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Immunoflourescent staining of cytoplasmic and spindle microtubules in mouse fibroblasts with antibody to τ protein

(tubulin/microtubule accessory proteins/networks)

JOE A. CONNOLLY*, VITAUTS I. KALNINS*, DON W. CLEVELAND†, AND MARC W. KIRSCHNER†

* Histology Division, Department of Anatomy, University of Toronto, Toronto, Canada M5S 1A8; and † Department of Biochemical Sciences, Moffett Laboratories, Princeton University, Princeton, New Jersey 08540

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ABSTRACT **τ protein isolated from porcine brain microtubules was further purified by electrophoretic elution from polyacrylamide gels and used to prepare antisera in rabbits. The antiserum to τ specifically stains mitotic spindles and a filamentous network within mouse fibroblasts when the indirect immunofluorescence technique is used. The staining of the filamentous network and mitotic spindles is identical to that observed when cells are treated with antiserum prepared against electrophoretically purified tubulin. The filamentous network observed with either serum is sensitive to Colcemid. Absorption of anti-τ serum with electrophoretically purified tubulin does not remove the immunoflourescent staining of the mitotic spindle, whereas absorption with electrophoretically purified τ protein does. Conversely, absorption of antitubulin serum with tubulin eliminates its ability to stain the mitotic spindle, whereas absorption with τ has no effect. We conclude that τ protein and tubulin are antigenically distinct proteins and that τ is an integral part of microtubules *in vivo*. These results also provide evidence that τ protein, or an antigenically related protein, is associated with microtubules not only in brain but also in other cell types.**

The development of procedures for the *in vitro* assembly of microtubules (1–3) has made possible the investigation of specific components in the polymerization process. Microtubules purified by successive cycles of assembly and disassembly have been found to contain, in addition to tubulin, a variety of accessory proteins whose presence is required under standard buffer conditions for assembly of tubulin (4–8). One of these accessory proteins, named τ, has been isolated from tubulin by phosphocellulose chromatography (4) and has recently been purified on the basis of its ability to stimulate tubulin polymerization *in vitro*. It comprises most of the microtubule-inducing activity recoverable following the removal of tubulin on phosphocellulose (9,‡). In the absence of τ, tubulin (under the standard ionic conditions) remains as a 6S subunit which is unable to polymerize into microtubules. In experiments testing competence of neural tubulin for growth onto flagellar nucleation sites, τ has been shown to be required for elongation as well as initiation of microtubule assembly (10). Because both the initial rate of microtubule growth and the final level of assembly are functions of τ concentrations, a stoichiometric rather than catalytic mode of action for τ is indicated.

Though τ has been found to copurify with microtubules through multiple cycles of assembly at constant stoichiometry with tubulin‡, the *in vivo* association of τ with microtubules has not been demonstrated. Indeed, other investigators have indicated that different accessory proteins (in particular high-molecular-weight proteins) may also be responsible for microtubule assembly in their preparations (5–8). Moreover, reports of the induction of microtubules or of microtubule-related structures from purified tubulin under nonphysiological solvent conditions or by the addition of polycations have raised the question as to whether these accessory proteins are in fact associated with microtubules *in vivo* (11–14).

We have attempted in this study to establish whether τ is present in cells outside brain tissue, and if so, to determine the spatial relationship between τ and polymerized microtubules in intact cells. In order to do so, antisera have been prepared to electrophoretically purified τ protein and to tubulin and used to examine the distribution of staining in mouse embryo fibroblasts by indirect immunofluorescence. Antisera to tubulin have previously been reported to specifically stain mitotic spindles (15) and Colcemid-sensitive fibrous networks in several cell types (16, 17). Furthermore, recent electron microscopic evidence indicates that microtubules are specifically labeled with antibodies to tubulin (18). We report here that identical staining patterns (which are consistent with the distribution of microtubules) are observed with antisera against τ and antisera against tubulin in both mitotic and interphase cells. In addition, reciprocal absorption studies indicate that τ protein and tubulin are antigenically distinct. We conclude that τ protein is present in microtubules in intact cells.

