Multiple Forms of Tubulin in the Cytoskeletal and Flagellar Microtubules of *Polytomella*

TIMOTHY W. McKEITHAN and JOEL L. ROSENBAUM
Department of Biology, Yale University, New Haven, Connecticut 06511. Dr. McKeithan's present address is the Department of Pathology, University of Chicago, Chicago, Illinois 60637.

ABSTRACT The alga *Polytomella* contains several organelles composed of microtubules, including four flagella and hundreds of cytoskeletal microtubules. Brown and co-workers have shown (1976, *J. Cell Biol.* 69:6–125; 1978, *Exp. Cell Res.* 117:313–324) that the flagella could be removed and the cytoskeletons dissociated, and that both structures could partially regenerate in the absence of protein synthesis. Because of this, and because both the flagella and the cytoskeletons can be isolated intact, this organism is particularly suitable for studying tubulin heterogeneity and the incorporation of specific tubulins into different microtubule-containing organelles in the same cell.

In order to define the different species of tubulin in *Polytomella* cytoplasm, a ^{35}S-labeled cytoplasmic fraction was subjected to two cycles of assembly and disassembly in the presence of unlabeled brain tubulin. Comparison of the labeled *Polytomella* cytoplasmic tubulin obtained by this procedure with the tubulin of isolated *Polytomella* flagella by two-dimensional gel electrophoresis showed that, whereas the β-tubulin from both cytoplasmic and flagellar tubulin samples comigrated, the two α-tubulins had distinctly different isoelectric points. As a second method of isolating tubulin from the cytoplasm, cells were gently lysed with detergent and intact cytoskeletons obtained. When these cytoskeletons were exposed to cold temperature, the proteins that were released were found to be highly enriched in tubulin; this tubulin, by itself, could be assembled into microtubules in vitro. The predominant α-tubulin of this in vitro–assembled cytoskeletal tubulin corresponded to the major cytoplasmic α-tubulin obtained by coassembly of labeled *Polytomella* cytoplasmic extract with brain tubulin and was quite distinct from the α-tubulin of purified flagella. These results clearly show that two different microtubule-containing organelles from the same cell are composed of distinct tubulins.

Many cells contain several functionally and structurally distinct microtubule organelles. It has not yet been resolved whether particular types of microtubules are composed of distinct tubulins or whether accessory proteins confer distinctive structure and function to microtubules composed of a universal tubulin dimer.

Microtubules from very different sources can copolymerize in vitro; for example, yeast (20, 27) and *Aspergillus* (8, 19) tubulin and even *Chlamydomonas* flagellar tubulin (31) copolymerize with brain tubulin. There appears to be no example in the literature showing failure of copolymerization in vitro— that is, utilization of only one type of tubulin in assembly when a second type of polymerization-competent tubulin is available as well. Despite this undiscriminating behavior of tubulin in vitro, there is no direct evidence in vivo that tubulin from one microtubule organelle can be used to build another type of organelle. Nevertheless, in some cases there is circumstantial evidence for a common tubulin pool; for instance, in many cells cytoplasmic or flagellar microtubules disassemble before formation of the mitotic spindle (3).

Despite the in vitro results, which suggest that tubulins may be completely interchangeable in assembly, in several organisms tubulin has been shown to exist in multiple forms in the same cell. For example, Bibring et al. (2) have detected two α-tubulins both in mitotic apparatus and cilia of sea urchins. Two-dimensional analysis of tubulin from *Aspergillus*—identified by copolymerization with brain tubulin—showed multiple tubulin spots (19). In several mutants resistant to benzimidazole derivatives, the position of the two major βs was changed, suggesting that a single gene codes for both forms of β-tubulin, presumably by posttranslational modification. However, certain minor β spots unaffected by the mutation may

represent one or more separate genes. Evidence from other mutants in *Aspergillus* suggests that there are at least two α-tubulin genes (14). Unfortunately, there is no evidence yet in this species that specific microtubules are assembled from particular forms of tubulin.

In a few cases, separate forms of tubulin have been associated with specific microtubule organelles. Stephens (23–25) has shown that different microtubule components of axonemes from sea urchin sperm or cilia are composed of tubulins with distinct amino acid compositions and peptide maps. An antibody against flagellar outer-doublet tubulin in *Naegleria gruberi* appears not to cross-react with cytoplasmic tubulin of the same cell, and pulse-chase experiments also suggest that newly synthesized outer-doublet tubulin does not exchange with the preexisting cytoplasmic tubulin pool (10, 11).

The primary goal of the present work has been to determine whether there are multiple forms of tubulin within a single cell at a given time and whether different forms are associated with particular types of microtubule organelles. This question was approached using the organism *Polytomella agilis*, a colorless alga that contains several microtubule organelles. Four typical flagella and basal bodies are found, and around the basal bodies are arrayed eight rootlets partially composed of microtubules. Along the sides of the rootlets arise hundreds of single microtubules, the cytoskeletal microtubules, which course just under the plasma membrane toward the cell posterior and appear to be responsible for maintaining the cell's oval shape (4, 5).

The multiplicity of microtubule organelles make the organism a favorable system for study. An additional advantage of the system is the fact that the flagellar and the cytoskeletal microtubules, simultaneously present in the cell, can each reassemble at least partially without protein synthesis (4, 6); this fact makes *Polytomella* a particularly suitable organism for studying tubulin heterogeneity and specificity of polymerization.

Our initial approach in this research has been to investigate tubulin heterogeneity by isolating flagellar and cytoskeletal tubulin and comparing them by two-dimensional electrophoresis. Cytoskeletal tubulin has also been polymerized in vitro. To determine the full number of tubulins in *Polytomella*, a cell extract has been mixed with brain tubulin and subjected to cycles of assembly and disassembly to isolate copolymerizing forms of tubulin.

MATERIALS AND METHODS

Culture of Polytomella

Axenic cultures of *P. agilis* Aragoa were obtained as a gift from Dr. David L. Brown, University of Ottawa, Ottawa, Canada. Cultures were grown at 25°C in a medium modified from that of Cramer and Meyers (7). Per liter, the medium contained the following: Na acetate, 2 g; K_2HPO_4, 1.32 g; KH_2PO_4, 1 g; Na_3 citrate $2H_2O$, 645 mg; $MgCl_2$, 144 mg; Arginine HCl, 100 mg; $MgSO_4 \cdot 7H_2O$, 25 mg; $CaCl_2 \cdot 2H_2O$, 20 mg; $FeCl_3 \cdot 6H_2O$, 3 mg; $CoCl_2 \cdot 6H_2O$, 1.3 mg; $MnCl_2 \cdot 4H_2O$, 0.8 mg; $Na_2MoO_4 \cdot 2H_2O$, 0.2 mg; $ZnCl_2$, 0.19 mg; $CuCl_2 \cdot 2H_2O$, 13.7 μg; Thiamine HCl, 20 μg; Vitamin B_{12}, 0.12 μg. Radioactively labeled cells were grown in low sulfur medium in which two thirds of the normal sulfate was replaced by chloride. In sulfur-free medium, all the sulfate was replaced by chloride. In all experiments ^{35}S as $H_2^{35}SO_4$ (sp act 43 Ci/mg), obtained from New England Nuclear (Boston, Mass.), was used as the label.

