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Actin Localization during *Fucus* Embryogenesis

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Embryogenesis in the Fucales serves as a model system for studying the acquisition of cellular and developmental polarity. Fertilized eggs bear no asymmetry, yet within 16 hours, a developmental axis is formed and the unicellular zygote germinates in accordance with this axis. Microfilaments (actin) play a crucial role in establishing the axis as evidenced by the inhibitory effects of cytochalasins on axis fixation. The cellular content of actin was determined by immunoblot, whereas the localization of F-actin was investigated using the fluorescent probe rhodamine phalloidin. Three isoforms of actin were detected in constant amounts at all developmental stages. Actin networks were found to be distributed uniformly in eggs and zygotes through the period of early zygote development when the polar axis was formed. However, as the polar axis became irreversibly fixed in space, actin was localized at the presumptive germination site by a cytochalasin-sensitive process. This correlation supports the proposal that actin networks play a critical role in axis fixation, and is consistent with our hypothesis that this process involves stabilization of membrane components by transmembrane bridges from the cell wall to the microfilament cytoskeleton.

INTRODUCTION

Many diverse processes involving motility and intracellular transport in animals utilize F-actin. Examples include muscle contraction, cell division, chemotaxis, vesicle transport, and membrane receptor mobility. In higher plants, whose cells are nonmotile, the functions of F-actin are not as well understood. It is reasonably certain that cytoplasmic streaming is driven by an actomyosin system and that tip growth in pollen tubes (Picton and Steer, 1982, 1983) relies upon apical microfilaments. Beyond these examples little is known.

Much more has been learned from studies of F-actin in lower plants. Plant microfilaments were first described in Characean algae (Nagai and Rebhun, 1966; Palevitz et al., 1974), and it was in this system that the actomyosin basis for cytoplasmic streaming was elucidated (Kamiya, 1981). Other studies in mosses and algae suggest that F-actin serves in nuclear migration (Saunders, 1986), chloroplast rotation (Klein et al., 1980), chloroplast localization (Blatt et al., 1980), polarized vesicle transport (Brawley and Quatrano, 1979), and embryonic polarization (Quatrano, 1973).

We are interested in the mechanisms by which zygotes of the brown alga *Fucus* establish an embryonic axis. The egg, both before and soon after fertilization, is radially symmetric. Yet within hours, the zygote establishes a developmental axis and commences highly localized growth that gives rise to a rhizoid protuberance. Many external vectors, most notably unilateral light, orient the developmental axis. One day after fertilization the pear-shaped zygote undergoes the first cell division, perpendicular to the growth axis, resulting in two cells of very different developmental fates. The rhizoid cell becomes the plant holdfast, whereas the thallus cell gives rise to the fronds.

Earlier research suggested that zygotic polarization in *Fucus* was an F-actin-mediated process (Quatrano, 1973; Brawley and Quatrano, 1979; Quatrano et al., 1979; Quatrano et al., 1985). The most convincing evidence was that cytochalasins, inhibitors of F-actin organization (MacLean-Fletcher and Pollard, 1980), specifically blocked axis establishment (Quatrano, 1973). Brawley and Robinson (1985) used nitrobenzoxadiazole (NBD)-phallacidin to localize F-actin in fucoid embryos and showed that F-actin was not localized in zygotes until the time of axis fixation. At that time and during rhizoid emergence, F-actin was localized in the rhizoid half of the zygote. To extend these original observations and further assess the role of F-actin in polarization, we report here the actin content at all stages of early *Fucus* development and the timing of actin localization relative to axis fixation. Two-dimensional gel analysis and fluorescent staining with the F-actin-specific probe rhodamine phalloidin (RhPh) have been used. The results indicate that G-actin is present as three isoforms at the same level in the egg and throughout early development. Until axis fixation begins, F-actin is arranged symmetrically in the cortex of the egg and zygote. As the axis becomes fixed, F-actin is localized at the future rhizoid

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end of the developmental axis. This redistribution of F-actin coincides with the acquisition of a fixed axis, and both processes are prevented by cytochalasin treatment. These findings have direct relevance to current models of polarization in *Fucus*.

RESULTS

Actin Identification and Developmental Analysis

To simplify protein analysis, we divided early development into a series of stages: mature, unfertilized eggs; fertilized, apolar zygotes (6 hr); polarized zygotes (12 hr); germinated zygotes (16 hr); and divided embryos (28 hr). The time in parentheses indicates the hours after fertilization at which zygotes were harvested for protein analysis. Multicellular embryos were also studied; 2-day (8-celled), 3-day (16-celled), and 8-day (50- to 100-celled) germlings were analyzed. Actin was identified at each developmental stage by immunoblot analysis using a monoclonal anti-actin antibody. The antibody bound strongly to a single band of protein at the appropriate apparent molecular mass, 42 kD (Figure 1). This protein was identical in size to chicken gizzard actin on one-dimensional immunoblots (data not shown). The weak association of the antibody with proteins of higher molecular weight may indicate the presence of actin-related molecules, as reported in *Chara* and pea chloroplasts (McCurdy and Williamson, 1987; Williamson et al., 1987). Actin was found to be present in *Fucus* at all stages of development, from egg to 8-day embryos. The relative amount of actin in cells was rather constant, although unfertilized eggs may contain slightly less than embryos.

