *et al.* 1996; Field and Kellogg 1999). Two mitosis specific kinases from *S. cerevisiae*, Gin4p and Kcc4p, regulate cell cycle progression and localize to the bud neck in a septin-dependent manner. The *S. cerevisiae* chitin synthases Chs3p and Chs4p have also been shown to interact with septins in budding yeast. Using septin-interacting proteins from *S. cerevisiae* to query the *A. nidulans* EST database has allowed identification of several putative septin interlogs (data not shown). Interestingly, potential orthologs of Gin4p, Hsl1p, Chs3p, and Chs4p were all highly expressed in the *A. nidulans* EST library. Two-hybrid analysis and immunoprecipitation are being used to identify septin-interacting proteins in *A. nidulans*. It is expected that a directed identification of interlogs by a combination of computational and experimental methods will significantly accelerate studies of septin function in *A. nidulans*.

ACKNOWLEDGEMENTS

This work was supported by the National Science Foundation grant 9904629 to MM. PJW was supported by the National Institutes of Health training grant in molecular and cellular mycology to UGA (AI07373).

Figure B.1. Alignment of *A. nidulans* and *S. cerevisiae* septins. Alignment of deduced amino acid sequences of septins from *A. nidulans* (AspA-E) with septins from *S. cerevisiae* (Cdc3-12, Spr3, Spr28, and Sep7) is shown. Alignment was generated using ClustalX 1.8 with default settings. Gaps have been introduced for optimal alignment and are indicated by dashes. The positions of G1 (GXXXXGKT), G3 (DTPG) and G4 (XKXD) nucleotide binding domains are indicated under the alignment. Darker shading indicates higher homology. GenBank accession numbers for *S. cerevisiae* genes are: Cdc3, AAB64515.1; Cdc10, CAA42339.1; Cdc11, AAB39301.1; Cdc12, AAB68863.1; Spr3, CAA97061.1; Spr28, CAA88498.1; Sep7, CAA98804.1.

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AspA : -----MSSAYNPIKLRKKKLVKGIQDC : 23
AspB : -----MGAGNAQTVTSKDPKAAAQAATDMKNVVRKLTGYGFANLPNWHRRKSVKGFNPN : 57
AspC : -----MAPVNETASP-----IGIANLPNQRHKIIVARQAAT : 32
AspD : -----MATTSSAPSTVFPFRS-----HWGPDSTITSIERKLLRPFQFN : 38
AspE : -----MSTPVPDSDPYARTESMTHRGRYSYASSYVSTVNNPRLRRRDPDPFN : 51
Cdc3 : MSLKKEQVSIKQDPQERQHDQFNDVQIKQESQDDHVDGQYNGTQNDSEFPAAESDVKVEPGLMGITSSQSKGQLVDPQPEIKFIRRIQINGYGFANLPKWHRRKSVKGFNPN : 121
Cdc10 : -----MDPLSSVQFAS-----YVGFDTITNGIERKLLRPFQFN : 34
Cdc11 : -----MSGIIDASSALKRKKHLRQITFT : 24
Cdc12 : -----MSAATAAAPPVFP-----VGISLDPNRYKIVNEEGQFT : 36
Spr3 : -----MMSKGSRLSDCPVEFPKIVSGFAEVIKIRQSSQGVAVDSHPKSPKELKRRRSSVFNKGCNRRDLLLNKKAQEIINTNSHGQDGLKKNLPRQELNANRQIDPT : 111
Spr28 : -----MFPMKDHS-----ALEQHTLSRDELRRKQYKQGLQS : 33
Sep7 : -----MSTATPPIINLFRKKKBEHRCITFT : 25

