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Cytokinesis in *Aspergillus nidulans* is controlled by cell size, nuclear positioning and mitosis

Tom D. Wolkow[1], Steven D. Harris[2] and John E. Hamer[1,*]

[1]Department of Biological Sciences, Purdue University, West Lafayette, IN 47907-1392, USA
[2]Department of Microbiology, University of Connecticut Health Center, Farmington, CT 06030-3205, USA

*Author for correspondence (e-mail: jhamer@bilbo.bio.purdue.edu)

SUMMARY

The mycelium of *Aspergillus nidulans* is composed of multinucleate cellular compartments delimited by crosswalls called septa. Septum formation is dependent on mitosis and requires the recruitment of actin to the site of septum formation. Employing a collection of temperature sensitive nuclear distribution (*nudA2*, *nudC3* and *nudF7*), nuclear division (*nimA5*, *hfaB3*), and septation (*sepD5*, *sepG1*) mutants, we have investigated the interdependency among nuclear positioning, mitosis, and cell growth in structuring the cellular compartments of *A. nidulans*. The cellular compartments of *nud*$^+$ strains were highly uniform with regard to nuclear distribution and averaged 38 μm in length. Incubation of *nud* mutants at semi-restrictive temperature resulted in aberrant nuclear distribution that appeared to direct the formation of variable-sized cellular compartments, ranging from 5 μm to greater than 81 μm. In germinating spores, the first septum forms at the basal end of the germ tube following the third round of nuclear division. Germlings must undergo mitosis in order to form a septum. Temperature-sensitive mitotic mutants were used to show that a single nuclear division is sufficient to activate septum formation, provided a critical cell size has been attained. In mitotic mutants and wild-type cells, delays in nuclear division resulted in the misplacement of the first septum. These results strongly support the role of mitotic nuclei in determining septal placement, and suggest that cell size control is post-mitotic in *A. nidulans*.

Key words: Septation, Nuclear positioning, Cell size, *Aspergillus nidulans*

INTRODUCTION

Following the completion of mitosis, cytokinesis occurs at a specific site in the cell cortex. This site organizes an array of cytoskeletal proteins, including actin and myosin, into a contracting ring at the midbody. In animal cells, the orientation of the actin ring is determined by the axis of the mitotic spindle (for review see Strome, 1993). In fungi, an actin ring precedes septum formation (Girbardt, 1979; Marks and Hyams, 1985). Genetic studies have identified a variety of conserved cytoskeletal proteins involved in the formation of the actin ring, together with novel gene products that may regulate its assembly (for review see Simanis, 1995). It is not clear, however, what parameters ultimately determine the site of cell division in fungi.

In *Saccharomyces cerevisiae*, spindle orientation is controlled by the presumptive bud site, which is organized early in the cell cycle (for review see Chant and Pringle, 1991). Mutations which perturb spindle orientation and nuclear positioning in *S. cerevisiae* do not affect bud site selection (Palmer et al., 1992; Li et al., 1993; Yeh et al., 1995). In fission yeast, it is also apparent that the mitotic spindle does not determine the site of septum placement or actin ring formation (Chang et al., 1996). A role for the nucleus in positioning the septum of *Schizosaccharomyces pombe* is not clear, since a mutation which perturbs nuclear position does not affect septum placement (Toda et al., 1983). However, a role for the premitotic nucleus in septum site positioning is favored because the nucleus is closely associated with the actin ring in both wild-type and morphologically abnormal cells (Chang and Nurse, 1996). Perhaps the strongest evidence for the role of the nucleus in septal placement comes from the classic study by Girbardt (1955). In the filamentous fungi, *Trametes gibbosa* and *Polystictus versicolor*, nuclear position and the resulting septal position could be displaced by removing cytoplasm from the basal end of fungal hyphae with a microneedle. Here, we address the role of nuclear position in septal placement in the multinucleate mycelium of *Aspergillus nidulans*.

Septation in *A. nidulans* is dependent on mitosis and actin ring formation (Harris et al., 1994). Unlike yeasts, the first two nuclear divisions in germinating spores occur in the absence of a septum, which generally forms after the third nuclear division. A block to the third nuclear division halts the formation of the first septum (Harris et al., 1994). In addition, septum formation in *A. nidulans* is highly asymmetric, partitioning the round spore body from an elongated, polarly growing germ tube.

Studies with the temperature-sensitive *sepA* mutant suggest that *A. nidulans* cells mark sites for septum formation. *sepA* mutants form large, aseptate, multinucleate hyphae at the

restrictive temperature. Trinci and Morris (1979) showed that when a *sepA* microcolony grown at 37°C is returned to permissive temperature, septa are laid down throughout the mycelium, and are spaced similarly to wild type (Trinci and Morris, 1979). Recently, *sepA* has been shown to be part of a new evolutionarily conserved gene family involved in cytokinesis and septation (Longtine et al., 1996; S. D. Harris, L. Hamer, K. Sharpless, and J. E. Hamer, unpublished results).

We have employed a combination of genetic and physiological approaches to demonstrate a clear role for nuclear positioning and mitosis on septum formation in *A. nidulans*. We show that nuclear distribution profoundly affects the position of the septum. We have also uncovered a cell size control for cell division in *A. nidulans*. In uninucleate cells, a threshold size is required to initiate DNA synthesis and to enter mitosis (Johnston et al., 1977; Carter, 1981; Nurse, 1975; for reviews see Nurse and Fantes, 1981; Fantes, 1984). Studies here suggest that cell size control is post-mitotic in *A. nidulans* and is a critical parameter controlling the onset of cytokinesis.

MATERIALS AND METHODS

Strains and growth conditions

Strains of *A. nidulans* used in this study are listed in Table 1. CM medium is 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, and nitrate salts are described in the appendix of Kafer (1977). For solid medium, 1.8% agar was added. Genetic techniques for *A. nidulans* are described by Harris et al. (1994).