In animal cells actin is not a single protein but is instead a family of related polypeptides that are regulated independently during early development (Lee et al., 1984; Davidson, 1986). To search for multiple actins in *Fucus*, proteins were separated by two-dimensional gel electrophoresis. Figure 2a shows the pattern of proteins resolved in two dimensions and visualized by silver staining. More than 200 proteins were clearly resolved. Immunoblot analysis of two-dimensional gels identified three isoforms of actin differing in charge (Figure 2b). These are labeled I, II, and III in Figure 2. Isoform II was the most heavily stained. Analysis of silver-stained two-dimensional gels from each stage of embryogenesis confirmed that all three isoforms were present throughout development (data not shown). No clear changes in relative staining intensity of the three actins were detected. The constant level of each isoform maintained in the cell suggests that all three function as authentic actins. This is the first direct demonstration of actin in *Fucus*.

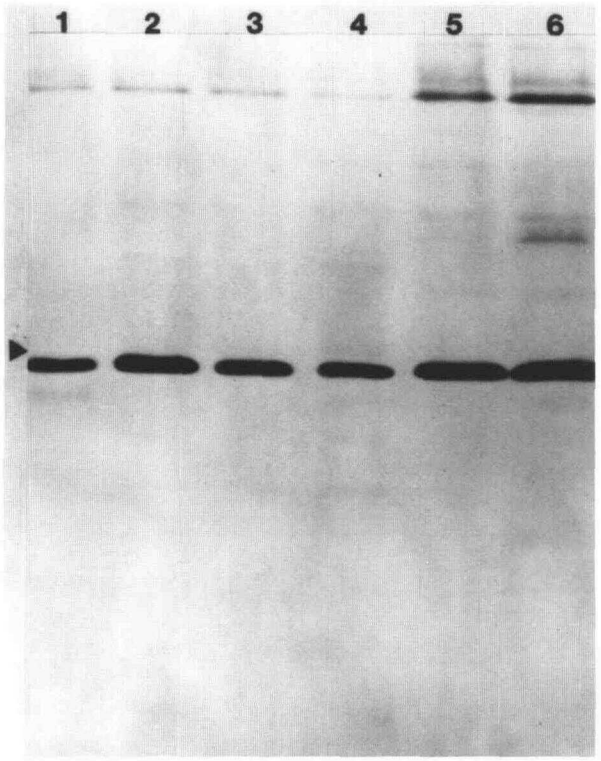

Figure 1. Immunoblot of Proteins from Early Developmental Stages Probed with Monoclonal Anti-Actin Antibody Diluted 1 to 1000.

Each lane was loaded with 15 μg of protein isolated from oogonia (lane 1), fertilized eggs (6 hr, lane 2), polarized zygotes (12 hr, lane 3), germinated zygotes (16 hr, lane 4), divided, two-celled embryos (28 hr, lane 5), and 15 to 20 celled embryos (3 day, lane 6). Arrow indicates a molecular mass of 43 kD. *Fucus* actin was identical to chicken gizzard actin (not shown)

F-Actin Localization

To investigate further the putative role of actin in development, we used RhPh to do an extensive analysis of F-actin localization during early embryogenesis. Because we cannot be certain that RhPh binds only to intact microfilaments, the stained filaments are referred to simply as F-actin. In a preliminary report Brawley and Robinson (1985) suggested that cortical F-actin became localized to the future rhizoid hemisphere at the time when the axis was fixed irreversibly in space. This study was hampered, however, by the high background autofluorescence using NBD-phallacidin and by a lack of direct correlation between the localization and the process of axis fixation in *Pelvetia*. Also, the time courses of axis fixation and germination were found to overlap to a considerable degree in *Pelvetia* (Figure 1 in Kropf and Quatrano, 1987). In this study, RhPh