MATERIALS AND METHODS

Gel Electrophoresis. Tubulin and τ were analyzed and purified by polyacrylamide gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate ($NaDodSO_4$) using the discontinuous system of Laemmli (19). Resolving gels were 7.5% in acrylamide and the stacking gels were 3%.

Purification of Antigens. τ protein was purified from porcine brain microtubules by phosphocellulose and hydroxyapatite chromatography by the procedure given by Witman *et al.* (10). The partially purified τ contained no visible tubulin and approximately 75% of this protein was in the form of four closely spaced bands which migrated on $NaDodSO_4$/acrylamide gels with apparent molecular weights between 55,000 and 62,000. The four major bands were localized in slab gels by staining, excised from the gel, electrophoretically eluted, and finally dialyzed against phosphate-buffered saline (phosphate/saline).

Tubulin, isolated initially by two cycles of polymerization and depolymerization by the method of Shelanski *et al.* (3), was subjected to final electrophoretic purification in the same manner.

Preparation of Antisera. Antiserum to τ was produced by injecting female New Zealand White rabbits intraperitoneally with 30 μg of τ protein in complete Freund's adjuvant once a week for 3 weeks. After an additional 2 weeks, the rabbits were boosted with 30 μg of the τ in complete Freund's adjuvant and bled 2 weeks after this booster injection. Preimmune sera were obtained from the same animals prior to immunization.

Abbreviation: $NaDodSO_4$, sodium dodecyl sulfate.

‡ Cleveland, D. W., Hwo, S.-Y. & Kirschner, M. W. (1977) *J. Mol. Biol.*, in press.

The preparation of antiserum to tubulin has been previously described (20).

Mouse Fibroblasts. Secondary cultures of mouse embryo fibroblasts were grown for 20 hr in Lab-Tek chambers (Miles Laboratories) in minimal essential medium (MEM) supplemented with 10% fetal calf serum.

For treatment of cells with Colcemid, the culture medium was removed and replaced with the same medium containing Colcemid at 0.5 μg/ml for 60 min prior to fixation of the cells.

Immunofluorescence. For immunofluorescent staining, cells grown in Lab-Tek chambers were rinsed quickly in phosphate/saline, fixed for 5 min in 3.5% paraformaldehyde in phosphate/saline (pH 6.6), washed four times in phosphate/saline, postfixed for 4 min in methanol and 2 min in acetone (both at −20°), and air dried. Cells were treated with antisera to tubulin (diluted 1:10) or to τ protein (diluted 1:30) and allowed to react at room temperature for 40 min in a humid chamber, washed four times in phosphate/saline, and then incubated for 30 min with fluorescein-conjugated goat antiserum to rabbit IgG (Hyland Laboratories) diluted 1:5 with phosphate/saline. The cells were than washed four times in phosphate/saline and mounted in 50% (vol/vol) glycerol in phosphate/saline (pH 7.5). Unless otherwise indicated, the pH of the phosphate/saline was 7.0. Cells were examined in a Zeiss microscope equipped with epi-fluorescent optics and interference filters and photographed on Kodak Plus-X Pan film developed in Diafine.

Absorption of Antisera. To absorb each of the antisera, we proceeded as follows: An aliquot of antiserum was diluted 1:10 in phosphate/saline. Electrophoretically purified antigen in phosphate/saline at a concentration of 1 mg/ml was then added in the ratio of 10 μl of antigen per 150 μl of diluted antiserum. This mixture was allowed to react for 90 min at room temperature. Anti-τ serum was then diluted 1:30 with phosphate/saline before use.

