Proc. Natl. Acad. Sci. USA
Vol. 84, pp. 3259–3263, May 1987
Cell Biology

Periodic crosslinking of microtubules by cytoplasmic microtubule-associated and microtubule-corset proteins from a trypanosomatid

(tubulin/*Crithidia fasciculata*/cytoskeleton/taxol)

GREGORY T. BRAMBLETT, SULIE CHANG, AND MARTIN FLAVIN

Laboratory of Cell Biology, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD 20892

Communicated by Keith R. Porter, January 15, 1987 (received for review September 19, 1986)

ABSTRACT **The dominant element in the cytoskeleton of *Crithidia fasciculata* is a peripheral corset of microtubules enclosing the cell body and closely underlying the plasma membrane. A lateral spacing of 50 nm is maintained by crosslinks, and microtubules may also be linked to the plasma membrane. We have characterized groups of polypeptides that associate with microtubules polymerized *in vitro* from the cytoplasm, or that are associated with the corset complex. They differ except for one of M_r 33,000 present in both groups. The corresponding native corset protein appears to be a dimer of M_r 66,000. These protein(s) copolymerize with brain tubulin, and the resultant polymer consists of pairs or small parallel bundles of microtubules, joined by periodic crosslinks spaced about 8.5 nm apart.**

Trypanosomatids display many unusual features of current interest to cellular and molecular biologists (1–3), but little is known about the composition of the cytoskeleton in these protozoa. Intermediate and actin filaments have not been identified, and the dominant element is a peripheral corset of microtubules enclosing the cell body and closely underlying the plasma membrane. These microtubules maintain a uniform lateral spacing of about 50 nm, and crosslinks can be seen between adjacent microtubules (4) and possibly also between microtubules and plasma membrane (2). Tubulin has been purified from cytoplasm, corset, and flagellar B tubule (5) and shown to exist in different states of posttranslational modification in these compartments (5, 6).

We report here a comparison between the cytoplasmic microtubule-associated proteins (MAPs) and the proteins associated with microtubule corsets, isolated from *Crithidia fasciculata*. Several of the corset proteins (COPs) have also been purified. Some of these fractions have the capacity to bundle microtubules, through the formation of closely spaced periodic crossbridges. A dimer of a 33-kDa polypeptide appears to be the major component of the bridges.

MATERIALS AND METHODS

Culture Conditions. *Crithidia fasciculata* (ATCC no. 11745) was maintained by twice-weekly transfer on complex medium no. 3 (7). Cells for harvest were grown in the same medium at 25°C, either with gentle rotary agitation in flasks containing 1 liter of medium or with forced aeration in carboys of 18 liters. Cells were harvested at a density of 2–3 $\times 10^7$ per ml by centrifugation for 10 min at 3000 $\times$ *g* at 2°C and washed twice with phosphate-buffered saline.

Preparation of Cytoplasmic MAPs. Cells were extracted by sonication as described by Russell *et al.* (5) for isolation of cytoplasmic tubulin, except that protease inhibitors [phenylmethylsulfonyl fluoride (0.5 mM), aprotinin (20 μg/ml), leupeptin (10 μg/ml), and soybean trypsin inhibitor (50 μg/ml)] were sometimes added to extraction buffer, and the lipid-free supernatant was centrifuged a second time, for 1 hr at 180,000 $\times$ *g* at 2°C. Tubulin plus MAPs was polymerized by addition of GTP to 1 mM, taxol (obtained from M. Suffness of the National Cancer Institute) to 20 μM, and 2% dimethyl sulfoxide (introduced with the taxol) (8, 9) followed by incubation for 40 min at 37°C. Polymer was harvested by centrifugation for 30 min at 45,000 $\times$ *g* at 30°C. Pellets were washed once by centrifugation after resuspension in taxol/PEMEG buffer (100 mM K Pipes, pH 6.9/2 mM EGTA/1 mM $MgSO_4$/0.1 mM EDTA/2 mM dithiothreitol/1 mM GTP/20 μM taxol/2% dimethyl sulfoxide). MAPs were solubilized by extraction of the pellet with taxol/PEMEG containing 0.6 M NaCl. MAPs were concentrated, from the supernatant after centrifugation at 30°C, with a Centricon filter (Amicon), dialyzed for 4 hr at 2°C against five changes of reassembly buffer (100 mM K Mes, pH 6.9/0.5 mM $MgSO_4$/1 mM EGTA/2 mM dithiothreitol), and centrifuged again at 45,000 $\times$ *g* at 2°C, and the supernatant was stored at −80°C. Heat-stable MAPs were those that remained in solution after the undialyzed preparation had been kept for 5 min in a boiling water bath.