Table 1. *A. nidulans* strains

Strain	Genotype
*A850**	*biA1;ΔargB::trpCΔB; methG1;veA1 trpC801*
*A28**	*biA1 pabaA6*
XX5†	*nudA2; pyrG89; wA2*
A01†	*nudC3; pabaA1 pyrG89; nicA2;wA2*
XX21†	*nudF7; pyrG89 yA2*
AJM2	*sepD5; argB2*
AJM29	*sepG1; pabaA6; sB3; chaA1*
SRF54‡	*biA1; ΔapsA::pyr4; ΔargB::trpCΔB*
AJC1.1§	*biA1;apsA1*
AJC1.7§	*biA1;apsB10*
ATW8	*pabaA6;pyrG89;nudF7;sepG1*
ATW9	*argB2;nudA2;sepD5*
ATW10	*argB2;pyrG89;nudF7;sepD5*
ATW11	*pabaA6;nudA2;sepG1*
*A781**	*nimA5;wA2*
SA253¶	*yA2 pabaA6;hfaB3*

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

†Obtained from Ron Morris, Department of Pharracology, University of Medicine and Dentistry of New Jersey, Picataway, New Jersey.

‡Obtained from Reinhard Fischer, Max-Planck-Institut für Terrestrische Mikrobiologie, Karl-von-Frisch-Str., D-35043 Marburg, Germany.

§Obtained from John Clutterbuck, Genetics Laboratories, Pontecorvo Building, University of Glasgow, Church Street, Glasgow G11 5JS, Scotland.

¶Obtained from Susan Assinder, School of Biological Sciences, University of Wales, Bangor, Gwynedd L57 2UW, Wales.

To observe phenotypes, supplemented CM liquid was inoculated with 1-5×10^4 conidia/ml, poured into a Petri dish containing glass coverslips, and incubated at 30°C for permissive temperature, 39°C for semi-restrictive, and 42°C for restrictive. For time course experiments monitoring the appearance of the first septum, cultures were grown at 39°C and coverslips were removed at hourly intervals and processed for microscopy. For each time point, 100 germlings were scored for the presence/absence of septa. All time course experiments were repeated three times with nearly identical results, and in most cases a typical experiment is shown. To measure intercalary compartment lengths, conidia were spread over the surface of solid CM, and then overlaid with a coverslip and germinated at 39°C for 20 hours. In this manner, hyphae began extension under the coverslip and then radiated over the top, adhering closely to the coverslips. Coverslips were lifted off the agar with tweezers and prepared for microscopy.

To observe the formation of the first septum in mitotic mutants and wild type, conidia were germinated in CM liquid for 7 hours at 37°C. To observe effects of hydroxyurea (HU) on positioning of the first septum, wild-type strain A28 was germinated in CM liquid on coverslips for 6 hours at 28°C. HU was added to the medium at a concentration of 45 mM. After three hours incubation in HU, germlings were released from the block and allowed to grow overnight.

Staining and microscopy

Fixing, staining, and microscopy methods are described by Harris et al. (1994). SYTO 16 live cell nucleic acid stain (Molecular Probes, Inc.) was added at 1 μM directly to coverslip cultures 15 minutes prior to observation.

Statistics

To test if a difference in intercalary length existed between each *nud* mutant and wild type, the nonparametric Mann-Whitney test was employed (Zar, 1984). A parametric test, such as the two-sample *t*-test or ANOVA, was not used because the samples did not come from normal populations and variance was not equal. Nonparametric tests do not require either of these criteria. To use the Mann-Whitney test, we ranked the data (measurements) and calculated the U and U′ value, which take the number of measurements and the sum of the ranks into consideration. The larger of the two values was compared to the respective critical value. If either value was equal to or greater than the critical value, Ho was rejected (α=0.05).

RESULTS

Cellular organization and asymmetric cell divisions in *A. nidulans*

The mycelium of *A. nidulans* is partitioned by crosswalls called septa. We use the term "compartment" to describe a cellular region delimited by septa. Thus, the fungal mycelium is composed of tip cells and intercalary compartments. Although cytoplasmic continuity between compartments is generally assumed due to the presence of a septal pore, nuclear division cycles are synchronous only within a compartment (Rosenberger and Kessel, 1967). Furthermore, active cell wall growth and nuclear division are generally confined to the tip cells, cell compartments undergoing branching to form new tips, and basally located cell compartments differentiating to form asexual reproductive structures (Katz and Rosenberger, 1971; S. Kaminskyj, T. Wolkow, S. D. Harris and J. E. Hamer, unpublished results). Thus, cellular compartments in *A. nidulans* appear to be functionally distinct.

Fig. 1 diagrams the asymmetric cell divisions in germinat-

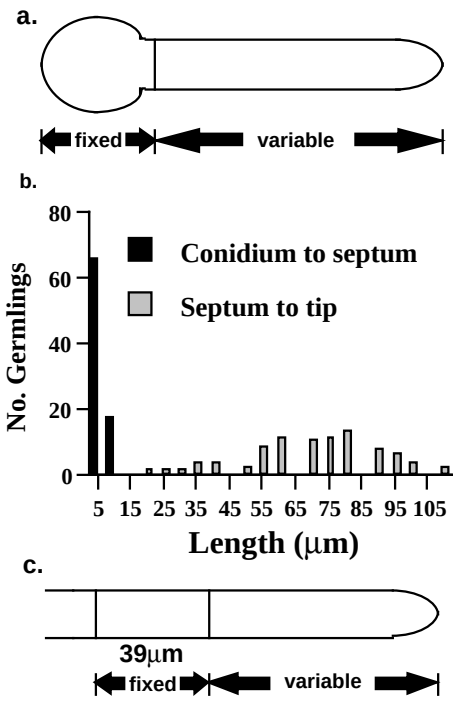

Fig. 1. Positioning of septa in *A. nidulans*. (a) Diagrammatic representation of the asymmetric placement of the first septum in germlings. Fixed describes the distance from the distal wall of the conidium to the septum (vertical line), and variable describes the distance from the septum to the hyphal tip. (b) Conidia were germinated for 8 hours, a time when 40% of germlings have a septum (Harris et al., 1994), and stained with Calcofluor. The distances from the distal conidium wall to the septum and from the septum to the hyphal tip where measured with a calibrated eyepiece micrometer in approximately 80 germlings. (c) Diagrammatic representation of the asymmetric placement of septa in vegetative hyphae. Fixed describes the distance (39 μm) between neighboring septa in hyphae (intercalary length), and variable describes the distance between the hyphal tip and the closest septum.

ing spores (germlings) and vegetatively growing hyphal cells of *A. nidulans*. In wild-type germlings, the first septum is formed during or following the third nuclear division (Harris et al., 1994). Germlings at this stage of growth are generally between 45 and 90 μm in length, and the positioning of the first septum occurs toward the basal end of the germ tube (Fig. 1a). Measurements of the septum-to-tip and conidium (distal wall)-to-septum distances in germlings having undergone one septation event are shown in Fig. 1b. Septum-to-tip distances varied from 20 μm to greater than 100 μm. In contrast, the conidium-to-septum distances were relatively constant, varying only slightly beyond 5 μm in some germlings. A mechanism must exist to determine the exact placement of the first septum in germlings.

