

Relationship of Actin, Microtubules, and Crosswall Synthesis During Septation in *Aspergillus nidulans*

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Studies of cytokinesis in animal cells demonstrate that microtubules play an important role in signaling the position of the actin-containing contractile ring and subsequent formation of the cleavage furrow. Septation in several fungi closely resembles animal cell cytokinesis in that a circumferential ring of actin is visible at the incipient division site. However, this does not necessarily mean that division is contractile since actin may also serve to localize septal wall synthesis. In addition, several studies in fission yeast have suggested that microtubules are dispensable for actin ring formation. We have used synchronized cells and fluorescence microscopy to follow actin structures, nuclear division and septal wall synthesis during septation in *Aspergillus nidulans*. Our data suggest that actin first appears at the septum site as a circumferential ring and that it later broadens and invaginates, forming an hourglass-shaped structure coincident with septal cell wall synthesis.

Depolymerization of microtubules early in septation prevents circumferential actin ring formation. Depolymerization of microtubules after circumferential actin ring formation blocks both the progression to invaginating bands and septal wall synthesis. In contrast to studies in yeast cells, our data suggest that microtubules are required for both the initiation and progression of septation in *A. nidulans*. *Cell Motil. Cytoskeleton* 38:373–384, 1997. © 1997 Wiley-Liss, Inc.

Key words: cytokinesis; chitin; mitotic spindle; filamentous fungi

INTRODUCTION

Cytokinesis in eukaryotic cells requires cytoskeletal elements and occurs through one of two basic mechanisms. The parent cell may be partitioned through the contraction of cytoskeletal elements, as is typically seen in animal cells. Alternatively, the parent cell may be partitioned through the localized deposition of cell wall material guided by cytoskeletal elements, as is typically seen in plant cells. In animal cells, a band containing actin and other proteins constricts inward from the periphery, eventually pinching the parent in two [reviewed by Rappaport, 1986; Satterwhite and Pollard, 1992; Fishkind and Wang, 1995]. Signals emanating from either astral microtubules [Rappaport, 1961; Devore et al., 1989], or midzone microtubules [Cao and Wang, 1996; Fishkind et al., 1996] are required to direct and perhaps maintain cleavage furrow formation [Wheatley and Wang, 1996]. Like cytokinesis, septation is coordinated with mitosis

and involves the recruitment of actin, in the form of a circumferential ring, to the incipient division site. However, there is no evidence that microtubules are important for signaling actin ring formation in fungi.

Fungi use both contractile and noncontractile mechanisms for cell division. The fission yeast, *Schizosaccharomyces pombe*, appears to use a contractile mechanism for cell division [reviewed by Simanis, 1995]. Actin appears

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as a thin circumferential ring over the premitotic nucleus [Marks and Hyams, 1985; Chang et al., 1996]. Near the end of mitosis, the actin staining condenses into a series of medial spots [Marks and Hyams, 1985] and septal wall material is deposited. During wall deposition, an actin-containing invagination can be seen [Chang et al., 1996], suggesting constriction of the plasma membrane may be taking place. A constricting actin ring can be clearly seen in the related yeast *S. japonicus* [Alfa and Hyams, 1990; Jochovia et al., 1991]. These findings, and the localization of tropomyosin at the division site [Balasubramanian et al., 1992], suggest that division occurs through the formation of a constricting actin ring. Studies with β -tubulin mutants demonstrate that an intact mitotic spindle is not required for the localization of the circumferential actin ring during septation in *S. pombe* [Chang et al., 1996]. However, recently it has been shown that the later stages of septum assembly in *S. pombe* may be controlled by the mitotic checkpoint that governs spindle assembly. Depolymerization of microtubules blocks the formation of a septum during synchronized cell division [Murone and Simanis, 1996] and genes implicated in the spindle assembly checkpoint are required to prevent unscheduled septation events [Fankhauser et al., 1993; Murone and Simanis, 1996].

The budding yeast, *Saccharomyces cerevisiae*, appears to use a noncontractile mechanism for septation. Early in the cell cycle a ring of chitin appears at the future site of septation and bud growth takes place through this ring. After nuclear separation, a disc of chitin forms within the ring to become the primary septum [reviewed by Bulawa, 1993]. Actin is seen as a collection of dots or patches at the neck during formation of the chitin ring and again later as the chitin disk is made [reviewed by Lew and Reed, 1995]. Rather than acting as a constricting ring, actin is thought to guide vesicles bearing cell wall material to the growing bud.

The mechanism of cytokinesis in multicellular filamentous fungi is less clear. Though these fungi partition their cytoplasm through septa, the septa are never digested. Thus, the "daughters" never separate. In addition, many fungal septa have pores allowing some cytoplasmic continuity between compartments [reviewed by Bracker, 1967; Gull 1978]. Microfilaments associated with septum formation have been reported in a few filamentous fungi [Girbardt, 1979; Hoch and Howard, 1980; Roberson, 1992]. In other cases, high concentrations of actin have been found at the sites where septa will form, but no evidence for contraction has been reported [Runeberg and Raudaskoski, 1986; Butt and Heath, 1988; Salo et al., 1989; Tanabe and Kamada, 1994]. Recently, presumed actin mutants of the basidiomycete *Coprinus cinereus* have been shown to be unable to make septa

[Tsukamoto et al., 1996] though, again, the exact role of actin is unclear.

Genetic and molecular studies have also been initiated on septation in the multicellular filamentous fungus, *Aspergillus nidulans* [Morris, 1976; Harris et al., 1994; Momany et al., 1995]. During the germination of the uninucleate conidia of *A. nidulans*, two to three rounds of mitosis occur in the absence of cytokinesis. A septum is generally formed after the third round of nuclear division (8-nuclei stage). The inability to septate at earlier divisions appears to be due to a control mechanism that prevents cytokinesis until germlings reach a critical threshold size [Wolkow et al., 1996]. After the formation of the first septum, dividing nuclei are found in tip cell compartments and a wave of nuclear division is followed by a wave of septation [Rosenberger and Kessel, 1967; Clutterbuck, 1970; Fiddy and Trinci, 1976]. Septation is dependent upon the preceding mitosis and a band of actin can be localized to the incipient division site. Cytochalasin A reversibly blocks septum formation suggesting that actin is required for septation [Harris et al., 1994].

