

Actin in the Preprophase Band of *Allium cepa*

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Abstract. F-actin has been identified in the preprophase band of *Allium cepa*. Cells attached to subbed slides were obtained from formaldehyde-fixed root tips digested in EGTA and Cellulysin. The air-dried cells were extracted in Triton X-100, treated with rhodamine-phalloidin, rinsed briefly in PBS, and viewed in the fluorescence microscope. Interphase cells contain a network of actin fibers that extends into all areas of the cytoplasm. During preprophase, the network is replaced by a band of fibers aligned in the position of the preprophase band. Colocalization of F-actin with rhodamine-phalloidin and microtubules with tubulin immunocytochemistry confirms that the

two bands are coincident. The actin appears to comprise a thin layer of fibers next to the plasmalemma. Like the microtubule preprophase band, the actin band narrows as preprophase progresses and disappears by midprophase. Fluorescent actin bands are not seen in fixed cells pretreated with excess unlabeled phalloidin before staining. They are also absent in roots exposed to cytochalasins B and D before fixation, but preprophase band microtubules at all stages of aggregation are still present. Colchicine treatment leads to the loss of both preprophase band microtubules and actin. The possible function of preprophase band actin is discussed.

THE preprophase band (PPB)¹ of microtubules (Mts), discovered more than 20 years ago by Pickett-Heaps and Northcote (12), encircles the cortex of most higher plant cells before mitosis and marks the zone at which the expanding cell plate, led by the phragmoplast, fuses with the parent plasmalemma (Pm) during cytokinesis (3, 4). Thus, positional information signified or encoded by the PPB appears to be critical in plant morphogenesis and the differentiation of a variety of cell types (3, 4, 9, 10, 12). Although the PPB disappears during prophase, it may still somehow influence the plane of division or signify a morphogenetically important change in cell organization that becomes active late in division. Despite its potential major role in plant development, the mechanisms responsible for PPB formation and operation remain unknown. It is therefore imperative that more information be gained about the organization and composition of the PPB and adjacent portions of the cortex and Pm. In the present paper, we report that, in root cells of *Allium cepa*, the PPB contains F-actin in addition to Mts.

Materials and Methods

Seedlings of *Allium cepa* L. CV White Portugal were grown in vermiculite as previously described (10). Root tips, 1 mm in length, were fixed for 1 h at room temperature in a solution containing 4% formaldehyde (freshly prepared from paraformaldehyde), 5 mM EGTA and 50 mM potassium phosphate buffer, pH 6.9. Fixative prepared with 50 mM Pipes buffer and 5 mM

Mg²⁺ (both used in Mt stabilization media; e.g., reference 2) showed no detectable differences in subsequent localizations. After the root tips were rinsed for 1 h in six changes of EGTA-phosphate, they were digested for 10 min in a solution consisting of 1% Cellulysin (Calbiochem-Behring Corp., San Diego, CA), 0.4 M mannitol, and 5 mM EGTA (17). After rinsing the roots once in EGTA-mannitol and twice in EGTA-phosphate (5 min each), they were gently squashed between an 18-mm square coverslip and a slide subbed with gelatin and chrom-alum. The coverslip was then carefully removed and the released cells were air dried on the slide overnight. The cells were then extracted in 0.5% Triton X-100 in PBS for 1 h, rinsed briefly in PBS, and then incubated for 45 min in the dark at room temperature in 9.0 µg/ml rhodamine-phalloidin (RP; obtained as a methanolic stock from Molecular Probes, Inc., Eugene, OR) in PBS. The cells were rinsed for 3 min in a few drops of PBS, mounted in a fresh drop of PBS under a coverslip, and viewed ~1 h later. Control slides were exposed to 0.5 mg/ml unlabeled phalloidin (Boehringer Mannheim Biochemicals, Indianapolis, IN) in PBS for 30 min before staining in RP.

