Tau Protein Function in Living Cells

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Abstract. Tau protein from mammalian brain promotes microtubule polymerization in vitro and is induced during nerve cell differentiation. However, the effects of tau or any other microtubule-associated protein on tubulin assembly within cells are presently unknown. We have tested tau protein activity in vivo by microinjection into a cell type that has no endogenous tau protein. Immunofluorescence shows that tau protein microinjected into fibroblast cells associates specifically with microtubules. The injected tau protein increases tubulin polymerization and stabilizes microtubules against depolymerization. This increased polymerization does not, however, cause major changes in cell morphology or microtubule arrangement. Thus, tau protein acts in vivo primarily to induce tubulin assembly and stabilize microtubules, activities that may be necessary, but not sufficient, for neuronal morphogenesis.

Neuronal processes are densely filled with parallel arrays of microtubules that act as scaffolds necessary for process extension (Daniels, 1975; Yamada et al., 1976) and serve as tracks for axonal transport (Schnapp et al., 1985). Studies using clonal cell lines that extend neuritic processes in culture have shown that neurite extension involves net microtubule assembly, and not simply rearrangement of existing microtubules into growing processes (Drubin et al., 1985; Gard et al., 1985; Olmstead, 1981). Microtubule assembly during neuronal process extension appears to involve the recruitment of tubulin from preexisting monomer pools into tubulin polymers (microtubules) (Drubin et al., 1985; Gard et al., 1985; Olmstead, 1981). It has been proposed that this assembly is driven by nontubulin protein factors that accumulate during process extension. Support for this proposal comes from the observation that extracts from mature rat brain tissue and from differentiated neuroblastoma cells contain higher levels of protein factors that promote microtubule assembly than do extracts from their respective less-differentiated counterparts (Nunez et al., 1975; Seeds and Maccioni, 1978).

Three major protein factors that promote microtubule assembly in vitro have been identified in brain extracts. These include the tau proteins of 50–70 kD (Weingarten et al., 1975; Cleveland et al., 1977), microtubule-associated protein 1 (MAP1) of ~330 kD, and microtubule-associated protein 2 (MAP2) of ~300 kD (for a review of MAP1 and MAP2 see Vallee et al., 1984). During neurite extension in PC12 pheochromocytoma cells, levels of tau protein correlate precisely with levels of assembled tubulin (Drubin et al., 1985). Furthermore, tau protein is bound to microtubules in PC12 cells (Drubin et al., 1986). These observations make tau protein a strong candidate for a factor that promotes microtubule assembly during process extension. Still, given the complex function that microtubules serve, it is not clear which associated proteins regulate polymer assembly and which regulate the interaction of microtubules with other components in the cell or serve some other function. Subtle questions about the importance of microtubule-associated proteins for polymer stability and dynamics have not been addressed in an in vivo context.

To test the activity of tau protein in vivo we have microinjected purified tau protein into living fibroblast cells (RAT1) that normally contain undetectable levels of this factor. Immunofluorescence shows that microinjected tau protein binds specifically to microtubules (Drubin et al., 1985). Two effects were demonstrated: first, tau causes a marked increase in the level of assembled tubulin. Even more dramatically tau protein increases both centrosome-nucleated and nonnucleated assembly in cells treated with low levels of microtubule depolymerizing drugs. Second, tau protein decreases the depolymerization rate of microtubules. These observations support the conclusion that tau protein promotes the assembly of microtubules that is required for neuronal process extension, and additionally stabilizes the resulting polymers.

Materials and Methods

Cell Culture and Microinjections

RAT1 fibroblast cells were cultured in DME supplemented with 10% calf serum (Gibco, Grand Island, NY). For microinjection experiments, cells were trypsinized and replated on poly-D-lysine coated coverslips. To compare microinjected cells with control sister cells, single cells were plated at low density ~15 h before microinjection. This allowed many cells time to divide once, often resulting in pairs of associated sister cells with similar morphologies. Microinjections were carried out exactly as described by Schulze and Kirschner (1986).
Figure 1. Localization of microinjected tau protein on RAT1 microtubules by double-label immunofluorescence. (A) Rhodamine antitubulin staining showing microtubule networks in RAT1 cells fixed 40 min after one cell (arrow) was microinjected with 2 mg/ml bovine brain tau protein. (B) Fluorescein anti-tau staining showing tau localization on microtubules in the tau-microinjected cells, and absence of tau in the uninjected cells shown in A. Bar, 10 μm.

