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Nuclear rotation and lineage specification in *Pelvetia* embryos

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Summary

The first division of *Pelvetia* zygotes is an unequal division which produces two cells with distinct developmental fates. The smaller rhizoid cell gives rise to the holdfast of the mature plant, and the larger thallus cell is the progenitor of the stipe and fronds. We have investigated the role of the cytoskeleton in determining the orientation of this invariant division. Prior to mitosis, microtubule-organizing centers (MtOCs) associated with the nuclear envelope undergo a precise realignment from transverse to axial with respect to the rhizoid/thallus axis. This is accomplished by a 90° rotation of the entire nuclear/MtOC complex. After rotation, each MtOC serves as a spindle pole during mitosis, and subsequently cytokinesis bisects the spindle. Both nocodazole and cytochalasin D cause incorrect alignment of MtOCs, indicating that both microtubules and microfilaments are required for nuclear/MtOC rotation. These inhibitors also result in aberrant orientation of the first division plane. Microtubules visualized by confocal microscopy connect the rotating nucleus to the apical cortex and may provide the force for rotation.

Key words: nuclear rotation, division plane, cytoskeleton, embryogenesis, *Pelvetia*, brown algae.

Introduction

Deterministic divisions, in which daughter cells have distinct fates, are essential for differentiation of tissues and specification of cell lineages during development. The clearest examples of deterministic divisions are unequal divisions producing morphologically distinct progeny, usually a larger and a smaller cell. In plant development, unequal, deterministic divisions control differentiation of guard cells in stomata, hair cells on roots, and sieve tube/companion cells in the phloem (Lyndon, 1990; Palevitz, 1986). These divisions are thought to partition unique sets of cytoplasmic determinants to the two cells and thereby induce distinct morphogenetic programs.

Partitioning of cytoplasmic determinants relies upon invariant positioning of the division plane. If the division plane is misaligned, determinants are segregated improperly and subsequent development is abnormal. In higher plants, orientation of division plane is determined by a membranous phragmosome bisecting the cytoplasm and/or by a preprophase band (PPB) in the cell cortex (for reviews see Gunning and Wick, 1985; Lloyd, 1991a,b). A PPB is a cortical hoop of microfilaments and microtubules that marks the division site where the centrifugally growing cell plate meets the parental wall. Although microtubules disappear before cytokinesis, the microfilaments remain and guide cell plate growth (Lloyd and Traas, 1988). Large vacuolated cells more commonly form a membranous phragmosome across the cell diameter; the nascent wall forms within the phragmosome and grows centrifugally to the parental wall. Positioning of PPBs and phragmosomes is dependent upon cytoskeletal filaments (Lloyd, 1991a). Invariant division planes are also important in development and differentiation of lower plants and algae, but many of these organisms do not form phragmosomes or PPBs. Instead, the division plane is thought to be determined directly by spindle orientation (Doonan et al., 1985; Kropf et al., 1990; Schmiedel et al., 1981), yet little is known concerning the mechanisms that position the spindle.

Zygotes of fucoid algae are excellent model organisms for investigating the mechanisms controlling spindle orientation and division plane in the absence of phragmosomes and PPBs. The first zygotic division in *Pelvetia* is an unequal, deterministic division oriented transverse to the embryonic growth axis, creating a smaller rhizoid cell and a larger thallus cell (Quatrano, 1978). The rhizoid cell is the progenitor of the holdfast, or root system of the plant, and the thallus cell gives rise to the fronds and stipe. Organelles are partitioned unequally at first division; mitochondria, Golgi, Golgi vesicles and cortical actin are preferentially localized in the rhizoid cell, the site of active tip growth (Brawley and Quatrano, 1979; Quatrano, 1972), whereas chloroplasts are partitioned mainly to the thallus cell, the progenitor of photosynthetic tissues (Brawley and Quatrano, 1979).

We have been studying the role of the cytoskeleton in early development and have speculated that mitosis and cytokinesis are oriented by two microtubule-organizing centers (MtOCs) closely associated with the nuclear membrane (Kropf et al., 1990). The orientation of the axis

defined by these MtOCs is at first transverse to the rhizoid/thallus axis, but prior to mitosis, MtOCs rotate 90° and serve as spindle poles. The mechanism of MtOC rotation and its relation to spindle alignment and division plane are the subjects of this report. Both microfilament- and microtubule-depolymerizing agents [cytochalasin D (CD) and nocodazole, respectively] disrupt rotation and cause misalignment of the first division plane, indicating that (1) rotation is dependent on the cytoskeleton and (2) MtOC orientation determines the plane of division. This mechanism for controlling division plane is distinctly different from that in higher plants, and instead resembles lineage specification during embryogenesis in some animals, in particular *Caenorhabditis elegan*s (Hyman, 1989). This similarity indicates that control of division plane by cytoskeletal-dependent nuclear rotation either evolved quite early in eukaryotes or is a striking example of convergent evolution.

Materials and methods

Algal culture

Pelvetia fastigiata receptacles were collected and shipped cold by Sea Life Supply (Sand City, CA). Receptacles were stored at 4°C for up to two weeks in the dark. To release fertilized eggs, receptacles were placed in the light at 14°C in artificial sea water (ASW; 0.45 M NaCl, 10 mM KCl, 9 mM $CaCl_2$, 16 mM $MgSO_4$, and 0.040 mg ml^{-1} chloramphenicol buffered to pH 8.5 with 10 mM Tris) for 4 hours or more, and then transferred to the dark (Jaffe, 1954). Eggs were fertilized as they exited the conceptacle and the time of fertilization (t=0) for a population was taken as 30 minutes after transferring receptacles to the dark. Zygotes were collected by filtration through nylon mesh, concentrated by vacuum suction and plated on No. 1 coverslips (18 mm, square) in plastic Petri dishes and grown at 14°C in unilateral illumination of 100 mmol (photons) m^{-2} s^{-1} using cool white fluorescent lamps (Sylvania-GTE, Falls River, MA). Under these conditions, zygotes attached to the coverslip within an hour and developed synchronously (Quatrano, 1980). The time courses of axis formation, axis fixation and germination were measured as previously described (Kropf and Quatrano, 1987), and cytokinesis was scored by adding ASW containing 1 M sucrose to plasmolyze the cells so that the partition wall could easily be visualized.

The effects of cytoskeletal inhibitors were investigated at all stages of the first cell cycle using CD (50 or 100 μg ml^{-1}) to disrupt microfilament function (MacLean-Fletcher and Pollard, 1980) and nocodazole (200 ng ml^{-1}) to depolymerize microtubules (Kropf et al., 1990). Both inhibitors were dissolved in dimethylsulfoxide (DMSO). Chronic and pulse treatment regimes were employed. In chronic treatments, inhibitor was added at the desired time and zygotes were incubated continuously in the presence of the drug. In pulse treatments, zygotes were exposed to the drug for 2 hours and then washed five times with ASW. To assess the effects of inhibitors on MtOC rotation, zygotes were fixed and prepared for immunofluorescence as described below.

