Development 126, 201-209 (1999)
Printed in Great Britain © The Company of Biologists Limited 1998
DEV0199

F-actin marks the rhizoid pole in living *Pelvetia compressa* zygotes

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Accepted 16 October; published on WWW 3 December 1998

SUMMARY

Spatial and temporal changes in F-actin during polarity establishment in *Pelvetia compressa* zygotes were investigated using vital staining with rhodamine phalloidin (RP). F-actin was localized to a patch in the cortex of young zygotes. When unilateral light was applied to induce a growth axis (photopolarization) in a population of zygotes, the cortical F-actin patches localized at the shaded pole (rhizoid pole of growth axis). Treatments that prevented photopolarization prevented localization of F-actin patches to the shaded pole. When the direction of the light treatment was reversed, the previous growth axis was abandoned and a new axis was established in the opposite direction. The F-actin patch repositioned to the new rhizoid pole within minutes of light reversal, indicating that F-actin was an immediate marker of the nascent growth axis. Repositioning probably occurred by disassembly of the initial patch and reassembly of a new one. The patch grew in size as zygotes developed, eventually becoming a ring just prior to rhizoid outgrowth. The rhizoid emerged at the site of the F-actin ring and, following germination, the ring was located in the subapical zone of the elongating tip.

Key words: Localized F-actin, Polarity establishment, Vital staining, *Pelvetia compressa*, Zygote polarity

INTRODUCTION

Zygotes of fucoid algae are model organisms for investigations aimed at understanding the establishment of initial developmental polarity. In most organisms, establishment of a primary axis (e.g. animal-vegetal axis in animals (Gilbert, 1988), micropyle-chalazal polarity in plants (Russell, 1993)) occurs during egg cell formation. In higher plants, the developing egg is enclosed within many layers of tissue, making polarity establishment difficult to investigate. In contrast, eggs of *Fucus sp.* and *Pelvetia compressa* are released in large numbers into seawater and are radially symmetrical cells without discernible axes. Following fertilization, fucoid zygotes establish a primary growth axis that determines the site at which a rhizoid will grow out from the cell (Kropf, 1997). Because zygotes sense vectorial cues in the intertidal environment when choosing this growth axis, axis orientation can be easily manipulated experimentally. For example, when zygotes are exposed to unilateral light, a commonly encountered vector in the intertidal environment, the rhizoid pole of the growth axis forms on the shaded side of the zygote. This process is known as photopolarization. The axis remains labile and its orientation can be changed by changing the direction of environmental vectors until approximately 8-10 hours after fertilization (AF). Shortly thereafter rhizoid outgrowth commences. The first cell division (approximately 1 day AF) is an asymmetric cleavage bisecting the growth axis and producing rhizoid and thallus cells of distinct morphology and cell fate (Fowler and Quatrano, 1995).

F-actin appears to play a fundamental role in several aspects of early development in fucoid zygotes, as indicated by experiments using agents that disrupt actin filaments (cytochalasins B or D, latrunculin B). In the presence of these agents, zygotes fail to photopolarize and, following drug removal, rhizoids emerge in random orientations with respect to the light vector (Quatrano, 1973; Love et al., 1997). Secretion (Hable and Kropf, 1998), dihydropyridine receptors (Shaw and Quatrano, 1996a), sulfated fucans (Novotny and Forman, 1974), and inward electrical current (Brawley and Robinson, 1985), all of which normally localize at the rhizoid pole, fail to localize in treated zygotes. Zygotes undergo multiple rounds of mitosis, but rhizoid outgrowth and cytokinesis are inhibited; chronic treatment therefore results in a spherical, multinucleate cell without detectable polarity (Bisgrove and Kropf, 1998).

In many cells, the distribution of F-actin reflects the inherent cellular polarity (Fowler and Quatrano, 1997; Gupta and Heath, 1997; Heslop-Harrison and Heslop-Harrison, 1992), but in polarizing fucoid zygotes F-actin has been reported to be uniformly distributed throughout the cellular cortex (Brawley and Robinson, 1985; Kropf et al., 1989; Shaw and Quatrano, 1996b). Asymmetry in the F-actin array has not been detected until just before rhizoid outgrowth when F-actin concentrates in a cortical cap at the rhizoid pole. In *P. compressa*, this localization is reported to occur at 11 hours AF (Brawley and Robinson, 1985), long after photopolarization is complete (4 to 5 hours AF; Hable and Kropf, 1998). There is, however, uncertainty regarding the organization in vivo of the F-actin

array in young fucoid zygotes. All investigations to date have been done on zygotes fixed with aldehydes, and it is clear that aldehyde fixation can induce artifactual changes in F-actin arrays in plant cells (Doris and Steer, 1996; Miller et al., 1996). We have therefore reinvestigated the structure, localization and function of F-actin in young *P. compressa* zygotes, with emphasis on the process of photopolarization. Using rhodamine phalloidin (RP) to visualize F-actin in living zygotes, we report that F-actin is organized into cortical patches by 3 hours AF, and these patches localize to the rhizoid pole during photopolarization. The F-actin patches are the earliest marker of developmental polarity thus far discovered.

MATERIALS AND METHODS

Plant material and culture

Sexually mature fronds of *P. compressa* (J. Agardh) De Toni were collected near Pigeon Point Lighthouse (North of Santa Cruz, CA). Receptacles were shipped cold and stored in the dark at 4°C for up to 3 weeks. To induce the release of gametes, receptacles were placed in light from above (100 μmol/m^{2}second) in artificial seawater (ASW; 0.45 M NaCl, 30 mM $MgCl_2{\cdot}6H_2O$, 16 mM $MgSO_4$, 10 mM KCl, 9 mM $CaCl_2{\cdot}2H_2O$, and 0.04 mg/ml choramphenicol buffered to pH 8.2 with 10 mM Tris base) for 4 hours or longer, rinsed with ASW and transferred to the dark for 30 minutes. Fertilization occurs as gametes are released, and the time of fertilization was considered to be 15 minutes after transfer to the dark. Zygotes were rinsed three times in ASW, with agitation, and plated on No. 1 coverslips in Petri dishes and placed in unilateral light. Zygotes attached to the coverslips by approximately 2-3 hours AF and germinated by approximately 10-12 hours AF. Unless otherwise noted, all procedures were performed at 17°C.

