Variable pathways for developmental changes in composition and organization of microtubules in *Physarum polycephalum*

LILIANKA SOLNICA-KREZEL, MAUREEN DICKINS-GILICINSKI*, TIMOTHY G. BURLAND and WILLIAM F. DOVE

McArdle Laboratory for Cancer Research, University of Wisconsin, 1400 University Avenue, Madison, WI 53706, USA

*Present address: Department of Pharmacology, University of Medicine and Dentistry of New Jersey, Robert Wood Johnson Medical School, 675 Hoes Lane, Piscataway, NJ 08854, USA

Summary

The development of uninucleate amoebae into multinucleate plasmodia in myxomycetes is called the amoebo-plasmodial transition (APT). During the APT in *Physarum polycephalum* the ability to form flagellar axonemes is lost; the astral, open mitosis is replaced by the anastral, closed mitosis; and cytoskeletal microtubules disappear. These changes are accompanied by alterations in the repertoire of expressed tubulins. Using immunofluorescence microscopy we have studied the timing of loss and accumulation of developmentally regulated tubulin isotypes in relation to other cellular events during the APT. We specifically asked whether changes in the composition of microtubules are correlated with changes in their organization. The plasmodium-specific $\beta_2$-tubulin can first be detected in microtubules of uninucleate cells after they become committed to plasmodium formation. However, rare cells are observed that exhibit $\beta_2$-tubulin at earlier or only at later stages of development. Amoeba-specific acetylated $\alpha_3$-tubulin disappears gradually during development. Individual cells differ in the timing of loss of this isotype: $\alpha_3$-tubulin is present in the majority of uninucleate cells, in a fraction of binucleate and quadrinucleate cells, and is absent from larger multinucleate cells. Cytoplasmic microtubules in uninucleate cells are organized by a single microtubule-organizing center (MTOC) juxtaposed to the nucleus. Binucleate cells and quadrinucleate cells exhibit variable numbers of MTOCs. Cytoplasmic microtubules persist during the APT until the stage of plasmodia containing at least 100 nuclei. The lack of a strict correlation between the changes in tubulin composition and changes in organization of microtubular structures indicates that accumulation of $\beta_2$-tubulin and disappearance of $\alpha_3$-tubulin isotypes are not sufficient to bring about reorganization of microtubules during development. Individual cells in a developing population differ not only in the succession of accumulation and loss of developmentally regulated tubulins, but also in the sequences of other cellular changes occurring during the APT.

Key words: microtubules, tubulin isotypes, development, *Physarum*

Introduction

Microtubule-based structures perform a variety of functions in eukaryotes. They participate in the separation of chromosomes, intracellular transport, flagellar and ciliary motility, and the establishment and maintenance of cell shape. A still unresolved problem is how this structural and functional heterogeneity of microtubular structures is generated.

One approach is to study the role of the major components of microtubular structures, i.e. tubulins. Most eukaryotic species exhibit multiple distinct $\alpha$- and $\beta$-tubulin isotypes, that can differ in primary sequence and/or in post-translational modifications. These distinct tubulins could provide specific structural and functional information for the morphogenesis of various microtubular arrays (Fulton and Simpson, 1976). However, whenever tested, tubulins that are highly divergent in sequence do not usually appear to be restricted to specific microtubular arrays (Lopata and Cleveland, 1987; Gu et al. 1988; Lewis et al. 1987; Joshi et al. 1987; but see Asai and Remolona, 1989). This indicates that a distinct tubulin may not be sufficient to determine the organization and function of the microtubular structure in which it is incorporated. However, in *Caenorhabditis elegans*, mutation of a gene encoding a distinct $\beta$-tubulin leads to conversion of microtubules containing 15 protofilaments into microtubules containing 11 protofilaments (Savage et al. 1989). Thus, some distinct tubulins may be necessary for the morphogenesis of a specific microtubular structure.

Acetylated tubulin isotypes are often found only in a subset of microtubules present in a cell (Sasse et al. 1987; Diggins and Dove, 1987). Microtubules containing acetylated $\alpha$-tubulin appear to be more stable than others, but it is not known whether a distinct function is associated with acetylation (Piperno et al. 1987; Webster and Borisy, 1989).