COPs Preparation. The complex of COPs was isolated as described by Russell *et al.* (5) for isolation of pellicular tubulin. The procedure involves sequential deflagellation, detergent extraction, and Polytron destabilization (5). Solubilization by sonication was enhanced by addition of 0.6 M NaCl and 1 mM $CaCl_2$ to the buffer (K. Gull, personal communication). To enrich the preparation in accessory corset protein (COPs), tubulin was depleted by discarding it after polymerization induced by dialysis against buffer plus 8 M glycerol (5). After dilution to reduce the glycerol concentration, COPs were fractionated by salt-gradient elution from a cation-exchange Mono S (Pharmacia) column.

Miscellaneous Procedures. Bovine brain microtubule protein was prepared by two cycles of polymerization, and pure tubulin (PC-tubulin) was prepared from it by phosphocellulose chromatography, as described (10). Protein determination was as described (11). $NaDodSO_4$/PAGE, using 9 or 10% polyacrylamide, and densitometric scanning of stained slab gels (12), as well as molecular weight standards (13), were also as previously described. For electron microscopy, 5 μl of sample (1–2 mg of protein per ml, or a 1:10 dilution) was applied to a 400-mesh carbon-over-Formvar- or collodion-coated grid. After 30 sec, samples were negatively stained by rapid sequential addition of 10 drops of 1% uranyl acetate. Grids were examined at 100 kV in a Phillips EM 410 microscope.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: MAP, microtubule-associated protein (in this paper, of cytoplasmic origin); COP, microtubule-corset protein; PC-tubulin, tubulin purified by phosphocellulose chromatography.

RESULTS

***Crithidia* MAPs and COPs.** Cytoplasmic MAPs were isolated by the procedure introduced by Vallee (8), which is based on the stabilized or enhanced binding of MAPs that occurs when microtubule assembly is promoted by taxol (14) and the selective solubilization of MAPs by high-salt extraction (8). We followed the protocol of Scholey *et al.* (9). Typical protein recoveries from 3×10^{11} cells were 2300 mg of total cell protein, 480 mg in high-speed supernatant, 6 mg of taxol-stabilized polymer, and 1 mg of high-salt MAPs. A conspicuous 95-kDa polypeptide in the polymer fraction (Fig. 1, lane 1) was not extracted by salt (lane 2). As indicated in Fig. 1, the most conspicuous MAP polypeptides (doublets in some cases) were at 130, 90, 65, 40, 36, and 33 kDa. Some additional MAPs were obscured by overlying tubulin, and we also do not mean to imply that other minor components in the high molecular mass region are unimportant. As with brain MAPs, a subset of *Crithidia* MAPs were relatively heat-stable (Fig. 1, lane 3).

COPs were solubilized from the purified microtubule corset apparatus by the method of Russell *et al.* (5). Protein recoveries from 6×10^{10} cells were 470 mg of deflagellated cell bodies, 30 mg of corset apparatus, and 20 mg of dialyzed sonicate supernatant, which yielded 3 mg of glycerol-induced tubulin polymer and 15 mg of tubulin-depleted COPs. As illustrated in Fig. 2, there were four conspicuous COP polypeptides (doublets in some cases), at 61, 43, 41, and 33 kDa. Two of these were easily purified from this simple mixture, as shown in lanes 3 and 4. A protein similar to COP-61 has also been purified from *Trypanosoma brucei* (15).