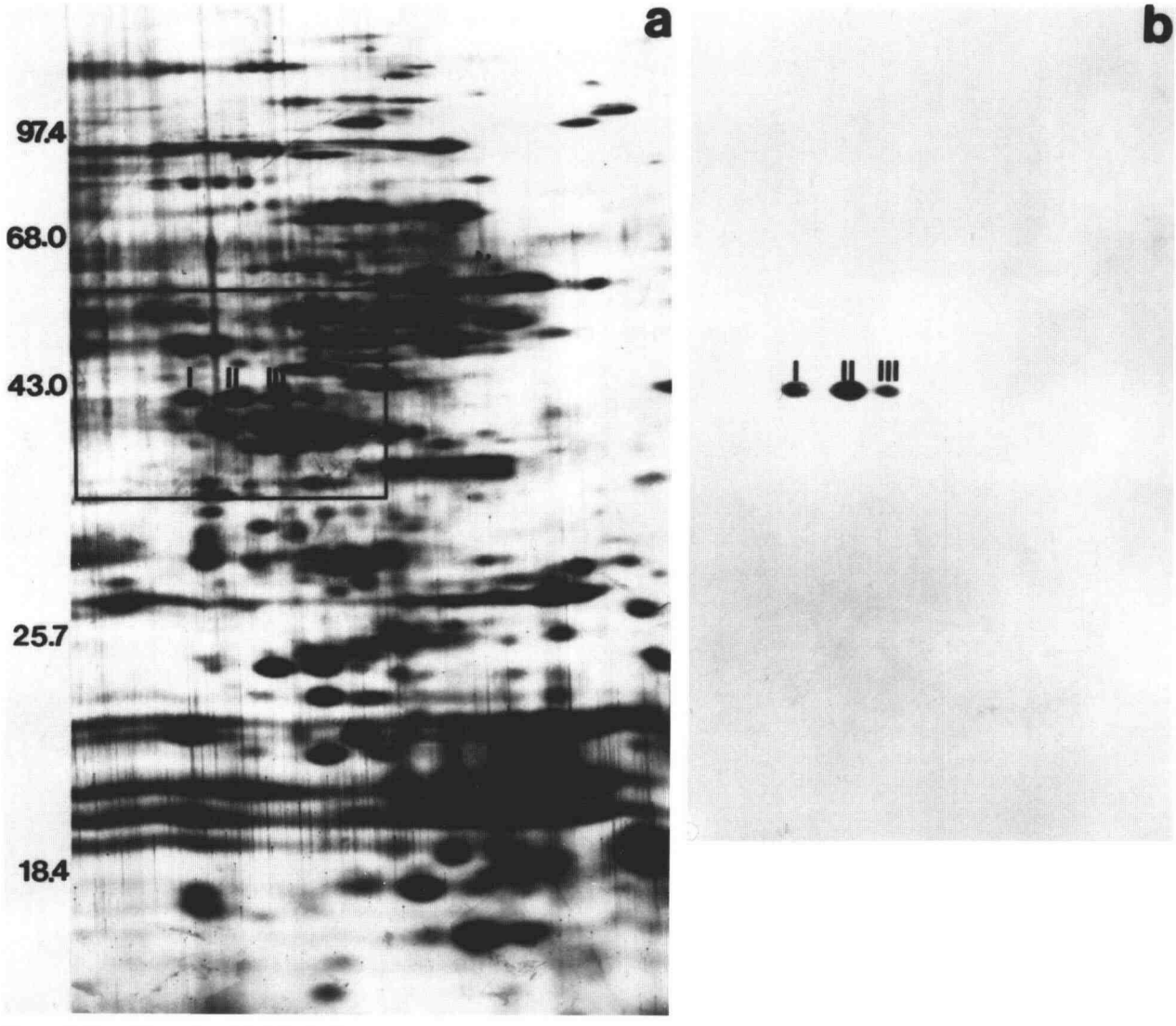

Figure 2. Two-Dimensional Gel Analysis.

(a) Silver-stained gel of protein from polarized zygotes (12 hr).
(b) Immunoblot showing three polypeptides recognized by the antibody. These polypeptides were identified unambiguously as the three spots in **(a)** by cutting out small, adjacent regions of a silver-stained gel. Each gel piece was destained with Farmer's reducer (Eastman-Kodak Co, Rochester, NY), blotted separately onto nitrocellulose, and probed with the antibody. Box indicates region that was subdivided and analyzed. Positions of molecular weight standards are indicated.

staining permitted us to filter out nearly all background autofluorescence (see "Methods"), and axis fixation was determined experimentally on the same cells as was the fluorescence localization.

We began by staining multicellular embryos bearing well-established rhizoids in which growth was strictly apical. As expected from studies of tip-growing fungi (Hoch and Staples, 1983; Heath, 1987), most RhPh staining was localized to the elongating tips (Figure 3a). This apical fluorescence was limited to a very fine cortical rim that extended typically approximately 10 μm back from the tip. In addition, a punctate fluorescence was found farther back (see also Figure 5). This spotty fluorescence did not appear to be in the cortex but instead spread throughout the cytoplasm. Treatment with excess phalloidin prior to staining prevented both components of fluorescence

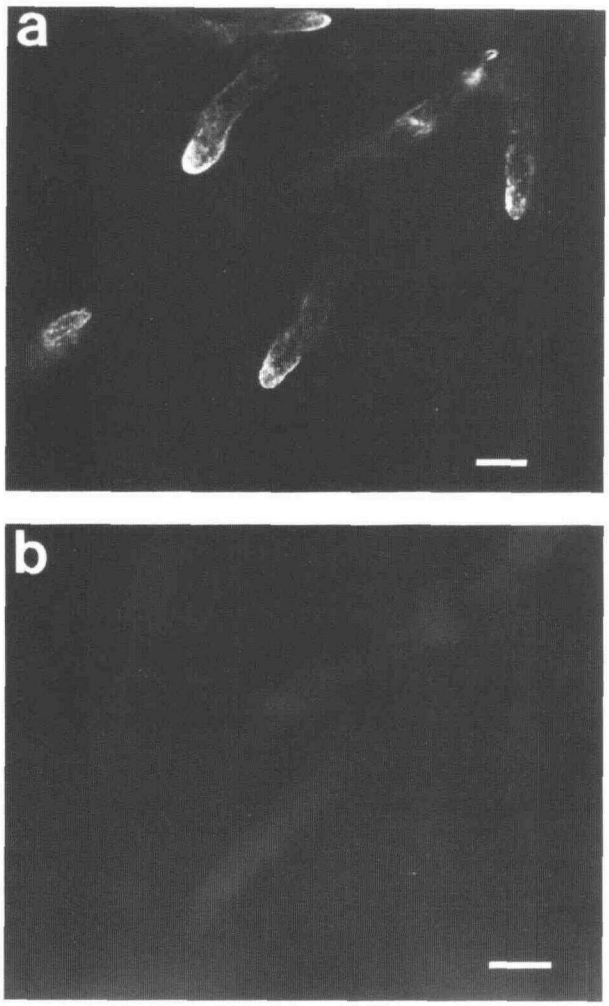

Figure 3. F-Actin Localization in Rhizoids.

(a) RhPh staining of rhizoids of 4-day-old embryos.
(b) 4-day-old embryos treated with excess unlabeled phalloidin before staining. Bar = 25 μm.

(Figure 3b). Thus, F-actin was localized clearly in the cortex at the tip and in plaques farther back.