In experiments utilizing cold treatment to depolymerize microtubules, dishes containing zygotes were placed at 0°C in an ice bath for 1 hour and then allowed to recover for varying lengths of time (1 second to 30 minutes) at 14°C. At the end of the recovery period, zygotes were fixed and prepared for immunofluorescence as described below.

Unless noted otherwise, all chemicals were purchased from Sigma Chemical Co., St Louis.

Immunofluorescence microscopy

Coverslips with attached zygotes were placed in a modified microtubule-stabilizing buffer (Doonan et al., 1985) for 10 minutes. Modified microtubule-stabilizing buffer contained 0.1 M piperazine-N-N -bis(2-ethanesulfonic acid) (Pipes) pH 6.8, 1 mM $MgCl_2$, 5 mM ethyleneglycol bis-(amino-ethyl ether) N, N, N^1, N^1-tetraacetic acid (EGTA), 0.01% Triton X-100 (v/v), and 20% glycerol (v/v). After this treatment, zygotes were fixed in one of two ways. For methanol fixation (Kropf et al., 1990), zygotes were placed in a solution of 64% absolute methanol (v/v), 30% glycerol (v/v), 5% DMSO (v/v), and 1% Triton X-100 (v/v). After 20 minutes the fixative was replaced by 1 ml of fresh fixative and embryos were rehydrated in increments by adding 0.25 ml of solution C [Kloareg and Quatrano, 1987; 100 mM NaCl, 20 mM $MgCl_2$, 2 mM KCl, 0.2% BSA (w/v), 10 mM 2-(N-Morpholino) ethanesulfonic acid (MES), 1 M sorbitol, and 1 mM EGTA adjusted to pH 5.8 with Tris base] every 5 minutes until the fixative was diluted to 50% strength. The embryos were then rinsed once in full-strength solution C and the cell wall was loosened by enzymatic digestion (see below).

In a second fixation protocol, zygotes were incubated for 20 minutes in FGT/MeOH which contained 80% modified microtubule-assembly buffer (80 mM KPipes, 5 mM EGTA, 1 mM $MgCl_2$, and 20% glycerol), 1.4% formaldehyde, 0.25% glutaraldehyde, 1.0 mM taxol, 0.2% Triton X-100, and 18% absolute methanol. FGT/MeOH was a modification of the FGT-fix of Gard (1991) and was mixed fresh before use. After fixation, embryos were transferred to absolute methanol at −20°C for at least 2 hours, rinsed briefly in modified phosphate-buffered saline [PBS; 137 mM NaCl, 2.7 mM KCl, 1.7 mM KH_2PO_4, 8.0 mM Na_2HPO_4, 30% glycerol (v/v), 0.1% sodium azide (w/v), and 0.1% BSA (w/v)] and incubated overnight at room temperature (T) in modified PBS containing 100 mM $NaBH_4$. Cells were rinsed and stored in solution C.

Cell walls were partially digested by overnight incubation in an enzyme cocktail of 7 mg ml^{-1} cellulase (CELF, Worthington Biochemical Corp, Freehold, NJ), 40 mg ml^{-1} hemicellulase (Sigma Chemical Company, St. Louis, MO), 6.25 units ml^{-1} alginate lyase (M-ase, a kind gift of Dr B. Kloareg, CNRS, Roscoff, France), and 0.1 mM phenylmethylsulfonylfluoride (PMSF) in solution C at room T. Cell walls were visibly distended and amorphous after this treatment.

After enzymatic wall digestion, fixed embryos were rinsed three times (5 minutes each) in modified PBS and incubated in blocking solution consisting of 2.5% (w/v) non-fat dry milk in modified PBS for 1 hour, rinsed three times in modified PBS (5 minutes each), incubated in 35 μl of anti- -tubulin (Amersham, Arlington Heights, IL) diluted 1:100 in modified PBS, rinsed three times in modified PBS, and incubated in 35 μl rhodamine-conjugated goat anti-mouse IgG (Organon Teknika Corp., West Chester, PA) diluted 1:50 in modified PBS. Incubation times in primary and secondary antibodies were 1 hour at room T for methanol fixed cells and 8 to 24 hours at 4°C for FGT/MeOH-fixed cells. Embryos were rinsed a final time in modified PBS, briefly dehydrated in absolute methanol and mounted in clearing solution consisting of benzyl benzoate and benzyl alcohol (2:1).

Angles of partition walls and MtOCs were measured on a Zeiss Axiovert 35 microscope (Carl Zeiss Co., Oberkochen, FRG) equipped with epifluorescence. Angles were measured with respect to the growth axis (defined as 0°) and recorded in 5° increments from −90° to +90° using a compass in an eyepiece. Fluorescence images were obtained on a MRC-600 laser-scanning confocal microscope (Bio-Rad laboratories, Richmond, CA) using a rhodamine filter set. A 16 nm band pass filter centered at 581 nm (Pomfret Research Optics Inc.,Orange, VA) was placed in front of the photomultiplier to reduce background autofluorescence. Digitally recorded images were stored on optical disks, and arranged for publication on a MacIntosh IIsi (Apple Computer Corp., Cupertino, CA) using Image 1.36 software.

Results

The time courses of successive events in the first cell cycle are shown in Fig. 1. The first cell cycle lasts approximately 24 hours and the first half of this period is devoted to establishing polarity (axis formation and axis fixation). This polarity is morphologically expressed as oriented growth and division in the second half of the cell cycle. A population of zygotes completed each of these stages in 4 hours or less; even so, some stages (e.g. germination and MtOC rotation) overlapped temporally. This report concerns the spatial orientation of the last three stages, MtOC rotation, mitosis and cytokinesis.

Rotation of the nuclear MtOC complex

Prior to germination, microtubules were associated with the entire surface of nuclear envelope (Kropf et al., 1990), but as the rhizoid emerged apparent MtOCs coalesced at two well defined foci (Fig. 2A) and microtubules extended from these foci into the elongating rhizoid apex. The linear axis defined by the MtOCs was at first transverse to the growth axis, but then rotated 90° and aligned with the growth axis (Fig. 2B,C). During rotation the nucleus elongated substantially and the MtOC rotating apically remained attached to the apical cortex via microtubules while the MtOC rotating basally lost its apical contacts. In the second cell cycle, this process was repeated in the rhizoid cell, but no rotation occurred in the thallus cell (Fig. 2D).