We are studying the role of distinct tubulin isotypes in the generation of microtubular diversity during the development of uninucleate amoebae into multinucleate, syncy-
tial plasmodia in the acellular slime mold *Physarum polycephalum*. This so-called amoebal–plasmodial transition (APT) provides a special opportunity to study the morphogenesis of microtubular structures. The APT involves cellular changes that include profound alterations in the organization of microtubular structures and changes in expression of tubulin isotypes. Amoebae exhibit microtubules in the cytoskeleton, the microtubule-organizing center (MTOC) and its associated centrioles, and the astral, open mitotic spindle (Havercroft and Gull, 1983). In developing cultures of apogamic strains, amoebae grow and divide until a critical cell density is reached. Then cells develop rather synchronously into plasmodia (Youngman et al. 1977; Solnica-Krezel et al. 1988). Developing cells enter a cell cycle 2.4 times longer than that of the amoeba, lose the ability to transform into flagellates, gain competence to form plasmodia at low cell density (commitment), and acquire the ability to ingest amoebae (Blindt et al. 1986; Bailey et al. 1987). This long transitional cell cycle terminates in the formation of a binucleate cell that grows by synchronous, intranuclear karyokinesis without cytokinesis; fusions frequently occur between cells with two or more nuclei (Anderson et al. 1976; Bailey et al. 1987). Cytoplasmic microtubules disappear, so that in the mature plasmodium the only microtubular structure detected is the closed mitotic spindle (Havercroft and Gull, 1985; Gull et al. 1985).

From seven distinct tubulin isotypes of *Physarum*, three are detected only in the plasmoid: Na1, Eo2 and b2. Two others, b1A and a3, are specific to the amoebal phase of the life cycle (Monteiro and Cox, 1987; Walden et al. 1989; Solnica-Krezel et al. 1988; Burland et al. 1983; Burland et al. 1984). Two of these developmentally regulated tubulins, a3 and b2, are of primary interest in relation to their possible role in the morphogenesis of microtubular structures. a3-Tubulin, an acetylated form of a1-tubulin, is detected only in the MTOC region of amoebae. Furthermore, when an amoeba transforms into a flagellate, a3-tubulin becomes more abundant and its distribution is expanded to flagellar axonemes and flagellar cone microtubules (Sasse et al. 1987; Diggins and Dove, 1987). These patterns of expression of a3-tubulin suggest a role in organization of microtubules.

The plasmoid-specific b2-tubulin exhibits only 83% sequence identity with the other two b-tubulins of *Physarum* (Burland et al. 1988). The high sequence divergence has the potential to determine functional specificity of this protein, although b2-tubulin is incorporated into both astral and anastral mitotic spindles, and into the cytoskeletal microtubules of cells developing into plasmodia (Diggins-Giliciński et al. 1989). In this study, we have tested the ability of b2-tubulin to be incorporated into yet another microtubular array of *P. polycephalum* — the flagellar axoneme, a structure highly distinct from other microtubular organelles.

We have used immunofluorescence microscopy to determine the timing of loss of a3-tubulin and accumulation of b2-tubulin, and to document their distribution among the various microtubular arrays that are observed during the APT. We show that there is no strict correlation between the changes in expression of a3- and b2-tubulins and the reorganization of cytoplasmic microtubules. In addition, we demonstrate b2-tubulin in flagellar axonemes. This provides further evidence that these distinct tubulin isoforms are not sufficient to change the organization of microtubules during development. We show cytoplasmic microtubules in developing plasmodia containing at least 100 nuclei. This demonstrates that the microtubular cytoskeleton is present during the APT in cells substantially larger than previously reported (Gull et al. 1985).

In studying a large number of cells undergoing the APT, we were also able to ask whether the succession of developmental events is identical in all developing cells. We confirm and expand the basic sequence of developmental events established earlier (Blindt et al. 1986; Bailey et al. 1987). However, we find that rather than a single, exclusive developmental pathway, there is heterogeneity among developing cells in relation to: (1) the timing of changes in expression of both b2- and a3-tubulins; (2) the reorganization of cytoplasmic microtubules; and (3) the sequences of physiological changes.

### Materials and methods

#### Reagents

Except as follows, all were purchased from Sigma Chemical Co. (St Louis, MO). Formaldehyde (EM grade) and Ladd-o-lac, LADD (Burlington, VT). Antibodies to mouse IgG: fluorescein-conjugated sheep antibody, Cappel Laboratories (Malvern, PA) and Sigma Chemical Co.; Texas Red-conjugated rabbit antibody, Cappel Laboratories and Texas Red-conjugated goat antibody, Jackson Immunoresearch Laboratories Inc. (West Grove, PA). Anti-mouse IgM fluorescein-conjugated rabbit antibody, Jackson Immunoresearch Laboratories. Antibodies to chicken IgG: fluorescein-conjugated rabbit antibody and Texas Red-conjugated goat antibody, Jackson Immunoresearch Laboratories.