During vegetative growth, further rounds of septation occur following successive rounds of nuclear division in polarly growing hyphal tip cells (Clutterbuck, 1970; Fiddy and Trinci, 1976). Fig. 1c illustrates that septa are always placed toward the basal end of the tip cell. This results in a long, variably sized hyphal tip cell, but uniformly spaced septa delimiting subapical compartments with a fixed intercalary length (Trinci and Morris, 1979; see below). Intercalary compartment length is not determined by intrinsic growth rate because this size is largely unaffected by tip cell extension rate or growth temperature (Trinci and Morris, 1979; see below). Thus, in both germinating spores and vegetatively growing hyphae, cell division is asymmetric due to the polarized confinement of apical growth.

Table 2. Genes and gene products

Gene	Gene product
nudA	Cytoplasmic dynein heavy chain (Xiang et al., 1994)
nudC	23 kDa protein of unknown function (Osmani et al., 1990)
nudF	Similar to the human *LIS-1* gene required for neuronal migration (Xiang et al., 1995a)
apsA	183 kDa coiled-coil protein similar to NUM1p of *Saccharomyces cerevisiae* (Fischer and Timberlake, 1995)

Nuclear positioning and the division site

To test if nuclear positioning influences the placement of septa, we attempted to displace the nuclei of growing hyphae and measure the resulting effects on the positioning of septa.

Nuclear positioning and migration are easily studied in filamentous fungi due to their multinuclear, multicellular structure and the identification of non-lethal mutations in both *A. nidulans* and *Neurospora crassa* that alter nuclear distribution (for review see Beckwith et al., 1995). Morris (1976) isolated several temperature-sensitive mutants of *A. nidulans* deficient in nuclear distribution (*nud*). We chose to study septal placement in strains carrying thermo-sensitive mutations in three different *nud* genes; *nudA2*, *nudC3*, and *nudF7*. Gene products have been identified and characterized for these genes (see Table 2). At restrictive temperature (42°C), nuclei of *nud* mutants fail to migrate into the extending hyphae of germinating spores, while at semi-restrictive temperatures (39°C) nuclei migrate into hyphae, but become aberrantly positioned (see below).

To ensure that the *nud* mutations did not interfere directly with the process of septation, we compared the kinetics of the first cell division event following spore germination (formation of the first septum) in wild type and *nud* mutants grown at 39°C. During these experiments, nuclear division rates in *nud* and wild-type germlings were highly similar (data not shown), confirming earlier results that *nud* mutations do not dramatically alter the kinetics of nuclear division (Osmani et al., 1990). In germlings of wild-type strains, the first septum is formed at approximately the time of the third round of nuclear division (Harris et al., 1994). Fig. 2 shows that 100% of *nudF7* and wild-type germlings formed septa by 9 hours at 39°C, and thus have identical kinetics of septum formation. *nudA2* and *nudC3* germlings initiated septum formation just slightly later, attaining 100% septa by 10.5 hours. Both wild type and *nud* mutants formed septa with a high degree of synchrony. From these experiments, we conclude that *nud* mutations did not affect the kinetics of septation at 39°C.

The positions of nuclei and septa in wild type and *nud* mutants are shown in Fig. 3. Following germination, nud^+ nuclei divided and moved into the extending germ tube (Fig.

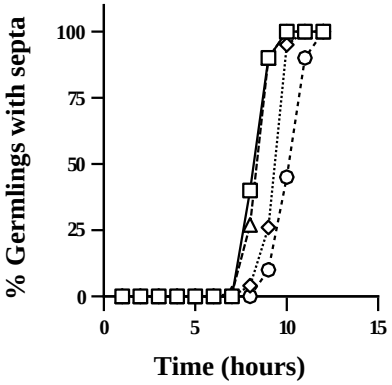

Fig. 2. Kinetics of septum formation in *nud* mutant and wild-type germlings. Conidia from strains A850(*nud*$^+$, □), XX5(*nudA2*, ◇), A01(*nudC3*, ○), and XX21(*nudF7*, △) were germinated on coverslips in complete media at 39°C. Coverslips were removed at various intervals, fixed and stained with a combination of Calcofluor and Hoechst 33258 to observe septa and nuclei (see Materials and Methods).

3a). As previously demonstrated (Beckwith et al., 1995), the nuclei of *nud* mutants accumulated in the conidium of germlings and failed to move into the extending germ tube at 42°C (Fig. 3b). At this restrictive temperature, *nud* mutants also failed to form an extensive branching mycelium. As anticipated, this same phenotype was observed for all three *nud* mutants used in this study (data not shown).

The vegetative mycelium of *A. nidulans* grown at 39°C contained cell compartments delimited by evenly placed septa (Fig. 3c). If unbranched, these compartments generally contained three nuclei that were evenly positioned throughout the mycelium. At the semi-restrictive temperature, nuclei from *nud* mutants were able to migrate into the extending germ tube, and the mutants were able to form an extensive, branching mycelium. However, Hoescht staining revealed that nuclei were not evenly distributed, but remained in clumps, containing 2 to >32 nuclei, throughout the mycelium (Fig. 3d). All *nud* mutants showed this phenotype at the semi-restrictive temperature (data not shown). We infer from these observations that growth at the semi-restrictive temperature allowed partial *nud*$^+$ gene function such that some nuclear migration could occur.