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AspA : RMVCSAETGRTTNNCKQVLLG-----KADDDATNAHLEGVNKPVTVEQLDD-----EETRFSTVDFDQGHQDNE : 99
AspB : VMVCSGKOKKSLNDRFN-----TSLYPKRAKAGPSHDIP-----KTVSOSISADLEEN-----GVNRFRTVDDPQGHQDNNND : 131
AspC : LMVCSGKOKTINLHLESTIKN-----YADHKRHQKQVDTVEEITKAELEEK-----FFKRFRTVDDPQGHQDNNNR : 106
AspD : VMVCSGKOKKSLNDRFASHLID-----SKGRITPNEFVRSSTETQVSHVLEEN-----GVNRFRTVDDPQGHQDNNND : 111
AspE : LLVCSGKOKKSLNDRFASKLALPP-----HKHPSRAPDEVEYDSHPASQGYTSHYLETID-----GERVGRFTVDDPQGHQDNNVDPI : 131
Cdc3 : LLVCSGKOKKSLNDRFASKLALPP-----EELANDQEEEGGEGHENQSQEQRRKVKIKSYESVLEEN-----GVNRFRTVDDPQGHQDNNND : 215
Cdc10 : LMVCSGKOKKSLNDRFASHLIDS-----ATGGDISALVTKTTEKISYHTVVED-----RVNRFRTVDDPQGHQDNNND : 107
Cdc11 : VMVCSGKOKKSLNDRFASHLID-----TTILLPTDTSTHIDLQREETVREDD-----EGVRFRTVDDPQGHQDNNND : 100
Cdc12 : VMVCSGKOKKSLNDRFASHLID-----RADGQCHQEPTRKTEVEDITRALDEEK-----HFRVRFRTVDDPQGHQDNNND : 109
Spr3 : LMVCSGKOKKSLNDRFASHLID-----LIDD-DIKENKPIIRYKSLVREGD-----GTHRFRTVDDPQGHQDNNND : 175
Spr28 : LLVCSGKOKKSLNDRFASHLID-----DDDKVTNNVTPENGAIEDIDPGYKTAHLSGLKLVTRKRYVNDLGLG-----VPTDFRTVDDPQGHQDNNND : 132
Sep7 : MLVCSGKOKKSLNDRFASHLID-----LLETKIFPKHQYKSNASISSNPEVKVIAPTKVSVFNSKNGIPSYSEFPMRANLFPQITTSLSLRGGNKDQKGFEMNDDTFFFNLMRSHGKRNDD : 145
GXXXGKTI-----DTPG

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AspA : A-SFGEIVGQYERQYDDIAEESRIKRNPRFRNRVGLVYIQT-----CHGRELDELMLRISPRVNVIPVIGKASSDPAIASSKLLMEDIEHY : 194
AspB : D-SWRPVENLQRYDAVBAENKVNRTNIDNRHACVYIQT-----CHSLKPDDEVMRDRHTKVNLPVIAKATDDEDSMFKKRLADLQHH : 225
AspC : D-SWQPIELQDDQHSVYIQQQPRR-TDKIDMRHACVYIQT-----CHLKPDDDEVMRDRSRVNLIPVIAKATDDEDSMFKKRLADLQHH : 200
AspD : STSWQPIELQDDQHSVYIQQQPRR-TDKIDMRHACVYIQT-----CHSLKPDDEVMRDRSRVNLIPVIAKATDDEDSMFKKRLADLQHH : 207
AspE : Q-LRGTQTESEKFEETNEEMKVVVSPGARQTHICTPLIDPSRLDENIAAABRAAAGTPRASDSKVLVLDENFDLQVLTWIGKTTVWPVISAATDARAYLAKAWDSIKKA : 250
Cdc3 : QKSWQPIELQDDQHSVYIQQQPRR-TDKIDMRHACVYIQT-----CHSLKPDDEVMRDRSRVNLIPVIAKATDDEDSMFKKRLADLQHH : 310
Cdc10 : SKANWPIELQDDQHSVYIQQQPRR-TDKIDMRHACVYIQT-----CHSLKPDDEVMRDRSRVNLIPVIAKATDDEDSMFKKRLADLQHH : 203
Cdc11 : P-SFPIELQDDQHSVYIQQQPRR-TDKIDMRHACVYIQT-----CHSLKPDDEVMRDRSRVNLIPVIAKATDDEDSMFKKRLADLQHH : 195
Cdc12 : X-ANQPVDVNDQDHSYVQEQPPYR-TKKPDRHVAVYIQT-----CHGKPDDEVMRDRSRVNLIPVIAKATDDEDSMFKKRLADLQHH : 203
Spr3 : AFTWPIELQDDQHSVYIQQQPRR-TDKIDMRHACVYIQT-----CHSLKPDDEVMRDRSRVNLIPVIAKATDDEDSMFKKRLADLQHH : 270
Spr28 : Q-SVVYKMDQDFANVKEEVRIKNTKTEQDQPVYIQT-----PRGKPDDEVMRDRSRVNLIPVIAKATDDEDSMFKKRLADLQHH : 227
Sep7 : SLCSSEMSVQEQDQHSVYIQQQPRR-TDKIDMRHACVYIQT-----CHGKPDDEVMRDRSRVNLIPVIAKATDDEDSMFKKRLADLQHH : 241
KXKD

