DEVELOPMENTAL BIOLOGY **194,** 246–256 (1998)
ARTICLE NO. DB978832

Is a Late Event during Polarization of *Pelvetia compressa* Zygotes

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Zygotes and embryos of the fucoid brown alga *Pelvetia compressa* undergo a series of asymmetric cleavages. We are interested in the developmental role of these cleavages and the mechanism controlling their alignment. To assess the importance of division plane alignment, the orientation of the first asymmetric division was altered and the effects on subsequent embryo elongation rates were analyzed. Although this division is normally oriented transversely (90°) to the growth axis, deviations up to 45° had no significant effects on embryo elongation. However, division planes that were parallel with the growth axis (0–45°) had drastic effects. Embryo elongation was severely inhibited and the wall often bifurcated and avoided the rhizoid tip. The orientation of the division plane is determined by the position of the centrosomes. We therefore investigated centrosomal position and function during the first cell cycle within the three-dimensional context of the cell. We found that, after karyogamy, microtubule organization changed from a radially symmetric circumnuclear array into a bipolar centrosomal array. The reorganization coincided with the migration of the centrosomes around the nucleus. The centrosomes separated slowly and asynchronously until they reached opposite sides of the nuclear envelope. At this time the centrosomal axis, defined by the position of the two centrosomes, was oriented randomly with respect to the cortical growth axis. The centrosomal axis then rotated into alignment parallel with the growth axis late in the first cell cycle. These results indicate that the growth axis and the centrosomal axis develop independently of each other and that the centrosomal axis does not align with the growth axis until just prior to mitosis.

INTRODUCTION

In multicellular eukaryotes a single cell, the zygote, ultimately gives rise to all of the different cell types in the organism. How this occurs is a fundamental question in developmental biology. Often diverse cell types are generated when a cell polarizes and then divides asymmetrically to produce nonidentical daughter cells. Such asymmetric divisions are important during early development in *Caenorhabditis elegans*, in neurogenesis in *Drosophila melanogaster* embryos, and during budding in *Saccharomyces cerevisiae* (Guo and Kemphues, 1996; White and Strome, 1996; Doe, 1996a,b). Asymmetric cleavages also occur during early development in the fucoid brown alga *Pelvetia compressa* (Kropf, 1997). We are interested in the developmental role of these cleavages and the mechanism controlling their alignment.

In fucoid algae, the egg is radially symmetrical, and developmental polarity is established soon after fertilization when the zygote attaches to the substratum and determines a rhizoid/thallus axis. The rhizoid/thallus axis defines the growth axis of the embryo, and elongation occurs via tip growth at the rhizoid pole. The growth axis aligns in accordance with a number of environmental vectors. Unidirectional light is commonly used in laboratory experiments to induce a growth axis parallel with the light vector (reviewed in Kropf, 1994, 1992). The first division is asymmetric and is oriented transversely to the growth axis, producing rhizoid and thallus cells with different developmental fates. The thallus cell gives rise to the stipe and fronds which are the photosynthetic and reproductive organs of the mature alga, while the rhizoid cell gives rise to the holdfast that adheres the alga to the substratum (reviewed in Kropf, 1997; Quatrano and Shaw, 1997).

The mechanism for specifying rhizoid and thallus cell fates remains uncertain. One possibility is that the fates are

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0012-1606/98 $25.00

determined by partitioning developmental determinants, although the identity of such molecules is presently unknown. Laser microsurgery experiments indicate that fate-determining molecules may be localized in the rhizoid and thallus cell walls of the polarized zygote, and the first division presumably partitions these determinants (Berger *et al.*, 1994).

Partitioning of determinants implies that division plane alignment is important for subsequent development. To test this, we analyzed the relationship between division plane and subsequent embryo morphology and elongation. Moderately misaligned divisions did not alter early embryogenesis, indicating that precise partitioning of putative determinants is not required. However, longitudinal divisions severely inhibited development, suggesting that the first division may be positioned to avoid bisecting the rhizoid apex.

How, then, is this division aligned? In cells with centrosomes, division plane orientation is determined by the position of the centrosomes (White and Strome, 1996). As in most eukaryotes, the centrosomes in fucoid algae are microtubule-organizing centers with centrioles (Bisgrove *et al.*, 1997; Motomura, 1994; Allen and Kropf, 1992). In *Fucus* and *Pelvetia*, the centriolar components of these centrosomes are acquired with the sperm at fertilization and are deposited on the nuclear envelope at karyogamy (Bisgrove *et al.*, 1997; Motomura, 1994). During the early stages of the first cell cycle, microtubules emanate from the nucleus in a radially symmetric array. Later, sometime prior to the first mitosis, the array reorganizes into a bipolar centrosomal array with microtubules emanating from the perinuclear centrosomes (Bisgrove *et al.*, 1997; Kropf *et al.*, 1990). In addition, the centrosomal axis, defined by a line drawn through the two centrosomes, is brought into alignment with the growth axis (Allen and Kropf, 1992). The spindle then forms from these aligned centrosomes and when the first division occurs it bisects the spindle and the zygote transversely. We have used immunofluorescence with antibodies that recognize centrin and tubulin to investigate the spatial and temporal aspects of centrosomal position and activity during early development.

MATERIALS AND METHODS

Culture

Sexually mature receptacles of *P. compressa* (J. Agardh) were collected near Pigeon Point Lighthouse, just north of Santa Cruz, California. Receptacles were shipped cold and stored at 4°C in the dark until use. To induce the release of fertilized eggs, receptacles were placed in the light (100 $\mu mol \cdot m^{-2} \cdot s^{-1}$) at 16°C in artificial seawater (ASW; 10 mM KCl, 0.45 M NaCl, 9 mM $CaCl_2$, 16 mM $MgSO_4$, and 0.040 $mg \cdot ml^{-1}$ chloramphenicol, buffered to pH 8.2 with 10 mM Tris base) overnight and then transferred to the dark for 30–45 min. The time of fertilization was considered the midpoint of the dark period. Zygotes were allowed to settle on coverslips where they adhered and were cultured in unidirectional light at 16°C. Unfertilized eggs were obtained by inducing gamete release into high-K^+ ASW (0.35 M KCl, 0.1 M NaCl, 9 mM $CaCl_2$, 16 mM $MgSO_4$, and 0.040 $mg \cdot ml^{-1}$ chloramphenicol, buffered to pH 8.2 with 10 mM Tris base) as previously described (Brawley, 1991).

Aberrant division planes were induced by pulse-treating zygotes 12–14 h after fertilization (AF) with either nocodazole (50 or 100 $ng \cdot ml^{-1}$) or oryzalin (0.3 or 0.6 μM), agents that depolymerize microtubules (Morejohn *et al.*, 1987). Final concentrations of inhibitors were obtained by diluting into ASW from stock solutions (10 $mg \cdot ml^{-1}$ nocodazole and 10 mM oryzalin) in dimethyl sulfoxide (DMSO). Final DMSO concentrations never exceeded 0.006%. Inhibitors were removed by rinsing zygotes five times in ASW. The position of the first division plane and embryo viability were determined by staining with fluorescein diacetate (FDA; Kropf *et al.*, 1990; Larkin, 1976), a substrate that is enzymatically cleaved into fluorescein inside cells. FDA was dissolved in acetone (5 $mg \cdot ml^{-1}$) and then diluted to 0.3 $\mu g \cdot ml^{-1}$ in ASW. After visualization, zygotes were rinsed five times with ASW. FDA treatment had no effect on embryo morphology or elongation rate. Zygotes and embryos were observed and photographed daily for 6 days on a Zeiss epifluorescence microscope equipped with digital imaging (Photometrics, Ltd., Tucson, AZ). Embryos were grouped into categories based on the type of first division (transverse, oblique, longitudinal, or bifurcated), and the mean elongation rate was calculated for each category. As the difference among the standard deviations for each category was significant, the data were transformed by taking the log, and an ANOVA was performed on the transformed data.