To examine the effects of asymmetric nuclear distribution on cell division in vegetative hyphae, we employed Calcofluor staining to visualize the placement of septa. Septa were present throughout the mycelium of *nud* mutants grown at semi-restrictive temperature. However, the lengths of intercalary compartments were variable, and occasionally short compartments were detected that completely lacked nuclei (Fig. 3c). To describe the variation in intercalary compartment size, we measured over 150 intercalary compartments in wild type and each *nud* mutant grown at 39°C. Fig. 4a shows that wild-type mycelia had compartment lengths that averaged 38 μm, close to the previously reported average of 39 μm (Trinci and Morris, 1979). Furthermore, wild-type mycelia displayed very little variance around this average (Fig. 4a; Table 3). In contrast, the intercalary compartment lengths of the three *nud* mutants grown at 39°C were strikingly different from wild type (Fig. 4b-d). The average compartment length for each *nud* mutant exceeded the wild-type value of 38 μm (Table 3). In addition, each *nud* mutant had a much wider distribution of intercalary compartment lengths. For example, we did not find any wild-type compartments shorter than 10 μm or greater than

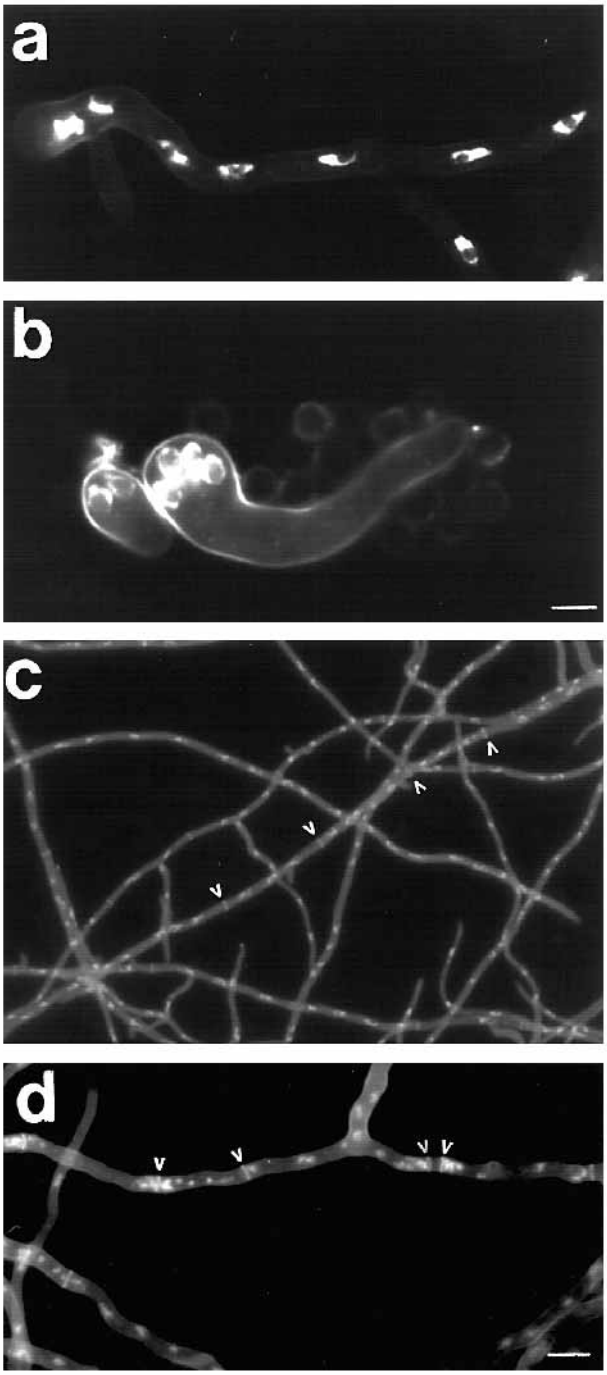

Fig. 3. Morphology of wild type and *nud* mutants of *A. nidulans*. (a and b) Conidia from A850(*nud*$^+$) and XX5(*nudA2*) were germinated on coverslips for 8 hours in CM at 42°C. Nuclei were stained with Hoechst 33258. (c and d) Conidia of A850(*nud*$^+$) and XX21(*nudF7*) were grown for 18 hours at 39°C. Strains were fixed and stained with both Hoechst 33258 and Calcofluor to show nuclear and septal positioning. Arrow heads identify the positions of septa. Bars: (a and b) 3 μm; (c and d) 6 μm.

