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Cell cycle-specific disruption of the preprophase band of microtubules in fern protonemata: effects of displacement of the endoplasm by centrifugation

TAKASHI MURATA* and MASAMITSU WADA

Department of Biology, Faculty of Science, Tokyo Metropolitan University, Minami-Ohsawa, Hachioji, Tokyo 192-03, Japan

*Author for correspondence

Summary

The preprophase band (PPB) of microtubules (MTs), which appears at the future site of cytokinesis prior to cell division in higher plant cells, disappears by metaphase. Recent studies have shown that displacement of the endoplasm from the PPB region by centrifugation delays the disappearance of the PPB. To study the role of the endoplasm in the cell cycle-specific disruption of the PPB, the filamentous protonemal cells of the fern *Adiantum capillus-veneris* L. were centrifuged twice so that the first centrifugation displaced the endoplasm from the site of the PPB and the second returned it to its original location. The endoplasm, including the nucleus of various stages of mitosis, could be returned by the second centrifugation to the original region of the PPB, which persists during mitosis in the centrifuged cells. When endoplasm with a prophase nucleus was returned to its original location, the PPB was not disrupted. When endoplasm with a prometaphase-telophase nucleus was similarly returned, the PPB was disrupted within 10 min of termination of centrifugation. In protonemal cells of *Adiantum*, a second PPB is often formed near the displaced nucleus after the first centrifugation. In cells in which the endoplasm was considered to have been returned to its original location at the prophase/prometaphase transition, the second PPB did not disappear even though the initial PPB was disrupted by the endoplasm. These results suggest that cell cycle-specific disruption of the PPB is regulated by some factor(s) in the endoplasm, which appears at prometaphase, i.e. the stage at which the PPB is disrupted in non-centrifuged cells.

Key words: *Adiantum*, cell cycle, centrifugation, endoplasm, fern protonema, microtubule, preprophase band.

Introduction

Microtubules (MTs) in plant cells play important roles in cell morphogenesis. Orientation of cortical MTs at interphase may regulate the orientation of cellulose microfibrils, which is considered to control the direction of expansion of the cell (Green, 1980). The preprophase band (PPB) of MTs, which develops at the G_2 phase and prophase of the cell cycle at the site of cytokinesis, is considered to regulate the plane of cell division (Gunning and Wick, 1985). The mitotic spindle distributes chromosomes to two daughter nuclei (Baskin and Cande, 1990). The phragmoplast builds a new cell plate during cytokinesis (Gunning, 1982).

The mechanism by which arrays of MTs are organized and disrupted during the cell cycle is of particular interest with regard to elucidation of the divergent roles of MTs in plant cells. At prophase, a PPB in the cortical cytoplasm has already formed and the mitotic spindle in the endoplasm begins to develop. By metaphase, the PPB has disappeared and the mitotic spindle has developed fully (Wick and Duniec, 1984). Pickett-Heaps (1969) proposed that MTs in the PPB are incorporated into the mitotic spindle. Indeed, injection of fluorochrome-labelled tubulin into prophase cells revealed that the same pool of tubulin could be used by the PPB and the mitotic spindle (Zhang et al. 1990). However, most aspects of the mechanism of the transition from the PPB to the mitotic spindle are unknown.

Recently, it was found that displacement of the endoplasm with the nucleus from the region of the PPB delayed the disruption of the PPB (Murata and Wada, 1991; Mineyuki et al. 1991a), an observation that suggests that close proximity of the endoplasm may be essential for disruption of the PPB. It is easily assumed but has not been demonstrated that the endoplasm acquires the capacity for disruption of the PPB in a stage-specific manner. In this study, we examined the effect of returning the endoplasm to its original location

at various stages of mitosis on the disruption of the PPB.