#### Cell culture

The *P. polycephalum* strain used in this study was the apogamic mutant CL (Cooke and Dee, 1974). Amoebae were cultured on DBL (Difco Bacto Liver) plates at 30°C, a temperature restrictive to plasmodial development, as described by Blindt et al. (1986). CL amoebae were induced to develop into plasmodia as described by Solnica-Krezel et al. (1988).

#### Induction of flagellate formation

Cells were harvested with 5 ml of deionized water per plate. The resulting suspension was shaken 2–3 h at 26°C, approx. 150 revs min⁻¹.

#### Immunofluorescence

Immunofluorescence of amoebal and developing cells was performed as described by Diggins-Giliciński et al. (1989) with the following modifications. A 5 ml sample of the fixative solution was applied per plate, cells were washed off only at the beginning of the 10-min fixation period, then directly pelleted by centrifugation. For staining with the 6-11B-1 monoclonal antibody, the concentration of Nonidet P-40 was increased to 0.04%. In order to fix cells that had been induced to form flagellates, a suspension of induced cells was centrifuged and the resulting pellet was resuspended in 10 ml of the fixative, incubated for 10 min at room temperature, and then treated as described above. The stained coverslips were mounted in 5% (w/v) n-propyl gallate, 10% (v/v) phosphate-buffered saline (PBS), 50% (v/v) glycerol, pH 8.5. Slides were observed with a Zeiss (Oberkochen, West Germany) Axioshot microscope using a total magnification of ×1000. Photomicrographs were taken with TMAX 400 ASA film.

### Results

#### Detection of b2-tubulin at different stages of plasmodium formation

During the APT b2-tubulin accumulates in uninucleate developing cells and is incorporated into microtubules of
Fig. 1. Detection of β2- and α3-tubulins at distinct cellular stages of the APT. A. Distinct cellular stages of the APT. The modified sequence of events is from Bailey et al. (1987). Abbreviations: A, amoeba; LF, cell that lost the ability to transform into flagellate; β2+, uninucleate cell positive for β2-tubulin; UD, uninucleate cell ingesting other cells; B, binucleate cell; Q, quadrinucleate cell; P, plasmodium containing more than four nuclei. B. Percentage of cells stained with the β2-tubulin antibody in distinct cellular stages of the APT, depicted in A. Numbers in parenthesis represent standard deviations between values obtained in different experiments. Different cell types were identified on the basis of phase-contrast and DAPI images; microtubules visualized with the general tubulin antibody KMX-1 were scored for β2-tubulin. C. The percentage of cells stained with the 6-11B-1 antibody (specific for the α3-tubulin) in distinct cellular stages of the APT. Different cell types were identified on the basis of phase-contrast and DAPI images, and assayed for whether their microtubular structures stained with the general tubulin antibody KMP-1 contained α3-tubulin.

Developmental changes in microtubules
Two conclusions can be drawn from these results. First, \( \beta_2 \)-tubulin accumulates during the APT, to a level detectable by immunofluorescence microscopy, in uninucleate cells usually after they lose the ability to transform into flagellates. Second, \( \beta_2 \)-tubulin accumulates during the APT before cells acquire the ability to ingest other cells.

Correlation between the expression of \( \beta_2 \)-tubulin and the commitment event

The above observations are consistent with the hypothesis that \( \beta_2 \)-tubulin accumulates during the APT in uninucleate cells around the time of commitment. However, establishing a temporal relationship between the commitment event and \( \beta_2 \)-tubulin expression by direct microscopic observation is not yet feasible, as there is no known

sayed contained this protein in their microtubules (Figs 6–8, below).

Fig. 2. Immunofluorescence detection and localization of \( \beta_2 \)- and \( \alpha_3 \)-tubulins in developing cells upon induction of flagellate formation. A. Phase-contrast image; note that most cells exhibit flagella; a round nonflagellated cell is indicated by the arrowhead. B. DAPI staining of the same field. Nuclei of flagellates are more elongated and stain more intensely in comparison to the one nonflagellate cell (arrowhead). C. \( \beta_2 \)-tubulin pattern. \( \beta_2 \) is detected in cytoplasmic microtubules of the nonflagellated cell, but is absent from all flagellates in the field. D. \( \alpha_3 \)-Tubulin pattern. \( \alpha_3 \)-Tubulin is present in flagellar axonemes and flagellar cones of all flagellates in the field, but is absent from the nonflagellated cell. Bar, 10 \( \mu \)m.