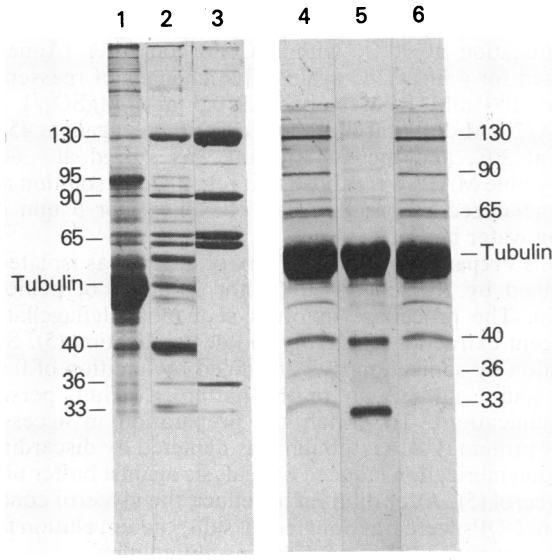

FIG. 1. NaDodSO$_4$/PAGE analysis of *Crithidia* MAPs, and their selective heat stabilities and abilities to copolymerize with PC-tubulin from brain. Major polypeptides are identified by their molecular mass in kilodaltons. A modified gel system gave somewhat different absolute mobilities in lanes 4–6. Lane 1: components of the polymer fraction obtained by incubating taxol with a high-speed supernatant fraction from *Crithidia*, as described in *Materials and Methods*. Lane 2: high-salt extract of the material in lane 1 ("taxol MAPs"); some MAPs are seen to have been obscured by the *Crithidia* tubulin in lane 1. Lane 3: MAPs that remained in the soluble fraction after heating (100°C, 5 min) the material in lane 2. For lane 4, brain PC-tubulin (1.7 mg/ml) was incubated with taxol MAPs (0.28 mg/ml; after removal of taxol and dimethyl sulfoxide by dialysis) in a final volume of 0.1 ml of reassembly buffer plus 2.5 mM GTP, for 35 min at 30°C. Ten microliters of the whole incubation mixture was applied to lane 4. Polymer fraction (a typically transluscent microtubule pellet), isolated by using an air-driven centrifuge operated for 20 min at 30 pounds per square inch at 25°C, was applied to lane 5. Ten microliters of the supernatant was applied to lane 6. No pellet was obtained from a control mixture lacking taxol MAPs.

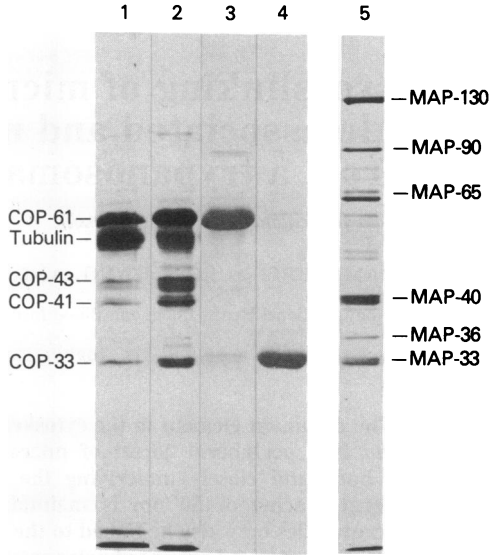

FIG. 2. NaDodSO$_4$/PAGE analysis of *Crithidia* COPs. Major polypeptides are identified by their molecular mass in kilodaltons, coupled to the appropriate acronym to indicate their origin. Lane 1 received 25 μg of the fraction solubilized from purified microtubule corset apparatus by sonication, after dialysis to remove salt. To enrich the associated proteins, pure tubulin was polymerized by dialysis against 8 M glycerol (5) and discarded; 25 μg of supernatant protein was applied to lane 2. After dilution to reduce the glycerol concentration, this mixture was fractionated on a cation-exchange (Mono S, Pharmacia) column. Fractions containing nearly pure COP-61 and COP-33 are shown in lanes 3 and 4. Lane 5 shows another preparation of cytoplasmic taxol MAPs (also shown in lane 2 of Fig. 1) run in the same slab gel for comparison.

COPs and MAPs are distinct sets of polypeptides (Fig. 2) with one possible exception, the 33-kDa component of each. Native COP-33 was eluted from a calibrated molecular sieve column (Superose 12, Pharmacia) with an apparent molecular mass of 65.8 kDa. It seems rather unlikely that asymmetry of a monomer would yield this precise value, and we tentatively conclude that native COP-33 is a homodimer.