Materials and methods

Plant material

The protonemata of *Adiantum capillus-veneris* L. were cultured as described by Murata and Wada (1991). In brief, spores of *Adiantum capillus-veneris* L. were sown between thin gelatin-agar films and cultured at 25°C in 3 ml of modified Murashige and Skoog's mineral salt solution for 1 day in the dark, and then for 6 days under red light (approx. 0.5 W m^{-2}). The protonemal cells grow towards the source of red light without cell division. For production of bent protonemal cells, the angle of incidence of red light was changed by 45-90 degrees 12-18 h before the induction of cell division.

Induction of cell division and centrifugation

The induction of cell division and centrifugation were performed basically in the same way as described previously (Murata and Wada, 1991). Seven days after sowing of spores, the resulting protonemata were irradiated continuously with blue light (BL; approx. 2 W m^{-2}) to induce the formation of the PPB and cell division. The protonemata were transferred into a custom-made centrifuge cuvette and centrifuged basipetally at 2800 *g* for 15 min, 12 h after the onset of BL, the time at which a PPB has formed in the apical region of about 90% of cells examined (Murata and Wada, 1991). The endoplasm was displaced to or near the bend in each cell, which had been made at about 100-200 μm from the tip by changing the angle of incidence of the red light (see Fig. 1A). At 14.5-15.3 h, the protonemata were centrifuged again acropetally at 1300-3500 *g* for 1-15 min to return the endoplasm to the apical region (Fig. 1A). All experiments were performed at 25°C.

Immunofluorescence microscopy

The methods used for immunofluorescence microscopy were the same as those described elsewhere (Murata and Wada, 1989). In brief, the protonemata were fixed in a solution of 8% paraformaldehyde, 0.2% picric acid, 1% dimethylsulfoxide, 5 mM ethyleneglycol bis(α-amino-ethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), and 0.5 mM phenylmethylsulfonyl fluoride dissolved in 0.1 M sodium phosphate buffer (pH 7.0). Protonemal cells were cut with a small piece of a razor blade, made permeable with a detergent solution, and treated with anti-tubulin antibody and then with a fluorescein-linked second antibody. The cells were mounted with mounting medium that contained 4′,6′-diamidino-2-phenylindole (DAPI), and observed under an epifluorescence microscope (Axioplan; Carl Zeiss, Oberkochen, Germany).

