Actin has multiple roles in the formation and architecture of zoospores of the oomycetes, *Saprolegnia ferax* and *Achlya bisexualis*

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Summary

Very similar changing patterns of actin are described with rhodamine-phalloidin labelling during the zoosporic life cycle of the oomycetes, *Saprolegnia ferax* and *Achlya bisexualis*. By comparing the changes with previously described ultrastructural and functional changes, we show that actin functions in numerous previously unrecognized processes.

Most spectacularly, the directed vesicle expansions of the cytokinetic system involve newly formed actin which outlines the developing zoospores. Disruption of this actin with cytochalasins leads to abnormal cleavage as witnessed by the formation of enlarged and irregular cysts. Prior to cytokinesis, two new types of organelle are synthesized and one, known as K bodies, clusters around the nuclei. These organelles are actin-rich during development and clustering, consistent with actin functioning in their positioning.

In the zoospores, actin is concentrated around the water expulsion vacuoles, indicating that they are contractile, and permeates the cytoplasm, probably with a skeletal role. This concept is supported by the first demonstration of actin specifically associated with a microtubular root in the secondary zoospore. Upon encystment there is a dramatic increase in stained actin in the form of peripheral plaques associated with the newly synthesized cell wall.

When the cysts germinate, a fibrillar actin cap, comparable to that previously described in hyphal tips, forms in the germ tube apex, but only after cell wall softening to permit germ tube protrusion. This sequence is consistent with the actin cap modulating turgor-driven expansion of the tip as previously discussed for hyphae.

In addition to disrupting cleavage-associated actin, cytochalasins show developmental stage, dose and drug (CE>CD>CB) specific effects on zoosporulation-related actin, which indicates that, contrary to previous suggestions, rhodamine-phalloidin staining is a useful indicator of actin behaviour in response to cytochalasins. These responses include differential effects on adjoining actin arrays, some of which are transient in the continued presence of the drugs, indicating a mechanism of drug adaptation.

Key words: actin, rhodamine-phalloidin, flagellate cells, cytokinesis, organelle positioning, cytochalasins, fungi, contractile vacuoles, tip growth, oomycetes.

Introduction

Differentiation during animal development involves many cellular shape changes mediated by both the cytoskeleton and selective cell-substrate interactions. In contrast, in plants and fungi many aspects of differentiation are mediated by selected cell wall extensibility with turgour pressure providing the force to produce cell expansion. Nevertheless, even in walled cells the cytoskeleton clearly plays a role in aspects of morphogenesis but the role is not easily deduced because of the concomitant behaviour of the cell wall (see reviews by Lloyd, 1982). An example of this situation is seen during tip growth of pollen tubes, root hairs and fungal hyphae where the locally extensible, newly synthesized apical cell wall yields to turgour pressure to generate the familiar tube shape. There is growing evidence that cytoskeletal elements, especially actin, play some role in this morphogenesis (see reviews by Heath, 1990a,b; Jackson and Heath, 1990a) but the role is not easily understood because of both concomitant changes in the cell wall and the fact that tip growth involves at least six coordinated activities, each of which could utilize cytoskeletal components (Heath, 1990b). Oomycetes, such as *Saprolegnia* and *Achlya* species, offer an unusual opportunity to clarify some of
the uncertainties because the hyphal tip can be induced to stop growing and produce biflagellate zoospores which, in turn, can reinitiate tip growth. The changes are rapid and highly reproducible and we know the major organizational changes (e.g. see Heath et al., 1971; Heath and Greenwood, 1970, 1971; Gay et al., 1971; Holloway and Heath, 1977a,b; Lehnen and Powell, 1991). These changes include new patterns of cell wall synthesis, organelle rearrangements, new organelle synthesis, cytokinesis, development and maintenance of motile cell (zoospore) shape and the initiation of new polarized cell growth. Such changes are common to many organisms. We previously exploited the 'natural experiments' in these changes, together with anti-microtubule inhibitor studies, to investigate the roles of microtubules (e.g. see Holloway and Heath, 1977a; Heath et al., 1982). In this report we extend our analysis by continuing our observations on the behaviour of actin in Saprolegnia hyphae (Heath, 1987, 1988; Jackson and Heath, 1989, 1990a,b; McKerracher and Heath, 1987; Heath and Kaminskyj, 1989) into the asexual reproductive phase of the life cycle of both Saprolegnia and the related genus, Achlya. We show that actin is indeed intimately involved in the processes mentioned above and also eliminate some of the alternatives on the way it functions during tip growth. Preliminary accounts of this work were presented at the International Society of Evolutionary Protistology meeting in Maryland in June, 1990 and the International Fungal Spore meeting in Georgia in August, 1991.