In this study we have investigated the dynamics of actin and cell wall accumulation at the division site and the role of microtubules in septation. Using synchronized cells and fluorescent microscopy, we show that actin first appears as a circumferential ring either over or near a nucleus. As septation proceeds, actin forms an invaginating band with cell wall deposition occurring in the wake or furrow of the band. Time course studies with the microtubule depolymerizing drug, benomyl, provide evidence that in *A. nidulans* there is a persistent requirement for microtubules for actin ring formation, invagination, and septal wall synthesis. These findings support a role for the mitotic spindle in septum formation and progression and are consistent with cytokinesis models developed in cultured animal cells where microtubules are continuously required for cleavage furrow formation [Wheatley and Wang, 1996].

MATERIALS AND METHODS

Aspergillus Strains and Growth Methods

For electron microscopy, wild-type strain A28 (FGSC) was grown for 48 hours at 37°C on cellulose membranes overlaid on complete agar 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). Trace elements, vitamins, nitrate salts, and amino acid supplements are described in the appendix to Kafer [1977]. BEN13 (biA1, AcrA1, benA15) was the gift of Berl Oakley (Ohio State University). For all other experiments, diploid strain A852 (biA1; Δ argB::trpC Δ B; methG1; veA1 trpC801/pabaA1 yA2; Δ argB::trpC Δ B;

veA1 trpC801) was used. Ten ml complete liquid medium supplemented with arginine was inoculated with $1-5 \times 10^4$ conidia/ml, poured into a Petri dish containing glass coverslips, and incubated at 30°C.

Staining and Microscopy

For electron microscopy, cultures were fixed in 2% sodium cacodylate-buffered glutaraldehyde (pH 7.2), postfixed in 2% osmium tetroxide and 1% uranyl acetate, washed in a graded acetone series, and embedded in Spurr's low viscosity resin. After thin sectioning, samples were stained with lead citrate. Micrographs were taken on a Philips 200 electron microscope. For all other experiments, cultures were fixed in 3.7% formaldehyde, stained, and photographed as described by Harris et al. [1994] except that Kodak P3200 film was used. An anti-actin monoclonal antibody (Amersham N350, Amersham, Arlington Heights, IL) with a FITC-coupled mouse secondary antibody (Sigma, St. Louis, MO) was used to detect actin. Calcofluor (a gift of American Cyanamid, Wayne, NJ) and Hoechst 33258 (Sigma) were used to stain for chitin and nuclei, respectively. Confocal microscopy was on a BioRad MRC-1000.

Septal Counts

For septal time course experiments (Figs. 2 and 5), 50–100 hyphal rings were scored for the presence of actin, actin and chitin, or chitin alone at each time point. To ensure that the lack of an actin ring was not due to poor staining, only data points from germlings that showed punctate cytoplasmic actin staining along the entire hypha were counted. To avoid erroneously counting punctate cytoplasmic actin as circumferential rings, only actin which clearly spanned the entire width of the hypha was counted as a ring. At the 11- and 12-hour time points there was usually no more than one septum per germling. By the later time points two or three septa could be seen in a single germling. All experiments were repeated three times with essentially identical results. Data sets for typical experiments are reported.

For the data in Table I, 100 germlings were counted for each time point and condition. As above, only germlings that showed punctate cytoplasmic actin staining along the entire germling were counted. Each germling was scored for the presence of an actin ring, a chitin ring, or no ring. Experiments were repeated twice with essentially identical results. Data sets for typical experiments are reported. The high number of rings staining for chitin in the earliest time point probably indicates a slightly longer incubation (11 hours, 15 minutes) relative to Figures 2 and 5. Because experimental and control samples were processed in parallel, this slight offset does not affect the interpretation.

TABLE I. Effect of Microtubule Depolymerization on the Progression of Septation*

Time	Ring		%
(hours)	Ben ^a	n ^b	Germlings
11	–	100	30
11	–	100	22
14	–	100	52
14	–	100	96
14	+	100	23
14	+	100	37

*Germlings were incubated for 11 hours, treated with benomyl, incubated for another 3 hours, and then fixed and stained. Germlings were scored either for the presence or absence of actin rings or for the presence or absence of chitin rings.

^aBen, benomyl; 100 μ l of 1 mg/ml benomyl dissolved in ethanol was added to experimental samples (+) for a final concentration of 10 μ g/ml. One hundred μ l of ethanol was added to negative controls (–).

^bn, number of germlings scored.

^cA, actin; C, chitin; A + C was not scored as a separate category in these experiments. Based on the data in Figure 2, at least some of the rings probably contained both.

Mitotic Block Experiments

For mitotic block time course experiments (Fig. 5), after 11 hours incubation at 30°C, 100 μ l of 1 mg/ml benomyl (a gift of the Dupont Company, Wilmington, DE) in ethanol was added per 10 ml culture for a final concentration of 10 μ g/ml. A volume of 100 μ l of ethanol was added to controls. Cultures were then incubated for an additional 0.5, 1, or 3 hours before hyphal rings were scored for the presence of actin, actin and chitin, or chitin as described for septal count experiments above. All experiments were repeated three times with essentially identical results. Data sets for typical experiments are reported.

Septal Position Counts

Flanking nuclear morphology and position were recorded for each ring containing only actin and for 50–100 rings containing actin and chitin or chitin alone using the same slides as for Figure 5. Elongated nuclei with dark spots (nucleoli) were counted as being in interphase (I). Condensed nuclei with no visible nucleoli were counted as in mitosis (M). Position relative to rings was designated as 1 if the ring bisected the nucleus or as 2 if the ring was flanked by nuclei. No repeatable differences in septal position relative to nuclei were seen at different time points (data not shown). Table II is a compilation of data from all time points used in Figure 5.