Triton-extracted cells were also stained with a rat monoclonal antibody against yeast tubulin (Accurate Chemical & Scientific Corp., Westbury, NY) diluted 100-fold in PBS, followed by fluorescein- or rhodamine-coupled rabbit anti-rat antibody (ICN Immunobiologicals, Lisle, IL) diluted 100-fold in PBS. Each antibody treatment lasted 15–45 min and was followed by three 5-min washes in PBS. The cells were then mounted in a medium containing 90% glycerol, 10% PBS, pH 8.0, and 5% *n*-propyl galate (Sigma Chemical Co., St. Louis, MO). In some experiments, cells were first stained with RP and suitable cells mapped and photographed. The coverslip was then removed, the slide reprocessed for tubulin immunocytochemistry using a fluorescein-tagged second antibody, and the same cells relocated and recorded. In other studies, the antitubulin protocol was performed first, after which the cells were stained with RP and viewed at both excitation wavelengths.

Root tips, 5 mm in length, were incubated for 3 h in several drops of 5 mM colchicine (Sigma Chemical Co.) in deionized water (d'H₂O) or 5 µg/ml cytochalasin B (CB; Aldrich Chemical Co., Milwaukee, WI) or cytochalasin D (CD; Sigma Chemical Co.). Stock solutions (1 mg/ml) of CB and CD were prepared in DMSO and diluted with d'H₂O before use.

1. *Abbreviations used in this paper:* CB, cytochalasin B; CD, cytochalasin D; DIC, differential interference contrast; Mt, microtubule; Pm, plasmalemma; PPB, preprophase band; RP, rhodamine phalloidin.

