Polarity of Midbody and Phragmoplast Microtubules

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ABSTRACT

A newly discovered method (Heidemann and McIntosh, 1980, Nature [Lond.], 286:517) for displaying the molecular polarity of microtubules (MTs) has been slightly modified and applied to the midbodies of cultured mammalian cells and the phragmoplasts of Haemanthus endosperm. The method involves the decoration of preexisting MTs in lysed cells with curved ribbons of tubulin protofilaments; the direction of curvature of these C-shaped appendages as seen in cross section reflects the intrinsic polarity of the MTs. In transverse sections of midbodies from HeLa and PtK cells, we find that essentially all the MTs in a given region of the structures have the same direction of hook curvature, and hence the same polarity. The midbody MTs that lie on one side of the spindle equator show the opposite polarity from those on the other side, indicating that the midbody is constructed from two families of antiparallel MTs. Midbody MTs are arranged with their fast-growing ends overlapping at the spindle equator, consistent with the hypothesis that the midbody is formed by the interdigitation of aster MTs. The polarities of the MTs from the phragmoplast of endosperm cells are the same as those found in the mammalian midbody. Our results eliminate one model for mitosis, but are consistent with others. The systematic and reproducible polarities observed favor the concept that MT polarity is an important factor in the formation and/or the function of these two mitotic structures.

Microtubules (MTs) possess an intrinsic polarity that derives from their construction out of asymmetric subunits polymerized head-to-tail (for a review, see reference 2). Functional manifestations of this polarity are found in the different rate constants for assembly and disassembly at the two ends of an MT (1, 3, 10, 20, 31) and in the polar orientation of dynein arms sometimes seen on flagellar MTs (1, 12, 33). Both of these properties have been used as probes for the polarity of MTs in particular cellular systems (4, 5, 12, 31). Recently, however, an alternate method for MT polarity determination has been described that is based upon the capacities of tubulin for polymorphic assembly. Burton and Himes (9) have identified conditions in which tubulin will form diverse protofilament bundles and abnormal wall junctions. In cross section, some of these aggregates display enantiomorphic images which have the potential of revealing the intrinsic polarity of the polymers (19). Heidemann and McIntosh (13) have found related conditions in which spontaneous tubulin assembly forms variegated protofilament bundles, but when the assembly reaction is seeded by MTs, the polymerizing material more closely resembles normal MTs. The preexisting MTs elongate in the usual fashion, but in addition, one observes the decoration of those MTs with C-shaped sheets of laterally associated protofilaments that make an unusual wall-to-wall junction with the true MT. Such sheets seen in cross section appear as hooks that curve clockwise or counterclockwise about the MT (Fig. 1a). When MTs of defined polarity, such as astral MTs and MTs grown from a basal body, are viewed from their fast-growing, or plus end, toward their slow-growing, or minus end (3), then ~90% of all hooks curve clockwise (13). We can therefore define a "right-hand rule" for relating hook curvature to MT polarity: curl the fingers of your right hand in the direction of hook curvature; your thumb will now point toward the minus end of the MT.

In this paper, we show that hooks can form directly on preexisting MTs in lysed cells, facilitating polarity determination for MTs not readily studied by a method requiring MT elongation. We have used these hooks to determine the polarities of the MTs in the mitotic midbody of mammalian cells and in the phragmoplast of Haemanthus endosperm. These systems were selected for study because the polarities of their MTs may be informative about mitotic mechanism. The midbody is an array of MTs found in telophase and postdivision animal cells. It is located in the zone between the separated chromosomes or sister nuclei and is believed to be derived from the interzone fibers of anaphase (22). Serial sections in both longitudinal (26, 29) and transverse orientation (7, 22-24) suggest that the midbody is constructed from two families of interdigitating MTs with a number of short MT fragments scattered throughout (Fig. 1b). The phragmoplast is the organelle that forms the cell plate, i.e., the wall that develops to achieve cytokinesis in plant cells. Phragmoplast origin is in...
most circumstances related to the spindle in a fashion reminiscent of the midbody (15), but there are many subtleties of phragmoplast formation and function that deny a strict homology (18). In this paper, we present data which define the polarities of midbody and phragmoplast MTs and relate the polarities observed to those predicted by several models for mitosis.