The effects of cytoskeletal inhibitors on centrosomal separation were investigated using cytochalasin D (CD; 50 or 100 $\mu g \cdot ml^{-1}$ diluted from 20 $mg \cdot ml^{-1}$ stock) to disrupt microfilament function (Kropf *et al.*, 1989; Cooper, 1987) and oryzalin (0.3 or 0.6 μM). The drugs were applied chronically from 4 h AF. CD-treated cells were harvested for immunolabelling 48 h AF and oryzalin-treated zygotes were harvested 18 h AF and centrosomal position was visualized by immunofluorescence.

Immunofluorescence Microscopy

P. compressa zygotes were fixed for 30 min at room temperature using one of two fixation protocols. In experiments in which microtubules alone were visualized, zygotes adhered to coverslips or unattached eggs in Eppendorf tubes were incubated 10 min at 16–20°C in 0.1 M Pipes, 1 mM $MgCl_2$, 5 mM EGTA (ethylene glycol-bis[β-aminoethyl ether]-N,N,N′N′-tetraacetic acid), 0.01% Triton X-100, 20% glycerol, pH 6.8, immersed 1–2 s in liquid N_2, and then transferred immediately to fixative containing 4% w/v paraformaldehyde (or 1.4% formalin), 0.0625% gluteraldehyde, 20% methanol, 1 μM taxol, and 0.1 mM *M*-maleimidobenzoyl *N*-hydroxysuccinimide ester (Pierce, Rockford, IL) in MTAB buffer (80 mM Pipes dipotassium salt, 5 mM EGTA, 1 mM $MgCl_2$, and 20% glycerol).

To visualize centrin or centrin and microtubules combined, zygotes adhered to coverslips were immersed 1–2 s in liquid N_2 then immersed immediately in fixative containing 3% paraformaldehyde, 0.5% gluteraldehyde in PHEM buffer (60 mM Pipes, 25 mM Hepes, 10 mM EGTA, 2 mM $MgCl_2$, pH 7.5) with 4% NaCl.

After fixation, coverslips were immersed a second time (1–2 s) in liquid N_2, then thawed either in the appropriate fixative or in −20°C methanol. Zygotes thawed in fixative were rinsed immediately with 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 (BSA)]. All rinses with mPBS consisted of 3 × 5-min washes. After rinsing, zygotes were incubated at room temperature (RT) overnight in mPBS with 5% Triton X-100 added

to extract pigments. Pigments were alternatively extracted from eggs and zygotes thawed in −20°C methanol by rinsing three times with −20°C methanol over several hours, then slowly rehydrating with mPBS lacking BSA.

Following pigment extraction, all samples were rinsed with mPBS and then incubated overnight at RT in mPBS containing 100 mM $NaBH_4$. The $NaBH_4$ was removed by rinsing with mPBS. Zygotes were then rinsed briefly in C medium (100 mM NaCl, 20 mM $MgCl_2$, 2 mM KCl, 0.2% BSA, 10 mM MES, 0.85 M sorbitol, 1 mM EGTA, with pH adjusted to 5.8 with Tris base) and then incubated 30 min at RT in a cocktail containing cell-wall-degrading enzymes [7 mg·ml^{-1} cellulase (CELF; Worthington Biochemical Corp., Freehold, NJ), 40 mg·ml^{-1} hemicellulase, 7 mg·ml^{-1} abalone gut extract, 0.1 mM PMSF (phenylmethylsulfonyl fluoride)] filtered through glass wool. After the enzyme treatment, zygotes were rinsed briefly in C medium, then with mPBS.

Samples to be labeled with monoclonal anti-centrin antibody 17E10 (a gift from Dr. Salisbury, Mayo Clinic Foundation, Rochester, NY) were incubated 5–10 min at 30°C in 0.005% w/v trypsin in mPBS, rinsed with protease inhibitors (0.01% w/v soybean trypsin inhibitor and 0.1 mM PMSF in mPBS), and then rinsed with mPBS. All samples were blocked overnight at RT in 2.5% w/v nonfat dry milk in mPBS, rinsed with mPBS, and incubated 4 h or longer at RT with primary antibodies [either monoclonal anti-centrin antibodies (diluted 1:100 in mPBS) or monoclonal anti-α-tubulin antibodies (DM1A; Sigma; diluted 1:100 in mPBS)]. Samples were rinsed in mPBS and incubated several hours in rhodamine-conjugated goat anti-mouse IgG (Cappel, Durham, NC; diluted 1:50 in mPBS).

Double-labeled zygotes were incubated first in anti-centrin antibodies, rinsed in mPBS, and then incubated in fluorescein-conjugated goat anti-mouse IgG (Cappel; diluted 1:50 in mPBS). They were then rinsed in mPBS, incubated in anti-α-tubulin antibodies, rinsed in mPBS, and incubated in rhodamine-conjugated goat anti-mouse IgG.

All samples were rinsed with mPBS, extracted with 100% methanol (3× 10-min rinses) and mounted in clearing solution (two parts benzyl benzoate to one part benzyl alcohol) containing 25 mg·ml^{-1} propylgalate. Fluorescence images were obtained on a MRC-600 laser-scanning confocal microscope (Bio-Rad Laboratories, Richmond, CA) using a narrow band pass (578–618 nm) filter set for rhodamine-labeled cells and a dual-channel filter set (K1/K2) for double-labeled cells.

RESULTS

Misalignment of the First Division Inhibited Embryo Elongation

To address the importance of aligning the first asymmetric division, the effects of division plane orientation on subsequent elongation rate and embryo morphology were measured. The orientation of the first division was altered by pulse-treating zygotes with nocodazole (50 or 100 ng·ml^{-1}) or oryzalin (0.3 or 0.6 μM) after they adhered to the substratum and prior to the first division. These agents depolymerize all microtubules in *Pelvetia* (Kropf *et al.*, 1990; S. Bisgrove, unpublished observations); they were chosen because they alter division planes without inhibiting axis formation or polar growth (Kropf *et al.*, 1990). After drug removal, the zygotes were allowed to develop for 4–6 days. During this period, alignment of the first division was measured and subsequent development of individual embryos was monitored daily.

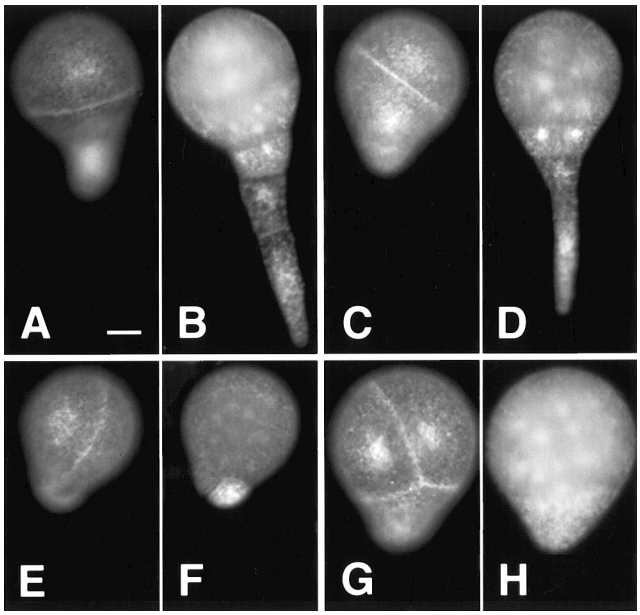

FIG. 1. Effect of misalignment of the first division plane on subsequent embryo morphology. Four embryos, each at two stages of development, are shown. (A, C, E, and G) Separate embryos representing four orientations of the first division plane following removal of microtubule inhibitors are shown. (B, D, F, and H) The same four embryos after 5 days of culture. See text for discussion. Cell walls and nuclei were visualized using FDA. Scale bar, 25 μm.

