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Cytoskeleton interactions in the ascus development and sporulation of *Sordaria macrospora*

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SUMMARY

The organization of actin during meiosis and sporulation in the ascus of the filamentous ascomycete *Sordaria macrospora* was determined by immunofluorescence without removal of the cell wall. Actin is present as a dense cortical network of microfilaments (MF) and plaques, a perinuclear shell of actin in prophase I of meiosis, and a complex array of MF involved in alignment of prespore nuclei and closure of spore cell membranes. The relationship of actin to the previously examined microtubule system of the ascus was determined by double-label immunofluorescence. The cytoskeletal inhibitors nocodazole, cytochalasin D and 2,3-butanedione monoxime were used to examine the roles of actin and myosin in ascus development. Microfilament and microtubule arrays are interdependant; disruption of one network results in abnormalities in the other. Both microfilaments and actin-myosin interaction are required for separation and migration of duplicated spindle pole bodies, septation and sporulation.

Key words: actin, myosin, nocodazole, cytochalasin, 2,3-butanedione monoxime, cytokinesis, ascomycete sporulation, fungal cytoskeleton, meiosis, development

INTRODUCTION

The components of the fungal cytoskeleton which have been most extensively studied are tubulin and actin. Microtubules (MT) in fungal cells, as in other systems, are involved in cell shaping and mechanical support (Thompson-Coffe and Zickler, 1992), spindle pole body (SPB) separation, nuclear divisions and migrations (Jacobs et al., 1988), and intracellular transport (Howard and Aist, 1980). The distribution and function of actin microfilaments (MF) in fungal cells are less well understood (Hohl, 1992). In many fungi, MF are labile to fixation, and the effects of such actin inhibitors as cytochalasin have frequently been difficult to interpret (Heath, 1990), while actin mutants show a wide range of phenotypes (reviewed by Solomon, 1991).

In budding and fission yeast, actin is present as cortical dots and cytoplasmic cables; the dots are suggested to be involved in budding and actin-membrane connections (Adams and Pringle, 1984), and in end growth and septum formation (Marks and Hyams, 1985; Alfa and Hyams 1990), while MF are probably required in vesicle transport (Lui and Bretscher, 1992, and references therein). In the hyphae of filamentous fungi, actin is mainly in peripheral plaques or dots near the plasma membrane; some species show a filamentous cap of apical actin associated with growing tips, or have longitudinal MF (reviewed by Heath, 1990). Hyphal actin is assumed to be important in tip growth and intracellular transport. Recent work (Heath and Harold, 1992) demonstrated actin involvement in the formation of zoospores in sporangia of oomycete fungi.

We previously studied the MT arrays and microtubule organizing centers (MTOC) in the ascus of a filamentous ascomycete, *Sordaria macrospora* (Thompson-Coffe and Zickler, 1992). This is a particularly favorable system for cytoskeletal analysis; the size of the ascus, and permeabilization methods which permit antibody penetration without cell wall digestion, provide both excellent detail in immunofluorescence and good preservation of labile cytoskeletal arrays. Ascus development (which proceeds from haploid mitosis and septation in the crozier, through karyogamy, ascus growth, meiosis, post-meiotic mitosis and the formation of eight ascospores within the mother cell; see Fig. 1) provides the opportunity to examine the role of the cytoskeletal elements throughout a complex morphogenetic cycle within a single cell.

The examination of actin organization in *Sordaria* asci, and its disruption by MT and MF inhibitors and an inhibitor of actin-myosin interaction, indicates that MF and MT systems are interdependent. MF arrays are involved in septum formation and possibly in the shape transition from the hooked crozier to the linear ascus; in mechanical support of meiosis I nuclei; in the separation and migration of SPBs and, finally, in the development of ascospores.

MATERIALS AND METHODS

Strains and growth conditions

Sordaria macrospora is a homothallic ascomycete; the strain used in this study was St Ismier FGSC 4818. Growth was on minimal medium (Zickler et al., 1984) in constant light conditions at 23°C. The recessive mutant *spo644* was isolated from protoplasts mutagenized with 1% ethyl methane sulfonate in isotonic buffer for 2 h. After regeneration, mycelia shooting abortive spores were easily detectable and *spo644* was isolated as a colony producing almost no wild-type spores.

Sample preparation and immunofluorescence

Asci were prepared as described (Thompson-Coffe and Zickler, 1992) with minor alterations necessary for good preservation of MF: briefly, fixed in 95 mM PIPES, pH 6.9, with 3.7% paraformaldhyde, 10 mM EGTA, 10 mM $MgSO_4$, 0.5 mM PMSF, and 0.3% Triton X-100 at room temperature for 20-30 min. Antibodies used were the N.350 anti-actin (Amersham, France) at a dilution of 1:10,000, Amersham anti-β-tubulin (1:1600), and the monoclonal anti-yeast tubulin YOL 1/34 (Kilmartin et al., 1982) at a dilution of 1:500. MPM-2 (Davis et al., 1983), which reacts with *Sordaria* MTOCs (Thompson-Coffe and Zickler, 1992), was used at 1:750 dilution. Secondary antibodies were Caltag FITC-conjugated polyvalent goat anti-mouse and Texas Red-conjugated goat anti-rat (Tebu, France), both at 1:250. Chromatin was visualized with 0.5 μg/ml 4′,6-diamino-2-phenylindole-dihydrochloride (DAPI, Boehringer-Mannheim).

Double staining for MT and MF was done routinely, but single staining was also performed; spindle pole bodies and spindles gave a leakage of signal between channels, and single labeling was necessary as a control. Double labeling also resulted in some loss of fine detail of the MF system.

Variations on fixation and processing

Actin MF in plants and fungi are difficult to preserve (Traas et al., 1987; Heath, 1990; McCurdy and Gunning, 1990); a wide variety of treatments was explored. Use of phosphate buffers instead of PIPES, or variations in pH, gave poor preservation. Saponin, at 0.5 mg/ml, could be used in place of Triton. Addition of lysine or *m*-maleimidobenzoyl *N*-hydroxysuccimide ester (Sonobe and Shibaoka, 1989) did not improve microfilament stability. Cold methanol postfix, reported necessary for good labeling with N.350 (McCurdy and Gunning, 1990), had no effect on the arrays observed. Fixation by methanol alone, or postfixation treatment with acetone, resulted in very poor preservation of MF.

Use of fluorochrome-conjugated phalloidins, prior to or following fixation, gave only background fluorescence; in other filamentous ascomycetes, phallotoxins have not revealed the network of fibers and dots commonly visualized by immunofluorescence (Bourett and Howard, 1992).

Drug treatments

Cytochalasin D (CD, Sigma) was dissolved in ethanol at 4 mM; dilutions from 4 to 10 μM in distilled water were made immediately before use. Nocodazole (Aldrich) was kept as a 5 mg/ml stock in dimethylsulfoxide (Aldrich) and used at 10 μM. The inhibitor of actin-myosin interaction 2,3-butanedione monoxime (BDM, Sigma) was stored in the dark and dissolved in distilled water at 10-20 mM the day of use. Since asci suffer morphological damage quickly upon removal from the perithecia, segments of cultures were floated, fungus side down, on solutions of distilled water with the appropriate drug and its solvent. Controls were done on asci incubated in distilled water with the solvent alone for the same length of time.

Cultures recovered well from nocodazole or CD treatment and resumed normal development, with spores formed and expelled. After BDM treatment, subsequent morphogenesis was extremely abnormal and spores were never expelled; even perithecial development was affected. BDM effects appear not to be reversible.

Electrophoresis and immunoblotting

Ascal bouquets were removed from the fruiting bodies and collected in 50 mM PIPES, pH 6.9, 10 mM EGTA, 10 mM $MgSO_4$, 0.5 M mannitol, 1 mM PMSF. Asci were extracted in 60 mM PIPES, pH 6.9, 10 mM EGTA, 2 mM $MgSO_4$, 1% Triton X-100, 1 mM PMSF at room temperature for 10 min, then centrifuged. Triton-insoluble pellets were boiled in sample buffer and applied to 10% or 6% SDS-PAGE gels. Triton-soluble proteins were precipitated with 2 volumes of −20°C ethanol and similarly treated. SDS-PAGE gels were stained with Coomassie blue. Proteins were transferred to nitrocellulose, and blots blocked for 2 h in Blotto (10 mM Tris, pH 7.4, 0.15 M NaCl with 3% nonfat dried milk and 0.1% Tween-20). The N.350 anti-actin (Amersham) was used at 1:2000. The monoclonal anti-myosin CC-212 (Klotz et al., 1986) was applied as full-strength culture supernatant; a mixture of 1:200 anti-β myosin (clone F26, Biosys) and 1:10000 anti-fast skeletal myosin (Sigma, clone MY-32) was also used. Blots were incubated overnight at 4°C; after washing, horseradish peroxidase-conjugated anti-mouse secondary (Amersham France) was used at 1:1000, for 2 h at room temperature. Blots were washed and developed using enhanced chemiluminescence (Amersham France).

RESULTS

The sexual cycle of *Sordaria* begins with the formation of a hook-shaped crozier cell, containing two haploid nuclei (Fig. 1). These undergo a simultaneous mitosis, with spindles positioned such that one daughter nucleus from each parent is present in the crook portion of the cell. Septa form on each side of the crook, resulting in a basal and a lateral cell flanking the binucleate ascus mother cell. Karyogamy takes place as the ascus mother cell begins to elongate, followed immediately by the long prophase of meiosis I. The ascus reaches its full length by the end of the first meiotic division. Meiotic spindles are positioned in the long axis of the cell; for the post-meiotic mitosis (PMM), the four spindles lie at right angles to the long axis of the ascus. The eight resultant nuclei are enclosed by cell membrane to form spores, which undergo a first mitosis prior to wall formation. The mature spores are ejected, perhaps by a turgor mechanism, through an opening at the ascus tip (the apical pore) which is formed following meiosis I.