RESULTS

Ultrastructure of the Septum

Ultrastructural studies of the specialized, thick septa that delimit asexual reproductive structures in *A.*

TABLE II. Effect of Microtubule Depolymerization on Septum Position*

Ring composition ^a	Ben ^b	n ^c	M-1	M-2	I-1	I-2	M/I-2
A	–	199	14%	19%	1%	66%	0%
A	+	223	30	31	3	36	0
A + C	–	100	0	0	0	100	0
A + C ^d	+	25 ^e	0	0	0	100	0
C	–	100	0	1	0	81	18
C	+	50 ^e	0	30	0	52	18

*Cells were incubated, fixed and stained as in Table I. Morphology and position of nuclei near hyphal rings was scored. Elongated nuclei were counted as interphase (I). Condensed nuclei were counted as mitotic (M). Position relative to rings was designated as 1 if the ring bisected the nucleus or as 2 if the ring was flanked by nuclei. No correlation of individual time points to nuclear/ring configuration was seen. Therefore, data from several time points were compiled for this table.

^aA, actin; A + C, actin + chitin; C, chitin.

^bBen, benomyl; 100 μ l of 1 mg/ml benomyl dissolved in ethanol was added to experimental samples (+) for a final concentration of 10 μ g/ml. One hundred μ l of ethanol was added to negative controls (–).

^cn, number of hyphal rings scored.

^dActin in rings very faint.

^eChitin in ring rare for benomyl-treated samples.

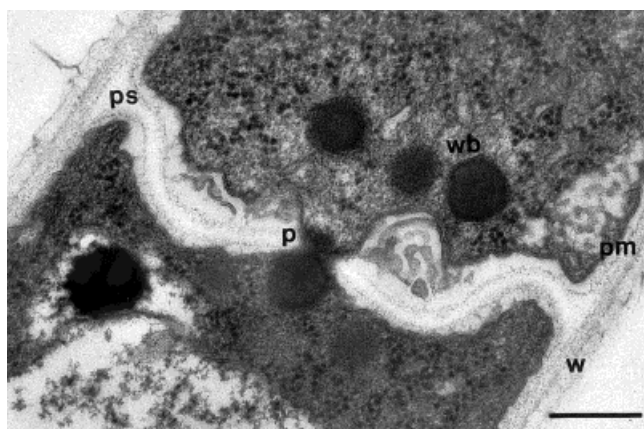


Fig. 1. Ultrastructure of the septum. A mature septum of *Aspergillus nidulans* was viewed by transmission electron microscopy. The septum has a trilaminar appearance and is surrounded by the plasma membrane. pm denotes plasma membrane; w denotes cell wall; p denotes septal pore; ps denotes primary septum; wb denotes Woronin Body. Scale bar = 0.25 μ m.

nidulans have been published [Mims et al., 1988; Sewall et al., 1990]. However, we could find no published electron micrographs of the septa that partition vegetatively growing hyphae of *A. nidulans*. Therefore, mature *A. nidulans* septa were examined by electron microscopy (Fig. 1). As has been previously noted for closely related fungi [reviewed by Bracker, 1967; Hunsley and Gooday, 1974; Gull, 1978], we observed that mature septa from *A. nidulans* have a central pore and are made of three well-defined layers. They are separated from the cytoplasm by the plasma membrane. Closely associated with

the septal pore were the membrane-bound organelles (Woronin bodies) thought to plug the pore after injury to adjacent hyphal compartments [Richle and Alexander, 1965; Collinge et al., 1978].

Temporal Relationship of Actin and Chitin in Septum Development

The presence of septa is generally detected either by a visible crosswall seen by brightfield optics or by Calcofluor-staining material seen by fluorescence microscopy. Calcofluor is a brightening agent that binds to fungal cell wall polymers containing β -linked glucans and chitin [Maeda and Isida, 1967]. Hereafter, we refer to all Calcofluor-staining material as chitin or as septal wall material. Previous work had shown that actin is required for septation and that actin and chitin sometimes colocalize at the septum in *A. nidulans* [Harris et al., 1994]. To find out when actin and chitin appear in septum formation, wild-type conidia were inoculated onto coverslips in complete medium and allowed to germinate. At hourly intervals, germlings were fixed and stained with an anti-actin monoclonal antibody, Calcofluor, and the dye Hoescht 33258 to visualize actin, chitin, and nuclei, respectively (Fig. 2). Under conditions employed in this study, the 11-hour time point corresponds to the eight-nuclei stage when the first chitin rings are appearing. Germlings grown for 10 hours showed neither actin nor chitin rings (data not shown). At 11 hours the majority of rings contained only actin. One hour later the majority of rings contained both actin and chitin. By the latest time point (16 hours) most rings contained only chitin.

Spatial Relationship of Actin and Chitin in Septum Development

Figure 3 shows micrographs representing progressive stages in septum formation. In early stages (Fig. 3A–C), actin rings were faint, sometimes punctate, and spanned the width of the hyphal cell. In addition, punctate actin staining persisted in the cytoplasm and actin remained concentrated at the tips of growing germlings (data not shown). The actin rings were sometimes located over mitotic nuclei (Fig. 3A) and sometimes between mitotic or interphase nuclei (Fig. 3B and C). Occasionally more than one faint ring could be seen along the length of a single hypha (data not shown). No chitin was detected. In later stages of septum formation (Fig. 3D–J), actin staining was more intense and chitin rings were clearly visible. Actin appeared as a broad band with a constriction in the middle, giving an hourglass shape (Fig. 3F–I). The chitin ring appeared to be located circumferentially to the actin-rich constriction. The chitin ring generally appeared to increase in thickness as the actin-rich constriction became smaller (Fig. 3H and J). Detection of this thickening of the chitin ring sometimes required viewing