Modest misalignment of the first division had little effect on morphology or growth. Zygotes in which the first division was correctly aligned transversely to the growth axis elongated an average of 0.8 μm/h via rhizoidal tip growth over 4–6 days (Figs. 1A, 1B, and 2). Zygotes in which the cell plate was oblique but did not bisect the rhizoid (45–67°; Figs. 1C, 1D, and 2) elongated at 0.7 μm/h, which was not significantly different from the transversely aligned controls ($P > 0.05$).

More severe misalignment (0–44°) resulted in longitudinal divisions in which the cell plate entered the rhizoid (Fig. 1E). The average rate of elongation in these embryos (0.1 μm/h) was significantly less than that of embryos with correctly aligned divisions ($P < 0.001$; Fig. 2). In embryos in which the division angle was less than 10° (4 embryos), no growth occurred at all. Embryos with larger division angles did elongate and growth rates were higher in embryos with larger division angles (11 embryos). Embryos with severely offset divisions often remained viable as they underwent multiple rounds of cell division despite the lack of elongation (Fig. 1F). In many zygotes that attempted to divide longitudinally, the cell plate bifurcated near the rhizoid, forming a three-celled embryo (Fig. 1G). The average rate of elongation in these embryos (0.2 μm/h) was also significantly reduced compared with that of embryos with normal

divisions ($P < 0.001$; Fig. 2). In some of these embryos the rhizoid cell failed to inherit a nucleus (Fig. 1G) and these embryos did not elongate, although they remained viable and the thallus cells underwent several rounds of mitosis (Fig. 1H).

These results indicate that division plane alignment is important for development. Because the orientation of the division plane is determined by spindle alignment which is, in turn, determined by centrosomal position, we conducted a detailed analysis of centrosomal position and activity.

Analysis of Centrosomes during Development

Zygotic microtubules reorganize from a circumnuclear array into a centrosomal array. Previously, we showed that some time prior to spindle formation, zygotic microtubules reorganize from a radially symmetric circumnuclear array into a bipolar centrosomal array in which microtubules emanate from two perinuclear centrosomes (Bisgrove *et al.*, 1997; Allen and Kropf, 1992; Kropf *et al.*, 1990). We have investigated the developmental progression of this reorganization (Figs. 3 and 4) using antibodies to centrin and α-tubulin. In unfertilized eggs and young zygotes, microtubules emanated uniformly from the nuclear periphery and extended toward the cortex in a radially symmetric, circumnuclear array (Figs. 3A and 4). At 4–6 h AF, microtubules in many zygotes emanated more prominently from a single site on the nuclear envelope (Figs. 3B and 4). Two foci of microtubules became apparent in zygotes 6–10 h AF (Figs. 3C and 4) and by 10–12 h AF few cells with one focus remained (Fig. 4). Double-labeling experiments with antibodies that recognize α-tubulin and the centrosomal protein centrin showed that the microtubule foci were coincident with centrin and were therefore likely to be centrosomes (Fig. 3D).

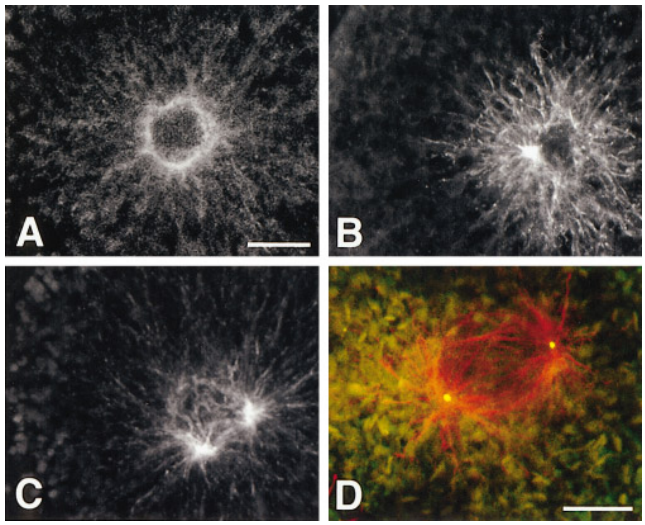

FIG. 3. Microtubule reorganization during the first cell cycle. Zygotes were stained with anti-α-tubulin antibodies (A, B, and C) or were double-labeled with anti-centrin and anti-α-tubulin antibodies (D). Microtubules initially emanated from the nuclear periphery in a circumnuclear array (A). As development proceeded, there was a transition to nucleation from one perinuclear focus (B) and then two perinuclear foci (C). (D) Centrin was localized at the perinuclear foci. This double-labeled image has been false-colored with centrin shown as two yellow foci and microtubules shown in red. Scale bars, 10 μm, and the bar shown in A also applies to B and C.

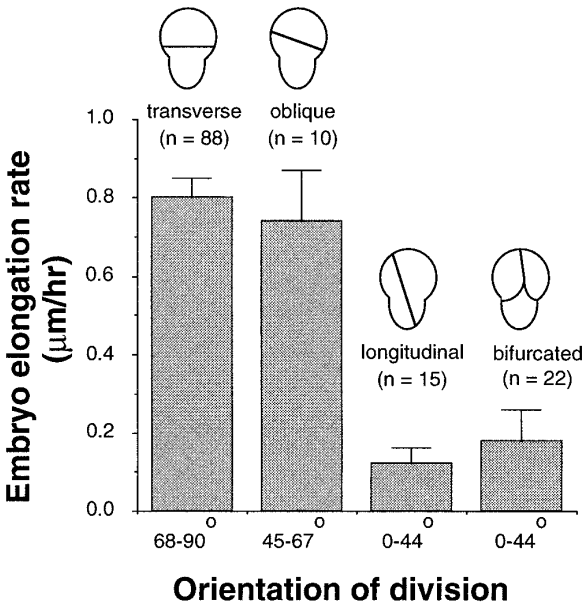

FIG. 2. Division plane orientation and embryo elongation rates. The angle of the first division with respect to the growth axis was measured on 135 embryos, and for each embryo the length was measured daily for 4–6 days. Data from two experiments were pooled. Bars show the mean ± standard error (SE).

Centrosomal separation is slow and asynchronous. Centrosomal position was monitored within the three-dimensional context of the cell. A series of optical sections through the nuclear region of each cell was collected and the distance separating the centrosomes was calculated trigonometrically as shown in Fig. 5A. Analysis of the time course of centrosomal separation indicated that separation was slow (Fig. 5B). Centrosomes in intermediate stages of separation were found at all time points. In addition, zygotes in all stages of the separation process were found at each time point between 4 and 12 h AF (Figs. 4 and 5B), indicating that separation was also asynchronous. As the centrosomes separated, the transition from circumnuclear to centrosomal microtubule arrays was also progressing (compare Fig. 5B with Fig. 4). By 10 h AF, many embryos had initiated polar growth.