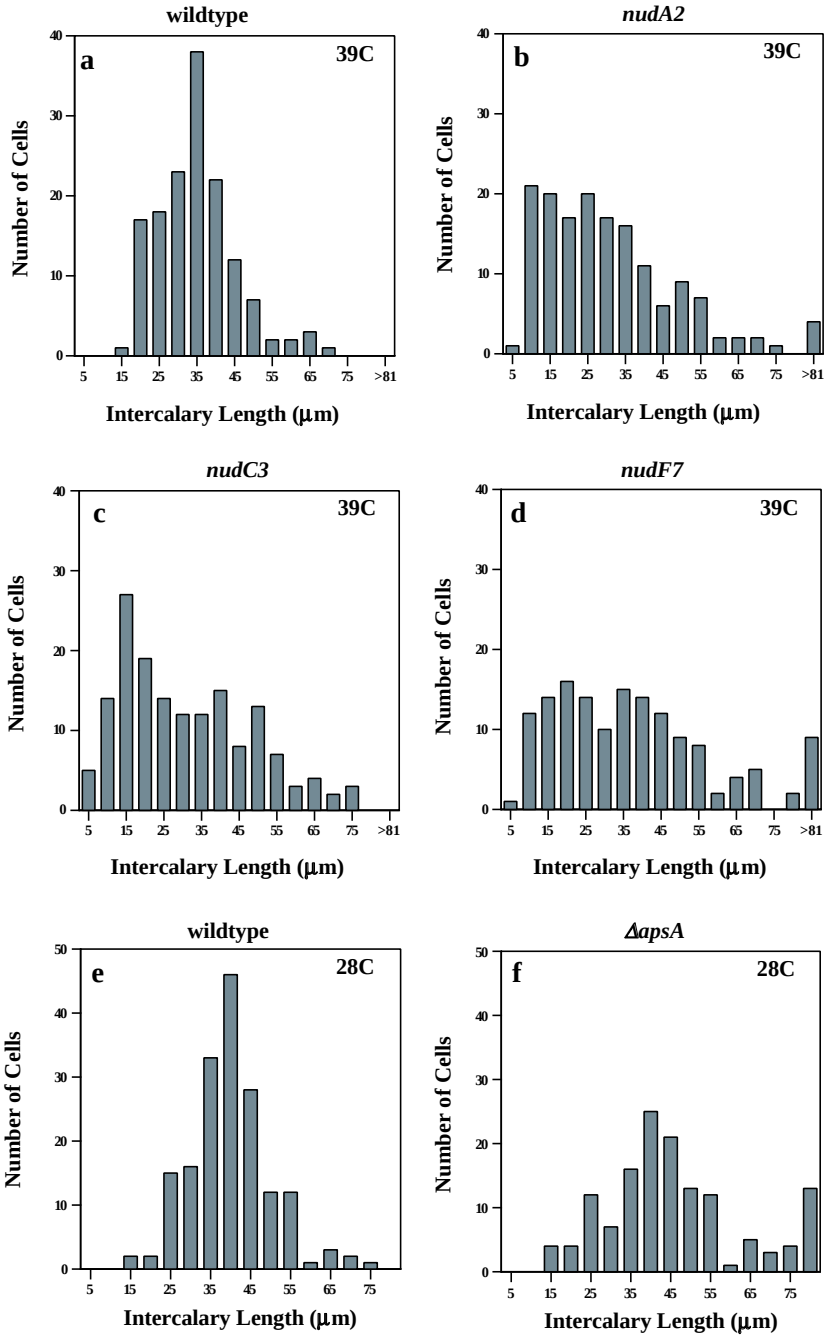

Fig. 4. Wild-type, *nud*, and *aps* mutant intercalary compartment sizes. A850 (*nud*$^+$), XX5(*nudA2*), AO1(*nudC3*), and XX21 (*nudF7*) were grown at 39°C for 18 hours, fixed, and stained with Calcofluor. A28 (*apsA*$^+$) and SRF54 (*ΔapsA*) were grown at 28°C for 18 hours, fixed, and stained with Calcofluor. Intercalary compartment lengths were measured with a calibrated eyepiece micrometer. Lengths were grouped and graphed in the following categories: <5, <10, <15, etc.

Table 3. Intercalary compartment length statistics

Genotype	Mean (μm)	s.d.	Variance
Wild type (39°C)	38.40	15.37	236
nudA2 (39°C)	46.95	42.30	1789*
nudC3 (39°C)	50.45	36.74	1350†
nudF7 (39°C)	47.26	29.90	895‡
Wild type (28°C)	37.36	10.33	106
ΔapsA (28°C)	44.18	23.16	536§
	150<*n*<200		

We found significant differences (α=0.05) in intercalary lengths between each *nud* mutant and wild type grown at 39°C using the Mann-Whitney test (Zar, 1984). No significant difference was found between *ΔapsA* and wild type grown at 28°C.

*Z = 36.12; †Z = 39; ‡Z = 66; §Z = 1.16.

75 μm (Fig. 4a). However, all three *nud* mutants shared a modest number of intercalary compartments with lengths both below 10 μm and above 75 μm (Fig. 4b-d). This large variance caused *nud* compartment lengths to differ significantly from those of wild type (Table 3). At permissive temperature, *nud* mutant compartment lengths were normal and averaged close to 38 μm (data not shown). Because each of these *nud* mutations disrupts a unique gene function (Table 2), it is unlikely that this effect at 39°C is gene specific. We infer from these data that the highly variable intercalary compartment lengths of *nud* mutants grown at 39°C are a consequence of asymmetric nuclear distribution.

In a genetic screen for mutants defective in asexual sporulation but not restricted for growth, Clutterbuck (1969) isolated

non-conditional anucleate primary sterigmata (*aps*) mutants (Table 2). *aps* mutants are unable to position nuclei correctly in the asexual reproductive structures known as conidiophores (Clutterbuck, 1994; Fischer and Timberlake, 1995). Although abnormal nuclear distribution is readily apparent in conidiophores, it is only moderately visible in the vegetative hyphae of *aps* mutants. Except for the occasional gaps devoid of nuclei, nuclear distribution in *aps* hyphae resembles that of wild type. Consistently, the intercalary length distribution of *ΔapsA* compartments resembled that of wild type, except for the population of compartments greater than 80 μm in length (Fig. 4e,f). Studies with the *apsA2* and *apsB3* mutants gave similar results (data not shown). In *ΔapsA* mutants, compartments with lengths greater than 80 μm coincided with the areas of hyphae devoid of nuclei. Table 3 shows that the average length of an *ΔapsA* cell was 44 μm. However, when the population of cells greater than 80 μm was not considered, this average was reduced to the wild-type average of 38 μm (data not shown). Irrespective of these large compartments, no significant difference was found between the lengths of wild-type and *ΔapsA* cell compartments (Table 3). Thus, the subtle vegetative nuclear distribution phenotype of the *ΔapsA* mutant did not disrupt septal placement significantly.

Nuclear distribution and positioning of septa

During phenotypic examination of the *nud* mutants grown at the semi-restrictive temperature, we noticed that nuclei tended to aggregate in clumps ranging in size from 2 to >32. We also observed that these nuclear clumps were commonly associated with a septum (Figs 3d, 5a). All *nud* mutants showed this phenotype at the semi-restrictive temperature. Staining of unfixed cells with the vital dye Syto16 demonstrated that this was not an artifact of fixation (data not shown). The occurrence of septa positioned near clumps of nuclei could have resulted from preferential septation near aberrantly distributed nuclei. Alternatively, septa may have influenced the distribution and spacing of nuclei. For example, at the semi-restrictive temperature, *nud* mutations may have caused aberrant septal placement, which in turn disrupted nuclear distribution.

To distinguish between these explanations, we constructed *nud* mutants lacking septa. Four septa-less *nud* mutants were constructed by crossing two *nud* mutants, *nudA2* and *nudF7*, with two late-acting *sep* mutants, *sepD5* and *sepG1* (Harris et al., 1994; Momany et al., 1995). Putative *nud,sep* double mutants were identified by their colony morphology at 42°C and confirmed by backcrosses to wild-type strains. *nud* mutants formed small, tight colonies at the restrictive temperature, whereas late-acting *sep* mutants formed larger, branching colonies. We did not observe any signs of direct gene interaction (i.e. suppression, lethality) between the *nud* and *sep* alleles, suggesting these gene products act independently. As anticipated, all double mutants formed small, tight colonies at 42°C, suggesting that the *nud* mutations are epistatic to late-acting *sep* mutations.

When grown at the semi-restrictive temperature, all four double mutants exhibited irregular nuclear distribution and clumping in the absence of septa (Fig. 5b,c). As described previously for the *nud* mutants grown at this temperature, anywhere from 2 to >32 nuclei were present in the clumps, and the lengths between each clump varied greatly (data not shown). Thus, the nuclear clumps that we observed in the various *nud* mutants are likely a consequence of the partial function of the *nud* gene products at 39°C. These clumps of nuclei most likely directed the placement of septa in close proximity.