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AspA : RLCVNFYDIEH-----D-----DEETVENAEKGLNFFIVGDFEVIDGRK--VRAKTPVYVLDNPHSHLAIAS : 266
AspB : SLDLQEGRYEILD-----DEETIAEWEKSKVFPVAVGQNSHATSDGRK--VRCGKFWLQDDEEHCQVKKIQ : 297
AspC : QKLNTPFIEH-----DEFAAAHARSMAAPVAVGQNSHATSDGRK--VRCGKFWLQDDEEHCQVKKIQ : 271
AspD : NAKMDFNDEILD-----DEE-RAVNAKVTIIPVAVGQNSHATSDGRK--VRCGKFWLQDDEEHCQVKKIQ : 277
AspE : NDDPLELTLTLDQEQEYTSSEGEDEEDGETSEAEADAAGETEGGHTKEETPEKAPESPTRQSEGGQDVGSAVLPFSSHPDKYSLQGDG--I-GRGKFWLQDDEEHCQVKKIQ : 367
Cdc3 : NHEEKFPYISND-----DAENSHLSERFSSVPAVAVGQNSHATSDGRK--VRCGKFWLQDDEEHCQVKKIQ : 382
Cdc10 : NFKVFPYDSEILD-----DEE-LELNRSRSITPVAVAVGQNSHATSDGRK--VRCGKFWLQDDEEHCQVKKIQ : 273
Cdc11 : NDDVNFYDIEH-----I-----SDEDEYTNMYRTLPPVAVGQNSHATSDGRK--VRCGKFWLQDDEEHCQVKKIQ : 269
Cdc12 : ERTFTPFLDAESKEDAKSGSN-----ISAAVEHARQTEAMPVAVGQNSHATSDGRK--VRCGKFWLQDDEEHCQVKKIQ : 285
Spr3 : DDPVCFYDSEVILN-----ATQD--FQKYFSSIAENYIFNPKDEK--VKGKFWLQDDEEHCQVKKIQ : 337
Spr28 : NRRVDFKSDTGLTALYIMDIDSS-----SAKSKYDNTKKEISFPVAVGQNSHATSDGRK--VRCGKFWLQDDEEHCQVKKIQ : 313
Sep7 : NVDKFEVDPED-----D-----DLESMEENQASLQFPALITSDTRDSEG-----RYVRSQVWLTSDDKISLKVILN : 310

