Proc. Natl. Acad. Sci. USA
Vol. 76, No. 11, pp. 5745-5749, November 1979
Cell Biology

Purification of cytoplasmic tubulin and microtubule organizing center proteins functioning in microtubule initiation from the alga *Polytomella*

(*in vivo* labeling/*in vitro* assembly/electron microscopy/turbidometry)

MARK E. STEARNS* AND DAVID L. BROWN†

Department of Biology, University of Ottawa, Ottawa, Ontario, Canada, K1N 6N5

Communicated by Keith R. Porter, August 31, 1979

ABSTRACT **Cytoplasmic tubulin and the microtubule organizing centers (MTOCs) for the cytoskeletal microtubule system of the flagellate *Polytomella* have been isolated. The isolated MTOCs serve as sites for the *in vitro* assembly of the purified tubulin protein. The major proteins (four polypeptides of molecular weights 190,000–210,000) functioning in this assembly have been extracted from the MTOCs and purified. Kinetic studies and experiments with *in vivo* ^{35}S-labeled MTOC proteins (or ^{35}S-labeled tubulin) demonstrate that these proteins function specifically in microtubule initiation and do not contribute to microtubule elongation. The results indicate that microtubule assembly *in vivo* is controlled by microtubule initiating proteins associated with the organelles termed MTOCs.**

Microtubule organizing centers (MTOCs) have been implicated in the regulation of microtubule assembly in many developing cells (1–3). MTOCs as diverse as animal cell centrosomes and kinetochores (4–8), yeast spindle pole bodies (9, 10), and *Polytomella* basal body rootlets (11) have been shown to promote the assembly of heterologous brain microtubule proteins *in vitro*. The precise mechanism of this site-initiated assembly is not understood, although trypsin digestion studies have indicated that the functional capacity of isolated spindle pole bodies (9, 10) and of basal body rootlets (12) resides in protein(s) or protein structures associated with MTOCs.

In all of the studies cited above (4–12), the microtubule protein(s) used in the *in vitro* assays was obtained from brain tissue of homeothermic animals. The validity of extrapolating these results to an understanding of MTOC-directed assembly *in vivo* depends to a large extent on whether the assembly properties of brain microtubules are uniformly shared by microtubule systems in such phylogenetically distant organisms as yeast and *Polytomella*. There is no compelling reason to assume that such uniformity exists; in fact, there are many indications to the contrary. Brain tubulin from the poikilothermic dogfish will self-initiate in the apparent absence of microtubule-associated proteins (13), a characteristic that is not shared by brain tubulin from homeotherms. The *in vitro* polymerization of microtubules from some non-neural cultured cells is promoted by microtubule-associated proteins that differ from those prominent in the brain systems (14, 15). Different microtubule systems within the same cell have been shown to differ greatly in their sensitivity to microtubule depolymerizing treatments (16, 17), and in one organism, the protozoan *Naegleria*, there is good evidence for the existence of tubulins specific to different microtubular organelles (18). The sum of these observations makes it likely that microtubule systems differ in their requirements for assembly within and between organisms and attempts to evaluate the role of MTOCs in the regulation of microtubule assembly should ideally utilize MTOCs and tubulin purified from a homologous source.

We report here procedures for the purification of cytoplasmic tubulin from *Polytomella* and a series of MTOC-associated proteins that function in microtubule initiation. It seems likely that MTOCs in general are composed in part of microtubule initiating proteins which operate *in vivo* in regulating the initiation of microtubule arrays.

