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Purification and characterization of sheep brain cold-stable microtubules

(tubulin/calmodulin/τ protein/cytoskeleton)

FABIENNE PIROLLET*, DIDIER JOB*, EDMOND H. FISCHER†, AND ROBERT L. MARGOLIS‡§

*Laboratoire de Biochimie Endocrinienne U244, Institut National de la Santé et de la Recherche Médicale U.S.M.G., BP 53X 38041 Grenoble, Cedex, France; †Department of Biochemistry, SJ-70, University of Washington, Seattle, Washington 98195; and ‡The Fred Hutchinson Cancer Research Center, Seattle, Washington 98104

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ABSTRACT **The isolation of cold-stable microtubules in high yields, described previously only from rodents, was extended to the brain of higher animals. Under optimal conditions, yields of 30 mg of cold-stable microtubules per 100 g of sheep brain could be obtained routinely. Material purified by two polymerization cycles displayed the same stability to cold temperature or to millimolar concentrations of calcium and the same lability to calmodulin and to ATP as did the purified material obtained from the rat [Job, D., Rauch, C. T., Fischer, E. H. & Margolis, R. L. (1982) *Biochemistry* 21, 509]. Furthermore, DE-52 chromatography of this material yielded a fraction that restored cold stability when added to cold-labile microtubules. Known to bind to calmodulin and to enhance microtubule assembly, τ proteins had no cold-stabilizing activity. Protein profiles of the cold-stabilizing fraction from sheep and rat brain were similar to one another but showed no protein bands corresponding to the τ proteins.**

Microtubules are fibrous elements found ubiquitously in eukaryotic cells, where they perform a wide variety of physiological functions. They are centrally involved in mitosis and in other motility-related phenomena such as the transport of subcellular organelles or changes in cell shape (for reviews, see refs. 1–3). To perform these tasks, they must be able to assemble at the proper time and place and with the proper orientation in response to specific physiological signals (1). Cytoplasmic microtubules are by and large highly labile, being in rapid equilibrium with their constituent tubulin subunits (4). Simultaneous aggregation and disaggregation at opposite ends can allow for the establishment of a steady state, during which subunits will flow from one end of the microtubule to the other (3, 5). This constitutes a treadmilling process that may be implicated in certain fundamental intracellular phenomena such as chromosome movement during anaphase (5, 6). Because lability allows for constant flux, it is likely that the equilibrium between the assembled and disassembled states may define the physiological function of microtubules. Indeed, microtubules locked in a nonequilibrium state by the drug taxol appear to lose their mitotic function (7).

The physiological mechanisms regulating microtubule assembly and disassembly are unclear at this time. There is evidence that pH (8), Ca^{2+} (9, 10), calmodulin (11–13), and the phosphorylation of microtubule-associated proteins (14–16) can affect to some extent microtubule behavior. We have been interested in a particular population of microtubules that has been designated as cold stable (13, 16–19). These microtubules, contrary to the more common cold-labile type, do not disassemble at 0°C or in the presence of millimolar concentrations of Ca^{2+} or when exposed to assembly-inhibiting drugs such as colchicine or podophyllotoxin. On the other hand, they do disassemble rapidly when exposed to ATP (16) or to substoichiometric amounts of calmodulin (13) or when subjected to shearing (19).

On the basis of these observations, we have proposed that disassembly at cold temperatures can be prevented by substoichiometric protein blocks randomly distributed along the polymer (19). A number of polypeptides appear to be involved; they seem to exist in the form of a large complex, but their individual contributions to microtubule cold stability have not been elucidated as yet.

In the past, all the work on cold-stable microtubules has been carried out on rat or mouse brain, from which only minute amounts of cold-stable microtubules could be isolated. Clearly, for any detailed biochemical characterization of the system, one would have to rely on a more abundant source of starting material. However, all attempts to reproduce in larger animals what had been done with rodents met with little success, in spite of the fact that stability to cold appeared to be a general phenomenon.

This manuscript describes a new procedure for the isolation from sheep brain of cold-stable microtubules having properties similar to those obtained earlier from the rat. From these cold-stable preparations, a group of polypeptides could be isolated displaying the same cold-stabilizing properties and size distribution as the proteins described earlier from rodents (19). Although their cold-stabilizing activity is greatly affected by calmodulin, the data presented here indicate that they are different from the τ proteins (20), a family of microtubule-associated proteins shown by Sobue *et al.* (21) also to react strongly with calmodulin.