Results

Disruption of the PPB by mitotic endoplasm

In protonemal cells centrifuged basipetally 12 h after

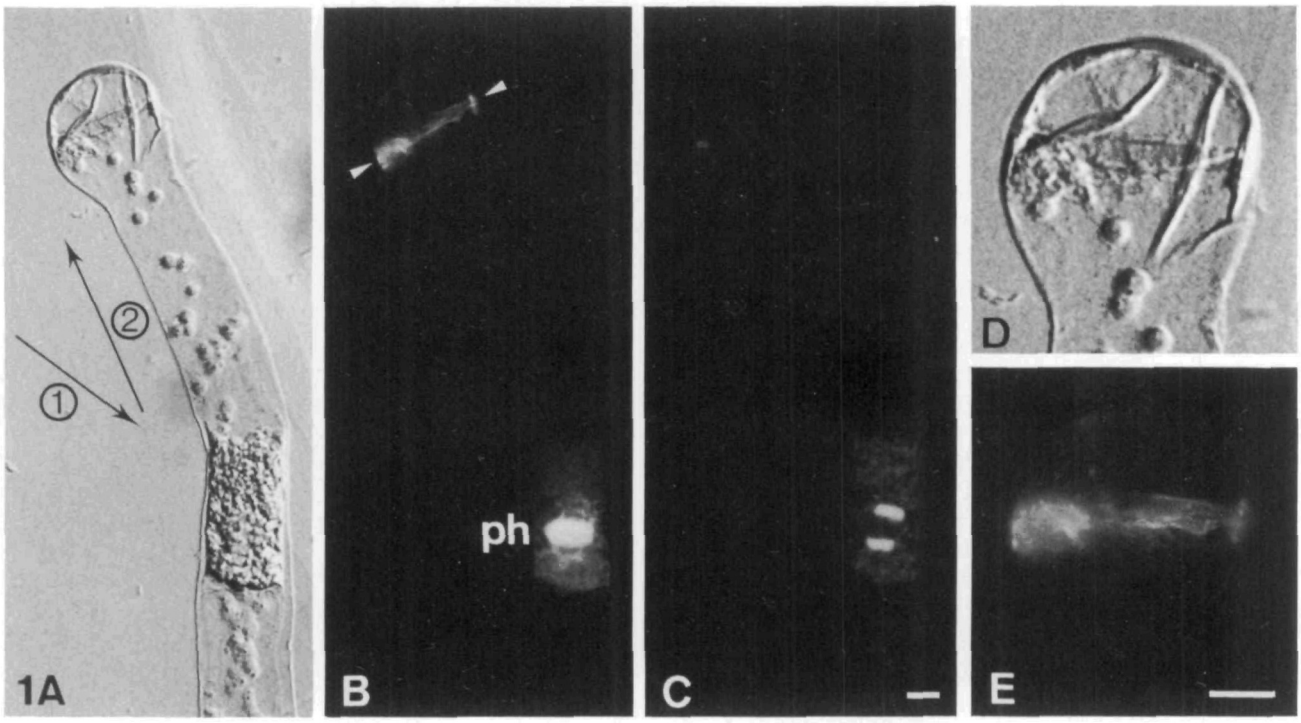


Fig. 1. Micrographs of a protonemal cell of *Adiantum capillus-veneris* L. The cell was centrifuged basipetally (arrow 1), 12 h after the onset of BL, and further cultured for approximately 3 h under BL. Before the second acropetal centrifugation (arrow 2). Bars, 10 μm. (A) Differential interference contrast image showing the distribution of organelles in the cell. (B) Staining with anti-tubulin. The PPB is indicated by arrowheads. ph, phragmoplast. (C) DAPI-stained image showing two daughter nuclei. (D and E) High-magnification images of the apical region of the same protonema as in A-C. (D) Differential interference contrast image showing a few organelles in the apical region. Lines within the cell are due to folding of the cell wall during preparation. (E) Staining with anti-tubulin of the same region as in (D). The PPB is obvious.

the onset of BL, the nucleus and most of the endoplasm that had been displaced by 100-200 μm did not return to the apical PPB region during mitosis (Fig. 1A, C), although oil droplets and a small amount of cytoplasm accumulated in the apical PPB region (Fig. 1D). As described previously, a PPB persists in the apical region during cell division (Fig. 1B, E; see also Murata and Wada, 1991). To prove that the presence of endoplasm is responsible for the disruption of the PPB, the protonemal cells that had been centrifuged basipetally were recentrifuged acropetally at 3500 *g* for 1 min 14.5-15.3 h after the onset of BL, when many cells had entered mitosis. Staining with anti-tubulin and DAPI made it easy to distinguish cells at interphase, prophase and telophase, even after centrifugation. However, we did not attempt to distinguish between cells at prometaphase and at anaphase, because we could not eliminate the possibility of the artificial disorientation of chromosomes by centrifugation.

In most cells at the stages from prometaphase to telophase, the endoplasm containing the nucleus could be returned to the apical region by a 1-min centrifugation (Figs 2-5). The mitotic spindle or the phragmoplast that was returned lay perpendicular or oblique to the cell axis. It did not rotate during a following incubation of 10 min. Just (1.8 min) after the termination of centrifugation, the PPB at the apical region was still visible in most cells, so that the PPB and the mitotic spindle or the phragmoplast were present simultaneously in the apical region (Figs 2 and 4). Microtubules at the PPB site are not polar MTs polymerized upon return of the spindle to the apex, since a similar array of MTs was observed when the spindle was sedimented incompletely or obliquely to the cell axis. No connection between MTs of the cell cortex and the spindle or the phragmoplast was observed. The PPB rapidly disappeared within 10 min of the termination of centrifugation (Figs 3, 5 and 9C). The time course of the disruption of the PPB was similar in cells at prometaphase-anaphase and in those at early telophase (Fig. 7). The disruption of the PPB by the endoplasm of dividing cells was also observed in cells that had been centrifuged at 1300 *g* for 5 min if the endoplasm was returned to the apical region (data not shown). Preprophase bands were not disrupted in cells in which the endoplasm was not returned to the apical PPB region, irrespective of the strength of the applied centrifugal force, indicating that the disruption of the PPB is related to sedimentation of the endoplasm and not to the strength of the centrifugal force.