Centrosomal separation requires microtubules but not F-actin. To investigate the roles of microfilaments and microtubules in centrosomal separation, zygotes were treated chronically from 4 h AF with CD to disrupt F-actin function (25 or 50 mg · ml^{-1}) or with oryzalin to depolymerize microtubules (0.3 or 0.6 μM). Although CD-treated zygotes did not

form rhizoids and were unable to complete cytokinesis, they underwent several rounds of mitosis forming spherical, multinucleate cells (Kropf *et al.*, 1989; Quatrano, 1973). The nuclei of 48-h-AF CD-treated zygotes had well-separated centrosomes and often contained multiple bipolar spindles. One spindle in such a cell is shown in Fig. 6A; the other spindles are out of the plane of focus. Oryzalin-treated zygotes, on the other hand, polarized and initiated rhizoidal growth, but failed to enter mitosis (Fig. 6B; Kropf *et al.*, 1990). Two closely associated centrosomes were present on the nucleus of 18-h-old zygotes (Fig. 6B, inset). Untreated controls sampled at the same stage had completed separation (data not shown). Centrosomal separation, therefore, depended on intact microtubules but not on functional F-actin.

Alignment of centrosomal and growth axes occurs late in the first cell cycle. Previous studies have shown that the centrosomal axis is aligned parallel with the growth axis prior to mitotic spindle formation (Allen and Kropf, 1992). To determine when this alignment occurred, we measured the angle (θ) between the centrosomal axis and the growth axis within the three-dimensional context of the cell (Fig. 7).

The centrosomal axis was randomly aligned during centrosomal separation and then became aligned parallel with the growth axis late in the first cell cycle, well after centrosomal separation was complete (Figs. 8 and 9). In zygotes sampled 10 and 12 h AF, centrosomes were found in various stages of separation (Figs. 8A and 8B) and were randomly aligned with respect to the growth axis (Figs. 9A and 9B). By 15 h AF, centrosomes in most zygotes had completed separation (Fig. 8C); however, they were still randomly aligned with respect to the growth axis (Fig. 9C). A few zygotes had entered mitosis, and in all but one of these (21/22), the spindle was aligned within 35° of the growth axis. By 18 h AF, centrosomal separation was complete in

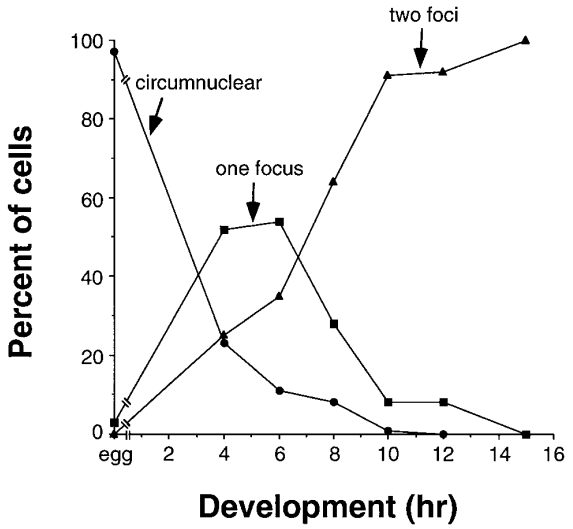

FIG. 4. Time course of microtubule reorganization. Eggs and zygotes were fixed and stained with anti-α-tubulin antibodies, and the percentage of cells with circumnuclear arrays and arrays with one or two foci of microtubules were scored. At each time point, 30–94 cells were sampled from two or three different populations.

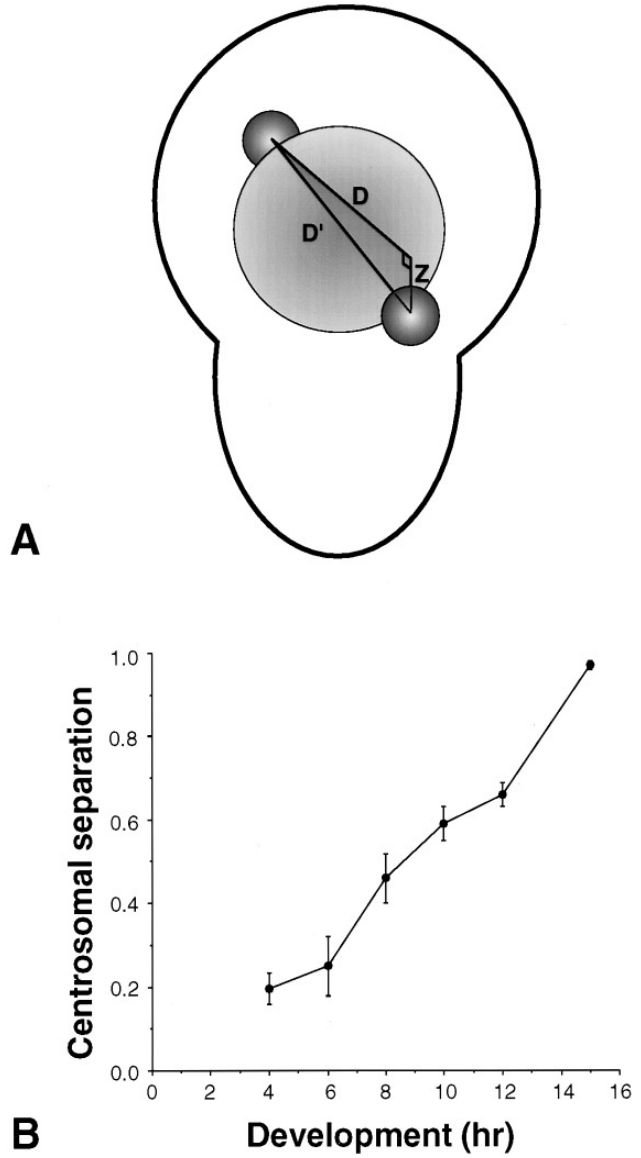

FIG. 5. (A) Analysis of centrosomal separation within the three-dimensional context of the zygote. A series of optical sections (1 μm thick) through the nuclear region of a fluorescently labeled zygote was collected by laser-scanning confocal microscopy and the distance separating the centrosomes was calculated trigonometrically. Centrosomal separation in the *z* dimension (line Z) was determined from the number of optical sections, and the distance between the centrosomes in the *xy* plane (line D) was measured from a projection of all sections. These were used as legs in a right triangle and the distance separating the centrosomes within the three-dimensional context of the cell (line D′) was calculated by the Pythagorean theorem. (B) Time course of centrosomal separation. Centrosomal separation was measured on 37–94 cells at each time point and normalized to nuclear diameter. Data points are means ± SE.

