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Nuclear and Cytoplasmic Tau Proteins From Human Nonneuronal Cells Share Common Structural and Functional Features With Brain Tau

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Abstract The heterogeneous family of tau proteins interacts with microtubules, actin filaments, and intermediate filaments. The tau isoforms have been shown to play a major role in neuronal polarity. However, tau-like proteins have been found in several other types of cells. Previous studies have also indicated the presence of a nuclear tau. The relationships between nuclear and cytoplasmic tau as well as the functional aspects of the nuclear tau are unknown. In this study, we demonstrate by reverse transcriptase polymerase chain reaction using specific primers that a transcript with features of neuronal tau is present in human fibroblast and Huh-7 hepatoma cell lines. Additionally, we present the first isolation and characterization of cytosolic and nuclear tau-like proteins from nonneuronal cells. Nonneuronal cytosolic tau components were isolated using the perchloric acid precipitation approach, while nuclear tau was isolated after selective extractions using high-ionic strength buffers. The cytoplasmic tau of nonneuronal cells is composed of at least three isoforms, whereas two main isoforms were detected in nuclear tau. Interestingly, the cytoplasmic and nuclear tau components exhibited the capacity to promote tubulin polymerization in vitro. Immunofluorescence studies using monoclonal anti-tau antibodies indicated a discrete distribution of tau protein in both the interphase and mitotic nucleus. In the latter, tau colocalized with the chromosomal scaffold. These studies, together with previous evidence on tau roles in modulating microtubule growth from centrosomes, and its role in the interaction patterns that stabilize the integrity of the cytoskeletal network, strongly support the idea that tau is a multifunctional protein involved in fundamental cellular processes. J. Cell. Biochem. 78:305–317, 2000.

Key words: microtubule-associated proteins; nuclear and cytoplasmic tau; structural characteristics; intracellular roles; chromosomal scaffold

Cytoskeletal polymers are interconnected to form a three-dimensional network. This network is highly dynamic, with the capacity to respond to the morphophysiological demands of the cell. The macromolecular interactions that stabilize this network and its intrinsic dynamics are the basis for numerous cellular processes [reviewed in Maccioni and Cambiazo, 1995]. However, the mechanisms on how the supramolecular components of the cytoskeleton are functionally interconnected and regulated are not well understood. There is increasing evidence that cytoplasmic tau, the low-molecular-weight microtubule-associated protein, plays a key role in regulating the organization and integrity of the cytoskeleton [Binder et al., 1985; Knops et al., 1991; Cowan and Hirokawa, 1992]. The heterogeneous family of neuronal tau is formed by a set of isoforms that share common peptide sequences [Wiche et al., 1991]. A main feature of tau protein is the presence of a tandem of three repeated sequences in the fetal brain immature form of tau and four repeats in the adult brain tau,

Abbreviations used: C_{tau}, cytoplasmic tau; N_{tau}, nuclear tau; DMEM, Eagle's modified Dulbecco's medium; DTT, dithiothreitol; FITC: fluorescein-isothiocyanate conjugate; NOR, nucleolar organizing regions; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PC-tubulin, phosphocellulose-purified tubulin; SDS, sodium dodecyl sulfate.

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which are directly involved in tau interaction with the tubulin/microtubule system [Lewis et al., 1988; Maccioni et al., 1989].

Studies using cDNA probes have shown that tau expression is modulated during brain development [Drubin et al., 1984]. Different tau-like proteins have been localized in the brain [Cowan and Hirokawa, 1992; Cross et al., 1993; Maccioni and Cambiazo, 1995] and oligodendrocytes [LoPresti et al., 1995]. However, tau-like proteins have also been localized in non-neuronal cells [Maccioni and Cambiazo, 1995; Henriquez et al., 1995]. Tau proteins associate with different cytoplasmic cytoskeletal filaments [Correas et al., 1990; Cross et al., 1993] and the cell centrosome [Lu and Wood, 1993]. Tau proteins play a role in controlling the assembly of microtubules over the centrosomes [Cross et al., 1996]. Additionally, tau has been localized in the cell nucleus, and may be involved in nuclear processes [Loomis et al., 1990; Thurston et al., 1997]. All these findings, taken together with the capacity of tau to interact with several cellular proteins in a regulatory context [Maccioni et al., 1995], and to self-associate under pathological conditions [Pérez et al., 1996], point to the complexity of the functional organization of this cytoskeletal protein.

Nuclear tau has been identified in neuroblastoma cells by immunofluorescence and Western blot assays, whereas cDNA sequencing studies demonstrated that the neuroblastoma cells expressed a 2-kb message that contained the entire tau coding region [Wang et al., 1993]. Tau protein localization in isolated nuclei of brain neurons was also reported [Brady et al., 1995]. Recent studies point to tau localization within the nucleolus of interphase cells and the nucleolar organizing regions (NORs) of acrocentric chromosomes in human cells [Thurston et al., 1996]. However, the extraction, molecular characterization, and functional features of tau from nuclear compartments have not been elucidated. In this study, we isolated tau components from cytoplasmic and nuclear tau preparations of human fibroblast and Huh-7 cells. It is noteworthy that both the cytoplasmic and nuclear tau isolated from nonneuronal cells induced in vitro microtubule assembly. Studies suggest that tau from nonneuronal cells shares common structural and functional features with brain tau.

MATERIALS AND METHODS

Isolation of mRNAs and Reverse Transcriptase Polymerase Chain Reaction

To obtain mRNAs from human Huh-7 hepatoma and human fibroblast, cells were grown in Eagle's modified Dulbecco's medium (DMEM) supplemented with 10% fetal calf serum (Life Technologies, Bethesda, MD), in a 5% CO_2 atmosphere. Confluent 175-cm^2 flasks were used for each preparation (approximately 2.0×10^6 cells per flask). Cells were detached mechanically and the mRNA was isolated using a mRNA isolation kit (PE Applied Biosystem, CA), following the instruction of the manufacturer. Yields ranging from 0.2 to 0.5 mg/ml of mRNA were usually obtained. First strand cDNA was synthesized using 100 ng of mRNA and a reverse transcriptase polymerase chain reaction (RT-PCR) kit (Stratagene, La Jolla, CA). The PCR amplification reaction was carried out in the presence of 1.5 mM $MgCl_2$ and 10 pmol of each primers (see primers below). Other conditions were according to the Taq polymerase provider (PE Applied Biosystem, Foster City, CA). The cycle was designed as follows: 30 s at 94°C; 30 s at 55°C; 60 s at 72°C. The amplifying reaction was repeated 35 times.