Fig. 3. Immunofluorescence detection and localization of \( \beta_2 \)- and \( \alpha_3 \)-tubulins in a flagellate that contains a vacuole with an ingested cell. A. Phase-contrast image. The flagella are out of focus. The arrowhead indicates a vacuole with an ingested cell. B. DAPI image of the same field. Note that the nucleus of the ingesting cell is bigger and stains less intensely than the nucleus of the ingested cell, and the nuclei of other flagellates in the field. C. \( \beta_2 \)-Tubulin pattern. \( \beta_2 \) is incorporated in the flagellar and cytoplasmic microtubules of the ingesting cell, but is excluded from the vacuole region and is absent from other flagellates in the field. D. \( \alpha_3 \)-Tubulin pattern. \( \alpha_3 \) is detected in the flagellar axonemes and flagellar cone, but absent from the cytoplasmic microtubules of the \( \beta_2 \)-positive flagellate. Bar, 10 \( \mu \)m.
positive cells (•) and multinucleate cells (○) in a population of developing cells is plotted as a function of the percentage of committed cells in the same population. By definition, the percentage of committed cells is a diagonal (—). The percentage of cells positive for β2-tubulin was determined by double-label immunofluorescence microscopy as the fraction of cells stained with the general anti-tubulin antibody KMX-1 that was also stained with the β2-tubulin antibody. On average 1500 cells on three microscope slides were assayed in each experiment; mean standard deviation ±1.5%. The percentage of multinucleate cells was established by phase-contrast and DAPI staining patterns. On average 1100 cells on three separate slides were assayed per experiment, mean standard deviation ±0.4%. The percentage of cells committed to plasmodium formation was determined by replating cells at a low cell density and subsequently scoring the resulting amoebal colonies and plasmodia with a dissecting microscope. On average 1100 cells on five plates were assayed in each experiment; mean standard deviation ±2.1%.

Detection and localization of α3-tubulin in intermediate cellular stages of the APT

The α3-tubulin isotype is formed by acetylation of α1-tubulin (Green and Dove, 1984; Diggins and Dove, 1987; Sasse et al. 1987). Acetylated α-tubulin from Physarum is specifically recognized by the 6-11B-1 monoclonal antibody raised against sea urchin sperm (Piperno and Fuller, 1985). This tubulin isotype has been detected in the amoebal and flagellate but not in the plasmodial stages of Physarum (Burland et al. 1983; Diggins and Dove, 1987; Sasse et al. 1987).

In order to estimate the stage of the APT at which α3-tubulin is lost, we performed a series of double-label indirect immunofluorescence experiments. The optimized fixation technique (see Materials and methods) resulted in essentially complete and uniform staining by the 6-11B-1 antibody of the MTOCs of interphase amoebae (99.5±0.5%; 6400 cells assayed; six experiments) and of mitotic amoebae (99.9±0.2%; 3600 mitotic cells assayed; six experiments), without significant background. This fixation technique was used in all further experiments with the 6-11B-1 antibody. In these experiments, developing cells were fixed in situ on plates, or else after the induction of flagellate formation. Cells were stained either with KMP-1 antibody (Birkett et al. 1985), or the β2-tubulin antibody to visualize all microtubules, and then with the 6-11B-1 antibody to stain microtubules containing α3-tubulin. The KMP-1 antibody recognizes an epitope in the Physarum α1-tubulin isotype that includes lysine 40, which, when acetylated, is recognized by the 6-11B-1 antibody. The acetylated protein is no longer recognized by the KMP-1 antibody (Walden et al. 1989). Thus, staining with KMP-1 provided a good control for the accessibility of the region of α-tubulin that receives the acetyl group. Additionally, cells were stained with DAPI. Different cell types were recognized by the phase-contrast and DAPI images and then assayed for the presence of α3-tubulin. All cells that transformed into flagellates exhibited the α3-tubulin isotype (Fig. 1C), in both flagellar axonemes and the flagellar cone as described earlier (Diggins and Dove, 1987; Sasse et al. 1987) (see also Figs 2 and 3). Additionally, one subset of the cells that failed to form flagellates contained detectable levels of α3-tubulin. These two observations indicate that α3-tubulin disappears during the APT usually after cells lose the ability to undergo the amoeba–flagellate transition. Interestingly, in the flagellates exhibiting β2-tubulin, α3-tubulin was detected only in the flagellar axonemes and cone, but not in an extensive array of other cytoplasmic microtubules present in this cell (Fig. 3).

