

Effect of Tau on the Vinblastine-induced Aggregation of Tubulin

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ABSTRACT Two microtubule-associated proteins, tau and the high molecular weight microtubule-associated protein 2 (MAP 2), were purified from rat brain microtubules. Addition of either protein to pure tubulin caused microtubule assembly. In the presence of tau and 10 μM vinblastine, tubulin aggregated into spiral structures. If tau was absent, or replaced by MAP 2, little aggregation occurred in the presence of vinblastine. Thus, vinblastine may be a useful probe in elucidating the individual roles of tau and MAP 2 in microtubule assembly.

Neuronal microtubules prepared by cycles of assembly and disassembly co-purify with two classes of microtubule-associated proteins: tau and the high molecular weight microtubule-associated proteins (MAPs) (9, 15). On polyacrylamide gel electrophoresis, tau migrates as a tetrad of polypeptides with molecular weights between 55,000 and 62,000, whereas the MAPs consist of two large proteins: MAP 1 (350,000 mol wt) and MAP 2 (300,000 mol wt) (3, 9). Tau and MAP 2 exhibit considerable structural differences in their amino acid compositions and sequences and also in their antigenic properties (3). Although tau and MAP 2 can independently catalyze tubulin polymerization into microtubules *in vitro*, no major differences in their mechanisms of action have yet been reported (16).

The antitumor drug vinblastine causes tubulin to polymerize *in vivo* and *in vitro* into spirals and paracrystals whose basic structural component resembles a microtubule protofilament (2, 12, 14). Recent work with a vinblastine analogue, vincristine, and an unresolved preparation of microtubule-associated proteins indicates that aggregation of tubulin into spirals requires these associated proteins (5). We show here that the two classes of microtubule-associated protein differ greatly in the nature of their effects on tubulin when vinblastine is present. Our results suggest that tau mediates large-scale aggregation of tubulin in the presence of vinblastine and that MAP 2 has little or no role in this process.

MATERIALS AND METHODS

Microtubule protein was isolated from the brains of adult male Sprague-Dawley rats by the method of Fellous et al. (7). Tubulin was purified from microtubule protein by chromatography on phosphocellulose (Whatman P11; Whatman, Inc.,

Clifton, N. J.) (13). Thermostable microtubule-associated proteins, consisting of tau and MAP 2, were prepared from microtubule protein, as described previously (7), and were resolved into tau and MAP 2 by gel filtration on Ultrogel ACA 34 (LKB Instruments, Inc., Rockville, Md.) (13). The purity of each protein was determined by acrylamide gel electrophoresis (10). The MAP 2 fraction was found to be free of tau, and the tau preparation contained all four of the reported bands (3) and a 1–2% contamination with MAP 2 (Fig. 1). For light-scattering experiments, tau, MAP 2, tubulin, and vinblastine were mixed in various combinations in cuvettes, and incubated at 37°C in a Carl Zeiss PM 6KS spectrophotometer (Carl Zeiss, Inc., New York) with an automatic thermostated four-sample changer. The turbidity was determined at 345 nm. All proteins were dissolved in a buffer consisting of 100 mM 2(N-morpholino)ethane sulfonic acid, pH 6.4, 1 mM ethyleneglycol-bis(β -aminoethyl ether)N,N'-tetraacetic acid, 0.1 mM ethylenediamine tetraacetic acid, 0.5 mM MgCl_2 , 1 mM guanosine-5'-triphosphate, and 1 mM β -mercaptoethanol. Protein concentrations were determined by the method of Lowry et al. (11). Samples for electron microscopy were placed on Formvar-coated, carbon-stabilized 400-mesh copper grids and negatively stained with 1% aqueous uranyl acetate before viewing with a Philips 301 electron microscope at an operating voltage of 60 kV.

RESULTS

When 9.09 μM tubulin and 0.70 μM MAP 2 were incubated together, normal microtubule assembly occurred, as indicated by a gradual increase in turbidity (Fig. 2A) and by the appearance of microtubules as seen by electron microscopy. If 10 μM vinblastine was then added to these microtubules, the turbidity declined within 5 min to a plateau value $\sim 25\%$ lower than the turbidity of the untreated microtubule sample. The plateau value remained unchanged during 1 h of observation. When vinblastine was added to a mixture of tubulin and MAP 2 before incubation, a small increase in turbidity was sometimes observed upon heating to 37°C; this increase was about one-third as great as that generated under the same conditions in