Vital staining of F-actin using RP

Zygotes and embryos adhered to coverslips were permeabilized by incubating with 0.01% w/v (0.1 mg/ml) saponin in ASW for 1 hour in the dark. Zygotes were then rinsed in saponin-free ASW and labeled with RP, a fluorescent probe that specifically binds F-actin (Drubin et al., 1993), by incubating a coverslip with zygotes in 45 μl of 6.6×10^{-7} M RP. A stock solution of 6.6×10^{-6} M RP was prepared in methanol and stored at –20°C. Prior to staining, the RP was desiccated and reconstituted in ASW to produce a working solution of 6.6×10^{-7} M. Cells were incubated in RP for at least 1 hour. Immediately prior to microscopic observations, cells were rinsed for 10 minutes in fresh ASW. Controls for the specificity of RP binding were conducted by incubating saponin-permeabilized living cells with an excess of unlabeled phallacidin (2×10^{-6} M) in ASW for 2 hours prior to staining with RP. Phallacidin has higher specificity than RP for F-actin and therefore should greatly reduce or eliminate RP staining. All procedures involving staining for F-actin were done at 17°C and staining took place in darkness. Viability of labeled zygotes was assessed by analyzing the percentage of cells that germinated.

The vital staining procedure described above required approximately 2-3 hours to complete. In order to stain cells at shorter intervals, the saponin treatment was shortened to 10 minutes and the staining was done with 1.2×10^{-6} M RP for 10 minutes. Since zygotes rarely germinated after staining with this high RP concentration, this protocol was considered non-vital.

Methanol fixation and immunolocalization of F-actin

Four, 6- or 8-hour-old zygotes were fixed in methanol at –80°C for 1 or 5 minutes. The methanol was replaced with fresh –80°C methanol every minute. The zygotes were then stained with RP by inverting coverslips over a drop of 1.3×10^{-6} M RP in ASW as described above. Immunolocalization of F-actin in methanol-fixed zygotes was carried out as previously described (Bisgrove and Kropf, 1998) using a mouse monoclonal anti-actin antibody raised against chicken gizzard actin (Clone C4; ICN Biomedicals, Aurora, OH), at a dilution of 1:100 in modified PBS (mPBS; 137 mM NaCl, 2.7 mM KCl, 1.7 mM KH_2PO_4, 8 mM Na_2HPO_4, 5% glycerol, 0.1% sodium azide, 0.1% bovine serum albumin) for 2 hours at room temperature. Zygotes were then rinsed in mPBS and incubated in rhodamine-conjugated goat anti-mouse antibody (Cappel, Durham, NC) at a dilution of 1:100 for 2 hours. Cells were rinsed and mounted in mPBS prior to observation.

Microscopy

Living RP-labeled zygotes were observed on an Olympus IMT2 inverted microscope equipped with epifluorescence using a narrow bandpass emission filter (605±27 nm band width filter; Chroma Technology, Brattleboro, VT). Although zygotes survived repeated imaging in epifluorescence, image quality was poor. In contrast, laser scanning confocal microscopy (LSCM) produced better images, but often damaged zygotes. For LSCM, zygotes labeled with RP or rhodamine-conjugated antibodies were examined using the Bio-Rad MRC-600 (Bio-Rad Inc., Richmond, CA) attached to a Nikon Optiphot. The 568 nm laser line was used with a 580 nm dichroic mirror and a 598±20 nm narrow bandpass emission filter (Chroma Technology Corp., Brattleboro, VT). An additional filter (580±16 nm; Pomfret Research Optics Inc., Orange, VA) was inserted in the optical path to reduce chloroplast autofluorescence. Z-series were collected at 0.5 μm or 1.0 μm steps using a linear signal output (non-enhanced). Laser irradiation often damaged zygotes and LSCM was therefore used primarily to collect representative images of F-actin staining.

Photolocalization of F-actin

To investigate the spatial relationship of F-actin and the growth axis, zygotes were plated onto glass coverslips and placed in unilateral light approximately 15 minutes AF. In order to determine the position of F-actin with respect to the shaded (rhizoid) pole, a coverslip was removed at the time of interest and zygotes were stained for F-actin, either vitally or non-vitally, as described above. The position of F-actin with respect to the orienting light vector was recorded as the angle θ, designated as the angle from the localized F-actin to the center of the cell to the shaded pole of the light vector. (When the patch and the shaded pole coincided spatially, θ was 0°.)

Percentage photolocalization of F-actin was calculated by subtracting the number of cells bearing F-actin localized on the lit hemisphere ($90°<|\theta|<180°$) from those containing F-actin on the shaded hemisphere ($0°<|\theta|<90°$) and dividing this number by the total number of cells scored. This quotient was then multiplied by 100 to give a percentage. Positive percentages indicated preferential localization of F-actin on the shaded hemisphere, while negative percentages indicated localization on the lit hemisphere.

To correlate the position of F-actin with the site of subsequent rhizoid outgrowth on individual cells, F-actin was stained at 6 hours AF and θ was measured as described above. The zygotes were allowed to develop and at 13 hours AF the position of rhizoid outgrowth was measured with respect to the orienting light vector; this angle was defined as ϕ.