Periodic Crosslinks Between Microtubules Polymerized from *Crithidia* MAPs and Brain Tubulin. Our initial experiments were carried out with buffer suitable for microtubule assembly from extracts of vertebrate brain. We used pure brain tubulin at concentrations where it does not assemble by itself, and *Crithidia* MAPs (the fraction shown in lane 2, Fig. 1) in proportion by weight to tubulin (1:6) similar to the proportion of brain MAPs found in twice-cycled brain microtubule protein. Fig. 1 shows the compositions of the mixture (lane 4) and of the resultant polymer (lane 5) and nonpolymerizing (lane 6) fractions. *Crithidia* MAPs were less effective than brain MAPs in promoting polymerization, bringing down only 5% instead of 50% of the tubulin under these conditions (residual taxol not removed by dialysis could also have played a role in this limited polymerization). MAP-33 and, to a lesser extent, MAP-40 were concentrated in the polymer fraction (Fig. 1, lane 5 vs. lane 6). It is of course possible that other MAPs, which appear enriched in the nonpolymerizing supernatant, also play a minor role in polymerization or crosslink formation (see below). Densitometric scans tentatively indicated that the following proportion of each MAP was in the polymer fraction: MAP-33, 48%; MAP-36, <11%; MAP-40, 14%; MAP-65, 4.5%; MAP-90 and -130, <3% each.

Electron microscopy of the polymer fraction revealed infrequent, normal-appearing microtubules, often aligned in

pairs in close and constant apposition throughout their entire lengths (Fig. 3*A*). At higher magnification (Fig. 3*B*), these could be seen to be joined by periodic crosslinks with a center-to-center spacing of about 8.5 nm. We measured the space between crosslinked microtubules at 7–11 nm (and the center-to-center spacing between tubulin subunits at 4.3 nm). Infrequently, in the same preparations, we also observed short series of periodic crosslinks with center-to-center spacing of about 17 nm (Fig. 3*C*).

Since *Crithidia* MAPs were only moderately effective in polymerizing brain tubulin, we carried out similar experiments in which MAPs were incubated with microtubules preassembled and stabilized with taxol. Electron microscopy revealed profuse arrays of tightly bundled microtubules with many clear sequences of crosslinks spaced at 8.5 nm.

Crosslinking of Microtubules by *Crithidia* COPs. Since we had isolated two of the COPs in nearly pure form, we also did preliminary experiments to determine how these would interact with brain tubulin and to address the question of whether COP-33 was the same protein as MAP-33, which appears to be a good candidate for a principal component (see Fig. 1, lane 5) of the crossbridges described above. Neither COP-33 nor COP-61 was observed to induce polymerization of pure brain tubulin.

However, both of the COPs apparently bound to, and sedimented with, brain microtubules preassembled with taxol (Fig. 4). The polymer with COP-33 consisted of loose bundles, suggestive of more intermittent crosslinking, as illustrated in Fig. 5*A*. Microtubules incubated without COPs were not bundled (Fig. 5*C*); they were also unstable, when these concentrations and manipulations were used, and were seen to be fracturing and depolymerizing. When COP-33 was present during taxol-induced polymerization, the microtubules were tightly bundled (data not shown) with frequent arrays of periodic crosslinks. Though less well-resolved than the crosslinks shown in Fig. 3 (conceivably because taxol