Materials and methods

Saprolegnia

Hyphae of Saprolegnia ferax (Gruith.) Thuret (ATCC no. 36051) were grown on dialysis membrane overlying a nutrient agar designated as OM by Heath and Greenwood (1968). Hyphal tips were induced to form zoosporangia by two different protocols, which gave identical results:

1. Portions of the margins of colonies containing many hyphal tips were floated off the membrane in liquid OM, picked up on a glass coverslip, covered by a drop of OM and incubated overnight, over water, in a Petri dish at approx. 4°C. These hyphae were then floated in a dilute salts solution (DS; Sistrom and Machlis, 1955), picked up on a coverslip and embedded in a thin layer of 0.5% water agar only at their most proximal ends. These colonies were further incubated for 2 h in fresh DS followed by a final incubation of 3 to 5 h (depending on developmental stage required) in more DS, all at 24°C.

2. The superior protocol involved dipping the proximal edge of the hyphae growing on the dialysis membrane in cool 2% water agar, which kept them attached throughout the DS transfers listed in (1) and subsequent staining.

Zoosporangia were fixed in either 6% formaldehyde in PIPES buffer with protease inhibitors and selected ions or 4% formaldehyde in PIPES alone for 5 min, as previously described (Heath, 1987). Both fixatives gave the same results. Subsequent rinses, rhodamine-phalloidin (RP) and mithramycin staining, mounting and observation were as described previously (Heath, 1980, 1987, 1988). Some sporangia were stained with Bodipy-phallacidin (Molecular Probes, Oregon), Rhodamine B or Nile Red, all with the same protocol and concentration (4 µM) as RP.

Zoosporangia were produced from colonies grown in liquid OM for 18 h followed by 5 × 1 h rinses in DS and a further 2.5 h (primary zoospores) or 6 h (secondary zoospores) in DS, all at approx. 24°C. Some secondary zoosporangia were examined from colonies incubated in DS for a further 13 h at 4°C followed by 40 min at approx. 24°C prior to fixation. Germinating cysts, mixed with zoospores and empty primary cysts, were produced via this latter protocol with a further 14 min at 24°C, followed by harvesting the free cells from the DS solution by centrifugation at 1,200 g_for 2 min and resuspension in OM for 1.7 h at 24°C. All zoospores and cyst populations were fixed by adding a volume of double strength fixative equal to the volume of DS or OM in the Petri dish. Medium and fixative were mixed by gentle swirling and left for a few minutes prior to harvesting by pipette into centrifuge tubes. Some preparations were mounted on slides in a 1:1 mixture of RP and Citifluor in phosphate-buffered saline (Marivac Ltd., Nova Scotia), others were rinsed (via centrifugation) and mounted in buffer and Citifluor. The total time in fixative varied between 5 and 23 min in different experiments but the results were similar in all variations of fixative and staining protocols, except that the longer fixative time failed to reveal the intracellular staining patterns in the zoospores for which 5 min total fixation seemed optimal.

Cytochalasin experiments were performed on hyphae grown on dialysis membrane overlying OM agar for 15 h, fastened to the membrane by agar as described above, then incubated for 2 × 1 h in DS followed by between 2.2 h and 22.2 h in DS containing cytochalasins and DMSO, all at approx. 24°C. Stock solutions contained 5 mg/ml (D) or 10 mg/ml (B and E) of cytochalasin in DMSO and were added to the DS to give the appropriate concentrations. For each cytochalasin treatment, 4-5 colonies were incubated in 10 ml of cytochalasin solution in the dark prior to fixation and RP staining. Spore sizes were measured from unfixed 22.17 h colonies observed with DIC optics and a video camera giving a screen magnification of ×1750. Median optical sections of spores in randomly picked fields of view were measured to the nearest mm with a rule from the screen. For aspherical spores the means of the maximum and minimum diameters were recorded. Diameters of empty, full but ungerminated and germinating cysts were combined because subjective observations of the separate population measurements showed no evident differences. The largest spore recorded from an un-treated population was 11.4 µm, thus the next largest size recorded (=12 µm) defined the lower limit of the 'large cyst' category.

All fluorescent observations were made with a 1.32 NA, x100 objective lens, a G2 filter cube for RP (Heath, 1988) and a V2 cube (excitation 395-446 nm, barrier 470 nm, dichroic mirror 460 nm) for mithramycin.

Developing sporangia were prepared for electron microscopy as previously described (Heath and Greenwood, 1971).

Achlya

Achlya bisexualis Coker, Q (ATCC no. 14524), was maintained on a complete nutrient agar, PYG (Kropf et al., 1984), at 22.5-24°C.

Extending hyphae and the various stages of zoosporogenesis were prepared by growing Achlya on dialysis tubing (Heath, 1987). Dialysis tubing was autoclaved in medium DMA3.2 (Schreurs et al., 1989) containing 0.4% locust bean gum.
gum (Sigma). Tubing was placed on DMA32 agar (2% w/v), (for hyphae) and PYG agar (for sporangiogenesis), and inoculated from the growing edge of a colony. After 20 h incubation, 5 x 10 mm rectangles of tubing were cut which included the edge of the hyphal mat as well as room for subsequent extension.