The pattern of fluorescence at earlier stages of development was investigated to determine when the localization of fluorescence could first be detected. During the first 7 hr postfertilization, the spherical zygotes stained brightly in the cortex and dimly throughout the endoplasm (Figure 4a). Most importantly, the entire fluorescence was distributed uniformly about the 7-hr-old zygote. A similar pattern of staining was found in younger zygotes and unfertilized eggs, which is consistent with the antibody determinations in the previous section. Depolymerization of F-actin prior to staining eliminated the cortical fluorescence (Figure 4b). Zygotes stained between 8 and 12 hr of development showed an increasingly asymmetric staining in the cortex, yet the entire circumference usually was stained. Just prior to germination, highly localized staining was observed in many zygotes (Figure 4c). At germination, all fluorescence was localized to a bright cortical rim at the rhizoid tip (Figure 4d), and the staining of the thallus had disappeared. From the time polarization began (8 hr) until germination (15 hr), we observed a gradual redistribution of fluorescence in which one region of the surface became gradually brighter at the expense of the opposing region (180° away).

The redistribution of cortical fluorescence prior to germination indicated that F-actin may participate in zygotic polarization. If this were true, we might expect the F-actin to accumulate at one end of the *Fucus* developmental axis, as previously reported for *Pelvetia* (Brawley and Robinson, 1985). It has long been known that neighboring zygotes establish an axis and germinate toward each other (Jaffe and Neuscheler, 1969). We have observed that polarizing zygotes lying close together commonly stain preferentially on the side toward their neighbor, which, presumably, was where the rhizoid would have emerged if the cell had been allowed to develop further (data not shown).

To be certain of this relationship, photopolarization was used to induce an axis of known orientation and the position of RhPh staining was correlated with the site of future rhizoid growth. When exposed to unilateral light, zygotes establish an axis along the light gradient and germinate on the shaded hemisphere (Kropf and Quatrano, 1987; Kropf et al., 1988b). Photopolarized zygotes showed two patterns of staining: they either fluoresced uniformly around the cortex or preferentially on the shaded hemisphere. (Fewer than 3% of zygotes stained preferentially on the lighted hemisphere.) In zygotes bearing asymmetric staining, fluorescence was most intense on the lower portion of the shaded hemisphere, nearest the substratum (Figure 5). Rhizoids normally grew from this region and attached to the substratum. In the cell shown, the fluorescence was mostly punctate.

A population of synchronously developing zygotes undergo polarization over a period of hours. The cells first form an axis and then irreversibly fix it in place. To determine whether this localization correlated temporally with axis formation or fixation, we measured the timing of these processes in detail. Axis formation was measured by placing zygotes in unilateral light for pulses of varying duration beginning at 1 hr postfertilization. This light pulse was followed by incubation until germination either in the dark or in uniform light. Under these conditions, zygotes were most sensitive to light orientation between 5 and 9 hr of development (Figure 6). During this period the percentage of zygotes that were polarized by the light (i.e. formed an axis) increased linearly. It should be noted that nearly 40% of the cells were polarized by a 2-hr light pulse from 1 to 3 hr of development. We do not know why this subpopulation of cells was so light-sensitive. Axis fixation was