Repositioning of F-actin

To determine whether or not F-actin repositioned to the new rhizoid pole when the direction of the orienting light was changed, zygotes were grown in unilateral light (L1) from 15 minutes AF until approximately 4 hours AF at which time the direction of the orienting light was reversed (L2). Following light reversal, zygotes were removed either every 10 minutes and non-vitally stained for F-actin or every hour and vitally stained for F-actin. In some experiments, repositioning of F-actin was correlated with photopolarization of the new growth axis. In these experiments, two coverslips containing zygotes were removed every 10 minutes following light reversal;

zygotes on one coverslip were non-vitally stained for F-actin and the other coverslip was placed in the dark to permit germination. The position of rhizoid outgrowth was used to quantitate the orientation of the growth axis at the time zygotes were moved to the dark. Photolocalization of rhizoid outgrowth (photopolarization) was calculated using the same formula as used for photolocalization of F-actin (see above).

Inhibitor studies

Pharmacological agents known to affect secretion (Brefeldin A), F-actin (latrunculin B) and microtubules (oryzalin) were applied to zygotes to observe their effects on photolocalization of F-actin. Stock solutions of inhibitors were prepared as follows: Brefeldin A, 2.5 mg/ml in ethanol; latrunculin B, 50 μM in DMSO; oryzalin, 10 mM in DMSO. Stock solutions were diluted to appropriate concentrations in ASW. In all experiments, the final solvent concentration was less than 0.1% and had no significant effect on development or F-actin localization.

To assess the effects of Brefeldin A, which prevents vesicle secretion and photopolarization in fucoid zygotes (Shaw and Quatrano, 1996b; Hable and Kropf, 1998), on photolocalization of F-actin, zygotes were plated on poly-L-lysine coated coverslips (175 μl of a 50 μg/ml solution was allowed to dry on 18 mm squares) and treated with 2.5 μg/ml Brefeldin A in unilateral light from 1 hour AF. At 4, 5 and 6 hours AF, coverslips with adhered zygotes were removed, rinsed in ASW for at least 10 minutes, and zygotes were stained using the vital staining protocol for F-actin. Germination was not inhibited in zygotes recovering from Brefeldin A treatment.

The effects of cytoskeletal disruption on F-actin was investigated by treating cells with latrunculin B, which disrupts F-actin function (Gupta and Heath, 1997; Love et al., 1997; Hable and Kropf, 1998), or oryzalin, which depolymerizes microtubules in *P. compressa* (Bisgrove and Kropf, 1998). Latrunculin B was applied at 10 nM and 30 nM to zygotes approximately 4 hours or 12 hours AF. Zygotes were stained following 1-6 hours of treatment. Zygotes were treated with 1 or 5 μM oryzalin from 2 to 6 hours AF or from 12 to 16 hours AF, respectively. Following treatments, zygotes were stained for F-actin using the vital staining protocol.

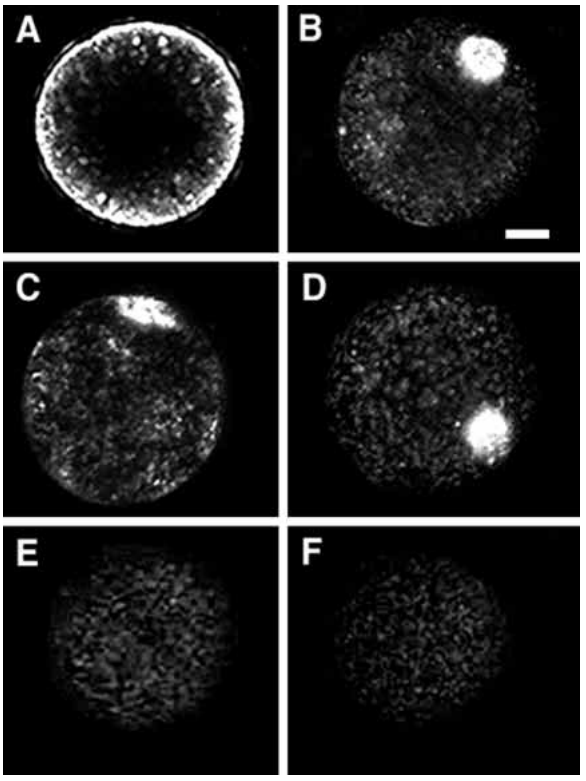

Fig. 1. F-actin is localized as a patch in young zygotes. Following aldehyde fixation, F-actin is uniformly distributed throughout the cortex (A). In contrast, zygotes fixed in methanol and stained with either RP (B) or anti-actin antibodies (C) show a localized patch of F-actin, as do living zygotes permeabilized with saponin and stained with RP (D). Pretreatment with excess phallacidin (2×10^{-6} M) eliminates all RP staining (E) in living cells, as does pretreatment with 10 nM latrunculin B (F). The punctate fluorescence throughout the cells is due to chloroplast autofluorescence. All images are projections of serial confocal sections. Bar, 25 μm.

RESULTS

F-actin is organized into cortical patches and rings in young zygotes

F-actin was uniformly distributed throughout the cortex of recently fertilized eggs and young zygotes that were preserved using conventional aldehyde fixation (Fig. 1A). Both diffuse and punctate labeling were observed within individual cells. In contrast, F-actin was localized to a cortical patch in zygotes preserved without aldehydes. Following methanol fixation, F-actin patches were observed in zygotes labeled with RP (Fig. 1B) or with anti-actin antibodies (Fig. 1C).

To investigate F-actin in living cells, zygotes were permeabilized with 0.01% saponin (Meiners et al., 1991) and labeled with RP. This treatment did not inhibit subsequent germination of the zygotes (Fig. 2), nor did it slow development noticeably (data not shown). F-actin was also organized into cortical patches in living zygotes (Fig. 1D), and pretreatment with phallacidin as a control for specificity eliminated all labeling (Fig. 1E). Treatment with 10 nM latrunculin B for 1 hour, which disrupts F-actin arrays (Hable and Kropf, 1998), completely eliminated subsequent labeling of zygotes with RP (Fig. 1F). Moreover, the patch disappeared in zygotes stained with RP and then treated with latrunculin B (data not shown).