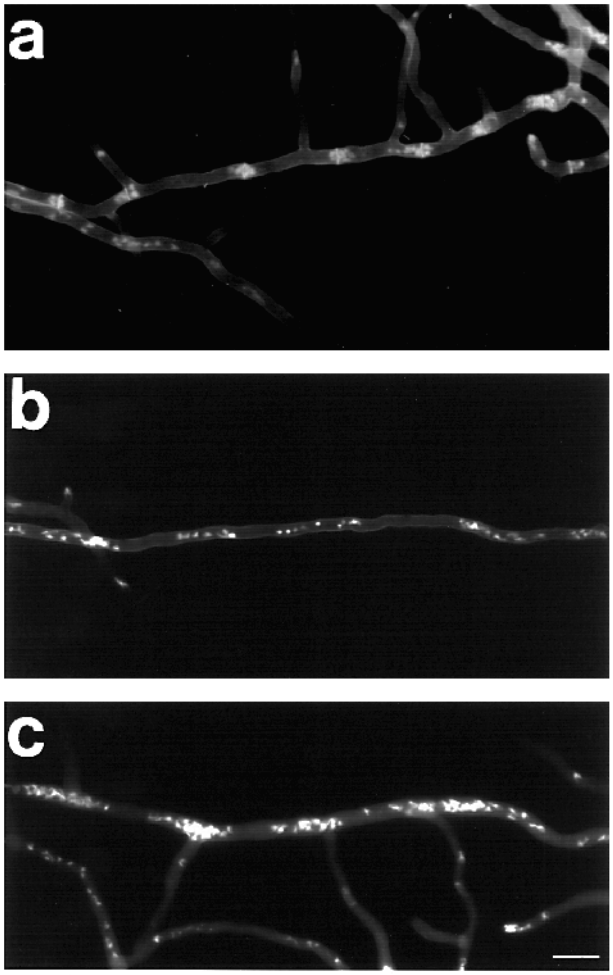

Fig. 5. Morphology of *nud* and *sep,nud* double mutants. ATS22 (*sepG1*) and AJM2 (*sepD5*) were crossed to XX21 (*nudF7*) and XX5 (*nudA2*). Double mutants were recovered and confirmed by genetic analysis (see text). Double mutants were germinated on coverslips for 20 hours in CM at 39°C. Specimens were fixed and stained with Calcofluor and Hoechst 33258. XX21 (*nudF7*) shows septa positioned in nuclear clumps. (a) *nudF7* (b) *sepG1,nudA2* (c), *sepD1,nudF7*. Bar, 6 μm.

Nuclear control of septation in *A. nidulans*

Harris et al. (1994) used HU block and release experiments to demonstrate that septum formation is dependent on the completion of the third nuclear division (eight nuclei stage). However, germlings with septa are generally longer than 45 μm and thus earlier nuclear divisions may fail to activate septation because a critical cell size has not been reached. To test this idea, we took advantage of the observation that mitotic mutants of *A. nidulans* are able to germinate and form elongated germ tubes (>45 μm) at the restrictive temperature, but are unable to complete mitosis. The *nimA* gene encodes a protein kinase (p79) (Osmani et al., 1991) which, in addition to the cyclin dependent kinase *nimX* (Osmani et al., 1994), is

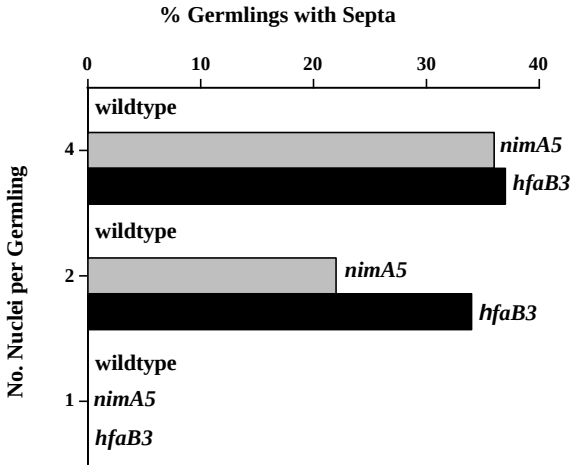

Fig. 6. Nuclear control of septation in *A. nidulans*. All strains were grown for 7 hours at 39°C, fixed, and stained with a combination of Hoechst 33258 and Calcofluor. Cells were counted and the percentage of germlings with one, two, or four nuclei and a septum was calculated for A28 (wild type), A781 (*nimA5*) and SA253(*hfaB3*). n>150.

required for successful passage through M phase (Osmani et al., 1987). At restrictive temperature (42°C), *nimA5* mutants arrest with a single interphase nucleus and do not form a septum (Morris, 1976; Harris et al., 1994). The *hfaB3* (high frequency of aneuploids) gene is required for correct chromosome segregation, and mutants arrest growth with a single abnormally shaped nucleus, characteristic of aneuploids (Upshall and Mortimore, 1984).

nimA5 and *hfaB3* mutants were germinated on coverslips at the semi-restrictive temperature, 37°C, for 7 hours. Under these conditions, wild-type germlings generally undergo three rounds of nuclear division and produce a septum toward the basal end of the extended germ tube. We found that *nimA5* and *hfaB3* mutants were slowed for mitotic progression at 37°C, undergoing only one or two nuclear divisions, although germ tube extension was comparable to wild type for the first 7 hours (data not shown). The frequency of septum formation in mitotic mutant and wild-type germlings containing one, two, or four nuclei was determined (Fig. 6). No wild-type germlings with two or four nuclei were observed to have septa (n>250). In contrast, more than 20% of *nimA5* and *hfaB3* germlings had a septum after one nuclear division. This fraction increased to more than 35% for each mutant if cells were incubated longer, allowing a second round of nuclear division. Similar results were obtained for the *bimD6* mitotic mutant of *A. nidulans* (S. D. Harris, unpublished results). We did not observe any wild-type, *nimA5*, or *hfaB3* germlings grown at 37°C having one nucleus and a septum. However, when grown at 42°C for 9 hours, we observed 16% of *hfaB3* germlings with only one nucleus and a septum. The septum traversed through a stringy Hoechst-stained nucleus, giving a 'cut' phenotype similar to some *S. pombe cdc* mutants (Samejima et al., 1993; Hirano et al., 1986).