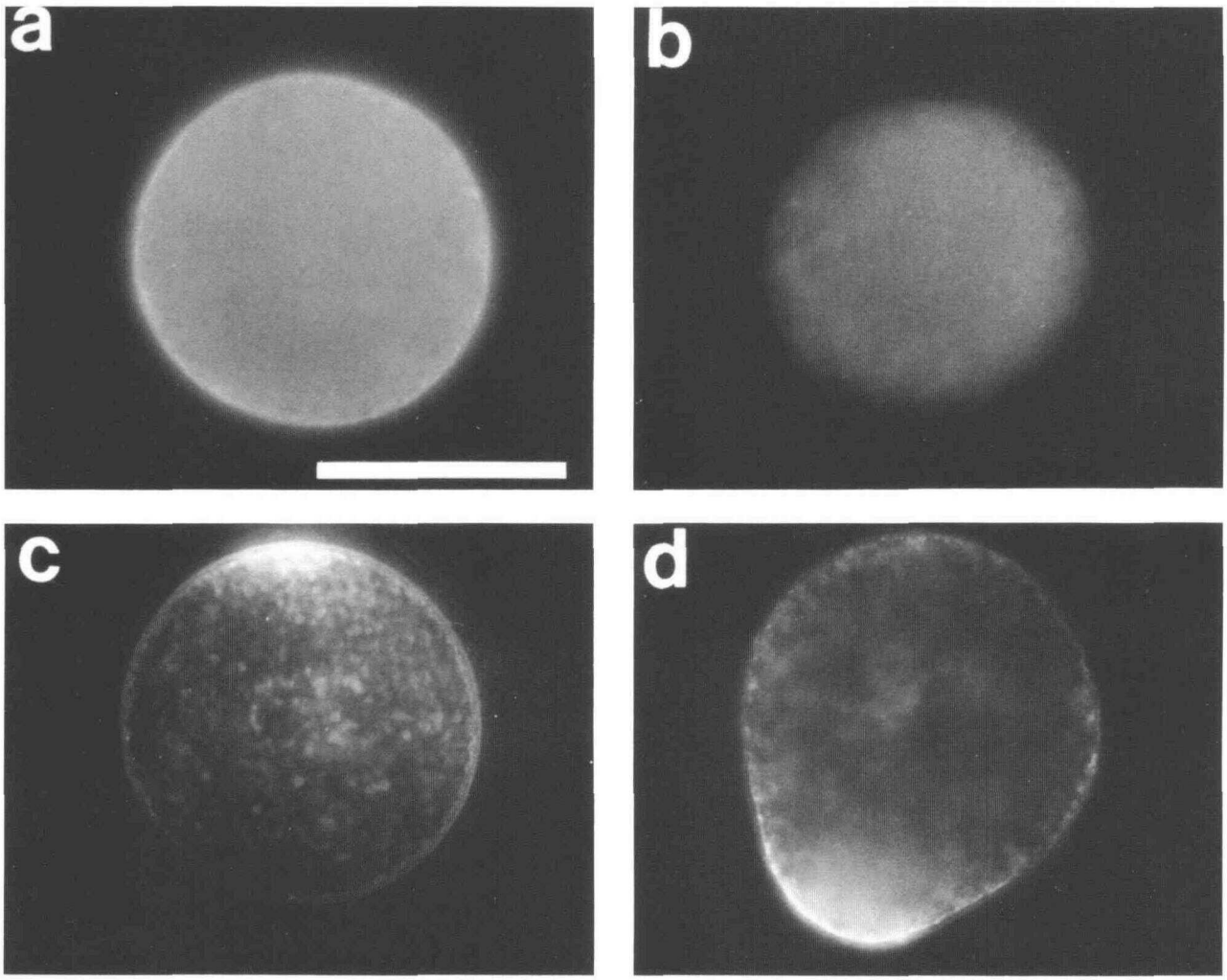

Figure 4. RhPh Staining of Zygotes during the 1st Day of Development.

(a) Seven-hour-old zygotes.
(b) Nine-hour-old zygotes treated with 0.6 M KI to depolymerize microfilaments prior to staining.
(c) Eleven-hour-old zygote.
(d) Sixteen-hour-old germinated zygote. Bar = 50 μm.

measured previously (Kropf and Quatrano, 1987) using two consecutive light pulses oriented 180° apart (see "Methods"). In that study we found that the percentage of cells bearing fixed axes increased from 30% at 8 hr to greater than 80% at 12 hr (Figure 6). The time course of axis fixation is quite reproducible.

The time course of F-actin localization was assayed by staining zygotes that had been treated with unilateral light continuously from 1 hr. Zygotes were scored for the location of staining: preferentially on the illuminated hemisphere, preferentially on the shaded hemisphere, or uniform. The percentage of zygotes preferentially staining on the shaded hemisphere increased simultaneously with, or just after, axis fixation (Figure 6). Nearly all of the other zygotes stained uniformly; very few stained brighter on the lighted side. In some experiments, axis fixation was measured on the same batch of cells used for staining. It was found that the percentage of cells with fixed axes was very similar to the percentage of cells bearing F-actin on the shaded hemisphere (data not shown).

The coupling between F-actin localization and axis fixation was tested further using cytochalasin D (CD). This inhibitor of actin filament organization prevents photopolarization (Quatrano, 1973), and we tested whether it also blocked the appearance of localized RhPh staining. Brawley and Robinson (1985) reported anomalous patterns of

F-actin distribution induced by cytochalasin treatment. Zygotes were grown 8 hr in uniform light and then placed in artificial seawater (ASW) containing 100 μg/ml CD and treated with unilateral light. At various times thereafter, cells were stained with RhPh, and the localization of fluorescence was scored with respect to the direction of the light treatment. At 17 hr, 33% of control cells in ASW containing 1% DMSO had fluorescence localized to the shaded hemisphere, compared with only 6% of CD-treated zygotes. DMSO alone delayed development and F-actin localization; 17-hr-zygotes in ASW had localized fluorescence and were well germinated (see Figure 6). Nonetheless, at 41 hr (33 hr after treatment began), DMSO controls had germinated and appeared as normal 2- to 4-celled embryos with fluorescence localized in the tip. By contrast, CD-treated zygotes were still unicellular and only 1% showed preferential staining on the shaded hemisphere; nearly 99% of the zygotes stained brightly and uniformly around the cortex. Similar results were obtained with cytochalasin B (CB). As an additional control, cytochalasins were shown to block axis fixation on the same batch of cells used for staining (see "Methods"). Thus, cytochalasin treatment prevented both axis fixation and F-actin redistribution toward the rhizoid site. The observation that cytochalasins did not eliminate fluorescence suggests that the actin network was not depolymerized by the drugs. There are now numerous reports of actin filaments in plants that are resistant to destruction by cytochalasins (for review, see Staiger and Schliwa, 1987).

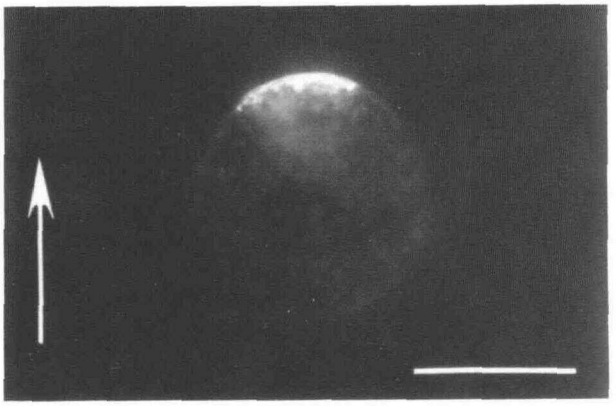

Figure 5. RhPh Staining of a 12-Hour-Old Zygote Treated with Unilateral Light from 1 Hour On.

Focal plane is just above substratum (coverglass) and localization of fluorescence on the dark side of the zygote is evident. Note also the punctate nature of the fluorescence. Arrow indicates direction of the light vector. Bar = 50 μm.

