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Tau protein binds to pericentromeric DNA: a putative role for nuclear tau in nucleolar organization

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Summary

The microtubule-associated tau protein participates in the organization and integrity of the neuronal cytoskeleton. A nuclear form of tau has been described in neuronal and non-neuronal cells, which displays a nucleolar localization during interphase but is associated with nucleolar-organizing regions in mitotic cells. In the present study, based on immunofluorescence, immuno-FISH and confocal microscopy, we show that nuclear tau is mainly present at the internal periphery of nucleoli, partially colocalizing with the nucleolar protein nucleolin and human AT-rich α-satellite DNA sequences organized as constitutive heterochromatin. By using gel retardation, we demonstrate that tau not only colocalizes with, but also specifically binds to, AT-rich satellite DNA sequences apparently through the recognition of AT-rich DNA stretches. Here we propose a functional role for nuclear tau in relation to the nucleolar organization and/or heterochromatinization of a portion of RNA genes. Since nuclear tau has also been found in neurons from patients with Alzheimer's disease (AD), aberrant nuclear tau could affect the nucleolar organization during the course of AD. We discuss nucleolar tau associated with AT-rich α-satellite DNA sequences as a potential molecular link between trisomy 21 and AD.

Key words: Nuclear tau protein, Satellite DNA, Pericentromeric heterochromatin, Nucleolus, NOR

Introduction

The microtubule-associated tau protein is involved in the polymerization and stabilization of neuronal microtubules, thereby participating in the organization and integrity of the cytoskeleton (Drechsel et al., 1992; Johnson and Hartigan, 1999). The functions of tau are regulated by post-translational modifications, among which the phosphorylation is one of the most well studied (Trinczek et al., 1995), because hyperphosphorylated variants of tau constitute one of the hallmarks of Alzheimer's disease (AD) (Grundke-Iqbal et al., 1986; Maccioni et al., 2001a). Tau, as well as other eukaryotic proteins, displays a wide functional diversity. Ultrastructural localization studies identified tau associated with ribosomes and somatodendritic compartments in certain areas of the central nervous system (Papasozomenos and Su, 1991). More recently, another non-microtubular localization of tau was identified in the nucleus of neuronal and non-neuronal cells (Lu and Wood, 1993; Lambert et al., 1995; Thurston et al., 1996; Cross et al., 2000). Some tau species have also been described associated with chromatin fractions (Greenwood and Johnson, 1995), or interacting with the plasma membrane (Brandt et al., 1995) and with the non-receptor tyrosine kinase Fyn (Lee et al., 1998). Even though tau is present mainly in neuronal cells, it has also been observed in non-neuronal cells, with a nucleolar localization in HeLa cells, non-transformed human fibroblasts and lymphoblasts (Thurston et al., 1996), as well as in human Huh-7 hepatoma cells (Cross et al., 2000). In all these non-neuronal cell types several isoforms of tau were detected with Tau-1 and Tau-5 antibodies. Purification of nuclear tau from Huh-7 hepatoma cells as well as from fibroblasts demonstrated that nuclear tau from non-neuronal cells share with neuronal tau the capacity to promote tubulin polymerization in vitro.

In mitotic cells, nuclear tau is associated with the nucleolar organizer regions (NORs), whereas in interphase cells tau displays a nucleolar localization at the dense fibrillar regions (Loomis et al., 1990; Thurston et al., 1996). The nucleus in eukaryotic cells is functionally compartmentalized. The best example of such compartmentalization is the nucleolus, where multiple loci of different chromosomes contribute to the formation of a functional nuclear compartment. Cohesion of the different NORs has an essential role in the formation of the nucleolus, which is the site of ribosomal biogenesis (Sullivan et al., 2001). NORs are present in the short arms of acrocentric chromosomes and correspond to arrays of GC-rich ribosomal DNA (rDNA) repeats immediately juxtaposed to the sequences that constitute the centromeres of acrocentric chromosomes. Centromeres of mammalian chromosomes are a specialized locus responsible for chromosome segregation during mitosis and meiosis (Rudd and Willard, 2004). They consist of tandemly repeated satellite DNA sequences (α-satellite in human cells and γ-satellite in murine cells), organized as