Transmission Electron Microscopy

Cells suspended in fresh medium were fixed by the addition of an equal volume of 1% glutaraldehyde in 50 mM sodium phosphate buffer at pH 7. The cells were immediately pelleted by gentle centrifugation. After a 2-h fixation at 20°C, the cells were washed twice with buffer and postfixed for 1 h in cold 1% osmium tetroxide in the same buffer. The cells were dehydrated in a graded acetone series and were infiltrated with Spurr's resin mix (22), which was then polymerized at 60°C for 18 h. Sections cut on a Sorvall Porter-Blum MT2 ultramicrotome (DuPont Co., Wilmington, Del.) were routinely stained with uranyl acetate and lead citrate before examination at 60 kV in a Philips 201 electron microscope.

Cytoskeletal microtubules polymerized in vitro were pelleted at 106,000 g for 20 min at 25°C (L2-65B ultracentrifuge, Ti 50 rotor, 40,000 rpm; Beckman Instruments, Inc., Palo Alto, Calif.), and the pellets were processed according to the tannic acid method of Begg and co-workers (1, 9).

Isolation of Flagella and Axonemes

750 ml of cells inoculated with ~10^5 cells/ml were grown to a density of 1–3 × 10^6/ml in 3 mCi $^{35}SO_4$, washed with sulfur-free medium by gentle centrifugation, pelleted, and resuspended in 40 ml of 10 mM HEPES, pH 7.4, with KOH, 5 mM $MgCl_2$, 2.5% sucrose. The cell suspension was chilled to 5–10°C and deflagellated by the addition of 8 ml of 25 mM dibucaine HCl (CIBA-GEIGY Corporation, Summit, N. J.) with vigorous stirring with a magnetic stirrer (28). The cells were pelleted at 1,000 g for 2 min (IEC model PR-6 centrifuge, rotor 253, 2,000 rpm, Damon/IEC Div., Needham Heights, Mass.). The supernates were again centrifuged at 2,300 g for 2 min to remove any remaining cells. The flagella in the supernates were pelleted at 12,000 g for 15 min (Sorvall RC-5 centrifuge, rotor SS-34, 10,000 rpm). To remove cellular debris, the pellet was resuspended in 4 ml of 10 mM HEPES, pH 7.4, with KOH, 1 mM $MgCl_2$, 5% sucrose (HMS) containing 25% Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden) and loaded onto two tubes with 6.3 ml of a 25–75% linear gradient of Percoll in HMS. The gradients were spun at 48,000 g for 40 min (L2-65B centrifuge, rotor SW 41, 20,000 rpm). The lowest major band, containing the flagella, in each tube was diluted with 9 ml of HMS and spun at 13,200 g for 20 min (Sorvall RC-5, HB-4 rotor, 9,000 rpm). The procedure from this point varied depending on whether flagella or flagellar axonemes were to be isolated. For flagellar isolation, the flagellar pellet was resuspended and washed three times in HMS using centrifugation at 13,200 g for 20 min. For axonemal isolation the flagella were instead resuspended in 10 ml of 10 mM HEPES, pH 7.4, with KOH, 5 mM $MgCl_2$, 0.5 mM EDTA, 25 mM KCl, 0.5% polyethylene glycol, 20,000 mol wt (HMEKP), a solution shown to preserve the motile capacity of many flagella (32). Flagellar membranes were removed by the addition of 0.1 ml of Nonidet P-40 (NP-40; Shell Co., London). The 10 ml of suspension was overlaid on 1 ml of HMEKP containing 40% sucrose and centrifuged at 13,200 g for 30 min (Sorvall RC-5, HB-4 rotor, 9,000 rpm). The pellet was resuspended in HMEKP and washed twice using centrifugation at 13,200 g for 20 min.

Deflagellation of Cells

Flagella were efficiently removed from *Polytomella* by brief vortex agitation in a fluted tube (6, 17). Most cells appeared uninjured by this procedure and could regenerate their flagella. This deflagellation method was not used for flagellar isolation, where the dibucaine procedure (see above) yielded purer samples.

Isolation and In Vitro Assembly of Cytoskeletal Tubulin

750 ml of cells, grown to 1–3 × 10^6 cells/ml in 3 mCi $^{35}SO_4$, were pelleted and washed twice with sulfur-free medium by gentle centrifugation. The cells were resuspended in 20 ml of sulfur-free medium and deflagellated in 5-ml aliquots by 1 min of vortex agitation in a fluted tube. The cells were washed twice more and resuspended in 30 ml of 10 mM 2(N-morpholino)ethane sulfonic acid (MES), pH 6.7, with KOH, 50 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA (MEMK), to which was added 30 ml of MEMK containing 10% dimethyl sulfoxide (DMSO), 0.67 mM phenylmethylsulfonyl fluoride (PMSF), and 6.7 μg/ml RNase. Cells at ~20°C were lysed by the rapid addition of 60 ml of MEMK containing 5% DMSO and 1% NP-40. The cell ghosts were immediately pelleted at 92 g for 10 min followed by 1,000 g for 2 min (IEC PR-6, rotor 253, 600 and 2,000 rpm).

The pellets were resuspended in 3 ml of ice-cold 10 mM MES, pH 6.7, with KOH, 50 mM KCl, 0.5 mM $MgCl_2$, 1 mM EGTA, 0.1 mM GTP (MEMKG) and, after 30 min, spun at 106,000 g for 30 min (Beckman L2-65B centrifuge, Ti 50 rotor, 40,000 rpm). The slightly hazy supernate was filtered through a 0.22-μm Millipore filter (Millipore Corp., Bedford, Mass.), to remove low-density membranous material that would interfere with the following step. To the filtrate was added 1.5 vol of ammonium sulfate saturated at 4°C. After 20 min, the precipitate was pelleted at 27,000 g for 20 min (Sorvall RC-5 centrifuge, 33–34 rotor, 15,000 rpm) to provide the final preparation.