For extending hyphae, rectangles were placed on fresh DMA32 and 1.5 h were allowed for recovery of growth to the normal extension rate of 4-5 μm/min. To prepare the various stages of zoosporogenesis, the cut hyphal mats on rectangles were placed on SS agar (1 mM potassium PIPES buffer, pH 6.5, 0.25 mM CaCl₂, 0.25 mM KCl and 0.5-0.8% Difco agar noble), and incubated in a humid box. The rectangles were transferred to fresh SS agar after 1 h to eliminate carry-over of nutrients. Hyphae on the edge of the cut rectangle extended into liquid surrounding the dialysis tubing and released spores 5-5.5 h later. Other hyphae remained on the tubing, were subjected to some dryness and released spores 5.5 to 6.5 h later. Development of some sporangia was abnormal with 2 discharge papillae but this did not affect actin distribution.

The actin cytoskeleton was visualized using RP as for Saprolegnia. All fluorescent observations used a 1.30 NA, x100 Neofluar objective and a Zeiss No. 15 filter set (excitation 540-552 nm, barrier 590 nm, dichroic mirror 580 nm).

Results

Overview of developmental stages
The asexual life cycles of Saprolegnia and Achlya species are very similar and are summarized in Fig. 1. Within this cycle there is synchrony but diverse stages do occur together. However, the distinctive morphologies of the stages of zoosporogenesis (Gay and Greenwood, 1966) and primary and secondary zoospores permit their identification. We shall describe Saprolegnia first, followed by Achlya for comparison.

Hyphal actin
Because of the actin specificity of RP-staining (Heath, 1987), we shall refer to RP-positive material as actin. Hyphae fixed during active tip growth contained an apical cap of fine fibrils which transformed sub-apically into coarser, more widely dispersed fibrils interspersed with peripheral plaques (Fig. 2). All of these structures were exclusively peripheral, as verified by optical sectioning (Heath, 1987). The sub-apical pattern of fibrils and plaques were uniform throughout the hyphae for many mm.

Sporangial actin
Early in sporangium development, prior to cross wall formation, the apical actin caps and most coarse sub-apical actin fibrils dispersed (Figs 3, 4). Fibrils persisted longer near the apex of the sporangium and were sparsely present during papillum formation (Figs 3, 4). The dominant forms of actin in the developing sporangia were residual peripheral plaques and a new population of spots deeper in the cytoplasm (Fig. 5).

The cytoplasmic spots were initially uniformly dispersed throughout the cytoplasm (Fig. 5) but concomitantly with cross wall formation some began to cluster (Figs 6-8). In a sample of 16 sporangia fixed at about the time of cross wall insertion, 3 had no cross wall and no clusters, 2 had no cross wall and a few small clusters and 11 had cross walls and larger and more abundant clusters. Clusters typically containing about 5 spots were associated with nuclei (Figs 6-8). In paired DIC and fluorescence micrographs of 18 sporangia, 73% of 143 clusters were associated with nuclei-like structures in the DIC images (e.g. Figs 6, 7). The remaining 27% may have been associated with nuclei above or below the focal plane because through-focus series were not taken. Most clusters lay between the nuclei and the...
plasmalemma, rather than on the inner side of the nuclei. The cytoplasmic spots, both clustered and dispersed, persisted throughout sporangium development until just prior to cytoplasmic cleavage. They were also stained by Bodipy - phallacidin but not by either Nile Red or Rhodamine B, both of which showed rather
Fig. 2. DIC (A) and fluorescence (B) images of a typical subapical (> 100 μm from tip) region of a RP-stained vegetative hypha. The optical plane of section is at the cell surface such that the peripheral actin plaques and prominent fibrils are clear (B).

Figs 3 and 4. Paired images of two young sporangia with the DIC images (A) focussed at the centre of the sporangia to show their early stages of development and the fluorescent images (B) focussed at the cell surface to show the abundant actin plaques and the almost complete absence of the fibrils (cf. Fig. 2). These sporangia appear to be at early stages of papillum formation (complete papillae are seen in Figs 12-14) as shown by the homogeneous cytoplasm in the slight apical protrusions. Note the greater abundance of residual actin filaments in these regions (arrow).

Fig. 5. An early stage in sporangium development. Both DIC (A) and RP (B) images are focussed at the centre of the sporangium. Cytoplasmic spots dispersed throughout the cytoplasm are evident in B.

Fig. 6. An unusually small sporangium at a later stage of development than that in Fig. 5. Dispersed cytoplasmic spots permeate the cytoplasm but three prominent clusters in B can be correlated with DIC images of nuclei (arrows) in A.

Fig. 7. A sporangium at a comparable stage of development to that shown in Fig. 6. Peripheral plaques (brackets, B) and dispersed cytoplasmic spots are present with numerous clusters predominantly located towards the cell periphery, adjacent to the DIC images of nuclei (arrows in A and B).