Cortical MF and MT arrays are interdependent

In the developing ascus, actin is organized as a cortical array of longitudinal MF with associated actin plaques, almost completely parallel to the MT network (Fig. 2A). Cortical MT are arranged in a dense parallel network converging on an apical MTOC (Fig. 2B). By confocal microscopy, the actin array is strictly cortical: no internal MF are seen (data not shown). A clear convergence of MF at the ascus tip is observed (Fig. 2C), but is often obscured by the strong anti-actin fluorescence of tip cytoplasm during the elongation process and by the higher concentration of actin dots at the apex (Fig. 2D).

MT organization at the apex differs over ascus development; until the end of meiosis I, the cortical MTOC is reac-

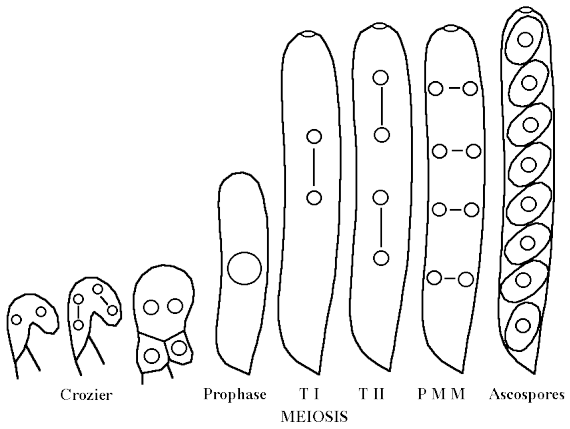


Fig. 1. Sexual cycle of *Sordaria macrospora*. Mitosis in the binucleate crozier is followed by septum formation delimiting the ascus mother cell. After karyogamy, the nucleus undergoes two meiotic divisions (TI and TII indicating telophase) followed by post-meiotic mitosis (PMM) and ascospore formation. Spores are expelled through the apical pore formed at the end of meiosis I.

tive with anti-tubulin and MPM-2 (Thompson-Coffe and Zickler, 1992). After meiosis I, the MTOC loses anti-tubulin and MPM-2 reactivity, and the apex is reorganized to form the apical pore. A concentration of MF and plaques covers the site in early pore development, and MF extend into the apical pore itself (Fig. 2E,F).

When asci are treated with 10 μM nocodazole, cortical MT arrays begin to depolymerize within 15 minutes (Fig. 3A). The cortical MF are also affected: whenever cortical MT are lost, the parallel MF network either is also depolymerized, or becomes extremely labile to fixation. Few cortical MF are seen in nocodazole-treated cells, although the peripheral plaques remain (Fig. 3B).

Treatment up to one hour with 10 μM CD alters the normal MF pattern but does not give complete disruption (not shown). To obtain more complete disruption, and to accumulate cells within the asynchronous population which were affected at fast steps in development, we incubated asci in 10 μM CD overnight prior to fixation and immunofluorescence. This treatment demonstrated that cortical MT organization is dependent upon the existence of functional MF. Overnight cytochalasin treatment causes extensive cortical MF disruption; peripheral actin plaques appear unaffected (Fig. 3C). Cortical MTs are not usually depolymerized, but the pattern of organization is changed (Fig. 3D), reminiscient of that seen in some morphological mutants of *Sordaria* (Thompson-Coffe and Zickler, 1992) or of asci in the early stages of nocodazole recovery.

When asci are treated overnight with 10-20 mM BDM, a concentration sufficient to depress the septation index of *Schizosaccharomyces pombe* in liquid culture (S. Wheatley, personal communication), cortical MF appear almost unchanged (Fig. 3E). The most obvious effect of this inhibitor of actin-myosin interaction on the cortical cytoskeleton is seen in MT organization. The normal array of parallel MT bundles is disordered; MT bundles are thicker, shorter and less numerous; there are many transverse and curving tubules. The cortical MTOC is either not visible in anti-tubulin immunofluorescence (Fig. 3F; compare with Fig. 2B), or seen as a small, faint dot. The MPM-2 reactivity of the MTOC remains in either CD or BDM treatment, but changes in shape; rather than a small circular spot at the apex (Fig. 3G), in BDM- or CD-treated asci it is greatly enlarged and uneven, with edges which appear fibrillar at the cytoplasmic face (Fig. 3H). This is noteworthy, since MPM-2 staining at the apex MTOC is unchanged after nocodazole disruption of the MT network (Thompson-Coffe and Zickler, 1992).

MF are not seen within the nucleus or the spindle, but actin surrounds the prophase I nucleus

In the *Sordaria* ascus, which is much larger than other fungal cells examined, we observed no visible MF, at this level of resolution, within the nucleus, spindle or SPB (data not shown). In double staining, spindles or SPBs are frequently visible in the channel used for actin; in single-label anti-actin experiments, however, no spindle or SPB is visible.

Actin is associated with the nuclear envelope in a stage-specific manner. During prophase of meiosis I, actin appears at the nuclear surface in patches which eventually extend nearly all around the nucleus (Fig. 4A,B). This perinuclear shell, which is not seen in any other prophase, is external by confocal microscopy (data not shown) and does not appear filamentous. The perinuclear prophase I actin seen in *Sordaria* is extremely stable in nature; it is unaffected by nocodazole, CD or BDM.

Actin and actin-myosin interaction are required for SPB separation

When asci are treated overnight with 10 μM CD, spindle pole bodies fail to separate (Fig. 4C). Two similarly oriented half-spindles are usually seen in this case; occasionally in spore mitosis only the closely apposed SPBs without spindle nucleation are found. Chromatin is in a prometaphase configuration, either at the nonpolar end of the double spindles or lying in an arc beneath unseparated SPBs (Fig. 4D).

In *Sordaria*, actin also appears to be necessary for at least the initial steps of SPB migration around the nuclear envelope following bridge break and separation of the doubled units. SPBs which lie a short distance apart and have no apparent physical connection either in MPM-2 (Fig. 4E) or anti-tubulin (Fig. 4F) immunofluorescence fail to continue their migration in 10 μM CD, and form parallel half-spindles engaging the same mass of chromatin. When asci are treated overnight with 10 or 20 mM BDM, unseparated SPBs and double half-spindles are also common (Fig. 4G,H). They are identical in appearance to those resulting from CD treatment, indicating an actinomyosin mechanism for SPB separation and migration.

In the newly delimited spores of *Sordaria*, part or all of the SPB detaches from the nuclear envelope to form an organizing center for the spore cortical MT network. This SPB is visible at the cell cortex until after first mitosis in the spore (Thompson-Coffe and Zickler, 1992; Fig. 5A). A new SPB, or a remnant of the translocated one, re-forms at the nuclear envelope and duplicates; it will later organize the mitotic spindle of the spore (Fig. 5B). In both BDM-

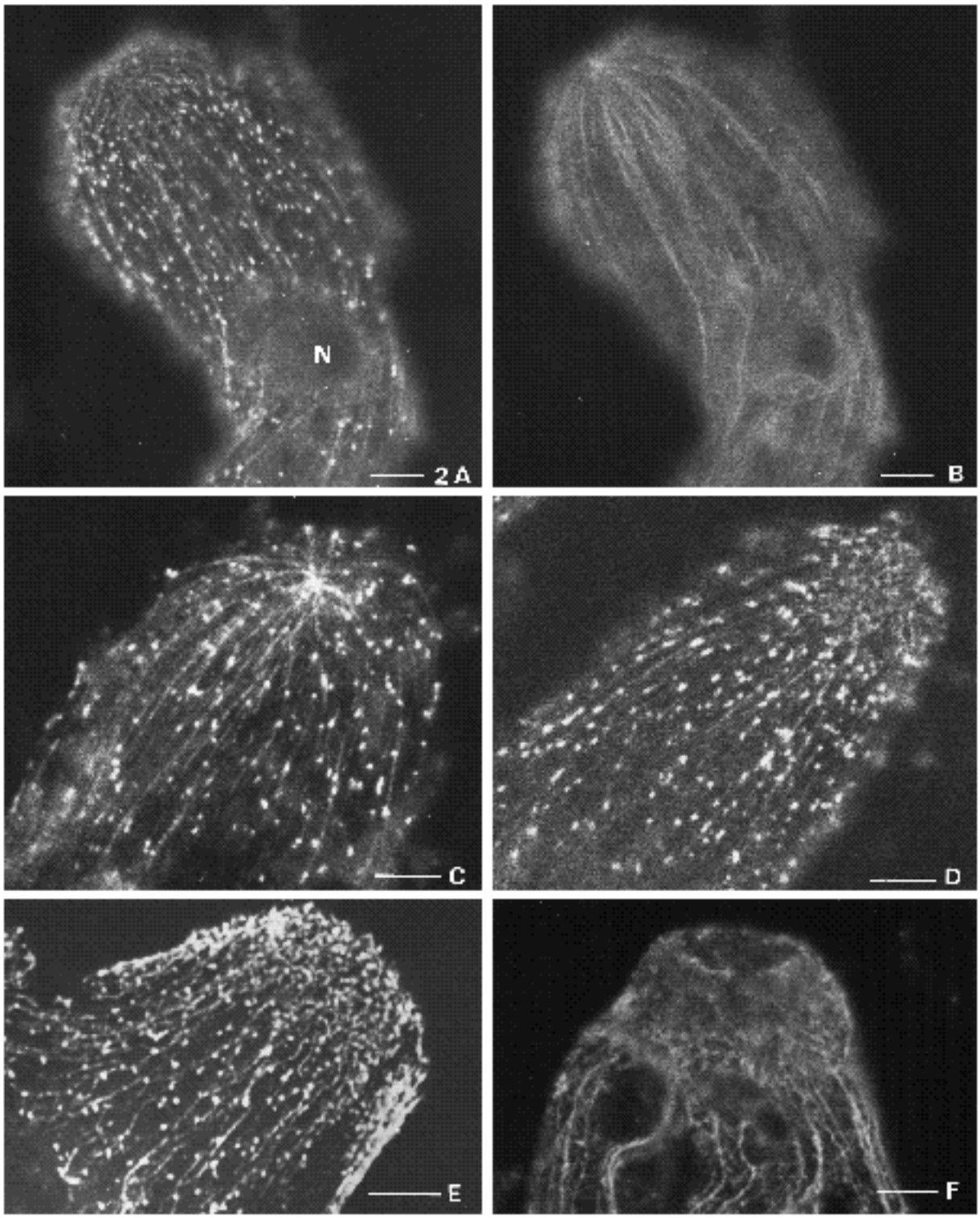


Fig. 2. (A) Cortical MF and actin plaques of the young ascus. MF run parallel to the MT array and are finer and denser above the nucleus. Actin plaques line the MF, and are most numerous between nucleus and apex. N, nucleolus. (B) Same ascus, cortical MT network. Apical MTOC is visible as a bright point of MT convergence. (C) MF of a prophase apex; an intersection point of MF is visible at the young ascus tip. (D) Frequently, a concentration of plaques and cytoplasmic anti-actin fluorescence marks the young apex. (E) Early apical pore formation, confocal view: a mesh of MF and actin plaques or particles covers the area which will become the pore. (F) MF run up to the PMM apical pore, which is rimmed with anti-actin fluorescence. A, B, D and E are double-stained; C and F are single-stained anti-actin. Bars, 5 μm.