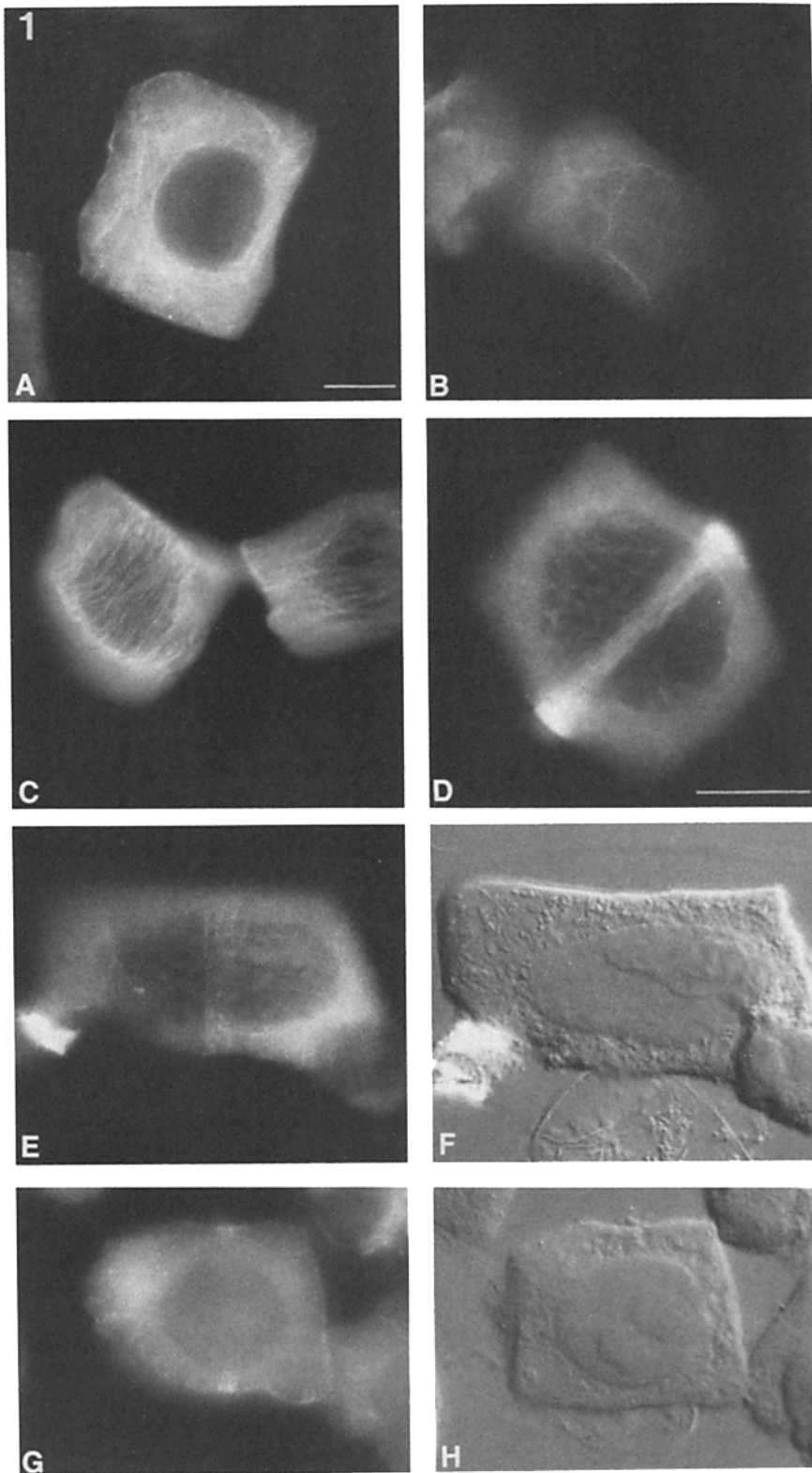


Figure 1. (A) RP fluorescence in an interphase root cell at mid-depth. Note the fibers that traverse the cell. (B) Cortical view of an RP-stained interphase cell. Note the randomly arranged fibers. (C) Tubulin localization (rhodamine second antibody) in an interphase cell. Note the transverse Mts in the cortex. (D) Higher magnification. Preprophase band microtubules (tubulin localization–rhodamine second antibody). (E) Cortical view of a preprophase cell stained with RP. Note the fluorescent band consisting of distinct fibers. (F) Same cell as in E, but viewed at mid-depth with DIC optics. (G) RP staining band in a preprophase cell viewed at mid-depth. (H) DIC image of the same cell shown in G. Bar, 10 μ m.

After treatment, the terminal 1-mm apices were excised, fixed, and processed as described above.

Cells were viewed on a Universal microscope (Carl Zeiss, Inc., Thornwood, New York) equipped with epifluorescence and differential interference contrast (DIC) optics. Most observations were made with a planapochromat, phase, 63 \times , 1.4 NA objective. Photographs were taken on Tri-X film (Eastman Kodak Co., Rochester, NY) and developed in diafine (Accufine Inc., Chicago, IL).

Results

Interphase root tip cells contain an elaborate meshwork of fine, RP-binding actin fibers similar to those reported elsewhere (2, 11). These fibers traverse all regions of the cytoplasm and many are closely associated with the nucleus (Fig. 1 A). Their random arrangement is especially evident in