MATERIALS AND METHODS

Purification of Cytoplasmic Tubulin. Swimmers of *Polytomella* were cultured as described (11), harvested in the exponential phase of growth (about 5×10^{10} cells per experiment), and washed twice in fresh medium. To remove contaminating flagellar tubulin proteins, the cells were double-deflagellated in the presence of 10 μg of cycloheximide per ml (19). The cell bodies were then homogenized in 1 ml of microtubule assembly buffer (MAB) [0.1 M 1,4-piperazinediethanesulfonic acid (Pipes)/1 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA)/1 mM $MgCl_2$/2 mM GTP/0.05% Triton X-100 at pH 6.4 and 4°C], using a teflon/glass Wheaton homogenizer. The homogenate was centrifuged for 30 min at 100,000 × *g* in a Beckman Ti 60 rotor and the supernatant containing cytoplasmic tubulin was applied to a 1.5 × 6 cm phosphocellulose column (20). The protein eluting with MAB from the heavily overloaded columns (>20 mg of protein per ml) was collected and dialized overnight at 4°C against MAB/4 M glycerol. To enrich for microtubule proteins, the dialyzed protein sample was made 2 mM in GTP and incubated at 33°C for 30 min, and the microtubules assembled were pelleted at 39,000 × *g* for 30 min at 33°C. The microtubule pellets were cold-solubilized (4°C, 30 min) in 1 ml of MAB and clarified by centrifugation at 39,000 × *g* for 30 min at 4°C, and supernatants (containing <10 mg of protein per ml) were again fractionated by phosphocellulose chromatography. Purified tubulin fractions were either immediately dialyzed against fresh MAB and used in assembly experiments or dialyzed against MAB/4 M glycerol and stored frozen at −10°C overnight. Before use in assembly studies, stored tubulin was dialyzed against MAB at 4°C to remove glycerol.

Purification of MTOC Proteins. The MTOCs for cytoplasmic microtubules in *Polytomella* (basal body rootlet complexes) were isolated and purified by using described methods

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Abbreviations: MTOCs, microtubule organizing centers; MAB, microtubule assembly buffer; TIPs, (micro)tubule initiating proteins.

* Current address: Department of Molecular, Cellular and Developmental Biology, University of Colorado, Boulder, CO.
† To whom reprint requests should be addressed.

(11), and assembly of microtubules onto intact MTOCs was assayed by electron microscopy. To obtain preparations containing (micro)tubule initiating proteins (TIPs), intact MTOCs were dialyzed for 20 min against 1 mM Tris·HCl, pH 8.0/0.1 mM EDTA/0.01% 2-mercaptoethanol at 4°C. The extracted MTOCs were pelleted (100,000 × *g* for 60 min) and the supernatant containing TIPs was dialyzed against MAB. The TIPs extract was then mixed with purified cytoplasmic tubulin (2 mg/ml) and incubated at 33°C for 10 min to promote microtubule assembly. In some of these experiments phosphocellulose-purified beef brain tubulin was used at this step. The microtubules assembled were pelleted, cold-solubilized in 1 ml of MAB as described above, and fractionated on a phosphocellulose column. Tubulin was eluted from the column with excess MAB and the TIPs bound to the phosphocellulose were eluted with MAB/0.55 M NaCl and then dialyzed against fresh MAB.

Microtubule Assembly Experiments. Protein concentrations at the various stages of purification were measured by the method of Hartree (21), samples were routinely assayed by sodium dodecyl sulfate slab gel electrophoresis (22), and the percentages of the different proteins were determined by densitometric scanning of 0.5% aqueous Coomassie brilliant blue 250-R stained gels.

Electron microscopy was carried out as described (11). Negative staining was used as a routine assay for the presence of microtubules in assembly studies.

Turbidimetric analysis of assembly was carried out at OD_{350} with a Gilford 2400 spectrophotometer equipped with cuvettes preequilibrated and maintained at the appropriate temperature.

In vivo labeling of tubulin and MTOC proteins was accomplished by growing *Polytomella* for 12 generations (≈48 hr) in the presence of 0.125 μCi of $^{35}SO_4$ (1000 mCi/mmol, New England Nuclear) (1 Ci = 3.7×10^{10} becquerels). The radioactive proteins were purified as described above and used in assembly studies. To monitor the kinetics of association and the stoichiometries of tubulin and the TIPs, samples were incubated at 33°C and at time intervals they were centrifuged at 39,000 × *g* for 30 min to pellet the assembled polymer. Radioactivity was determined by suspension of pellets in Aquafluor and liquid scintillation counting (Beckman, LA-233). Protein concentration in the pellets (see Figs. 5 and 6) was routinely determined from a linear standard curve comparing amounts of protein and cpm.