Fig. 8. A sporangium at a slightly earlier stage than those shown in Figs 6 and 7 showing parfocal images stained with mithramycin (A) for nuclei and RP (B). Whilst most cytoplasmic spots are still dispersed, some are clustered with nuclei (arrows in B).

weak (Nile Red) or very bright (Rhodamine B) diffuse cytoplasmic staining.

In order to identify the cytoplasmic spots in pre-cleavage sporangia, we compared the numbers and distribution of actin spots and organelles. There were 125 (mean = 124.5 ± 17.2, n = 4) spots per median optical section per sporangium. A median longitudinal section of a similar sporangium prepared for electron microscopy showed 290 dispersed dense bodies (Fig. 10) (Gay and Greenwood, 1966), 8 clustered K bodies (Fig. 9) (Heath and Greenwood, 1971; Holloway and Heath, 1977a), 241 dispersed bars (Fig. 9) (Gay and Greenwood, 1966) and 70 unidentified and previously unreported dispersed “dark vesicles” (Fig. 10). The latter were characterized by a moderately stained lumen and apparently radially arranged material on their surfaces (Fig. 10).

Septa judged to be undergoing synthesis, based on the stage of development of the cytoplasm, were associated with a high concentration of peripheral plaques and diffuse cytoplasmic actin (Fig. 11). The plaques persisted until all plaques were lost late in sporogenesis but the diffuse actin dispersed rapidly.

One of the most conspicuous arrays of actin in developing sporangia was associated with cytoplasmic cleavage. As the cleavage vacuoles began to outline incipient zoospores, their membranes associated with sheet-like arrays of actin which formed polygonal patterns in optical section (Figs 12-15). These arrays initially co-existed with the peripheral plaques and cytoplasmic spots (Figs 12, 13) but later the cleavage actin dominated (Figs 14, 15). The nearly cleaved zoospores were permeated by a low concentration of diffuse actin which was heterogeneous as if excluded from organelles (Fig. 15). This diffuse cytoplasmic actin persisted after cytokinesis (Fig. 16), but the actin formerly associated with the cleavage vacuole membranes (which became part of the zoospore plasmalemma) was lost (Fig. 16). The cleaved, pre-release zoospores each contained a single hollow sphere of actin (Fig. 16).

Throughout zoosporulation, the organization of actin elsewhere in the colonies, including in the sporangium-bearing hyphae, remained unchanged.

Primary zoospores

Released zoospores retained their diffuse cytoplasmic actin (Figs 17, 18) but the spheres of actin (Fig. 17) were absent. However, similarly sized regions devoid of cytoplasmic actin coincided with water expulsion vacuoles (wev) in DIC images (Fig. 17). The plasmalemmata of released zoospores bore uniformly distributed fine spots of much lower intensity and size than the peripheral plaques found in walled cells. (Figs 17, 18). There was also a low level of stain in the flagella (Fig. 18).

Primary cysts

Primary cysts contained dramatically more actin than zoospores as shown by their relative fluorescence in a single field of view (cf. Figs 21, 25). This actin formed peripheral plaques, which were always present (Figs 19, 20), and an eccentric cluster of about 5 similarly sized internal spots (Fig. 19), which were not always present (Fig. 20). Younger cysts (with colonies in DS for shorter times) more frequently lacked the clustered spots than cysts from older colonies. Serial optical sections through 15 cysts (e.g. Figs 19, 20) revealed 69.9 ± 8.7 peripheral plaques per cyst.

During excystment of secondary zoospores, the cytoplasm contained a low level of diffuse actin with small punctate regions (Fig. 21). No actin remained in the empty cysts (Figs 21, 25).

Secondary zoospores

The level of staining in secondary zoospores was consistently lower than in either primary or secondary cysts (Figs 22, 25, 28). However, all secondary zoospores had diffuse cytoplasmic actin with inhomogeneities indicating exclusion from unidentified subcellular compartments (e.g. Figs 23, 24) and about half had fine spots on the plasmalemma (Fig. 25). Spores fixed for more than 5 min showed no other staining, but shorter fixations consistently showed strong staining around the wev and a linear structure running along the groove towards the opposite end of the cell (Figs 22-25). The wev is located on one side in the anterior of the cell...
(Holloway and Heath, 1977b). Thus the linear structure was posterior and in 14 out of 18 spores it lay in the opposite side of the cell. It appeared either as a line of 4 or 5 rather irregular dots interconnected with less intensely stained material (Fig. 22), or as a simple linear structure (Figs 23-25).

Fig. 9. Portion of the cytoplasm from a developing sporangium, showing K bodies with fibrillar contents and undulating membrane (k), bars (b), a dense body (db), a nucleus (N), kinetosomes (K) and associated microtubular roots (r). Bar, 1 μm.

Fig. 10. Another portion of cytoplasm from the sporangium shown in Fig. 9, showing dense bodies (db) and dark vesicles with amorphous contents and smooth membranes (V) and apparently radially arranged material on their surfaces (arrows). Bar, 1 μm.