Large-scale preparations from up to 32 liters of cells were performed in a similar fashion by scaling up each step of the procedure, except that the cells were not deflagellated. Cells were harvested with a De Laval cream separator (De Laval Separator Co., Poughkeepsie, N.Y.; model 104, Cow to Can) and washed with fresh medium. In aliquots, the cells were resuspended in MEMK in not less than 1/32 the original volume and lysed as described above; further steps were similar to those described for small volumes. In experiments to demonstrate polymerization of the cytoskeletal tubulin, the final supernate was not precipitated with ammonium sulfate but was instead dialyzed against MEMKG buffer containing 4 M glycerol and 1 mM dithiothreitol for 4 h at 4°C. The sample was made 2 mM GTP and incubated at 37°C for 20 min, and microtubules were pelleted at 106,000 g for 20 min (Beckman L2-65B, Ti 50 rotor, 40,000 rpm). The pellet was resuspended in MEMKG at 4°C and, after 30 min, spun at 27,000 g for 30 min (Sorvall RC-5, SS-34 rotor, 15,000 rpm). The supernate from this last centrifugation was heated to 37°C for 30 min, and the microtubules were pelleted at 35,000 g for 30 min.

Polymerization of Tubulin from Radioactive Polytomella Homogenate with Calf Brain Tubulin

750 ml of cells labeled with 3 mCi $^{35}SO_4$ were pelleted by low-speed centrifugation, washed by centrifugation with sulfur-free medium, and washed once with polymerization mix (PM) containing 100 mM PIPES, pH 6.9, with KOH, 2 mM EGTA, 1 mM MgCl₂. One quarter of the cells were used for the remainder of the experiment. The cells were pelleted and resuspended in 1.5 ml of PM at 40°C containing 0.1 mM GTP and 1 mM PMSF and lysed by the addition of 75 μl of 10% NP-40. After 30 min, the lysate was spun at 6,000 g for 30 min (L2-65B, Ti 50, 40,000 rpm). Avoiding the cloudy layer at the top we mixed 0.65 ml of the supernate with 1.3 ml of PM containing 8 M glycerol and 2 mM GTP and with 0.65 ml of calf brain tubulin in PM that had been isolated by two cycles of temperature-dependent assembly and disassembly. The mixture was incubated at 37°C for 30 min and then centrifuged at 106,000 g for 30 min. The pellet was homogenized with 0.4 ml of PM, containing 0.1 mM GTP, at 4°C. After 30 min the solution was centrifuged at 106,000 g for 30 min. The supernate was mixed with an equal volume of PM containing 8 M glycerol and 2 mM GTP. After incubation at 37°C for 30 min, the mixture was spun at 106,000 g for 30 min. The pellet was homogenized in 0.2 ml of PM containing 0.1 mM GTP at 4°C and, after 30 min, was centrifuged at 106,000 g for 30 min to yield the supernate as the final product.

Protein Determination

Protein determinations were performed by the method of Lowry et al. (14) as modified by Schacterle and Pollack (18), using bovine serum albumin as a standard.

SDS-Urea PAGE

Proteins prepared for electrophoresis by the method of Laemmli (12) were loaded onto slab gels (20 × 15 × 0.15 cm) of the Laemmli formulation with the following alterations: (a) SDS was deleted from both stacking and separation gels and (b) the separation gel was composed of a 6–16% linear acrylamide gradient and a 3–8 M linear urea gradient. After electrophoresis, slab gels were fixed in 12.5% TCA, stained with Coomassie Blue, dried on a Hoefer (model SE 540) gel drier (Hoefer Scientific Instruments, San Francisco, Calif.), and exposed for autoradiography on Kodak X-Omat x-ray film.

Two-dimensional Electrophoresis

Two-dimensional electrophoresis was performed by the method of O'Farrell (16) with the exceptions that the catholyte was 5 mM Ca(OH)₂, 10 mM NaOH, and that tubes of 3.5-mm inner diameter were used for the first dimension. Samples were also prepared according to O'Farrell, except that samples prepared from pellets of whole cells by the method of O'Farrell were then centrifuged at 22°C at 106,000 g for 30 min (L2-65B, Ti 50, 40,000 rpm). The pIs of the polypeptides were determined by slicing the isoelectric focusing gels into 5-mm-long sections, eluting with 0.5 ml of 10 mM KCl, determining the pH, and fixing and staining the individual slices.

Isoelectric Focusing Slab Gel

Isoelectric focusing slab gels (20 × 15 × 0.15 cm) were made by pouring 20 ml of 10% acrylamide, 40 mM glutamic acid, and, after polymerization of this plug, pouring 45 ml of gel mixture of the O'Farrell (16) formulation, except that the

ampholines consisted of 20% pH 3.5–10, 20% 4–6, and 60% 5–7. Electrophoresis in slab gels was performed as with tube gels, except that they were run at 600 V for 1 h and 1,000 V for 23 h. Gels were fixed in 12.5% TCA, washed extensively in 10% acetic acid, and dried and autoradiographed as with SDS gels (see above).

RESULTS

The organization of microtubule organelles in *Polytomella* has been described by D. L. Brown et al. (5). Fig. 1 a shows a phase micrograph of *Polytomella* demonstrating its oval shape and the presence of four anterior flagella. The electron micrograph in Fig. 1 b shows the two rootlets and three of the four anterior basal bodies. Radiating from the sides of the rootlets are numerous cytoskeletal microtubules, which lie just under the cell membrane.

Two-dimensional electrophoresis of flagella (Fig. 2 a and c) or flagellar axonemes revealed two prominent spots corresponding to the α- and β-tubulins. However, near the major α spot were found three less prominent spots that approximately comigrated in the SDS dimension with the major α spot and in the isoelectric focusing dimension had pIs similar, but not identical, to that of the major α spot; these were tentatively identified as a minor flagellar α components. In order of most basic to most acidic, the spots were named α1 through α4, with α3 being the most prominent in the flagella. The α4 spot was relatively minor but increased in quantity with prolonged storage of the sample; this suggests that it may be an artifactually modified form of α3. Only one distinct β spot (β1) was seen in two-dimensional gels; however, a tailing of the β1 spot in the acidic direction was found to be attributable to several minor bands, as discussed below.

Two-dimensional electrophoresis of whole cells (Fig. 2 b and e) and of whole cells mixed with flagella (Fig. 2 d) showed the presence of spots in whole cells that comigrated with α1, α2, α3, and β1 of flagella. However, in this case (in contrast to that of flagella), α1 was by far the major spot. Moreover, in deflagellated cells (Fig. 2 f), the α3 spot was diminished relative to that in nondeflagellated cells (Fig. 2 e). Because cytoskeletal microtubules are the most numerous microtubules in *Polytomella*, they presumably contain most of the tubulin in whole cells. For this reason it was assumed that α1 corresponded to the α-tubulin comprising cytoskeletal microtubules. In addition to the difference in isoelectric point, α1 was found to migrate slightly faster than α3 in the SDS dimension.