MATERIALS AND METHODS

Tubulin Preparation

Microtubule protein (MTP) was prepared from bovine brain by two cycles of temperature-dependent polymerization and depolymerization in a buffer containing 0.1 M 2-[N-morpholino]ethane sulfonate (MES), pH 6.5, 1 mM MgCl₂, 1 mM EGTA, 1 mM GTP, and 8 M glycerol (30, 34). An MTP pellet was resuspended in 0.5 M PIPES, pH 6.9, 1 mM MgCl₂, 1 mM EDTA, and 1 mM GTP at 0°C. We call this buffer 0.5 PAMEG. The resuspended MTP was cycled once through assembly and disassembly in 0.5 PAMEG by the customary temperature changes and then spun at 250,000 g for 3 h at 4°C to remove oligomers and retard spontaneous polymer initiation. The resulting high speed supernatant, which we will call tubulin, was frozen in small aliquots and kept in liquid nitrogen for subsequent use. Protein concentrations were determined by the method of Bradford (6).

Cells

HeLa cells were grown on coated microscope slides in petri dishes using Eagle's minimum essential medium supplemented with 10% calf serum (Grand Island Biological Co. [GIBCO] Grand Island, New York) and 1% nonessential amino acids (GIBCO). The slides used for cell growth were coated with Teflon (Spray-Mate, dry lubricant, 3 M Co., St. Paul, Minn.) and then dipped in 1 mg/ml polylysine, rinsed in H₂O, dried, and sterilized with ultraviolet light. PtK₂ cells were similarly grown but in Ham's F12 (GIBCO) plus 10% fetal calf serum.

Cell Lysis and Growth of Hooks

Cells were rinsed quickly with warm 0.5 PAMEG and then treated with a mixture containing 1% Triton X-165, 0.5% deoxycholate, 0.02% sodium dodecyl sulfate, 2.5% dimethyl sulfoxide (DMSO), and 0.5 mg/ml tubulin in 0.5 PAMEG at 37°C for 30 min. This solution lyses the cells, stabilizes cellular MTs, and induces hook formation (13; for detailed instructions in making this detergent mixture, see 14).

Isolation and Treatment of Cilia

Tetrahymena pyriformis was grown in 0.75% proteose peptone (Difco Laboratories, Detroit, Mich.), 0.75% yeast extract, 1.5% sucrose, 1 mM MgSO₄, 0.05 mM CaCl₂, 2 mM KH₂PO₄, and 30 μg/ml Sequestrene (Ciba-Gegy Corp., Pharmaceuticals Div., Summit, N. J.). Cells in midlog growth were deciliated by a 2-min treatment with 4 mM Dibucaine (Pfaltz & Bauer Inc., Stamford, Conn.). The cells were pelleted at 800 g for 5 min; then the cilia present in the supernate were sedimented at 17,000 g for 15 min, washed in 0.1 M PIPES, 1 mM EGTA, 1 mM MgCl₂, 1 mM GTP, pH 6.9 (0.1 PAMEG), and resuspended in 0.5 PAMEG. Cilia were then incubated in a buffer containing 2.5% DMSO and 2 mg/ml tubulin in 0.5 PAMEG with or without 0.1% Triton X-100 for 20 min at 34°C to induce the formation of hooks on the ciliary MTs.

Preparation and Treatment of Endosperm Phragmoplasts

Fruits of Haemanthus katherinae Baker were generously provided by W. T. Jackson (Dartmouth College, Hanover, N. H.). Endosperm cells were gently squeezed onto slides coated with Teflon, polyllysine, and a thin layer of 0.1% agar in water with 3.5% glucose. The cells were allowed to settle and spread at room temperature for 20–30 min in a wet chamber. They were then treated with a mixture containing 2.5% DMSO, 0.04% Saponin (Sigma Chemical Co., St. Louis, Mo.) or 0.3% polyoxyethylene-20 cetly ether (Brij 58, Sigma Chemical Co.), and 0.5 mg/ml tubulin in 0.5 PAMEG for 30 min at 37°C to lyse the cells and form hooks.

Electron Microscopy

Cells and cilia were fixed with 2% glutaraldehyde in 0.1 PAMEG for 30 min, washed twice in 0.1 M cacodylate buffer, pH 7.1, and then postfixed with 1% OsO₄ in the same cacodylate buffer. In some cases 1% tannic acid was added to the glutaraldehyde-containing buffer. Dehydration, including en bloc staining with uranyl acetate and phosphoglutonic acid and embedding were carried out according to a standard procedure (11). The cilia were embedded as a pellet in an Eppendorf tube while the cells were flat embedded between two Teflon-coated slides separated by chips of coverslips to serve as spacers. Specimens were sectioned on an ultramicrotome (DuPont Instruments-Sorvall, DuPont Co., Newtown, Conn.; or LKB Instruments, Inc., Rockville, Md.) and observed either in a Philips EM 300 or a JEOL 100C electron microscope. From the cutting of the sections through the printing of the electron micrographs, the original orientation of the sections was always considered to assure that the final pictures were not mirror images of the true structures. Handedness of the hooks was scored on prints with a final magnification of about 50,000. Fig. 1a shows some of the images observed and the ways that we classified them.