Micrographs of typical *nimA5* germlings grown at 37°C are shown in Fig. 7. For both *nimA5* and *hfaB3* germlings (not shown), we only observed septa in cells with extended germ tubes, and in every case the septum partitioned two daughter

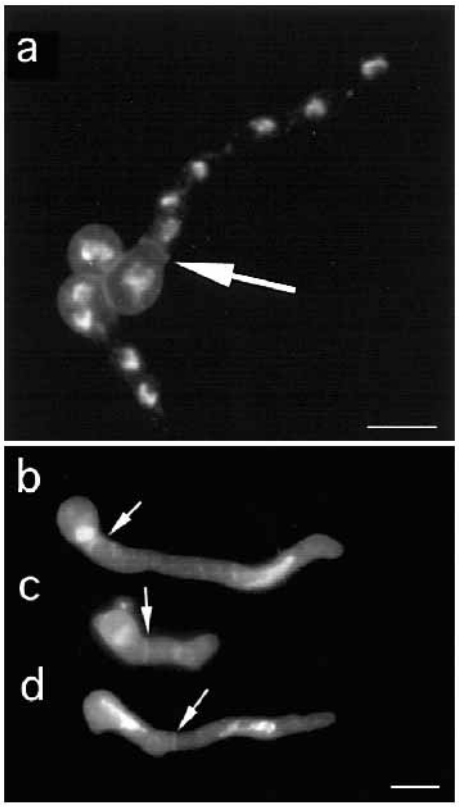

Fig. 7. Septum formation in wild type and *nimA5* mutants. All strains were grown for 7 hours at 37°C and fixed and stained with a combination of Hoechst 33258 and Calcoflour. (a) A28 (wild type). Germlings with 2, 4 and 8 nuclei are shown. Septum formation never occurs in wild-type cells with 2 or 4 nuclei. A germling with eight nuclei contains a septum positioned at the basal end of the germ tube. (b-d) A781 (*nimA5*). Growth at the semi-restrictive temperature (37°C) slowed nuclear division without affecting germ tube extension in these germlings. Nuclei migrated into the germ tube and the septum is positioned between the two nuclei. The septum is frequently displaced towards the hyphal tip. Bars: (a) 5 μm; (b-d) 4 μm.

nuclei. We conclude that a single nuclear division is sufficient to trigger septation in *A. nidulans* germlings, provided a critical cell size has been attained.

In *nimA5* mutants, we frequently observed displacement of the septum toward the middle of the germ tube (Fig. 7c,d). One explanation for this displacement is that nuclear migration has triggered septum formation further up the germ tube. To test this hypothesis, we germinated wild-type spores for 6 hours to break dormancy, and then incubated them in HU for 3 hours. HU blocks DNA synthesis without affecting nuclear migration or germ tube extension (Bergen and Morris, 1983). This treatment permitted nuclei arrested in S-phase to migrate into the extending germ tube. Germlings were then washed free of the drug and allowed to continue growth overnight. Following fixation and Calcofluor staining, the conidial-to-septum distances were determined as previously described (see Fig. 1). Instead of forming septa within a restricted distance of 5-10 μm, Fig. 8 shows that this treatment displaced the septum in a significant fraction (48%) of the cells. These results suggest that displacement of nuclei can alter the position of the first septum.

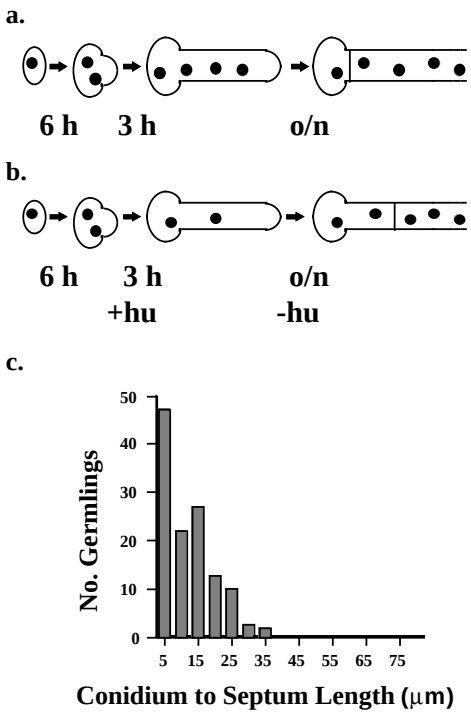

Fig. 8. Delays to nuclear division displace the first septum towards the tip. (a) Diagram of control results. The septum of untreated germlings was positioned at the basal end of the germ tube. (b) Diagram of HU treated germlings. Germlings were allowed to germinate, blocked with HU for six hours, and then washed free of the drug and allowed to form septa (see text for details). The septum of treated germlings was positioned toward the hyphal tip. (c) Graph of the conidium to septum lengths of HU treated germlings (compare with lengths of untreated germlings in Fig. 1b). All germlings were fixed, stained with Hoechst 33258 and Calcofluor, and the conidium (distal wall)-to-septum distances were determined (see Materials and Methods).

DISCUSSION

Cellular morphogenesis requires a controlled interplay of directed cell growth (polarity), nuclear movement, mitosis, and cytokinesis. These events must be coordinated such that cell growth occurs in concert with cell division, and that each cell receives a requisite amount of genetic and cytological content. In the most genetically amenable yeasts and filamentous fungi, many of the individual biochemical components that drive each of these events are well known and appear to be evolutionarily conserved among eukaryotes. It now becomes important to determine how these various cellular events are coordinated to achieve a seemingly infinite array of different cell morphologies. We have used a collection of temperature sensitive mutations in well defined gene products to investigate the organizational pattern of cell division in *A. nidulans*.

The role of the *nud* genes is to direct nuclear migration and distribution in *A. nidulans*. Support for this comes from observations that nuclear division (Xiang et al., 1994), mitochondrial movement, and tip growth (Xiang et al., 1995a) in *A. nidulans* are unaffected by mutations in *nud* genes. In addition, deletion mutants of the dynein heavy chain (*ΔnudA*) are viable in *A. nidulans* (Xiang et al., 1995b). When incubated at a semi-restrictive temperature, *nud* mutants produced a similar phenotype consisting of clumped arrays of nuclei throughout the extended mycelium, regardless of the presence of septa. Likewise, all three *nud* mutations had the same effect on septation: the formation of aberrantly spaced septa. These results suggest that partial function of the *nud* gene products at the semi-restrictive temperature caused abnormal nuclear distribution which subsequently led to aberrant septal placement. We could find no evidence for a direct role of *nud* genes in septum formation, either by observing the kinetics of septum formation in *nud* mutants, or by looking for genetic interactions in a subset of *sep,nud* double mutants. The collection of *sep* and *nud* mutants continues to grow and genetic interactions may ultimately be found. The evidence presented here supports the hypothesis that the effect of the *nud* mutations on septum formation is an indirect effect of aberrant nuclear positioning.