To verify that the electrophoretic spots tentatively identified as tubulins were, in fact, tubulins by their ability to polymerize into microtubules, experiments were conducted to copolymerize ^{35}S-labeled extract of *Polytomella* with calf brain tubulin. Cells were lysed at 4°C, and the supernate from a high-speed centrifugation was used. A mixture containing ^{35}S-labeled extract at 1.9 mg/ml and brain tubulin at 1.8 mg/ml was subjected to two complete cycles of polymerization and depolymerization. Fig. 3 a represents a stained SDS slab gel and Fig. 3 b, the autoradiogram of the same gel, showing each of the fractions from this procedure. The stained gel (Fig. 3 a), on which the brain microtubule components were predominant, demonstrated that the high molecular weight microtubule-associated proteins (MAPs), which ordinarily copolymerize with brain tubulin, were lost at the first polymerization step. The autoradiogram (Fig. 3 b) shows the labeled *Polytomella* proteins and demonstrates that *Polytomella* tubulin was highly enriched by this procedure after a single cycle of polymerization and was even further enriched by a second cycle of assembly and disassembly. In the twice-cycled microtubule protein, *Polytomella* tubulin was so preponderant that other bands were only
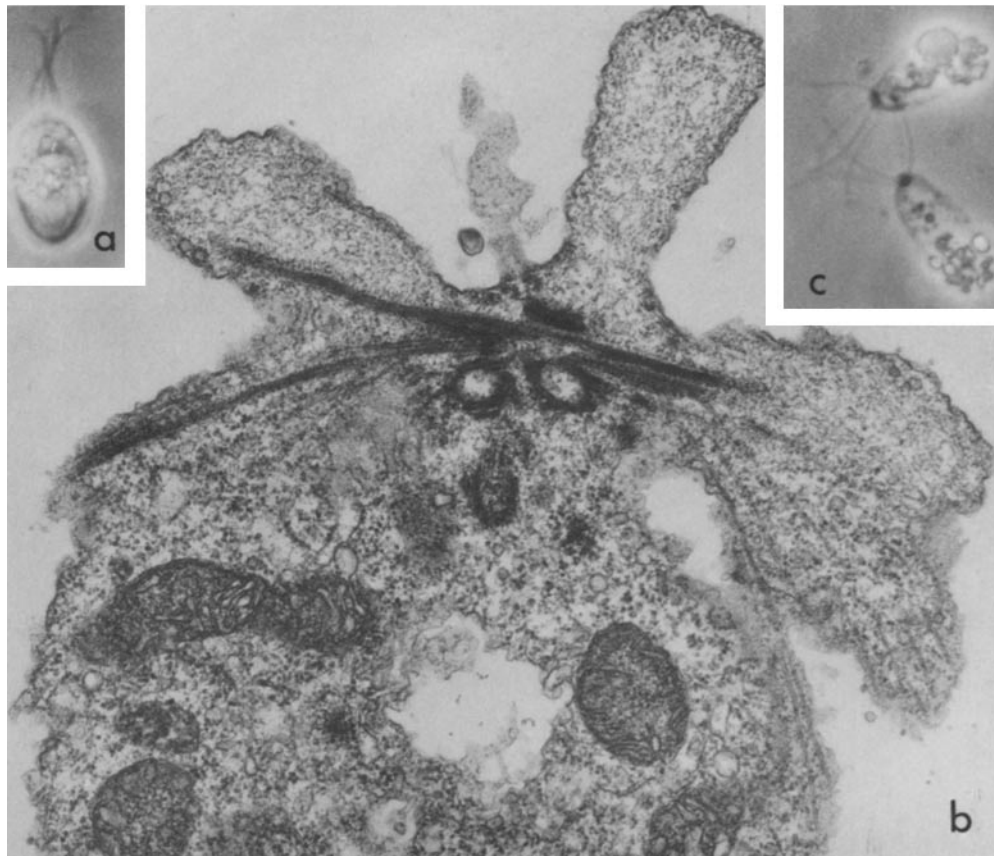
FIGURE 1 Micrographs of *Polytomella agilis* showing distribution of organelles. (a) Phase light micrograph. × 1,100. (b) Electron micrograph of oblique section of anterior portion of cell. × 34,000. (c) Phase light micrograph of cell "ghost" made by gently lysing the cell in Triton X-100. See Materials and Methods. × 1,100.

faintly visible. Approximately 5% of the radioactive label in the cells remained in the polymerized microtubule pellet after one cycle of assembly and disassembly with brain tubulin as a carrier. More than 50% of the protein label from the first cycle remained after a second cycle. When the polymerized proteins were examined by two-dimensional electrophoresis and autoradiography (Fig. 3c), the same three spots (α1, α2, and α3) were observed in the α-tubulin region as had been seen in whole cells; this observation confirmed that these spots were, in fact, tubulins. Of the three, α1 (the tubulin assumed to compose the cytoskeletal microtubules) was the major α-tubulin to polymerize.

The initial homogenization of the cells at 4°C presumably depolymerized the labile cytoskeletal microtubules; however, the supernate obtained from centrifugation of this homogenate must also have included any tubulin in the cell that was unpolymerized, and this tubulin may have been retained in the subsequent assembly steps. To isolate specifically only the tubulin that had been in the polymerized form in the intact cell, we devised a second method to purify tubulin of the cytoskeletal microtubules of the cell.

It had been observed that when cells were quickly but gently lysed with a nonionic detergent, a cell ghost persisted that retained the cell outline (Fig. 1c) but that was considerably more transparent by phase microscopy than was the original cell. The decrease in phase density was presumably attributable to loss of the cytosol proteins and much of the membranous components from the cell. These "ghosts" contained nuclei and starch grains, as well as flagella and rootlet complexes. The

cytoskeletal microtubules, which lie just beneath the plasma membrane and are responsible for maintaining the oval shape of the intact cell (4), were presumably the structures that produced the faint outline seen at the former site of the cell membrane and that enabled the ghost to retain the original shape of the cell. (By electron microscopy [data not shown], the structures remaining in the cell ghosts were shown to be the cytoskeletal microtubules, and other microtubule organelles starch grains, small quantities of membranous organelles, and variable numbers of ribosomes.) The ghosts gradually disintegrated, probably as a result of gradual depolymerization of the cytoskeletal microtubules. Consistent with this notion was the observation that ghosts were considerably stabilized by glycerol, DMSO, and polyethylene glycol, all of which stabilize microtubules, and were destabilized by cold, a treatment that depolymerizes microtubules.