Protein Preparation
FITC-BSA was obtained from Sigma Chemical Co., (St. Louis, MO). Bovine brain tau protein was separated from MAP2 in heat-stable microtubule-associated proteins by chromatography on Sepharose 4BCL (Pharmacia Fine Chemicals, Piscataway, NJ) as described in detail in Herzog and Weber, 1978, and was concentrated using an ultrafiltration unit (Amicon Corp., Danvers, MA).

Immunofluorescence
Cells were washed briefly with PBS (0.13 M NaCl, 2 mM KCl, 8 mM Na2HPO4, 2 mM KH2PO4, pH 7.2) and then immersed for 10 min at room temperature in 80 mM Pipes, pH 6.8, 5 mM EGTA, 1 mM MgCl2, and the detergent 1% Empigen BB (Albright and Wilson, Whitehaven, Cumbria, UK), 0.3% glutaraldehyde. The cells were next rinsed briefly in PBS and then incubated in PBS containing 1 mg/ml NaBH4 for 7 min. Before and after each antibody addition the cells were washed five times in PBS containing 1% BSA and 0.1% Tween 20 over a 10-min period. Antibodies were diluted in this same wash buffer. For double-label immunofluorescence, fixed and permeabilized cells were treated for 30 min with affinity-purified anti-bovine brain tau protein antiserum (Drubin et al., 1984; Pfeffer et al., 1983) (1:200 dilution), for 15 min with DMct-I tubulin monoclonal antibody (Blose et al., 1984) (1:200 dilution, a gift of Dr. Steven Blose, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), and for 15 min with a mixture of fluorescein-conjugated goat anti-rabbit antiserum and rhodamine-conjugated goat anti-mouse antiserum (1:50 and 1:200 dilution, respectively). Coverslips were mounted in 90% vol/vol glycerol, 20 mM Tris pH 7.9.

Microinjected cells were identified using a fluorescein filter. Cells were photographed on hypersensitized Kodak Tech-Pan (2415) film using the fluorescein filter as described by Schulze and Kirschner (1986). After bleaching the fluorescein image for several minutes to eliminate possible effects of "bleed through" of the fluorescein signal into the rhodamine channel, the tubulin staining pattern was photographed using a rhodamine filter.

Microtubule Quantitation
Relative microtubule mass in sister cells with similar morphologies was quantitated in two ways. For cells treated 30 min in nocodazole, total microtubule lengths were digitized by projecting images from negatives onto a GTCO (Rockville, MD) digitizing pad interfaced with an IBM personal computer. For cells with more dense microtubule arrays (cells treated for <30 min with 33 μm nocodazole) relative microtubule mass was determined by densitometry of negatives containing images of mitotic sister cells using a Zeinet soft laser densitometer (Biomed Instruments, Inc., Chicago, IL).

Results
Microinjected Tau Protein Binds Specifically to Microtubules in Living Cells
The antitubulin immunofluorescent staining pattern of a field of RAT1 fibroblast cells is shown in Fig. 1 A. The cell designated by an arrow was microinjected with a 2 mg/ml solution of purified bovine brain tau protein to ~10% the cell volume 40 min before fixation. (Tau protein was purified from bovine brain to give the usual four major subspecies by the procedure of Drubin and Kirschner [1986] based on the method of Herzog and Weber [1978]). The anti-tau immunofluorescent staining pattern of the same field of cells shown in Fig. 1 A is shown in Fig. 1 B. The three uninjected cells are devoid of tau immunofluorescence even though the affinity-purified tau antiserum employed in these studies binds tightly to tau protein isolated from rat PC12 cells (Drubin et al., 1985) and brain. The cell microinjected with tau protein, on the other hand, is stained brightly by the affinity-purified tau antibody. The tau staining pattern in the microinjected cell is identical to the corresponding tubulin staining pattern shown in Fig. 1 A. We conclude that the microinjected tau protein is bound to microtubules in RAT1 cells, a result further supported by the observation that pretreatment of the cells with drugs in-
hibiting tubulin polymerization abolishes the filamentous tau immunofluorescence pattern (not shown). The fixation procedure preserves all known elements of the cytoskeleton, and in no case was tau staining observed in association with any structures other than microtubules.