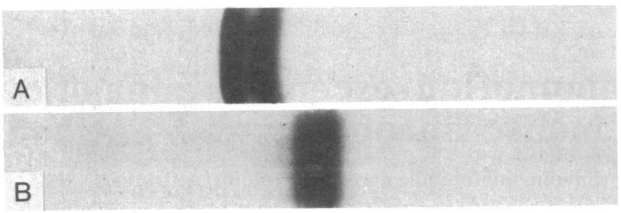

FIG. 1. Polyacrylamide gels of (*A*) purified tubulin (35 μg) and (*B*) purified τ protein (30 μg). Electrophoresis is from right to left.

RESULTS

τ protein, which copurifies with tubulin through repeated cycles of polymerization and depolymerization, was initially separated from tubulin on phosphocellulose. The major activity for promotoing microtubule assembly has been found to be due to a set of closely related polypeptides that migrate between 55,000 and 62,000 molecular weight on $NaDodSO_4$/polyacrylamide gels.‡ The partially purified τ preparation, as obtained after phosphocellulose and hydroxyapatite chromatography, though devoid of tubulin, contains small amounts of several other polypeptides of higher and lower molecular weights. The τ peptides were, therefore, further purified by gel electrophoresis. After elution of the protein from gels, the purified τ was seen to consist of the four closely spaced polypeptides and to be free of any other detectable proteins (Fig. 1). This elec-