To purify tubulin from cytoskeletal microtubules, we lysed cells to form ghosts, which we then pelleted. The pellet was resuspended in cold buffer to depolymerize the labile cytoskeletal microtubules, and, after a second centrifugation, the supernatant protein was concentrated by ammonium sulfate precipitation. SDS electrophoresis of samples from the various steps in the procedure (Fig. 4a) showed that tubulin was highly enriched in the pellet of cell ghosts and was their most prominent component. The supernate obtained after cold treatment of the ghosts was even more highly enriched with tubulin. Tubulin was obtained in reasonable yield by this method, for ~3% of the radioactive label in the whole cell remained in the final tubulin sample.
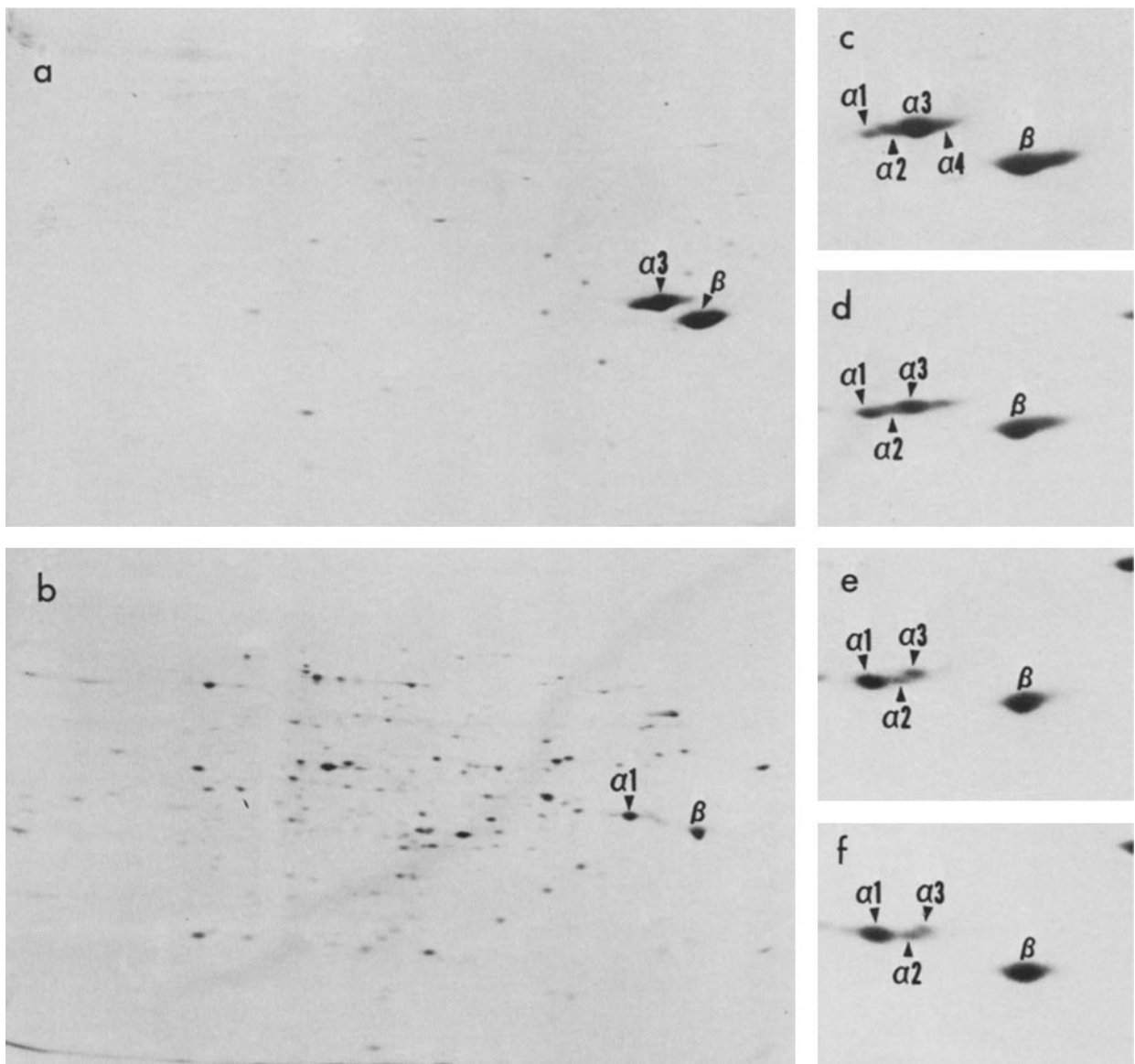
FIGURE 2 Two-dimensional (2-D) electrophoresis of flagellar and whole-cell samples. All panels are autoradiographs of 2-D gels of samples labeled with $^{35}SO_4$. Gels *a* and *c* are of flagella; *b* and *e* are of whole cell proteins; *f* is of protein from deflagellated cells; and *d* is a mixture of samples of flagella and of whole-cell protein. *a* and *b* show the entire 2-D gel; in *c–f* only the tubulin region is shown. All samples were continuously labeled with $^{35}SO_4$ except for *b*, which was pulse labeled.

Two-dimensional electrophoresis of the cytoskeletal tubulin demonstrated the presence of $\alpha1$, $\alpha2$, and $\alpha3$; again, $\alpha1$ was the major α-tubulin component (Fig. 4 *b*). This can be compared to the two-dimensional map of flagella (Fig. 4 *c*), in which $\alpha3$ is the major α-tubulin, and a mixture of the two samples (Fig. 4 *d*).

To confirm that the doublet seen on SDS electrophoresis of the final sample (Fig. 4 *a*, lane *13*) was in fact tubulin, protein was isolated in bulk by the procedure described above and was dialysed against buffer containing glycerol and then subjected to two cycles of polymerization and depolymerization without carrier brain tubulin. The extract became turbid under polymerization conditions, and the final pellet, when examined by electron microscopy, revealed smooth-walled single microtubules (Fig. 4 *e*).