**Tau Protein Promotes Microtubule Assembly in Fibroblast Cells**

The ability to introduce tau protein into living RAT1 cells that normally lack this factor makes possible an evaluation of the in vivo effects of tau protein on microtubule assembly and microtubule stability. Immunofluorescence results like that shown in Fig. 1 A usually give the impression that cells microinjected with tau protein contain higher microtubule densities than uninjected neighboring cells. The possible extent of an increase in microtubule density caused by tau microinjection ultimately is limited by the size of the unassembled tubulin pool. Estimates for the proportion of tubulin in the unassembled state range from 10–50% (Hiller and Weber, 1978; Caron et al., 1985).

We have used two approaches to further study the effects of microinjected tau protein on the extent of microtubule assembly in RAT1 cells. In both approaches one cell in a pair of mitotic sister cells was microinjected with tau protein and the microtubule arrays in both cells were compared by immunofluorescence. Sister cells were used because they have similar morphologies (Solomon, 1981) and similar microtubule patterns (Fig. 2 A). We have noted that with this cell line comparisons between sister cells are much more accurate than general population comparisons. For the first strategy, normal RAT1 sister cells were examined. For the second strategy, the pool of unassembled tubulin was artificially increased by incubating the cells in nocodazole. This increases the maximum possible augmentation in assembled tubulin that can be achieved by tau microinjection, and thus provides an opportunity for a more dramatic visualization of the effect of tau protein. In both cases a 2 mg/ml tau microinjection concentration was employed to create a tau-to-tubulin molar ratio in RAT1 cells similar to that found in PC12 cells after tau induction during neurite extension (assuming that 10% of a cell volume can be microinjected and that a cell contains 2 mg/ml tubulin) (Hiller and Weber, 1978). As a control for
Figure 3. (A) Histogram showing the distribution of microtubule mass ratios between cells microinjected with FITC-BSA, and their respective sister cells. The arrow indicates the interval containing the arithmetic mean, and the asterisk shows the interval into which the cell pair in 2A fell. (B) Histogram showing the distribution of microtubule mass ratios between cells microinjected with 2 mg/ml FITC-BSA (see Fig. 2B for FITC-BSA fluorescence). While the microtubule networks in the two uninjected cells have been greatly thinned compared with cells not exposed to nocodazole, while astral microtubules were absent from cells not microinjected with tau. Furthermore, the cells microinjected with tau protein contained longer microtubules and apparently higher densities of free microtubules than their uninjected counterparts. The consistency of this affect between cells treated for 1 h and cells treated for 2 h with 0.4 μM nocodazole suggests that this is a steady state phenomenon. Microinjection of 2 mg/ml FITC-BSA did not affect microtubule assembly under these conditions (not shown).

Table 1. Microtubule Assembly and Stability in RAT1 Cells Microinjected with Tau Protein

| Tau Protein Stabilizes Cellular Microtubules |
|-------------------------------------------|
| An expectation arising from the demonstration that tau protein promotes microtubule assembly in vivo is that microtubules coated with tau protein in RAT1 cells will be more stable than those without tau protein. Microtubule stability can be evaluated operationally by incubating cells in high levels of nocodazole, a drug blocking microtubule assembly (Salmon et al., 1984). For these experiments RAT1 cells were microinjected with 2 mg/ml tau protein or 2 mg/ml FITC-BSA, and were treated 60 min later with 33 μM nocodazole for the times indicated below. The cell marked with an arrow in Fig. 5A was microinjected with 2 mg/ml FITC-BSA (see Fig. 5B for BSA fluorescence) and treated for 1 min with 33 μM nocodazole. The microtubule networks in both cells shown in Fig. 5A have been greatly thinned compared with cells not exposed to nocodazole. Generally the majority of microtubules disappears within 1 min of treatment with nocodazole and the remaining microtubules disappear more slowly over the next 30-40 min. Microinjection of 2 mg/ml FITC-BSA into the RAT1 cell shown in Fig. 5A did not affect the extent of microtubule disappearance after 1 min with nocodazole as compared with the neighboring uninjected cell. The cell indicated with an arrow in Fig. 5C, however, was microinjected with 2 mg/ml of tau protein and then exposed to nocodazole for 1 min (see Fig. 5D for tau immunofluorescence). While the microtubule networks in the two uninjected cells have been dramatically thinned by nocodazole treatment, the microtubule network in the tau-containing cell seems unaffected by nocodazole. To determine whether tau protein blocks microtubule dis-|
assembly completely or alternatively slows the process, longer nocodazole treatment times were employed. Fig. 6 shows pairs of sister cells treated for different times with 33 μM nocodazole. In each case the cell microinjected with 2 mg/ml tau protein is designated with an arrow. After 1 min of nocodazole treatment (Fig. 6A, as was shown for the cells in Fig. 5C), the cell containing tau protein has a significantly higher density of microtubules than the cell without tau. After 3.5 min (Fig. 6B) of nocodazole treatment a slight decrease in microtubule density is now apparent in the microinjected cell, especially toward the interior of the cell. Finally, after 30 min of nocodazole treatment (Fig. 6C) the tau-microinjected cell has a greatly reduced microtubule network, although more microtubules remain in the microinjected cell than in the neighboring uninjected cell. Eventually virtually all microtubules disappear from microinjected and uninjected cells (not shown). Thus, tau protein slows that rate of disassembly but does not fully inhibit tubulin dissociation.