MATERIALS AND METHODS

Preparation of Crude Extract. Sheep brains (about 100 g per brain) were packed in ice immediately after slaughter and processed within 30 min. All procedures were performed at 0–4°C except where indicated. Brain tissue was homogenized for 10 sec in a Waring blender set at the lowest speed in buffer designated MEM buffer (100 mM 4-morpholineethanesulfonic acid, pH 6.75/1 mM EGTA/1 mM $MgCl_2$) and 1.5 mM $CaCl_2$. Although the proportions of buffer to brain material were varied as indicated under *Results*, a ratio of 1.4:1 gave optimal yields of cold-stable microtubules. The homogenate was allowed to settle for 1 min, EGTA was added to a final concentration of 3.0 mM, and the mixture was blended for an additional 5 sec.

Assembly and Isolation of Microtubules. The extract was centrifuged at 150,000 × *g* for 30 min, and the supernatant was carefully collected. Microtubule assembly was initiated at 30°C. For all assembly measurements, turbidity was monitored at 350

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Abbreviation: MEM buffer, 100 nM 4-morpholineethanesulfonic acid, pH 6.75/1 mM EGTA/1 mM $MgCl_2$.

§To whom reprint requests should be addressed.

nm with a temperature-controlled UVICON 810 spectrophotometer. After 100–120 min, the extract was chilled to 0°C for 30 min and centrifuged at 200,000 × *g* for 90 min through a 50% (wt/vol) sucrose cushion in MEM buffer. After suspending the pellets in the same buffer to a final concentration of *ca.* 10 mg of protein per ml, the suspension was centrifuged at 5,000 × *g* for 5 min at 0°C. The supernatant was sheared at 0°C by repeated passage through a syringe fitted with a 26-gauge needle and recentrifuged at 50,000 × *g* for 20 min. The supernatant was collected for further use.

Other Isolation Procedures. Cold-stable microtubules from rat brain were isolated as described (13, 19); τ polypeptides were prepared from purified bovine brain microtubules as described by Cleveland *et al.* (20). A protein fraction showing cold-stabilizing activity was isolated from purified cold-stable microtubules by methods to be detailed elsewhere (unpublished data). In brief, sheared cold-stable microtubule supernatants were made 1.0 mM in free $CaCl_2$ and passed through a DE-52 cellulose column. The flow-through fraction containing the activity was used for subsequent experiments and gel electrophoresis analysis.

RESULTS

Isolation of Cold-Stable Microtubules. Analysis of microtubule assembly in crude-extract supernatant solutions from sheep brain revealed that addition of guanine nucleotides was not required for optimal polymerization (Fig. 1), as established for the rat system (16). However, addition of either GDP (Fig. 1) or GTP (data not shown) did alter the kinetics of assembly, always delaying initiation. At increasing ratios of buffer to tissue during homogenization, the initiation lag became more and more pronounced, even though final yields in assembled material were the same.

During the assembly reaction, aliquots were removed at 5-min intervals and chilled to 0°C to determine the quantity of cold-stable microtubules present. The data clearly show that at different buffer-to-brain-tissue ratios, very different yields of cold-stable material were obtained. At a 0.5:1 ratio, cold-stability was at most 10% after 2 hr (Fig. 1*A*), whereas it increased to 50–55% at a 1.4:1 ratio (Fig. 1*B*). However, the ratio of cold stability to total polymer did not vary appreciably with time during assembly for any condition.

The differences in stability to cold observed at different buffer-to-tissue ratios may be related to the presence of salts in the brain extract. The conductivity of the most concentrated solution was 11 mS and of the most dilute extract was 8.5 mS. Likewise, increasing the conductivity of a purified solution of cold-stable microtubules to a value greater than 10 mS by addition of NaCl substantially suppressed cold stability (data not shown). At an optimal buffer-to-tissue ratio of 1.4:1, ≈30 mg of purified cold-stable microtubules could be obtained per 100 g of brain.

Recycling of Cold-Stable Microtubules. To obtain cold-stable microtubules, the crude-extract supernatant solution was assembled at a buffer-to-tissue ratio of 1.4:1, optimal for cold stability. After 1 hr, the turbid solution was brought to 0°C for 10 min so that only cold-stable polymers would remain. The chilled solution was layered onto a 50% sucrose cushion and centrifuged at 30°C; cold-stable microtubules were recovered in the pellet. For further purification, the pellet was suspended in 0°C buffer, sheared, centrifuged in MEM buffer, and then carried through a second assembly cycle as just described.