The tubulins present in the various fractions could be com-

pared by electrophoresis in an isoelectric focusing slab gel of (*a*) flagella, (*b*) tubulin isolated from cell supernate by polymerization with brain tubulin, and (*c*) tubulin derived from solubilized cytoskeletons of cell ghosts (Fig. 5). The tubulin components in the two nonflagellar samples were virtually indistinguishable, $\alpha1$ being the major α-tubulin; $\alpha2$ and $\alpha3$ were also present. Conversely, in the flagellar preparation, $\alpha3$ was the major form seen, with $\alpha1$ and $\alpha2$ present as minor components. The major βs of all three samples exactly comigrated in this isoelectric focusing gel, as demonstrated by the presence of only a single band in the lanes in which mixed samples were electrophoresed. Four minor βs, 2 through 5, were clearly seen in the flagellar sample but were much less prominent in the cytoskeletal sample. These minor βs were consistently observed in flagellar samples, and, of the four, $\beta3$ and $\beta4$ were present in greatest abundance. The apparent
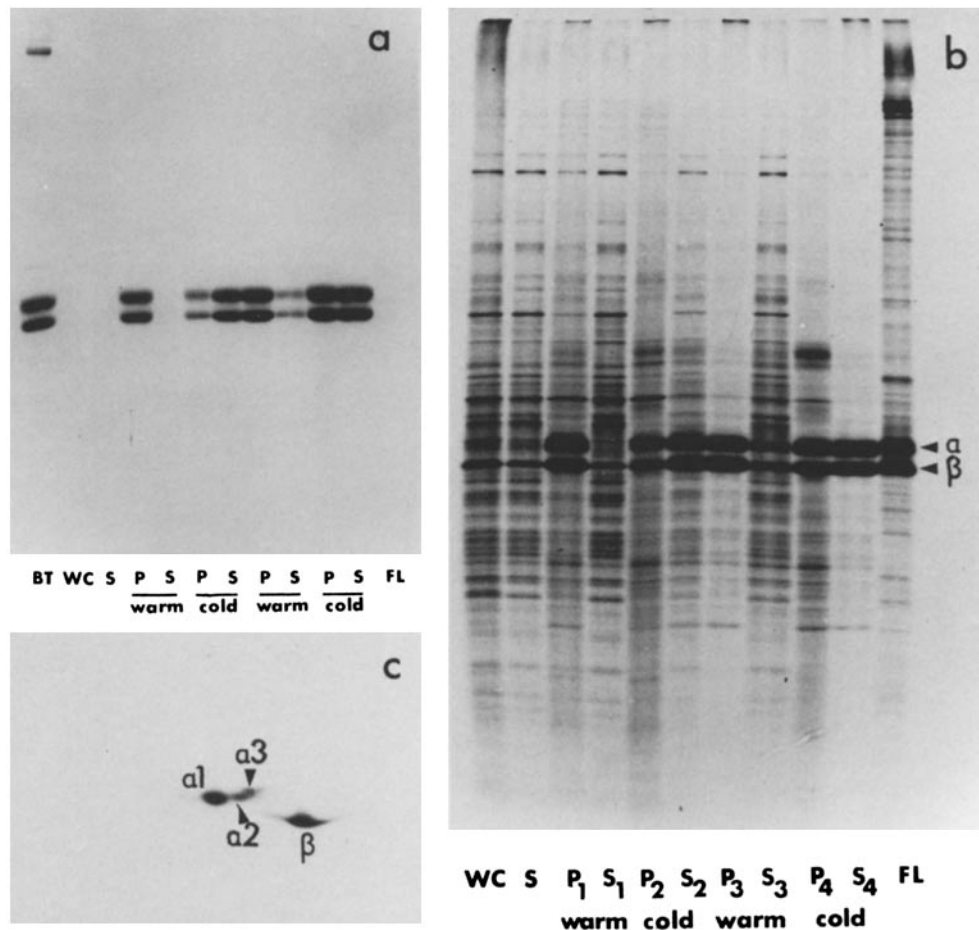
FIGURE 3   Polymerization of *Polytomella* tubulin in cell supernates with carrier brain tubulin. *Polytomella* supernate labeled with $^{35}SO_4$ was mixed with unlabeled calf brain tubulin and subjected to two complete cycles of polymerizationand depolymerization. Equal numbers of radioactive counts were loaded on each channel. (*a*) Coomassie Blue–stained gel, which shows predominantly the calf brain microtubule proteins. (*b*) Relatively enlarged autoradiograph of the same gel, showing *Polytomella* proteins. From left to right: channel *BT*, initial brain tubulin sample before polymerization with *Polytomella* protein (not included in autoradiograph); channel *WC*, whole-cell protein of *Polytomella*; channel *S*, *Polytomella* high-speed supernate. The next eight channels show pairs of pellet *P* and the supernatant *S* after centrifugation of the following four samples: (*1*) mixed sample after polymerization in the warm, (*2*) after cold depolymerization of the resuspended microtubule pellet, (*3*) after polymerization in the warm of the cold supernate, and (*4*) after the cold depolymerization of the second microtubule pellet (twice-cycled microtubule protein). The final channel *FL* is a ^{35}S-labeled flagellar standard. (*c*) Tubulin region of 2-D gel autoradiograph of final cold supernate (twice-cycled microtubule protein).

isoelectric point of α1 was pH 5.5, of α3 was 5.4, and of β1 was 5.2, although the exact values obtained appeared to depend somewhat on the precise electrophoretic conditions.

DISCUSSION

These results indicate that there is considerable heterogeneity of tubulin in *Polytomella* and that the principal α-tubulin tubulin component of cytoskeletal microtubules (α1) is different from the principal α component of flagella (α3). On the other hand, the principal β components of the two organelles are indistinguishable by either SDS electrophoresis or isoelectric focusing.

Preparation of Cytoplasmic Tubulin

Two methods were used to isolate tubulin from the cytoplasm. Polymerization of a mixture of brain tubulin with a supernate from homogenized cells resulted in a striking enrichment in *Polytomella* tubulin. The high yield shows that *Poly-*

tomella tubulin can not only efficiently polymerize under these conditions but can also proceed through cycles of assembly and disassembly. Because the cell supernate was added at a high concentration to the brain tubulin, it is possible that *Polytomella* tubulin polymerized into microtubules composed exclusively of *Polytomella* tubulin; however, because the tubulin in crude supernates alone does not appear to polymerize, the algal and bovine tubulin probably copolymerize to form microtubules of mixed composition. All the major α species and even the minor β forms appear to be capable of polymerization under these conditions.

Copolymerization with brain tubulin has been used previously in several studies to identify and purify tubulins: *Aspergillus* (8, 19) and yeast (20, 27) tubulins were identified by this method, and *Chlamydomonas* flagellar tubulin synthesized in vitro from isolated polysomes was also identified by copolymerization (28).