In nonflagellated developing cells, the α3-tubulin epitope was localized to the MTOC region as in myxamoebae (Figs 5–7). The majority of the uninculate, β2-positive cells exhibited detectable levels of α3-tubulin (Fig. 1C). However, the intensity of the α3-tubulin signal exhibited by uninculate β2-tubulin-positive cells was routinely lower than that of β2-tubulin-negative cells (Fig. 6). Only 50% of the uninculate cells that ingested other cells contained detectable levels of α3-tubulin in MTOCs stained with KMP-1. The proportion of α3-tubulin-positive cells was smaller among binucleate cells (Figs 1C and 6); only 10% of the quadrinucleate cells contained this tubulin isotype (Figs 1C and 7). All cells containing more than four nuclei were negative for the α3-tubulin isotype (not shown).
Several conclusions may be drawn from these results. First, α3-tubulin disappears gradually during development. Second, developing cells differ with respect to the timing of loss of this protein: some of them lose detectable α3-tubulin very soon after the loss of ability to form flagellates, but some cells may exhibit this tubulin as late as the quadrinucleate stage.

Correlation between the disappearance of α3-tubulin and the commitment to plasmodium formation

Knowing that α3-tubulin disappears during the APT as cells lose the ability to transform into flagellates and before they start to express β2-tubulin, we investigated whether the loss of α3-tubulin was coincident with the commitment event. The percentage of committed cells in a developing population was compared with the percentage of cells stained by the KMP-1 antibody that were negative for α3-tubulin. Table 1 presents results of these studies: in one developing culture, the proportion of cells negative for α3-tubulin was significantly higher than the proportion of committed cells. In the remaining six cultures, the percentage of α3-tubulin-negative cells was significantly lower than the proportion of cells committed to plasmodium formation. This result, together with the observation of heterogeneity in stage of loss of α3-tubulin among developing cells, is consistent with the view that the loss of α3-tubulin starts during the APT generally after the commitment event. However, some cells may lose the acetylated α-tubulin epitope before commitment.

Reorganization of cytoskeletal microtubules during the APT

In an amoebal cell, cytoskeletal microtubules radiate from a single MTOC localized near the nucleus (Havercroft and Gull, 1983). We studied the organization of cytoplasmic microtubules in cells developing into plasmodia. As in amoebal cells, uninucleate cells expressing β2-tubulin exhibited one MTOC juxtaposed to a characteristic enlarged nucleus (Fig. 5). Binucleate cells possessed one to three MTOCs (Fig. 6). Quadrinucleate cells displayed one to five or more MTOCs (Fig. 7). In larger multinucleate cells, microtubules are nucleated by multiple MTOCs.

Table 1. Correlation between the loss of α3-tubulin and the commitment event

<table>
<thead>
<tr>
<th>Culture</th>
<th>% Committed cells x±S.D.</th>
<th>% α3-Negative cells x±S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amoebae</td>
<td>0±0</td>
<td>0.5±0.5</td>
</tr>
<tr>
<td>Developing cells</td>
<td>0.1±0</td>
<td>4.3±2.3</td>
</tr>
<tr>
<td>6.5±1.1</td>
<td>4.5±0.6</td>
<td></td>
</tr>
<tr>
<td>11.9±1.1</td>
<td>8.5±0.6</td>
<td></td>
</tr>
<tr>
<td>12.9±2.3</td>
<td>0±0</td>
<td></td>
</tr>
<tr>
<td>13.7±0.6</td>
<td>0.2±0.3</td>
<td></td>
</tr>
<tr>
<td>16.4±3.1</td>
<td>15.7±0.6</td>
<td></td>
</tr>
<tr>
<td>21.3±1.5</td>
<td>7.8±1.9</td>
<td></td>
</tr>
</tbody>
</table>