constitutive heterochromatin (Karpen and Allshire, 1997; Csink and Henikoff, 1998; Murphy and Karpen, 1998). The distribution and organization of these centromeric sequences at the periphery of the nucleolus as well as that of active and inactive rDNA within the nucleolus do not occur in a random fashion. Variations are observed depending on the cell type and cell metabolic activity as well as on the stage of the cell cycle. In neuronal cells, centromeres are found clustered and attached as large aggregates to the surface of the nucleolus, so that the majority of neuronal nucleoli contain a characteristic ring of nuclear heterochromatin. Large arrays of inactive methylated rDNA repeats have been observed clustered inside the nucleoli at the periphery, close to the ring of centromeric heterochromatin (Manuelidis, 1984; Akhmanova et al., 2000). Although the link between the nucleolus and centromeric heterochromatin has not been clearly defined, it has been suggested that centromeric perinucleolar heterochromatin and the associated proteins have an important role in the regulation of the nucleolar structure (Carmo-Fonseca et al., 2000).

It has been recently demonstrated that tau can bind to double- and single-strands of DNA (Hua and He, 2003; Krylova et al., 2005) in an aggregation-dependent and phosphorylation-independent manner (Hua and He, 2002). This interaction protects DNA from denaturation in vitro (Hua and He, 2003). Some of the most recent observations describe the capacity of tau protein to induce a conformational change on the DNA at a ratio of one tau molecule per 700 bp of DNA. This observation suggests that the mass ratio of tau to DNA is important during the complex formation (Qu et al., 2004).

In an attempt to gain an insight into the functionality of nucleolar tau, in this study we analyzed the capacity of tau to interact with pericentromeric heterochromatin. Using immunofluorescence, immunoFISH, confocal microscopy and gel retardation techniques we demonstrated that nucleolar tau partially colocalizes with human pericentromeric α-satellite DNA sequences in human fibroblasts, lymphoblasts and HeLa cells. Moreover, tau directly binds to human α- and murine γ-satellite sequences forming specific protein-DNA complexes with these pericentromeric DNA sequences. In this report, we discuss the possibility that tau, through its association with satellite DNA sequences, participates in the organization of the nucleolar structure and/or in the heterochromatinization of a fraction of ribosomal genes.

Results

Nuclear tau localizes at the border of the nucleolus

It has been shown that lysine residue K9 of histone H3 is methylated in centromeric satellite DNA sequences organized as constitutive heterochromatin (Henikoff, 2000; Richards and Elgin, 2002; Sims et al., 2003). Anti-dimethylK9H3 antibodies bind to constitutive heterochromatin, and they are excluded from the center but not the periphery of the nucleolus (Peters et al., 2003). We have used immunofluorescent confocal microscopy to analyze the distribution of tau protein compared with dimethylK9H3 in interphase human fibroblasts and HeLa cells. For all fluorescence experiments we used the monoclonal Tau-1 antibody that recognizes an unphosphorylated tau epitope at amino acids 189-207. Tau-1 detects small amounts of tau and has been used previously to study nuclear tau (Loomis et al., 1990; Wang et al., 1993; Thurston et al., 1996).

As shown in Fig. 1, tau was clustered as small dots in non-transformed fibroblasts, whereas it appeared as larger strongly fluorescing spots in transformed HeLa cells. As denoted in Fig. 1C′,F′, in both cases tau appeared to be predominantly present at the border of the nucleolus either partially overlapping (in the case of human fibroblasts) or in close proximity to (in the case of HeLa cells) DNA labeled by anti-dimethylK9H3 antibodies. Results obtained with an antibody directed against nucleolar protein nucleolin confirmed the nucleolar localization of tau in human fibroblasts and HeLa cells (Fig. 2). As previously described (Dranovsky et al., 2001), nucleolin appeared concentrated at the internal periphery of nucleoli of interphase cells. In both cell types, a fraction of tau colocalized with nucleolin at the interior of the nucleolus (Fig. 2G,J), this colocalization being more important at the interior periphery of the nucleolus especially in the case of non-dividing primary fibroblasts (Fig. 2E-G) compared with proliferating HeLa cells (Fig. 2H-J).