The second method used to isolate cytoplasmic tubulin involved gentle cell lysis to preserve a cell ghost in which cold
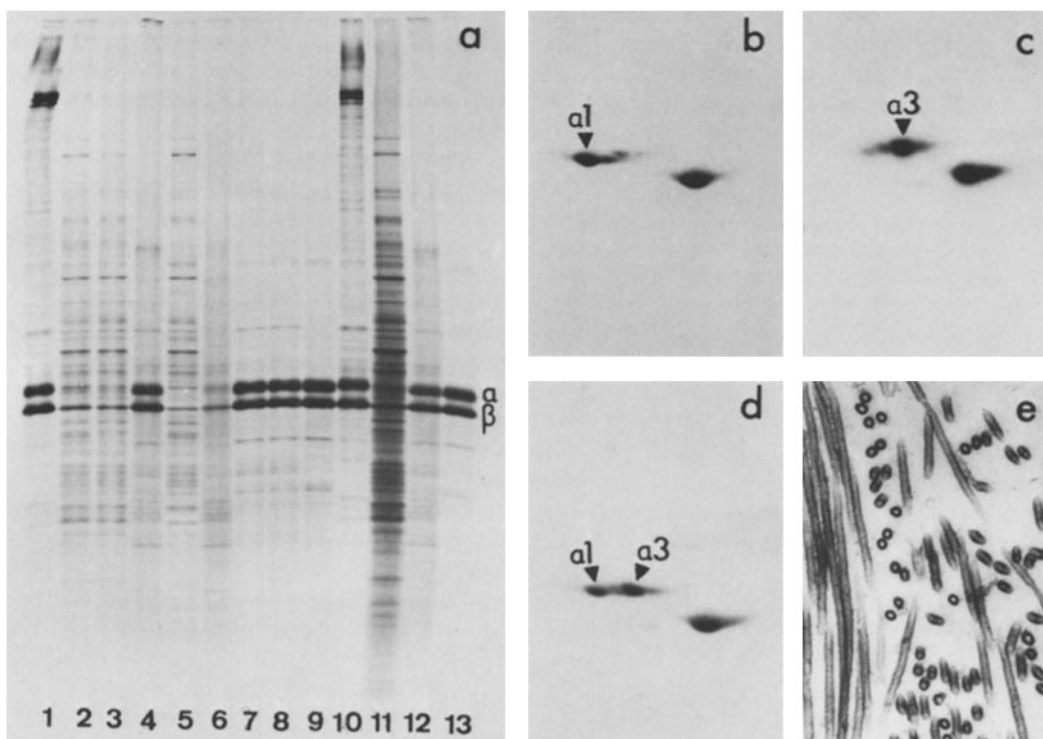
FIGURE 4 Purification of *Polytomella* cytoplasmic tubulin from preparation of cell ghosts. (*a*) Autoradiograph of SDS urea gel showing steps in purification from a sample of $^{35}SO_4$-labeled cells. Channels *1-10* contain equal numbers of radioactive counts. Channels *1* and *10*, flagellar standard; *2*, whole cells; *3* and *11*, deflagellated cells; *4* and *12*, cell "ghosts"; *6* and *7*, pellet and supernate, respectively, after homogenization of ghosts in the cold; *8*, supernate clarified by Millipore filtration; *9* and *13*, final sample, ammonium sulfate precipitate of supernate. Channels *11-13* summarize the experiment; *11* and *12* are more heavily loaded to better show increasing enrichment of tubulin in going from deflagellated cells (*11*) to cell ghosts (*12*) to final sample (*13*). (*b*) Tubulin region of 2-D gel of cytoplasmic tubulin. (*c*) Tubulin region of flagellum. (*d*) Mixture of flagellar sample and cytoplasmic tubulin sample. (*e*) Electron micrograph of microtubules polymerized from sample prepared from cytoskeletal microtubules essentially as in *a*. × 46,000.
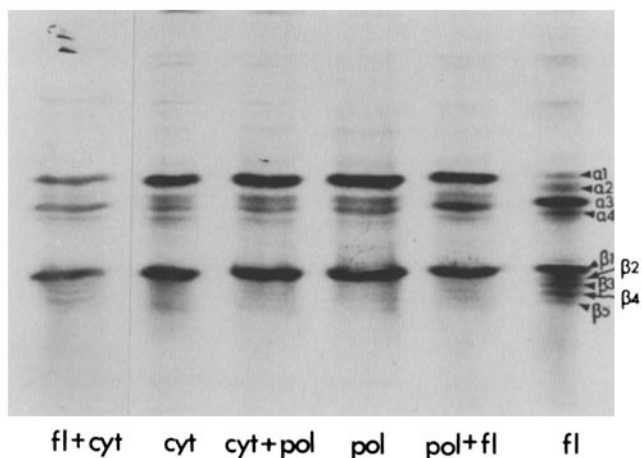

FIGURE 5 Autoradiograph of isoelectric focusing slab gel of flagellar sample, *fl*; cytoplasmic tubulin prepared from cell ghosts, as described in Fig. 4, *cyt*; and cytoplasmic tubulin prepared by polymerization with cold carrier calf brain tubulin, *pol*. The channels each contain, as labeled, one of these samples or a mixture of two of the samples.

labile microtubules were maintained intact. Isolating these ghosts and then shifting to conditions that depolymerize microtubules yielded a highly enriched tubulin fraction that was capable of assembly without addition of carrier brain tubulin. This appears to be the first report of polymerization of tubulin

obtained by such a scheme; however, tubulin has recently been isolated by a similar method from tissue culture cells (21). The preparation of tubulin from cell ghosts has the advantage that MAPs may be coextracted with the tubulin; accordingly, the method may be useful in identifying any proteins that bind to microtubules in vivo but that do not efficiently copolymerize with them in vitro. The gentleness and simplicity of this procedure are likely to help preserve the ability of tubulin and its associated proteins to assemble.

Electrophoretic Evidence for Tubulin Heterogeneity

Two-dimensional electrophoresis of intact flagella and axonemes revealed no differences in their respective tubulins. In addition to the major α spot, α3, the flagellar sample also contains minor amounts of α1 and α2. Although it is conceivable that the presence of these minor species is attributable to contamination of the flagellar preparation by some other microtubule organelle such as the rootlet complex, their consistent presence in apparently constant amounts in flagellar samples, which appear very pure by light microscopy, argues that they are real components of the flagellum. Similarly, two-dimensional electrophoresis of *Chlamydomonas* flagella has also revealed minor α-tubulin components with more basic pI than the major α3 component (13). At present, it is not known whether particular microtubules or protofilaments are enriched in α1 or α2, or in the minor forms of β.