To confirm that the perinuclear foci were indeed MtOCs, microtubule depolymerization and regrowth experiments were conducted. Microtubules were depolymerized by cold treatment (0°C for 60 minutes) and then allowed to reassemble by warming to 14°C. Neither microtubules nor the perinuclear centers were detectable in cold-treated zygotes by indirect immunofluorescence (Fig. 3A), but within a minute of warming a brightly staining focus reappeared on the nuclear envelope and short microtubules were associated with the center (Fig. 3B). Within a few minutes a second center was organized (Fig. 3C). Both foci had microtubules associated with them and the lengths of the microtubules increased with time (Fig. 3D).

The MtOCs appeared to be physically attached to the nuclear envelope. During rotation the nucleus elongated and became kidney-bean or spindle shaped with MtOCs attached at the poles. In kidney-bean-shaped nuclei, the MtOCs appeared to be pulling their respective regions of the nuclear envelope toward the apex (Fig. 4A,B). The tight association between nucleus and MtOC was confirmed in lysed embryos. Embryos treated with hypoosmotic solutions lysed at the rhizoid tip owing to the relatively weak apical cell wall. In lysed cells, MtOCs remained attached to the extruded nucleus (Fig. 4C). Thus, the nucleus and MtOCs can be considered a single entity, referred to as a nuclear MtOC complex.

Zygotes of fucoid algae appear to be radially symmetric about the growth axis. If this were true, there would be no preferential direction of rotation when zygotes attached to a coverslip were observed from above or below. This hypothesis was tested by measuring the angle of the MtOC axis with respect to the growth axis in zygotes viewed from beneath the coverslip. Zygotes and 2-celled embryos were fixed and prepared for immunofluorescence at times when the nuclear MtOC complex was expected to be in mid-rotation. The orientation of the MtOC axis was scored over 180° in 5° intervals, with 0° being axial and 90° (and −90°) being transverse (Fig. 5). Thus, a fully rotated nuclear MtOC complex was recorded as 0°, and the unrotated complex was 90° (−90°). MtOC angles were equally distributed over positive and negative values in both zygotes (Fig. 5A) and in rhizoid cells of two-celled embryos (Fig. 5B). There was no preferential direction of rotation as judged by the Sign test, supporting the hypothesis that the young embryos are radially symmetric.

Effects of cytoskeletal inhibitors on MtOC alignment

To investigate the roles of these microfilaments and microtubules in rotation, MtOC orientations were measured after treatment with specific inhibitors. Cytochalasin D (CD) prevents actin polymerization (MacLean-Fletcher and Pollard, 1980; Cooper, 1991) and inhibits microfilament-dependent

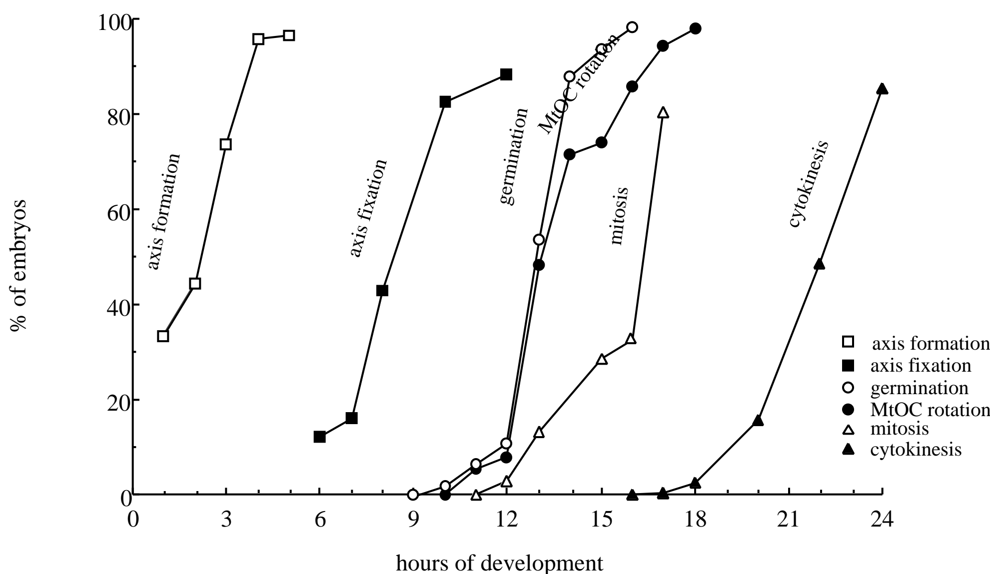

Fig. 1. Developmental stages of the first cell cycle. All embryos were grown in ASW at 14°C in unilateral illumination. Axis formation is the establishment of a labile axis which later becomes irreversibly set in place during axis fixation.