RESULTS

Cytoplasmic Tubulin Purification. The composition, as determined by gel electrophoresis, of the fractions obtained at various stages in the purification of *Polytomella* cytoplasmic tubulin is shown in Fig. 1. Ten to 15% of the protein from the high-speed supernatant of the total cell homogenate coelectrophoresed with a tubulin standard (Fig. 1, lane a). This corresponds to a tubulin concentration of 2–2.5 mg/ml, which should be sufficient for *in vitro* microtubule assembly. However, in this fraction, only nonspecific aggregates formed under assembly conditions in the presence or absence of glycerol. Fractionation of the high-speed supernatant on phosphocellulose, followed by extensive dialysis of the unbound fraction against MAB/glycerol, produced a tubulin-containing fraction capable of microtubule assembly. Gels of this unbound fraction (Fig. 1, lane b) exhibited a profile indistinguishable from that of the high-speed supernatant (compare Fig. 1, lanes a and b), suggesting that the phosphocellulose treatment removed (minor) components that interfered with microtubule assembly. Both the phosphocellulose treatment and the presence of 4 M glycerol were essential for the preparation of an assembly-competent tubulin fraction. One temperature-dependent cycle of assembly and disassembly of this fraction (Fig. 1, lane b) produced a sample of microtubule proteins containing about 55% tubulin (Fig. 1, lane c). A second phosphocellulose chromatographic step yielded a purified tubulin fraction (Fig. 1, lane d) containing only traces of other proteins. In MAB, in the absence of glycerol, this purified tubulin was assembly-incompetent at concentrations up to 4 mg/ml.

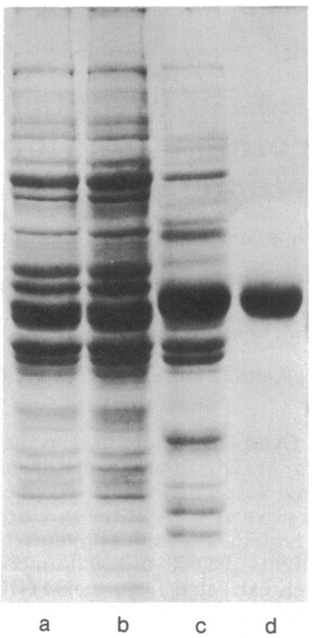

FIG. 1. Sodium dodecyl sulfate gels of purification steps of *Polytomella* cytoplasmic tubulin. Lanes: a, 100,000 × *g* supernatant of total cell homogenate crude extract; b, unbound protein eluted from first phosphocellulose treatment of crude extract; c, tubulin-enriched fraction after one cycle of assembly and disassembly; d, purified tubulin obtained by phosphocellulose chromatography of sample c.

Purification of TIPs. The capacity of the isolated, intact MTOCs (rootlets attached to basal bodies) to initiate the assembly of purified cytoplasmic tubulin (at 1 mg/ml) was examined first by electron microscopy. As shown in Fig. 2, large numbers of microtubules were initiated at sites on the rootlet MTOCs. Negative staining showed that all of the microtubules assembled were in association with MTOCs. For a complete description of the MTOC structure and the pattern of associated cytoplasmic microtubules in *Polytomella* see ref. 23.

The brief dialysis of intact MTOCs in low-ionic-strength buffer extracted a mixture of many proteins (Fig. 3, lane a). As we have previously reported (12), the extracted MTOCs no longer initiated assembly whereas the extract initiated assembly of purified tubulin. The major proteins in the MTOC extract functioning in microtubule initiation were selected by incubating the extract with purified tubulin under assembly conditions for 10 min and then sedimenting the microtubules formed. Sodium dodecyl sulfate gels of these pellets (Fig. 3, lane b) showed a large tubulin band and a minimum of four protein bands in the high molecular weight (190,000–210,000) range. These proteins, which we term TIPSs, were separated from tubulin by phosphocellulose chromatography (Fig. 3, lane c) and were shown to reinitiate the assembly of purified tubulin incompetent to self-initiate microtubule assembly (Figs. 4–6).