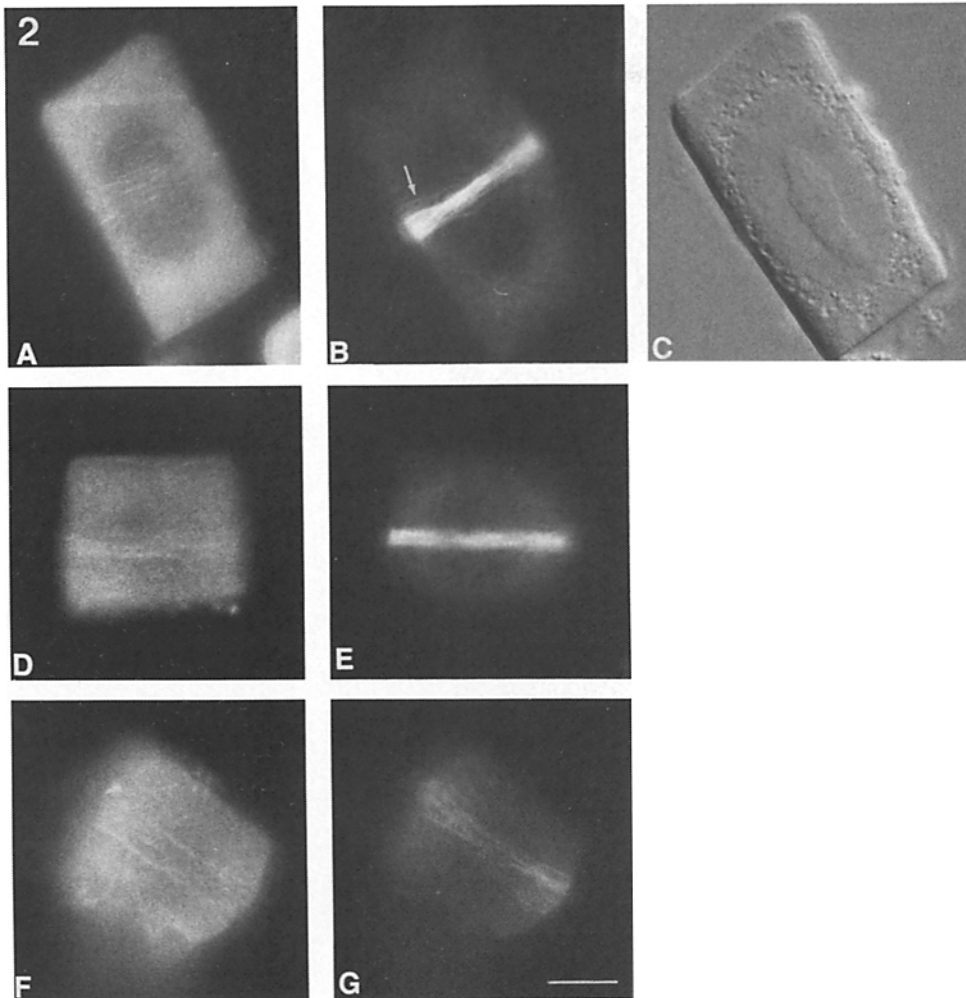


Figure 2. Colocalization of F-actin (with RP) and tubulin (with anti-tubulin and fluorescein-tagged second antibody) in three different root cells. (A, D, and F) RP fluorescence. (B, E, and G) Microtubules. (C) DIC; same cell as in A and B. Note the similarity in the position of some actin fibers and Mts (arrow) in A and B. Areas of dissimilar orientation or localization are also evident, however (F and G). The bright cell fragment evident at the lower right of A was dislodged during reprocessing for tubulin localization (B and C). Bar, 10 μ M.

paradermal views of the cell cortex (Fig. 1 B). In contrast, cortical Mts have a predominantly transverse arrangement when visualized by tubulin immunocytochemistry (Fig. 1 C).

When cells about to enter mitosis (as recognized by the irregular or stellate shape of the nucleoli in DIC [Fig. 1, F and H]) are stained with RP, another actin array is seen. Such cells, which almost always contain an Mt PPB when stained for tubulin (Fig. 1 D), often contain a band of actin fibers as well (Fig. 1 E). The actin band encircles the cortex (Fig. 1 G) and is arranged in the same circumferential position as the PPB, as judged by comparison with companion slides stained for tubulin. The specificity of the actin staining is established by the absence of RP fluorescence in cells pretreated with excess unlabeled phalloidin (data not shown). Fluorescence of interphase actin fibers is also lost under these conditions.

Although comparison between cells stained with RP and those reacted for tubulin indicates that the actin and Mt bands are coincident, this point was specifically demonstrated by two colocalization protocols. In the first procedure, cells were initially subjected to tubulin immunocytochemistry using a fluorescein-tagged second antibody and then stained with RP. Observations at both excitation wavelengths show that the Mt and actin bands occupy the same position (data not shown). Slides stained only for tubulin

were checked for low levels of fluorescein excitation at the green wavelengths used for rhodamine, and none was found. However, in order to preclude this possibility, a second procedure was used in which the RP localization was done first and preprophase cells were photographed and their positions noted. The coverslip was then removed, the slide subjected to tubulin labeling (again with fluorescein), and the same cells were relocated. We again found that the actin and Mt bands are coincident (Fig. 2).