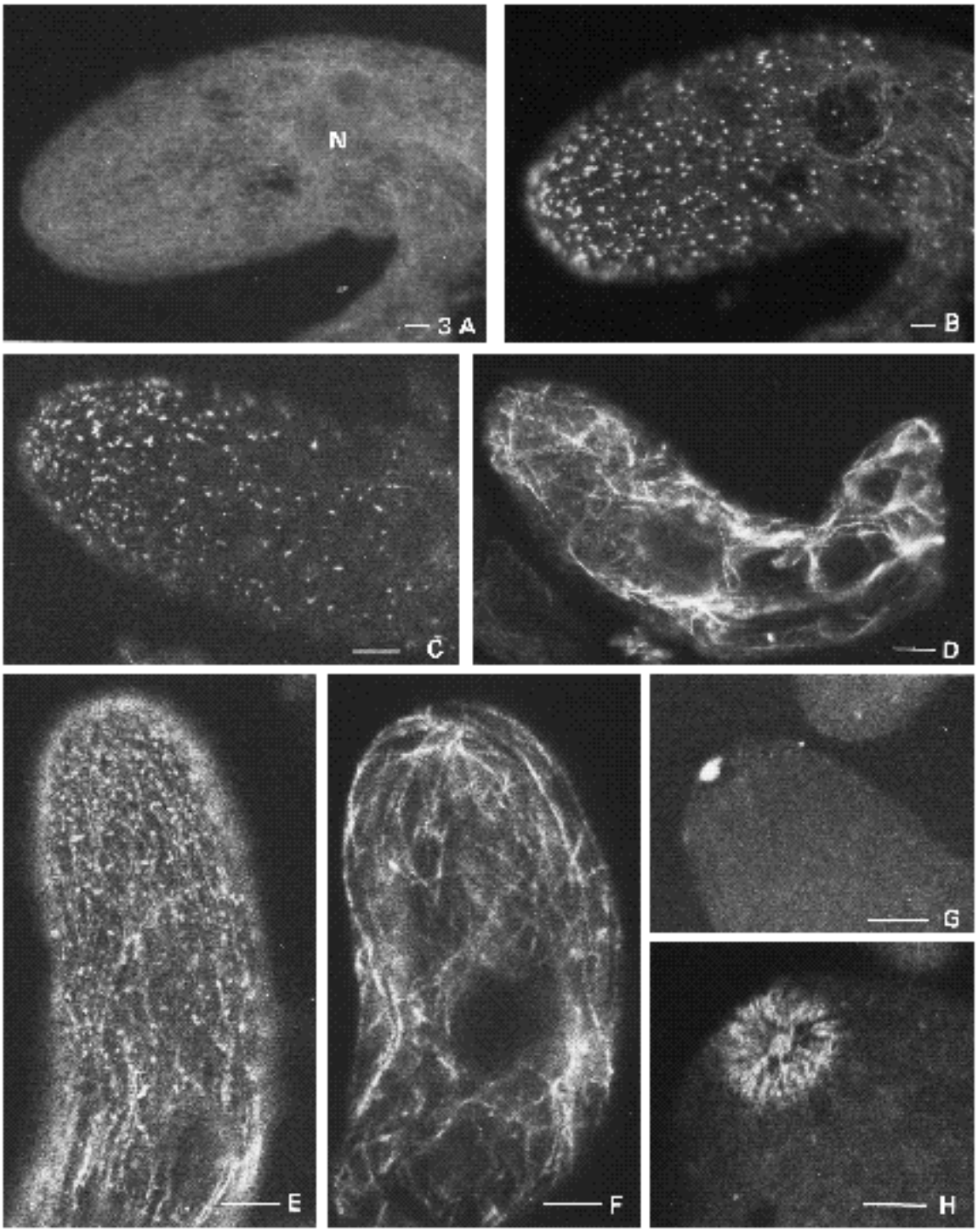


Fig. 3. (A) Anti-tubulin of nocodazole-treated ascus. N indicates nucleus; darker circle within is the nucleolus. Cortical MT are depolymerized; compare with Fig. 2B. (B) Anti-actin of same ascus. Most MF above the nucleus are lost; peripheral plaques remain. Compare with Fig. 2A, same age ascus. (C) Anti-actin of ascus after overnight treatment with CD. Cortical MF are disrupted; few fibers remain. (D) Anti-tubulin of ascus treated overnight with 10 μM CD; cortical MT are chaotic and no apical MTOC is visible. Compare with Fig. 2B, same stage ascus. (E) Overnight treatment with 20 mM BDM does not greatly affect cortical MF organization (compare with Fig. 2A). (F) Anti-tubulin of BDM-treated ascus. As after CD treatment, cortical MT organization is bizarre and unfocused; compare with Fig. 2B. (G) MPM-2 immunofluorescence marking of the apical MTOC in untreated ascus. The rounded shape is identical to that seen in nocodazole-treated asci. (H) MPM-2 apical markage of BDM-treated cell. A wide, fibrous-appearing circle of reactivity covers the ascus apex. A, B, C, G and H are from double-labeled slides; D, E and F are single-labeled. Bars, 5 μm.

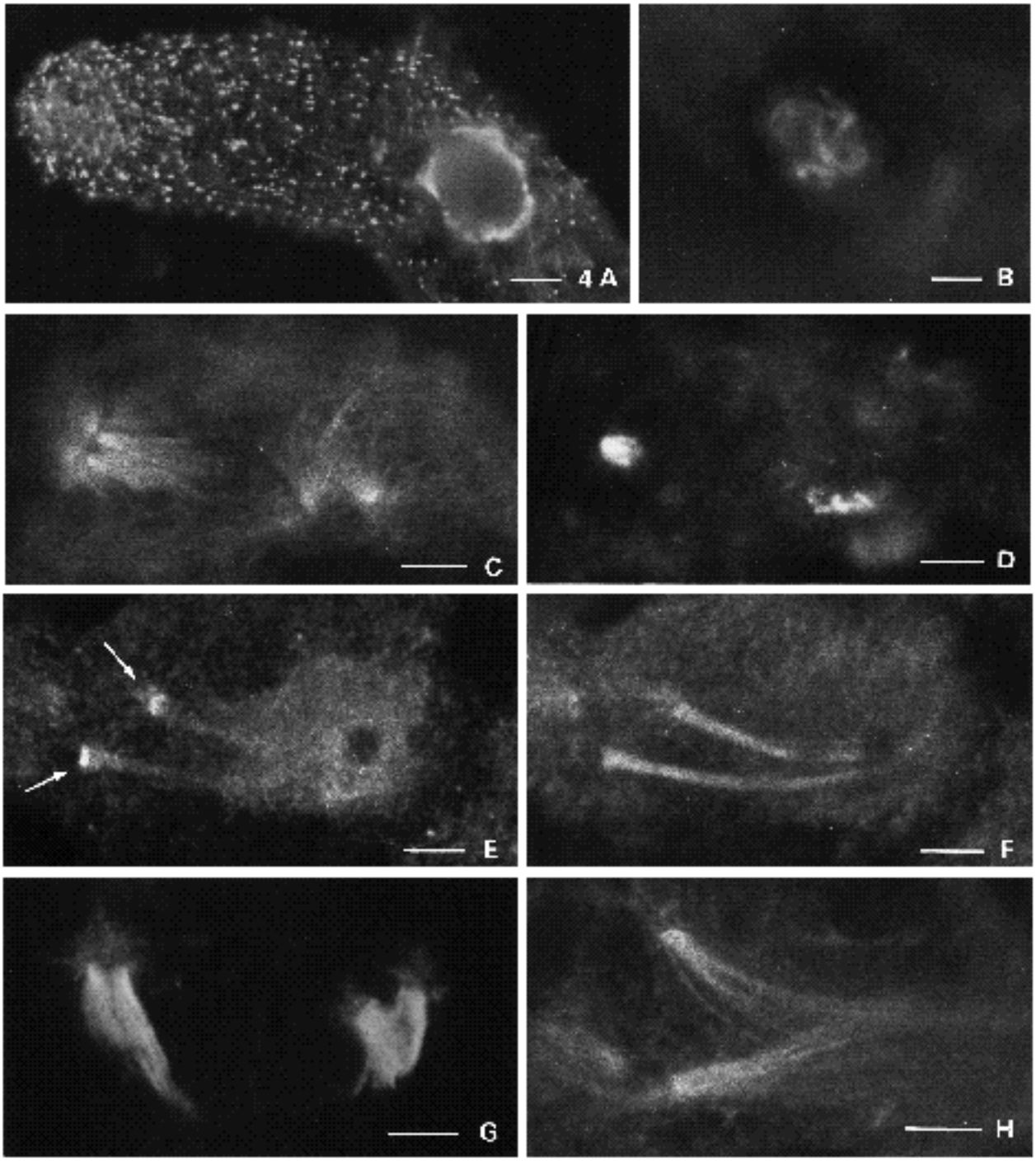


Fig. 4. (A) A ring of anti-actin reactivity surrounds the prophase I nucleus during meiotic pairing. (B) DAPI of same ascus. (C) Anti-tubulin of ascus following overnight CD treatment. SPBs of the meiosis II spindles have failed to separate and migrate around the nuclei; parallel half-spindles have formed. Note that the SPBs on the right appear slightly more separated than those on the left. (D) DAPI of nuclei in C. Note that chromatin of the right half-spindles appears more extended than that engaged by the spindle on the left. (E) MPM-2 immunofluorescence of SPBs in a CD-treated PMM cell; arrows indicate SPBs. SPBs are seen as distinctly separate, having migrated along the stretched nuclear envelope prior to blockage. (F) Anti-tubulin of the nucleus shown in E. The half-spindles, separate at the polar ends, curve toward the same chromatin mass. (G) Anti-tubulin of BDM-treated ascus, showing blockage of SPB separation and half-spindles similar to CD-treated nuclei in C, D. (H) Anti-tubulin of BDM-treated ascus, showing a partial migration and blockage, as in CD-treated ascus of E, F. A, E and F are double-stained samples; C, G, and H are single-stained. Bars, 5 μm.