Although the major tubulin found in the cell body either by copolymerization of brain tubulin with cell supernates or by assembly of tubulin from isolated cytoskeletons is $\alpha 1$, the cytoplasm also contains minor amounts of $\alpha 2$ and $\alpha 3$. A possible explanation of the fact that some $\alpha 3$ is found to polymerize with brain tubulin is that $\alpha 3$ may be present in the cell body as part of the pool for flagellar regeneration. Surprisingly, however, small amounts of this $\alpha 3$ spot are also observed in the microtubules prepared from cell cytoskeletons. Because the cells were deflagellated before preparation of cell ghosts and because the few flagellar microtubules that escape attachment should be stable, and therefore sedimentable, under the conditions used to solubilize the tubulin from the ghosts, it seems unlikely that tubulins from intact flagellar are responsible for the presence of the $\alpha 3$ spot. It is possible that (a) a small amount of $\alpha 3$ identical to that in flagella is also found in cytoskeletal microtubules, (b) a polypeptide that fortuitously comigrates with flagellar $\alpha 3$ is a component of nonflagellar microtubules, or (c) unpolymerized $\alpha 3$ in a flagellar tubulin pool is present in a pelletable aggregate.

That there is exact coelectrophoresis of $\beta 1$ from flagellar and cytoskeletal microtubules raises the interesting possibility that a single β-tubulin is a part of the tubulin in both microtubule structures despite their distinct α-tubulins.

Evidence for heterogeneity of tubulin has been presented by several research groups. The most interesting category of heterogeneity is that occurring within a single cell type, because tissue-specific tubulins—analogous to isozymes—may have no relevance to specificity of polymerization in vivo. Stephens (23–25) has extensively studied the tubulins of sea urchins by amino acid analysis and peptide mapping. Within the sperm flagellum, significant differences in both α- and β-subunits were observed between central-pair and outer-doublet tubulins. Furthermore, in both sperm flagella and cilia from gastrula-stage embryos, subtle differences were noted between the tubulins from the A- and B-subfibers. Major differences were observed between tubulin from unfertilized eggs and that from gastrula cilia; in this case, a stage-specific difference, as opposed to an organelle-specific difference, cannot be ruled out. Although these results are quite convincing, the subunits were isolated from one-dimensional SDS gels and thus may have been contaminated by small quantities of proteins that comigrate with tubulin. Although these contaminants would undoubtedly be minor in quantity, if by chance a simple tryptic peptide, such as lysine, arginine, or a dipeptide, happened to be present in multiple copies in the digest of a contaminant protein, the contaminating peptide may have artifactually appeared in the map.

The extensive tubulin heterogeneity reported by Stephens suggests that there may be tubulin heterogeneity in Polytomella not detected by two-dimensional electrophoresis. Species of tubulin that differ only in uncharged residues or that have compensated differences in charged residues are unlikely to be resolved by isoelectric focusing. Indeed, amino acid sequencing of the N-terminal tryptic peptides of actin by Vandekerckhove and Weber (26) have revealed differences in heart and skeletal muscle actins although they exactly comigrate on isoelectric focusing gels.

Isoelectric focusing of flagellar samples to less than equilibrium results in splitting of the $\alpha 3$ tubulin into a very tight pair of bands (results not shown). The separation decreases with increasing time of electrophoresis. It has not been established whether these represent distinct polypeptides.

Evidence for tubulin heterogeneity was also obtained by Kowit and Fulton (10, 11) in Naegleria, where tubulin from the flagella is apparently antigenically distinct from cytoplasmic tubulin. During flagellar growth, only newly synthesized tubulin, and apparently not the preexisting cytoplasmic tubulin, is incorporated into the flagella. These results are compatible with the present finding of a difference in α-tubulins of flagellar and cytoskeletal microtubules.

Heterogeneity of tubulin has been shown electrophoretically by Bibring et al. (2) and by Morris and co-workers (15, 19). By genetic analysis the latter group showed that a single gene codes for the two major β species, whereas minor β forms appear to be encoded by separate genes. Similarly, the major α-tubulin is coded by a single gene, but minor α species are separately encoded. Tubulin heterogeneity in Aspergillus appears to be attributable in part to posttranslational modifications and in part to the expression of multiple tubulin genes. Unfortunately, neither group has shown that specific microtubules are composed of distinct forms of tubulin.

The present work leaves several questions unanswered. First, are the differences in α tubulins attributable to separate genes or to chemical modification of a common precursor? If the first possibility is true, isolation of mRNA and translation in vitro might show synthesis of both forms in vitro. If the second possibility holds, appropriate radioactive labeling studies might show a precursor-product relationship between the common precursor and the various tubulin products. Alternatively, sequencing the tryptic peptides that differ between the tubulins could decide the question. Tryptic mapping might also show whether the β-tubulins in flagellar and cytoskeletal microtubules are in fact identical.

In both Polytomella and Chlamydomonas (13) there is evidence that the principal flagella tubulin, $\alpha 3$, is synthesized as a precursor. When tubulin synthesis is stimulated by deflagellation, the principal α-tubulin synthesized in both species is more basic than $\alpha 3$, and, in fact, exactly comigrates on two-dimensional gels with $\alpha 1$, the cytoskeletal α-tubulin. Pulse-chase experiments in Chlamydomonas further support the existence of a precursor to flagellar $\alpha 3$ tubulin. Perhaps even stronger evidence for the synthesis of flagellar $\alpha 3$ tubulin as a precursor comes from studies in which mRNA, isolated from Chlamydomonas (13) and Polytomella (McKeithan, T. W., C. D. Silflow, P. A. Lefebvre, and J. L. Rosenbaum, manuscript in preparation) at times after deflagellation and translated in the reticulocyte lysate system, directs the in vitro synthesis of an α-tubulin that comigrates on two-dimensional gels with the $\alpha 1$, not the mature $\alpha 3$ found in the flagellum. Together, these results suggest that flagellar $\alpha 3$ tubulin is synthesized as a precursor that comigrates with the cytoskeletal $\alpha 1$ tubulin, and that it is modified posttranslationally before or during assembly into the flagellum (13).

It is unclear whether microtubules in Polytomella show specificity of polymerization by incorporating, as they polymerize, only the appropriate species of tubulin. That is, do different tubulin dimers fail to copolymerize in vivo, either because of incompatibility of the dimers themselves or because of tubulin-binding proteins that confer specificity on the polymerization reaction? Several alternative mechanisms can be suggested. First, perhaps there is only one tubulin pool, and the tubulin is chemically modified after incorporation into the microtubule; this model does not demand that a growing microtubule distinguish among tubulin dimers. Another possibility is that microtubule organelles may be formed, in normal

development, at a distinct time during which only dimers of the appropriate category are synthesized. Finally, the tubulins may be spatially compartmentalized so that only the appropriate tubulin dimers are available for polymerization. Techniques have been developed to separate and quantitate tubulin in polymerized and unpolymerized forms in a cell. Studies using this technique with cells regenerating flagella in the absence of protein synthesis may be able to distinguish among some of these possibilities.

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