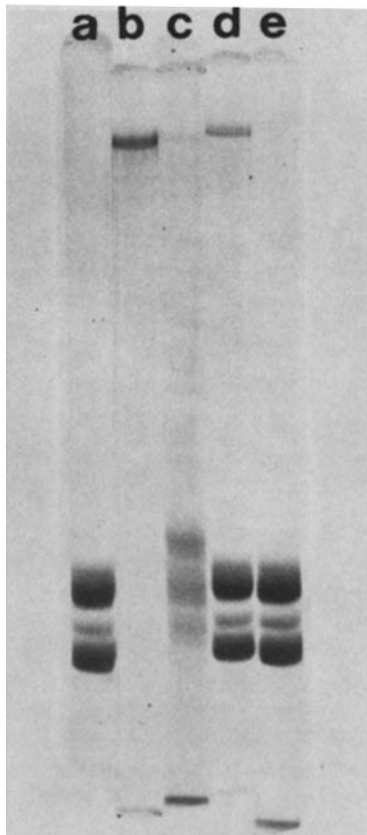


FIGURE 1 Electrophoretic analysis of tubulin and microtubule-associated proteins from rat brain. Samples of tubulin and microtubule-associated proteins from rat brain were reduced and carboxymethylated with sodium iodoacetate (4) and subjected to electrophoresis on discontinuous 6% polyacrylamide gels in the presence of 0.1% sodium dodecyl sulfate (10). The gels were stained with fast green and photographed. The direction of electrophoresis is from top to bottom. The gel samples were as follows: a, tubulin purified on phosphocellulose; b, MAP 2; c, tau; d, tubulin (1.00 mg/ml) incubated for 30 min at 37°C with MAP 2 (0.20 mg/ml); e, tubulin (0.96 mg/ml) incubated for 30 min at 37°C with tau (0.11 mg/ml). The microtubules polymerized in the samples shown in gels d and e, as observed by turbidimetry and electron microscopy, whereas a sample identical to that shown in gel e generated spiral structures when 10 μ M vinblastine was added.

the absence of vinblastine (Fig. 2A). In many experiments, no increase in turbidity was observed when samples of tubulin containing MAP 2 were incubated at 37°C in the presence of 10 μ M vinblastine. These results demonstrate that vinblastine inhibits MAP 2-catalyzed microtubule assembly and, in addition, induces partial disassembly of these microtubules.

Tau's effects were very different from those of MAP 2. Although 1.06 μ M tau induced rapid polymerization of 9.09 μ M tubulin (Fig. 2B) into normal looking microtubules (Fig. 3A), the addition of 10 μ M vinblastine to these samples caused the turbidity to decline by 50% within 1 min and then to increase rapidly after 2 min, until, at 10 min after addition of the drug, the turbidity reached a value three times as great as that of the untreated microtubule sample. Similarly, when vinblastine was added to a sample of tau and tubulin before incubation, a very large increase in turbidity occurred upon heating to 37°C (Fig. 2B). The increase in turbidity due to vinblastine was much larger than that observed when microtubules were formed in the same system in the absence of

vinblastine. The rate of increase in turbidity after the addition of vinblastine was approximately the same whether the drug was added before or after microtubule assembly. When the drug-treated samples were examined by electron microscopy, the only organized structures visible were spirals (Fig. 3B), circles, and paracrystalline assays, whose common structural element was a filament 40–50 Å in diameter. In samples in which tau and tubulin had formed microtubules as seen by turbidimetry and electron microscopy, addition of vinblastine to such microtubule preparations caused the almost complete disappearance of microtubules and the appearance of large numbers of spirals. Samples lacking tau, that is, containing only tubulin or tubulin with vinblastine, showed no increase in turbidity and contained no organized structures when viewed in the electron microscope. In other experiments, we found that the rate of increase in turbidity was directly proportional to the concentrations of tau, tubulin, and vinblastine and that, at concentrations <5 μ M, vinblastine inhibited microtubule assembly without inducing tubulin aggregation.

DISCUSSION

Although both tau and MAP 2 could catalyze microtubule assembly, the properties of these two proteins, in the presence of vinblastine, differed from one another in two respects (Fig. 2). First, the turbidity generated by tubulin, MAP 2, and vinblastine was much less than that generated when tubulin and MAP 2 formed microtubules. In contrast, tubulin and tau generated a turbidity in the presence of vinblastine that was much greater than that seen when they formed microtubules. Second, addition of vinblastine to MAP 2-catalyzed microtubules caused only disassembly, whereas addition of the drug to tau-catalyzed microtubules caused substantial aggregation. These observations indicate a much greater difference between