Several lines of evidence suggest that it is the position of mitotic nuclei that ultimately influences septal placement. First, septum formation in *A. nidulans* is dependent on mitosis. In growing hyphal cells, parasynchronous waves of nuclear division are closely followed by septum formation (Clutterbuck, 1970; Fiddy and Trinci, 1976), and when assayed as germlings, mitotic mutants of *A. nidulans* that block at the G_2/M boundary fail to form septa when incubated at restrictive temperature (Harris et al., 1994). Secondly, Harris et al. (1994) demonstrated that germlings with four nuclei could not form septa when further nuclear division events were blocked with either HU or benomyl. These studies convincingly showed that germling size alone was not sufficient to trigger septation and that passage through mitosis was required to activate septum formation. However, these experiments did not eliminate the possibility that any nuclear division could activate septum formation, provided it occurred in the right place or in the appropriately sized hyphal cell or germ tube.

Incubation of two mitotic mutants (*nimA5* and *hfaB3*; G_2/M transition) at a semi-restrictive temperature produced a significant fraction of binucleate germlings with a single septum. Similar effects have been observed with other M-phase mutants of *A. nidulans* (S. D. Harris, unpublished results). In addition, we noted that in septated germlings, the septum was always placed between the two nuclei. These results are most readily explained by the hypothesis that a delayed nuclear division cycle in these germlings resulted in germ tube growth and septation following the first nuclear division. We hypothesize that wild-type germlings do not form a septum until a critical cell size has been attained and mitosis has occurred. A model to illustrate this hypothesis is shown in Fig. 9. The model proposes that septum formation is triggered by mitotic nuclei during germ tube extension. However, septum formation is delayed until a mitotic nucleus is properly positioned in the extending hyphal cell, a time coincident with the third nuclear division. Earlier nuclear divisions are prevented from activating septum formation due to a control mechanism that inhibits cytokinesis until a critical cell size is reached. We propose that cell size control is mediated by the presence of a negative regulator of septation with a tip-high concentration gradient. The formation of the septum is thus dependent on signals generated/diluted during cell extension and growth (cell size), and upon signals emanating from mitotic nuclei in the germ tube.

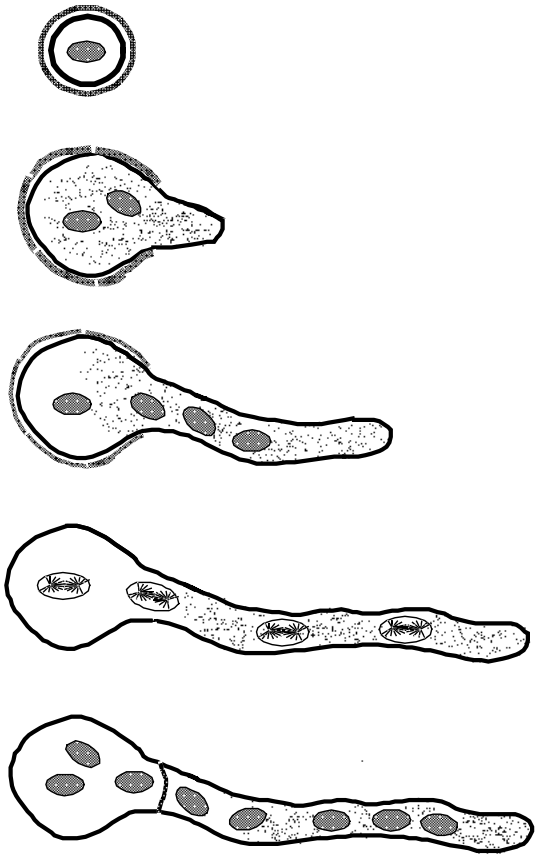

Fig. 9. A model for septum formation in germinating conidia of *A. nidulans*. The presence of a septation inhibitory mechanism with a tip-high gradient prevents the first two nuclear divisions from activating septation. Upon germ tube extension and consequent dilution of the inhibitory mechanism from basal hyphal regions, the third mitotic event initiates septum formation in an asymmetric fashion. Further septation events are initiated by nuclear divisions spatially separated from the inhibitory gradient.

This model accounts for the dependency of septation with mitosis (Harris et al., 1994), and the finding that a single mitotic division is sufficient to activate septation, provided germ tube extension and nuclear migration have occurred. In addition, the presence of a tip-high concentration gradient inhibiting septation explains why cell division in growing hyphal cells and germinating spores is asymmetrical. Finally, this model predicts that mitotic nuclei can activate septation in all areas of the mycelium, other than the region proximal to the tip. This prediction is also consistent with our experimental observations. However, it remains to be determined how nuclei position septation sites. In fungi, the nuclear membrane remains intact during mitosis, suggesting that either a signaling mechanism may exist or that a direct physical interaction between the nucleus and the cell cortex may be involved.

The model proposes that cytokinesis in *A. nidulans* is inhibited until a critical cell size is reached. This regulation may be mediated by a cytokinesis inhibitor with a tip-high concentration gradient. Tip-high gradients of calcium are known in fungi (Jackson and Heath, 1993), although no direct role in septation has been found. In *S. pombe* cell size regulates mitosis and is mediated by regulatory phosphorylation of $p34^{cdc2}$ (Russell and Nurse, 1987; Featherstone and Russell, 1991; Lundgren et al., 1991; Parker et al., 1991, 1992). It remains to be determined if regulatory phosphorylation of p34 affects cell size and cytokinesis in *A. nidulans*.

The results described here support a model where cell size, together with mitosis, controls the ability of *A. nidulans* germlings to undergo septation. Although mitosis has been well studied in *A. nidulans*, no other evidence for a cell-size control has been found. In budding yeast, a cell size control is evident close to START, while in fission yeast, size control is exerted primarily at G_2. In both systems, continued cell cycle progression is delayed until a critical cell size is reached. Evidence here suggests that in *A. nidulans* vegetative hyphae, cell size control is post-mitotic and may control the onset of cytokinesis. It remains to be determined whether this type of cell size control will be generalized to other polynuclear cells. The life cycle of *A. nidulans* involves alternating cycles of uninucleate spore and multinucleate hyphal cell production (Clutterbuck, 1969; Doonan, 1992). Shifting the cell size control from a pre-mitotic step (G_1/G_2) to a postmitotic step (cytokinesis) may be a simple mechanism for producing multinucleate hyphae from uninucleate spores.

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