The percentage of cells committed to plasmodium formation was established as described for Fig. 1. The percentage of cells negative for α3-tubulin was established by double-label indirect immunofluorescence microscopy. Cells were stained with KMX-1 antibody recognizing all microtubules in the cell, and with 6-11B-1 antibody recognizing only α3-tubulin. During subsequent microscopic observations the percentage of cells negative for α3-tubulin was established as a fraction of cells that were stained with KMX-1 antibody but not with 6-11B-1 antibody. Each line represents separate culture, x, mean value of three separate estimations; S.D., standard deviation of x.
Fig. 6. Organization of cytoplasmic microtubules and detection of α3-tubulin in binucleate developing cells. A,B,C. Phase-contrast images; D,E,F, DAPI images; G,H,I, β2-tubulin patterns; J,K,L, α3-tubulin patterns. A,D,G,J. Binucleate cell with cytoplasmic microtubules organized by one MTOC, α3-tubulin is virtually absent. B,E,H,K. Binucleate cell in which cytoskeletal microtubules are organized by two MTOCs. α3-Tubulin is detected in dot-like structures associated with each MTOC. C,F,I,L. Binucleate cells in which three MTOCs organize cytoplasmic microtubules, the 6-11B-1 antibody stains a dot-like structure in only one of them. Note the intensity of staining by the 6-11B-1 antibody and the comma-like appearance of the MTOC in the uninucleate β2-tubulin-negative cell present in the field. Bar, 10μm.
cells, microtubules formed a very fine and uniform meshwork in which MTOCs were not readily observed. This arrangement of microtubules persisted during further development and could be detected even in some plasmodia containing more than 100 nuclei (Fig. 8). However, no microtubules were observed in the mature macroplasmodium, in agreement with previous studies (Havercroft and Gull, 1983). The structure stained by the 6-11B-1 antibody changed its morphology during development: from a 'comma-like' structure in the majority of uninucleate cells to a 'dot-like' structure in some uninucleate and multinucleate developing cells (Figs 5, 6 and 7). In some binucleate and quadrinucleate cells, a3-tubulin was detected only in a subset of MTOCs (Figs 6 and 7).

Discussion

This work has demonstrated that in a population of P. polycephalum amoebae undergoing apogamic development individual cells exhibited variable sequences of developmental changes. Transitional cells varied in respect of: (1) the timing of the loss and accumulation of developmentally regulated tubulins; (2) reorganization of cytoplasmic microtubules; and (3) sequences of physiological changes.

Changes in tubulin expression during the APT

Plasmodium-specific β2-tubulin accumulated to a detectable level in uninucleate cells, usually after they became committed to plasmodium formation and before they acquired the ability to ingest other cells. The detection of β2-tubulin in a few cells that retained the ability to transform into flagellates can be interpreted in either of two ways. First, these cells may have been exceptional cells in which β2-tubulin accumulated to a detectable level before the commitment event. Second, these β2-tubulin-positive flagellates may have represented rare cells that retained the ability to form flagella even beyond the point of commitment to plasmodium development. The latter possibility was supported by the detection of rare flagellates positive for β2-tubulin with a vacuole containing an ingested cell. Additionally, some developing cells were delayed in their expression of β2-tubulin, as a few β2-tubulin-negative cells that ingested other cells were observed. The detection of rare binucleate cells negative for β2-tubulin could indicate that indeed some cells accumulate this protein only after binucleate formation; by contrast, they could represent cells that had been incorrectly identified as containing two nuclei, or were incompletely stained.

α3-Tubulin disappeared gradually during the APT. This process started only after the loss of the ability to form flagella. Individual cells lost α3-tubulin at any time in development between commitment (or even before), until the stage of quadrinucleate plasmodia. There are two possible explanations for the apparent loss of α3-epitope during the APT. First, α3-tubulin was absent from such non-staining MTOCs. Second, this isotype was still present in these MTOCs, but was masked by proteins expressed at this stage of development. Previous work demonstrated the absence of α3-tubulin in the plasmodium, by Western blotting or autoradiography of total proteins (Diggins and Dove, 1987; Burland et al. 1983) and purified plasmodial tubulins (Sasse et al. 1987). We are therefore confident that α3-tubulin disappears during the APT. The only uncertainty that remains is whether α3-

Fig. 7. Organization of cytoplasmic microtubules and detection of α3-tubulin in a quadrinucleate cell. A, Phase-contrast image; B, DAPI staining of the same cell; C, β2-tubulin pattern, cytoplasmic microtubules radiate from at least five MTOCs; D, α3-tubulin pattern, α3-tubulin is detected in only two out of five MTOCs visible in the cell. Bar, 10 μm.