Assembly Studies with Purified Proteins. Fig. 4 shows by turbidimetric analysis the effect of various concentrations of TIPs (0.1–0.4 mg/ml) in promoting the assembly of fixed

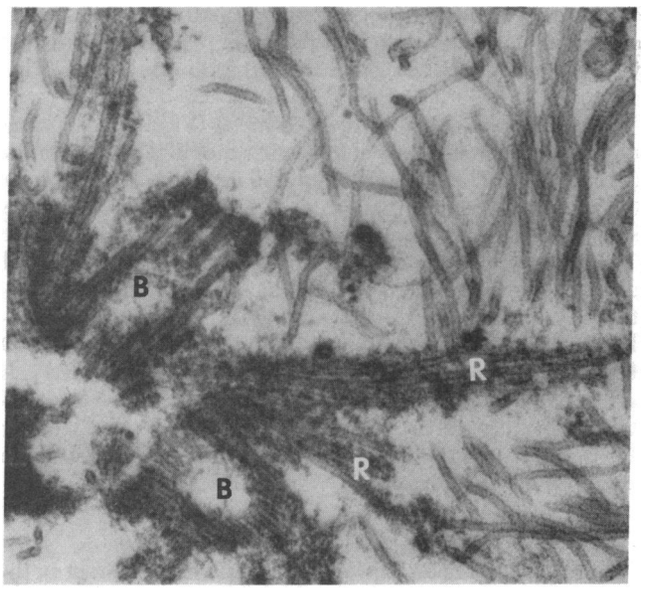

FIG. 2. Thin-section electron micrograph showing that intact, isolated MTOCs (rootlets) are sites for initiation of microtubule assembly from purified *Polytomella* cytoplasmic tubulin that was incompetent to self-initiate assembly. B, Basal body; R, rootlet. (×60,000.)

concentrations of purified tubulin (2.0 or 3.0 mg/ml). Increasing amounts of TIPs (or of tubulin) clearly increase the initial rates of assembly, producing a considerable change in optical density, as much as 0.05 unit for 2 mg of tubulin per ml after a 5-min incubation. Raising the tubulin level (3 mg/ml) increased the initial rates of assembly at 5 min (reducing the effect that TIPs have on initiation) and increased the OD at equilibrium. Increasing the concentration of TIPs above 0.2 mg/ml at either tubulin concentration did not result in a significant increase in OD at equilibrium, indicating that the primary effect of TIPs is on microtubule initiation. At low concentrations of TIPs (0.1 mg/ml) equilibrium was not attained by 30 min, and even longer times of incubation did not result in the maximal equilibrium values. Possible explanations of this result include a progressive denaturation of tubulin with incubation time and a limiting microtubule number concentration. The optimal temperature for maximal assembly of *Polytomella* microtubule proteins was 33°C. At 33°C and 0.2 mg of TIPs per ml, the critical concentration of tubulin required for assembly was 0.16–0.18 mg/ml. All of the protein-association studies described below were carried out at the optimal assembly temperature by using TIP concentrations ≥0.2 mg/ml. In these experiments >90% of the tubulin protein was in polymer form at equilibrium.

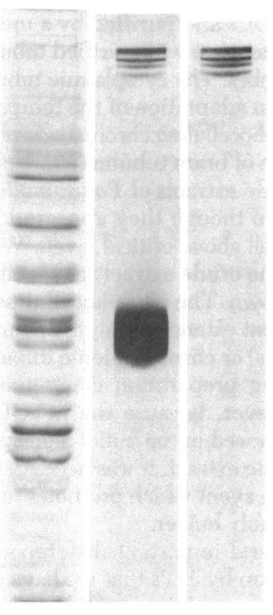

FIG. 3. Sodium dodecyl sulfate gels of purification steps of TIPs from isolated *Polytomella* MTOCs. Lanes: a, total Tris/EDTA extract of MTOCs; b, prominent TIPs from the total extract which have associated with tubulin to form microtubules; c, purified TIPs (molecular weights, 190,000–210,000) obtained by phosphocellulose chromatography of sample b.