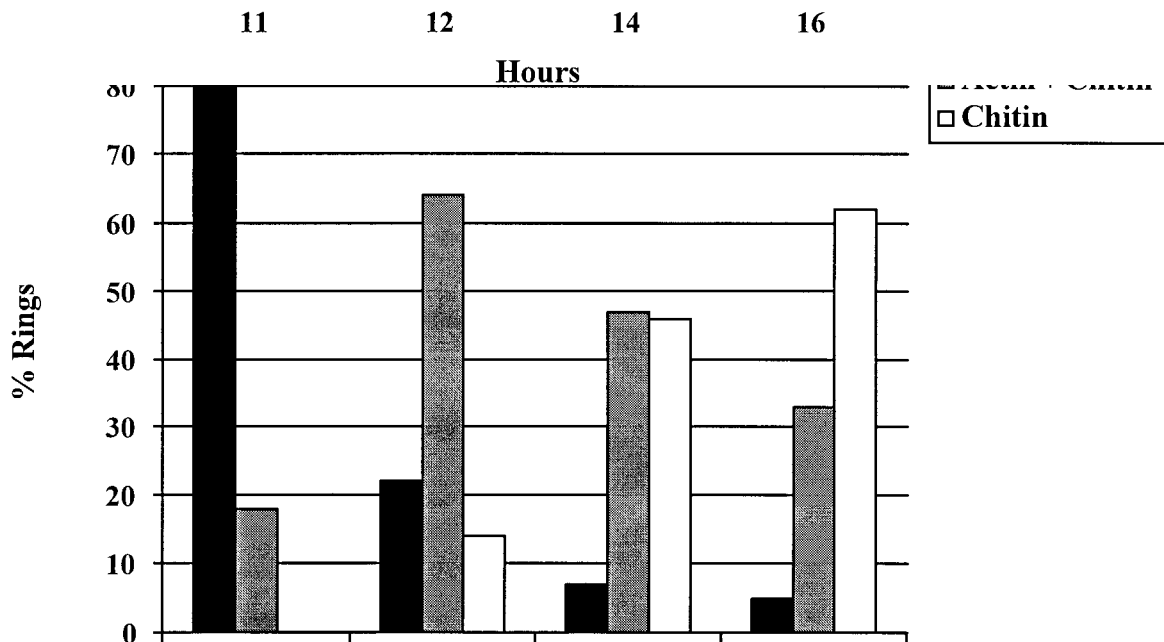


Fig. 2. Temporal order of septum development. Conidia (asexual spores) were inoculated into complete medium and incubated at 30°C. At hourly intervals, germlings were fixed and stained to localize actin, chitin, and nuclei. The 11-hour time point represents the eight-nuclei stage, the time of the appearance of the first septa. The presence of actin and/or chitin was scored in 50–100 hyphal rings.

through several focal planes and so is not obvious in all micrographs (data not shown). In the final stages of septum formation, the chitin ring was present without an actin ring or band (Fig. 3K, L). In many cases, a region with no cytoplasmic actin staining was seen immediately adjacent to the chitin ring (Fig. 3L). Confocal microscopy confirmed the hourglass pattern of actin staining (Fig. 4) and revealed a higher concentration of actin at the leading edge of the central invagination. Confocal examination of germlings stained for both actin and chitin also confirmed the location of the chitin ring circumferential to the actin constriction (data not shown).

Role of Microtubules in Septation

Previous work has shown that septation in *A. nidulans* requires a mitotic division after a requisite size is reached [Harris et al., 1994; Wolkow et al., 1996]. In *A. nidulans*, nuclear condition can be determined by phase microscopy or fluorescence microscopy after staining with DAPI or Hoescht's. Interphase nuclei appear elongated with visible nucleoli while mitotic nuclei are much smaller and have no visible nucleoli [Clutterbuck, 1970; Orr and Rosenberger, 1976]. To investigate the relationship of actin and chitin ring formation to mitosis, germlings were grown to the point of the appearance of

the first chitin rings (11 hours) and then treated with the antimicrotubule drug benomyl. Benomyl depolymerizes microtubules, blocking nuclei in mitosis while germling growth continues [Orr and Rosenberger, 1976; Sheir-Neiss et al., 1978; Oakley and Morris, 1980]. After 0.5, 1 and 3 hours incubation in benomyl, ring composition and nuclear condition were scored (Fig. 5).

We anticipated three possible outcomes of destabilizing microtubules in septating germlings: (1) if microtubules have no role in septation, we expected benomyl would have no effect on circumferential actin ring formation, invagination, or septal wall deposition. In this case, the number of circumferential actin rings (A), invaginating actin rings with chitin (A + C), and chitin rings (C) would increase over time after benomyl treatment in the same manner as untreated controls; (2) if microtubules are required for the initiation of septation, but not its progression, we expected the number of circumferential actin rings (A) would decrease after benomyl treatment relative to controls, but that circumferential actin rings already formed at the time of benomyl addition would progress to invaginating actin rings with chitin (A + C) and to chitin rings (C), as in untreated controls; and (3) if microtubules are required only for the progression of septation, but not its initiation, we ex-