Actin bands of various dimensions have been seen. In some cells, a rather broad array of transverse fibers appears amongst the random interphase cortical elements (Fig. 3 A). In others, the band is quite narrow (Figs. 1, E and G, and 2 D) and few if any interphase fibers remain. Comparison of band width, nucleolar morphology, and chromatin condensation (using Hoechst 33258 fluorescence; data not shown) indicates that the band begins as a broad array. Thus, its formation mirrors that of the Mt PPB, which is also initially quite wide but narrows as preprophase progresses (18). This conclusion is supported by our colocalization studies which show that broad actin bands accompany broad Mt bands, and both appear to narrow coordinately.

Although the actin and Mt bands are coincident, they do differ in some respects. The RP fluorescence of the actin band is relatively weak (Figs. 1 E and 2, A, D, and F), and

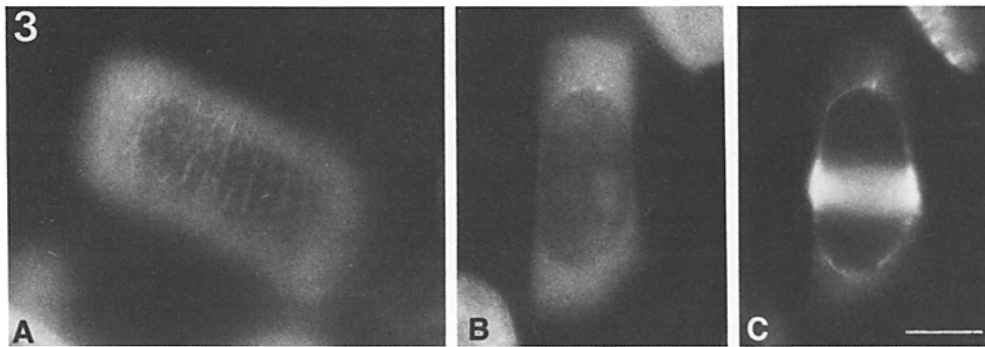


Figure 3. (A) Broad array of transverse actin fibers characteristic of an early PPB. (B) Root cell treated with CD for 3 h before fixation. No RP-staining PPB fibers are evident. (C) Same cell, tubulin localization (fluorescein second antibody). A prominent Mt PPB is present. Bar, 10 μ M.

through-focusing indicates that the actin fibers occupy a thin layer near the Pm. In contrast, mature PPBs are several Mts deep (e.g., references 3, 10, 12) and exhibit intense fluorescence after tubulin localizations using either rhodamine- or fluorescein-tagged second antibodies (Figs. 1 D, 2, B and E, and 3 C). Although PPB Mts are often seen in prophase cells, our observations indicate that the actin band may disappear somewhat earlier. This observation may explain why most ($\sim 70\%$) but not all cells with PPBs fluoresce with RP. Lastly, although the Mt and actin bands are coincident, individual actin fibers and Mts are not necessarily colocalized or coaligned (Fig. 2, F and G).

As already noted, RP fluorescence in the PPB, as well as that associated with other actin fibers, is lost when fixed cells are pretreated with unlabeled phalloidin. Likewise, F-actin fluorescence disappears in cells exposed to CB or CD before fixation (Fig. 3 B). However, PPB Mts at all stages of aggregation are still present (Fig. 3 C). Both PPB actin and Mts are absent in preprophase cells treated with colchicine, while cortical Mts but not actin fibers are lost in interphase cells exposed to this agent (data not shown).

Discussion

Our evidence indicates that the PPB of *Allium* contains F-actin in addition to Mts. The actin band seen in our preparations appears at a stage before mitosis when the PPB is present, and it disappears thereafter. Moreover, the location of the actin band is coincident with that of the PPB. The specificity of the RP staining is affirmed by the lack of binding in cells pretreated with cytochalasins or excess unlabeled phalloidin. Our results are further strengthened by the localization of actin in the PPB using antiactin immunocytochemistry (Marc, J., personal communication).