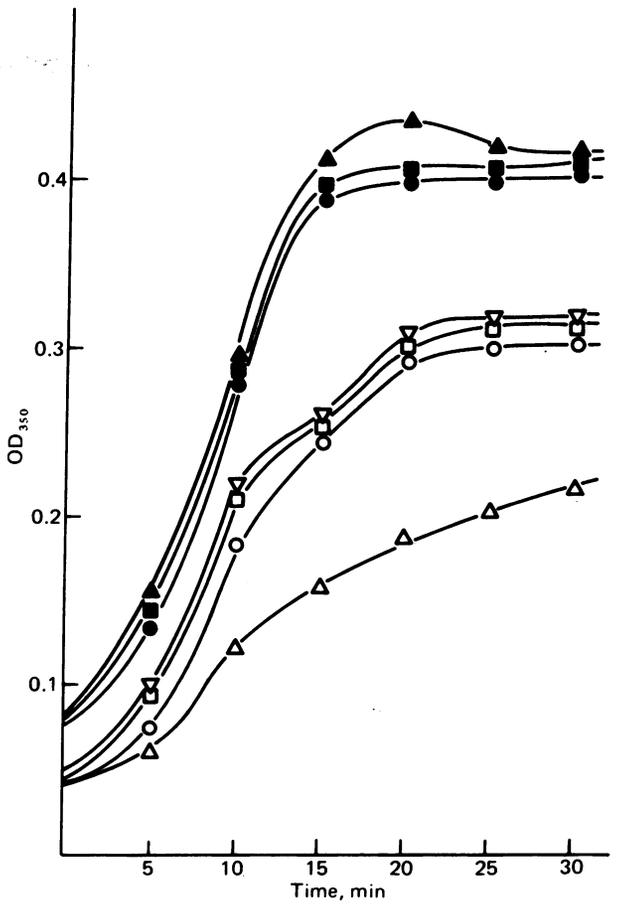

FIG. 4. Turbidimetric analysis of assembly at 33°C in samples containing purified tubulin at 2 mg/ml and TIPs at 0.1 (△), 0.2 (○), 0.3 (□), and 0.4 (▽) mg/ml and with purified tubulin at 3 mg/ml and TIPs at 0.2 (●), 0.3 (■), and 0.4 (▲) mg/ml.

Interactions of TIPs and Tubulin. The turbidimetric results have been confirmed and extended to yield information on the stoichiometries of TIPs and tubulin by using *in vivo* ^{35}S-labeled proteins and by monitoring the time course of appearance during assembly and the amounts of both proteins present in sedimented microtubules. Fig. 5 shows that with a fixed tubulin concentration (2.0 mg/ml), all of the available [^{35}S]TIPs at 0.2, 0.3, and 0.4 mg/ml were incorporated into the microtubules during the first 5 min of assembly. Note that this assembly corresponds to a very small change in turbidity (Fig. 4), which we interpret to represent the initiation phase of microtubule assembly. Microtubule pellets taken at periods after 5 min showed no increase in the amounts of [^{35}S]TIPs and measurements of the [^{35}S]TIPs in the corresponding supernatants