Fig. 11. Basal region of a developing sporangium judged by its cytoplasmic appearance (A) to be close to the time of septum synthesis. The RP image was exposed to highlight the high concentration of actin associated with the septum (arrow, B). × 562.

Fig. 12. Sporangium at a later stage than those shown in Figs 5-8, as indicated by the mature apical papillum (large arrow, A). In the images near the surface (A, B) peripheral plaques (brackets, B) and cytoplasmic spots clustered with nuclei (small arrows, A and B) are evident but less intensely stained than comparable structures in Figs 5-7. Actin is beginning to be associated with the cleavage vacuoles (double arrows in A and B) and is more clearly seen as the somewhat diffuse polygonal arrays in the deeper focal plane in C (arrows).

Fig. 13. Sporangium at a comparable stage of development to that in Fig. 12. Cleavage vacuoles, with associated actin, are clear between nucleate (N) masses of cytoplasm (arrows A and B). Peripheral plaques are still evident in B but cytoplasmic spots are less prominent.
Fig. 14. Sporangium at a stage inferred to be later than those in Figs 12 and 13. All cytoplasmic spots and peripheral plaques are absent (B) and the cleavage-associated polygonal arrays are sharper than in the earlier stages (B), although the cleavage vacuoles themselves are not well contrasted in the DIC image (A).

Fig. 15. Sporangium at a comparable stage to Fig. 14 but with much more clearly detectable cleavage vacuoles in the DIC image (arrows, A). Actin is associated with the cleavage vacuoles (arrows, B). The difference in clarity of the cleavage vacuoles in the DIC images between Figs 14A and 15A may be related to unexplained fixation-induced cytoplasmic shrinkage, since such is evident in Fig. 15. Note the diffuse but inhomogeneous staining permeating the cytoplasm of the incipient zoospores in B. Note also the abundant peripheral plaques and fibrils in the hypha lying across the sporangium in B.

Fig. 16. Post-cleavage sporangium. The primary zoospores have begun to assume their pyriform shape (e.g. arrows in A) and flagella profiles are detectable (circled in A). The image in B is from a slightly different focal plane and shows diffuse, but inhomogeneous, cytoplasmic staining and spherical concentrations of actin, some of which can be correlated with "depressions" in the DIC image, which most likely represent water expulsion vacuoles (arrows in B, correlate to A).

Secondary cysts
Older cultures contained many unstained empty cysts (presumably primaries) and full ones (Fig. 25). We assume that the latter were secondary cysts. Their staining pattern was similar to that of primary cysts with similar presence and absence of the eccentrically located clusters of cytoplasmic spots (Fig. 25). The only difference was that these clusters were typically larger than in primary cysts (compare Figs 19 and 25) with as many as 20 spots detected per cluster.
Germinating cysts

Germinating cysts initially looked very similar to cysts, showing peripheral plaques with and without a cluster of cytoplasmic spots. However, from the earliest sign of germ tube (or exit tube) formation, they developed a fine peripheral array of filaments enclosing the germ tube apex (Figs 26, 27). In spite of an intensive search, this array, which is comparable to the apical cap of hyphae, was never found in cysts which lacked a protuberance of the wall, i.e., an incipient germ tube. As the germ tube elongated, the peripheral plaques in the cyst persisted and an apical cap and sub-apical plaques and cables typical of hyphae developed in the germ tube (Fig. 28).

Achlya actin behaviour

The behaviour of actin during active tip growth and zoosporogenesis in Achlya was similar to that in Saprolegnia. Growing hyphae contained an apical cap and sub-apical peripheral plaques and cables (Fig. 29). The cap and cables were lost early in sporangium formation and replaced by cytoplasmic spots, both dispersed and clustered (Fig. 30). Actin concentrated around the developing septa of the sporangia (Fig. 31). During cleavage, actin associated with the cleavage vacuoles, forming polygonal boundaries; the cytoplasmic spots were barely detectable (Fig. 32). After cleavage, the cleavage actin became less prominent (Fig. 33) and the incipient zoospores contained diffuse cytoplasmic actin and, contrary to Saprolegnia, clustered cytoplasmic spots (Fig. 34). This pattern persisted during zoospore release (Fig. 35). Primary cysts contained much more stained actin than the zoospores, mostly in the form of peripheral plaques (Figs 36, 37) with some cysts containing clustered cytoplasmic spots (Fig. 37).

Cytochalasin treatments

The effects of cytochalasins were determined for Saprolegnia only. Cytochalasins D and E (but not B at up to 25 μg/ml) caused dose-dependent and stage-specific changes in both actin patterns and development.

Cytochalasin D at 5 μg/ml caused no obvious morphological effects at any stage of the life cycle but sporangia did appear a little shorter and wider than normal (cf. Fig. 40). However, there were two transient changes in actin staining unique to this treatment. After 2.7 h in the drug (= 4.7 h in DS), sub-sporangial hyphae lost all fibril staining, and only the peripheral plaques remained (Fig. 38), but by 5.2 h in the drug all hyphae again showed normal staining patterns (Fig. 39). Similarly, in sporangia, after cross wall maturation and prior to cleavage, staining was very light or totally absent (Fig. 40). Adjacent sporangia at earlier and later developmental stages, and subtending hyphae, all stained strongly and normally.