RESULTS

Polarity-revealing Hooks Can Form on Preexisting Microtubules

In our earlier work with conditions for hook formation, MT appendages were found predominantly, if not entirely, on the newly polymerized portion of the MTs studied (14). Here we have asked if conditions could be found in which hooks would form on preexisting MTs. Isolated cilia were used as a system in which the preexisting MTs could be unambiguously distinguished from the newly grown polymer, although these MTs with so many microtubule-associated proteins (MAPs) may not form hooks as well as other MTs. After a 20-min incubation of cilia with tubulin at 2 mg/ml in 0.5 PAMEG plus 2.5% DMSO some hooks are seen (Fig. 2). Shorter incubation times are not adequate to produce hooks in this system. These conditions are similar to the ones used earlier, except that the time of incu-
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The equivalence of the graphs, given the variability seen not the products of MT elongation. Fig. 4 ashows a single cross on either side of the midregion are therefore preexisting MTs, elongated during lysis in hook-forming buffer. The MTs seen staining material and by the peak in MT number, have not at the midregion of the midbody, as marked both by the darkly 500400 300 0 1 2 3 400 500 600 700 800 900 μm

bination is longer. This change seems to favor hook formation relative to MT elongation.

A detailed structural analysis of midbodies exposed to our lysis conditions has confirmed that hooks can add to preexisting MTs. The midregion of a midbody is characterized by an accumulation of electron-dense material in between the MTs, was taken as a marker (0 point on the abscissa); the distance from this area was determined by counting the sections in either direction, assuming that the thickness of a silver section is ~900 A.

The midbodies of HeLa cells contain more MTs than those of PtK, and the courses that they follow are somewhat less divergent (Fig. 4c). We have therefore used HeLa cells for the bulk of our MT polarity data collection. Five telophase HeLa cells treated to form hooks have been serially sectioned, and the frequencies of hook handedness have been tabulated at different positions relative to the midregion. The hooks seen are qualitatively similar to those found in PtK cells. The relative numbers of HeLa cell hooks of each hand, presented in Table II, provide convincing evidence that the polarities of HeLa midbody MTs are the same as those found in PtK cells.

Polarity of Phragmoplast MTs

Fig. 5 a and b show cross sections of a phragmoplast from a lysed cell of Haemanthus endosperm: Fig. 5 a shows a section cut while approaching the cell plate; Fig. 5 b is on the far side. As with the midbody the predominant hook hand switches as one passes the midregion of the structure. Table III presents data from two cells relating hook handedness to position on the phragmoplast. It is evident that the polarities of phragmoplast MTs as seen with this assay are the same as those of the midbody: the plus ends of the MTs are overlapping in the middle of the structure.

DISCUSSION

The tabulated frequencies of hook handedness for both midbody and phragmoplast MTs display a convincing consistency, supporting the assertion that our method is a dependable determination of MT polarity. The data suggest that all MTs on one side of the midbody have a single polarity, while those on the other side have the opposite orientation. Because MT number is known to be higher in the midregion of the midbody than on either side, the totality of available facts supports the
FIGURE 4 Cross sections through a PtK₂ (a and b) and a HeLa (c) cell midbody treated with the hook-forming mixture. (a) When looking towards the center of the midbody mostly counterclockwise hooks are found. Sometimes a clockwise one can be seen on an MT which has also counterclockwise hooks attached to it (arrows). × 51,000. (b) The midregion of the same midbody as in a. The electron-dense material present in this area seems to interfere with the formation of hooks, because only a very few can be seen in the regions where that material is common. × 46,000. (c) HeLa cell midbody. The cross section was taken after passing through the midregion. Outward from the midbody, mainly clockwise hooks are displayed. × 46,000.
FIGURE 5 Part of the phragmoplast of a Haemanthus endosperm cell in cross section, lysed as described in Materials and Methods. Approaching the cell plate (a) one sees mainly counterclockwise hooks, while sections on the far side of the forming cell plate reveal predominantly clockwise hooks (b). × 36,000.
The observed polarity of the midbody MTs can be related to some models for mitosis in which MT polarity is considered. Our observations, together with those of Heidemann and McIntosh (13) are consistent with the models of Nicklas (27) and of Margolis et al. (21). They are inconsistent with the model of McIntosh et al. (25) in which interdigitating astral MTs are anticipated, but it is predicted that they would slide into an arrangement with polarities opposite to those we observe (Fig. 6). This model should for this and other reasons (28) be discarded, although the ideas of MT polarity and specific interactions between antiparallel MTs upon which it is based are still likely to be important in mitotic mechanism.