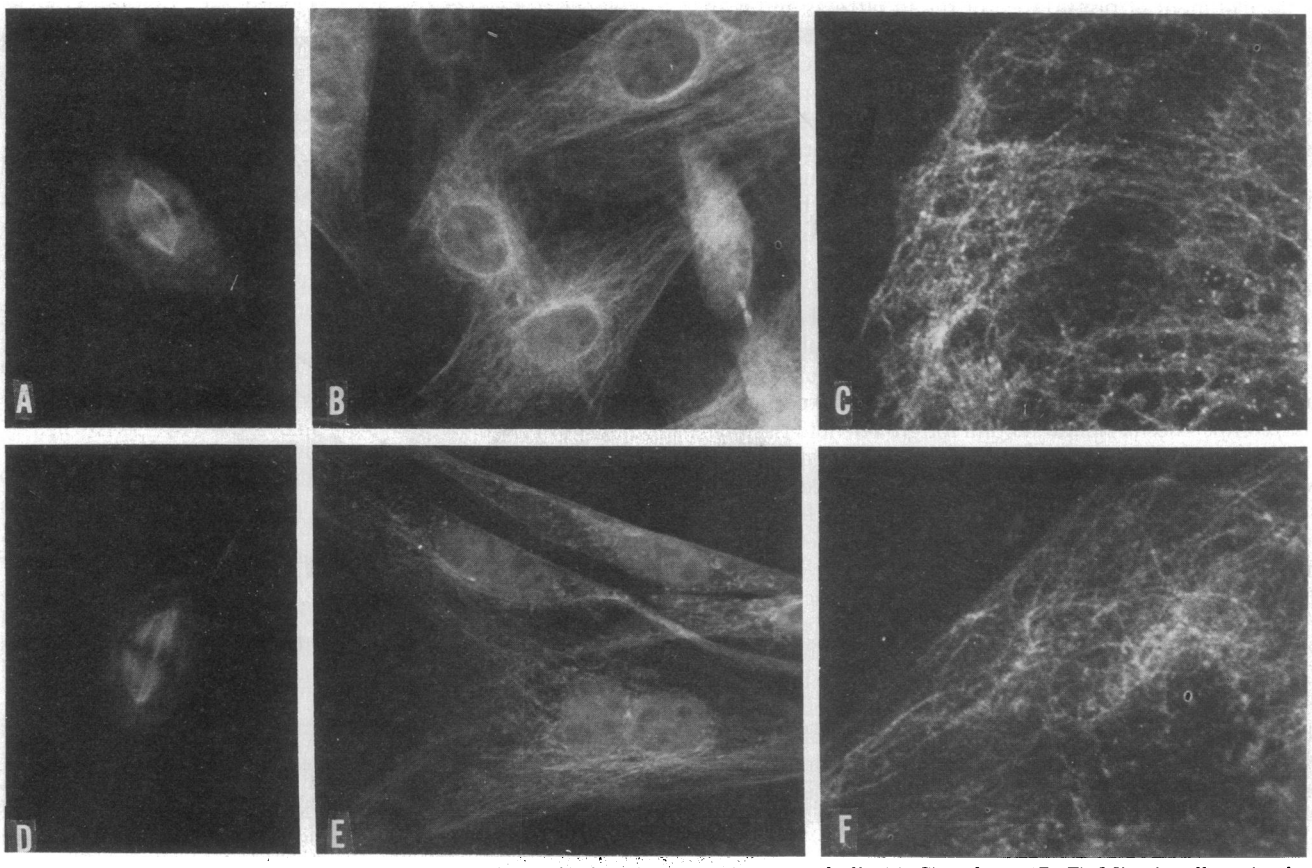

FIG. 2. Immunoflourescent staining of mouse embryo fibroblasts with antisera to tubulin (*A–C*) and to τ (*D–F*). Mitotic cells stained (*A*) with antitubulin and (*D*) with anti-τ. (×640.) Interphase cells stained (*B*) with antitubulin and (*E*) with anti-τ. (×640.) Higher magnification of interphase cells stained (*C*) with antitubulin and (*F*) with anti-τ. (×1920.)

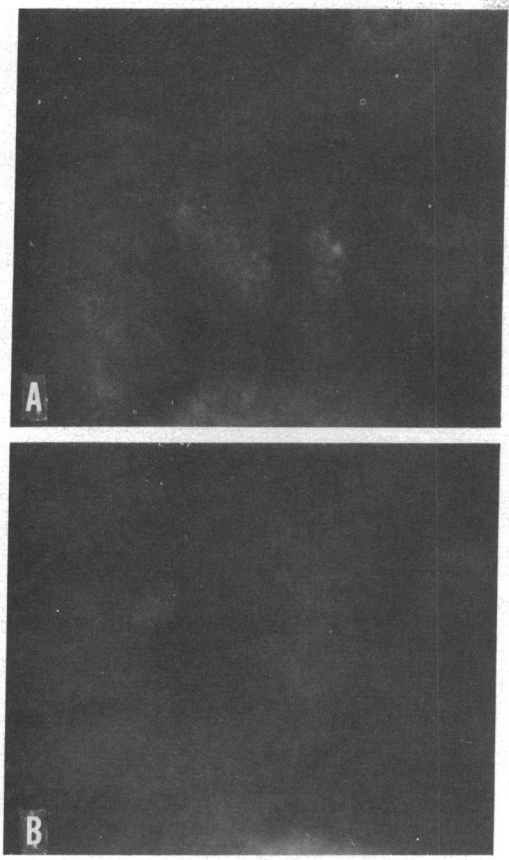

FIG. 3. Immunoflourescence of mouse embryo fibroblasts pretreated with Colcemid prior to fixation and then stained with (*A*) antiserum to tubulin or (*B*) antiserum to τ protein. (×1920).

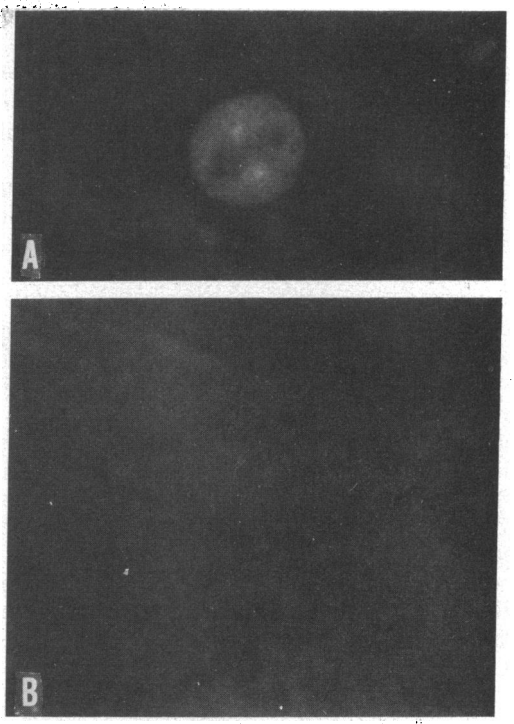

FIG. 5. Immunoflourescence of mouse embryo fibroblasts treated with serum obtained prior to immunization with τ protein. (*A*) A mitotic cell (×640); and (*B*) an interphase cell (×1920.)

trophoretically purified τ was used for subsequent immunization of rabbits.