We can now turn our attention to the possible function of PPB actin, and three alternatives deserve particular attention. The first possibility involves an actin role in PPB formation. That is, an actin-based system could be responsible for reorganizing the interphase cortical Mt array into the PPB. It is still unclear whether the PPB forms from pre-existing cortical Mts or is instead polymerized from tubulin dimers as the old interphase array disassembles. If reorganization of intact Mts is involved, it is possible that this process requires an interaction between actin, Mts, the Pm, and associated proteins. However, certain observations render this possible function unlikely. First, although PPB actin is lost in cells treated with CB or CD, PPB Mts at all stages of

aggregation (i.e., broad as well as narrow bands) are still present. Second, we would expect that if actin guides the rearrangement of cortical Mts into the PPB, it would still be present in the absence of Mts. That the actin band is absent after colchicine treatment indicates that PPB Mts are responsible for actin aggregation and not vice versa. Finally, if we assume that an actin-based process establishes the PPB, we must still identify the factors that initially cluster the actin.

The second possible function centers on the potential role of the PPB in division plane determination. Although the PPB cannot directly influence the expanding phragmoplast-cell plate complex during telophase because it has long since disappeared, it could still determine the zone of plate fusion along the Pm. Recent evidence clearly shows that the phragmoplast/plate edge responds to this zone as it reaches the cortex (9). It is therefore possible that the PPB alters the cortex or Pm in a manner that is manifested during telophase. For example, proteins which interact with F-actin or Mts in the phragmoplast (2, 4; Palevitz, B. A., manuscript submitted for publication) may be left behind on the Pm by the PPB. Alternatively, the PPB could concentrate ion channels (e.g., Ca^{2+} or K^{+}) in the Pm which, when active during telophase, set up electrical currents that guide the cell plate. Although Mts and associated proteins are capable of moving membrane material (16), PPB actin could also function in this process. This could explain the sensitivity of cell plate alignment to CB and phalloidin (8). Recent evidence indicates that actin-based systems cluster ion channels in polarizing fucoid eggs (1) and dividing moss cells (15). The latter system is especially relevant to our work (8-10) because the site of an inwardly directed Ca^{2+} current moves as the cell plate rotates toward its fusion site along the Pm (14).

The last alternative is that the actin has no specific function and its presence in the PPB is a fortuitous consequence of the aggregation of PPB Mts. In this model, cortical actin, perhaps derived from the random fibers in the interphase cortex reported here or the thin microfilament-like elements recently seen along cortical Mts in freeze-substituted material (e.g., reference 6), are trapped and aligned as Mts aggregate in the PPB. Our indications that not all PPBs contain actin might support this last hypothesis. It is noteworthy, however, that under the conditions used in this study, RP staining was not detected in the large kinetochore fibers of mitotic cells, although it does build up in the phragmoplast during cytokinesis (Palevitz, B. A., manuscript submitted for publication). Thus, Mt aggregation per se is not sufficient for F-actin accumulation. It would be of interest to look for

RP fluorescence in other plant cells that contain clustered cortical Mts, such as differentiating tracheary elements undergoing localized wall deposition. Weak RP fluorescence was recently reported beneath wall thickenings in *Cobaea* seed hairs, but it is not filamentous (13).

It is clear that we still do not know how that PPB operates or whether it is merely a manifestation of a more basic morphogenetic system responsible for division plane determination (e.g., reference 3). However, it may be significant that a band of F-actin is also present in the division plane of fungal cells, although it appears just before and during septation rather than before karyokinesis (5, 7). Unlike the PPB, the fungal band does not contain Mts. Further information on the content and organization of the phragmoplast/cell plate, PPB, and coincident "charmed" zones in the cortex and Pm should help clarify the mechanism and control of cytokinesis in plants.

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