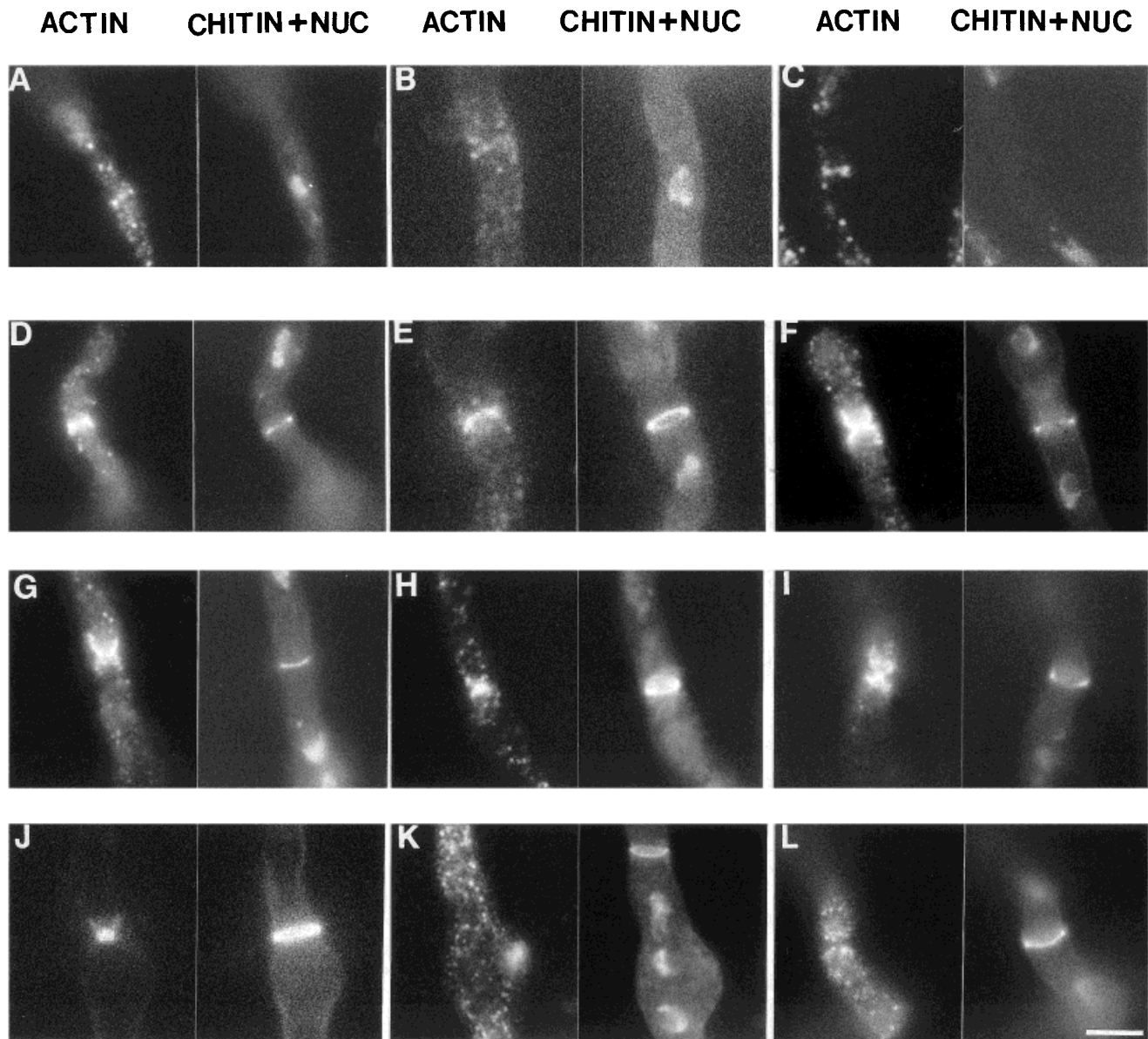


Fig. 3. Stages in septum development. Micrographs representing various stages in septum formation were assembled in the presumed temporal order. Left and right panels show the same cell: **left panels**, actin localization; **right panels**, chitin and nuclear localization. The early actin ring is faintly visible: over a mitotic nucleus (**A**), between mitotic nuclei (**B**, only one nucleus is in field of view); and between

interphase nuclei (**C**). The actin ring becomes more prominent and the chitin ring becomes visible (**D**, **E**). Actin pinches inward as the chitin ring thickens (**F**–**J**). The actin ring disappears leaving a disk of chitin at the septum (**K**). Cytoplasmic actin is displaced away from the septum, possibly by secondary wall growth (**L**). Scale bar = 5 μ m.

pected that after addition of benomyl, the number of circumferential actin rings (**A**) would be the same or higher, but invaginating actin rings with chitin (**A** + **C**) and chitin rings (**C**) would be lower relative to untreated controls.

In control cultures, 4–9% of germlings contained mitotic nuclei at all time points. As expected, in benomyl-treated cultures the number of germlings containing mitotic nuclei rose rapidly: 40–50% of germlings had mitotic nuclei after a half-hour incubation and 58–74%

had mitotic nuclei after 3 hours incubation. At 14 hours, only 22% of rings in controls stained for actin alone. The remaining 78% stained for chitin (Fig. 5A). In contrast, 66% of rings in benomyl-treated cultures stained for actin alone and only 34% stained for chitin (Fig. 5B). This increase in the percentage of actin-staining rings and decrease in the percentage of chitin-staining rings could have resulted from either a rise in the number of actin rings or a drop in the number of chitin rings in the

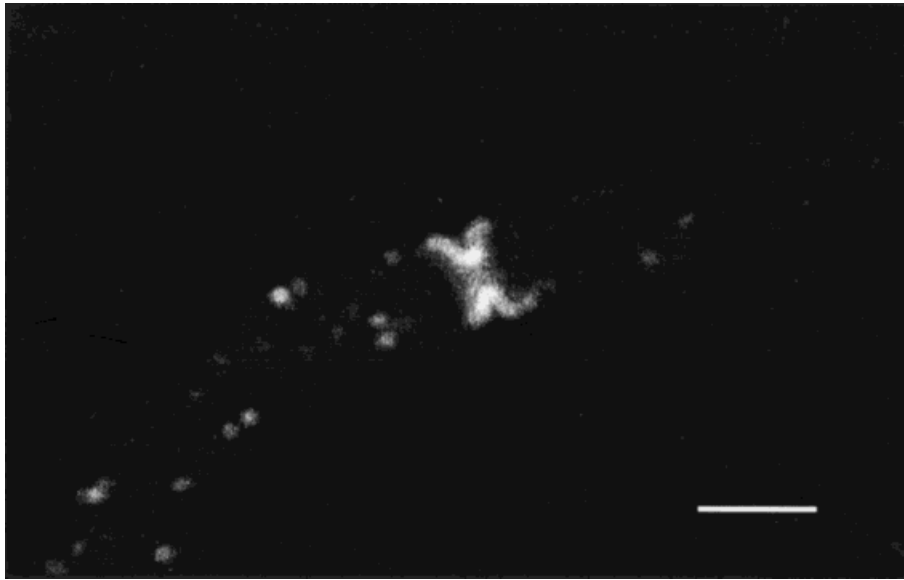


Fig. 4. Actin at the septum by confocal microscopy. Actin in a developing septum (hourglass stage) was viewed by confocal microscopy. A medial section is shown. Scale bar = 5 μ m.

population. To distinguish these possibilities, we counted actin rings and chitin rings as a percentage of the total number of germlings (rather than as a percentage of the total number of rings) in control and benomyl-treated cultures (Table I). After 3 hours incubation (14 hours postinoculation), half the germlings in untreated controls were initiating the synthesis of new septa ($A = 52\%$) and virtually all germlings had at least one mature septum ($C = 96\%$). In benomyl-treated cultures at the same time, there was little change from the time of addition of benomyl (11 hours). Only 23% of germlings were initiating the synthesis of new septa (A) and only 37% of germlings had mature septa (C). We conclude that polymerized microtubules are required for both formation of the circumferential actin ring and for progression to the invaginating actin ring with septal wall synthesis.