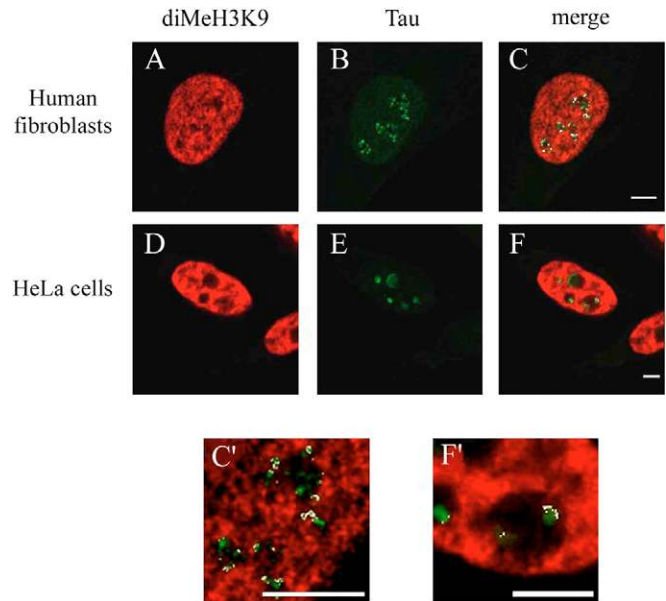

Fig. 1. Nuclear tau localizes at the border of the nucleolus. Colocalization of endogenous tau with heterochromatin was analyzed by immunofluorescent technique and confocal microscopy in human skin fibroblasts and HeLa cells. Each row represents a single optical section of the same nucleus. Left panels (A,D) correspond to diMeH3K9 distribution revealed with rabbit anti-diMeH3K9. Middle panels (B,E) show subnuclear tau distribution detected with the Tau-1 monoclonal antibody. Merged images of tau with diMeH3K9 are shown on right panels for human fibroblasts (C) and HeLa cells (F) with double-labeled pixels displayed in white. (C′,F′) Enlarged views of C and F showing the overlap between tau and diMeH3K9 at the nucleolus in white. Scale bar, 5 μm.

A fraction of nucleolar tau colocalizes with pericentromeric α-satellite DNA

Alpha-satellite DNA is a repetitive DNA sequence present at the centromere region of each normal human chromosome and consist of megabases of ~171-bp monomers arranged either in a highly homogeneous, multimeric organization or in a more heterogeneous monomeric form lacking this higher order periodicity (Willard, 1991; Warburton et al., 1996). In order to determine if the partial colocalization of tau with diMeK9H3