Cold-labile microtubules were prepared by allowing tubulin to assemble in crude-extract supernatant solutions at a 0.5:1.0 buffer-to-tissue ratio. The assembled material was centrifuged through a 50% sucrose cushion at 30°C.

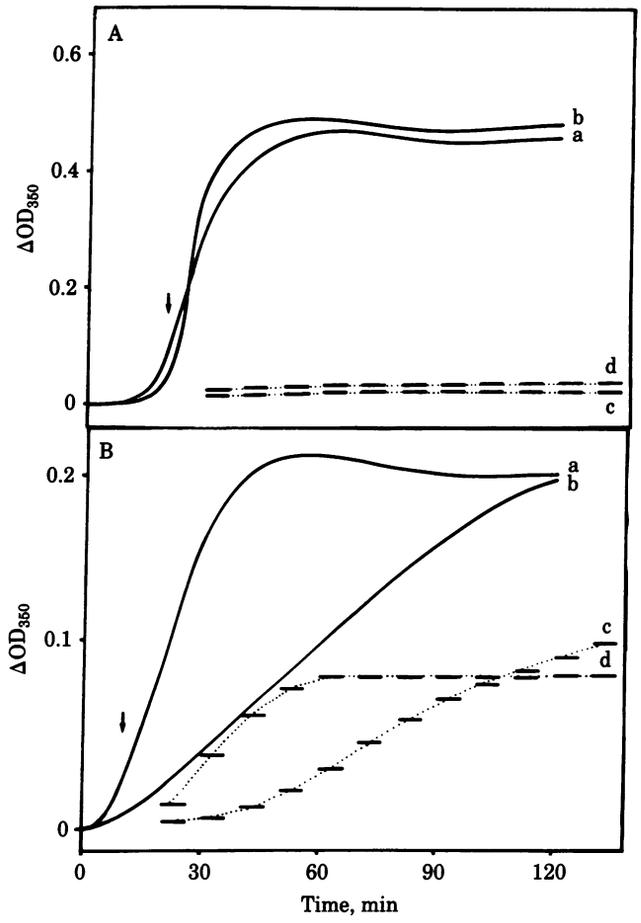

FIG. 1. Effect of buffer-to-tissue ratio on sheep brain crude-extract microtubule assembly and cold stability. Supernatant fractions from high-speed centrifugation were warmed to 30°C and monitored for microtubule assembly by changes in turbidity. Aliquots were removed at 10-min intervals during assembly and chilled to 0°C. The turbidity was measured to determine the residual levels of cold-stable microtubules. Assembly was carried out at a buffer-to-brain tissue ratio (vol/wt) of 0.5:1 (*A*) and 1.4:1 (*B*). Curves: a, assembly with no nucleotide added; b, assembly with 1.0 mM GDP; c and d, levels of cold-stable material remaining when aliquots from samples a and b were brought to 0°C, respectively.

A comparison of the assembly properties and stability of the two types of purified microtubules is shown in Fig. 2*A*. Differences observed were reproducible and persisted after additional assembly/disassembly cycles. As with the rat system, cold-stable microtubules never exceeded 50–60% of the material present. In all cases, there was a characteristic difference in the initiation of assembly, which occurred always more rapidly with the cold-stable population.

Further tests were made to determine whether the sheep cold-stable microtubules behaved similarly to the rat brain material in the presence of destabilizing agents. Free Ca^{2+}, up to a concentration of 1 mM, was without effect at 30°C but caused rapid disassembly in the presence of 1.0 μM calmodulin. Disassembly also was observed with 0.5 mM ATP and occurred almost immediately either in the presence of 10 mM free Ca^{2+} or in 1.0 mM free Ca^{2+} at 0°C. Similar responses have been observed with rat brain cold-stable microtubules (13, 16, 19, unpublished data).