The primers to amplify the four human tau binding repeats [Goedert et al., 1989] were named R1 through R4. The primer sequences were: R1: 5′ primer (5′-ctg cag aca gcc ccc gtg ccc-3′), 3′ primer (5′-ccc gcc tcc cgg ctg cac gaa-3′); R2: 5′ primer (5′-aag gtg cag ata att aat aag-3′), 3′ primer (5′-gcc gcc tcc cgg gac gtg ttt-3′); R3: 5′ primer (5′-agt gtg caa ata gtc tac aaa-3′), 3′ primer (5′-gcc acc tcc tgg ttt atg atg-3′); R4: 5′primer (5′-cag gtg gaa gta aaa tct gag-3′), 3′ primer (5′-tcc tcc gcc agg gac gtg ggt-3′). To amplify the amino terminal domain of tau, the following set of primers was used, and designated as TN: 5′ primer (5′-atg cac caa gac caa gag ggt-3′), 3′ primer (5′-gct gcg atc ccc tga ttt tgg-3′). Amplification products were resolved in a 2.5% agarose gel, and sized using a 100-bp DNA Ladder (Life Technologies).

Protein Electrophoresis and Western Blot Analysis

Enriched tau fractions from both the cytoplasm and the nucleus were characterized in 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) [Laemmli, 1970].

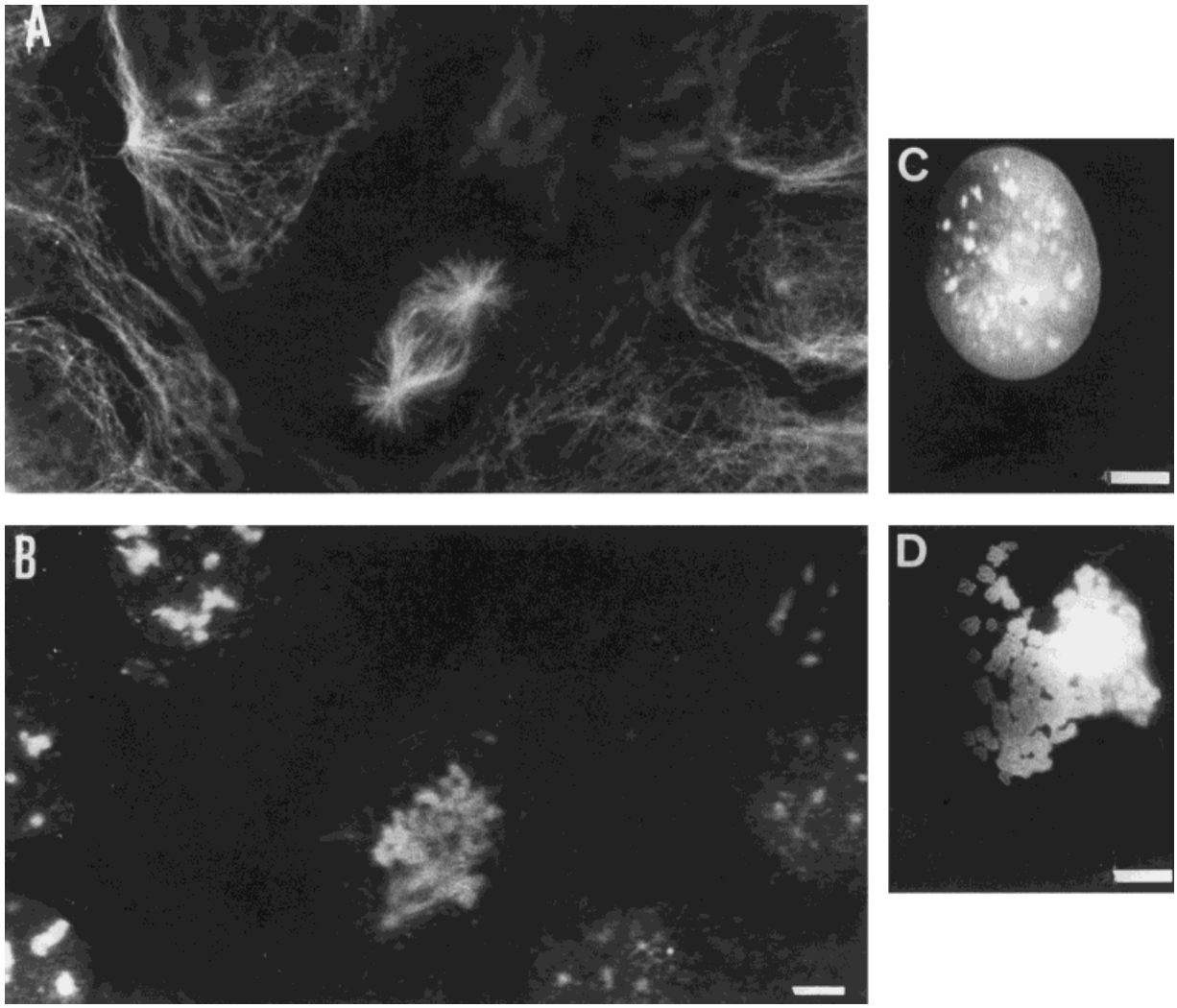

Fig. 1. Double immunofluorescence showing tau colocalization with chromosomes at metaphase. Huh-7 cells were extracted with Triton X-100, fixed as described in Materials and Methods and incubated with a monoclonal anti-tubulin antibody **(A)** or with the polyclonal antibody TRS.1.2 against native tau **(B)**. Rhodamine-conjugated second antibodies were used to reveal tau staining, whereas an FITC-conjugated immunoglobulin was used to visualize antitubulin staining. Simple immunofluorescences showing the tau distribution within an isolated nucleus **(C)** and chromosomes **(D)** from human Huh-7 hepatoma are also shown. A spotlike staining of the cell nucleus was evidenced. The anti-tau antibody also revealed the localization of tau with the chromosomal scaffold in these cells. Scale bars = 5 μm in B and 1 μm in C,D.

Gels were visualized with Coomassie blue staining, or blotted into nitrocellulose. Nitrocellulose membranes were prepared and probed as described [Cross et al., 1993] using either the monoclonal clone tau-2 (dilution 1:1,000) that recognizes human tau (provided by Dr. L. Binder), or the polyclonal anti-tau TRS.1.2 antibody directed to the first tau repeat, which has been characterized previously [González et al., 1995, 1998; Cross et al., 1996]. Blots were revealed with second antibodies conjugated with peroxidase.