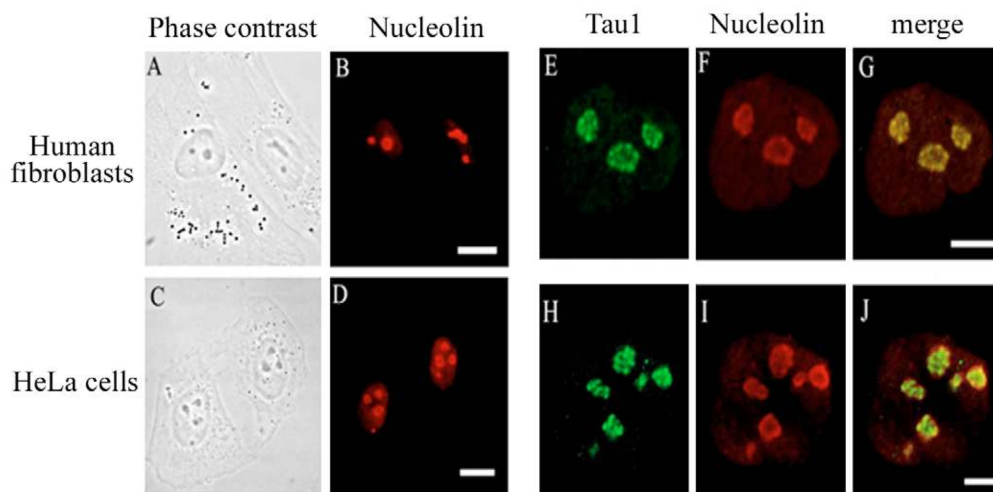

Fig. 2. Nuclear tau partially colocalizes with nucleolin. Colocalization of endogenous tau with nucleolar protein nucleolin was analyzed by immunofluorescence and confocal microscopy in human skin fibroblasts and HeLa cells. Each row represents a single optical section of the same nucleus. Upper panels (A,B), show phase-contrast image (A) and fluorescent nucleolin distribution (B) in human fibroblasts. Lower panels (C,D), show phase-contrast image (C) and fluorescent nucleolin distribution (D) in HeLa cells. (E,H) Tau distribution revealed with Tau-1 monoclonal antibody. (F,I) Subnuclear nucleolin distribution detected with nucleolin C23 polyclonal antibody. Merged images of tau with nucleolin are shown on the right for human fibroblasts (G) and HeLa cells (J). Bars, 10 μm (A-D); 5 μm (E-J).

observed in human fibroblasts corresponds to a partial colocalization of tau with human α-satellite DNA, we used immunoFISH and confocal microscopy. In Fig. 3 we show the immunoFISH results obtained in human non-transformed fibroblasts (Fig. 3A-C), lymphoblasts (Fig. 3D-F) and in transformed HeLa cells (Fig. 3G-I). Tau protein appeared to be not randomly distributed but rather displayed a spatial organization clustered as small dots in the case of non-transformed human fibroblasts and lymphoblasts, or as larger spots in the case of HeLa cells (Figs 1, 2). In all cases we noticed a distinct relationship between tau and clusters of α-satellite-labeled pericentromeric DNA, with tau being systematically very close to pericentromeric heterochromatin of non-transformed cells, as well as partially overlapping with a portion of fluorescently labeled α-satellite DNA in transformed and non-transformed cells.

In Fig. 4 we show non-confocal conventional fluorescence microscopy images of human fibroblasts (Fig. 4A-E) or HeLa cells (Fig. 4F-J). The merged images of Hoechst 33258 and tau labeling (Fig. 4D,I) confirm the nucleolar localization of tau in these two cell types. The merged images of tau and α-satellite DNA localization indicate that in all cases, tau (green) appears to be at the center of a 'group' of neighboring clusters of centromeric α-satellite sequences (red), with tau partially colocalizing with these sequences.