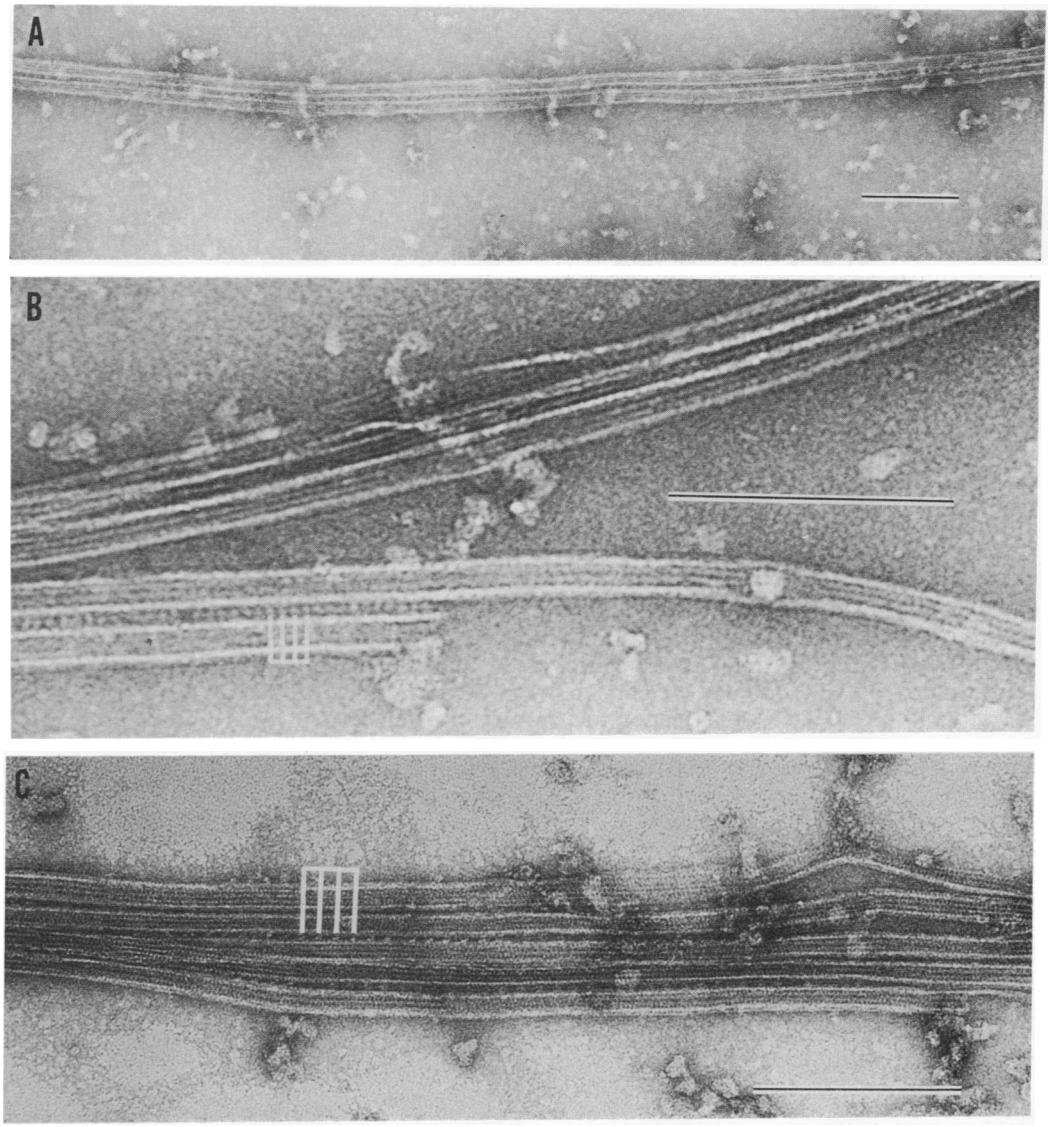

FIG. 3. Periodic crosslinks between microtubules polymerized *in vitro* from brain PC-tubulin and *Crithidia* cytoplasmic taxol MAPs (see Fig. 1 legend). (*A*) PC-tubulin (1.9 mg/ml) was incubated for 30 min at 37°C with taxol MAPs (0.36 mg/ml; freed of taxol by dialysis) in reassembly buffer containing 2.5 mM GTP, and an aliquot was applied directly to a grid. Microtubules were infrequent and were about equally divided between single microtubules and pairs in close and constant apposition through their entire length. (*B*) Polymer was first harvested in an air-driven centrifuge and resuspended in reassembly buffer with GTP. Periodic crosslinks (indicated by the comb) were clearly seen at this higher magnification. Center-to-center spacing between crosslinks is about 8.5 nm. (*C*) Resuspended polymer was diluted with 10 volumes of reassembly buffer plus GTP and promptly applied to a grid. The center-to-center spacing between crosslinks in this small microtubule bundle is about 17 nm. (In *A–C*, bar = 200 nm.)