In most prophase cells, the endoplasm was not displaced by centrifugation for 1-5 min. However, in a small fraction of prophase cells, the endoplasm was displaced to the apical PPB region. In such cells, the apical PPB was not disrupted even 10 min after the termination of centrifugation (data not shown, see below).

To displace the prophase endoplasm in almost all cells, the duration of centrifugation (at 3500 *g*) was extended to 15 min. Protonemal cells remained able to divide even after such prolonged centrifugation (data not shown). The endoplasm with the prophase nucleus was displaced and returned to the apical PPB region in most cells (Fig. 6A). At 10 min after the termination of centrifugation the apical PPB remained in 74 cells out of 76 cells examined (Figs 6B, C and 9A).

Persistence of a second PPB

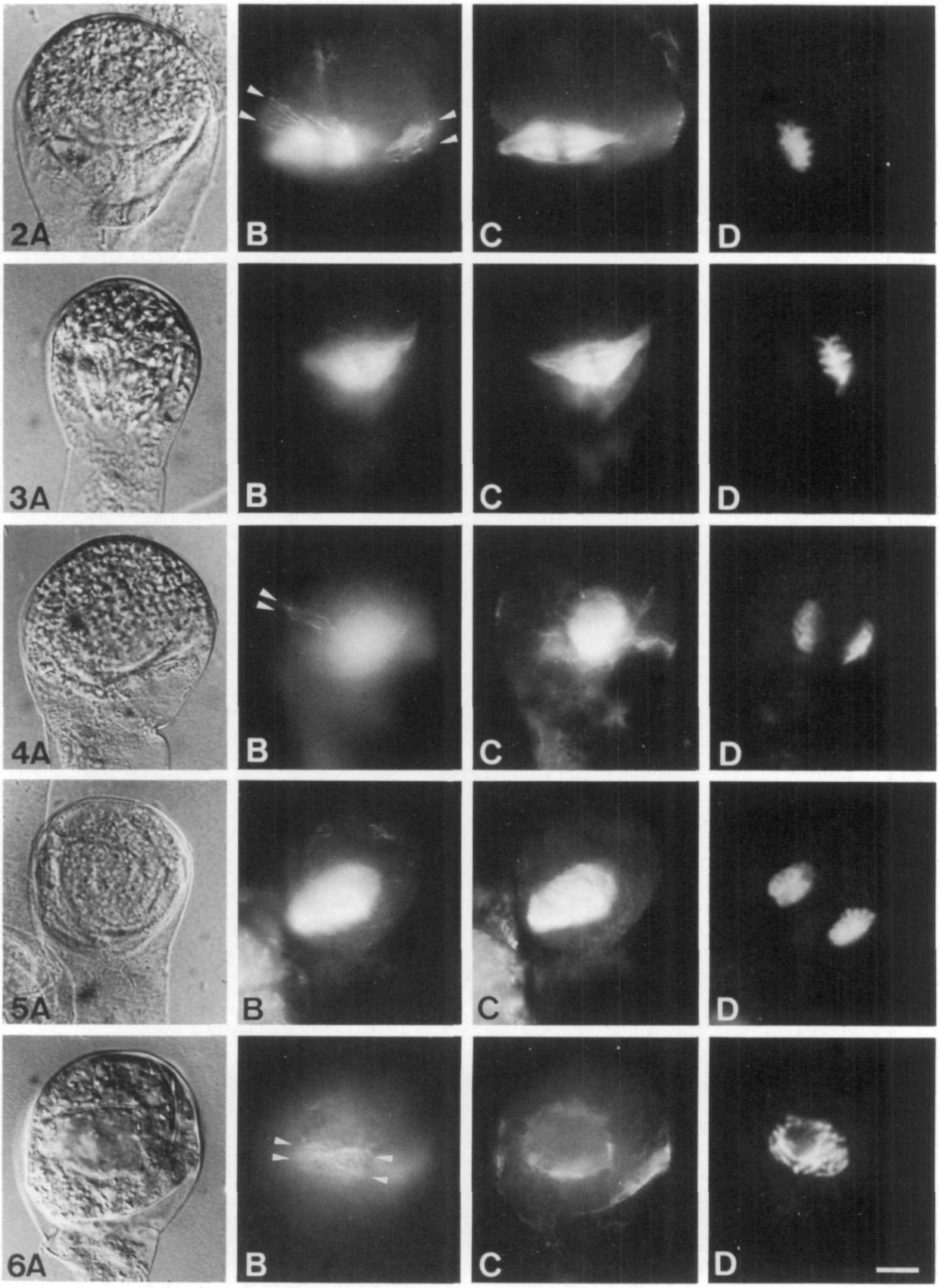
As reported previously (Murata and Wada, 1991), a PPB forms near the displaced nucleus (at the cylindrical region) in many centrifuged cells at prophase and this PPB has also disappeared by metaphase. The effects of centrifugation on the disappearance of such a secondarily formed PPB were also examined. When protonemata were centrifuged acropetally at 3500 *g* for 15 min, so that the prophase endoplasm was displaced from the region of the second PPB and returned to the apical region, the second PPB persisted for 10 min after the termination of centrifugation in prophase cells (data not shown, see below). Even in the cells that were at or beyond prometaphase, the second PPB often remained intact (Fig. 8). These cells are considered to pass through the prophase/prometaphase transition during or after the 15-min acropetal centrifugation because, without the second centrifugation, the second PPB disappears at early prometaphase and does not reappear in prometaphase-telophase cells. In such centrifuged cells with a prometaphase-anaphase nucleus in the apical region and a second PPB in the cylindrical region, the apical PPB disappeared in 13 cells out of 16 cells observed (Figs 8B and 9B). These results suggest that the endoplasm acquires the capacity for disruption of the PPB at prometaphase.