In order to correlate actin and chitin ring formation with position of the mitotic nucleus, we scored nuclear position and condition along with ring composition (Table II). Although movement of nuclei within hyphal compartments could have confounded this analysis, benomyl also blocks nuclear movement in *A. nidulans* [Oakley and Morris, 1980]. We recognized five distinct configurations: mitotic nuclei bisected by a ring (M-1); mitotic nuclei flanking a ring (M-2); interphase nuclei bisected by a ring (I-1); interphase nuclei flanking a ring (I-2); and mitotic and interphase nuclei flanking a ring (M/I-2). The majority of actin rings in untreated cultures were flanked by interphase nuclei (I-2), not surprising given that only 5% of the germlings contained mitotic nuclei. As expected, following benomyl treatment, the percentage of actin rings flanked by interphase nuclei fell while the

percentages of actin rings bisecting a mitotic nucleus or flanked by mitotic nuclei rose. However, rings staining for both actin and chitin were always found between interphase nuclei, even after benomyl treatment (I-2). In the benomyl-treated cultures, the actin staining was faint and never showed the hourglass shape. Thus, compromised microtubules appear to prevent invagination of the actin ring. These results suggest that the actin ring persists through mitosis, about 5 minutes in *A. nidulans* [Bergen and Morris, 1983], and that actin ring invagination and chitin deposition occur postmitotically. The observation that only mature septa (those staining for chitin alone) were found between mitotic and interphase nuclei (M/I-2) is in agreement with observations that nuclei within a septal compartment are synchronized [Fiddy and Trinci, 1976].

In order to verify that the effects of benomyl treatment on septation were mediated by microtubule depolymerization rather than by some cryptic function of the drug, we used the benomyl-resistant strain BEN13. This strain carries a mutation in β -tubulin which confers resistance to benomyl [Sheir-Ness et al., 1978; Oakley and Morris, 1980]. In contrast to wild-type (Table I), BEN13 cells treated with benomyl were still able to make septa (Table III). This result clearly shows that the benomyl block of septation in wild-type cells is mediated through its effect on microtubules.

DISCUSSION

These studies clarify the temporal and spatial relationship of actin and chitin ring formation, demon-

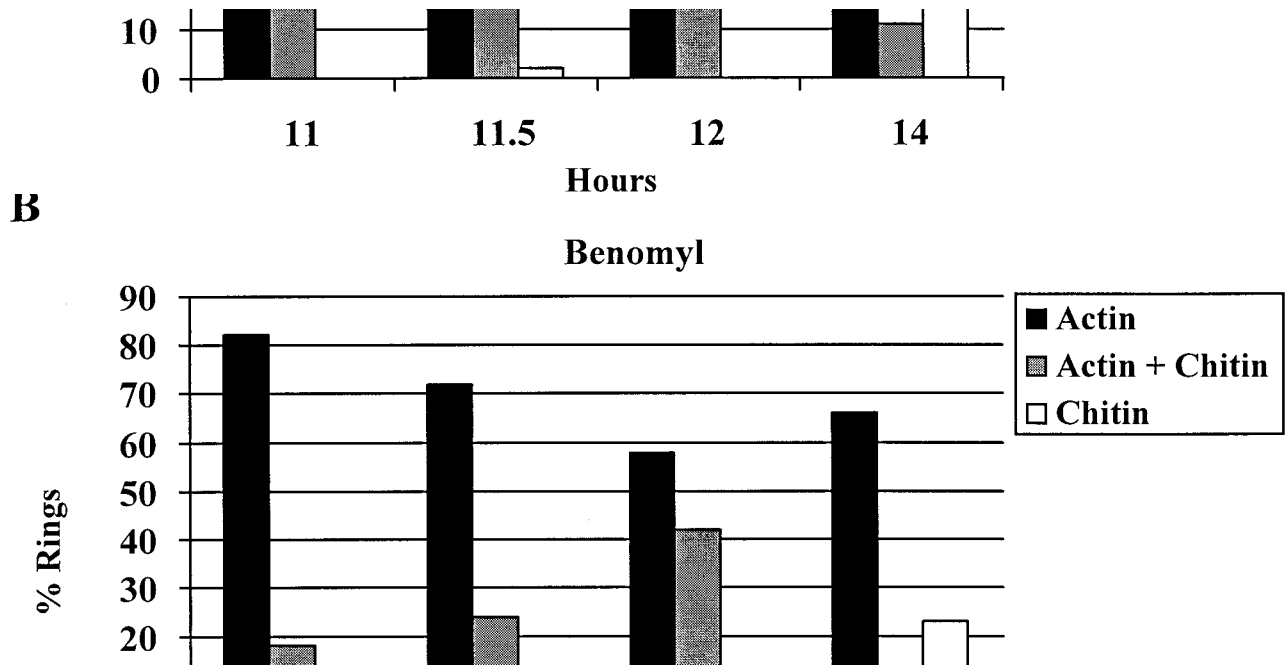


Fig. 5. Percentage of actin rings increases with mitotic block. Conidia (asexual spores) were inoculated into complete medium and incubated at 30°C. At the 11-hour time point (the point when the first septa are made) either ethanol alone (A) or 10 µg/ml Benomyl in ethanol (B) was added to identical cultures. At 0 time (11-hour time point) and after 0.5, 1, or 3 hours incubation, cultures were fixed and stained to localize actin, chitin and nuclei.

TABLE III. Effect of Microtubule Depolymerization on the Progression of Septation in the Benomyl-Resistant Strain BEN13*

Time (hours)	Ben ^a	n ^b	Ring composition ^c	% Germlings
7	–	100	C	0
11	–	100	C	68
11	+	100	C	69

*Germlings were incubated for 7 hours (the point just prior to the formation of the first septum for this strain), treated with benomyl, incubated for another 4 hours, and then fixed and stained. Germlings were scored either for the presence or absence of chitin rings.