Tau directly interacts with human α-satellite DNA sequences

The previously described capacity of tau to interact with DNA

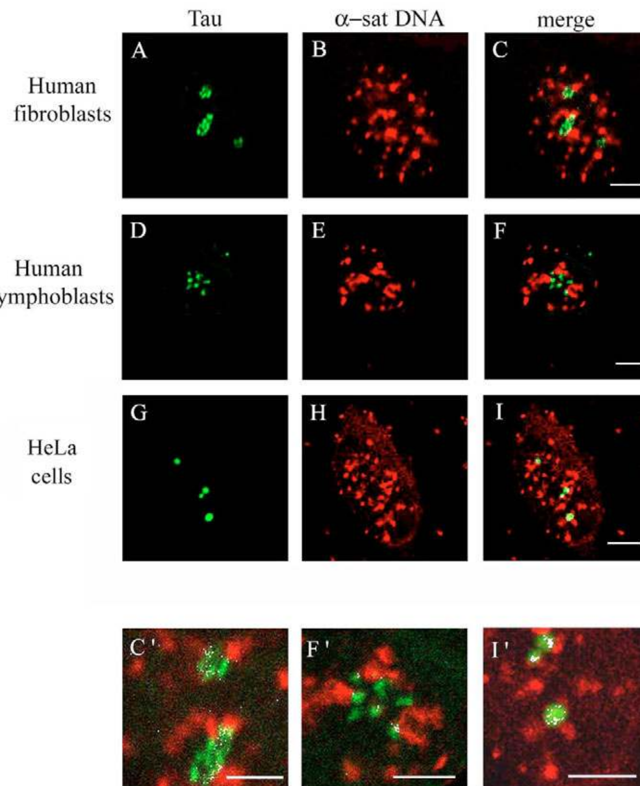

Fig. 3. Tau partially colocalizes with pericentromeric α-satellite DNA. Colocalization of endogenous tau with pericentromeric α-satellite DNA was studied by immuno-FISH and confocal microscopy in human skin fibroblasts, human lymphoblasts and HeLa cells. Each row represents a single optical section of the same nucleus. Left panels (A,D,G) correspond to subnuclear tau distribution detected with the Tau-1 monoclonal antibody. Middle panels (B,E,H) show α-satellite DNA repeats distribution revealed by FISH using fluoRED labeled α-satellite p82H plasmid as a probe. Merged images of tau with pericentromeric α-satellite DNA are shown on right panels for human skin fibroblasts (C), human lymphoblasts (F) and HeLa cells (I) with double-labeled pixels displayed in white. C′,F′,I′ are enlarged views of C,F,I respectively. Bars, 5 μm.

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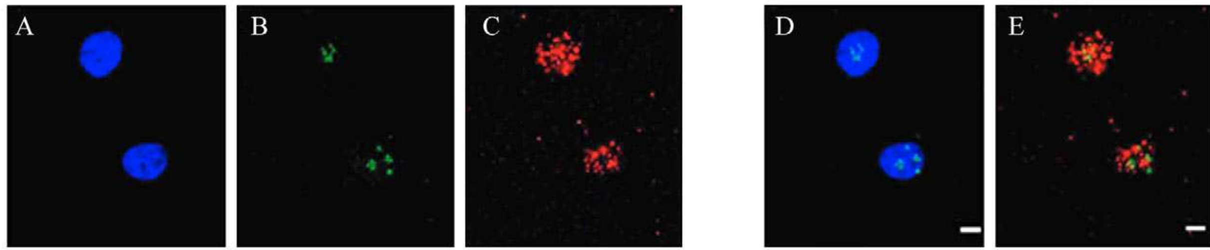

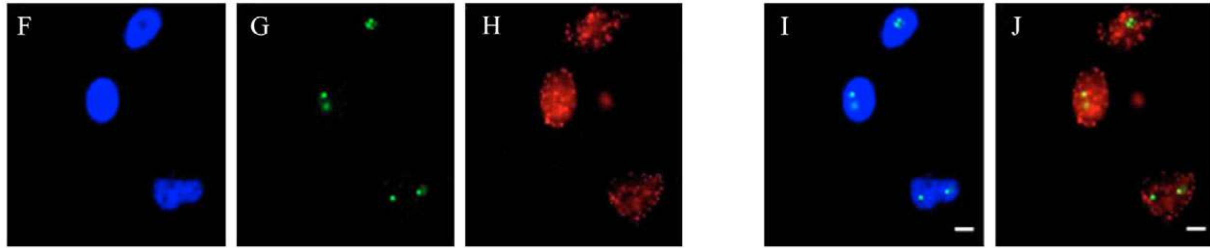

Fig. 4. Tau localizes inside the nucleolus and partially colocalizes with pericentromeric α-satellite DNA. Non-confocal conventional fluorescence microscopy was used to analyze total DNA distribution and colocalization of tau with α-satellite DNA in human skin fibroblasts and HeLa cells. (A and F) Total DNA distribution in cells revealed with Hoechst 33258. (B,G) Subnuclear tau distribution detected with the Tau-1 monoclonal antibody. (C,H) Distribution of α-satellite DNA repeats revealed by FISH using α-satellite p82H plasmid as a probe. Merged images of tau with Hoechst 33258 are shown in panels D (human skin fibroblasts) and I (HeLa cells) and merged images of tau with pericentromeric α-satellite DNA are shown in panels E (human skin fibroblasts) and J (HeLa cells). Bars, 10 μm.