Orientation of cell division

In cells centrifuged twice, mitotic spindles or phragmoplasts lay perpendicular to the cell axis as described above. Orientation of cell plates was also observed. When spindles or phragmoplasts were returned by 1-min centrifugation (at 3500 *g*), nearly longitudinal cell plates, connecting the top and the neck of the apical swollen region of cells were often observed (data not shown). The result suggests that in such cells the orientation of cell plates is determined according to that of the spindle or the phragmoplast and that the PPB site could not guide the growing cell plate, in contrast to Ôta's (1961) experiment.

Discussion

The PPB was disrupted by the return of the endoplasm during the second centrifugation in a stage-specific manner (Fig. 9). We cannot deny the possibility that the disruption of the PPB may be caused by centrifugal damage, resulting in the activation of a protease or an increase in intracellular levels of calcium ions, which might disrupt the PPB. However, it is likely that the disruption was caused by the restoration of some factor(s) that had been displaced in centrifuged cells and is essential to the disruption of the PPB in non-


2A
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Figs 2-6. Effects of the second, acropetal centrifugation (3500 *g*, 1 min in Figs 2-5; 3500 *g*, 15 min in Fig. 6) on disruption of the apical PPB in mitotic cells. PPBs are indicated by arrowheads. Differential interference contrast images showing return of organelles (A); images stained with anti-tubulin, of cortical (B) and endoplasmic (C) regions, and DAPI-stained images of the nuclei (D), are shown. Bar, 10 μm. **Fig. 2.** A metaphase cell 1.8 min after the termination of centrifugation. **Fig. 3.** A metaphase cell 10 min after the termination of centrifugation. **Fig. 4.** A telophase cell 1.8 min after the termination of centrifugation. **Fig. 5.** A telophase cell 10 min after the termination of centrifugation. **Fig. 6.** A prophase cell 10 min after the termination of centrifugation.