Tubulin was initially purified by two cycles of polymerization and depolymerization, followed by an analogous final purification by electrophoresis (Fig 1), prior to its use for the immunization of rabbits. The antiserum obtained has been previously characterized (20, 21) and reveals specific staining of mitotic spindles and other microtubule-containing structures.

To determine the relative spatial distributions of the τ protein and of tubulin in mouse embryo fibroblasts, we treated the cells with rabbit anti-τ serum or rabbit antitubulin serum, and then with fluorescein-conjugated goat anti-rabbit IgG. When fibroblasts were treated with antitubulin, both mitotic spindles and filamentous cytoplasmic networks were clearly stained (Fig. 2 *A–C*), as expected. When mouse fibroblasts were treated with antiserum to τ, specific staining was also observed, indicating that a protein antigenically similar to τ (which was isolated from brain) exists in mouse fibroblasts. Moreover, the specific staining pattern observed with this antiserum is very similar to that obtained with antiserum to tubulin. As with antitubulin serum, both the mitotic spindle and a filamentous cytoplasmic network are clearly stained (compare Fig. 2 *A–C* with 2 *D–F*). The similarity in specific staining patterns observed indicates that τ and tubulin have the same distribution in mouse fibroblasts.

To ensure that the network seen with antiserum to τ protein represents microtubules, we first exposed mouse fibroblasts to Colcemid (0.5 μg/ml for 60 min) (22) and then treated them with antiserum to τ or antiserum to tubulin. In neither case could stained filaments be observed in the cytoplasm (Fig. 3).

To eliminate the possibility of crossreactivity between the two antisera, the following absorption studies were performed. When antitubulin serum was absorbed with electrophoretically purified tubulin, the staining of spindles was abolished and only very faint staining of centriolar areas remained (Fig. 4*A*). Absorption of this antiserum with electrophoretically purified τ protein, on the other hand, did not significantly reduce the

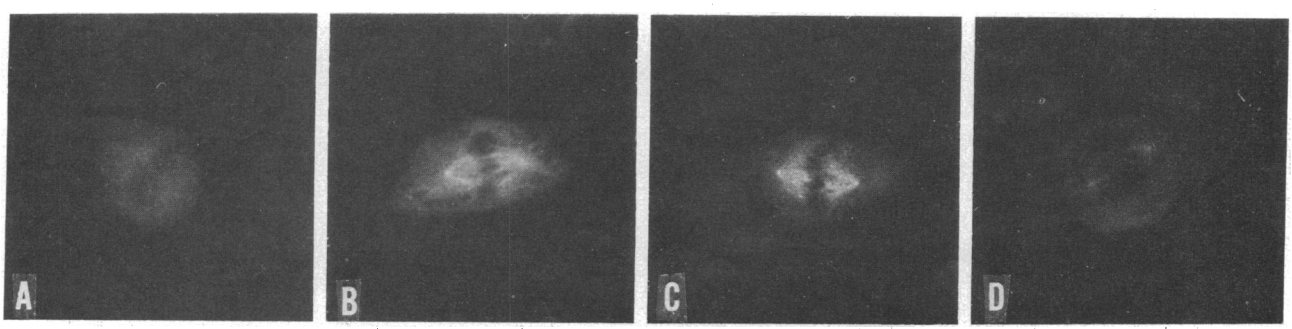

FIG. 4. Immunoflourescent staining of mouse embryo fibroblasts in mitosis treated with antisera absorbed with tubulin or absorbed with τ prior to incubation. (*A*) Antitubulin serum absorbed with tubulin; (*B*) antitubulin serum absorbed with τ protein; (*C*) anti-τ serum absorbed with tubulin; and (*D*) anti-τ serum absorbed with τ protein. (×640.)