(Hua and He, 2003; Krylova et al., 2005; Hua and He, 2002) and the partial colocalization of tau protein with α-satellite DNA observed in Figs 3 and 4, led us to investigate the capacity of tau to directly bind and form protein-DNA complexes with human α-satellite DNA sequences. In an attempt to address this question, we performed electrophoretic mobility-shift assays with purified tau protein and a 700 bp radioactively labeled DNA probe containing α-satellite sequences. The tau protein used in these gel-shift experiments was prepared from normal bovine brain and purified as described in the Materials and Methods. The degree of purity of tau protein, especially the absence of contamination with other microtubule-associated proteins (MAPS), was evaluated by SDS-PAGE and western blot. All the bands observed after Coomassie Blue (CB) staining correspond to tau protein as detected with the Tau-5 monoclonal antibody (Fig. 5A).

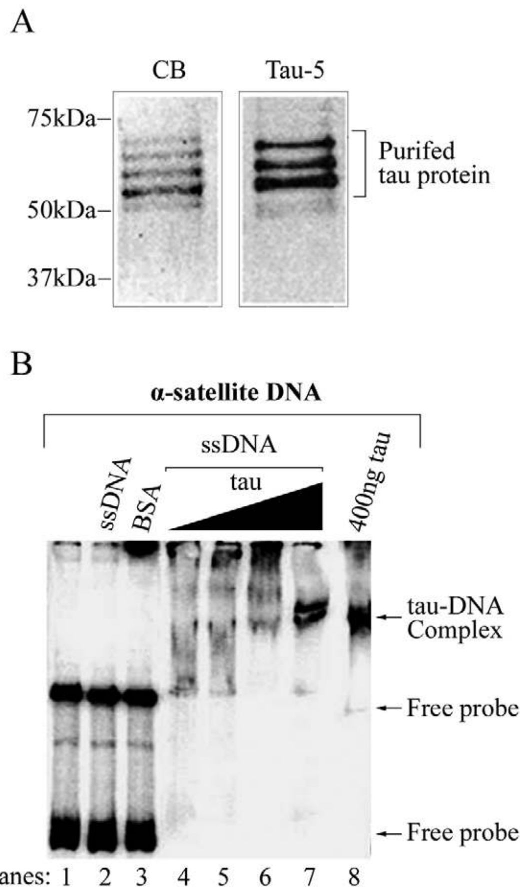

Fig. 5. Tau protein associates with α-satellite DNA. The association of tau protein with α-satellite DNA was analyzed by electrophoretic mobility shift assay (EMSA). (A) Purified tau protein from bovine brain was resolved by 12% SDS-PAGE and then stained with Coomassie Blue (CB) or immunodetected with Tau-5 monoclonal antibody to confirm the absence of contaminants. (B) Different concentrations of purified tau protein (100 ng, 200 ng, 300 ng and 400 ng) were incubated with the ^{32}P-labeled α-satellite DNA probe of 700 bp. The incubations were performed in the presence of 0.5 μg unlabeled sonicated salmon sperm DNA (ssDNA) used as random non-sequence specific competitor DNA in 50 mM NaCl buffer. The EMSA gels were dried and radioactivity was visualized using PhosphorImager and the computer program ImageQuant. The arrows indicate the shift observed for the labeled probe owing to the interaction of the probe with tau and the free probe.

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For EMSA experiments we used four different concentrations of tau protein, each one of the four samples was incubated with a constant amount of radioactively labeled α-satellite probe, in the presence of an excess of unlabeled sonicated salmon sperm DNA as random non-sequence specific DNA competitor. A substantial retardation of the probe was observed in the presence of 100 ng tau, corresponding to the lowest amount of tau used here (Fig. 5B, lane 4). At 100, 200 and 300 ng tau, retardation of the free probe coincided with the formation of a tau-α-satellite complex that migrated as a smear. At 400 ng tau, the shift of the probe was evident and the formation of a highly retarded tau-α-satellite complex was clearly observed (Fig. 5B, lane 7). As a control, such a shift was not observed when the α-satellite DNA probe was incubated with equivalent amounts of BSA (Fig. 5B, lane 3). Also, the pattern of the formation of the tau-α-satellite complex remained unchanged regardless of the presence or absence of unlabeled ssDNA as competitor (Fig. 5B, compare lane 7 with lane 8). Overall, the results obtained here demonstrated that tau was able to form protein-DNA complexes with human α-satellite DNA sequences, and strongly suggested that tau could be interacting with these sequences with a certain specificity.