DISCUSSION

The three isoforms of actin recognized by the antibody on two-dimensional immunoblots suggest strongly that actin is a small family of closely related proteins in *Fucus*. By comparison, *Chara* giant internodal cells have one major actin isoform of 43 kD as well as two minor spots (Williamson et al., 1987), *Chlamydomonas* flagella contain a single isoform (Piperno and Luck, 1979), and *Arabadopsis* seedlings contain five isoforms (Wlliamson et al., 1987). Multiple isoforms are also common in animal embryos (Durica and Crain, 1982). Like animal cells, higher plant cells also contain multiple actin genes (Meagher et al., 1983; Shah et al., 1983; Drovin and Dover, 1987) and our results raise the possibility that the same is true in *Fucus*. Presently, we are isolating actin genes and cDNAs from *Fucus* libraries for analysis. With these probes, we will examine the localization of actin mRNA during polarization by in situ hybridization and begin to analyze the actin gene family and its expression during embryogenesis.

RhPh has proven to be an immensely useful tool for visualizing F-actin in fungal and plant cells (Lloyd, 1988). Recently, researchers have used this probe to follow F-actin throughout the cell cycle and have found microfilament arrays associated with the phragmoplast and preprophase band (Palevitz, 1987; Seagull et al., 1987), structures previously thought to contain only microtubules, as well as an F-actin network surrounding the spindle (Schmit and Lambert, 1987). In higher plants RhPh shows distinct fibers and cables in the cytoplasm, but in fungi a bright cortical rim, plaque, or cap is seen at the elongating apex (Hoch and Staples, 1983; Adams and Pringle, 1984; Heath, 1987), much as we have found in germinated *Fucus* embryos. Brawley and Robinson (1985) observed the same structures in *Fucus* and *Pelvetia* using NBD-phallacidin. The absence of distinct fibers may indicate that the actin cytoskeleton is a fine meshwork at the tip. This apical F-actin cytoskeleton is involved intimately in tip growth and morphogenesis (Picton and Steer, 1982; Doonan, et al., 1988) and probably serves in the polar transport of vesicles to the apical plasmalemma (Brawley and Quatrano, 1979; Picton and Steer, 1981; Brawley and Robinson, 1985). In some fungi, subapical fibers (Hoch and Staples, 1985; Heath, 1987) are clearly visible, but we were unable to see them in *Fucus*, perhaps because of the opaque, brown pigmentation of the cell interior.

Our results support the premise that, in *Fucus*, the localization of F-actin to the presumptive rhizoid plays an important role in axis fixation. This conclusion rests upon two lines of evidence: (1) F-actin redistributes to the presumptive rhizoid site at the time the axis becomes irreversibly fixed, and at any given time the percent of cells with localized actin is similar to the percent with fixed axes. It should be noted that the F-actin localization described herein is the earliest macromolecular asymmetry known to

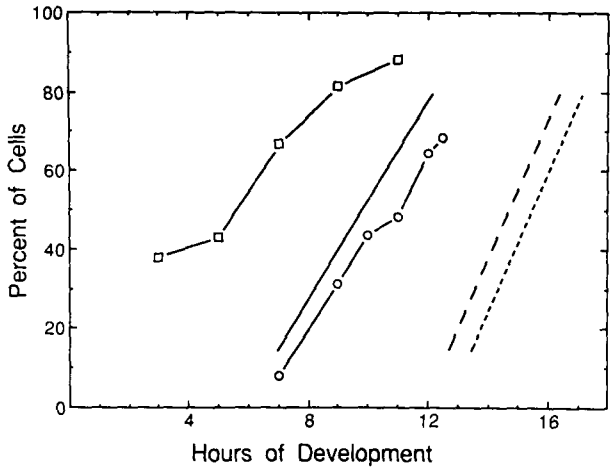

Figure 6. Timing of Early Developmental Processes.

To measure axis formation (□), fertilized eggs were exposed to unilateral light from 1 hr until the time indicated on the abscissa. They were then allowed to germinate in uniform light. F-actin localization (○) was scored as the percentage of zygotes that stained preferentially on the shaded hemisphere with RhPh after being treated with unilateral light from 1 hr until the time indicated on the abscissa. The timing of axis fixation (solid line), germination (long dashed line), and Ca^{2+} localization as assayed with chlorotetracycline (short dashed line) were reported previously (Kropf and Quatrano, 1987) and are depicted by lines without data points.

arise during *Fucus* development. (2) Cytochalasins, previously shown to prevent fixation (Quatrano, 1973), also prevent the localization of F-actin at the presumptive rhizoid. This inhibition is not due to a nonspecific developmental arrest; cytochalasins neither affect the normal progression of changes in protein synthesis (Kropf et al., 1988a), nor prevent sulfation of wall polysaccharides (Brawley and Quatrano, 1979). Thus, it is not merely the presence, but the redistribution, of F-actin that is critical for axis fixation.

The mechanism by which F-actin localizes at the presumptive rhizoid is unknown. In animal cells numerous types of motility and intracellular transport are associated with a flow of cortical actin fibers. These include cytokinesis, cell migration, extension of the neuronal growth cone, and lymphocyte capping. Bray and White (1988) suggest that the flow of cortical actin is driven by a localized relaxation of surface tension that causes a bulk cortical movement from regions of lower tension toward regions of higher tension. A similar mechanism may operate in *Fucus* if tension were relaxed preferentially on the lighted hemisphere (presumptive thallus) of the zygote. This process would be quite similar to capping on lymphocytes. Alternately, localization may be accomplished by a preferential degradation of F-actin at the presumptive thallus. At present, we cannot distinguish between these two or other possibilities.