Cytochalasin D (10 μg/ml) also induced shorter and wider sporangia as well as more abundant intercalary sporangia. Hyphal fibrils did not stain throughout the experiments (Fig. 41) but hyphal plaques appeared more abundant than in controls (compare Fig. 41 with Fig. 2). Cleavage vacuole membranes did not stain, but pre-release zoospores showed normal diffuse cytoplasmic actin while lacking the peri-wev staining. Primary cysts showed normal staining but more appeared unstained. Many of the cysts were larger (mean diameter 12.67 ± 3.44 μm, n = 50; largest cyst = 20.5 μm; % large cysts = 48.0) and more irregular (cf. Figs 43, 44) compared with the remarkably uniform control populations (Fig. 42: mean diameter 9.83 ± 0.81 μm, n = 56; largest cyst = 11.4 μm; % large = 0). In contrast, germ tubes through which secondary zoospores were released in the drug were of normal size and shape, even on enlarged cysts (Fig. 43).

Increasing the concentration of cytochalasin D to 20 μg/ml elicited similar but more dramatic effects than observed with 10 μg/ml. Again, enlarged cysts (mean diameter 12.80 ± 3.34 μm, n = 72; largest cyst = 22.8 μm; % large cysts = 48.6), with normal germ tubes, were produced but many fewer sporangia and spores formed (Fig. 45). The cytoplasm in most hyphae was abnormally clumped and deformed, suggesting general toxicity.

Cytochalasin E at 5 μg/ml produced comparable results to cytochalasin D at 10 μg/ml. Terminal sporangia were apparently shorter and wider, intercalary sporangia were abundant and hyphae showed a high density of peripheral actin plaques but no fibrils throughout the incubation period. Spore production and cyst sizes were normal, as were the actin patterns throughout zoosporulation and in the cysts. Only the peri-wev actin in the pre-release primary zoospores was not observed.

At 10 μg/ml, cytochalasin E totally suppressed sporulation (Fig. 46). Hyphal tips swelled but probably only prior to addition of the drug. No cross walls, papillae or zoospores formed. After 2.7 h in the drug, the swollen hyphal tips (incipient sporangia) contained peripheral actin plaques and a few isolated cytoplasmic spots and the subtending hyphae had abundant plaques but no fibrils (Fig. 47). After 5.2 h in the drug, the aborted sporangia appeared dead. In subtending hyphae actin formed large aggregates and needle shaped structures (Fig. 48).

Discussion

The present observations indicate unexpected new functions for actin during cytokinesis and zoospore morphogenesis and in the structure of the mature zoospores. They also clarify and reinforce previously suggested functions in hyphal growth.

Cytokinesis

Actin forms spectacular polygonal arrays coincident with cleavage vacuoles. The formation of these arrays during cleavage, their assembly at the end of cytokinesis, and the induction of abnormally large zoospores (inferred from the large cysts) by actin-disrupting cytochalasin treatments, strongly implicate them as a...
In *Saprolegnia*, cytokinesis is effected by directed vacuole expansion (Gay and Greenwood, 1966; Gay et al., 1971) guided, at least in part, by kinetosome-vacuole expansion (Gay and Greenwood, 1966; Gay et al., 1971). The morphology of the sheet-like arrays of actin associated with the cleavage vacuoles is most consistent with a role in directing their (presumably) osmotically driven expansion by appropriately localized differential expansions and/or contractions. This role differs in detail from the well known contractile ring operating in animal cell cytokinesis (see Schroeder, 1975) but both must involve controlled actin-based force generation and membrane linkages. It differs more significantly from aiding vesicle translocations in plant phragmoplasts (Kakimoto and Shibata, 1987; Schmit and Lambert, 1987, 1990). However, it is directly applicable to the recently re-described vesicle expansion-based cytokinetic apparatus in the zoosporangia of another oomycete, *Phytophthora cinnamomi* (Hyde et al., 1991), a genus in which zoospore cleavage sensitivity to cytochalasins has previously been described (Oertel and Jelke, 1986). Actin involvement in oomycete cytokinesis fits the emerging generality that cytokinesis in all systems apparently involves actin interacting with cleavage membranes (Hepler and Bonsignore, 1990; Wick, 1991).