^aBen, benomyl; 100 μ l of 1 mg/ml benomyl dissolved in ethanol was added to experimental samples (+) for a final concentration of 10 μ g/ml. One hundred μ l of ethanol was added to negative controls (–).

^bn, number of germlings scored.

^cC, chitin.

strate dynamic changes in actin that are consistent with a contractile mechanism for septation, and suggest a role for microtubules in the initiation and progression of septum development. Time course experiments (Fig. 2) showed that most early septa were composed of actin alone. After 1 hour, most septa were composed of both actin and chitin. By our latest time point, most septa were composed of only chitin. We interpret this to mean that during septum development an actin ring is the precursor to the chitin ring and that actin and chitin are both present during an intermediate stage of septum development. The presence of the actin ring before the chitin ring is consistent with the observation of Harris et al. [1994] that Cytochalasin A reversibly inhibits septation. It is possible that the faint actin rings we observed before chitin deposition were not precursors to septa, but other hyphal actin structures such as the actin plaques or perinuclear caps reported in some filamentous fungi [Butt and Heath, 1988; Heath, 1990]. This seems unlikely, however, because the actin rings we observed were not visible 1 hour before the formation of the first septa (data not shown), spanned the entire width of the hypha (Fig. 3A–C), and were not consistently positioned surrounding nuclei as perinuclear caps would be. Observations on a population of germlings undergoing the earliest septation event were also consistent with the view that the actin rings are precursors to the chitin rings seen in mature septa. The proportion of germlings with actin rings at 11 hours was very close to the proportion with chitin rings at 11.5 hours, as would be expected if each actin ring gave rise to a chitin ring (37% and 40%, respectively, data not shown). The appearance of a ring containing actin, followed by a ring containing actin and chitin, followed by a ring containing chitin alone, is identical to the order of events seen during the initial stages of the contractile divisions of the fission yeast *S. pombe* [Marks and Hyams, 1985; Chang et al., 1996].

The morphology of actin during the intermediate stage of septum formation (when both actin and chitin are present) suggests a contractile mechanism. Actin appears as a broad band with a constriction in the middle giving an hourglass shape (Fig. 3D–J). A similar conformation of the actin band was thought to indicate a contractile actin mechanism in the fission yeast *S. japonicus* [Alfa and Hyams, 1990]. Confocal microscopy (Fig. 4) confirmed the hourglass appearance of the actin band and revealed more concentrated actin staining at the leading edge of the invagination. This concentration of actin is reminiscent of the cleavage furrows of animal cells undergoing contractile divisions [reviewed by Satterwhite and Pollard, 1992; Fishkind and Wang, 1995].

Interestingly, the “hourglass” was always seen with a ring of chitin located circumferentially to the central actin constriction (Fig. 3D–J and data not shown). Generally, the smaller the actin constriction, the thicker the chitin ring (Fig. 3H, J and data not shown). The relative positions of actin and chitin suggest that chitin synthesis takes place at the leading edge of the actin invagination. As the actin ring invaginates, it may pull the membrane inward and chitin may be deposited in its wake. Alternatively, the centripetal thickening of the chitin ring may push the membrane and actin forward.

The chitin synthases are known to be membrane-bound enzymes that receive monomer on the cytoplasmic face of the membrane and extrude polymer to the outer face [Cabib et al., 1983]. Therefore, septum development takes place between the membrane and the cell wall, outside the cytoplasm where no cytoskeletal elements reside (Fig. 1). Without the actin invagination, activation of cell wall biosynthetic enzymes would likely lead to general thickening of the wall rather than the highly localized synthesis of septation. Gooday and Schofield [1995] have proposed that chitin synthases may be activated by membrane stress caused by turgor in apical cell growth. If the chitin synthases are sensitive to membrane stress, the pulling inward of the plasma membrane by actin contraction could serve to activate chitin synthesis at the septum. Localization of chitin synthases and actin ring components is needed to understand this process more fully.

The apparent redistribution of cytoplasmic actin away from the area around late-stage septa was seen repeatedly (Fig. 3L). This may result from the addition of secondary wall layers flanking the chitinous middle layer of the septum, so that cytoplasm no longer abuts the chitin disk (see Fig. 1). The punctate appearance of actin at the border of the actin-free zone was also seen repeatedly. It may indicate that actin is directing the transport of new material used to make the secondary septal wall. We

cannot, however, rule out the possibility that this pattern of actin staining is an artifact of fixation. Double staining experiments with reagents to detect actin and secondary wall material will resolve this question.

Previously, Harris et al. [1994] concluded that microtubules are not required for septation in *A. nidulans*. This observation was based on septum staining with Calcofluor following the addition of benomyl. In *A. nidulans*, benomyl blocks mitosis prior to the onset of anaphase. Nuclei arrest with condensed chromatin and high levels of the nimA and p34 kinases [Osmani et al., 1991a,b]. Here, by carefully following actin ring formation and cell wall deposition, we have uncovered two essential roles for microtubules during septum formation. Microtubules appear to be required both for the initiation of cytokinesis and for its progression in *A. nidulans*.

The requirement of microtubules for the initiation of cytokinesis is suggested by benomyl's ability to prevent the formation of new actin rings in germlings competent to undergo cytokinesis (Table I). Previously Wolkow et al. [1996] showed that nuclear positioning influences septal positioning in *A. nidulans* mycelia. Taken together, these results are consistent with cytokinetic models which invoke the position of the mitotic spindle as determining the eventual site of actin ring formation. Our findings are in contrast to findings in fission yeast where actin rings are capable of forming in the apparent absence of a mitotic spindle [Chang et al., 1996; Gould and Feoktistova, 1996]. However, our studies do not define the position of the actin ring relative to mitotic nuclei. The majority of actin rings were found between interphase nuclei (Table II). With benomyl treatment there was a roughly equivalent increase in actin rings bisecting mitotic nuclei and in actin rings flanked by mitotic nuclei. It remains possible that actin ring formation may be activated by adjacent mitotic nuclei or that actin rings are not formed in any one position relative to mitotic nuclei.