In both methanol-fixed and living zygotes, the percentage of cells that labeled was highly variable, ranging from 5% to 75%. Most labeled cells had a single F-actin patch, but multiple patches were observed in up to 15% of labeled zygotes. All subsequent experiments were conducted with saponin-permeabilized zygotes labeled with RP.

Changes in the F-actin array during development were examined in living zygotes. The patch of F-actin was small (<15 μm in diameter) in a 3-hour-old zygote (Fig. 3A), and increased to greater than 15 μm in diameter by 8 hours AF (Fig. 3B). The patch resolved into a cortical ring just prior to germination (Fig. 3C). The transition from small to large patch occurred between 6 and 8 hours AF, and from large patch to ring between 8 and 10 hours AF (Fig. 4). In germinated zygotes, the ring was located subapically in the rhizoid, usually 5-10 μm behind the elongating tip. The ring appeared loosely organized in a recently germinated zygote 10 hours AF (Fig. 3D), but became more tightly focused by 14 hours AF (Fig. 3E). Patches and rings were observed in dark-grown zygotes (Fig. 3A-C) and in zygotes photopolarized with unilateral light (Fig. 3D,E). Zygotes polarized by the presence of a neighbor (group effect) also had subapical rings (Fig. 3F).

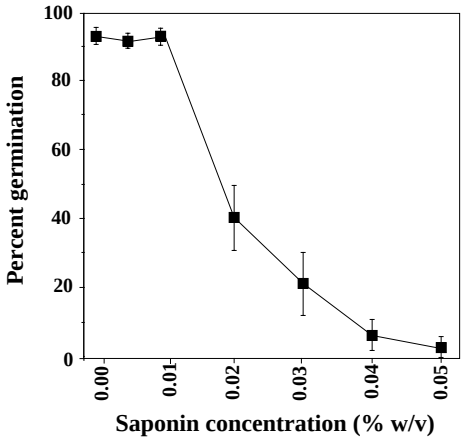

Fig. 2. Germination of zygotes following treatment with saponin of varying concentrations and vital staining with RP. Cells were incubated in ASW-saponin for 1 hour from 4 to 5 hours AF and stained for one hour with RP. All incubations took place in total darkness. Bars are s.e.m. *n*=450 cells in 5 replicate experiments.

Several F-actin arrays were present in the apical rhizoid cell of multicellular embryos. Cortical F-actin was present at the previous crosswall, and F-actin cables extended longitudinally from this array to the subapical region containing the ring (Fig. 3G). The F-actin ring was present only in the tip-growing apical cell of the rhizoid, and not in other embryonic cell types.

A fully intact microtubule cytoskeleton did not appear to be needed to maintain the organization of the F-actin arrays. Treatment with 1 μM oryzalin caused depolymerization of all conspicuous microtubules (Bisgrove and Kropf, 1998), but had little effect on F-actin patches or rings (data not shown). However, at higher concentrations of oryzalin (5 μM), the F-actin ring reorganized into focal masses in the cortex of the cell (Fig. 3H) and growth was inhibited. These disruptive effects may be related to depolymerization of a minor, but stable, microtubule population, or they may be completely unrelated to microtubule depolymerization.

Although some labeled zygotes developed following laser irradiation with a 568 nm line (see Fig. 3A and B, D and E), many zygotes were damaged, making LSCM of limited use for repeated observation of F-actin in living cells. Conventional epifluorescence microscopy was less damaging and therefore was used to investigate the spatial relationship between the patch and the growth axis.

The F-actin patch localizes to the rhizoid pole during photopolarization

The presence of an F-actin ring at the germination site suggested that F-actin may mark the rhizoid pole of the developing growth axis in young zygotes. To test this possibility, the position of the F-actin patch was monitored during photopolarization. Zygotes were grown in unilateral light beginning 15 minutes AF and stained with RP at times thereafter. F-actin patches were not consistently observed in zygotes less than 3 hours old, but at 3 hours AF many zygotes had cortical patches and the patches were nearly randomly distributed with respect to the light vector (Fig. 5A). Between