Although the biochemical data do not address the question of localization, they do provide further insight into the role of actin in polarization. If the thallus F-actin depolymerizes during fixation, one might expect a net increase in actin monomer content. However, as our fluorescent study indicates, F-actin may only reorganize, not depolymerize, during fixation. The constancy of actin monomer levels and pattern of isoforms throughout the first days of embryogenesis implies that the cells carefully regulate actin pools, regardless of the distribution of the F-actin networks. Clearly, changes in actin content are not involved in polarization.

How might actin filaments function in determining the growth axis? Recently, we reported that the cell wall surrounding *Fucus* zygotes must be present for transit through axis fixation (Kropf et al., 1988b). Protoplasts were unable to fix an axis. Because cytochalasin treatment is the only other condition known to prevent fixation, we suggested that a transmembrane link between actin filaments and the cell wall at the future rhizoid site denotes axis fixation. The current findings support this hypothesis. F-actin localizes at the presumptive rhizoid pole as fixation proceeds, and we believe that these cortical, localized filaments could form cross-bridges to components of the cell wall. Such connections have been documented in plant (Heath and Seagull, 1982; Lancelle et al., 1987) and animal (Horowitz et al., 1986) cells and are usually indirect linkages involving accessory proteins (such as actin-binding proteins) and integral membrane proteins (Horowitz et al., 1986). If such a complex were involved in axis fixation, this process would be prevented by disruption of the wall or the actin cytoskeleton, as observed. Once the axis is fixed, however, another mechanism, perhaps localized metabolic activation, maintains the orientation of the growth axis. Wall removal or cytochalasin treatment after fixation does not disrupt a preformed axis (Quatrano, 1973; Kropf et al., 1988b).

The localized filaments probably also serve to transport vesicles to the presumptive site prior to germination. Quatrano and Stevens (1976) found that a highly sulfated fucan, fucoidan, first appears in the cytoplasm at 6 to 10 hr and is transported to the future rhizoid in vesicles and secreted locally into the wall at 12 hr. This localized secretion accounts for the accumulation of egg jelly (Schroter, 1978) and plasma membrane patches (Peng and Jaffe, 1976) on the shaded hemisphere of ungerminated zygotes. We believe that these vesicles are transported from the perinuclear dictyosomes to the presumptive rhizoid along the localized filaments. Indeed, cytochalasins block vesicle transport in *Fucus* (Brawley and Quatrano, 1979). If these vesicles also bear Ca^{2+} channels, their localized insertion could account for the subsequent accumulation of membrane-associated Ca^{2+} at the time of rhizoid emergence (Kropf and Quatrano, 1987).

METHODS

Plant Material, Media, and Reagents

Fucus distichus (L.) Powell was collected from Yaquina Head, OR, and stored in the dark at 4°C for up to 1 week. Fertilized eggs were shed from mature receptacles over a period of 1 to 2 hr by standard procedures using osmotic shock (Quatrano, 1980). Zygotes were collected by sedimentation, washed three times in ASW and plated in plastic Petri dishes. To collect unfertilized eggs, mature receptacles were induced to release intact oogonia by shedding in Ca^{2+}-free ASW containing 10 mM EGTA. Each oogonium contained eight unfertilized eggs. Cultures were incubated at 14°C in constant illumination of 50 μmol (photons)$\cdot m^{-2} \cdot s^{-1}$ in a seed germinator (Hoffman Manufacturing, Albany, OR) using cool-white fluorescent lamps (Sylvania-GTE, Fall River, MA). Zygotes settle and attach to the substratum within minutes and develop synchronously under these conditions (Quatrano, 1980).

The ASW contained 450 mM NaCl, 10 mM KCL, 9 mM $CaCl_2$, 30 mM $MgCl_2$, and 16 mM $MgSO_4$. It was buffered to pH 7.5 with 10 mM Tes and contained 40 $\mu g \cdot ml^{-1}$ chloroamphenicol to prevent bacterial contamination.

All reagents were purchased from Sigma, St. Louis, MO, unless otherwise noted.

Protein Labeling and Gel Electrophoresis

Zygotes were plated as a confluent monolayer in 60-mm plastic Petri dishes and grown until the beginning of the developmental stage of interest. At that time the ASW was replaced by 2 ml of fresh ASW containing 100 μCi/ml $Na_2{}^{14}CO_3$ (New England Nuclear Research Products, Boston, MA). The dish was placed in a CO_2 trap and zygotes were labeled for either 4 or 6 hr. At the end of the labeling period, zygotes were washed twice with 4 ml of ASW, harvested in 2 ml of extraction buffer, and frozen at −20°C. Intact oogonia were labeled using a similar protocol. Extraction buffer contained 10 mM Tris-Hcl (pH 7.5), 0.1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 0.5% Triton X-100, 0.5% SDS, 1% β-mercaptoethanol, and 0.5% insoluble polyvinylpyrrolidone. At a convenient time, cells were homogenized and protein was precipitated by 5 volumes of acetone (−20°C) and collected by centrifugation at 16,000*g* for 10 min. After four additional rounds of homogenization and centrifugation, the straw-colored pellet was dried and proteins were extracted from the acetone powder in sample buffer (0.5 M Tris, pH 6.8, 10% SDS, 20% glycerol, and 10% β-mercaptoethanol). Proteins were resolved in one dimension using SDS-PAGE (12.5% acrylamide) or in two dimensions (O'Farrell, 1975) using isoelectric focusing (16 hr at 800 V) followed by SDS-PAGE and were silver-stained (Bio-Rad, Richmond, CA).