Tau forms protein/DNA complexes with murine γ-satellite DNA sequences

Considering the fact that tau protein was able to form a protein-DNA complex with human α-satellite-DNA sequences, we investigated the capacity of tau to bind to the closely related murine pericentromeric γ-satellite-DNA sequences. Major murine satellite repeats (corresponding to γ-satellite sequences) share with the human α-satellite sequences the same centromere localization, the same (A+T)-rich base content and the same heterochromatin structure (Joseph et al., 1989; Choo, 1997; Craig et al., 2003; Guenatri et al., 2004). In order to investigate the capacity of tau to interact with murine γ-satellite DNA, we performed EMSA experiments under the same conditions as those used in Fig. 5B except that a 936 bp radioactively labeled γ-satellite DNA fragment, containing four γ-satellite repetitive units, was used as a probe instead of human α-satellite DNA used in Fig. 5B.

As previously observed with the human α-satellite DNA, a substantial retardation of the murine γ-satellite DNA probe was observed at the lowest (100 ng) concentration of tau protein (Fig. 6A, lane 4), coinciding with the formation of a smear of tau-α-satellite complex. The shift of the murine γ-satellite probe was total at 400 ng tau (Fig. 6A, lane 7). At this concentration, a highly retarded tau-γ-satellite complex was clearly formed regardless of the presence or absence of an excess of unlabeled ssDNA as a competitor (Fig. 6A, compare lane 7 with lane 8).

In order to verify the presence of tau in the corresponding protein/DNA complex, we carried out an EMSA experiment followed by western blotting. For this purpose, two concentrations of tau (100 and 200 ng) and one concentration of protein HMG-I were incubated either with the ^{32}P-labeled γ-satellite probe (Fig. 6B, lanes 1-5), or with the unlabeled γ-satellite probe (Fig. 6B, lanes 6-13) in the presence of unlabeled ssDNA as in Fig. 6A. The monoclonal Tau-1 antibody revealed the presence of tau protein (Fig. 6B, lanes 6 and 7) in the tau-γ-satellite complex. This reaction was specific because nothing was revealed with the anti-tau antibody in lane 8 containing HMGI and γ-satellite DNA, or in lane 9