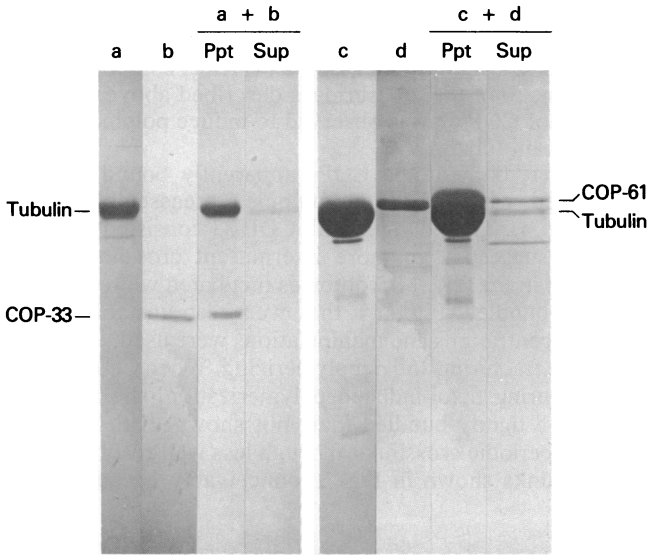

FIG. 4. Quantitative binding of two purified COPs to taxol-stabilized microtubules. The two purified COPs shown in Fig. 2 were desalted by repeated Centricon filtration. Taxol-polymerized microtubules were prepared by incubating PC-tubulin for 30 min at 37°C in reassembly buffer plus 20 μM taxol and 2.5 mM GTP. Polymer was centrifuged down and resuspended in the same buffer. Microtubules (0.18 mg/ml) were incubated for 30 min at 37°C in the same buffer with COP-33 at 0.05 mg/ml, and, after centrifugation, aliquots of precipitates (Ppt) and supernatant (Sup) were added to lanes a + b as indicated. Equivalent single additions of microtubules or COP-33 were added to lanes a and b, respectively. The procedure using COP-61 (lanes c, d, and c + d) was the same, except that the microtubule concentration was 1.8 mg/ml, and COP-61 was 0.25 mg/ml.

interferes with visualization), spacing appeared to be the same as with the MAPs-induced polymer: the center-to-center distance between links was 8.5 nm, and the interval between adjacent microtubules was 7–9 nm. Thus, the available evidence suggests that COP-33 is the same protein as MAP-33, though we have not yet determined whether the latter is also a dimer in solution.

Polymers formed when taxol-preassembled microtubules were incubated with COP-61 have also been seen as loose bundles (Fig. 5*D*). However, we have not seen periodic crosslinks, and, with highly purified COP-61, bundling has not been consistently observed, suggesting that it may require the presence of traces of impurities.

Fig. 5*B* shows that the polymer formed from taxol and *Crithidia* corset tubulin is in the form of ribbons, as has been reported for the glycerol-induced polymer of tubulin from all three subcellular compartments (5). Since we used highly purified corset tubulin, this propensity is probably inherent in the structure of the tubulin itself.

DISCUSSION

We report here the beginning of an investigation of an unusual cytoskeleton, a subpellicular microtubule corset. Although corset-like arrays of limited extent or distribution are known in other cells, we have chosen a trypanosomatid because it exemplifies this type of cytoskeleton and, so far, has been found to lack other identifiable cytoskeletal elements. *Crithidia fasciculata* provide an easily cultivated nonpathogenic species. We hope to identify the components of this cytoskeleton and the way in which they are assembled and perhaps ultimately to reconstruct the apparatus *in vitro*.

We tentatively identified four major non-tubulin polypeptides (COPs) from purified corset complexes, designated (in relation to their apparent size in kDa) COP-61, -43, -41, and -33 (we should stress that the nature of the association of these with the corset is undefined). COP-61 and -33 were obtained nearly pure, and native COP-33 was tentatively shown to be dimeric. Because vertebrate brain tubulin is most completely studied, we first examined the *in vitro* interaction of COPs with microtubules from this source. COP-33 bound quantitatively and stabilized aggregated microtubules into tight parallel bundles. Similar bundling (reviewed in ref. 16) has been attributed to several proteins, sometimes with little potential relation to cellular microtubules, and has attracted relatively little interest because, in contrast to actin (17), bundles are not a characteristic form of cellular microtubules. The more interesting feature of bundling by COP-33 was that it yielded periodic crosslinks between adjacent microtubules (see below).

A second initial approach was to isolate cytoplasmic MAPs, which might be a pool destined for future corset (in the case of MAP-33) or mitotic spindle microtubules, or might be associated with other or as-yet-unrecognized non-corset interphase microtubules. The method of Vallee (8), using taxol to promote polymerization and MAP binding, proved satisfactory, even though with *Crithidia* the polymers were ribbons rather than microtubules. Although not all of the components have yet been characterized as authentic MAPs, the point we wish to make here is that the major MAP polypeptides were different from COPs with the possible exception of MAP-33, which was also the principal MAP that copolymerized with brain tubulin. The polymer consisted of tightly coupled pairs or small bundles, joined by periodic crosslinks with a center-to-center spacing of about 8.5 nm, corresponding roughly to one crosslink per tubulin dimer. A periodicity of 17 nm was also observed, albeit less frequently.