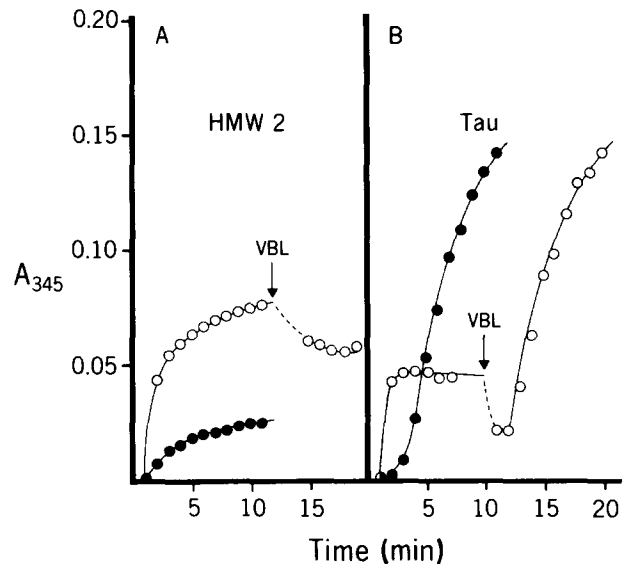


FIGURE 2 Effect of vinblastine on tubulin polymerization in the presence of MAP 2 (A) and tau (B). 500- μ l aliquots of tubulin (1.0 mg/ml) were incubated at 37°C with MAP 2 (0.212 mg/ml) (A) or tau (0.063 mg/ml) (B) in the presence (●) or absence (○) of 10 μ M vinblastine. In some experiments, microtubules were assembled in the absence of vinblastine and, at the time indicated by the arrow, vinblastine (VBL) was added, to a final concentration of 10 μ M. Development of turbidity was monitored at 345 nm.

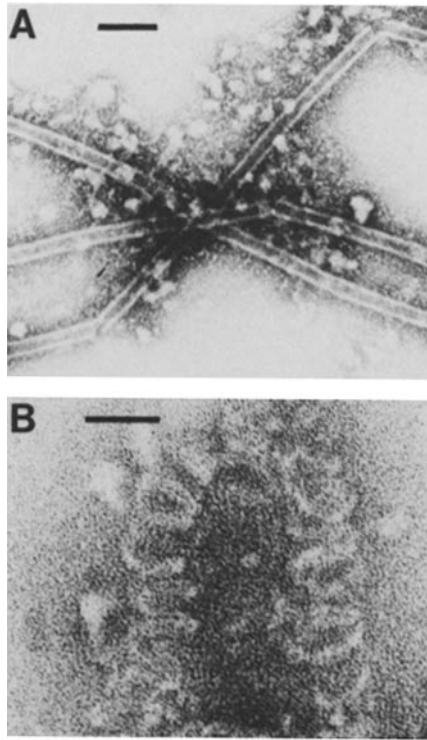


FIGURE 3 A, polymerization of tubulin in the presence of tau. Tubulin (0.89 mg/ml) was incubated at 37°C in the presence of tau (0.18 mg/ml). Microtubule assembly was monitored by turbidity at 350 nm. After microtubule assembly was complete, a drop of the sample was placed on a copper grid and stained as described in the text. Bar, 100 nm. $\times 84,000$. B, polymerization of tubulin in the presence of tau and vinblastine (11 μ M). Aggregation was monitored by turbidimetry at 350 nm. After aggregation was complete, a drop of sample was placed on a copper grid and stained as described. Bar, 50 nm. $\times 204,000$.

the functional characteristics of tau and MAP 2 than has been reported to date.

The difference between the MAPs was not just because, as in the experiment shown in Fig. 2, MAP 2 was present at a lower molar concentration than was tau. In similar experiments, we have found that concentrations of tau as low as 0.58 μ M induced substantial aggregation in the presence of 10 μ M vinblastine. In contrast, MAP 2-microtubules partially disassembled, with no further aggregation, in the presence of 10 μ M vinblastine, even when the MAP 2 concentration was 1.40 μ M.¹

When comparing two proteins such as tau or MAP 2, which are heterogeneous and which may contain less active or inactive components, it is difficult to be certain that one is actually comparing truly equivalent amounts of the proteins, even when the two proteins are present in equimolar concentrations, as derived from reasonable molecular weight estimates (3, 9). However, if we use, as a criterion for functional concentration, the initial rate at which each protein can catalyze the assembly into microtubules of a given concentration of tubulin, we have observed that, in experiments in which the activity of tau was about one-third that of the sample of MAP 2 to which it was compared, the tau-containing microtubule sampled showed a large increase in turbidity after addition of vinblastine, whereas the MAP 2-containing samples showed a large decrease.¹ Thus, it appears that the large-scale increase in turbidity upon addi-

tion of vinblastine is a property restricted to samples of tubulin which contain tau.