For nocodazole-treated pairs of cells the ratio of microtubule mass in microinjected vs. uninjected sisters was determined roughly by densitometry for time points at which a large number of microtubules are present. When the number of microtubules was small, microtubule mass was accurately determined by digitometry of total microtubule length. Table 1 shows the average ratios of microinjected to uninjected microtubule mass for pairs of sister cells. The difference is largest after 1 min of nocodazole treatment, when most of the RATI cell microtubules are lost. At this time four times as much microtubule mass remains in cells containing tau than remains in uninjected cells. Again, FITC-BSA has no affect on microtubule retention. At 3.5 and 12 min of nocodazole treatment, a further reduction in the microtubule networks is seen in both cells. However this still leaves three times as much tubulin in the polymerized state in tau-containing cells. Lastly, at 30 min (Fig. 6C) there still remains twice as much tubulin in cells containing tau than remains in uninjected cells or cells containing FITC-BSA.

Discussion

Tau protein was originally defined biochemically as a factor that coassembled with tubulin and promoted microtubule assembly in vitro (Weingarten et al., 1975; Cleveland et al., 1977). It was later shown to be rather specific to neuronal cells and to be associated with microtubules in neuritic processes by immunofluorescence (Drubin et al., 1986). In brain, tau is preferentially localized in axons rather than in dendrites or cell bodies (Binder et al., 1985; Peng et al., 1986), but in certain brain pathologies, such as Alzheimer's disease, it can be found in unusual structures called neurofibrillary tangles (Wood et al., 1986; Kosik et al., 1986; Grundke-Iqbal et al., 1986). Despite the evidence for a close association of tau protein with microtubules, the difficult histochemistry and the high density of microtubules in neuronal cells made it difficult to assess whether tau protein was also associated with other major cytoskeletal elements such as intermediate filaments or actin filaments. Such possible associations have been inferred by in vitro experiments (Carlier et al., 1984; Seldon and Pollard, 1983; Runge et al.,...
Figure 5. Stabilization of RAT1 microtubules by microinjected tau protein. (A) Rhodamine antitubulin staining of RAT1 cells treated for 1 min with 33 μM nocodazole 60 min after one cell (arrow) was microinjected with FITC-BSA. (B) Fluorescein fluorescence showing FITC-BSA presence in the microinjected cell shown in A. (C) Rhodamine antitubulin staining of RAT1 cells treated for 1 min with 33 μM nocodazole 60 min after one cell (arrow) was microinjected with 2 mg/ml bovine brain tau protein. (D) Fluorescein anti-tau staining showing tau localization on microtubules in the tau-microinjected cell shown in C. Bar, 10 μm.

1979) and could also play a role in pathological conditions. However, as shown here, when tau protein is injected into fibroblast cells, it associates primarily with microtubules and not actin or intermediate filaments. It is possible that in neuronal cells specific proteins could mediate its interaction with other components of the cytoskeleton. No preference is shown for any subsets of microtubules in the cell.

The importance of tau as an assembly-promoting factor rests on its in vitro activity, which it shares with other associated proteins, and circumstantially, on its induction by nerve growth factor in pheochromocytoma cells at the time of neurite extension (Drubin et al., 1985). In the absence of a specific drug for inhibiting tau activity, the best test for tau function would be to genetically or pseudogenetically alter tau expression. We have chosen to use fibroblast cells because of the low or absent basal level of tau expression, and because of the favorable morphology which allows a semi-quantitative assessment of the degree of microtubule assembly. We have, in effect, performed a complementation experiment to study tau function in vivo. Starting with fibroblast cells that contain undetectable levels of tau protein, tau protein was microinjected at physiological levels (similar to the levels in PC12 cells after treatment with nerve growth factor (Drubin et al., 1985) and effects on microtubule assembly were examined.