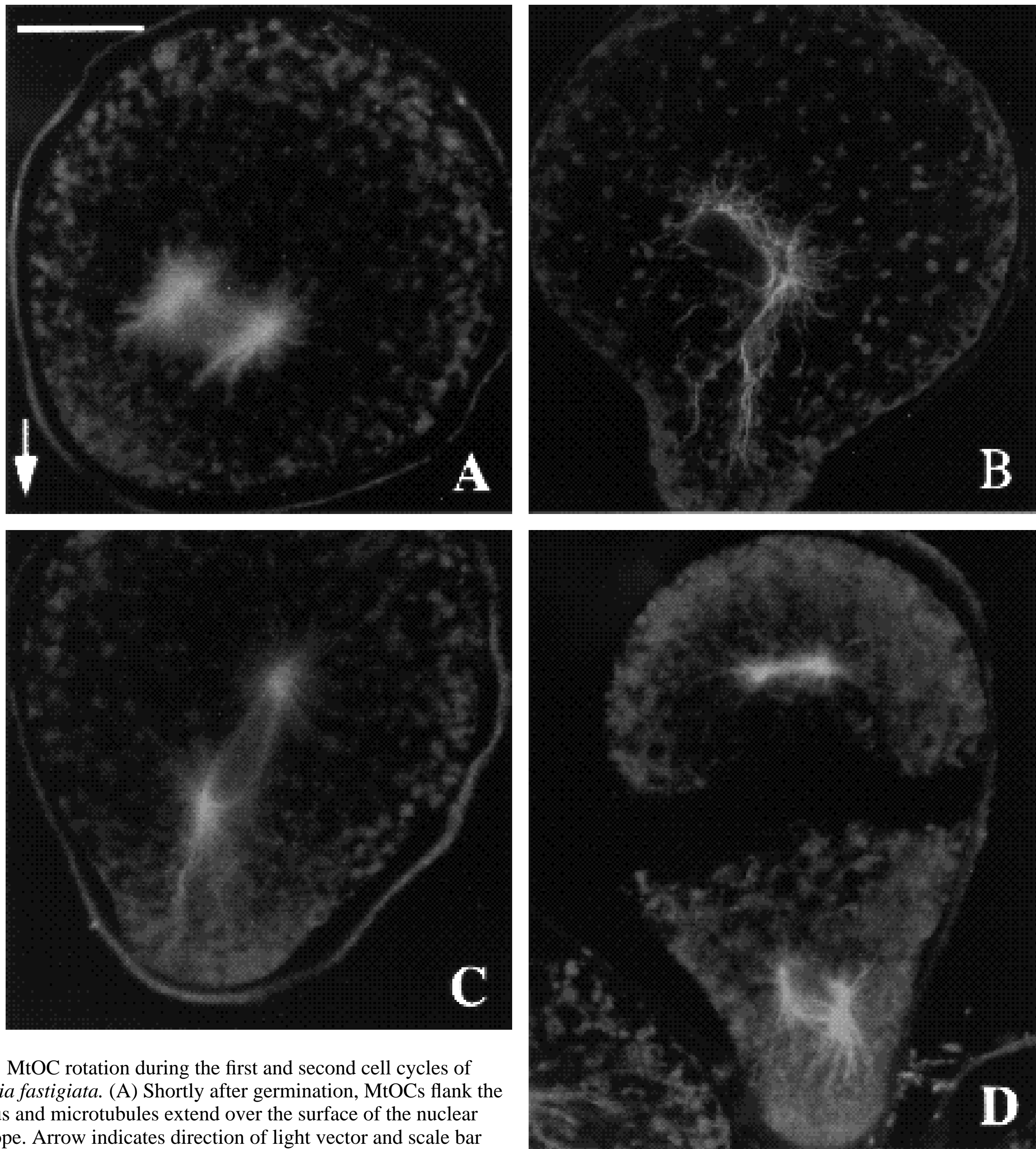

Fig. 2. MtOC rotation during the first and second cell cycles of *Pelvetia fastigiata.* (A) Shortly after germination, MtOCs flank the nucleus and microtubules extend over the surface of the nuclear envelope. Arrow indicates direction of light vector and scale bar represents 25 µm. (B) In mid-rotation microtubules extend from the leading MtOC into the cortex of the rhizoid. (C) The leading MtOC remains connected to the rhizoid cortex by microtubules at the completion of rotation. (D) In the 2-celled embryo, MtOCs in the rhizoid cell rotate but those in the thallus cell do not. Rhizoid MtOCs are in mid-rotation.

processes. The effects of chronic CD treatment on MtOC alignment were investigated as follows. CD was added to duplicate dishes of photopolarized zygotes at hourly intervals prior to and during rotation. At 24 hours postfertilization, zygotes were fixed and the orientation of the MtOC axis was measured. Control zygotes in ASW (or in ASW containing 0.5% DMSO) completed rotation by 16 hours and MtOCs were properly aligned with the growth axis (Fig. 6A). By contrast, MtOCs in zygotes treated with CD were poorly aligned. When CD was added anytime prior to the start of rotation, MtOCs were randomly oriented at 24 hours (Fig. 6B). Treatments begun during the rotation period had progressively less effect, and MtOC alignment was more normal when CD was added at the end of rotation (16 hours, Fig. 6C). Even so, CD treatment after rotation did disrupt MtOC orientation (compare Fig. 6A and C) indicating a possible role for microfilaments in maintaining orientation of the nuclear MtOC complex in later stages of the cell cycle. Analysis of variance in MtOC angles confirmed the temporal differences in CD effects; variance in MtOC angles was least for controls, greatest for zygotes treated early, and progressively declined with later addition (Fig. 6D). The variance in