The spirals generated by the combination of tau, tubulin, and vinblastine in this study were very similar to those reported by other authors after the addition of *Vinca* alkaloids to microtubule protein in vitro (5, 6, 8, 14). Donoso et al. (5) have recently demonstrated that such spiral formation requires the presence of microtubule-associated proteins. They did not fractionate their MAPs preparations, however, and, in view of our results, it is likely that the aggregation they observed was due to tau. Fig. 1 shows a sample of tubulin to which tau was added (gel e). This sample was able to polymerize into microtubules and to form spirals in the presence of vinblastine, whereas pure tubulin, shown in gel a, was completely inactive. Nevertheless, the tau bands in gel e are invisible and the two samples are electrophoretically identical. It is therefore possible that tau could be present in a microtubule preparation, influencing its assembly properties and remaining undetected.

It has been proposed, based on ultrastructural studies, that vinblastine-induced spirals consist of a single chain of tubulin dimers arranged end-to-end as in a microtubule protofilament (6, 8). If this is true, our results suggest that this type of spiral formation is facilitated by tau and that tau may be involved in facilitating the formation of longitudinal connections between tubulin dimers in protofilaments. The occasional appearance of para-crystalline arrays in our preparations of tau, tubulin, and vinblastine suggests that the true picture may be more complex and that either tau or vinblastine could induce interactions between tubulin dimers in adjacent spiral filaments as well as within the filaments. Whatever is the precise nature of the interaction of tau with tubulin, our results suggest that the interaction of MAP 2 with tubulin is different. Whether this is due to MAP 2 having a different effect on tubulin than does tau, or whether they have the same effect unless vinblastine is present, or whether vinblastine prevents the binding of MAP 2 to tubulin, cannot be determined from these data and remains the subject of future investigation.

In summary, our results show that, in the presence of vinblastine, tau and MAP 2 differ greatly in their ability to polymerize tubulin and suggest that vinblastine may be a useful probe in future studies to distinguish between the roles of the individual microtubule-associated proteins in the regulation of microtubule assembly.

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REFERENCES

1. Amos, L. A. 1977. Arrangement of high molecular weight associated proteins on purified mammalian brain microtubules. *J. Cell Biol.* 72:642-654.
2. Bryan, J. 1971. Vinblastine and microtubules. I. Induction and isolation of crystals from sea urchin oocytes. *Exp. Cell Res.* 66:129-136.
3. Cleveland, D. W., S.-Y. Hwo, and M. W. Kirschner. 1977. Physical and chemical properties of purified tau factor and the role of tau in microtubule assembly. *J. Mol. Biol.* 116:227-247.
4. Crestfield, A. M., S. Moore, and W. H. Stein. 1963. The preparation and enzymatic hydrolysis of reduced and S-carboxymethylated proteins. *J. Biol. Chem.* 238:622-627.
5. Donoso, J. A., K. M. Haskins, and R. H. Himes. 1979. Effect of microtubule-associated proteins on the interaction of vincristine with microtubules and tubulin. *Cancer Res.* 39:1604-1610.
6. Erickson, H. P. 1975. Negatively stained vinblastine aggregates. *Ann. N. Y. Acad. Sci.* 253:51-52.

¹ Ludueña, Fellous, McManus, and Nunez. Manuscript in preparation.

7. Fellous, A., J. Francon, A.-M. Lennon, and J. Nunez. 1977. Microtubule assembly *in vitro*. Purification of assembly-promoting factor. *Eur. J. Biochem.* 78:167-174.
8. Fujiwara, K., and L. G. Tilney. 1975. Substructural analysis of the microtubule and its polymorphic forms. *Ann. N. Y. Acad. Sci.* 253:27-50.
9. Kim, H., L. I. Binder, and J. L. Rosenbaum. 1979. The periodic association of MAP 2 with brain microtubules *in vitro*. *J. Cell Biol.* 80:266-276.
10. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)*. 227:680-685.
11. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265-275.
12. Marantz, R., and M. L. Shelanski. 1970. Structure of microtubular crystals induced by vinblastine *in vitro*. *J. Cell Biol.* 44:234-238.
13. Nunez, J., A. Fellous, J. Francon, and A. M. Lennon. Competitive inhibition of colchicine binding to tubulin by microtubule-associated proteins. *Proc. Natl. Acad. Sci. U. S. A.* 76: 86-90.
14. Warfield, R. K. N., and G. B. Bouck. 1974. Microtubule-macrotubule transitions: intermediates after exposure to the mitotic inhibitor vinblastine. *Science (Wash. D. C.)*. 186: 1219-1221.
15. Weingarten, M. D., A. H. Lockwood, S.-Y. Hwo, and M. W. Kirschner. 1975. A protein factor essential for microtubule assembly. *Proc. Natl. Acad. Sci. U. S. A.* 72: 1858-1862.
16. Zingsheim, H.-P., W. Herzog, and K. Weber. 1979. Differences in surface morphology of microtubules reconstituted from pure brain tubulin using two different microtubule associated proteins: the high molecular weight MAP 2 proteins and tau proteins. *Eur. J. Cell Biol.* 19:175-183.