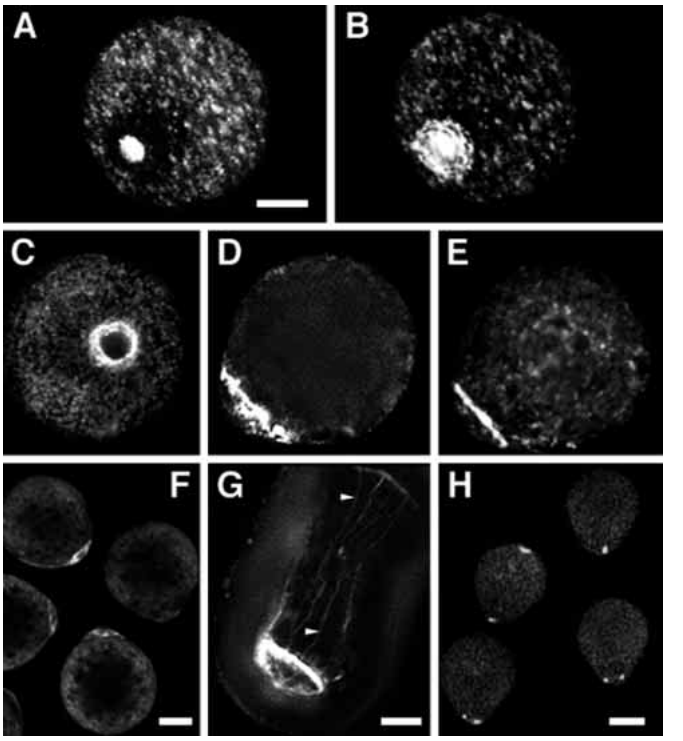

Fig. 3. Changes in the F-actin array vitally stained with RP. (A) 3 hour-old, dark-grown zygote showing a small, localized F-actin patch. (B) 5 hours later (8 hours AF) the F-actin patch has increased in diameter (same cell as in A). (C) Dark-grown zygote with a F-actin ring viewed end on at 10 hours AF. (D) Recently germinated zygote with loosely organized ring viewed edge on at approximately 10 hours AF. (E) By approximately 14 hours AF, the ring becomes sharply defined (same cell as in D). A-E, Bar, 20 μm. (F) The subapical F-actin ring is present in zygotes polarized in response to the group effect. Bar, 40 μm. (G) The ring persists in the apical cell of the rhizoid in a 5-day-old embryo. Note cables (arrowheads) extending from the cell plate toward the tip. Bar, 15 μm. (H) Treatment with 5 μM oryzalin induces focal masses of F-actin in 16-hour-old zygotes. Bar, 50 μm. All images are projections of serial confocal sections; this makes rings viewed edge-on appear as lines.

3 and 4 hours AF the F-actin patches localized to the shaded hemisphere, and by 5 hours AF the F-actin patches were on the shaded hemisphere in nearly all zygotes.

To determine whether F-actin localization to the shaded hemisphere depended on a growth axis being established in accordance with the light vector, photopolarization of the axis was inhibited by treatment with the secretory inhibitor Brefeldin A (Hable and Kropf, 1998). Zygotes treated from 1 hour AF had patches by 4 hours AF but the patches remained randomly distributed with respect to a light vector (Fig. 5B), indicating that photopolarization of the zygotes was required for photolocalizaton of F-actin patches to the shaded pole.

The spatial relationship between the orienting light vector and the F-actin patch was examined in greater detail by measuring the angle θ defined by the center of the patch, the center of the cell, and the shaded pole of the orienting light vector in 6-hour-old zygotes (Fig. 6A). The absolute value of θ was less than 25° in over 95% of zygotes, indicating that the F-actin patch was tightly localized to the rhizoid pole. In

contrast, the F-actin patch was nearly randomly positioned in zygotes grown in the dark (Fig. 6B).

If the F-actin patch marks the rhizoid pole, then the rhizoid should grow out from the patch site. To test this possibility, zygotes were grown in unilateral light until 6 hours AF, labeled with RP, and the angle θ was measured as before. The zygotes were placed in the dark and allowed to germinate and the angle ϕ, defined by the germination site, the center of the cell and the shaded pole of the orienting light vector, was measured on the same zygotes. Zygotes were well germinated at 13 hours AF, indicating that labeling protocol did not slow development. The position of the F-actin patch at 6 hours AF strongly correlated with the position of rhizoid outgrowth (Fig. 7). In most cases θ and ϕ were near 0°, but importantly, when θ varied from 0 it accurately predicted ϕ. Thus, the position of the F-actin patch predicted the site of germination more precisely than the orienting light.

F-actin repositions when the direction of orienting light is changed

The orientation of the growth axis in young zygotes is labile and can be changed in response to environmental cues until just before germination when it becomes fixed in place (Quatrano, 1973; Kropf, 1997). To determine whether the F-actin patch in young zygotes repositions to the new rhizoid pole when the orientation of the axis is changed, young zygotes were exposed to two successive unilateral light vectors from opposite directions. Zygotes were treated until 4 hours AF with unilateral light from direction 1 (L1), the direction of light was reversed 180° (L2) and zygotes were stained with RP every 10 minutes thereafter. At the time of light reversal, F-actin was localized to the shaded hemisphere of L1, and it remained there for approximately the first 40 minutes in L2 (Fig. 8A). Abruptly, between 40 and 50 minutes following light reversal, the F-actin patch repositioned to the hemisphere shaded during L2. During repositioning, a small percentage of zygotes had two F-actin patches, one at the shaded and one at the lighted pole (Fig. 8B). Patches were never observed on the flanks of the zygote, between the two poles. Repositioning of the F-actin patch in individual zygotes was also observed on approximately the same time course (*n*=3, data not shown).

Light reversal was also used to investigate the temporal relationship between photopolarization and F-actin patch localization. Every 10 minutes following light reversal, the zygotes on one coverslip were stained for F-actin and those on a second were placed in the dark overnight to permit germination. The position of rhizoid outgrowth with respect to L1 was measured on zygotes on the second coverslip, and used as a measure of axis orientation, and hence photopolarization, at the time zygotes were placed in the dark. The experiment was conducted twice, and in both cases repositioning of the F-actin patch was coincident with photopolarization in accordance with L2 (Fig. 9A). In one population of zygotes, photopolarization with L2 and F-actin repositioning occurred between 40 and 50 minutes following light reversal (consistent with data in Fig. 8A), while in the other population they were delayed until approximately 2 hours after light reversal. That

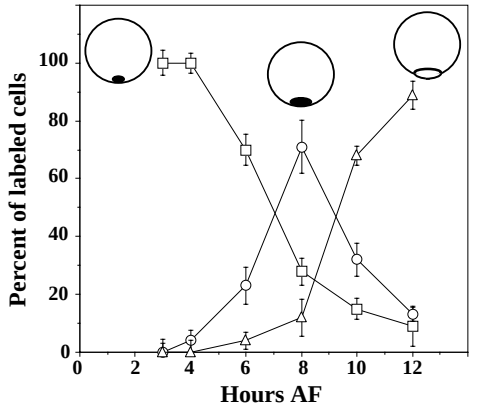

Fig. 4. Morphological changes in the F-actin array in a population of cells. F-actin undergoes a transition from a patch <15 μm in diameter (squares) to a larger patch >15 μm in diameter (circles) between approximately 6 and 8 hours AF. The larger patch then becomes a ring (triangles) between approximately 10 and 12 hours AF. Percentages are calculated from the number of cells that labeled at each time point. Bars are s.e.m. *n*=265 labeled cells in 3 replicate experiments.