Immunofluorescence

Human hepatoma cells, Huh-7, or human fibroblasts were grown over coverslips, and processed as described elsewhere [Henríquez et al., 1995]. The extracted cells were fixed in cold methanol for 15 min and coincubated for 2 h with the polyclonal TRS.1.2 anti-tau antibody (dil. 1:200) and a monoclonal antibody against β-tubulin (Sigma Chemical Co., St. Louis, MO) at a dilution of 1:500 for the experiment of Figure 1, or with the monoclonal an-

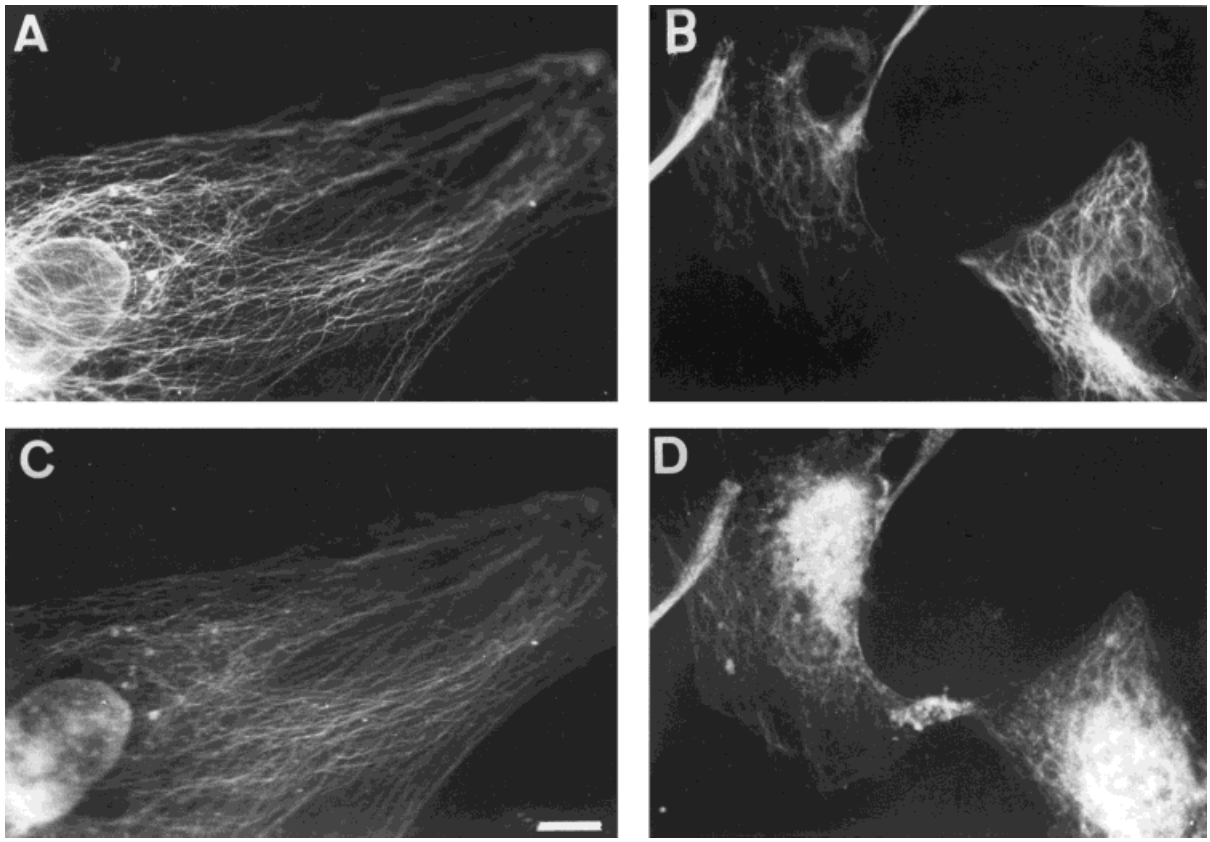

Fig. 2. Double immunofluorescences of interphase human fibroblasts **(A,C)** and Huh-7 hepatoma cells **(B,D),** using a polyclonal anti-tubulin (A,B) and the tau-2 monoclonal antibody (C,D). Rhodamine-conjugated second antibodies were used to reveal tubulin staining, whereas an FITC-conjugated immunoglobulin was used to visualize anti-tau staining. Scale bar = 5 μm.

tibody tau-2 (dil. 1:500) and a polyclonal antibody against bovine brain tubulin (dil. 1:200) in the experiment of Figure 2. For visualization of the immunostained material, coverslips were coincubated with fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG to localize the cellular components stained with the monoclonal antibody, and with rhodamine-(TRITC)-conjugated anti-rabbit IgG, at a 1:200 dilution, to localize components stained with the polyclonal antibodies.

Nuclei and Condensed Chromosomes Preparation

Cells were incubated with 10 μg/ml of nocodazole for 1 hour or with 0.6 μg/ml of colcemide for 6 h. Subsequently, cells were detached and swollen in a hypotonic 0.05 M KCl solution for 20 min at 37°C. The cell nucleus was spun onto coverslips at 200 *g* for 2 min and fixed in cold methanol (−20°C). These coverslips were used for immunofluorescence with the anti-tau antibodies. For staining of condensed chromosomes, the swollen-cell suspension was placed on a microscope glass slide and dried. Both preparations were subjected to immunofluorescence as described in the previous section.