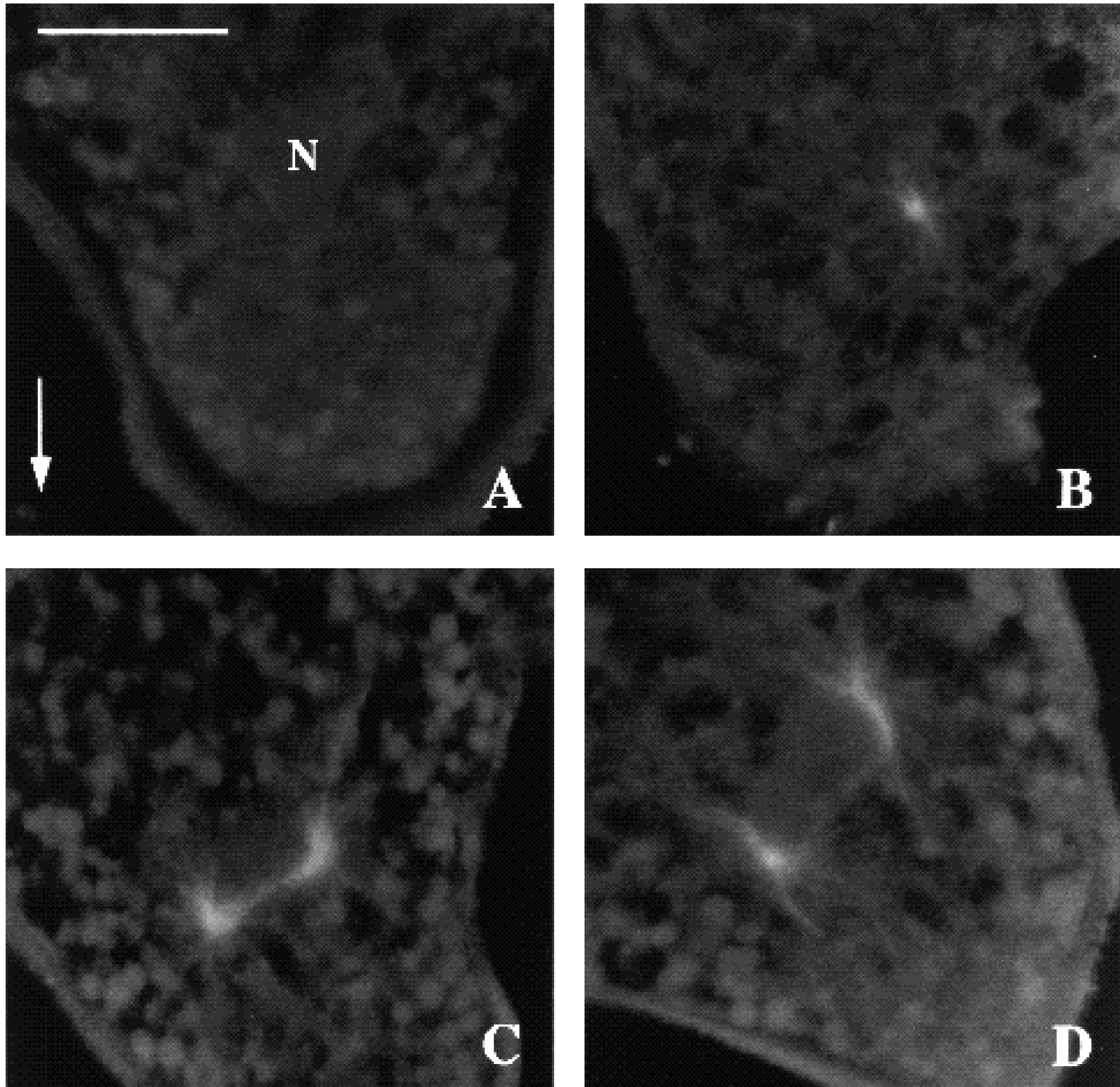

Fig. 3. Recovery of microtubules after cold treatment. (A) MtOCs and microtubules are not observed after a 1 hour treatment with 0°C ASW. N = nucleus. Arrow indicates direction of light vector and bar is 25 μm. (B) Less than 1 minute after cells are warmed to 14°C, a single MtOC is apparent. (C) A few minutes later, both MtOCs can be detected and short microtubules radiate over the nuclear surface. (D) Microtubules steadily increase in length and by 10 minutes they extend toward the rhizoid cortex. Cells shown are the rhizoid cell of the two-celled embryo; identical results are obtained using the zygote.

the control population was different from that in CD-treated populations at a significance level of $P<0.005$ according to the Bartlett test.

Experiments were designed to test whether MtOCs in CD-treated zygotes formed in random alignment with respect to the growth axis, or instead formed in proper orientation (transverse to the light vector) and rotated incorrectly. Zygotes were grown for 5 hours in unilateral light to induce an axis and then treated with CD. At hourly intervals beginning at 10 hours, zygotes were fixed and MtOC angles were measured. As soon as MtOCs could be clearly identified (10 hours), their orientation was random with respect to the developmental axis; at no time were MtOCs preferentially transverse to the growth axis (data not shown).

The effects of pulse (2 hour) treatments with nocodazole or CD were investigated in zygotes between 5 and 14 hours old. Both CD (Fig. 7C) and nocodazole (Fig. 7D) disrupted MtOC orientation when compared to untreated controls (Fig. 7A) or controls mock treated with 0.5% DMSO (Fig. 7B). CD had the greatest effect when the treatment was applied

during the period of rotation, yet treatments before or after rotation also caused abnormal MtOC alignment (Fig. 7E). By contrast, nocodazole was equally effective at all treatment times (Fig. 7F). Nocodazole treatment resulted in a greater variance in MtOC angle than did CD treatment (compare Fig. 7E and F).

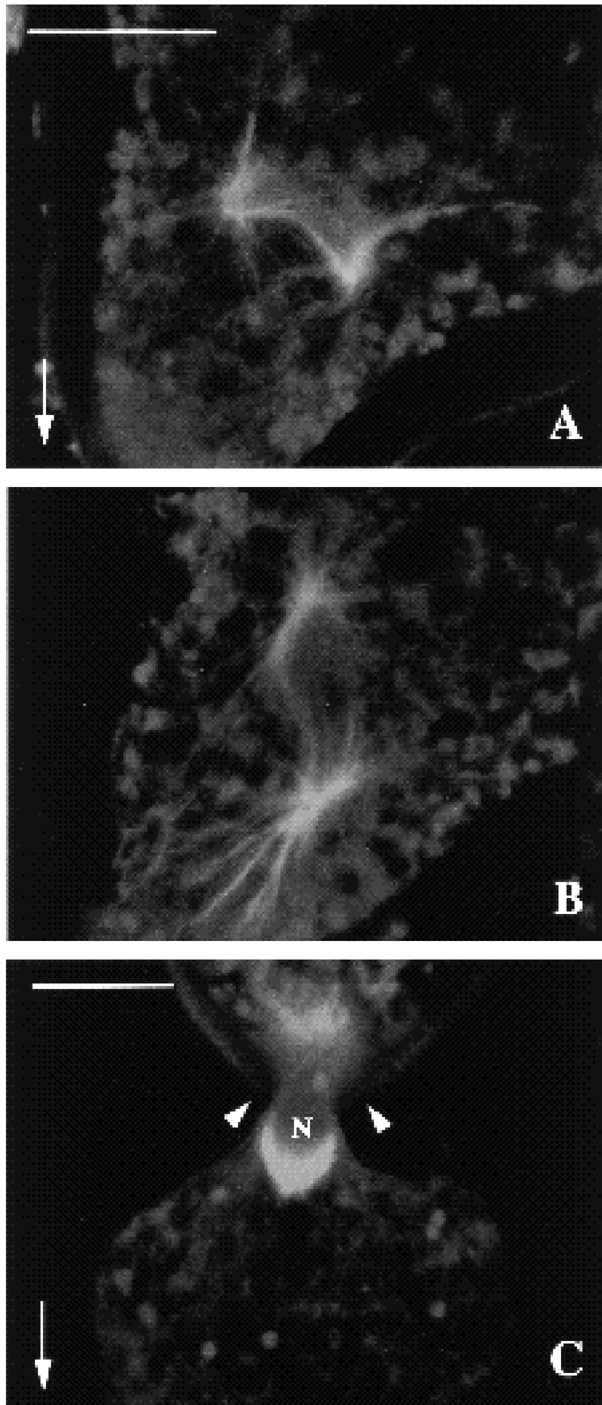

Fig. 4. MtOCs are attached to the nuclear envelope. (A) Nucleus in rhizoid cell before MtOC rotation. The nucleus appears curved (kidney-bean shaped) with concave side toward the rhizoid tip. Arrow represents direction of light vector and bar is 25 μm. (B) Nucleus of rhizoid cell towards the end of MtOC rotation. Microtubules appear to pull leading MtOC and nuclear attachment site toward the rhizoid tip. (C) A lysed rhizoid cell. Cytoplasm and the nucleus have been expelled from the rhizoid tip, and MtOC material remains attached to the nucleus (N). Arrowheads indicate the area of cell wall rupture.

Mitosis and cytokinesis

By the end of rotation, the nucleus had elongated along the growth axis and MtOCs resided at the poles (see Fig. 2C). As zygotes entered mitosis the metaphase spindle was typically cylindrical and measured approximately 25-30 μm by 5 μm (Fig. 8A). Spindle poles were clearly visible as spheres surrounded by a halo of astral microtubules. The migrating chromosomes in anaphase appeared as two unstained bands (Fig. 8B). Distinct nuclei became visible at telophase, and microtubules extended from the single, distal MtOC on each nucleus toward the division site on the parental wall (Fig. 8C). Microtubules were not associated with the division plane and a phragmoplast was not evident.