Actin was identified by transferring one- and two-dimensional gels onto nitrocellulose using a trans-blot apparatus (Bio-Rad). The blot was probed with a monoclonal antibody raised against chicken gizzard actin (Amersham Corporation, Arlington Heights, IL), and bound antibody was visualized by enzyme-linked immunodetection with horseradish peroxidase (Vectastain, Vector Laboratories, Inc., Burlingame, CA).

Fluorescence Microscopy

Fertilized zygotes were plated in ASW as a monolayer on No. 1 cover glasses (VWR Scientific, Inc., San Francisco, CA) and incubated at 14°C until the desired developmental stage. During incubation, developing zygotes attached to the coverslips, which were then processed through the treatments described below. At the time of interest, individual coverslips were fixed for 10 min in buffered ASW (ASW, 30 mM Pipes, pH 7.5) containing 3% formaldehyde. After a brief rinse in buffered ASW, cells were placed in a permeabilization solution (0.1% Triton X-100 in buffered ASW) for 10 min and rinsed again prior to staining. Coverslips were stained for 3 min with RhPh (Molecular Probes, Eugene, OR), a fluorescent probe used to localize F-actin (Wieland and Govindan, 1974; Wulf et al., 1979). RhPh was applied at a final concentration of 8.25×10^{-7} M achieved by a 1 to 4 dilution of RhPh stock (3.3×10^{-6} M in methanol) with buffered ASW. The staining pattern achieved with this working solution of RhPh was identical to that obtained with other RhPh preparations containing less methanol. Reduced methanol solutions were made by diluting the stock 1 to 24 in buffered ASW and by lyophilizing the stock prior to resuspension in buffered ASW. Stained coverslips were rinsed for 5 min with buffered ASW and mounted in rinse containing 0.1% *n*-propyl gallate to prevent photobleaching of rhodamine (Giloh and Sedat, 1982).

Specificity of RhPh binding was tested by incubating fixed, permeabilized cells with unlabeled phalloidin (250 μg/ml in buffered ASW) for 1.5 hr prior to staining with RhPh. As an additional control, growing cells were incubated in KI, which disrupts F-actin organization (Dancker et al., 1975; Hoch and Staples, 1983; Heath, 1987). Cells were treated with 0.6 M KI for 90 min immediately prior to fixing and staining.

RhPh was used to investigate the relationship of F-actin localization to photopolarization. In these experiments, cells were grown on coverslips in ASW in unilateral light beginning 1 hr after fertilization. This light treatment induced a polar axis. At various times thereafter, coverslips bearing attached zygotes were stained and the location of the fluorescence with respect to the orienting light vector was recorded. To correlate these results with events in photopolarization, the time courses of axis formation and fixation were analyzed. Axis formation was assayed by incubating zygotes in unilateral light for various periods beginning at 1 hr postfertilization. At the end of the light treatment, cells were allowed to germinate in uniform light or in the dark. The next day the position of rhizoid outgrowth was scored with respect to the orienting light, either on the shaded or lighted hemisphere. Rhizoids polarized by the light emerged from the shaded hemisphere, whereas those unaffected by the light treatment grew from either hemisphere in equal numbers. Therefore, to calculate the percentage of zygotes polarized by the light, the number of cells bearing rhizoids on the lighted hemisphere was subtracted from the number bearing rhizoids on the shaded hemisphere, and this difference was divided by the total number of cells (>200) and multiplied by 100 ([no. rhizoids on shaded − no. rhizoids on lighted/total no. rhizoids] × 100). The procedure to measure axis fixation has been described previously (Kropf and Quatrano, 1987). Briefly, zygotes were grown in dishes in unilateral light for 8 hr; this treatment was designated light orientation 1 (LO1). At 8 hr and at hourly intervals thereafter, a separate dish was rotated 180° (light orientation 2 [LO2]), and the position of subsequent

rhizoid outgrowth was scored. If the axis were fixed in space during LO1, rhizoids grew from the original dark side (shaded during LO1); if the axis were still labile at the time of rotation, rhizoids grew from the hemisphere shaded during LO2. By scoring the position of rhizoid outgrowth in hundreds of cells, the percentage of cells bearing fixed areas at the time of rotation was determined.

The relationship between F-actin localization and axis fixation was further investigated using cytochalasins. These drugs disrupt axis fixation (Quatrano, 1973), and their effect on F-actin localization was investigated. Cytochalasins B and D (CB and CD, respectively) were maintained as 10 mg/ml stocks in DMSO. Zygotes were grown 8 hr in uniform light and then placed in unilateral light in CB or CD (100 μg/ml in ASW). Four hours later, and at various times thereafter, zygotes were stained with RhPh and the localization of fluorescence with respect to the light vector was scored. As controls, zygotes were treated with 1% DMSO. Drug treatments were not given earlier than 8 hr because of reduced cell viability.

Cells stained with RhPh were observed and photographed using a Universal microscope (Carl Zeiss, Thornwood, NY). Due to the large amount of autofluorescence within these cells from phenolics and chlorophyll, a narrow pass band filter (emission = 580 nm, band width = 20 nm, blocked x-ray to far IR: Pomfret Research Optics, Orange, VA) was used in conjunction with a Zeiss rhodamine filter set. All photography was done using Tri-X Pan 400 film (Eastman Kodak, Rochester, NY). Exposure times ranged from 10 to 45 sec. The film was developed in Microdol X (Eastman Kodak).

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