Cytosolic and Nuclear Tau Isolation

Huh-7 cells were grown to confluence on DMEM supplemented with 10% fetal calf serum in 30 flasks of 175 cm^2 (3×10^8 total cells), whereas human fibroblasts were primary cultures originated from samples of scrotal epithelium from foreskin of healthy boys undergoing treatment for phymosis at the University Hospital. Cells were used for the preparation of both nuclear and cytoplasmic tau. Cells were detached mechanically, washed in saline phosphate, collected by low-speed centrifugation, and resuspended in the following hypotonic buffer: 10 mM HEPES-NaOH pH 7.9, 1.5 mM

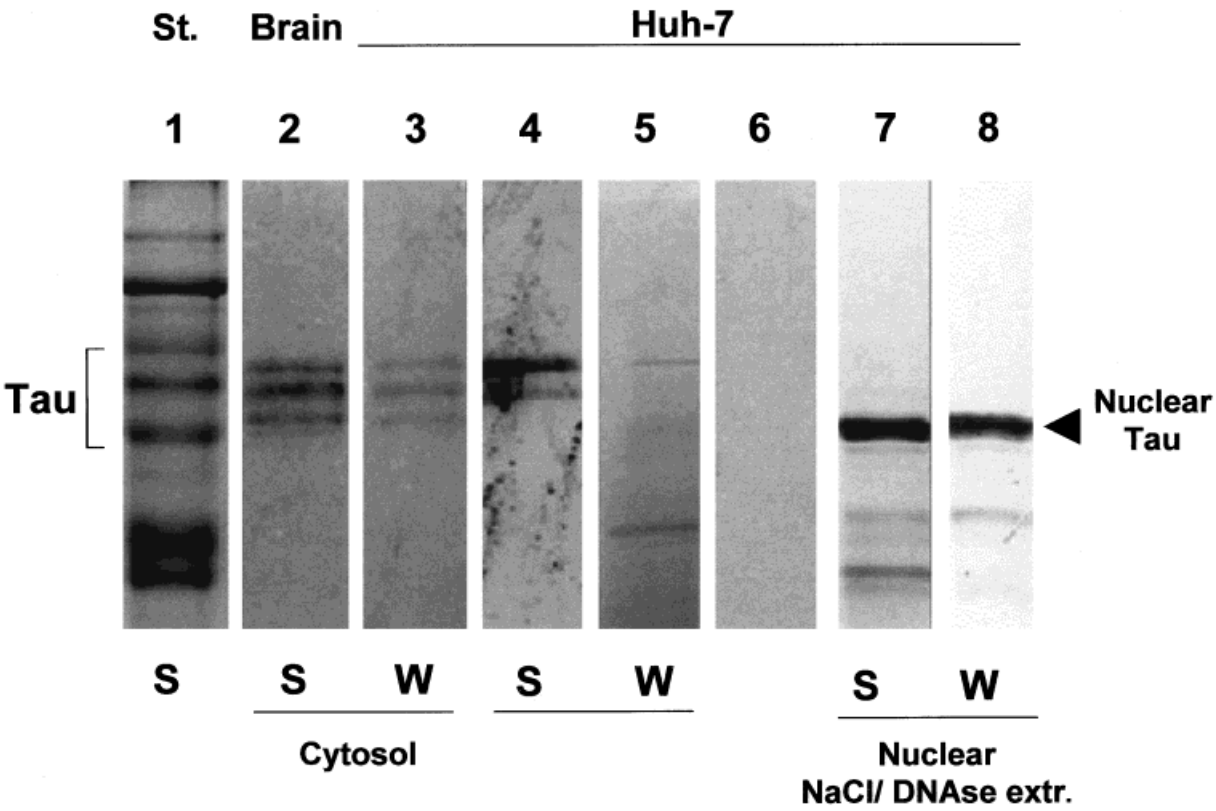

Fig. 3. Electrophoretic analysis of cytoplasmic and nuclear tau extracted from Huh-7 hepatoma cells. To analyze cytoplasmic tau, cells were extracted in hypotonic buffer 10 mM HEPES-NaOH, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT plus anti-proteases, incubated on ice, and adjusted to 0.5% Tween-20. The supernatants containing tau were subjected to a partial purification after boiling and perchloric acid extractions. Isolated tau (20 μg) was analyzed by gel electrophoresis (lane 3) and Western blot using tau-2 antibody (lane 4). The cell nuclei were isolated, subjected to extraction with Nuclear Buffer plus 0.1% NP-40, and subjected to boiling and the perchloric acid extraction procedure. The solubilized material containing several nuclear proteins was analyzed by electrophoresis (lane 5) and Western blots (lane 6). To analyze and purify nuclear tau, a nuclear fraction of human hepatoma Huh-7 cells was extracted with Nuclear Buffer in the presence of 0.1% NP-40. Samples were subjected to treatment with DNAase I followed by 1 M NaCl. The solubilized protein was analyzed by electrophoresis (lane 7) and by Western blots using the monoclonal tau-2 (lane 8). Lane 2 represents electrophoresis of controls using bovine brain tau (40 μg). Molecular weight markers with kilodaltons in parentheses are shown in lane 1: a2-macroglobulin (190), β-galactosidase (108), fructose-6-phosphate kinase (89), pyruvate kinase (77), fumarase (61), lactic dehydrogenate (41), triose phosphate isomerase (36). S, stained gels; W, Western blots. Additional details are indicated in Materials and Methods.

$MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol (DTT). Aprotinin (0.2 μg/ml), leupeptin (2 μg/ml), pepstatin (1 μg/ml), and 100 μg/ml PMSF were added to the buffer before use. Unless otherwise indicated, all buffers were supplemented with these antiproteases. The cell suspension was incubated for 15 min on ice, then 200–300 ml of Tween-20 per total volume in flasks was added to reach a final concentration of 0.5%. The suspension was vortex mixed vigorously for 10 s, and the nuclei were separated from the cytoplasmic fraction by centrifugation at 200 *g* for 10 min.

After cell extractions in the hypotonic buffer, soluble protein of the cytoplasmic fraction was subjected to further purification by boiling and perchloric acid extractions. Cytoplasmic fraction was boiled for 5 min, denatured proteins were collected by centrifugation, and the supernatants were extracted with 2.5 % perchloric acid [Farias et al., 1992]. After the separation of denatured proteins, the supernatants were neutralized by exhaustive dialysis against 5 × 1 l of 10 mM MES, pH 6.8. According to experimental conditions of Figure 3 (lanes 5,6), the isolated nuclear pellets were resuspended in 1 mM HEPES-NaOH, pH 7.9, 1.5 mM $MgCl_2$, 1 mM KCl, and 0.5 mM DTT and anti-proteases (Nuclear Buffer) plus 0.1% NP-40, incubated in ice for 15 min and digested

with 70 μg/ml DNAase I for 30 min at 37°C without further addition of 1 M NaCl. Samples were subjected to boiling and perchloric acid extraction, and aliquots of soluble were analyzed by electrophoresis.

In a parallel experiment for the nuclear tau purification according to experimental conditions of Figure 3 (lanes 7,8), nuclear pellets were washed two times in 0.25 M sucrose and resuspended in Nuclear Buffer plus 0.1% NP-40. Nuclear samples were then incubated in ice for 15 min and were further digested with 70 μg/ml DNAase I for 30 min at 37°C. Then 1M NaCl was added to the digested mix to give a final volume of 1 ml to allow tau solubilization. Samples were subjected to boiling and perchloric acid extractions as described above. Both neutralized cytoplasmic and nuclear fractions were concentrated by freeze drying and were used for the experiments. Protein concentration of tau, tubulin, and from crude preparations were determined as described previously [Maccioni et al., 1989].