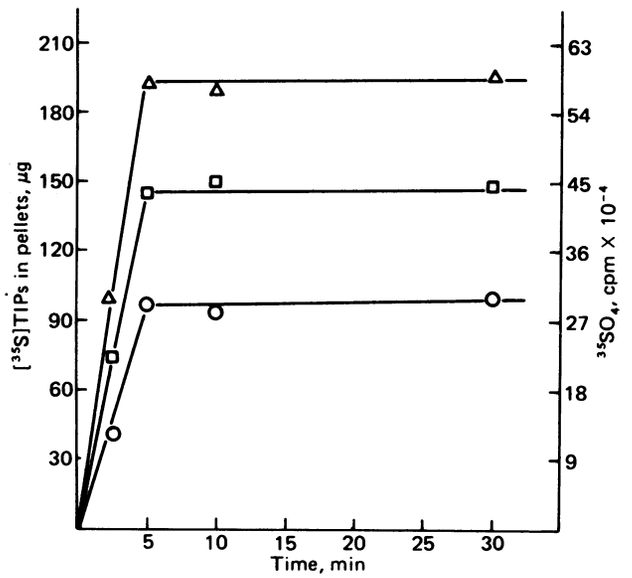

FIG. 5. Sedimentation analysis of TIPs–tubulin association with a fixed tubulin concentration (2 mg/ml) and ^{35}S-labeled TIP concentrations of 0.2 (○), 0.3 (□), and 0.4 (△) mg/ml. The mixtures were incubated at 33°C for 5, 10, or 30 min before centrifugation to pellet the microtubules. Note that the sample sizes for these experiments and those in Fig. 6 were 0.5 ml.

showed only background levels of radioactivity. We interpret these results to indicate that, in the presence of increasing amounts of TIPs, more microtubules were initiated to assemble and that the elongation phase of microtubule assembly (from 5 to 30 min) is due to the addition of tubulin dimers alone. The

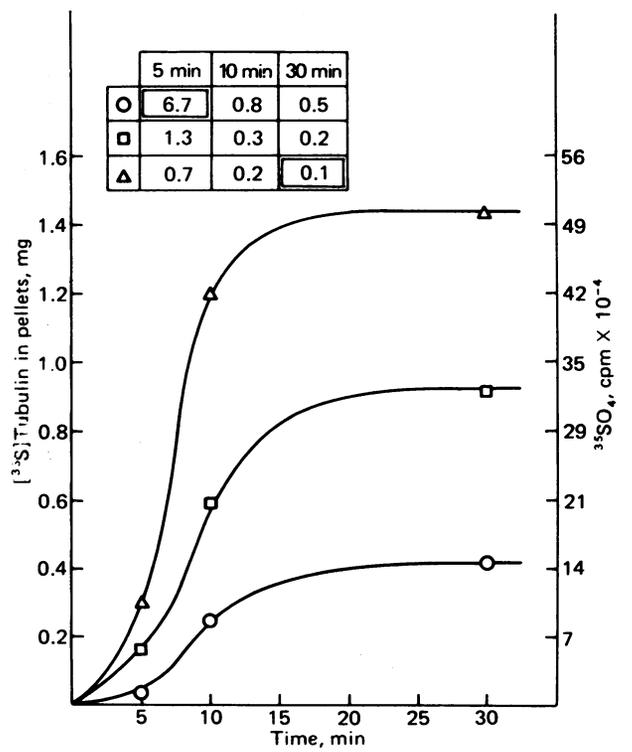

FIG. 6. Sedimentation analysis of TIPs–tubulin association with a fixed TIP concentration (0.2 mg/ml) and ^{35}S-labeled tubulin concentrations of 1.0 (○), 2.0 (□), and 3.0 (△) mg/ml. Incubation was at 33°C for 5, 10, or 30 min before sedimentation of microtubules. (*Inset*) Ratios of TIPs to tubulin by weight in the microtubules sedimented at the 5-, 10-, and 30-min incubation times.

converse experiments, using a fixed concentration of TIPs (0.2 mg/ml) and increasing concentrations of [^{35}S]tubulin (Fig. 6), yielded results consistent with the turbidimetric studies (Fig. 4).