390 L. Solnica-Krezel et al.
Fig. 8. Organization of cytoplasmic microtubules in a developing plasmodium. A. Phase-contrast image, note the edge of the thick plasmodium and numerous nuclei. B. β2-tubulin pattern, microtubules form a fine meshwork in which MTOCs are not easily observed. Bar, 10 μm.

tubulin vanishes before or only after cytoplasmic microtubules are lost. A Western blot analysis cannot distinguish between these two possibilities in our particular system for two reasons. First, only trace amounts of α3-tubulin are detected in amoebae by Western blotting (Diggins and Dove, 1987; Sasse et al. 1987). This precludes a meaningful quantitative analysis of α3-tubulin levels during the APT. Second, the large populations of developing cells required for a blotting analysis would contain cells at different stages of development. This analysis would not provide enough resolution to determine whether α3-tubulin is lost before or after the loss of cytoplasmic microtubules (see Solnica-Krezel et al. 1988, for discussion). Therefore, Western blot analysis will not strengthen our conclusions obtained with histochemical methods. The fact that KMP-1, which recognizes the same region of α-tubulin as 6-11B-1, stained those structures not stained by 6-11B-1 indicated that this region of α-tubulin was accessible. However, we cannot exclude the possibility that it was the acetylated form of α-tubulin that was specifically blocked during the APT. We consider this possibility unlikely, as it fails to explain why only some of several MTOCs co-existing in a single cell (Figs 6 and 7) were blocked.

The loss of the 6-11B-1 epitope could result from: (1) loss of acetylase activity; (2) increase in deacetylation rate; or (3) disappearance of the substrate for acetylation. Two lines of evidence argue against the third possibility. First, the tubulin isotype containing Lys40 and Tyr44, the substrate for the acetylation in amoebae, is detected in
plasmodium (Walden et al. 1989). Second, two other α-tubulin isotypes with potential acetylation sites are expressed in plasmodia at high levels (Walden et al. 1989).

Our demonstration that β2-tubulin accumulates and α3-tubulin starts to disappear during development at the uninucleate stage is comparable with earlier studies showing that the synthesis of myosin heavy chains switches from amoebal to plasmodial type in transitional uninucleate cells (Uyeda and Kohama, 1987). However, it is not clear whether individual transitional cells also differ in their timing of accumulation and loss of stage-specific myosins.

The gradual acquisition of plasmodial-specific and concomitant loss of amoebal-specific transcripts occurs over several nuclear division cycles as amoebae develop into plasmodia (Sweeney et al. 1987). Since Sweeney et al. (1987) studied populations of developing cells rather than single cells, one cannot distinguish whether the gradual changes are due to: (1) nonhomogeneity of populations; (2) gradual changes in the expression of a specific gene occurring in perfect synchrony in all developing cells; or (3) gradual changes in the expression of a specific gene in individual cells at different developmental stages in different cells. Our data support the last explanation of gradual changes in levels of some developmentally regulated gene products during the APT.

Reorganization of the cytoskeleton during the APT

The organization of cytoplasmic microtubules in uninucleate developing cells was indistinguishable from that of amoebae. In binucleate cells, one to three MTOCs radiated cytoplasmic microtubules. The number of MTOCs per cell increased as the number of nuclei per cell increased, but differed between plasmodia that contained equal numbers of nuclei. The variation in the number of MTOCs in larger plasmodia containing the same number of nuclei is probably a consequence of the fact that they arise from binucleate cells with varying numbers of MTOCs.

Cytoplasmic microtubules persisted during development and were present in plasmodia containing in excess of 100 nuclei. This contrasts with previous studies demonstrating a lack of cytoplasmic microtubules in plasmodia containing 16 or 32 nuclei (Gull et al. 1985). One possible explanation for this discrepancy is that we have studied developing plasmodia grown on plates, rather than plasmodia developing in a liquid medium after enrichment for committed cells by glass-bead column chromatography (Gull et al. 1985). It is possible that plasmodia growing in liquid disassemble the cytoskeletal microtubules at earlier stages of the APT than those developing on an agar surface. More likely, large plasmodia arise faster on an agar surface because of frequent fusions, which would be inhibited in liquid cultures. Therefore, plate-grown plasmodia contain cytoplasmic microtubules because they represent an earlier developmental stage than their counterparts from liquid cultures.

The role of distinct tubulins in the reorganization of cytoplasmic microtubules

The plasmodium-specific, highly divergent β2-tubulin is not limited to the intranuclear mitotic spindle of mature plasmodia but, remarkably, in transitional cells is incorporated into cytoskeletal and astral mitotic spindle microtubules. Thus, this developmentally regulated tubulin is not sufficient to bring about changes in microtubule organization (Diggins-Glicinski et al. 1989). Here we have confirmed and extended these observations, showing that whenever expressed in developing cells that retained the ability to transform into flagellates, β2-tubulin was incorporated into all microtubules of this cell type, even into flagellar axonemes. Therefore, during the APT, β2-tubulin participates in all the known microtubular structures of P. polycephalum.