and CD-treated asci the separation of the post-PMM SPB from the nucleus frequently fails to occur. Spores at this stage in CD- or BDM-treated cultures often show no apparent cortical SPB and a reduced cortical MT network (Fig. 5C). Spores are frequently blocked at mitosis with unseparated SPBs (Fig. 5D); when SPBs fail to detach from the

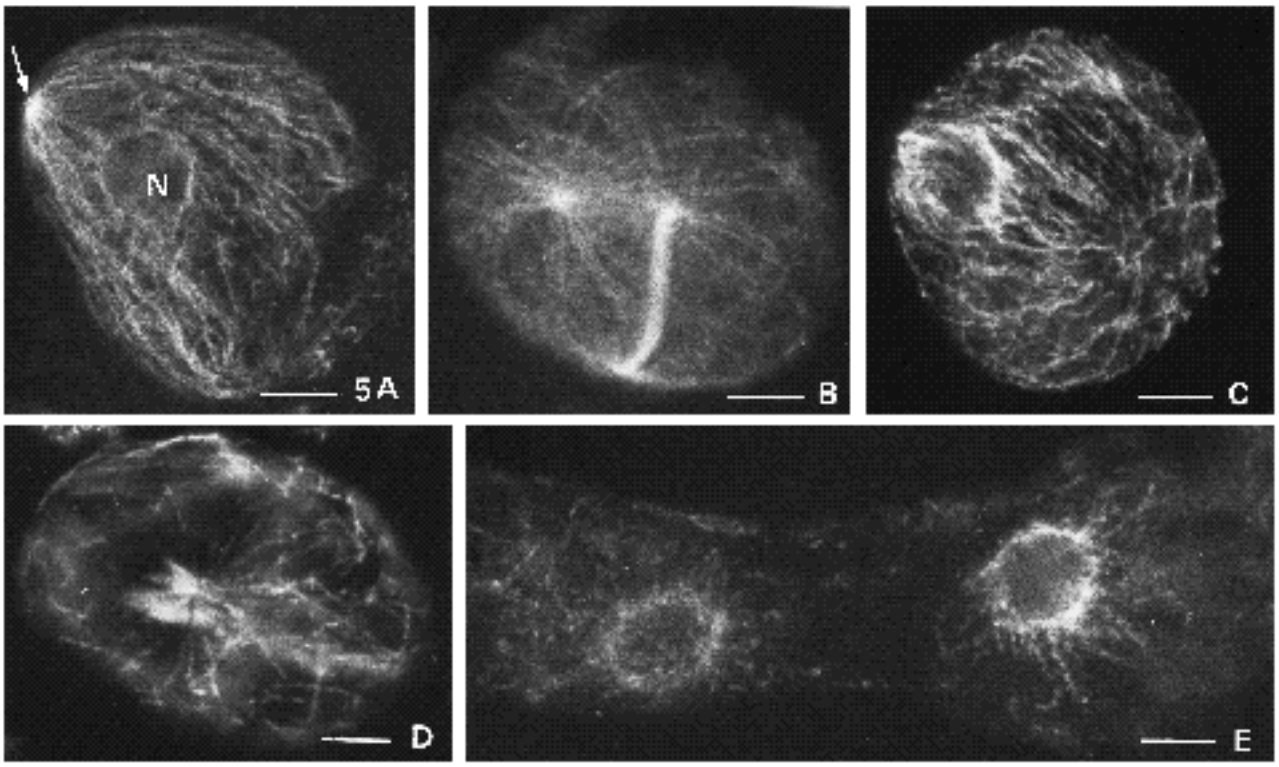


Fig. 5. Anti-tubulin immunofluorescence. (A) MT of untreated spore. Nucleus (N) is recentered and cortical SPB (arrow) is clearly visible. (B) MT arrays of untreated spore at mitosis. All three SPBs are active. While the spindle SPBs appear to nucleate some cytoplasmic MTs, the majority of the cortical MTs originate at the SPB lying above the spindle, near the cell membrane. (C) BDM-treated spore, analogous to that in A. No cortical SPB is visible. Cortical MTs do not show the normal organization; the nucleus has not centered and shows high anti-tubulin reactivity. (D) BDM-treated spore at mitosis. The SPB appears to have detached from the nuclear envelope, but does not organize an abundant cortical array as in B. The nuclear SPBs have failed to separate and the parallel half-spindles are located high in the cortex rather than at the cell center; most of the meager cortical cytoskeleton is nucleated from these SPBs. (E) MT organization at the nuclear envelope of CD-treated ascus, post-meiosis II. The MT halos are identical to those seen following low-nocodazole treatment on prophase or telophase nuclei. All samples single-labeled. Bars, 5 μm.

nuclear envelope, an undifferentiated blur of anti-tubulin fluorescence is seen at the cell cortex at this stage.

The nuclear envelope of *Sordaria* is a site of MT nucleation at prophase and at the telophase/interphase transition; when asci are treated with 10 μM nocodazole to disassemble MT and then incubated in 0.5 μM nocodazole a further 1.5 h to allow limited regrowth at MTOCs, nuclear envelopes at the proper stage show halos of short MT (Thompson-Coffe and Zickler, 1992). Following overnight CD treatment, the same effect is seen: nuclei in prophase asci have MT halos (Fig. 5E). In BDM-treated asci, prophase MT halos are also found, although to a lesser extent.

The prolonged drug treatments also permitted us to find points at which actin and actin-myosin interaction were required for normal morphogenesis; treated cultures are deficient in the initial formation of asci, and in the development of spores.

Actin and actin-myosin interaction are required for normal morphogenesis

In untreated cells, MT in the crozier are few, heavily bundled, and mainly arise from the nuclear envelope (Thompson-Coffe and Zickler, 1992). Actin is present both as plaques and fibers; fibers are seen at the crook and tip of the premitotic crozier (Fig. 6A), and at the site of septum formation by anaphase of crozier mitosis (Fig. 6B,C). Plaques are mainly at the tips of croziers (Fig. 6C,D) during division and the subsequent crozier/ascus shape transition. Following ascus formation, coarse actin cables form, which are particularly numerous between nucleus and apex (Fig. 6E). There are many MF at the ascus tip, where general actin fluorescence is high. Peripheral plaques are also seen. At this point in development there is little if any coincidence between the MF and MT arrays. Slightly older asci show less tip fluorescence and more numerous and finer cortical MF (Fig. 6F), which are parallel to the cortical MT array.

MF and actin-myosin interaction are necessary for the crozier/ascus transition (see Fig. 1). When 10 μM CD or 20 mM BDM is applied to cultures at crozier stage, no further development occurs: asci do not form until after washout of CD, or not at all following BDM treatment. Blockage occurs primarily at the two-nucleus stage prior to crozier mitosis: of 426 croziers examined, 73% had arrested at this point. Among the remaining 23%, 7% either showed three nuclei, or one nucleus in mitosis with the other apparently blocked at SPB separation. Cells with four nuclei were