staining of mitotic spindles with the antitubulin antibody (Fig. 4*B*). Conversely, when the mouse fibroblasts were treated with anti-τ serum absorbed with electrophoretically purified tubulin, there was no significant reduction in the staining of mitotic spindles (Fig. 4*C*). However, absorption of this same serum with electrophoretically purified τ protein removed most of the spindle staining. The background fluorescence showing some residual staining at centriolar regions (Fig. 4*D*) was similar to that obtained with the preimmune serum (Fig. 5*A*). These results indicate that antiserum to τ does not crossreact with tubulin, that antiserum to tubulin does not crossreact with τ, and that τ and tubulin are antigenically distinct. The staining observed with antiserum to τ cannot be due, therefore, to antibody against any possible contaminating tubulin in the τ protein used for immunization.

When mouse fibroblasts were treated with preimmune sera from the same rabbits used to prepare antisera to τ or to tubulin, neither mitotic spindles nor cytoplasmic networks were stained (Fig. 5 *A*, *B*). Several of the preimmune sera did, however, stain the centriolar region of the mitotic spindle (Fig. 5*A*). We have observed this previously with other preimmune rabbit sera and believe it to be due to attachment of the IgG to the centriolar region and to be unrelated to antigenic specificity.

DISCUSSION

Antisera prepared against purified τ protein stain the same structures as those stained by antisera to tubulin. Both mitotic spindles and the filamentous cytoplasmic networks of interphase cells are stained with anti-τ or antitubulin antisera. The staining of cytoplasmic microtubules by either antiserum is abolished by pretreatment of the cells with Colcemid.

The methods of preparation of the τ protein used for the immunization ensure that it is not contaminated with tubulin. τ is completely separated from tubulin by phosphocellulose and hydroxyapatite chromatography as judged by gel electrophoresis. The τ fraction further purified by $NaDodSO_4$/gel electrophoresis is clearly separated from tubulin contamination (Fig. 1). Absorption studies using antisera prepared against electrophoretically purified τ or tubulin indicate that the two proteins are antigenically distinct. Neither the absorption of anti-τ serum with purified tubulin nor the absorption of antitubulin serum with purified τ diminishes the staining of mitotic spindles. Moreover, peptide mapping studies confirm that there are no apparent structural similarities between τ and tubulin (23,‡). Because both antisera were nonprecipitating, Ochterlony double diffusion tests could not be performed to verify further the specificity of the antisera.

In vitro experiments indicate that τ is incorporated into the microtubule in a fixed stoichiometry with tubulin throughout the length of the microtubule (10). If microtubules formed *in vivo* are identical to those polymerized from brain extracts *in vitro*, then one would predict that identical immunofluorescent staining patterns would be observed with antisera to τ or to tubulin. The results reported here support the hypothesis that τ is associated throughout the full length of microtubules *in vivo*. The present study further indicates that τ is not preferentially localized in the region of nucleation sites.

The *in vivo* function of τ remains to be investigated. *In vitro* studies have indicated that under roughly physiological conditions τ is required for assembly of microtubules from purified tubulin. Hence, τ could be utilized by the cell to regulate the extent of microtubule polymerization. τ, a minor protein component, could thus control the polymerization of a much larger amount of cellular tubulin. Indeed, the microheterogeneity of τ, which appears to be nonartifactual,‡ could reflect a variety of levels of such regulation. Alternatively, though τ induces polymerization *in vitro*, it could serve another function *in vivo*, possibly functioning in intracellular motility or transport or in the interaction of microtubules with other cellular components. In any case, the results presented here indicate that τ is an integral protein component of the microtubule lattice and is probably widely distributed in various cell types.

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