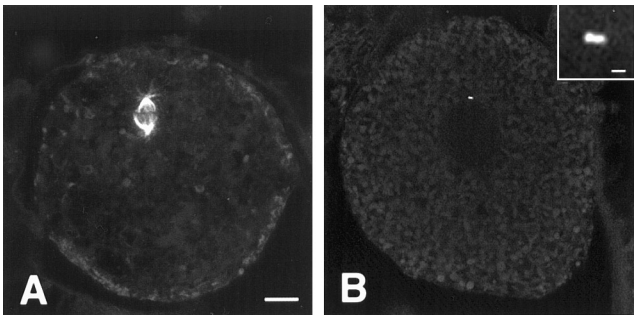

FIG. 6. Effects of CD or oryzalin on centrosomal separation. (A) Zygote treated from 4 to 48 h AF with CD. One spindle in this multinucleate cell is shown by microtubule immunofluorescence. (B) Zygote treated from 4 to 18 h AF with oryzalin germinated, but centrin immunofluorescence showed that the two centrosomes remain close together on the nuclear envelope. Rhizoid is down and to the right. Inset is a magnification of the two closely associated centrin foci. Scale bar shown in A is 10 μm, and also applies to B. Scale bar in the inset, 1 μm.

all zygotes and most (48/55) had entered mitosis. Again, centrosomes in premitotic zygotes were randomly aligned with the growth axis, while in mitotic zygotes, the spindles were aligned with the growth axis (Figs. 8D and 9D). Spindle alignment in mitotic cells is better at 18 h than at 15 hr, indicating that alignment continues during mitosis.

In the rhizoid cell, centrosomes separate before they align. As the next division in the rhizoid lineage also occurs transverse to the growth axis, we investigated the timing of the alignment of the centrosomal and growth axes in the rhizoid cell. At telophase of the first division, the rhizoid nucleus had a single centrosome positioned on the side of the nucleus closest to the rhizoid tip (Fig. 10A). This centrosome duplicated (Fig. 10B) and the two centrosomes migrated apart uniformly (Fig. 10C) until they reached positions on opposite sides of the nuclear envelope. At this stage, the centrosomal axis was oriented transversely to the growth axis (Fig. 10D). A rotational alignment of 90° occurred after centrosomal separation was complete. One of the centrosomes migrated toward the rhizoid apex while the other moved basally (Fig. 10E), bringing them into alignment parallel with the growth axis of the embryo (Fig. 10F).

Divisions in the rhizoid lineage are aligned in accordance with the rhizoid tip. F-actin in the rhizoid tip is involved in division plane alignment in the zygote (Allen and Kropf, 1992; Kropf *et al.*, 1989). Because actin in the rhizoid cell of the two-celled embryo is localized to both the rhizoid tip and the cell plate from the first division (Kropf *et al.*, 1992), either site could provide the positional information for centrosomal and subsequent division plane alignment. To determine which site the division plane preferentially aligned with, the spatial relationship between the growth axis and the division plane was altered by changing the direction of rhizoid growth after the first division. This was accomplished by changing the light vector by 90°; rhizoids are negatively phototropic and therefore grew away from the new light vector (Fig. 11). Divisions that occurred after the change in growth direction were aligned transversely to the new growth axis and were not correlated with the orientation of the first division plane (arrows in Fig. 11).

DISCUSSION

Division Plane Alignment

The first cleavage in fucoid algae produces two morphologically and physiologically distinct cells with different developmental fates (Kropf, 1992, 1994), suggesting that division plane orientation is important for partitioning determinants. Our results indicate that *Pelvetia* zygotes are capable of tolerating some misalignment of the first division. It is, therefore, unlikely that the cell plate precisely partitions cytoplasmic determinants that are segregated in broad do-

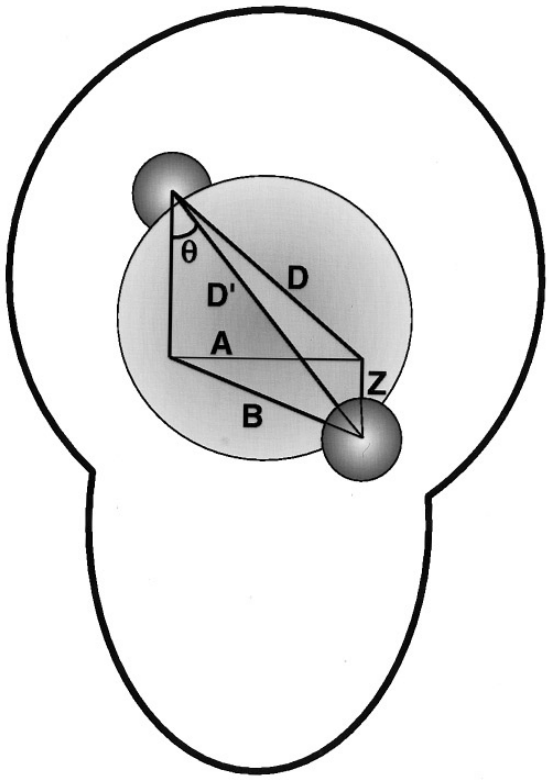

FIG. 7. Method used to calculate alignment of growth and centrosomal axes. The angle θ was calculated trigonometrically on cells that had initiated polar growth as follows. First, the distance separating the centrosomes along a line perpendicular to the growth axis (line A) was measured on a projected image constructed from a series of optical sections. The distance Z was obtained from the number of optical sections separating the two centrosomes, and the length of the hypotenuse (line B) was calculated. The true distance separating the centrosomes (line D′) was calculated as described in Fig. 5A. Line D′ and line B were then used as legs in a right triangle, and θ was calculated using the formula $\sin \theta = B/D'$.

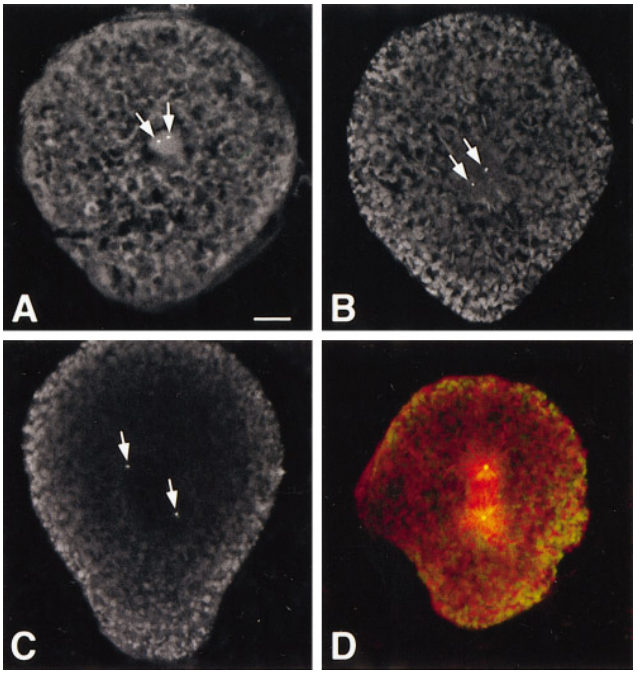

FIG. 8. Centrosomal separation, alignment, and spindle formation during the first cell cycle. The rhizoid/thallus axis is oriented from the top to the bottom in all images. Zygotes were stained with anti-centrin antibodies (A, B, and C) or were double-labeled with anti-centrin and anti-α-tubulin antibodies (D). Centrosomes located close together were found in random alignments with respect to the growth axis (A and B) as were fully separated centrosomes in premitotic cells (C). In mitotic zygotes, spindles were aligned with the growth axis, and centrin labeled the spindle poles (D). D is false-colored with centrin shown in yellow and microtubules shown in red. Scale bar, 10 μm.