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AspA : ALGSHADKELDHDFLVNYRTEKSKSVGDAATG----- : 304
AspB : MDIRTHDEEKHEHNNHLNYSRDKTQMGVAQDPSV----- : 335
AspC : IDIRTHLDLHTDEEQHAYRAQQMETRKFPG----- : 304
AspD : PDIRTHQDILHTSQHHTFRAKQIALKSSAANG----- : 315
AspE : SVFSEWRSEHREASRVWHEWRTSRNHRDAIAS----- : 402
Cdc3 : LLIKQFTEEKERSKILNYSRDKAKLGIKQDNSV----- : 420
Cdc10 : PDIRTHQDILHTSYHHTFRAKQIALKEN----- : 306
Cdc11 : ALGISHHDKNYHEILNYSRTEASGESVAASIRPNLTKINGSSSSITTRNTN----- : 328
Cdc12 : LLLFNYLDLSTQEMHHTVYRRLRGHENTGEGN----- : 322
Spr3 : TDFDNVLDVSEEDYBCKRSEMLRLLKARDCLTKSVDTEBQRFLEEMNFDEIENKLN----- : 405
Spr28 : IALGSHQKELVNNVLDNYSRTEKSKSVGDAATG----- : 351
Sep7 : VVFGSHQDFKELTONLHNYRSEKSSVANAEEIGPNSKTRGNSAPSLNSFASLISTQFPNSQTLANNLRADTPRNQVSGNFKENEYENGEHDSANBQEMSPVRLQREIKQENEN : 431

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AspA : -----TDSINPELATQSVRLKEEQRRREKLRBIKLVQREIAEKRQELARESGLREIARMARASQGEAAEAEQ----- : 379
AspB : -----FKEVNPVAVGQNSHATSDGRK--VRCGKFWLQDDEEHCQVKKIQ-----GKRGKFWLQ : 428
AspC : -----BARPKLDNPKFKHEENLKKFTDQVLEKSRFRQWQKLIARLDRLLNKLEATHAAVYVFPFSVYARSSIVGLTWIQHSSRERERKPARLIDS----- : 399
AspD : -----HTSGSRFSPSADRSLRNSRQVMTNGY----- : 343
AspE : -----P-----KPRSPGRTGPPFARRPRNRYTFRLSLTLKSTFSLIQPTFPSSPISLLVRCSDGASALAPLIA----- : 469
Cdc3 : -----FKEPDPISQLEKTLHEAKLAKLEIEMKTVQKQVSEKELQKSETELPARKHEKMEKLTQKLAEDDKKQLQELSINSASPNNVHSPVPTKKGFLR----- : 520
Cdc10 : -----ANSRSHAMSSNAIQR----- : 322
Cdc11 : -----FPKQSNINNDVLPASDMHQSTGENNETYMTREEQLREERLKAPEERVOQLLKRQELGREKEIREARLEKREKIKQEE----- : 415
Cdc12 : -----BDFTLPAIAPARKLSHNPYKKEENALKKYFTDQVKAERFRQWQNSVNRIRLNGDLEIQG-KVKKLEEQVKSQVKKSHL----- : 407
Spr3 : -----YKCYEIIMKTVMKVATEWDPFPIRQLAKKKNLNSREISKFRDWKSLFMRQENFNQELQLNHLKENIQLEQDLEYKLLKLGSSNSHSDATLVNWHKR----- : 512
Spr28 : -----YSYIDETSRSVSNSTRNSASRTLGNPDTNDENAYQHKREIDEKNRIEDYQRKDLLEKMLAAPQNKV----- : 423
Sep7 : LIRSIKTESPPFLNSPDLPERTLRNISETPVYVLRHERLAPQKLEELBAQSAKELKQRKQELREKAEKLEKREKLINQKLNKSSSSINSLQGSTSRIKKNMTYDLASIASGRD : 551

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Figure B.2. Phylogenetic analysis of septins from *A. nidulans* and other fungi.

Phylogenetic tree was constructed using neighbor-joining. Bootstrap values >50% based on 1,000 replicates are shown above the line. *A. nidulans* septins are underlined.

GenBank Accession numbers for *S. cerevisiae* and *A. nidulans* sequences are as for

Figure1. GenBank accession numbers for other fungal genes are: Spn1, AAB53692.1;

Spn2, CAB57440.1; Spn3, AAB53691.1; Spn4, CAB11495.1; Spn5, CAB11273.1;

Mde8, CAA19121.1; Spcc584.09, CAB41232.1 (all from Field *et al.* 1999); Cacdc3 ,

S43298; Cacdc10, P39826; Abs1, ABZ82019.1; Mcs1, CAB61437.1; Nccdc10,

CAC09398; Pbs1, AJ132791.1. No GenBank numbers are available for Ums1 sequence

(personal communication from Michael Bolker, Universitaet Marburg) and Abs2

(Ospina-Giraldo *et al.* 2000). Abbreviations are: Ab, *A. bisporus*; An, *A. nidulans*; Ca, *C.*

albicans; Mc, *M. circinelloides* ; Nc, *N. crassa*; Pb, *P. brassicae*; Sc, *S. cerevisiae* ; Sp, *S.*

pombe. All septins are from ascomycetes except where indicated by footnote.