The data from the labeling experiments (Figs. 5 and 6) were used to determine the stoichiometric ratios of TIPs to tubulin (Fig. 6 *inset*). These values, expressed on a weight basis, show the predicted decrease in the ratio of TIPs to tubulin with time and with increasing concentrations of tubulin protein available. The two extremes (the 5-min sample with tubulin at 1.0 mg/ml and the 30-min sample with tubulin at 3.0 mg/ml) show a 6- to 7-fold variation in the TIPs-to-tubulin ratio. By using molecular weights of 200,000 as the average for TIPs and 110,000 for tubulin (and assuming that the four TIPs bands seen on gels are equally represented in the microtubules formed), the extreme ratios convert to 3.7 mol of TIPs per mol of tubulin dimer for the 5-min sample and 1 mol of TIPs per 13 mol of tubulin dimer in the 30-min sample.

DISCUSSION

The MTOC has been viewed as an important element in the spatial and temporal regulation of microtubule assembly *in vivo* (1–3). Some properties of the MTOC regulatory system have been demonstrated in previous *in vitro* studies of microtubule assembly. For example, the timing of spindle microtubule assembly appears to be related to an activation or maturation of the centrosome (5, 7, 24). Similarly, the number of microtubles assembled by isolated yeast spindle pole bodies has been shown to vary with the position in the cell cycle (10). There is good evidence that isolated MTOCs possess a limited number of microtubule initiating sites (8–10) and that the positioning of the initiating sites in the MTOC could confer the spatial control that results in a specific pattern of microtubules within a complex array (12).

In this paper we report on the isolation of what we believe to be a key component of the MTOC-regulatory system: a series of proteins (termed TIPs) that initiate the assembly of homologous cytoplasmic tubulin. The TIPs were removed from intact, isolated MTOCs and purified by a method that relies on their capacity to associate with purified tubulin and to promote microtubule assembly. The cytoplasmic tubulin of *Polytomella* was purified by an adaptation of the temperature-dependent cycling and phosphocellulose chromatographic techniques used in the purification of brain tubulin (20). Unlike brain extracts, however, the crude extracts of *Polytomella* did not assemble microtubules even though they appeared to contain tubulin concentrations well above critical levels. Whether this reflects the presence in the crude extracts of specific inhibitory components is not known. The phosphocellulose chromatographic step and subsequent extensive dialysis apparently removed any inhibitory factor(s) or corrected ionic imbalance, producing a tubulin-containing preparation competent to assemble microtubules. However, because the MTOCs (the basal body rootlets) were removed in the initial high-speed centrifugations used to prepare the extract, it was necessary at this stage to include glycerol, an agent which promotes tubulin self-assembly (25), in the assembly buffer.

There are several important differences between the microtubule initiation by TIPs that we have described here and the microtubule assembly promoted by the various microtubule-associated proteins (see ref. 26 for a recent review) purified from brain microtubules. The two major groups of microtubule-associated proteins, the tau and the high molecular weight proteins, are required in stoichiometric amounts proportional to tubulin polymer and thus affect both the rate and total extent of polymer formed. We have shown, for example, that purified

microtubule-associated protein 2 associated with tubulin at a constant stoichiometry (approximately 1:7) to regulate the initial rate and final amount of microtubule assembly at equilibrium (27). The ratio of TIPs to tubulin, on the other hand, can vary many-fold without affecting the extent of assembly. The initial rate of assembly is dependent on the concentrations of both TIPs and tubulin, whereas the total amount of polymer formed (within limits) is a function of the tubulin concentration alone. These results and those from the kinetic studies of labeled TIPs–tubulin association suggest that TIPs are present at a constant amount per microtubule regardless of the length of the microtubule assembled. Clearly, the maximal length of the microtubules formed in the absence of stabilizing microtubule-associated proteins may be limited by such uninteresting factors as the progressive denaturation of tubulin, and, hence, the minimal possible ratio of TIPs to tubulin that one might expect would not be achieved. However, by extending the basic procedures established in this paper it should be possible to select microtubule-associated proteins from *Polytomella* extracts and to examine their involvement in a homologous MTOC-initiated assembly system. Results from this approach should more accurately reflect the normal minimal ratios of TIPs to tubulin required for microtubule assembly.