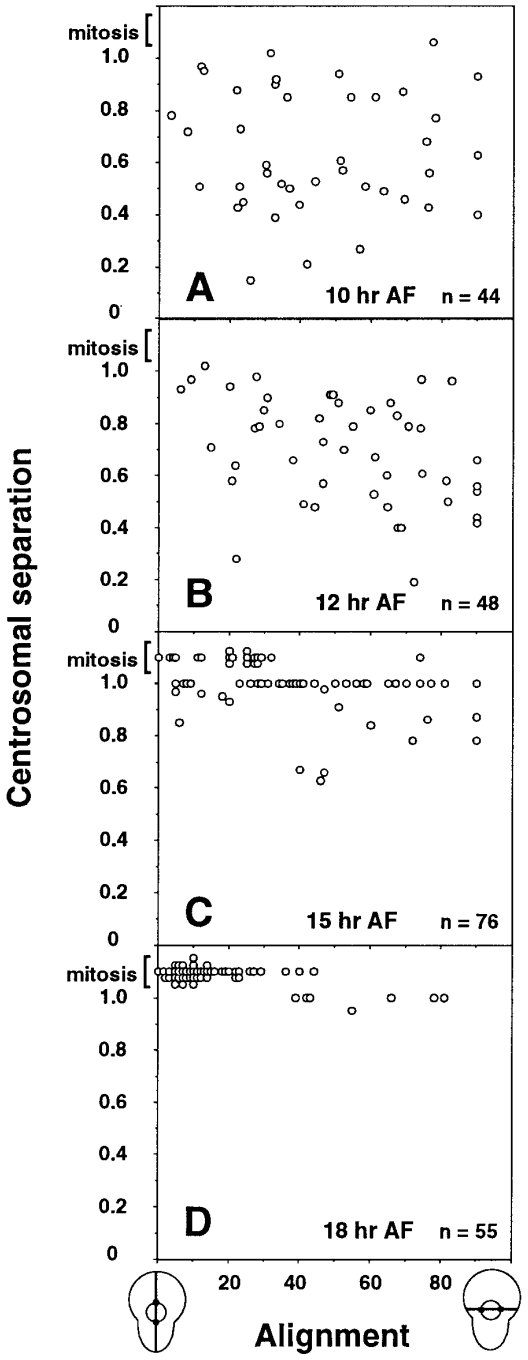

FIG. 9. Centrosomal separation and alignment during the first cell cycle. For each zygote centrosomal separation, normalized to nuclear diameter, was plotted against the angle of alignment with respect to the growth axis. Each point represents a single zygote. In zygotes sampled 10 (A) and 12 h AF (B), centrosomes were found in various stages of separation and were randomly aligned with the growth axis. At 15 (C) and 18 h AF (D), centrosomes in premitotic zygotes were fully separated and randomly aligned, while spindles in mitotic zygotes were preferentially aligned with the growth axis.

mains. Instead, if fate-determining factors are differentially partitioned, they must be tightly associated with the cortical poles of the zygote because only longitudinal divisions blocked rhizoid elongation. There is evidence that suggests determinants may exist in the cell wall. Using laser microsurgery on two-celled embryos, Berger *et al.* (1994) have shown that the cell wall of the rhizoid cell can induce a thallus cell to assume a rhizoid-like morphology. It would be interesting to determine whether this inducing activity is tightly localized to the rhizoid pole.

The first division plane may also be positioned to avoid bisecting the growth zone in the rhizoid apex. A cell plate that intersected the growing tip would be expected to interfere with elongation of that apex. To continue elongating, the rhizoid would have to extend new cell wall in pace with the growing tip, which seems unlikely. Alternatively, following a longitudinal division, apical elongation could cease entirely, or the embryo could organize a new tip from one or both of the daughter cells. Our findings indicate that longitudinally oriented cell plates often bifurcated, as if avoiding the tip region. When the cell plate did bisect the

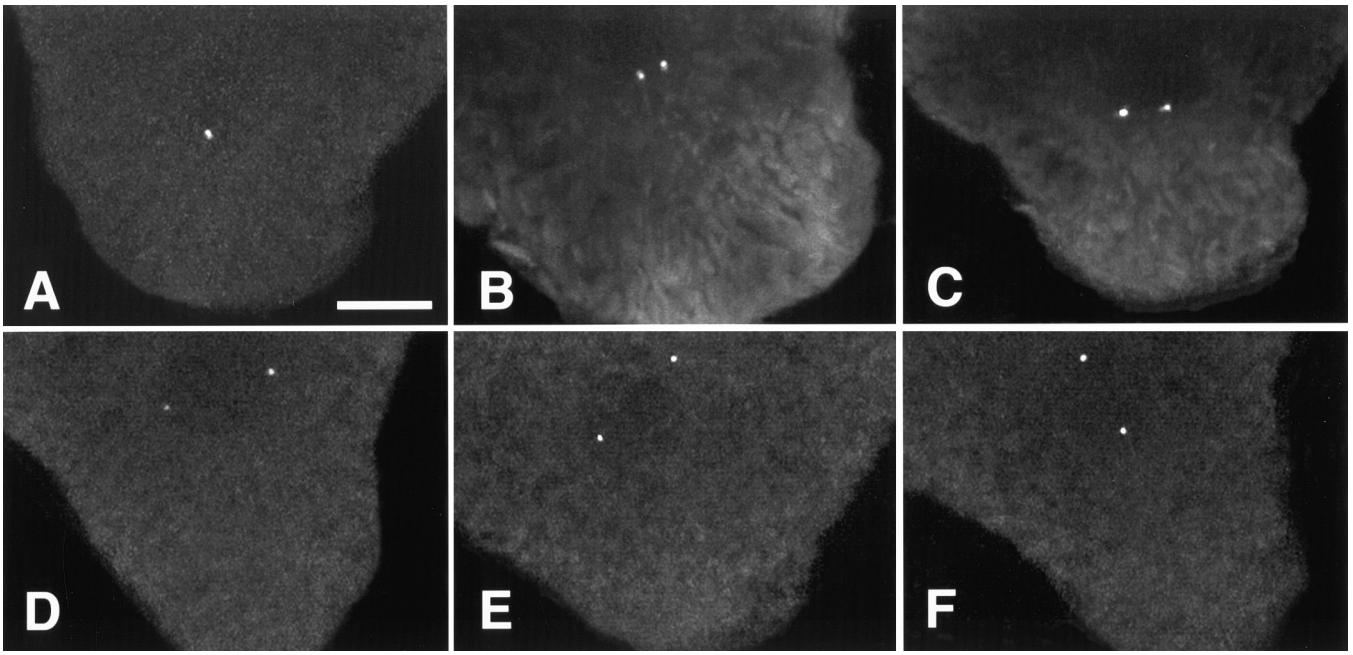

FIG. 10. Centrosomal separation in the rhizoid cell of a two-celled embryo. Zygotes were stained with anti-centrin antibodies (A–F). At telophase of the first division, the rhizoid nucleus acquired a single centrosome (A), which duplicated (B), and the two centrosomes moved apart from each other evenly (C) until they reached positions on opposite sides of the nucleus and were transverse to the growth axis (D). Fully separated centrosomes rotated (E) into axial alignment (F). Rhizoid is down and slightly to the right in all images. Scale bar, 10 μm.

rhizoid apex ($n = 4$), tip growth was arrested, but new tips were not initiated.

In contrast, Shaw and Quatrano (1996) reported that longitudinal divisions in zygotes of a closely related alga, *Fucus distichus*, correlated with the formation and elongation of two rhizoids. The apparent discrepancy in the two sets of data may relate to the inhibitors used. Division plane orientation in their experiments was altered following treatment with the Golgi inhibitor brefeldin A. Brefeldin A inhibited rhizoid growth by disrupting the secretory pathway, and it is possible that multiple secretory sites at the cell surface were organized upon release from the drug. In our experiments, treatment with oryzalin and nocodazole did not abolish polar secretion at the tip and rhizoids continued to elongate. After release from these drugs, rhizoids were actively growing and longitudinal divisions that bisected the growth site stopped growth. Because secretion was not disrupted, multiple secretory sites were not initiated. Regardless of the mechanism, both sets of data support the hypothesis that longitudinal division planes perturb development.