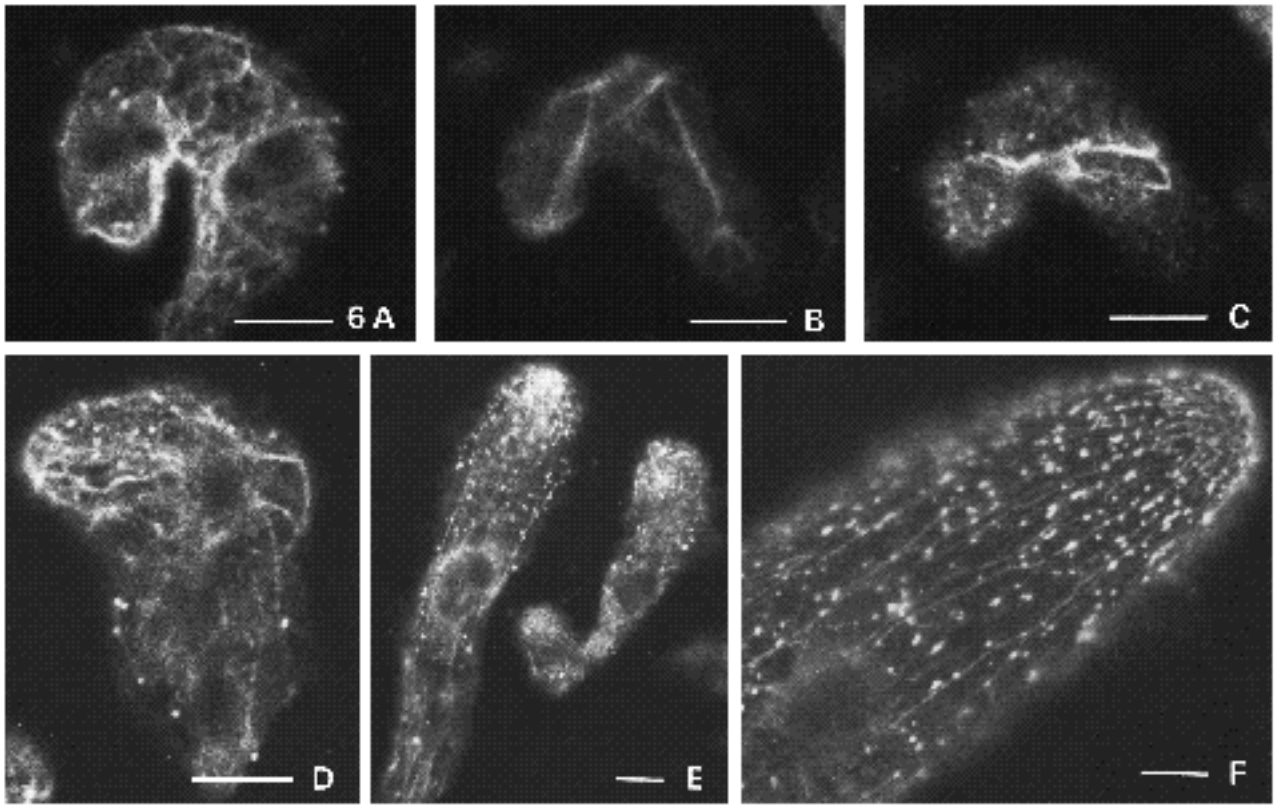


Fig. 6. (A) Anti-actin immunofluorescence of binucleate crozier prior to mitosis. MF are most abundant in the crook and between the nuclei. (B) Anti-tubulin of crozier mitosis; spindles are positioned to leave two daughter nuclei in the crook, which will become the ascus mother cell. (C) Anti-actin of the crozier in B. MF are seen at the future septa sites, and plaques are more numerous at the tip of the cell. (D) Older crozier. Coarse MF run between the nucleus and the outside of the crook; they are numerous between nucleus and tip. (E) Young asci after karyogamy, in early prophase I. MF are finer and more numerous; there is diffuse fluorescence at the tips. (F) Slightly older ascus, shown from above the nucleus to apex. Cytoplasmic and particularly tip anti-actin fluorescence is lower; an increased number of MF parallel the organizing MT array of the cortex at this stage. All samples double-labeled. Bars, 5 μm.

seen in 2% of the croziers and probably represent a population affected by the drug after SPB separation but before septation. No significant differences were seen between CD and BDM treatments. MT appeared normal, but no MF were seen at sites where septa should have formed.

Karyogamy also appeared to be affected. In untreated cells, haploid nuclei lie side by side in the ascus mother cell (see Fig. 1), move into a vertical arrangement as the young ascus begins elongation, then fuse. While only rare ascus mother cells with two nuclei are seen in control cultures, 12.5% of the 426 croziers (in other words, nearly half the post-mitotic cells seen following drug treatment) had two laterally aligned nuclei, indicating a significant delay or blockage prior to karyogamy.

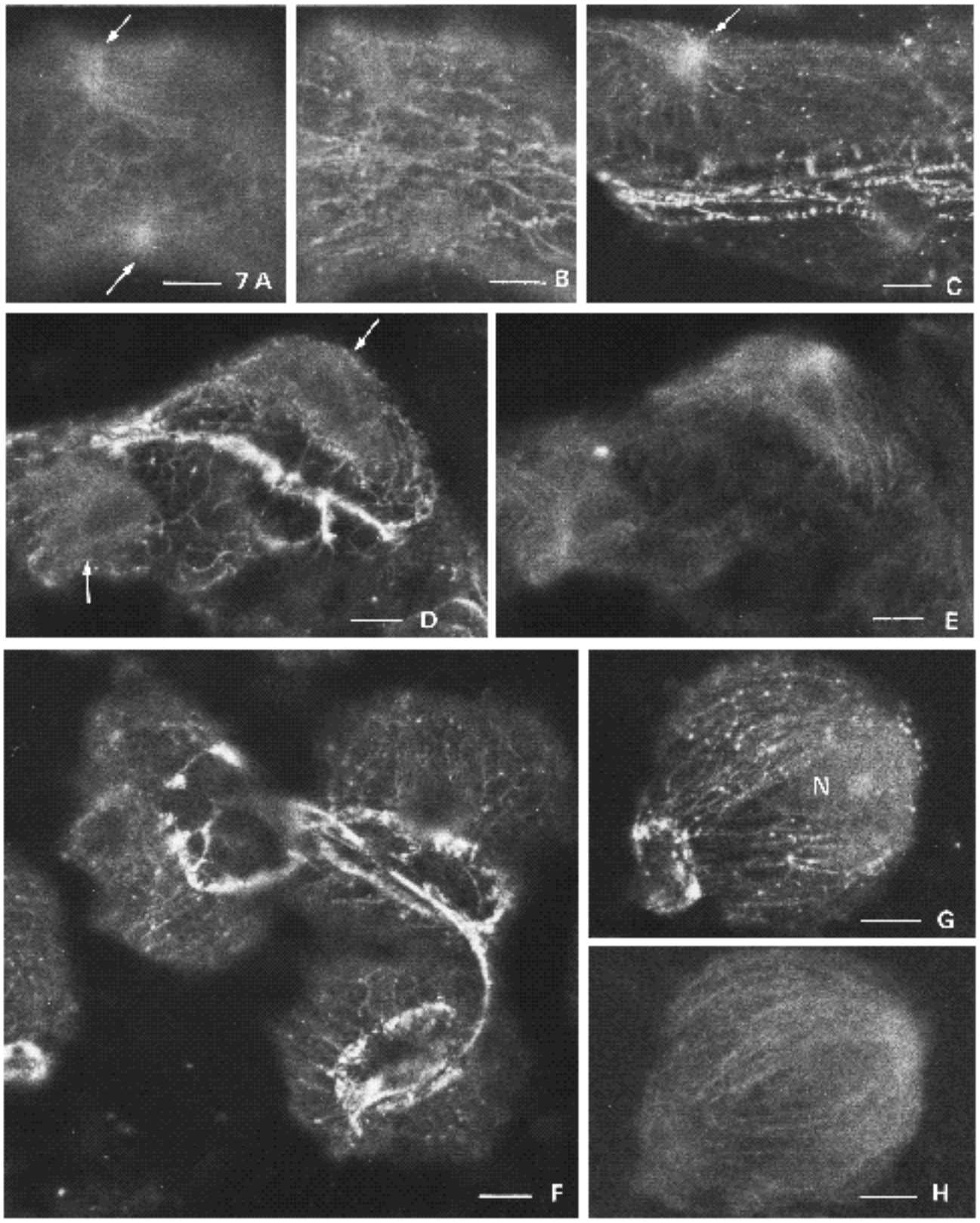
Actin and myosin are involved in spore delimitation and alignment

Sporulation in *Sordaria* (see Fig. 1) and related species follows a post-meiotic mitosis (PMM) in which proper spindle alignment is crucial for correct spore formation (Zickler and Simonet, 1980; Raju, 1992). During PMM the SPB changes orientation, with one portion becoming perpendicular to the nuclear envelope; this portion will organize a large aster curving around both sides of the telophase nucleus. The nuclei migrate to the ascus cell membrane, four on each side; shortly thereafter they are realigned to lie in a single row of eight, with SPBs next to the membrane. The future spore cell membrane (derived from

Fig. 7. (A) Anti-tubulin, telophase of PMM. Arrows indicate the SPBs approaching the plasma membrane of the ascus. (B) Anti-actin of A. MF strands appear along the division midline. (C) Anti-actin showing shift of daughter nuclei relative to one another across the division midline. The MF strands are now much heavier and more parallel. Arrow indicates the top daughter nucleus. Apparent SPB markage is due to double-staining for tubulin (see Materials and Methods). (D) Slightly more advanced stage showing more coalesced MF rope between two shifted daughter nuclei (arrows). Fine MF run upwards toward the MT halo of the nuclei. (E) Anti-tubulin of D; MT halo extends widely outward from SPB. (F) Anti-actin of early MF cages. The MF rope has narrowed considerably in diameter and widely open cages of MF have appeared at the bases of the three young prespores; these cages straddle the MF rope connecting the forming cells. An array of MF now surrounds the entire prespore. (G) Anti-actin of closing cage; N indicates the nucleus. This spore has been separated from its rope. The cage is narrowed, apparently contracting. The elaborate array of MF around the young spore is more clearly visible than in F. (H) Anti-tubulin of spore shown in G. MT positioning is similar but not identical to MF distribution. MT ends curve in, engaged by MF cage, as spore closure occurs. All samples double-labeled. Bars, 5 μm.

invaginations of a double membrane laid down at the ascus plasmalemma between meiosis II and the PMM), descends along the MT halo to enclose the new cell. Part of the SPB then detaches from the nuclear envelope and remains near the plasma membrane of the future spore (Raju, 1980; Thompson-Coffe and Zickler, 1992).

At late anaphase of PMM, as the nuclei near the ascus cell membrane, a new array of MF appears in the cell. Ini-



tially visible as several parallel strands (Fig. 7A,B,C), it runs the length of the ascus at the midline of the division. Fine MF extend upward from this rope toward the apical SPB, enclosing the MT halo (Fig. 7D,E). It is at this point that realignment of the eight nuclei occurs. During their movement, the forming spores retain their connection to the MF rope. The rope itself appears to extend from the base of the ascus to the apex, where it is anchored at the apical pore (not shown); any structure to which it might be attached at the ascus base is not visible, since the basal linkage between asci of the same perithecium is destroyed during sample preparation. The rope position is shifted by nuclear realignment to the base of the row of forming spores, and the strands which compose the rope become intertwined.

Immediately after realignment, a cage of MF forms at the point where the MT halo reaches the actin rope (Fig. 7F). This MF cage, which engages all the spore MT ends, is initially a loose, open structure; as the cell membrane closes at the base of the young spore, the ends of the MT halo curve inward and the actin cage also becomes more and more narrow, apparently constricting (Fig. 7G,H). When the nucleus is recentered and the spore membrane completely closed, the actin cage is reduced to a prominent dot at the base of the cell, connecting the spores to the dense rope of longitudinal MF.

Actin MF in the forming spore are first seen as the delicate arrangement of fibers and plaques which appears before cage formation. After the nucleus recenters, the young spore shows fine MF in a longitudinal array like that of the MT; these are labile and faint. As the spore matures, the actin fibers disappear while the plaques remain; in the binucleate spore only plaques are present.