Although the biochemical composition of the diverse structures functioning as MTOCs is not known, the demonstration that the microtubule initiating capacity of isolated spindle pole bodies of yeast (9, 10) and rootlet MTOCs of *Polytomella* (12) is sensitive to brief trypsinization, in combination with the findings of this study that MTOCs consist in part of TIPs, encourages generalization. Obviously, further work is required to determine whether TIPs are specifically localized on the MTOCs of *Polytomella in vivo* and whether similar proteins are components of the diverse MTOCs of other organisms.

This work was supported by National Sciences and Engineering Research Council grants to D.L.B.

1. Pickett-Heaps, J. D. (1969) *Cytobios* **1**, 257–280.
2. Porter, K. R. (1966) in *Principles of Biomolecular Organization*, Ciba Foundation Symposium (Little Brown, Boston), pp. 308–345.
3. Bouck, G. B. & Brown, D. L. (1976) *Annu. Rev. Plant Physiol.* **27**, 71–94.
4. McGill, M. & Brinkley, B. R. (1975) *J. Cell Biol.* **67**, 189–199.
5. Snyder, J. A. & McIntosh, J. R. (1975) *J. Cell Biol.* **67**, 744–760.
6. Telzer, B. R., Moses, M. J. & Rosenbaum, J. L. (1975) *Proc. Natl. Acad. Sci. USA* **72**, 4023–4027.
7. Gould, R. R. & Borisy, G. G. (1977) *J. Cell Biol.* **73**, 601–615.
8. Gould, R. R. & Borisy, G. G. (1978) *Exp. Cell Res.* **113**, 369–374.
9. Byers, B., Shriver, K. & Goetsch, L. (1978) *J. Cell Sci.* **30**, 331–352.
10. Hyams, J. S. & Borisy, G. G. (1978) *J. Cell Biol.* **78**, 401–414.
11. Stearns, M. E., Connolly, J. A. & Brown, D. L. (1976) *Science* **191**, 188–191.
12. Stearns, M. E. & Brown, D. L. (1978) *Proc. Int. Congr. Electron Microsc. 9th* **2**, 270–271.
13. Langford, G. M. (1978) *Exp. Cell Res.* **111**, 139–151.
14. Nagle, B. W., Doenges, K. H. & Bryan, J. (1977) *Cell* **12**, 573–586.
15. Weatherbee, J. A., Luftig, R. B. & Weihing, R. R. (1978) *J. Cell Biol.* **78**, 47–57.
16. Behnke, O. & Forer, A. (1967) *J. Cell Sci.* **2**, 169–192.
17. Tilney, L. G. & Gibbins, J. R. (1968) *Protoplasma* **65**, 167–179.
18. Fulton, C. & Simpson, P. A. (1976) in *Cell Motility*, eds. Goldman, R., Pollard, T. & Rosenbaum, J. L. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), Book C, pp. 987–1005.
19. Brown, D. L. & Rogers, K. A. (1978) *Exp. Cell Res.* **117**, 313–324.
20. Weingarten, M. D., Lockwood, A. H., Hwo, S.-Y. & Kirschner, M. W. (1975) *Proc. Natl. Acad. Sci. USA* **72**, 1858–1862.
21. Hartree, E. F. (1972) *Anal. Biochem.* **48**, 422–427.
22. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685.
23. Brown, D. L., Massalski, A. & Patenaude, R. (1976) *J. Cell Biol.* **69**, 106–125.
24. Weisenberg, R. C. & Rosenfeld, A. C. (1975) *J. Cell Biol.* **64**, 146–158.
25. Lee, J. C. & Timasheff, S. N. (1975) *Biochemistry* **14**, 5183–5187.
26. Kirschner, M. W. (1978) *Int. Rev. Cytol.* **54**, 1–71.
27. Stearns, M. E. & Brown, D. L. (1979) *FEBS Lett.* **101**, 15–20.