¹septin from basidiomycete

²septin from zygomycete

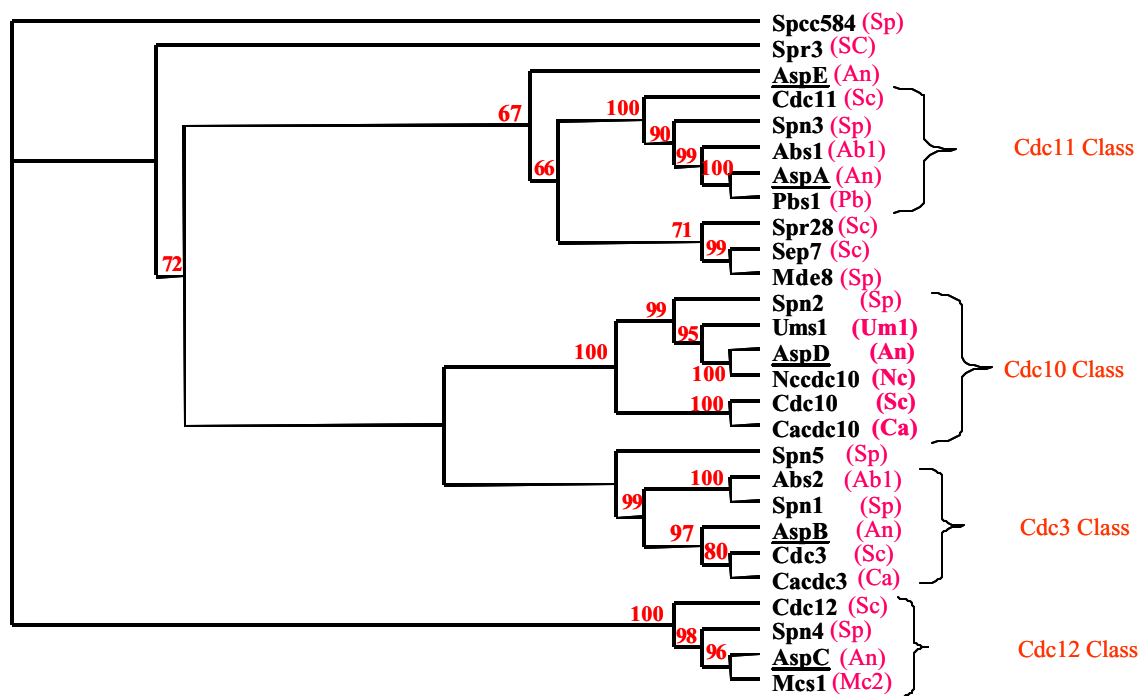


Figure B.3 Comparison of intron-exon structure of septin genes from *A. nidulans* and other fungi. White boxes represent introns and gray boxes represent exons. The predicted positions of GTPase domains are indicated by black boxes and of coiled-coil domains by stippled boxes. Beginning of the first G1 GTPase domain is indicated by a vertical dashed line. Intron positions are shown relative to predicted amino acid sequence of exons. Dashed lines connecting introns between *aspD* and *spn2* or *nccdc10* indicate identical positions relative to predicted amino acid sequence of exons.