Use of a Protein Fraction to Induce Cold Stability. After disassembly of cold-stable microtubules, a fraction capable of conferring cold stability to microtubules could be obtained by

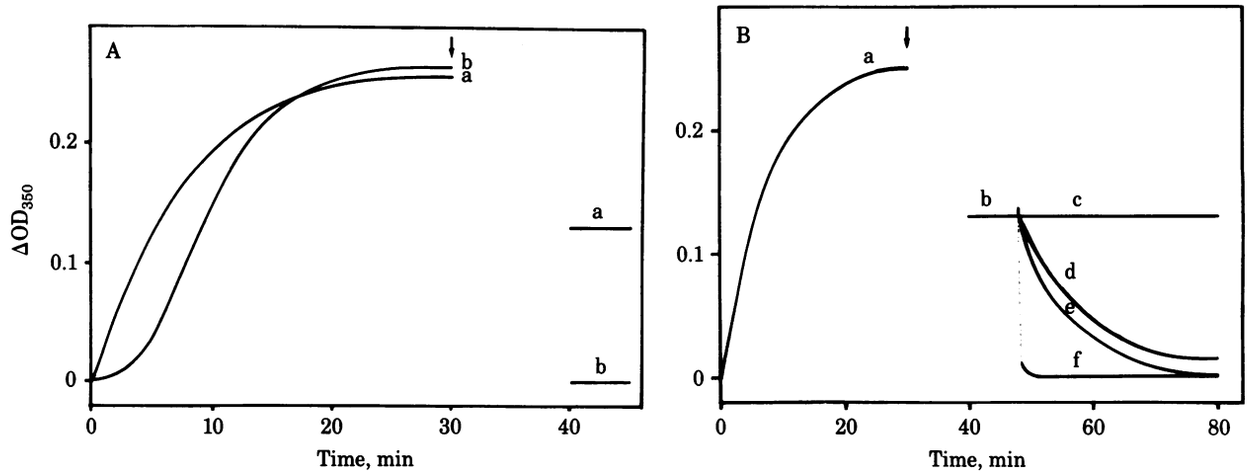

FIG. 2. Properties of purified cold-stable microtubules. Sheep brain microtubules (purified by three assembly cycles; 2.5 mg/ml) were assembled in MEM buffer containing 0.5 mM GTP at 30°C. (*A*) Behavior of cold-stable microtubules (curve a) is compared to that of similarly prepared cold-labile material (curve b). At the time indicated by the arrow, aliquots from each sample were removed and chilled at 0°C for 10 min. The resulting level of cold stability is shown. (*B*) After cold-stable microtubules were assembled as above (curve a), 25 μM podophyllotoxin was added at the time indicated by the arrow, and the sample was brought to 0°C for 10 min. Curve b represents the remaining level of cold-stable material measured after rewarming the solution to 30°C. To that sample, the following additions were made: 1 mM free Ca^{2+} (curve c), 1 mM free Ca^{2+}/1 μM beef brain calmodulin (curve d), 0.5 mM ATP (curve e), and either 10 mM free Ca^{2+} at 30°C or 1.0 mM free Ca^{2+} at 0°C (curve f).

passage of the disassembled protein through a DE-52 cellulose ion-exchange column (see legend of Fig. 3 for details). The active material emerged from the column in the flow-through fraction, while the bulk of the tubulin and microtubule-associated protein was retained, as reported for the mouse brain system (18). Readdition of this fraction to cold-labile microtubules (isolated as described in the legend of Fig. 1*A*) produced substantial cold stability even when added at a low ratio (0.075:1.0) to cold-labile microtubule protein (Fig. 3*A*). Curiously, the DE-52 eluate fraction diminished the extent of polymerization relative to the control in the first assembly cycle but greatly augmented the extent of assembly in the second cycle. The reason for this effect is not known; it was not seen with rat brain microtubules similarly assayed (unpublished data).

Because cold-stable microtubule-associated proteins present in the DE-52 flow-through fraction migrated on gels in the same molecular weight range as the τ proteins (refs. 19 and 20; see also Fig. 4), purified τ proteins were assayed to determine whether they could confer cold stability. Though the τ proteins isolated by the method of Cleveland *et al.* (20) did enhance the assembly of cold-labile microtubule protein, they did not induce any detectable cold stability in either of two assembly cycles, even when added at a high ratio (0.25:1.0) to total microtubule protein. Neither τ1 nor τ2 protein displayed any demonstrable activity.