In Vitro Tubulin Polymerization Assay

Pure tubulin was subjected to an in vitro polymerization assay as described [Gaskin et al., 1974]. Tubulin (1.4 mg/ml), was prewarmed with 90–120 μg of nuclear or cytoplasmic tau (obtained from human fibroblasts and Huh-7 cells) in 0.1 M MES, pH 6.8, 1 mM $MgCl_2$, 0.5 mM EGTA, and 1 mM DTT. After a few seconds, GTP was added to reach a final concentration of 1 mM. The reaction was carried out in 200 μl final volume, and the progression of polymerization was monitored by following the increase in the absorbency at 340 nm. To assess the nature of assembled polymers, the cuvettes with the polymerization reaction were placed on ice for 1 min, the absorbency was recorded immediately, and the reassembly was analyzed at 37°C. Routinely, electron microscopy was used to visualize the assembled polymers, as described by Maccioni et al. [1989].

RESULTS

Tau Identification in Neuronal and Nonneuronal Cell Lines

Using single-stranded cDNAs obtained from Huh-7 human hepatoma and human fibroblast as a template, discrete sequences containing the four repetitive binding domains on the adult human neuronal tau [Goedert et al., 1989; Andreadis et al., 1992] covering a protein domain from amino acids 256 through 367 were amplified. Each subdomain contained the respective binding repeats and regions of inter-repeat, and spanned a sequence of 31 amino acids or 93 nucleotides. As shown in Figure 4, after each sequence amplification using the specific pairs of primers, named as R1, R2, R3, and R4, specific amplification products of the expected size were obtained. To assess another tau segment upstream of the binding repeats, a 106 amino acid N-terminal domain, from amino acids 31 to 137 of the adult human tau sequence, was amplified using the specific pair of primers designated as TN (see Materials and Methods). As shown in Figure 4, corresponding to the cDNA from Huh-7 and human fibroblast, an amplification product of the expected size (300 nucleotides) was obtained after the PCR reaction for Huh-7 hepatoma cells and human fibroblasts. Controls carried out without the presence of RT in all the RT-PCR experiments gave negative results, supporting the specificity of the reaction. To assess whether the set of amplified sequences are an integral part of the conservative tau domain containing the tandem of repeats, a sequential amplification from the first segment through the fourth segment containing the binding repeats was carried out. For that purpose, the primer R1 read in the forward direction with the respective reverse primer was used in consecutive amplification reactions. The results showed an additive amplification of the tandem of four sequences from each of the tau segments, from 90 bp in the segment expressing a single repeat up to 360 bp for the four tandem repeats (data not shown). Peptide maps (n = 3) of protein digested with the endoprotease Lys-C, using the procedure of Cleveland et al. [1977], showed striking similarities in the peptide patterns of purified brain tau and cytoplasmic taulike protein isolated from human fibroblasts (data not shown).

Immunofluorescence Studies

The staining of the cell nucleus was demonstrated with different types of anti-tau antibodies. Both the monoclonal tau-2 and the polyclonal antibody TRS.1.2 against tau clearly stained the nucleus in human Huh-7 hepatoma and fibroblast cells. A double immunofluorescence with the monoclonal anti-tubulin antibody and the TRS.1.2 anti-tau antibody is

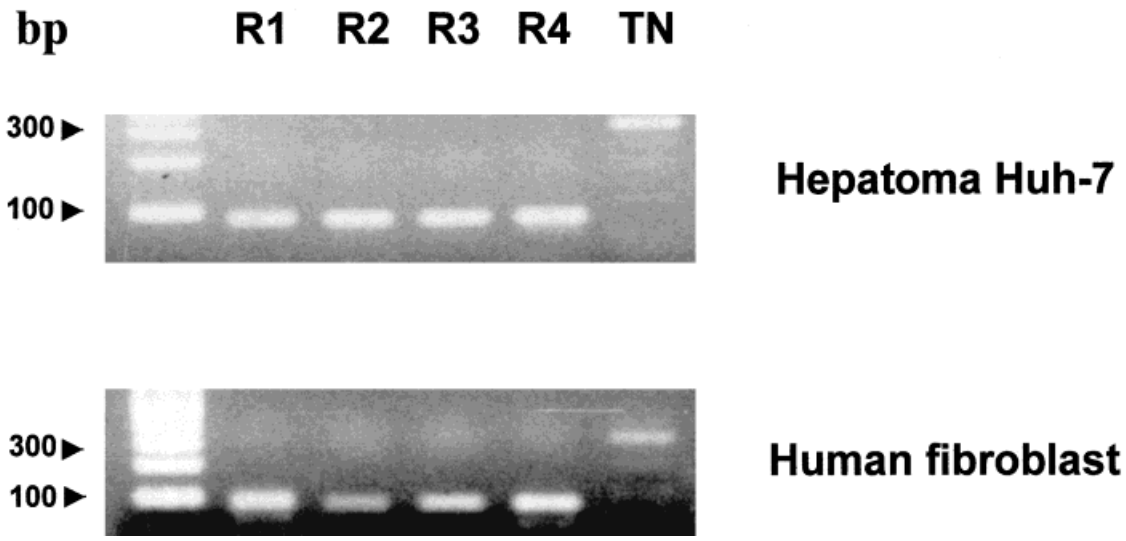

Fig. 4. Amplification of the repetitive binding motifs of tau from neuronal and nonneuronal cells in culture on the basis of reverse transcriptase polymerase chain reaction analysis. The amplification products resolved in 2.5% agarose gels from either hepatoma Huh-7 or human fibroblasts are shown. R1 to R4 denotes the amplification corresponding to segments from the first through the fourth repetitive sequence, and TN denotes amplification corresponding to an amino-terminal moiety of tau. To size the amplification products, 100 bp of a DNA ladder was used (left lane). Other details are in the Materials and Methods section.

shown in Figure 1. As expected, the anti-tubulin antibody stained some cytoplasmic microtubules in interphase cells and the mitotic spindle of a dividing cell (Fig. 1A). In contrast, as revealed by the rhodamine staining, the anti-tau antibody (Fig. 1B) stained preferentially the condensed chromosome in the dividing cell, whereas some discrete nuclear staining was evidenced with the anti-tau antibody in the surrounding interphase cells. To better visualize nuclear staining and tau distribution in the interphase cells, cell nuclei were isolated and further stained with the anti-tau antibodies. The isolated nuclei appeared clear and homogeneous. As shown in Figure 1C, tau displayed a discrete distribution within the interphase nucleus. To further assess localization of tau with condensed chromosomes, isolated chromosomes from these cells were also stained with the anti-tau antibody. In this study, tau appears to colocalize with the scaffold of the condensed chromosome with a rather discontinuous distribution (Fig. 1D). Similar findings were obtained by using the tau-2 monoclonal anti-tau antibody. Human fibroblasts at interphase were also double-stained with monoclonal tau-2 antibody and anti-tubulin antibody, thus revealing colocalization of the tau-like protein along microtubules (Fig. 2).