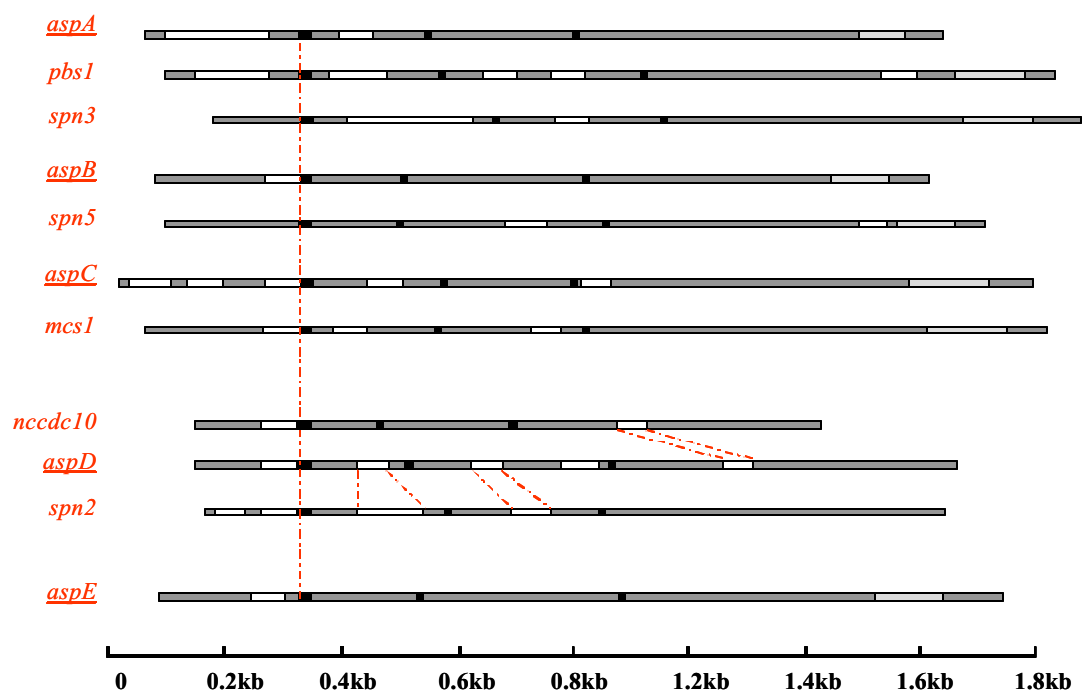


Figure B.4 Genomic organization of *A. nidulans* septin genes. Probable locations of *A. nidulans* septin genes are shown superimposed on the current *A. nidulans* map of chromosomes I, II, III and VIII (www.Aspergillus-genomics.org). Aligned boxes represent contigs and shadowing represents cosmids that contain repeats.