**Organelle arrangements during zoospore development**

Punctate actin is widely reported in diverse fungi (Heath, 1990b), but, like the peripheral plaques in *Saprolegnia* and *Achlya* hyphae, it is consistently located exclusively in the peripheral cytoplasm, adjacent to the plasmalemma. The cytoplasmic spots formed in the sporangia are a novel arrangement for fungal actin. They look similar to the RP-positive vesicles which Grolig et al., (1990) showed to be rhodamine, not phalloidin, positive in *Mougeotia*. Such is not the case in the sporangia because neither Nile Red nor Rhodamine B stained the cytoplasmic spots, yet both stained the vesicles of *Mougeotia* (Grolig et al., 1990). Furthermore, Bodipy-phallacidin gave identical staining to RP, yet its fluorophore is anionic, in contrast to cationic rhodamine, and thus unlikely to react in a similar way with any specific cytoplasmic organelle. We conclude that the cytoplasmic spots are indeed actin-rich.
Because neither conventional fixation nor freeze substitution adequately preserves actin in electron microscopic preparations of *Saprolegnia* (Heath and Kaminskyj, 1989), it is not possible to identify directly the ultrastructural equivalents of the cytoplasmic spots. Consequently, we used a correlative approach. Organelles preexisting in the hyphal cytoplasm can be ruled out because their morphology, abundance and distribution do not match the cytoplasmic spots (Heath and Greenwood, 1971). The newly formed dense bodies and bars (Gay and Greenwood, 1966; Gay et al., 1971; Heath and Greenwood, 1970) are too numerous and incorrectly distributed. Only K bodies (Heath and Greenwood, 1971; Holloway and Heath, 1977a; Lehn and Powell, 1989, 1991) coincide in both abundance and distribution with the cytoplasmic spots clustered with the pre-cleavage nuclei and only the previously unreported “dark vesicles” (Fig. 10, cf. similar vesicles in pollen tubes (Kappler et al., 1986)) correlate with the dispersed spots. The clustered spots in some of the
primary and secondary cysts also correlate with the known behaviour of K bodies (Holloway and Heath, 1977a; Lehnen and Powell, 1989, 1991). We speculate that the cytoplasmic spots are, in fact, actin filaments attached to the surface of the K bodies and “dark vesicles” and that these filaments may be artefactually concentrated during fixation (possibly yielding the radial material on the vesicle surfaces) by collapsing to their attachment points from a more diffuse actin network. Such a diffuse network may be a precursor to that seen later in the zoospores. Organelle-associated actin with possible roles in organelle positioning and morphogenesis is becoming a widely reported phenomenon (Abe et al., 1991; Henderson and Locke, 1992a and b; Bourett and Howard, 1992) which may also relate to previously described fungal cytoplasmic matrices (McKerracher and Heath, 1986, 1987).

**Actin in the motile cell**

Studies of flagellate cells have concentrated on organelles or microtubules. Actin has been largely ignored. Nevertheless some cells use actin filaments to anchor and orientate flagella (Kleine and Clark, 1980; Uyeda and Furuya, 1987, 1989), to produce specialized cellular processes (Pagh and Adelman, 1982), for vesicle morphogenesis (Brugerolle and Bricheux, 1984) or for intracellular transport and cytokinesis (Cohen et al., 1984; Hirano et al., 1987; Metenier, 1984; Kersken et al., 1986a). Its expected role in the wev is controversial. Patterson (1980) suggested that the vacuole is contractile, implying a role for perivacuolar actin and myosin, yet the data are conflicting (c.f. Allen and Fok, 1988) with evidence for (Cohen et al., 1984) and against (Metenier, 1984; Kersken et al., 1986b; Cho and Fuller, 1989) the presence of actin and similarly contradictory results from the effects of inhibitors (Cohen et al., 1984; Kersken et al., 1986a). Our results, showing actin around the vacuole of both primary and secondary zoospores and the demonstration of myosin 1 around Acanthamoeba wevs (Baines and Korn, 1990), support a role for actin in wev function. Our actin labelling in the pre-released, but not released, primary zoospores suggests that it is easily dissociated during processing, possibly explaining negative results in previous studies.

The second novel concentration of zoospore actin is that associated with one of the microtubular roots. The linear actin in the end of the secondary zoospore opposite the water expulsion vacuole coincides with the octet microtubular root (Holloway and Heath, 1977b; Barr and Allan, 1985; Barr and Desaulniers, 1989; Hardham, 1987). Because the staining pattern varies from linear to interconnected dots, it may represent a site of attachment of an actin population which undergoes variable contraction. This actin is probably connected to the third novel arrangement, the diffuse array permeating the cytoplasm and outlining intracellular structures in both zoospore types. We suggest that zoospore cytoplasm is permeated by actin connected to the octet and the plasmalemma (the spots on the zoospore surface). Such an array may help generate cell shape and organelle distributions (Holloway and Heath, 1977a) as well as amoeboid excystment of the secondary zoospores (Holloway and Heath, 1977b).

In addition to the above actin arrays, zoospores must also contain high levels of G actin because there is a dramatic difference in RP-stainable (presumably F) actin (mostly peripheral plaques) between the zoospores versus cyst stages. Since encystment takes approximately 1 min (Holloway and Heath, 1974), de novo synthesis is unlikely. Because the cyst, unlike the zoospores, is supported by a cell wall, such polymerization is unlikely to relate to structural integrity. This is simply another example of the consistency of association between plaques and walled cells.