TABLE I. Flow Chart of Cytoplasmic Tau Purification From Hepatoma Huh-7 Cells

Step	Total protein (mg)	Purity (%)
1. Huh-7 cells	3×10^8 cells (30 flasks)	
2. Cell supernatant (1.2 ml)	12.8	
3. Boiling step	0.91	56[a]
4. Perchloric acid extraction	0.18	84[b]

[a]Purity was determined by densitometric scans of tau relative to the total protein in stained gels after SDS-polyacrylamide gel electrophoresis.
[b]The purified tau protein contained three isoforms, as analyzed by gel electrophoresis. Calculations considers the three tau components.

Tau Isolation From the Cytoplasm and Nucleus

Partially purified tau was obtained from human fibroblasts and Huh-7 cell lines. Tables I and II briefly summarize the steps used to isolate cellular tau on the basis of a fast and reliable purification procedure. A preparation enriched in cytoplasmic tau from these cells (>84% purity), containing the three tau isospecies, was obtained. Nuclear tau was purified from the isolated nuclei with >72% purity (Table 2). Minor contaminants were detected by

TABLE II. Flow Chart of Nuclear Tau Purification From Hepatoma Huh-7 Cells

Step	Total protein (mg)	Purity (%)
1. Huh-7 isolated cell nuclei	(3×10^8 cells)	
2. Nuclear extract (1.2 ml) (Nuclear Buffer plus NP-40/DNAase/1 M NaCl)	3.6[a]	
3. Boiling step	0.24	43
4. Perchloric acid extraction	0.09	72

[a]This value corresponds to soluble protein. Total protein in the nuclear extract was higher considering a fraction of insoluble material obtained after extraction.

the Coomassie staining procedure, as revealed in Figure 3. Interestingly, when the Huh-7 cellular extractions were carried out in hypotonic buffers with a moderate ionic strength, without addition of NaCl, tau was only found in the cytoplasmic fraction. Thus, lanes 3 and 5 in Figure 3 show protein profiles of the cytoplasmatic and nuclear protein fractions obtained after boiling and perchloric acid extractions, resolved in 8% SDS-PAGE and further stained with Coomassie brilliant blue. Lane 2 corresponds to Coomassie-stained cow brain tau used as a control, whereas lane 3 represents the staining pattern of cytosolic tau-like protein isolated from Huh-7 cells. Part of the same gel was transferred onto nitrocellulose and probed with the tau-2 antibody. As shown in lane 4, the antibody revealed the presence of a highly stained lower-migration band and a faster-migrating protein. Several analyses resolved the more stained band in a doublet, thus indicating three major isoforms of cytoplasmic tau. Minor differences in staining of these bands may reflect a differential sensitivity of cytoplasmic tau isospecies toward the antibody, among other interpretations. However, no staining with anti-tau antibody was revealed in the proteins extracted from the nucleus under low ionic strength conditions (Fig. 3, lane 6), although some protein bands were stained in the Coomassie-stained gels (Fig. 3, lane 5).

Because nuclear protein was not extracted under the previous conditions, protein extractions from isolated nuclei were carried out after digestion with DNAase I and addition of 1 M NaCl to the isolation buffer. As shown in Figure 3 (lane 7), Coomassie blue staining of the gel revealed a protein doublet obtained after the salt extraction from isolated nuclei of hepatoma Huh-7 cells. Interestingly, this tau doublet exhibited a faster migration than cytoplasmic tau, and migrated with an apparent molecular weight of 45 kDa. After transferring the proteins onto nitrocellulose, the membranes showed tau reactivity when probed with the monoclonal anti-tau antibody tau-2 (Fig. 3, lane 8). The polyclonal antibody against tau gave similar results. In all cases there was a specific reaction of the antibodies against the double protein bands around 45 kDa blotted in the nitrocellulose membrane. Similar results were obtained with human fibroblasts (data not shown).

In Vitro Polymerization of Tubulin

To examine whether the tau-enriched fractions obtained from the nucleus and cytoplasm were functional in promoting microtubule assembly, in vitro tubulin polymerization assays were performed. The turbidimetric assay was carried out using both cytoplasmic and nuclear fractions obtained from human fibroblasts and Huh-7 cells. Figure 5A shows the assembly progress in the presence of enriched tau fractions from both the cytoplasm (C_{tau}) and the nucleus (N_{tau}) from human fibroblasts. The control shows that tubulin by itself did not polymerize (Tub.). Tau fractions obtained from the cytoplasm (C_{tau}) and the nucleus (N_{tau}) of hepatoma Huh-7 cells were also efficient in promoting tubulin assembly (Fig. 5B). Because these preparations were highly enriched in tau, the possibility that minor contaminants on the tau samples may account for the effects in promoting tubulin assembly is unlikely. In both assembly studies, electron microscopy confirmed that the polymers formed were normal microtubules as evidenced by electron microscopy (Fig. 6 A,B). This was confirmed by cold reversibility of preformed polymers from samples obtained from the in vitro assays.