Comparison of Proteins on $NaDodSO_4$/Polyacrylamide Gels. Sheep-derived proteins containing cold-stabilizing activity migrated on $NaDodSO_4$ gel chromatography with a pattern iden-

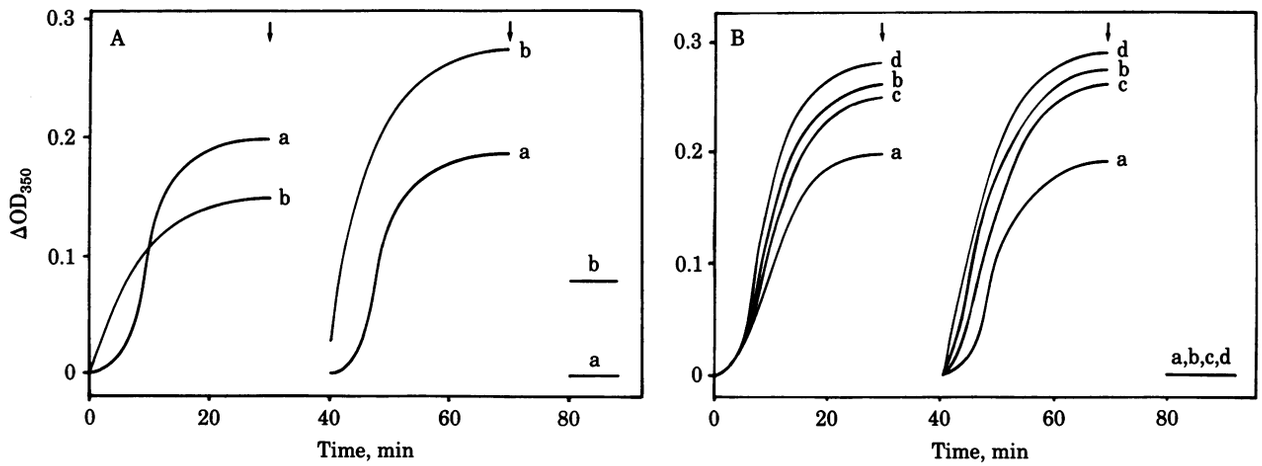

FIG. 3. Effects of a purified cold-stabilizing fraction and of τ proteins on microtubule assembly and cold stability. (*A*) Reconstitution of cold stability with the flow-through eluate from the DE-52 column. Twice recycled cold-stable microtubule protein (8 mg) was disassembled and then passed through a 3-ml DE-52 cellulose column at 4°C in MEM buffer containing 2 mM $CaCl_2$. Curves: a, control with three-cycle-purified cold-labile beef brain microtubule protein (1 mg/ml) alone; b, in the presence of 75 μg of protein from the DE-52 column flow-through fraction. At the time indicated by the arrows, the samples were chilled to 0°C for 10 min; additionally, at the time indicated by the second arrow, 25 μM podophyllotoxin was added to prevent further assembly and to reveal the cold-stable level. (*B*) τ1 and τ2 proteins isolated from beef brain microtubules were added to three-cycle-purified cold-labile beef brain microtubule protein (1 mg/ml). Assembly was carried out as described in *A*, with no additions (curve a), 250 μg of τ1 protein (curve b), 250 μg of τ2 protein (curve c), and 125 μg each of both τ1 and τ2 proteins (curve d). The samples were chilled to 0°C for 10 min (first arrow), and 25 μM podophyllotoxin was added to prevent further reassembly (second arrow).

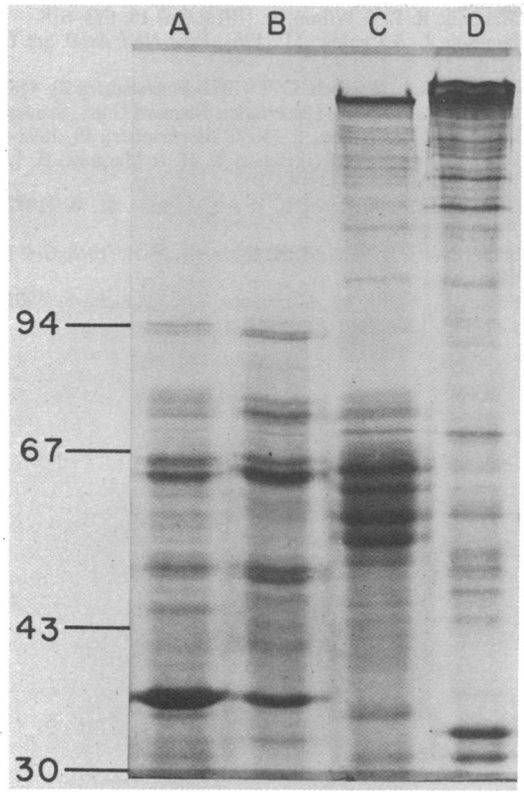

FIG. 4. NaDodSO$_4$ gel electrophoresis (21) of cold-stabilizing factors isolated from both sheep (lane *A*) and rat (lane *B*) brain cold-stable microtubules by DE-52 cellulose column chromatography as described in the legend of Fig. 3. These are compared to τ1 (lane C) and τ2 (lane D) proteins prepared from beef brain microtubules. Proteins (50 μg) were loaded in each lane, and the gels were stained with Coomassie blue R. Molecular weight standards are shown $\times 10^{-3}$.

tical to the cold-stabilizing microtubule proteins isolated by the same procedures from rat brain. In both instances, no major band corresponding to the τ proteins could be observed, consonant with the above results, which demonstrated that τ proteins lacked cold-stabilizing activity.