As shown in Table I, injected tau protein increases microtubule levels in RAT1 fibroblast cells by an average of 44% over levels found in morphologically similar sister cells. The magnitude of this assembly induction, though small, could be limited by the pool of available tubulin. In PC12 cells the pool of available tubulin increases before tau induction and thus the tau induction can produce a larger increase in microtubule assembly (Drubin et al., 1985). To observe a more dramatic tau effect in RAT1 cells, we artificially increased the pool of unassembled tubulin by nocodazole treatment. When tau protein was injected into cells subsequently
treated with nocodazole, we observed a dramatic increase in assembly as compared with uninjected controls. Increases in microtubule length and in the number of astral and free microtubules were observed. The apparent promotion of free microtubule formation in response to tau microinjection suggests that tau presence in axons could promote centrosome-independent microtubule assembly predicted by electron microscopic studies (Chalfie and Thomson, 1979; Bray and Bunge, 1981).

While tau protein promotes microtubule assembly in RAT1 cells, it does not cause obvious morphological changes over the 3-h time course examined (beyond 3 h, microinjected tau protein cannot be detected by immunofluorescence due, presumably, to protein degradation). Therefore, while neurite extension requires microtubule assembly, increased microtubule assembly is not sufficient to cause neurite extension. Additional factors must be required to utilize newly formed microtubules for process extension. While tau protein microinjection failed to induce a neuritic morphology, it did show that this factor can promote the increased microtubule assembly observed during nerve growth factor-induced morphogenesis.

**Consequences of Tau Binding for Microtubule Dynamics**

The presence of tau protein on microtubules in RAT1 cells reduces drastically the apparent dissociation rate of tubulin from microtubules as judged by the procedure of Salmon et al., 1984, in which cells are incubated in very high concentrations of nocodazole. When we examined the effect of microinjected tau protein, microtubules containing tau protein were found to depolymerize more slowly in response to high concentrations of nocodazole (Fig. 5 and 6, Table I). This result suggests that the increased stability of neuritic microtubules in differentiated PC12 cells (Black and Greene, 1982; Heideman et al., 1985) is at least partially due to the presence of tau protein.

The disappearance of microtubules from normal fibroblast cells treated with high nocodazole levels (Fig. 5 and 6) does not appear to occur by a progressive shortening of all microtubules from the cell periphery toward the centrosome. Instead, the number of microtubules decreases with time without evidence for disassembly intermediates. This behavior was also observed recently in monocytes in which it
is possible to count and measure every microtubule in a cell (Cassimeris et al., 1986). In monocytes and in fibroblasts most microtubules are lost quickly in response to nocodazole treatment but a subset of microtubules is lost at a significantly slower rate. The rate-limiting step appears to be initiation of depolymerization, not dissociation of tubulin subunits from depolymerizing microtubules. These observations can be explained if the disappearance of microtubules in fibroblast cells results from a transition from a stable or growing state to a rapidly shrinking state, as suggested by the model of dynamic instability (Mitchison and Kirschner, 1984).

Tau protein slows the rate of microtubule loss without allowing visualization of disassembly intermediates. Thus it appears that tau stabilizes microtubules primarily by decreasing the transition rate to the rapidly disassembling state. Recent in vitro studies have in fact shown that microtubule-associated proteins can have an inhibitory effect on such transitions (Horio and Hotani, 1986).

The fact that tau protein microinjection into growing RAT1 cells causes a significant increase in microtubule assembly and stability suggests that these cells normally lack tau-like factors at levels found in differentiated PC12 cells. Thus it may be that tau-like factors are characteristic of neuronal cells that have adopted a differentiated morphology. In support of this, assembly behavior of microtubules in fibroblast cells (Schulze and Kirschner, 1986) is similar to that found in vitro with tubulin lacking associated proteins (Mitchison and Kirschner, 1984). Unfortunately we know nothing about the presence of MAP proteins in RAT-1 cells. The subset of microtubules in monocytes and fibroblasts that have increased stability could, however, contain selectively bound tau-like factors. General lack of tau-like factors in proliferating fibroblast cells may explain why only tubulin mutants have been obtained when attempts were made to genetically identify drug resistant cells in culture.

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