DISCUSSION

Tau protein was initially characterized as a neuronal protein binding specifically and in a reversible fashion to microtubules [Matus, 1988]. However, previous studies have demonstrated the presence of tau-like proteins in a

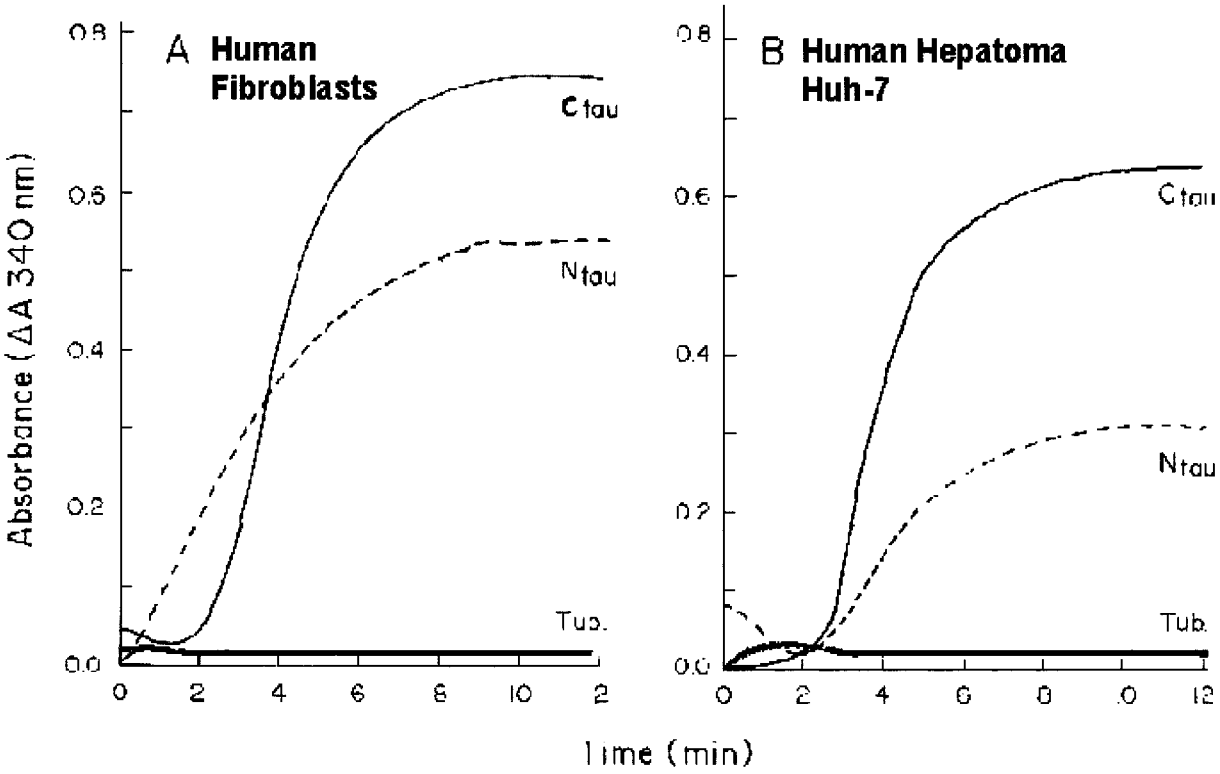

Fig. 5. Microtubule assembly induced by cytoplasmic and nuclear tau. **A:** Samples of 0.2 ml of PC-tubulin (1.4 mg/ml) were induced to polymerize in the presence of 90 μg of partially purified cytoplasmic tau (solid line, C_{tau}) or nuclear tau (dashed line, N_{tau}) from human fibroblasts. Control tubulin alone in the absence of tau components was also analyzed (Tub.). The time course of assembly was monitored by the turbidity change at 340 nm after the addition of 1 mM GTP to samples in 0.5-ml cuvettes. **B:** Equivalent samples of PC-tubulin were allowed to assemble in the presence of either 90 μg of C_{tau} or N_{tau} from hepatoma Huh-7 cells, and the assembly monitored as indicated above. Additional details are indicated in Materials and Methods.

set of nonneuronal cells in culture [Cross et al., 1993] by using several specific anti-tau antibodies. Additionally, there is cumulative evidence that tau [Cross et al., 1993; Henríquez et al., 1995; Maccioni and Cambiazo, 1995] as well as other microtubule-associated proteins (MAPs) [Cunningham et al., 1997] interacts with microtubules and stress fibers. The association of tau with neurofilaments [Heinmann et al., 1985; Miyata et al., 1986] and vimentin filaments [Capote and Maccioni, 1997] has been also reported. Thus, the tau-like components may play a role in mediating cellular interactions among these components of the cytoskeleton in neuronal and nonneuronal cells [Griffith and Pollard, 1982; Cross et al., 1993; Knowles et al., 1994; Maccioni and Cambiazo, 1995]. Recent studies have shown that a tau-like protein plays a role in the outgrowth of microtubules from centrosomes [Cross et al., 1996]. These studies, together with the reports on nuclear tau components [Loomis et al., 1990], indicate that tau is a multifunctional protein with a major cellular relevance.

In the present study, we found that the tau-like components identified in several cultured cells share common structural and functional features with the known brain tau [Goedert et al., 1989]. In addition, we have purified nuclear tau from isolated nuclear preparations using high ionic strength (1 M NaCl) buffers. This has enabled us to further characterize nuclear tau. Using specific sets of primers, we were able to demonstrate that at least neuronal htau40 variant is present in nonneuronal cells, such as human hepatoma Huh-7 cell line and human fibroblasts. The repetitive sequences that were amplified are unique for the brain tau [Kosik et al., 1989]. Brain tau contains the small binding motifs also found in neuronal MAP-2 [Lewis et al., 1988; Lee, 1990]. Interestingly, the present RT-PCR reaction data sup-

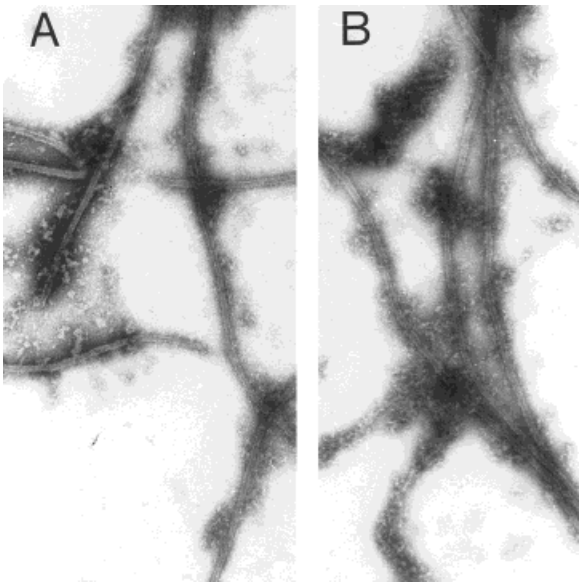

Fig. 6. Electron microscopy of microtubules obtained from in vitro assembly assays described in Figure 5. Tubulin assembly was promoted with cytoplasmic tau protein purified from human fibroblasts **(A)** or from the Huh-7 human hepatoma cells **(B)**.

port the hypothesis that the putative tau proteins from nonneuronal cells share common binding repeats with the brain tau. This observation support several reports that have demonstrated a role of tau in microtubule interactions in a variety of cell types [Cross et al., 1993; Knowles et al., 1994; Henríquez et al., 1995]. Primers that allowed amplification of segments of cDNAs, encoding for tau segments that include the interrepeat domains along the binding motifs, were used in this study. In this context, it is worth pointing out that the developmentally regulated interrepeat region is functionally involved in modulating the capacity of tau to induce microtubule assembly [Goode and Feinstein, 1994]. Other reports also indicate that flanking regions downstream of the binding repeats appear to be critical for an effective interaction of the binding motifs on tau [Gustke et al., 1994].