No strict correlation existed between the apparent loss of α3-tubulin from the MTOC region of developing amoebae and disassembly of cytoskeletal microtubules during the APT. First, α3-tubulin was no longer detected in cells containing more than four nuclei, whereas MTOCs and cytoplasmic microtubules persisted during development until at least the 100-nucleus stage. Second, in some small plasmodia, α3-tubulin was detected only in a subset of several MTOCs in the cell. Therefore, detectable amounts or accessibility of α3-tubulin in an MTOC seem not to be necessary for the function of nucleating cytoskeletal microtubules.

The levels of acetylated α-tubulin increase dramatically when an amoeba transforms into a flagellate (Diggins and Dove, 1987, Sasse et al. 1987). In developing cultures, all cells that could be converted to flagellates, also exhibited α3-tubulin. In flagellates containing β2-tubulin, acetylated α-tubulin was detected only in a subset of microtubules: in flagellar axonemes and flagellar cones, but not in other cytoplasmic microtubules. The observations that α3-tubulin disappeared during the APT only after the loss of the ability to transform into flagellates, and was differentially localized to flagellar axonemes and flagellar cones of flagellates, are consistent with the hypothesis that α3-tubulin is necessary for the amoeba–flagellate transition.

The preferential localization of α3-tubulin to flagellar cone microtubules is observed in Chlamydomonas flagellates (LeDizet and Piperno, 1988; Holmes and Dutcher, 1989), but this is the first time it has been clearly documented in Physarum. This segregation of α3-tubulin to a subset of cytoplasmic microtubules could be more easily observed in β2-tubulin-containing flagellates that exhibited a more extensive cytoplasmic microtubular network than that typical of flagellates formed from amoebal cells.

Alternative sequences of developmental changes

The α3- and β2-tubulin antigens served as developmental markers to establish the sequence of different intermediate stages during the APT. Two inferences were drawn from our data. First, cells that are positive for α3-tubulin and negative for β2-tubulin usually represent earlier stages of the APT than cells that are both α3-tubulin- and β2-tubulin-positive. Second, the cells containing both α3- and β2-tubulins represent earlier stages of development than cells that are α3-tubulin-negative and β2-tubulin-positive (Fig. 1). Correlative studies between the expression of α3- and β2-tubulins and the commitment event demonstrated that usually α3-tubulin is lost and β2-tubulin accumulates after the commitment event. Thus, a typical developing cell first loses its ability to form a flagellate and becomes committed to plasmodium formation (these two events cannot be ordered by this method). Next, the developing cell acquires the ability to ingest other cells; subsequently it forms a binucleate cell. These conclusions are comparable to those obtained by other workers (Anderson et al. 1976; Blindt et al. 1986; Bailey et al. 1987). However, we have expanded the known sequence of events by establishing when cells acquire the ability to ingest other cells. We have also detected cells that trans-
formed into flagellates while expressing β-tubulin and ingesting other cells (Fig. 3). This indicates that, whereas the above sequence of changes is the most frequent one, it is not absolute, and certain cells may undergo developmental changes in a different order. Detection of such low frequency events was possible only because very large numbers of developing cells were monitored. Thus, developing cells are heterogeneous with respect to the timing of changes in expression of developmentally regulated tubulins, reorganization of microtubular structures and the order of physiological changes. We speculate that the latter changes are a consequence of heterogeneity in accumulation and loss of stage-specific gene products that execute these processes.

What is the significance of the variable developmental pathways exhibited by individual cells during the APT in P. polycephalum? Variable developmental pathways are probably not an idiosyncrasy of development resulting from an apogamic mutation in the matA locus. The fact that other genera of myxomycetes exhibit natural conversion between apogamy and heterogamy (Collins et al. 1983) indicates that the apogamic APT represents a normal process in the slime molds. Lack of precision may be inherent in this and indeed in other developmental processes.

We are grateful to Dr G. Piperno for his generous gift of 6-11B-1 antiserum, and to Dr J. Dee and Dr J. Bailey for helpful discussions and sharing unpublished data. We thank Linda Clipson for help with the illustrations, and Rex Kochanski for comments on the manuscript.

Our research is supported by Core grant CA-07175 and Program-Project grant in Tumor Biology CA-23076 from the National Cancer Institute. L.S.-K. has been the recipient of Wisconsin Power and Light Foundation Fellowship in Cancer Research dedicated to the memory of employees and retirees of Wisconsin Power and Light Company who suffered or died as a result of cancer. M.D.-G. has been supported by predoctoral training grant 5-T32-CA09135.

References


(Revised 2 February 1999 – Accepted 29 March 1999)