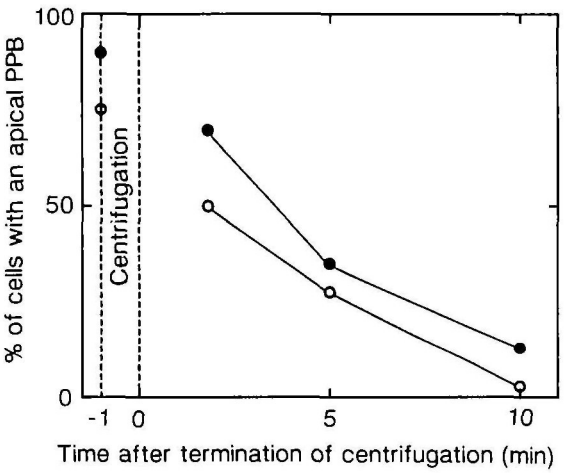

Fig. 7. Time courses of the disruption of the apical PPB after returning the endoplasm by centrifugation (3500 *g*, 1 min). The data at −1 min indicate frequencies of apical PPBs before centrifugation (14.9-15.3 h after the onset of BL). Filled circles indicate results from cells at prometaphase-anaphase. Open circles indicate results from early-telophase cells in which the diameter of the phragmoplast is smaller than that of the cylindrical region of the cell. Numbers of cells examined at each point are as follows. For prometaphase-anaphase cells: 39, 56, 71 and 33 cells at −1, 1.8, 5 and 10 min, respectively. For early-telophase cells: 24, 48, 54 and 46 cells at −1, 1.8, 5 and 10 min, respectively.

centrifuged cells. The basis of this hypothesis is as follows. (1) The disruption was stage-specific; (2) the first basipetal centrifugation did not disrupt the PPB (Murata and Wada, 1991); and (3) the centrifuged cells remained capable of division.

There are various plausible candidates for the factor(s) that is restored by the return of endoplasm. One such candidate is the mitotic spindle. If tubulin molecules from the PPB are incorporated into the mitotic spindle, the PPB may disintegrate as a result of the lack of tubulin molecules that is due to competition for tubulin with the mitotic spindle. However, the PPB was not disrupted when the endoplasm with a prophase nucleus, around which the mitotic spindle had begun to

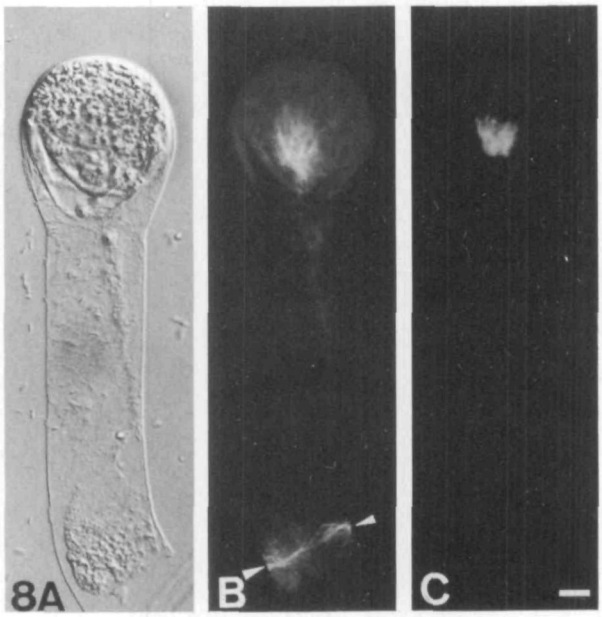

Fig. 8. Fluorescence micrographs showing that the PPB persisted in the cylindrical region of a prometaphase cell, ten minutes after the termination of a 15-min acropetal centrifugation (3500 *g*). (A) Differential interference contrast image. (B) Staining image with anti-tubulin. Arrowheads indicate the secondarily formed PPB. (C) DAPI-stained image showing prometaphase nucleus at the tip. Bar, 10μm.

develop (Wick and Duniec, 1984), was returned to the PPB. This result does not support the hypothetical competition for tubulin. To obtain more convincing information about the role of the mitotic spindle in the disruption of the PPB, we tried to return the endoplasm without the mitotic spindle to the apical PPB region. However, to date, such attempted manipulations have failed.