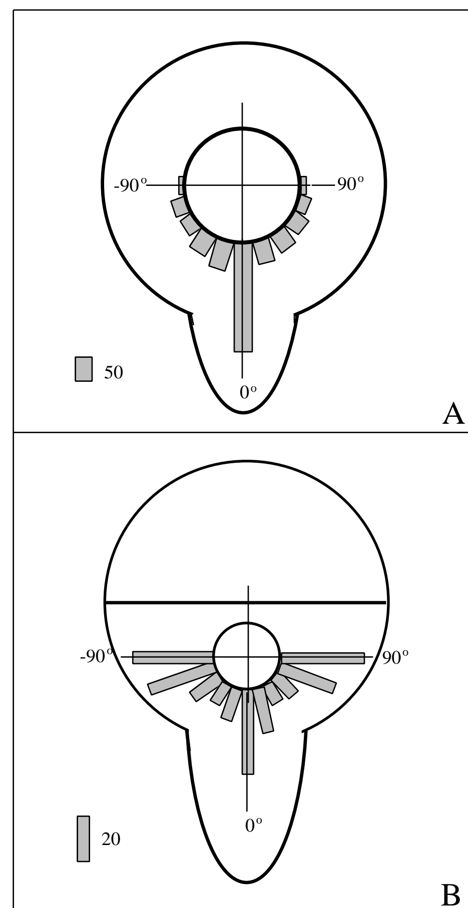

Fig. 5. Direction of rotation of nuclear MtOC complex in the first and second cell cycles. Bars represent number of embryos with MtOCs at specified angle. Data were collected in 5° intervals, but for simplicity are pooled and shown in 15° intervals. (A) First cell cycle. Most zygotes have completed rotation (0°) by 14 hours; MtOCs still in the process of rotating are equally dispersed over positive and negative angles. n = 538. (B) Second cell cycle. Most embryos in the second cell cycle are in the early stages of MtOC rotation (90° and −90°) at 27 hours; again MtOCs are equally dispersed over positive and negative angles. n = 272 embryos.

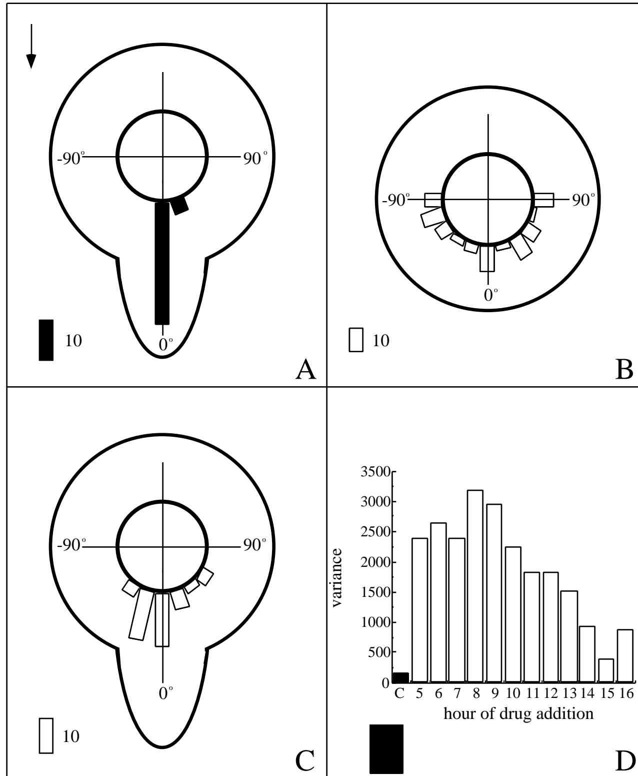

Fig. 6. MtOC angles in zygotes chronically treated with CD. (A) Untreated control embryos at 16 hours after fertilization. $n = 31$. (B) Chronic CD treatment beginning at 8 hours. Cells do not germinate in the presence of CD. $n = 50$. (C) Chronic CD treatment beginning at 16 hours. Zygotes had germinated prior to treatment. $n = 41$. (D) Variance of MtOC angles in zygotes chronically treated with CD. Abscissa indicates time of drug addition; C is untreated control. Angles were measured with respect to the polarizing light vector rather than the growth axis because zygotes treated early did not form a rhizoid.

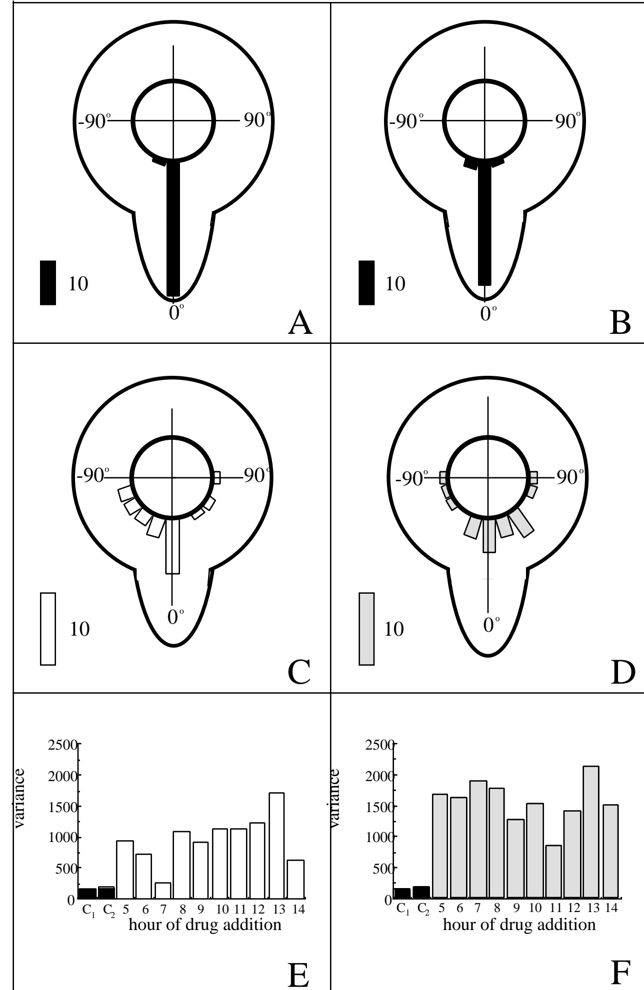

Fig. 7. Effects of 2 hour pulse treatments with CD or nocodazole on rotation of the nuclear MtOC complex. (A) MtOCs are fully rotated at 16 hours in untreated controls. $n = 29$ (B) Treatment with 0.5% DMSO from 5 to 7 hours. $n = 29$. (C) Treatment with CD from 12 to 14 hours. $n = 19$. (D) Treatment with nocodazole from 12 to 14 hours. $n = 21$. (E) Variance in MtOC orientation of CD-treated zygotes. (F) Variance in MtOC orientation of nocodazole-treated zygotes. C_1 = ASW control, C_2 = DMSO-treated control.