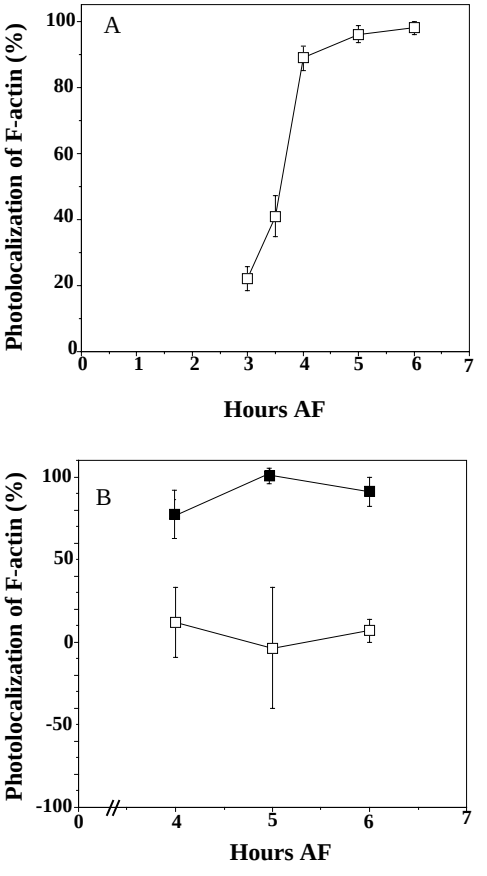

Fig. 5. Photolocalization of F-actin. (A) F-actin patches in a population of zygotes are not well photolocalized at 3 hours AF (random distribution is 0% photolocalization) but become photolocalized to the shaded hemisphere between 3 and 4 hours AF (all patches on the shaded hemisphere = 100% photolocalization). *n*=65 cells in 3 replicate experiments. (B) Treatment with 2.5 μg/ml Brefeldin A to inhibit photopolarization results in a nearly random distribution of F-actin patches (white squares) compared to untreated controls (black squares). *n*=60 cells in 2 replicate experiments. Bars are s.e.m.

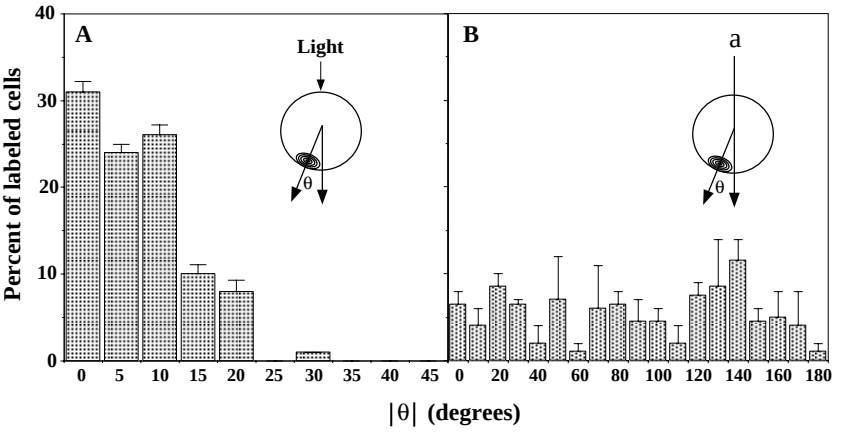

Fig. 6. (A) Spatial correlation between the orienting light vector and the position of the F-actin patch in 6-hour-old zygotes. The absolute values of θ were close to 0°, indicating that the F-actin patch was preferentially located near the shaded pole. *n*=156 cells in 3 replicate experiments. (B) F-actin patches in dark-grown zygotes were nearly randomly positioned with respect to an arbitrary vector (a). *n*=41 cells in two replicate experiments. Bars are s.e.m.

F-actin repositioned on a time course that was indistinguishable from photopolarization strongly indicates that the cortical F-actin patch is an immediate marker of the rhizoid pole. During repositioning F-actin was located at either pole but not at intermediate positions along the flanks of the zygote, and subsequent germination also occurred preferentially near the poles (Fig. 9B).

DISCUSSION

Assessment of the vital staining protocol

Saponin is a naturally occurring glycoside that permeabilizes eukaryotic cell membranes (Agarwal and Rastogi, 1974). Although its mechanism of action is poorly understood, it has been used extensively to load fluorescent molecules into living cells (Wassler et al., 1990; Huang and Liang, 1994; Meiners et al., 1991). We have used saponin permeabilization to load RP into *P. compressa* zygotes, and several observations indicate that this procedure gives an accurate representation of F-actin structure and localization. First, RP labeling is specific for F-actin; pretreatment with an excess of unlabeled phallacidin or with latrunculin B abolishes all staining. Second, the labeled F-actin remains dynamic; patches and rings change in morphology with time and the labeled patch repositions with a changed light vector. Third, following RP labeling, zygotes continue to develop, indicating that the staining protocol is not cytotoxic. Finally, F-actin patches, very similar to those observed in living zygotes, are also observed in young zygotes fixed in methanol and labeled with RP or with anti-actin antibodies.