DISCUSSION

The purpose of this investigation was three-fold: (*i*) to extend previous studies that had been carried out only in rat or mouse brain to the brain of larger animals so that sizeable amounts of cold-stable microtubules could be obtained; (*ii*) to demonstrate that observations that had been made only in rodents could be reproduced in other species—that is, that cold stability was widespread and, therefore, potentially of general significance; and (*iii*) to see whether some of the observed effects could be attributed to the τ proteins because Sobue *et al.* (22) have shown that these proteins react strongly with calmodulin.

Cold-stable microtubules could be isolated in high yields from sheep brain. Many of their properties are similar to those described previously for microtubules from rat brain: both are stable to millimolar concentrations of Ca^{2+} and to assembly-inhibiting drugs but rapidly disassemble when exposed to micromolar concentrations of Ca^{2+}/calmodulin or to ATP. Conditions necessary to duplicate in higher animals what had been done with rodents are now clear: (*i*) brains must be processed rapidly after sacrificing the animals; (*ii*) the system is sensitive to ionic strength; and (*iii*) microtubule assembly must be allowed to proceed spontaneously without the addition of glycerol or dimethyl sulfoxide. (Although these reagents greatly increase the viscosity of the protein solutions, they inhibit the formation of cold-stable microtubules.)

In a purified system, there must be a proper ratio of tubulin to the microtubule-associated proteins that are responsible for cold stability. This point deserves further elaboration. Webb and Wilson (18) have proposed that cold stability depends on the addition of a factor that would stabilize a constant amount of tubulin, independent of its total concentration, once saturation had been reached. By contrast, we have proposed a model based on the presence of substoichiometric amounts of the associated proteins, the main factor being the mean number of blocks per microtubule (19). This model predicts that addition of tubulin to partially cold-stable preparations would lead eventually to the disappearance of cold stability. It is easy to demonstrate that this actually happens both in crude extracts and in purified preparations. Thus, addition of purified tubulin to a crude extract substantially decreases the yield of cold-stable material obtained. Because ionic strength plays an important role in determining the number and length of microtubules formed during assembly, it thus affects the mean number of blocks per microtubule and, hence, the overall cold stability.

The profiles of the proteins involved in cold stability from rat and sheep brain are qualitatively similar, although a protein of 35,000 molecular weight seems to be more abundant in the sheep fraction. A number of these proteins have been designated as STOPs (Stable-Tubule-Only Proteins) because they are found only in those purified microtubules that show cold stability (19). Although they appear to be incorporated into a single complex (unpublished data), it is not known which of these polypeptides is responsible for the observed activity or what other regulatory properties the entire complex might have.

Although the molecular weight of the polypeptides isolated from DE-52 chromatography is in the range of that of the τ proteins (*ca.* 60,000) it is evident that the two protein groups are distinct. Indeed, they do not comigrate on gels nor do they have overlapping functions: purified τ proteins cannot generate cold stability, and we show elsewhere that, unlike the τ proteins, material in the DE-52 column flow-through cannot induce assembly of purified 6S tubulin (unpublished data).

Assembly and disassembly of microtubules are, of course, highly regulated phenomena, and it is quite probable that the extensive studies that have been carried out *in vitro* cannot adequately express the complexity of the physiological system because of the absence of certain regulatory components. Nonetheless, the mechanism by which microtubules may control various cellular processes in response to intracellular and extracellular signals is becoming increasingly clear. Regulation of microtubule behavior by Ca^{2+}/calmodulin appears to occur both through direct interaction and by promoting a calmodulin-dependent phosphorylation (unpublished data). As these may represent distinct functions, a dual role for calmodulin can be envisaged in the attachment of microtubules to cellular elements and in the translocation and disassembly (3) of cold-stable microtubules as they approach the poles of the mitotic apparatus.

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