Our observations on the phragmoplast must be regarded as preliminary. While many MTs and hooks have been scored for polarity, the phragmoplast appears to be a more complex concept that the midbody is constructed from the interdigitation of two antiparallel families of MTs. The identity of the polarity of the astral and the midbody MTs in each half spindle is consistent with the hypothesis that the midbody arises through the interdigitation of MTs. We imagine that at some time during mitosis a fraction of the astral MTs from opposite poles bind to one another to form the bundles seen as interzone fibers at anaphase, and that these bundles ultimately form the midbody itself.

Table I

<table>
<thead>
<tr>
<th>Cell</th>
<th>Clockwise hooks</th>
<th>%</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>ai</td>
<td>357</td>
<td>96.0</td>
<td>372</td>
</tr>
<tr>
<td>ao</td>
<td>36</td>
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<td>100</td>
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<tr>
<td>bi</td>
<td>157</td>
<td>86.3</td>
<td>182</td>
</tr>
<tr>
<td>bo</td>
<td>9.1</td>
<td>80.9</td>
<td>88</td>
</tr>
</tbody>
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Data from two cells, a and b. The subscripts i and o indicate the region of the midbody under examination: i implies sections taken on the way in toward the midregion, o implies sections cut on the way out.

Table II

<table>
<thead>
<tr>
<th>Cell</th>
<th>Clockwise hooks</th>
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<th>Total</th>
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</thead>
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<td>110</td>
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<tr>
<td>bi</td>
<td>163</td>
<td>94.2</td>
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<tr>
<td>bo</td>
<td>15</td>
<td>8.8</td>
<td>170</td>
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<tr>
<td>ci</td>
<td>132</td>
<td>96.4</td>
<td>137</td>
</tr>
<tr>
<td>co</td>
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<td>1.6</td>
<td>61</td>
</tr>
<tr>
<td>di</td>
<td>64</td>
<td>2.7</td>
<td>2,406</td>
</tr>
<tr>
<td>eo</td>
<td>7.2</td>
<td>219</td>
<td>236</td>
</tr>
</tbody>
</table>

Data from five cells, a, b, c, d, and e. The subscripts i and o are as defined in the legend to Table I.

Table III

<table>
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<th>Cell</th>
<th>Clockwise hooks</th>
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<th>Total</th>
</tr>
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<td>96.3</td>
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<td>ao</td>
<td>31</td>
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<tr>
<td>bi</td>
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<td>97.3</td>
<td>300</td>
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<tr>
<td>bo</td>
<td>19</td>
<td>2.1</td>
<td>901</td>
</tr>
</tbody>
</table>

Data from two cells, a and b. The subscripts i and o are as defined in the legend to Table I.

Fig. 6 MT polarity in metaphase (a), anaphase (b and c), and telophase (d) as proposed by McIntosh et al. (25) in their model for mitosis. MTs are represented by lines; the kinetochore MTs by dotted lines. The stippling indicates the material that is, according to the model, transferred from the poles to the midregion of the midbody. The polarity of MTs with respect to their organizing centers is indicated by arrowheads. The results from the work of Bergen et al. (4) have shown that the fast-growing (plus) ends of MTs are distal to the organizing center, meaning that the arrowhead depicts the plus end of these MTs. In telophase (d), the polarity of the interzonal MTs, proposed by McIntosh et al. (25), is opposite to the one we found in this paper (d'); the MTs are oriented with their plus (fast-growing) ends embedded in the osmiophilic material at the midregion of the midbodies.
structure than the midbody, and only two cells have been successfully processed. We include these preliminary data here because of their striking similarity to corresponding data from the midbody. The equivalence of the MT polarities supports the idea that the two structures arise by a common mechanism (15). The phragmoplast, however, is capable of lateral growth, especially in cambium cells. This growth appears to involve the initiation of new MTs from the edge of the growing cell plate. A microtubule-organizing capacity for the phragmoplast is further suggested by the ultraviolet microbeam irradiations performed by Inoué (17). If phragmoplast MTs are initiated at the cell plate, then they are upside down with respect to their organizing center when compared to astral and flagellar MTs. We plan an extensive study of this organelle in different stages of formation to explore this enigmatic possibility.

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REFERENCES