When asci are treated with 10 μM nocodazole for 1-1.5 h, the cortical MF network is lost (or destabilized to fixation) after depolymerization of MT; the rope and cages of MF, however, remain and appear mostly unaltered (Fig. 8A,B). Treatment with 4-10 μM CD for 30-90 min causes

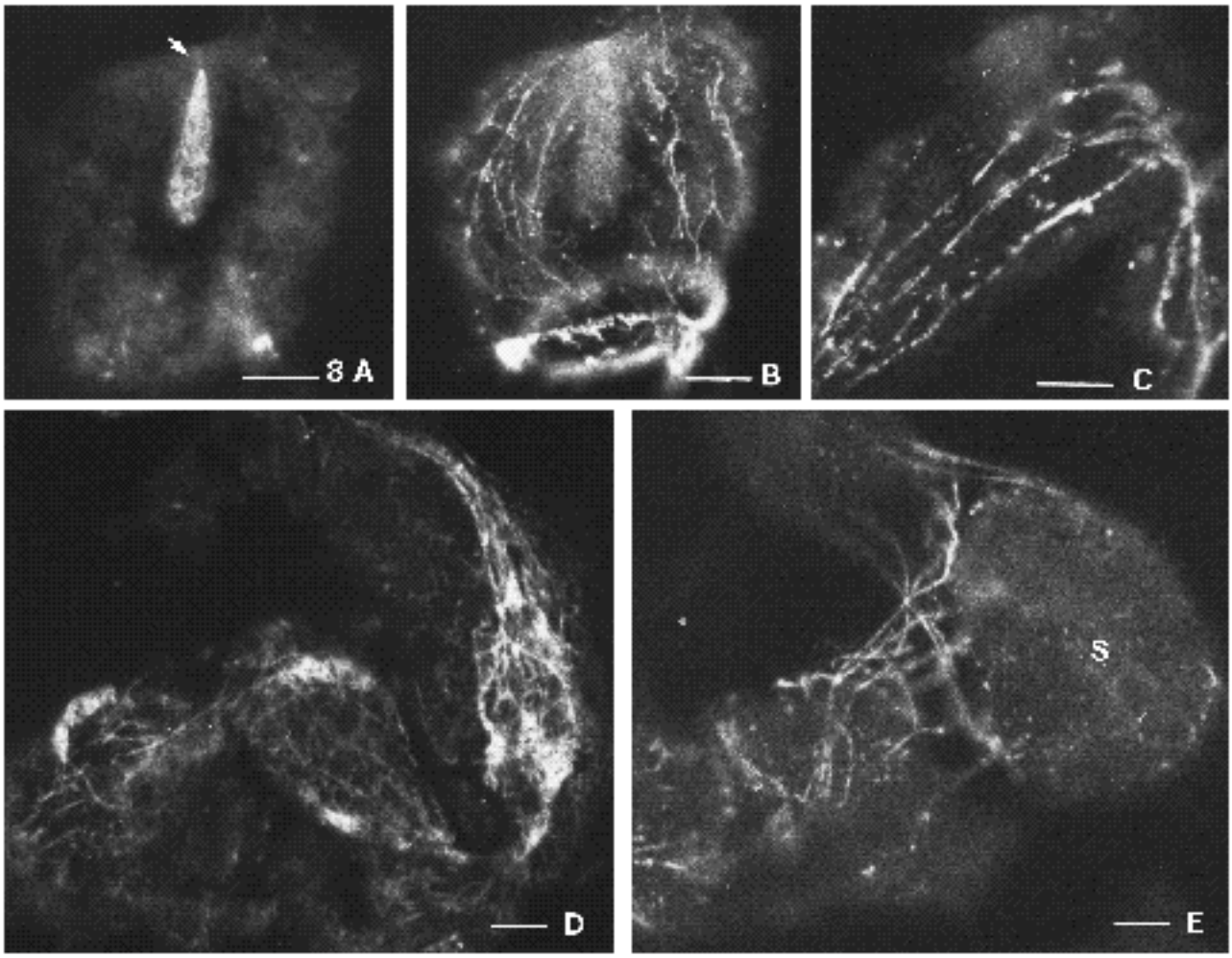


Fig. 8. (A) DAPI of nocodazole-treated prespore; arrow indicates SPB. (B) Anti-actin of A. After nocodazole treatment, no MT are seen but rope and cage MF appear near normal; MF extend upward from cage to SPB, as in control cells. (C) Cage and rope MF of ascus treated overnight with CD. Structures appear thinned (compare with Fig. 7F) and no MF mounting upward from the rope are found. (D) Rope of BDM-treated ascus. The MF of these three prespores have not narrowed into the normal structure (see Fig. 7F); open, abortive cages are seen on the left. (E) Mature spores (S) from BDM-treated culture. The MF that normally form a compact rope are separate strands. A, B, double-labeled; C, D, E, single-labeled. Bars, 5 μm.

varying degrees of cortical MF disruption, from slight to severe, without affecting cage/rope morphology. Overnight CD treatment causes severe disruption of cortical MF; ropes and cages remain but are thinned and fragile in appearance (Fig. 8C). The cages seen are of varying diameter, from widely open to constricted. Few spores are formed; but due to CD prevention of SPB separation, it is impossible to tell if this is because cells fail to progress through PMM, or because the few cage/rope assemblies seen are inefficient.

After treatment with the inhibitor of actin-myosin interaction, BDM, the cortical MF appear nearly unchanged. The MF of the rope and cage are severely affected by this drug; in early stages of rope formation the MF or MF bundles fail to aggregate into a coherent rope, but remain as separate strands (Fig. 8D). Cage formation is rare, and those seen are also composed of few MF bundles and mostly are of the earliest stage, widely open. In formed spores, the connecting 'rope' often appears, instead, as a network of unconnected fibers (Fig. 8E). In BDM-treated cells but not those treated with CD, spores with a variable number of nuclei, as well as anucleate spores, were formed.

In order to examine the relationship between rope, cage and spore formation, we used *spo644*, a morphological mutant with abnormal sporulation. The round ascus shows a disordered cortical MT array (Fig. 9A). Due to the balloon shape of the ascus, the PMM spindles align randomly (Fig. 9B), and abnormal spores are formed. These vary in size and may be anucleate or multinucleate.

The cortical MF network in *spo644* is more wild-type than the MT array; MF run in a parallel, longitudinal fashion, while the short MTs are mostly transverse. At the time when the MF rope first appears in the wild-type, *spo644* telophase nuclei are randomly located; this appears to retard rope formation so that the initial steps are visible. The MF are first visible at the base of the ascus. In the wild-type they extend almost immediately, unbroken, from base to apex along the midline of the PMM, but in *spo644* there is no midline (Fig. 9C,D). The rope meanders through the ascus, sometimes branching extensively as it travels from one division nucleus to another. Frequently formation of a normal pattern is incomplete, the rope never reaching the apex (Fig. 9E,F). When a nucleus is correctly located near the ascus membrane and the MF rope, a near-normal cage forms. Except in asci with an almost normal morphology, more cylindrical than round, the step of spore nucleus alignment does not occur.

To determine whether myosin, the molecule proposed to be the main site of action of BDM in its inhibition of actin-myosin interaction (Higuchi and Takemori, 1989; Yagi et al., 1992), was present in *Sordaria* asci, electrophoresis and Western blotting were done for both actin and myosin. In 10% polyacrylamide gels, a polypeptide comigrating with rabbit muscle myosin heavy chain was present in Triton-insoluble extracts of asci (Fig. 10, lane B), but not in the Triton-soluble portion. A 45 kDa polypeptide was seen in both soluble and insoluble fractions. Actin was blotted from 10% gels, using the N.350 antibody (Fig. 10, lane C). It reacted with a single band comigrating with the 45 kDa molecular weight marker, and more faintly with a band around 85 kDa; this reactivity pattern was also obtained in *Chlamydomonas* with N.350 (Harper et al., 1992).

A monoclonal antibody specific for smooth muscle and nonmuscle myosins from a wide range of organisms, CC-212 (Klotz et al., 1986), reacted with three bands in the 200 kDa range, blotted from a 6% gel (Fig. 10, lane D). The mixture of fast and slow anti-skeletal muscle myosin identified a single band at 205 kDa, as well as an apparent degradation product which migrated more quickly (Fig. 10, lane E). This latter pattern was identical to that obtained, much more weakly, with Amersham anti-pan myosin (not shown), which also recognizes both fast and slow isozymes.

DISCUSSION

As part of an ongoing project examining the distribution and function of cytoskeletal elements in ascus and spore development, we have used anti-actin immunofluorescence to locate MF arrays in *Sordaria*, and inhibitory drugs to examine their functions. The MF of this part of the ascomycete life cycle are more complex that those of the fungal vegetative cycles so far described.

The peripheral plaques common in ascomycetes (Kilmartin and Adams, 1984; Marks and Hyams, 1985; Alfa and Hyams, 1990), basidiomycetes (Hoch and Staples, 1983; Roberson, 1992) and zygomycetes (Butt and Heath, 1988) are present throughout ascus development in *Sordaria*, associated with an extensive cortical array of longitudinal MF parallel to the MT network. At the level of resolution used, the actin plaques appear to be located along the MF just below the cell membrane.

There has been much discussion as to the ultrastructural equivalent of the actin plaques in fungi. Hoch and Staples (1983) noted that their localization in *Uromyces* germ tubes correlated with that of filasomes, which are surrounded by a fibrillar mesh which binds anti-actin antibody in immunogold EM (Roberson, 1992). However, other MF-associated granules are seen in fission yeast (Kanbe et al., 1989) and peripheral plaques are present in fungi lacking filasomes (Heath and Harold, 1992).

Kilmartin and Adams (1984) noted that plaque localization throughout the yeast cell cycle implied an involvement in new cell wall deposition, but that they are also seen in areas where no growth is occurring. Adams and Pringle (1984) speculated that they represented points of attachment of the MF cytoskeleton to the plasma membrane, and perhaps a transmembrane linkage to the cell wall.