The same spacings were observed (although with less resolution) with COP-33, and it seems likely, though not proven, that MAP-33 and COP-33 are the same and constitute at least the major component of the crosslinks. Although this protein spans 7–11 nm between microtubules, in contrast to the 25-nm crosslinks seen in the corset *in vivo*, it seems a likely candidate as a key element in the corset apparatus.

While this work was in progress, a report appeared (16) that described taxol-polymerized microtubules from a nematode, with similar periodic crosslinks. The principal non-tubulin polypeptide in the nematode polymer had an apparent molecular mass of 32 kDa. The nematode results differ (16, 18) principally in that the native crosslinking protein appears to be monomeric (using the same criterion by which we find the molecular mass of native COP-33 to be 66 kDa) and the dimensions of the crosslinks do correspond to some seen (in mechanosensory neurons) in the living animal. If the protozoan and nematode protein prove to be the same, the evolutionary span would suggest a widespread role for similar proteins in relation to invertebrate microtubules.

We dedicate this paper to the memory of our colleague Dr. Adavi S. N. Murthy.

1. Vickerman, K. & Preston, T. M. (1976) in *Biology of the Kinetoplastida*, eds. Lumsden, W. H. R. & Evans, D. A. (Academic, New York), Vol. 1, pp. 35–130.
2. McGhee, R. B. & Cosgrove, W. B. (1980) *Microbiol. Rev.* **44**, 140–173.
3. Vickerman, K. (1986) *Nature (London)* **322**, 113–114.
4. Paulin, J. J. & McGhee, B. (1971) *J. Parasitol.* **57**, 1279–1287.
5. Russell, D. G., Miller, D. & Gull, K. (1984) *Mol. Cell Biol.* **4**, 779–790.
6. Chang, S., Bramblett, G. T. & Flavin, M. (1986) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **45**, 1676 (abstr.).
7. Evans, D. A. (1978) in *Methods of Cultivating Parasites in Vitro*, eds. Taylor, A. E. R. & Baker, J. R. (Academic, New York), pp. 62–63.