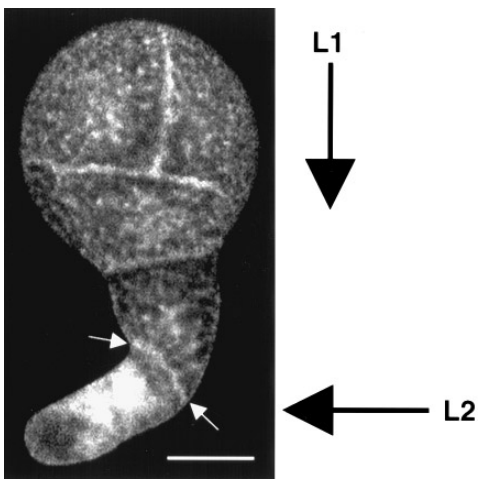

FIG. 11. Cell plates in the rhizoid cell preferentially align in accordance with the rhizoid tip. Rhizoid bending was induced by rotating the unidirectional light from L1 to L2 after the first division. Negatively phototropic rhizoids grew away from L2. The cell plate that formed after the change in growth direction (arrows) was aligned transversely to the new growth axis. Cell plates were stained with FDA. Scale bar, 50 μm.

The Centrosomal and Growth Axes Develop Independently

We previously postulated that centrosomes may first align transversely to the growth axis and then rotate a full 90° into parallel alignment (Allen and Kropf, 1992). This would imply that the two axes share spatial information during development. Our current findings, however, do not support this hypothesis and instead indicate that the two axes develop independently.

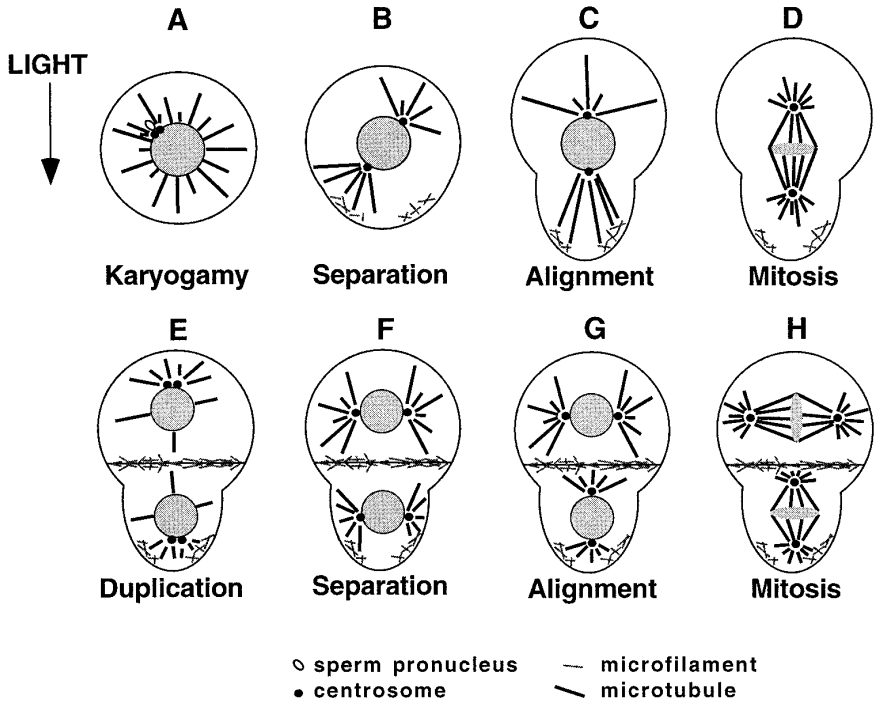

FIG. 12. Centrosomal positions during the first (A–D) and second (E–H) cell cycles in *Pelvetia*. (A) Centrioles are deposited on the nuclear envelope at karyogamy (2 h AF). (B) Over the next few hours, the centrosomes migrate apart from each other until they reach opposite sides of the nuclear envelope. During this time, the cortical growth axis orients in response to perceived vectors, such as unidirectional light (arrow), and rhizoid growth begins about 10 h AF. (C) Alignment of the centrosomal axis occurs just prior to or during mitosis (18 h AF). (D) The mitotic spindle is aligned with the cortical growth axis and the plane of the first cell division bisects the spindle and the zygote transversely. (E) The single centrosome of each daughter duplicates immediately after division. (F) The two centrosomes in each cell migrate apart from each other until they reach positions on opposite sides of the nuclear envelope. At this stage the centrosomal axes in both rhizoid and thallus cells are oriented transverse to the growth axis. (G) In the rhizoid cell there is a 90° rotational alignment that occurs after centrosomal separation is complete. This rotation brings the centrosomes in the rhizoid cell into alignment parallel with the growth axis of the embryo. (H) The second mitotic spindle in the thallus cell forms transverse to the growth axis while the second spindle in the rhizoid cell forms parallel to it.

The centrosomal axis is established at karyogamy with the deposition of centrioles from the fertilizing sperm onto the egg nuclear envelope (Fig. 12A; Bisgrove *et al.*, 1997; Motomura, 1994). Sperm entry also marks a site in the cortex of the zygote, but whether this site establishes an initial growth axis is unknown (Kropf, 1997). After fertilization, vectors such as unidirectional light orient the growth axis (Jaffe, 1968), but our findings show that vectors have no effect on the centrosomal axis (Fig. 12B). Therefore, the centrosomal axis is randomly oriented with respect to an induced growth axis. The growth axis and the centrosomal axis remain spatially independent until mitosis.

The developmental time courses and cytoskeletal dependencies of the two axes provide additional evidence that they are independent. Centrosomal separation is a slow and asynchronous process, whereas the growth axis develops rapidly and synchronously (Kropf, 1997; W. E. Hable and D. L. Kropf, personal communication); it is permanently fixed in space by 10 h, before centrosomal separation is complete (Fig. 12B). The cytoskeletal requirements of the two axes are also different. Microtubule antagonists inhibit development of the centrosomal axis but not the growth axis, whereas F-actin antagonists block establishment of the growth axis. Taken together, these data indicate that positional cues are not being transduced from the growth axis to the centrosomal axis as they develop.

Alignment of Centrosomal and Growth Axes

Alignment occurs by a rotation of the nucleus so that the centrosomal axis aligns parallel with the cortical growth axis (Figs. 12C and 12D; Allen and Kropf, 1992). We report here that this alignment is a late event, occurring at or just prior to the onset of mitosis. This is as late as alignment can possibly occur in order to ensure an axial spindle and transverse division plane. In fact, it appears that final adjustments are still being made during mitosis.

Alignment occurs about 3 h after the completion of centrosomal separation and 8 h after rhizoid/thallus axis fixation and the initiation of polar growth. As the rhizoid/

thallus axis is initially labile, it is reasonable that the centrosomes are not aligned until after the rhizoid/thallus axis is permanently fixed in place. However, a reason for delaying alignment for 8 h or more after fixation of the growth axis is not clear. One possibility is that the cues that initiate alignment are linked to the cell cycle and rotational alignment is triggered as the zygote progresses into mitosis.