To assure that we have identified nonneuronal taulike components that are molecularly similar to neuronal tau, a set of specific primers were designed based on the human neuronal tau sequence [Goedert et al., 1989; Andreadis et al., 1992], to amplify a domain (106 amino acids) from the N-terminal region of this protein. As seen in Figure 4, a fragment of the expected size was found when using cDNAs from Huh-7 or human fibroblast as templates. These results strongly suggest that at least some neuronal tau isotypes are present in nonneuronal cell lines. These data support previous evidence showing that the cytosolic nonneuronal tau components contain a set of isoforms with apparent molecular weights of approximately 62 kDa, as inferred from the calibration curves, in agreement with that estimated for the neuronal tau [Kanai, 1989; Goode and Feinstein, 1994]. The present data on the capacity of the nonneuronal component to efficiently induce microtubule assembly of purified brain tubulin provide a functional support on the role of tau-like proteins in nonneuronal cells.

The results presented in this article further our understanding of the putative role of taulike proteins within the nucleus. A discrete distribution of tau in the interphase nucleus was shown. In a set of distinct experiments, different anti-tau antibodies stained the cell nucleus in various cell lines and primary cultured cells, with a pattern similar to that observed in Figure 1. Moreover, bright spots were usually observed in the staining of nuclear tau. Nuclear tau staining has been consistently revealed in several other reports [Cross et al., 1996; Thurston et al., 1996]. Our data using two different anti-tau antibodies also show that nuclear tau colocalizes with the chromosomal scaffold. This observation highlights the association of tau with nuclear components, whereas previous reports only indicated a discrete distribution of tau components. Furthermore, in the present studies, isolated chromosomes and cell nuclei preparations were used to avoid any possible interference with cytoplasmic tau, again supporting the finding that there is nuclearly localized tau that associates with the chromosomes.

In addition to the immunological analysis of tau within the cell nucleus, we determined that these proteins also share common physical and functional properties with the neuronal tau. For this purpose, a partial isolation of nuclear and cytoplasmic tau from nonneuronal cell lines was carried out. Boiling followed by a dilute perchloric acid extraction allowed us to obtain preparations enriched in cytoplasmic tau from human hepatoma Huh-7 and fibroblasts cells. It is noteworthy that this procedure was sufficient to extract soluble cytoplasmic tau, but not the nuclear tau. Under these

conditions, nuclear tau remained insoluble. However, after incubation with 1 M NaCl and DNAase before the boiling and perchloric acid steps, soluble nuclear tau was extracted from the nucleus.

The presence of at least three isoforms of cytoplasmic tau from nonneuronal cells and two isoforms in nuclear tau suggest that these isoforms could be functionally significant in both the cytoplasm and the cell nucleus. As for the nuclear tau, two major isoforms were detected with a similar electrophoretic migration, even though it is possible that each of these bands may contain various isospecies that comigrate in a normal SDS-PAGE. These isoforms were strongly stained by the monoclonal tau-2 and polyclonal antibodies. However, nuclear tau displayed a higher mobility as compared with cytoplasmic tau. The apparent molecular weights estimated for both tau variants are in agreement with the true molecular weight predicted from human tau sequence data [Goedert et al., 1989; Andreadis et al., 1992]. Both nuclear and cytoplasmic tau from nonneuronal cells contained the tandem of four repeats; therefore, the differences in electrophoretic migration were not a consequence of extended repeats. The faster migration of the set of nuclear tau isoforms may be explained by a lower molecular weight, or on the basis of a decreased level of postranslational modifications, a finding that remains to be elucidated. As a matter of fact, nuclear tau isoforms exhibited electrophoretic mobilities comparable with the fastest migrating form of the cytoplasmic tau.

The interesting observation that high ionic strength buffers were necessary to release tau from the nucleus indicates that it is tightly bound to chromatin [Thurston et al., 1996]. However, we cannot discard the possibility that it could be bound to RNA at the nucleolar organizing centers [Thurston et al., 1996]. However, because nuclear tau seems to be distributed as a part of the scaffold of the condensed chromosome, it is likely that the protein remains associated with DNA. As indicated above, no previous reports point to the functional aspects of nonneuronal tau species. In the present studies in human nonneuronal cells, the fact that the protein can induce in vitro polymerization of pure tubulin is noteworthy. This assay and electron micrographs clearly indicate that the enriched tau fraction obtained from cultured cells specifically interact with tubulin, thus promoting its assembly into microtubular polymers. The cytoplasmic fraction promoted assembly to a higher extent. This difference may be caused by postranslational modifications, an aspect that needs further investigation. In all cases, microtubules assembled with either nuclear or cytoplasmic tau were cold sensitive and formed normal microtubules. Our studies indicate that cytoplasmic and nuclear forms of tau are structurally and functionally similar to the known brain tau protein. These results together with previous studies [reviewed in Maccioni and Cambiazo, 1995] indicate that tau is a multifunctional protein. At the cytoplasmic compartment, tau appears to play a role in modulating microtubule stability [Cowan and Hirokawa, 1992] and in mediating interactions between the different components of the cytoskeletal network [Cross et al., 1993; Henríquez et al., 1995], whereas nuclear tau could play a structural role in the nuclear architecture [Stein et al., 1998], observations that demand an in-depth evaluation. These facts could have important implications for our understanding of Alzheimer′s disease, because abnormally modified tau is a major molecular protagonist in this disease [Grundke-Iqbal et al., 1986]. These results strongly suggest that an abnormal function of tau, or changes in tau distribution among cellular compartments, e.g., nuclear, cytoplasmic, and so on, could have profound effects in the cell physiology in different cell types.

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