We favor the idea of a MF-membrane linkage because of the extensive association of plaques with cortical fibers in *Sordaria*, but would suggest that more than one type of actin-associated plaque is present, as demonstrated in *Schizosaccharomyces* (Kanbe et al., 1989). In *Sordaria*, plaques cluster in areas of growth but are also present in areas where no growth is known to occur. While their localization in the binucleate spore may indicate a role in wall deposition, this is less probable for the plaques located behind the ascus tip; a similar situation is seen in sporangia of oomycetes, where an adhesion plaque anology is favored (Heath and Harold, 1992). It seems likely that the apparently homogeneous plaques seen in immunofluorescence represent different populations with differing functions.

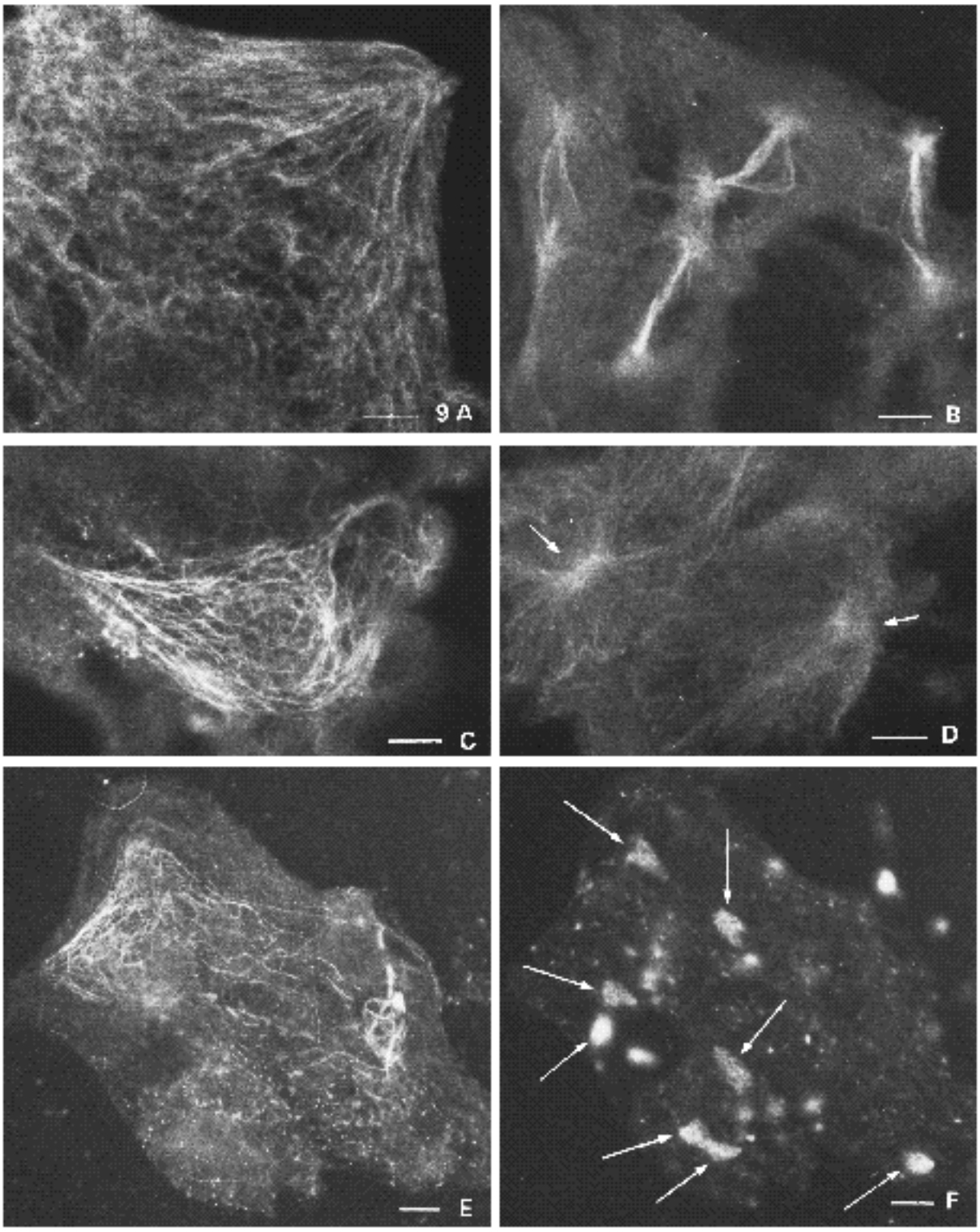


Fig. 9. *spo644*, untreated. (A) Anti-tubulin of *spo644* ascus tip. The rounded ascus shows many short transverse tubules (compare with Fig. 2B). (B) Anti-tubulin of the PMM; the four spindles are randomly arranged in this mutant (see Fig. 1), resulting in aberrant spore nuclei positions. (C) Anti-actin of prespore nuclei, comparable to Fig. 7D. A mass of separate MF strands loops across and between the nuclei. (D) Anti-tubulin of C; arrows indicate SPBs. (E) Anti-actin of MF 'rope' in *spo644*; (F) shows the DAPI of this ascus, arrows indicating prespore nuclei. Four nuclei appear involved in the mass of MF at the upper left of the ascus; three nuclei in the lower left have never been reached by MF, while a dense tangle of MF at the lower right portion has no associated nuclei. All samples double-labeled. Bars, 5 μm.

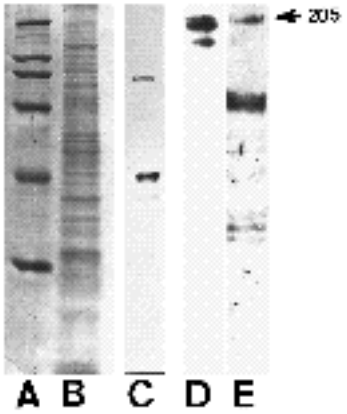


Fig. 10. Electrophoresis and immunoblotting of *Sordaria* ascal proteins. Lane A, molecular mass markers in 10% poylyacrylamide gel. From top: 205 kDa, 116 kDa, 97.4 kDa, 66 kDa, 45 kDa, 29 kDa. Lane B, Coomassie blue stain of Triton-insoluble fraction from a population of asci ranging from prophase I to sporulation. Lane C, Immunoblot of the same sample, incubated with monoclonal anti-actin. Lanes D and E, same sample separated on 6% gels and blotted with CC-212 and the mixture of anti-fast and slow myosin antibodies. Position of 205 kDa marker in 6% gel is indicated on the right.

As the ascus matures the number of plaques lessens. It may be that the links between the dense cortical array of MF and the plasma membrane are disassembled because the MF of the network will be recruited for rope and cage formation during sporulation. After the PMM, the amount of cortical actin lessens as the actin array involved in spore alignment, cleavage and linkage is elaborated.

The cortical MF are probably also involved in vesicular transport necessary for polarized growth, as previously suggested in hyphae (reviewed by Heath, 1990). In budding yeast, a number of studies (reviewed by Lui and Bretscher, 1992) provide strong evidence that MF and actin-binding proteins are required for polarized growth and vesicle transport. In *Sordaria* cortical MF are unlikely to have a role in the mechanical support of cell shape; *spo644* MF, while somewhat distorted to follow the balloon morphology, are longitudinal and fairly well organized. Cell shape appears to be maintained by the cortical MT network (Thompson-Coffe and Zickler, 1992).

In the crozier of *Sordaria*, actin fibers and plaques are involved in septum formation. Those nuclei forming spindles at the time of CD or BDM addition appear to complete the process but do not septate. MF are apparent in the future zone of septation by anaphase of crozier mitosis in untreated cells and persist after telophase. The actin belt seen at the septum site does not broaden and constrict during this process, unlike that of *Schizosaccharomyces japonicus* (Alfa and Hyams, 1990). Septum formation in fission yeast, however, precedes cell separation, whereas in *Sordaria* septal pores providing some physical continuity persist after nuclear division and septation (Beckett, 1981), and the septating cell does not become constricted during the division.

The delay in karyogamy caused by CD or BDM treatment implies actin-myosin involvement in nuclear migration; nuclei are blocked in the lateral configuration. In later stages of ascus development, treated asci were often observed to have nuclei in unusual orientations, either too close following a division or too widely separated following a migration (data not shown). The presence of MT halos around telophase and prophase nuclei in CD- or BDM-treated cultures also suggests a retardation of nuclear migration; this organization on the nuclear envelope is normally transient and probably involved in positioning of nuclei relative to one another and to the apex prior to and after division (Thompson-Coffe and Zickler, 1992). Its persistence in CD- or BDM-treated cells together with the aberrant nuclear positioning support a block in nuclear migration.

BDM has been used to inhibit tension development in intact and skinned muscle fibers (Horiuti et al., 1988); from biochemical data, it was shown to suppress the ATPase action of heavy meromyosin in both the presence and absence of actin (Higuchi and Takemori, 1989), indicating an inhibitory effect of BDM on the myosin head. From X-ray diffraction studies, it interferes with actin-myosin interaction by altering the binding of myosin heads to actin filaments (Yagi et al., 1992). In *S. pombe*, 20 mM BDM treatment significantly depresses septation index (S. Wheatley, personal communication). Ohta et al. (1991) have reported that in the fungus *Physarum*, the comigration of nucleus and centrosome is inhibited by cytochalasin, but not nocodazole. Watts et al. (1987) found that budding yeast mutant in MYO1 (a myosin II type) were deficient in nuclear migration and cytokinesis. From our data, both functional MF and actin-myosin interaction are necessary for septation and normal nuclear migration in *Sordaria*.

The involvement of actin in centrosome separation and motility has been shown in other studies (Euteneuer and Schliwa, 1985; Schatten et al., 1988; Callaini and Riparbelli, 1992), but to our knowledge this is the first study showing an actin-myosin interaction in separation and initial migration of SPBs. In sea urchin first mitosis (Schatten et al, 1988), initial migration was not noted to be affected by CD treatment although centrosome separation was prevented. In *Drosophila*, however, cytochalasin B treatment arrested the migration of duplicated and separated centrosomes (Callaini and Riparbelli, 1992), as shown here for SPBs. In *Sordaria*, the effect of inhibition of MF or actin-myosin interaction on SPBs was clear; each crozier or ascus continued development after drug treatment until the following nuclear division, at which point most SPBs failed to separate or migrate normally. The treatment with CD or BDM affected not only the separation of duplicated SPBs, but also the separation of the SPB from the nucleus during spore development and its migration to the spore cell membrane.