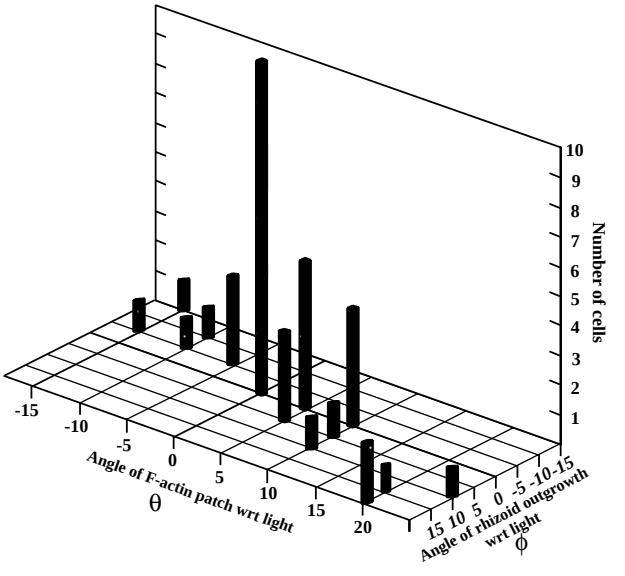

Fig. 7. The position of the F-actin patch correlates with the position of subsequent rhizoid outgrowth. Single cells were stained for F-actin at approximately 6 hours AF and θ measured. At 13 hours AF, the position of the rhizoid outgrowth with respect to the light vector (ϕ) was measured on the same cells. In most cases, θ and ϕ were near 0° and when θ varied from 0°, it accurately predicted ϕ. *n*=36 cells.

F-actin is an early marker of the rhizoid pole

Fertilized eggs begin to become competent to perceive light gradients at approximately 2 hours AF and, when grown in unilateral light, an entire population photopolarizes in accordance with the light by 4 hours AF (Hable and Kropf, 1998). Photolocalization of F-actin occurs on the same time course; F-actin is nearly randomly distributed in zygotes 3 hours AF, but is clearly localized to the shaded pole by 4 hours AF (see Fig. 5A). When the direction of orienting light is changed, F-actin localizes to the new rhizoid pole on a time course that is indistinguishable from photopolarization (see Fig. 9). Taken together, these data demonstrate that F-actin is an early marker of the rhizoid pole.

There are several plausible mechanisms by which F-actin may reposition following reversal of the direction of unilateral light; these include translocation of the patch through the cortex (Hird, 1996), contraction of an actomyosin network (Roegiers et al., 1995), or disassembly/reassembly of the patch. We favor the latter possibility and postulate that during polarization in response to newly perceived environmental cues, the F-actin patch at the old rhizoid pole disassembles and a new patch assembles at the new rhizoid pole. Two observations strongly support this mechanism; (1) patches were found at either pole, but not at intermediate positions, during repositioning, and (2) a few percent of zygotes had two patches, and in these zygotes the F-actin patches were positioned with one near each pole of the light vector (see Fig. 8B), as if one were disassembling while the other assembled. Disassembly/reassembly of F-actin patches occurs very rapidly

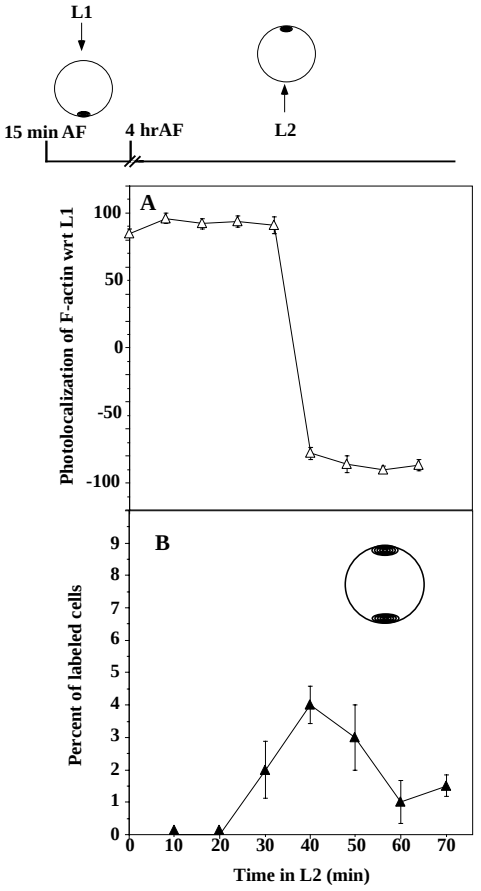

Fig. 8. Localized F-actin can be repositioned in response to a new light vector. (A) The direction of unilateral light was reversed (L1 to L2) at 4 hours AF and photolocalization of F-actin with respect to L1 was measured every 10 minutes thereafter as described in Materials and methods. (+100% photolocalization = all patches on the hemisphere shaded during L1; −100% photolocalization = all patches on the hemisphere shaded during L2; and 0% photolocalization = random patch distribution.) F-actin repositioned to the new shaded pole 40-50 minutes after light reversal. *n*=290 cells in 3 replicate experiments. (B) Percentage of cells with two patches following light reversal. 32 of 290 total cells had two patches. Bars are s.e.m.