The requirement of microtubules for the progression of cytokinesis is suggested by benomyl's ability to block already formed circumferential actin rings from invagination and the deposition of chitin (Fig. 5; Table I). In addition, in benomyl-treated germlings, the hourglass pattern of actin staining was never seen and rings containing both actin and chitin were only rarely seen (Table II, data not shown). The rare actin and chitin rings showed only faint punctate actin staining and presumably resulted from the persistence of rings that contained both actin and chitin at the time of benomyl addition. Interestingly, recent studies in cultured animal cells have demonstrated that the midzone microtubule bundles are continuously needed for cleavage furrow formation [Wheatley and Wang, 1996]. In these studies, depolymerization of

microtubules with nocodazole caused furrowing to halt or even regress.

We can envision three ways in which the depolymerization of microtubules may block the progression of septation. First, progression may be controlled by the spindle assembly checkpoint [reviewed by Murray, 1995]. Thus, in addition to halting further progression of mitosis, the spindle assembly checkpoint may also prevent further progression of septation by arresting actin ring invagination and chitin deposition. Interestingly, a component of the spindle assembly checkpoint identified in *S. cerevisiae* as Bub2 [Hoyt et al., 1991], is highly similar to *S. pombe* Cdc16 which plays a role in septation [Fankhauser et al., 1993]. *Cdc16* mutants undergo additional rounds of septation at the restrictive temperature without completing another round of mitosis. It will be of interest to see if a similar gene product in *A. nidulans* signals actin ring invagination in response to microtubule assembly.

Second, continued septum development beyond actin ring formation may require exit from mitosis, i.e., there may be a postmitotic checkpoint for septum progression. A requirement to exit mitosis is strongly suggested by the observation that invaginating actin rings with chitin are always flanked by interphase nuclei (A + C, Table II). A postmitotic checkpoint which blocks cell separation if nuclei are not properly placed on either side of the division site has been suggested by *S. pombe* actin ring mutants [Chang et al., 1996] and *S. cerevisiae* dynein mutants [Li et al., 1993; McMillan and Tatchel, 1994]. Perhaps a similar postmitotic checkpoint blocks actin invagination and chitin deposition rather than cell separation in *A. nidulans*. Finally, it remains possible that a microtubule-mediated transport process is also required for completion of septation. Future studies will investigate these possibilities.

Based on the data presented here we propose the following model for septum formation in *A. nidulans* (Fig. 6). After the germling has reached the requisite size [Wolkow et al., 1996], a microtubule-dependent signal from the mitotic nucleus causes the formation of the actin ring (Fig. 6A–C). The actin ring persists through mitosis, nuclear separation and return to interphase. Late in mitosis or early in interphase, a microtubule-dependent signal allows septation to progress (Fig. 6D). The actin ring becomes more prominent and constricts at the center giving an hourglass shape (Fig. 6E). As actin pinches inward, chitin synthesis takes place at the leading edge of the invagination. As the actin ring invaginates, chitin is deposited in its wake (Fig. 6E, F). Actin becomes less pronounced, pulls back from the developing septum, and eventually disappears, leaving the new septal cell wall with its chitinous middle layer (Fig. 6G, H).

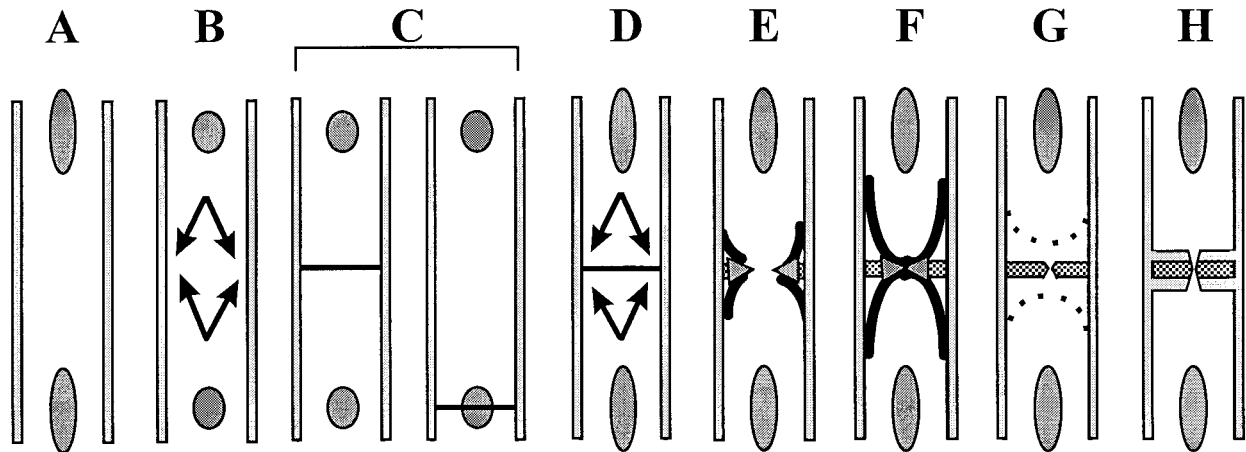


Fig. 6. A model of septum development. After the germling passes a size threshold, a signal from the mitotic nucleus triggers actin ring formation (A, B). The actin ring forms either between nuclei or directly over a nucleus (C). The actin ring persists through mitosis and return to interphase. A signal from the late mitotic or interphase nucleus (D) triggers actin condensation and invagination. The actin ring thickens to a band and begins to pinch inward giving an hourglass shape. Chitin synthesis takes place at the leading edge of the invagination. As the actin band pinches inward it leaves chitin in

its wake (E, F). Actin becomes punctate, pulls back from the developing septum (G), and eventually disappears, leaving the new septal cell wall with its chitinous middle layer (H). Shaded ovals represent interphase nuclei; shaded circles represent mitotic nuclei; thick solid lines and broken lines represent actin; arrows represent a signal from the nucleus; small gray triangles represent activated chitin synthase; stippled bands represent chitin (the primary septum); gray bands represent other septal wall material (the secondary septa).

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