**Hyphal actin**

Growing hyphae of Saprolegnia contain an apical filamentous actin cap and sub-apical peripheral spots and cables (Heath, 1987). Similar arrays in Achlya hyphae extend the generality of this pattern among the oomycetes.

The actin cap may function in morphogenesis by...
Fig. 38. Surface focal planes of sub-sporangial hyphae from a colony incubated for 2.7 h in DS containing 5 μg/ml cytochalasin D. Peripheral plaques are present but the normal fibrils are absent (cf. Figs 2 and 39).

Fig. 39. Surface focal plane of a sub-sporangial hypha from a colony incubated in DS containing 5 μg/ml cytochalasin D for 5.2 h. Plaques and fibrils are present in normal abundance.

Fig. 40. Typical sporangium from the same colony as Fig. 38. The RP image (B) is exposed such that the actin in the hypha below the cross wall and an adjacent hypha is grossly overexposed (cf. Figs 38, 39) yet virtually no staining is detectable in the sporangium.

Fig. 41. Surface focal plane of a hypha incubated in 10 μg/ml cytochalasin D for 2.7 h, showing increased abundance of plaques and absence of fibrils.

Fig. 42. Empty cysts from a normal colony. Note uniform diameter. A full cyst (c) bears a germ tube. Other germ tubes are detectable but out of focus on the empty cysts (arrows).

Figs 43 and 44. Enlarged and irregular cysts bearing normal sized germ tubes from a colony incubated in 20 μg/ml cytochalasin D for 22.2 h.

Fig. 45. Margin of a colony incubated in 20 μg/ml cytochalasin D for 22.2 h. Rather few spores or empty sporangia are present but hyphal tips are swollen and bear undischarged sporangia. Bar, 100 μm.

Fig. 46. Margin of a colony incubated in 10 μg/ml cytochalasin E for 22.2 h. No spores or empty sporangia are seen and the hyphal tips are distorted. Bar, 100 μm.

controlling apical expansion (Jackson and Heath, 1990a) but could also generate or maintain factors essential to establishing polarity (Heath, 1990b). During cyst germination, a non-polarized cell becomes polarized and produces a germ tube or exit tube. If actin caps establish polarity they should form before germ tube emergence. They did not. They only formed when the cyst wall had been softened to permit localized expansion, consistent with the morphogenic role (Jackson and Heath, 1990a). However, the formation of normal exit tubes on enlarged cysts in cytochalasins is contrary to this suggestion. Jackson and Heath (1990a) showed that 20-50 μg/ml of cytochalasin E was needed to disrupt hyphal actin caps, compared with the 10 μg/ml which totally suppressed zoosporulation. It seems likely that the actin caps in both hyphae and exit tubes are more resistant to cytochalasins than the cytokinesis-associated actin, possibly due to differing actin binding proteins (Cooper, 1987).

The sub-apical actin cables in hyphae parallel the long axes of both the hyphae and the elongated mitochondria and nuclei. These organelles lose their elongated shape and orientation along the hyphae (Heath and Greenwood, 1971) at the same time as cables are lost in the developing sporangia. This correlation is consistent with a role for the cables in generating organelle shape and orientation and is the first indication of their possible function.

Our results also help explain the role of the peripheral actin plaques. Because they form so abundantly when new cell walls develop at encystment (cf. also Temperli et al., 1990), it may be inferred that they function in wall synthesis. However, their abundance in sub-apical regions of hyphae, where wall synthesis is minimal (Fevre and Rougier, 1982), and absence in growing tips (Heath, 1987; Fig. 29), where synthesis is...
Actin-cytochalasin-phalloidin interactions

Tang et al. (1989) questioned the value of RP staining in because filasomes are unknown in oomycetes (Heath et al., 1989). However, our observations of cytochalasin-induced rearrangements of RP staining patterns, show the technique is indeed informative. For example, the transient loss of actin bundles, but not plaques in the hyphae, in the continued presence of the drug (5 μg/ml cytochalasin D) shows that cells can accommodate to cytochalasins. This, and the correlated cable loss and plaque increase (cytochalasin D and 5 μg/ml cytochalasin E) (cf. Schliwa, 1982) and crystal-like aggregate formation (10 μg/ml cytochalasin E), show that cytochalasin effects cannot be explained by a simple F-actin capping model. Conversely, the transient lack of all RP staining in developing sporangia (5 μg/ml cytochalasin D) suggests a transient cytochalasin-induced change in fixation permeabilization of the cell membrane or wall.

The present study has suggested that actin plays previously unrecognized roles in the asexual zospore life cycle of the aquatic organisms, Saprolegnia ferax and Achlya bisexualis. Many of these roles are relevant to our understanding of general aspects of cell biology and open up new avenues of investigation in terms of cytoplasmic mechanics. They also reinforce the idea that these organisms are well suited to further studies of cell development and function.

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