Cytokinesis required both microfilaments and microtubules. Treatment of young zygotes with either 50 μg/ml CD or 200 ng/ml nocodazole inhibited cytokinesis completely (Fig. 9), and zygotes remained single celled for at least 3 days. The period during which cytokinesis was sensitive to inhibition differed for the two treatments. Nocodazole inhibited cytokinesis when added anytime up to the beginning of division, but had progressively less effect when added later. CD, on the other hand, inhibited cytokinesis maximally only if added before 16 hours of development, well in advance of cell division. Between 16 and 20 hours the effectiveness of CD declined nearly linearly, such that 80% of zygotes treated with CD at the onset of cytokinesis (20 hours) completed division.

The role of cytoskeletal filaments in determining the orientation of division was investigated using 2 hour treatments with CD or nocodazole. [Chronic treatments could not be used because they inhibited cytokinesis (Fig. 9).] The experimental design was similar to that described above for measuring effects on rotation. Zygotes were treated for 2 hour intervals between 8 and 16 hours postfertilization and the orientation of the partition wall was measured with respect to the growth axis. In untreated zygotes and in zygotes mock-treated with 0.5% DMSO, the wall formed transverse to the growth axis (Figs. 10A and B, respectively). A 2 hour treatment with CD (Fig. 10C) or nocodazole (Fig. 10D) increased the variability in wall angle. The effect on division angle was independent of developmental age at the onset of treatment, and nocodazole generally had a greater effect than CD (Fig. 10E,F). The variance in wall angle in controls was different from that in CD- or nocodazole-treated zygotes at a significance level of $P<0.005$ according to the Bartlett test.

Discussion

Nuclear MtOC rotation

Cells of brown algae contain centrioles associated with pockets in the nuclear membrane (Brawley and Quatrano,

Fig. 8. Mitosis in the first cell cycle. (A) Metaphase spindle is aligned with the growth axis. Arrow indicates direction of light vector. (B) At anaphase, migrating chromosomes appear as two unstained bands. (C) The nuclear envelope is present on daughter nuclei at telophase, and microtubules radiate toward the division site. Bars represent 25 μm.

1979; Neushul and Dahl, 1972), so it is likely that in *Pelvetia* each perinuclear MtOC contains paired centrioles and therefore constitutes a centrosome. In lower plants and algae, microtubules originating from perinuclear centrosomes are thought to function in nuclear positioning (McNaughton and Goff, 1990) and nuclear transport (Doonan et al., 1986; Schmiedel et al., 1981). Our findings indicate that this population of microtubules is also involved in nuclear rotation and spindle alignment. Spindle alignment in zygotes of *Laminaria*, a related brown alga, is determined by centrosomal migration (Motomura, 1991), but the mechanism of movement and the factors controlling alignment have not yet been investigated. By contrast, the results of our study indicate that in *Pelvetia* force is produced by a cytoskeletal-based system and that the force is exerted at the site of MtOC attachment to the nuclear membrane.

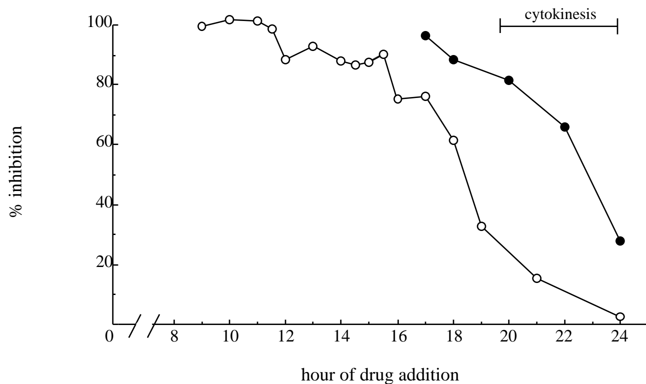

Fig. 9. Inhibition of cytokinesis by chronic treatment with cytochalasin D or nocodazole. Inhibitor was added at times indicated and cell division was scored at 36 hours. Percentage inhibition was calculated as (No. not divided/No. total)×100. (○) Treatment with 50 μg ml^{-1} CD, n = 1658. (●) Treatment with 200 ng ml^{-1} nocodazole, n = 682.

Immunofluorescence images of rotating nuclei provide the strongest evidence that rotational force is applied at the MtOCs; MtOCs appear to be pulled toward the apex and, by virtue of being firmly attached to the nuclear membrane, distort the morphology of the nucleus. As rotation proceeds, only the MtOC moving apically remains attached to the rhizoid cortex by microtubules; microtubules associated with the MtOC moving basally (toward the thallus pole) radiate in all directions. This observation is consistent with the hypoth-

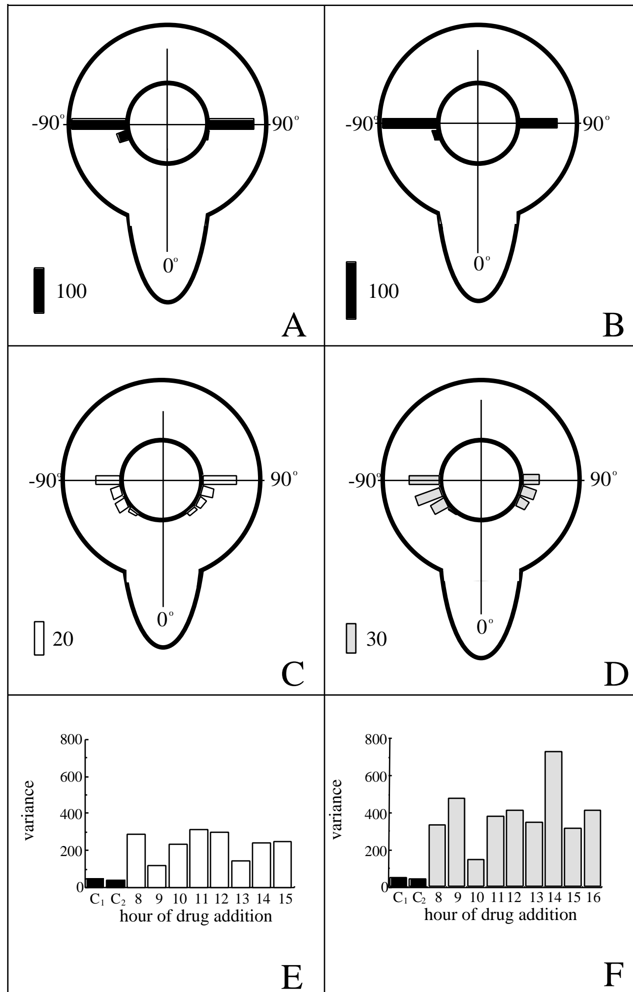

Fig. 10. Effects of 2 hour treatment with CD or nocodazole on division angle. Bars represent number of embryos with partition wall at specified angle. Data were collected in 5° intervals, but for simplicity are pooled and shown in 15° intervals. (A) Control zygotes grown in ASW, $n = 234$. (B) Zygotes treated with 0.5% DMSO from 8 to 10 hours, $n = 200$. (C) Treatment with CD from 12 to 14 hours, $n = 64$. (D) Treatment with nocodazole from 9 to 11 hours, $n = 101$. (E) Variance in wall angles in CD-treated embryos. (F) Variance in wall angles in nocodazole-treated embryos. C_1 = ASW controls, C_2 = controls treated with 0.5% DMSO.

esis that force is transduced to the MtOCs via microtubules anchored in the apical cortex.