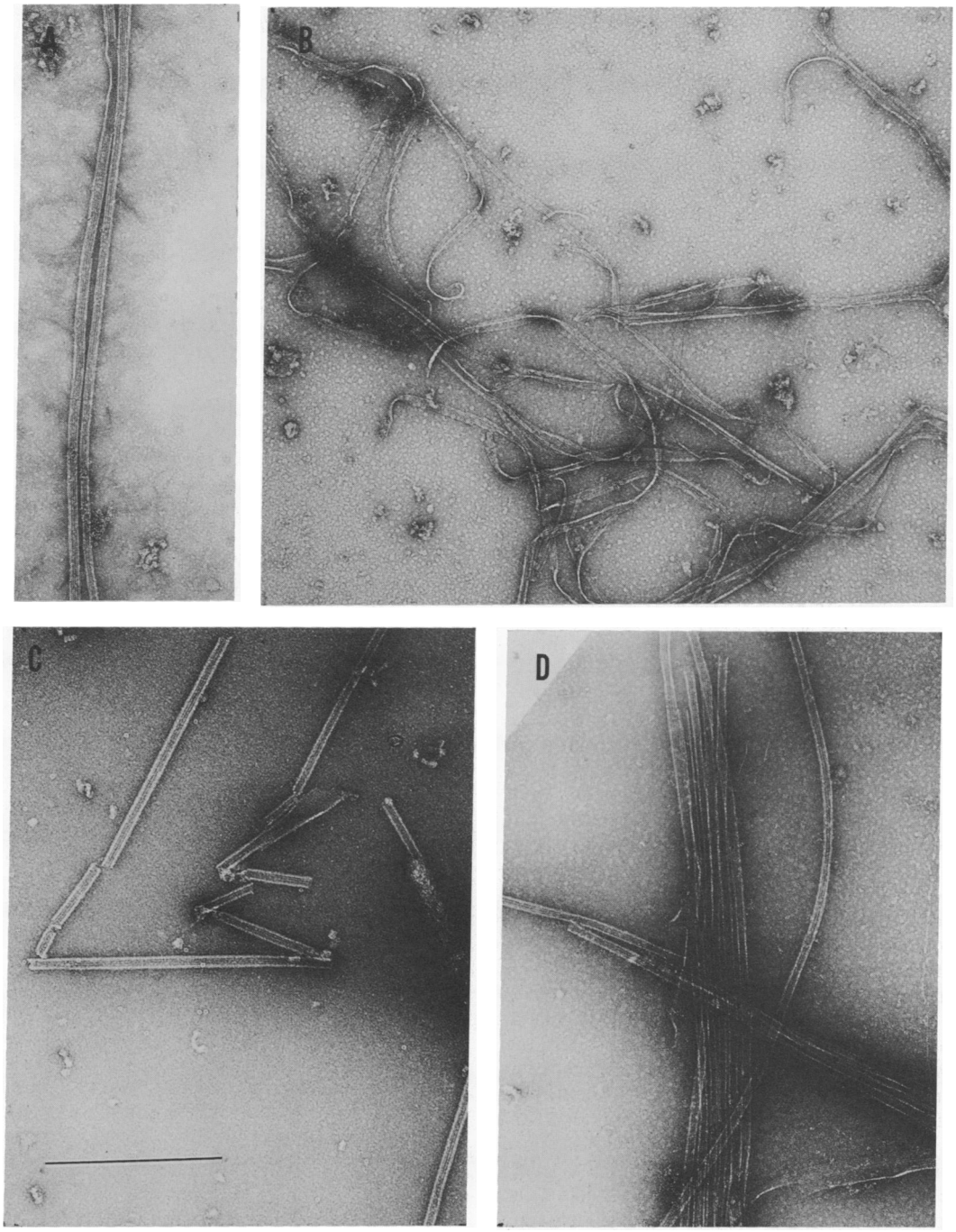

FIG. 5. Polymers formed in the presence of various tubulins and corset proteins. (*A*) Taxol-stabilized microtubules were prepared from bovine PC-tubulin and washed (as in the legend to Fig. 3), then incubated (200 μg/ml) for 30 min at 37°C in reassembly buffer plus taxol and GTP with COP-33 (50 μg/ml). As shown, microtubules were often seen in more loosely associated, or infrequently crosslinked, pairs. (*B*) Taxol-induced polymer from pure *Crithidia* corset tubulin. The corset tubulin that had been discarded from the sample that had been applied to lane 2 of Fig. 2 was carried through a second cycle of temperature- and glycerol-dependent assembly. After removal of glycerol by dialysis, this very pure tubulin was incubated with taxol and GTP in the usual way. The resultant polymer appears to consist entirely of ribbons. (*C*) Taxol microtubules were incubated as above without any COP fraction; at this dilution (200 μg/ml) microtubules are unstable and are seen to be fracturing and depolymerizing. (*D*) Taxol microtubules were incubated (as in *A*) with COP-61 (250 μg/ml). Microtubules were in somewhat loosely-associated bundles. [Magnification is the same in *A–D*; bar (in *C*) = 500 nm.]

8. Vallee, R. B. (1982) *J. Cell Biol.* **92,** 435–442.
9. Scholey, J. M., Neighbors, B., McIntosh, J. R. & Salmon, E. D. (1984) *J. Biol. Chem.* **259,** 6516–6525.
10. Kumar, N. & Flavin, M. (1982) *Eur. J. Biochem.* **128,** 215–222.
11. Murthy, A. S. N. & Flavin, M. (1983) *Eur. J. Biochem.* **137,** 37–46.
12. Murthy, A. S. N., Bramblett, G. T. & Flavin, M. (1985) *J. Biol. Chem.* **260,** 4364–4376.
13. Tsuyama, S., Bramblett, G. T. & Flavin, M. (1986) *J. Biol. Chem.* **261,** 4110–4116.
14. Albertini, D. G., Herman, B. & Sherline, P. (1984) *Eur. J. Cell Biol.* **33,** 134–143.
15. Stieger, J. & Seebeck, T. (1986) *Mol. Biochem. Parasitol.* **21,** 37–45.
16. Aamodt, E. J. & Culotti, J. G. (1986) *J. Cell Biol.* **103,** 22–31.
17. Pollard, T. & Cooper, J. A. (1986) *Annu. Rev. Biochem.* **55,** 987–1035.
18. Aamodt, E. & Culotti, J. (1986) *J. Cell Biol.* **103,** 402a (abstr.).