A supply of energy from mitochondria is another candidate. Sonobe (1990) characterized an ATP-dependent cortical MT-depolymerizing factor in homogenates of cultured tobacco cells. Since this factor is present throughout the cell cycle, he suggested that the increase in the concentration of ATP at mitotic phase causes the stage-specific disruption of cortical MTs and the PPB. Close proximity of mitochondria may be essential for the disruption of the PPB.

In animal cells, phosphorylation of proteins by $p34^{cdc2}$ kinase is considered to be involved in the disruption of interphase MTs at mitosis (Verde et al. 1990; Lamb et al. 1990). In plant cells, such a kinase is located within the PPB itself (Mineyuki et al. 1991b). Therefore, the kinase should not be displaced from the region of the PPB by centrifugation as performed in this study. However, if it is activated by the mitotic endoplasm and the active form of the kinase triggers the disruption of the PPB, the PPB may be disrupted when the mitotic endoplasm is returned to the region of the PPB.

In conclusion, we have shown that the endoplasm is

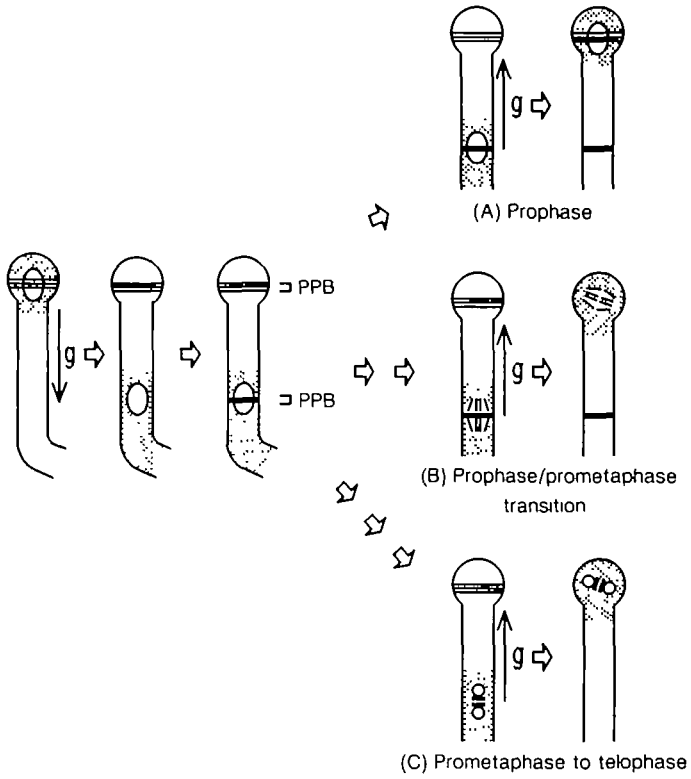

Fig. 9. Schematic representation of the effects of centrifugation on the disappearance of the PPBs in *Adiantum* protonemata. Ovals within cells indicate nuclei. Stippled regions indicate endoplasm. Transverse lines indicate PPBs. The first centrifugation displaces the nucleus and the endoplasm from the apical region (left). At prophase, a secondarily formed PPB has formed around the displaced nucleus and has not yet disappeared. The apical PPB, formed before the first centrifugation, persists after the second centrifugation (A). At the prophase/prometaphase transition, the second PPB in the cylindrical region still remains before and after the second centrifugation, but the apical PPB disappears during incubation after the second centrifugation (B). From prometaphase to telophase, the second PPB in the cylindrical region disappears before the second centrifugation and the apical PPB disappears during incubation after the second centrifugation (C).

essential to the stage-specific disruption of the PPB in *Adiantum* protonemal cells. Various mechanisms to explain the role of the endoplasm remain to be examined.

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