The force for rotation is likely provided by microtubule- or microfilament-dependent movements, but at present the data do not discriminate between these two possibilities. Inhibitor studies indicate that disruption of either the microtubule or microfilament network interferes with rotation. Microtubules linking MtOCs to the apical cortex are certainly positioned appropriately for force generation by microtubule-dependent motors (kinesin, dynein or others), or by changes in the polymerization state of microtubules. Depolymerization of anchored microtubules would result in apical movement of the attached MtOCs. However, until the distribution of endoplasmic F-actin is clarified, the possibility remains that an actomyosin system drives rotation. Although endoplasmic F-actin has not been detected, its existence has been inferred from studies showing that cytochalasin inhibits secretory vesicle transport from perinuclear Golgi to the rhizoid cortex (Brawley and Quatrano, 1979). Previous studies using rhodamine phalloidin to label F-actin in fucoid algae have only detected F-actin in the cellular cortex where it forms a fine cortical meshwork lacking distinct cables (Brawley and Robinson, 1985; Kropf et al., 1989). In germinated zygotes, F-actin staining is preferentially localized at the rhizoid apex. We have recently begun to reinvestigate the location and structure of F-actin in *Pelvetia* zygotes, and find that the rhizoid cortex contains an interwoven meshwork of actin cables just beneath the plasma membrane (Kropf et al., 1992). This network girdles the rhizoid and is probably the foundation of the cortical cytoskeleton. Again, no endoplasmic F-actin has been detected. Based upon these observations, we prefer a working model in which the F-actin is involved in anchoring microtubules in the apical cortex and that the anchored microtubules drive rotation. Cytochalasins would then inhibit rotation by disrupting the cortical cytoarchitecture of the rhizoid.

Cytokinesis

The mechanism by which the zygotic cytoplasm is cleaved at first division is unknown. Cytokinesis occurs predominantly by furrowing, but not strictly so since vesicles do coalesce with the growing partition membrane (Brawley and Quatrano, 1979). Inhibitor studies demonstrate that cytokinesis is inhibited by CD added before 16 hours, but not by CD addition just prior to cytokinesis. (The lack of effect of later additions is probably not related to poor uptake because CD inhibits rhizoid growth almost immediately.) By contrast, cytokinesis is completely inhibited by nocodazole at all times up to the start of cleavage (20 hours). Interestingly, rhizoids of nocodazole-treated zygotes elongated to the length that they would normally obtain by first division and there they stopped, indicating coordinate control of growth and division. These data indicate that furrowing and formation of the partition wall in *Pelvetia* is immediately dependent on microtubules, but not microfilaments. Cytokinesis in higher plant cells is also primarily dependent upon microtubules which form a double ring structure (phragmoplast) at the leading edge of the centrifugally growing cell plate (Gunning and Wick, 1985). However, microtubules in *Pelvetia* zygotes do not reorganize into a phragmoplast during cytokinesis, and are not closely associated with the invaginating furrow (Brawley et al., 1977). Instead, microtubules originating from the distal side of the telophase nuclei extend into the cortex at the division site. Recently, we have discovered a hoop of actin cables at this cortical division site (Kropf et al., 1992). This ring persists long after division is completed and, unlike the homologous structure in animal cells, does not contract during cytokinesis. As postulated above for nuclear rotation, cytokinesis may involve an interaction between cortical actin cables and endoplasmic microtubules, but as yet the specific mechanism of cytokinesis in plant cells lacking a phragmoplast is unknown.

MtOC orientation determines division plane

As is the case in animal cells, cytokinesis in lower plants and algae that lack PPBs and phragmosomes is thought to be

spatially oriented by the spindle (Doonan et al., 1985; Gunning and Wick, 1985). In general, the cleavage furrow (cell plate) bisects the spindle. What, in turn, controls orientation of the spindle? Our results indicate that in *Pelvetia*, spindle position is determined by two MtOCs that rotate from transverse to axial alignment prior to mitosis. This hypothesis is supported by inhibitor studies demonstrating that disruption of MtOC alignment is associated with abnormal division plane. Furthermore, there is a direct correlation between the degree to which MtOCs are misaligned and the degree to which partition walls are skewed; variances in both parameters were greater after nocodazole treatment than after CD treatment. Confirmation of the chain of causality from rotation of the nuclear MtOC complex to spindle alignment to division plane will require measurements of MtOC and division angles on individual, living zygotes. Toward this goal, we are presently attempting to microinject fluorescently tagged tubulin into zygotes.

Control of division plane orientation in *Pelvetia* embryos is strikingly similar to that in the nematode, *Caenorhabditis elegans*. The first zygotic division in *C. elegans* is also an unequal, deterministic cleavage forming a larger AB cell, and a smaller P_1 cell. The P_1 cell then divides transverse to the AP axis, while the AB cell divides axially (Hyman and White, 1987). These are the same patterns of division in *Pelvetia*, with the thallus cell substituting for AB and the rhizoid cell for P_1. The mechanisms controlling these invariant divisions are also quite similar. The transverse divisions in P_0 and P_1 are oriented by rotational events. In P_0 the spindle forms transversely and rotates axially, and in P_1 cells the nucleus rotates 90° bringing centrosomes into an axial orientation (Strome, 1989; Hyman, 1989). Hyman (1989) investigated the mechanism of centrosome rotation in P_1 using laser ir-radiation and proposed that the centrosome moving apically is pulled by microtubules connecting it to the apical cortex. Although the structures that anchor the microtubules in the cortex of *C. elegans* are unknown, they may in fact be cortical microfilaments as we believe for *Pelvetia*. The presence of microtubule-dependent nuclear rotations in embryos of such distantly related organisms as *C. elegans* and *Pelvetia* most likely indicates that this mechanism for determining division orientation arose quite early in the evolution of eukaryotes. Alternatively, it may represent the convergent evolution of very similar mechanisms to control unequal, deterministic cleavages in these two organisms.

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