MT organization in the cortex was affected in those asci which underwent elongation in either BDM or CD. MPM-2 reactivity at the cortical MTOC showed an alteration of shape, indicating that the actin prominent in the ascus tip at this time, in interaction with myosin, serves to establish the initial shape of this MTOC or to maintain that form once established. The form is not MT-dependent; nocodazole treatment does not affect MPM-2 reactivity. The shape of MTOCs affects the number and orientation of MT, presumably by altering the number and orientation of nucleating sites (reviewed by Brinkley, 1985). The broadened arc shape of the MTOC in BDM- or CD-treated asci may explain the disorganization of the cortical MT and the resultant loss of the MTOC as a point of convergence in anti-tubulin immunofluorescence.

The degree of interdependence among cytoskeletal com-

ponents shown here and by others (Menzel and Schliwa, 1986; Agosti and Stidwell, 1992) underscores the difficulty of interpreting effects of cytoskeletal inhibitors, especially when only one component is examined after treatment. Alterations in MF were reported for the siphonous green alga *Bryopsis* when exposed to MT-disrupting agents (Menzel and Schliwa, 1986) and in *Hydra* treated either with nocodazole or low temperature (Agosti and Stidwell, 1992). In the case of *Bryopsis*, as in *Sordaria*, cortical MT and MF networks are extensive, longitudinal, and closely aligned, while in *Hydra* the MT and MF networks do not coincide.

Actin filaments have been described, in several systems (Traas et al., 1987; LaFountain et al., 1992, and references therein), as present at spindle poles and within the spindle; however, this is frequently interpreted as being due to movement of soluble proteins into the exclusion volume of the open spindle (Sanger et al., 1989) rather than as demonstrating a functional role. In closed fungal mitoses, actin was not found in the nucleus (Kilmartin and Adams, 1984; Butt and Heath, 1988; Alfa and Hyams, 1990). Our results conform with those obtained with other fungi; no MF were seen in the division spindle or SPB.

Interphase perinuclear actin shells have been described in the zygomycete *Neozygites* (Butt and Heath, 1988) and in mutants of *S. pombe* (Marks et al., 1986). The *Sordaria* perinuclear actin, like these shells, appears not to be filamentous and has no obvious connection to cytoplasmic MF. In contrast, it is present only on meiotic prophase I nuclei. The shell is proposed by Butt and Heath (1988) to be an interphase storage form utilized for septum formation. That in *S. pombe* mutants (Marks et al., 1986) is seen only when the normal actin array is lost at restrictive temperature. However, the prophase I shell in *Sordaria* forms in the presence of an extensive cortical MF network. The most obvious explanation is that this shell serves to reinforce the enlarged nucleus during bivalent pairing. The diameter of the nucleus in *Sordaria macrospora* increases from 4.2 μM in leptotene to 9 μM by late pachytene (Zickler, 1977); it would not be surprising if support of the nuclear envelope was necessary. This enlargement is peculiar to first meiotic prophase; in agreement with a reinforcement role, no other nucleus has an actin ring during ascus development.

The coincidence in time of the spore cell membrane descent with the formation and narrowing of the MF cage at the base of the MT halo suggests an interaction between MT, MF and membrane in the closure of the spore, in a manner analogous to cytokinesis in animal cells. The contractile nature of the cage is shown by its narrowing during spore delimitation in untreated cells, and by the results seen after treatment with the inhibitor of actin-myosin interaction, BDM: while in CD-treated cells the rope and cage are thinned, the cage narrows; in BDM treatment, most cages observed stay open. The fact that BDM-treated cells often show spores with aberrant numbers of nuclei while CD-treated cells do not implies that the constriction of the MF cage ensures proper membrane enclosure of cytoplasm containing a single nucleus. The existence of the dense cortical MF reaching into the apical pore, where the rope of MF linking the formed spores is anchored, suggests that the MF rope may be involved in propelling the linked spores out through the apical pore. BDM-treated cultures, which have abnormal MF ropes, never expel matured spores.

In oomycete sporangia, MF outline developing vegetative spores, and cytochalasin treatment results in abnormal cleavage and diminished zoospore production (Heath and Harold, 1992). The actin pattern seen is completely different from that in *Sordaria*; the zoospore is surrounded by a polygonal array reminiscient of MF seen during cellularization in *Drosophila* embryos (Warn and Magrath, 1983). The MF of the oomycete sporangium do not appear to constrict or contract, and are lost after spore enclosure. The cleavage actin probably directs localized expansion or contraction of vacuoles which will form the spore cell membranes, rather than acting in contractile-ring fashion (Heath and Harold, 1992).

In *Sordaria* the telophase nuclei of the PMM lie in rows of four, opposite one another; after the MF rope appears, they are realigned on one side of the ascus, and the rope is shifted from a central position to the base of the spores, opposite the SPB. In wild-type cells we observe that it is attached at the apical pore and, from observation of *spo644*, we assume that the rope forms from base to apex of the ascus. We propose a simple model for realignment based on the following idea: the rope strands are fixed at one end (presumably at the apical pore) and not at the other; in this case, a lateral slippage between MF in the strands of the rope and two twists of the strands would produce alignment of the nuclei. A diagrammatic representation of this process is shown in Fig. 11. The lateral slippage of MF places the nuclei in a staggered formation. A first torsion puts the two rows at an angle of 90°, while a second twist aligns them as seen in the ascus, displacing the rope opposite the SPBs, and intertwining the MF strands. The few intermediate forms seen in our study agree with this model (see Fig. 7D; lateral displacement of prespore nuclei along the rope strands). Slippage of actin fibers relative to one another in an MF bundle has been demonstrated in the acrosomal process in *Limulus* sperm (DeRosier and Tilney, 1984); in this case extension and twisting motion are produced by alteration of intrafilament bonds within a coil of actin fibers, constrained at one end. Possibly, in *Sordaria* the twisting motion is imparted by actin-binding proteins of the fascin type (reviewed by Pollard and Cooper, 1986) which form crossbridges as the filaments slip past one another. Such a mechanism would result in a change in diameter of the rope, which is seen here.

What is the role of actin-myosin interaction in rope formation? If an actinomyosin mechanism closes the contractile cage, it is not surprising that most cages in BDM-treated cultures appear open. It is more surprising that ropes, in such cultures, often remain as separate strands rather than forming the bundle normally seen. Myosin is unlikely to be the only agent which forms the crossbridges necessary for twisting, since spores in BDM cultures often undergo alignment. The ascomycete in which the most myosin research has been done, budding yeast, now has four identified myosins (reviewed by Cheney and Mooseker, 1992); of these, three are unconventional myosins. MYO3 is of the ameboid myosin-I type and thus capable of crosslinking actin filaments. A similar myosin in *Sordaria* may be involved in crosslinking the MF strands of the rope after

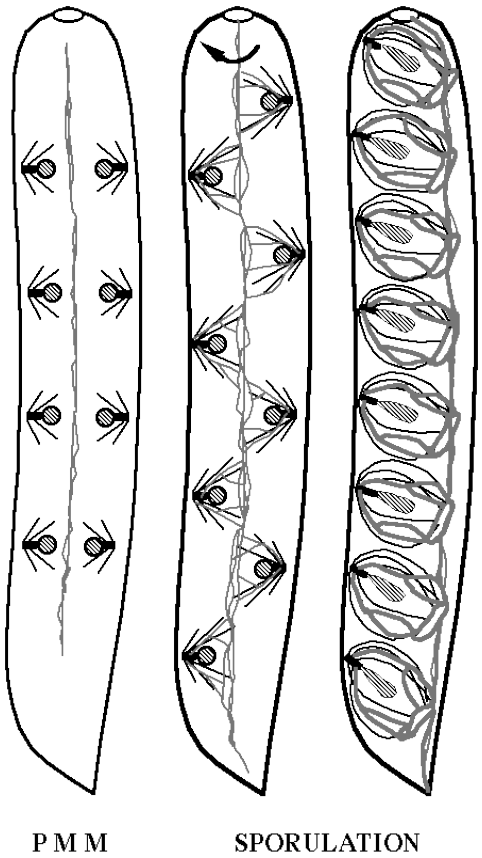


Fig. 11. Schematic drawing of cytoskeleton during sporulation. Straight, black lines indicate MT; curving gray lines represent MF. SPBs are shown as black rectangles, and nuclei as barred circles. PMM: anaphase nuclei approach the plasma membrane, longitudinal actin fibers appear at the division midline. Sporulation: MF strands extend and thicken, engaging ends of the developing nuclear MT halo. Slippage between MF shifts the nuclei into a staggered position across the ascus. As MF extend upwards toward the SPBs, one row of nuclei is shifted around the long axis of the cell (arrow at top of ascus indicates rotation). MF cages are formed, engaging cortical spore MT ends and descended spore cell membranes; aligned spores remain attached to the MF strands, now twisted into a compact rope.

spore alignment. In Triton-insoluble extracts of *Sordaria* asci, three protein bands of the same molecular mass range as yeast MYO1 (Watts et al., 1987) and MYO2 (Johnson et al., 1991) react with the CC-212 anti-myosin (which recognizes smooth mucle and nonmuscle myosins only), suggesting that more than one isoform may be present; only one of these three bands is reactive with anti-skeletal muscle antibodies.

The involvement of actin in centrosome, basal body or SPB separation and migration appears to be widespread; it is reported in organisms as diverse as echinoderms (Schatten et al., 1988), insects (Callaini and Riparbelli, 1992), birds (Boisvieux-Ulrich et al., 1990), and, as shown by this study, fungi, where actin-myosin interaction is required. Similarly, cytochalasins affect nuclear migration in *Drosophila* (Zalokar and Erk, 1976), *Physarum* (Ohta et al., 1991) and *Sordaria*, while myosin has been shown to be involved in migration of ascomycete nuclei (Watts et al., 1987; this study).

The rope and cage MF which mediate spore nucleus realignment, cytokinesis, and possibly spore release from the ascus, have not been described for any other organism. It is likely that such MF arrays are a general feature of ascomycetes, such as *Neurospora*, in which the morphology of sporulation is similar to that of *Sordaria*.

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