Although the mechanism of rotational alignment in *Pelvetia* is unknown, two models by which spindles can be oriented in dividing cells have been described. In one, microtubules emanating from the centrosomes interact with the cell surface, and polymerization of these microtubules exerts force on the spindle poles. The spindle ultimately positions where the net force from the astral microtubules on the spindle poles is zero (Bjerknes, 1986).

Alternatively, in *S. cerevisiae* and *C. elegans* spindle alignment is postulated to occur via microtubule motor proteins acting on astral microtubules that interact with localized F-actin in the cell cortex. A cortical patch of actin and capping protein is thought to serve as a site to attach a dynactin complex containing dynein. Ratcheting by dynein, a minus-end-directed motor, along the astral microtubules pulls one centrosome toward the cortical site (White and Strome, 1996; Palmer *et al.*, 1992). During rotational alignment in *Pelvetia*, the nucleus often appears bent or crescent shaped, as if it is being pulled toward the rhizoid cortex (S. Bisgrove, unpublished observations; Allen and Kropf, 1992). For this reason, we propose that rotational alignment in *Pelvetia* occurs by a pulling force on the centrosomes, similar to the mechanism proposed to occur in *S. cerevisiae* and *C. elegans*.

Division Plane Alignment in the Second Cell Cycle

The next few divisions in the rhizoid lineage also occur transverse to the growth axis. Unlike the first cell cycle, there is a defined spatial relationship between the centrosomal and the growth axes in the second cell cycle. At telophase of the first division, the rhizoid nucleus acquires a single centrosome positioned on the same side of the nucleus as the rhizoid tip. After duplication (Fig. 12E), the two centrosomes migrate apart uniformly, and when completely separated they are oriented transversely to the growth axis (Fig. 12F). In the rhizoid cell, centrosomes in midseparation are always aligned transversely to the growth axis and only fully separated centrosomes are found in other orientations. Therefore, alignment in the rhizoid cell is accomplished via a full 90° nuclear rotation that occurs after centrosomal separation is complete (Figs. 12G and 12H). The fact that centrosomes do not align during separation in the rhizoid cell is interesting because it indicates that the cues that trigger rotational alignment in the first cell cycle are transient.

When rhizoids of divided embryos are induced to change their growth direction, cell divisions in the apical, tip-growing cell align transversely to the new growth axis, independent of the orientation of the previous division plane. Therefore, the rhizoid tip serves as a positional cue for rotational alignment in rhizoid cells, even though F-actin is present both in the rhizoid tip and at the cell plate from the first division (Kropf *et al.*, 1992). We presume that the first zygotic division would also be altered if rhizoid growth direction were changed prior to division, but this experiment is not feasible because the rhizoid is very short at the time of first division and growth direction cannot be accurately assessed. Thus, we propose that rotational alignment of the centrosomal axis is always oriented by the position of the growing tip.

ACKNOWLEDGMENTS

We thank Drs. Whitney Hable, Lilian Alessa, Brent Bisgrove, and David Gard for critically reviewing the manuscript. This work was supported by NSF Award IBN 95066094 to D.L.K.

REFERENCES

Allen, V. W., and Kropf, D. L. (1992). Nuclear rotation and lineage specification in *Pelvetia* embryos. *Development* **115,** 873–883.

Berger, F., Taylor, A., and Brownlee, C. (1994). Cell fate determination by the cell wall in early *Fucus* development. *Science* **263,** 1421–1423.

Bisgrove, S. R., Nagasato, C., Motomura, T., and Kropf, D. L. (1997). Immunolocalization of centrin in *Fucus distichus* and *Pelvetia compressa* (Fucales, Phaeophyceae). *J. Phycol.* **33,** 823–829.

Bjerknes, M. (1986). Physical theory of the orientation of astral mitotic spindles. *Science* **234,** 1413–1416.

Brawley, S. H. (1991). The fast block to polyspermy in fucoid algae is an electrical block. *Dev. Biol.* **144,** 94–106.

Cooper, J. A. (1987). Effects of cytochalasin and phalloidin on actin. *J. Cell Biol.* **105,** 1473–1478.

Doe, C. Q. (1996a). Asymmetric cell division and neurogenesis. *Curr. Opin. Genet. Dev.* **6,** 562–566.

Doe, C. Q. (1996b). Spindle orientation and asymmetric localization in Drosophila: Both inscuteable? *Cell* **86,** 695–697.

Guo, S., and Kemphues, K. J. (1996). Molecular genetics of asymmetric cleavage in the early *Caenorhabditis elegans* embryo. *Curr. Opin. Genet. Dev.* **6,** 408–415.

Jaffe, L. F. (1968). Localization in the developing *Fucus* egg and the general role of localizing currents. *Adv. Morphol.* **7,** 295–328.

Kropf, D. L. (1992). Establishment and expression of cellular polarity in fucoid zygotes. *Microbiol. Rev.* **56,** 316–339.

Kropf, D. L. (1994). Cytoskeletal control of cell polarity in a plant zygote. *Dev. Biol.* **165,** 361–371.

Kropf, D. L. (1997). Induction of polarity in fucoid zygotes. *Plant Cell* **9,** 1011–1020.

Kropf, D. L., Berge, S. K., and Quatrano, R. S. (1989). Actin localization during *Fucus* embryogenesis. *Plant Cell* **1,** 191–200.

Kropf, D. L., Coffman, H. R., Kloareg, B., Glenn, P., and Allen, V. W. (1993). Cell wall and rhizoid polarity in *Pelvetia* embryos. *Dev. Biol.* **160,** 303–314.

Kropf, D. L., Jordan, J. R., Allen, V. W., and Gibbon, B. C. (1992). Cellular polarity in *Pelvetia* zygotes: Studies of intracellular pH and division alignment. *Curr. Top. Plant Biochem. Physiol.* **11,** 143–152.

Kropf, D. L., Maddock, A., and Gard, D. L. (1990). Microtubule dis-

tribution and function in early *Pelvetia* development. *J. Cell Sci.* **97,** 545–552.

Larkin, P. J. (1976). Purification and viability determinations of plant protoplasts. *Planta* **128,** 213–216.

Morejohn, L. C., Bureau, T. E., Mole-Bajer, J., Bajer, A. S., and Fosket, D. E. (1987). Oryzalin, a dinotroaniline herbicide, binds to plant tubulin and inhibits microtubule polymerization in vitro. *Planta* **172,** 252–264.

Motomura, T. (1994). Electron and immunofluorescence microscopy on the fertilization of *Fucus distichus* (Fucales, Phaeophyceae). *Protoplasma* **178,** 97–110.

Palmer, R. E., Sullivan, D. S., Huffaker, T., and Koshland, D. (1992). Role of astral microtubules and actin in spindle orientation and migration in the budding yeast *Saccharomyces cerevisiae. J. Cell Biol.* **119,** 583–593.

Quatrano, R. S. (1973). Separation of processes associated with differentiation of two-celled *Fucus* embryos. *Dev. Biol.* **30,** 209–213.

Quatrano, R. S., and Shaw, S. L. (1997). Role of the cell wall in the determination of cell polarity and the plane of cell division in *Fucus* embryos. *Trends Plant Sci.* **2,** 15–21.

Shaw, S. L., and Quatrano, R. S. (1996). The role of targeted secretion in the establishment of cell polarity and the orientation of the division plane in *Fucus* zygotes. *Development* **122,** 2623–2630.

White, J., and Strome, S. (1996). Cleavage plane specification in *C. elegans:* How to divide the spoils. *Cell* **84,** 195–198.

Received for publication October 20, 1997
Accepted December 15, 1997