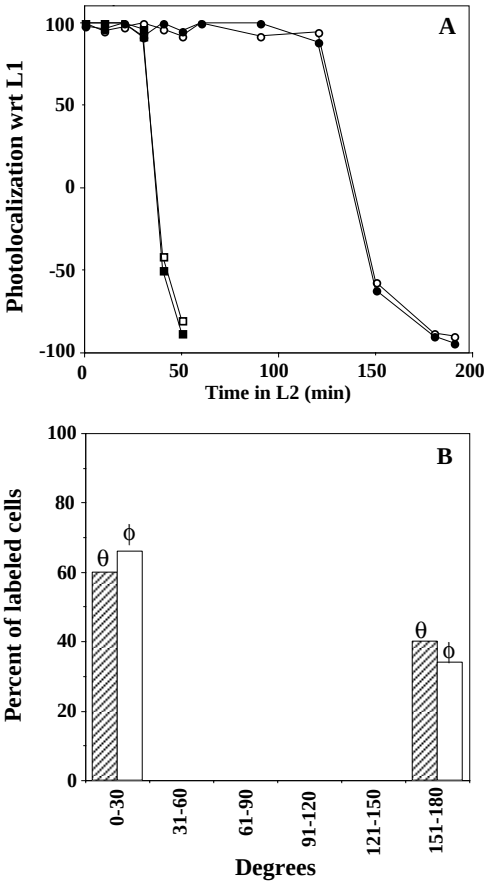

Fig. 9. Temporal (A) and spatial (B) relationships between photopolarization and repositioning of the F-actin patches following light reversal. (A) Photolocalization of F-actin (white symbols) and photolocalization of the rhizoid pole (photopolarization, black symbols) were measured as described in Materials and methods. (+100% photolocalization = all patches or rhizoids on the hemisphere shaded during L1; −100% photolocalization = all patches or rhizoids on the hemisphere shaded during L2; and 0% photolocalization = random patch or rhizoid distribution.) Squares and circles are two separate populations of zygotes. *n*=220. (B) Positions of F-actin patches (θ, hatched columns) and rhizoid outgrowths (ϕ, open columns) with respect to L1 were measured on the population represented by squares in (A). F-actin and rhizoids were located at either the shaded or lit pole, but not in intermediate positions along the flanks of the zygote. *n*=100.

in budding yeast (<5 minutes; Ayscough et al., 1997), and in fucoid algae disassembly/reassembly presumably can occur many times over during the first 8-10 hours of development while the axis is labile.

The existence and function of F-actin patches in very young zygotes are uncertain. When fertilized eggs are grown in unilateral light, patches are first detected about 3 hours AF and they are initially randomly distributed with respect to the light vector (see Fig. 5). These patches may mark the rhizoid pole of a pre-existing axis, perhaps induced at fertilization. In *Cystosira barbata*, a brown alga related to *P. compressa*, the sperm entry site becomes the rhizoid pole of the growth axis in zygotes grown in the dark (Knapp, 1931). We are therefore presently investigating whether there is an F-actin patch at the sperm entry site in *P. compressa*, and whether this site is an initial rhizoid pole that is abandoned when the zygotes polarize in response to environmental vectors. If so, patches may persist at the sperm entry site in Brefeldin A-treated zygotes because the zygotes cannot photopolarize; this would account for the random positioning of patches in zygotes exposed to unilateral light in the presence of Brefeldin A.

The signal transduction pathway that leads to F-actin patch assembly at the nascent rhizoid pole is unknown. It is likely that other proteins (possibly actin-binding proteins) localize to the rhizoid pole before F-actin patch assembly and are needed to recruit F-actin (Fowler and Quatrano, 1997). During bud formation in yeast, site selection proteins (Bud2p, Bud5p etc.) localize polarity establishment and maintenance proteins (Cdc42p, Bem1p etc.), which in turn localize cytoskeletal proteins, including F-actin (Pringle et al., 1995). Local ion fluxes may also be involved in assembly of the F-actin patch in *P. compressa*. Ca^{2+} preferentially enters the rhizoid pole of young zygotes (Robinson and Jaffe, 1975) and the cytosolic H^+

activity is highest there (Kropf et al., 1995). Ca^{2+} and H^+ fluxes are known to trigger F-actin assembly during fertilization of sea urchin eggs (Begg et al., 1996), and may play a similar role in F-actin patch assembly at the nascent rhizoid pole in *P. compressa* zygotes.

Assembly of an F-actin patch at the nascent rhizoid pole may be an essential step in defining this site during polarization. Several investigations have shown that agents which disrupt F-actin prevent photopolarization (Quatrano, 1973; Love et al., 1997; Hable and Kropf, 1998), and we find that although F-actin patches are still present when photopolarization is inhibited with Brefeldin A, they do not localize to the shaded pole. Once the patch is assembled, it may provide a foundation for recruiting additional molecules and processes to the rhizoid site over the next several hours (Kropf, 1997). Recruitment of dihydropyridine receptors (Shaw and Quatrano, 1996a), sulfated fucans (Novotny and Forman, 1974), inward electrical current (Brawley and Robinson, 1985), and secretion (Hable and Kropf, 1998) to the rhizoid pole is dependent on functioning F-actin. F-actin may also anchor mRNAs at the rhizoid pole (Jeffery, 1985; Yisraeli et al., 1990), but as yet no mRNAs that localize to this site have been identified in fucoid zygotes (Bouget et al., 1996).

Localized F-actin marks an incipient growth site in other tip growing cells including germinating pollen grains (Heslop-Harrison and Heslop-Harrison, 1992), budding moss gametophytes (Quader and Schnepf, 1989), branching fungal hyphae (Bachewich and Heath, 1998), and budding yeast (Pringle et al. 1995). The role of F-actin has been most extensively investigated in budding yeast, where it has been shown to recruit many proteins, including those involved in local secretion (Sec4p, Sec6p, and Sec8p), to the growth site during bud formation (Pringle et al., 1995; Ayscough et al., 1997) and mating type projection (Ayscough and Drubin, 1998).

Subapical rings associated with rhizoid growth

At germination, the F-actin patch reorganizes into a subapical ring which persists during subsequent tip growth. Rings of F-actin have also been observed subapically in fern protonema (Kadota and Wada, 1989) and at the incipient growth site during hyphal branching (Bachewich and Heath, 1998). Although the function of the ring is unclear, its localization behind the fragile apex makes it unlikely to play a significant role in tip reinforcement (Kropf et al., 1998). Instead, it is attractive to speculate that it may in some way help to define the expanding dome and separate it from the subapical rhizoid domain. A subapical ring of tight adhesion between cortical F-actin, plasma membrane and cell wall has been observed in *P. compressa* rhizoids (Henry et al., 1996). This ring of tight adhesion may serve as a boundary that helps to maintain localization of specific plasma membrane constituents. For example, exclusion of cellulose synthase complexes from the membrane at the apical dome may help maintain the apical wall in an pliable state.

We wish to thank Sherryl Bisgrove and Whitney Hable for constructive criticisms on the manuscript, and Uday Tirlapur for initial observations of patches and rings in fixed material. This work was supported by NSF awards IBN-9506094 and IBN-9808711 to D. L. K.

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