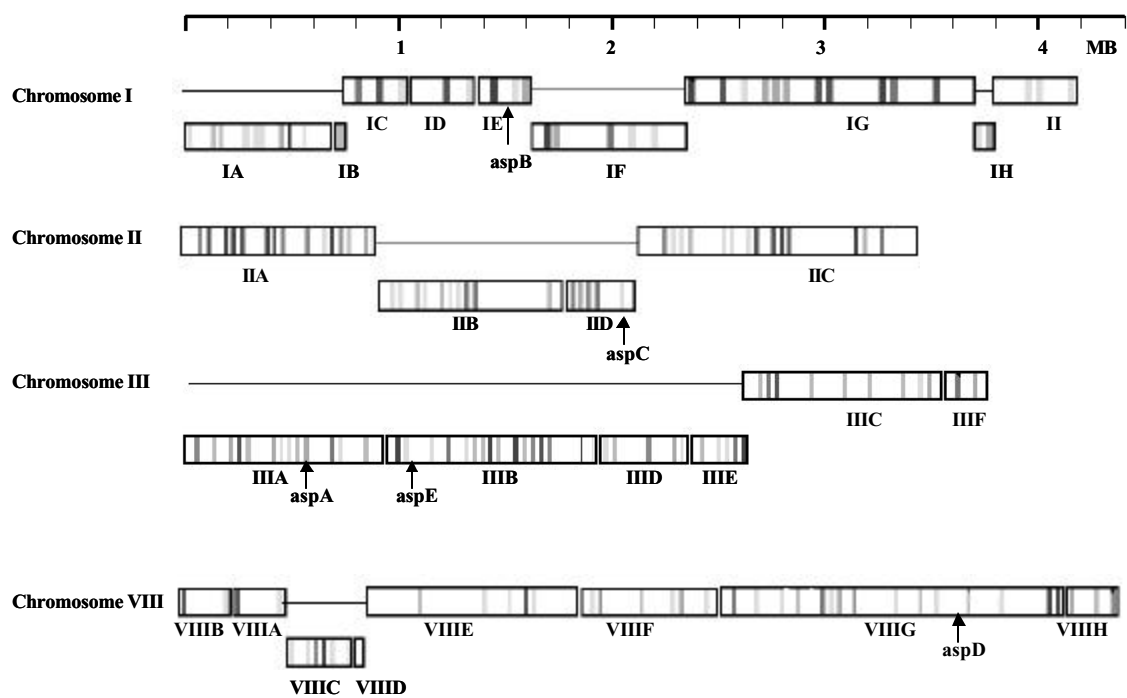
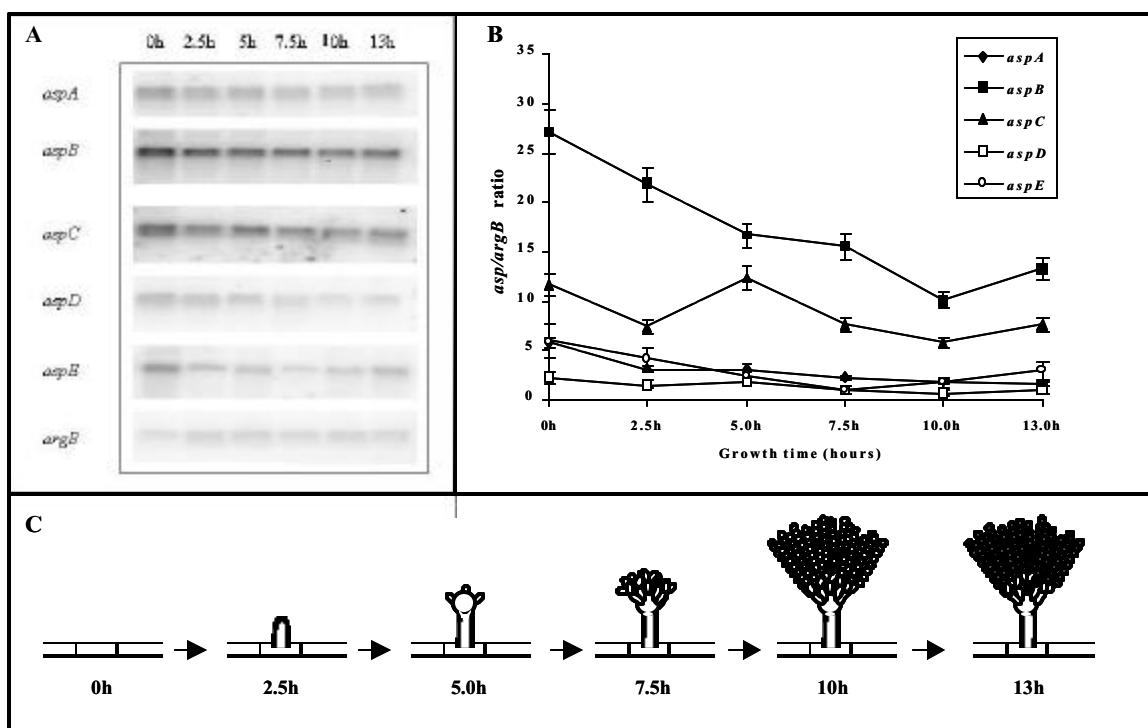


Figure B.5 Expression of *A. nidulans* septin genes during conidiation. (A) Hybridization of *A. nidulans* septin genes to polyA-RNA isolated from synchronized cultures undergoing conidiation. (B) Plot of signal intensities read from blots in (A). Error bars show standard deviation of three replicate reads. (C) Diagram representing conidial stages at each time point.



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