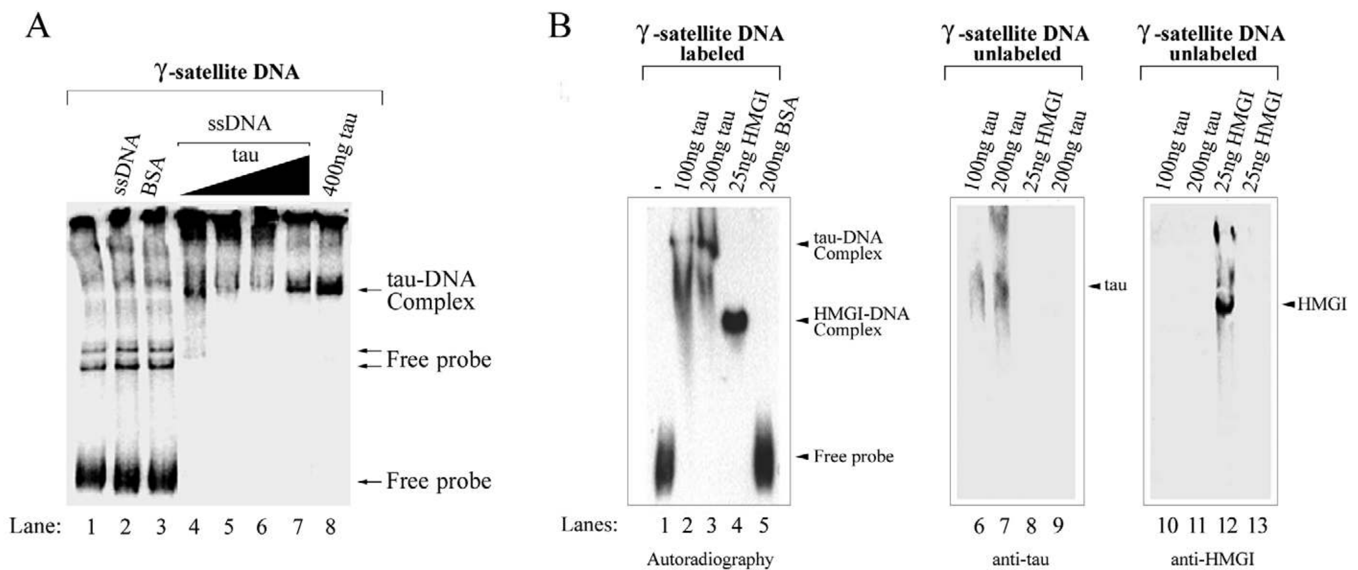

Fig. 6. Association of tau protein with murine γ-satellite DNA. Electrophoretic mobility shift assays (EMSA) were performed to analyze the interaction of purified tau protein with murine γ-satellite DNA sequences. (A) Different concentrations of purified tau protein (100 ng, 200 ng, 300 ng and 400 ng) were incubated with the ^{32}P-labeled γ-satellite DNA probe of 936 bp in the presence of 0.5 μg ssDNA as random non-sequence specific competitor DNA. The gels were dried and the radioactivity was visualized using PhosphorImager and the computer program ImageQuant. (B) Indicated amounts of tau protein or HMGI protein were incubated with the γ-satellite DNA probe, either labeled (lanes 1-5) or unlabeled (lanes 6-13) in the presence of 0.5 μg ssDNA unlabeled competitor. No satellite DNA was added in lanes 9 and 13. The complexes were resolved in a non-denaturing TBE-polyacrylamide gel. The gel was either, dried and autoradiographed (lanes 1-5) or transferred to a nitrocellulose membrane and immunoblotted using either the Tau-5 monoclonal antibody (lanes 6-9) or an anti-HMGI polyclonal antibody (lanes 10-13). The arrows indicate the shift observed for the complexes owing to the interaction with tau and the free-labeled probe.

containing 200 ng of tau alone without DNA. By contrast, the polyclonal anti-HMGI antibody revealed the presence of HMGI in the HMGI-γ-satellite complex (Fig. 6B, lane 12). This reaction was also specific because no bands were revealed in lanes 10 and 11 containing tau protein and γ-satellite DNA or in lane 13 containing 25 ng of protein HMGI without DNA.

Tau-satellite DNA interaction is apparently mediated by AT-rich DNA sequences

In order to ascertain the binding specificity of tau for these pericentromeric satellite DNA sequences, and to test the capacity of tau to bind other DNA fragments of the same size containing random sequences, we carried out competition experiments. In these experiments, a constant concentration of tau (400 ng), was incubated with an excess (25×, 50× or 75×) of different unlabeled DNA probes of equivalent sizes corresponding to: *f*-ECFP, sonicated poly dI/dC, sonicated poly dG/dC, α-satellite and γ-satellite (see Materials and Methods). The corresponding unlabeled DNA probes were incubated with tau before adding the radioactively labeled γ-satellite (Fig. 7A) or α-satellite (Fig. 7B) probes. In Fig. 7A, we show the competition experiments obtained for the tau-γ-satellite complex. As expected, competition was observed in the presence of an excess of unlabeled γ-satellite fragment (Fig. 7A, compare lane 2 without competitor with lanes 11 and 12 with competitor DNA). Under the same conditions, no competition was observed in the presence of an excess of unlabeled *f*-ECFP (Fig. 7A, lanes 3 and 4) or unlabeled sonicated poly dG/dC (Fig. 7A, lanes 8-10). However, competition was observed in the presence of an excess of unlabeled sonicated poly dI/dC (Fig. 7A, lanes 5-7), even though this competition was not as strong as that obtained in the presence of unlabeled γ-satellite DNA. Very similar results were obtained during competition experiments obtained for the tau-α-satellite complex (Fig. 7B, lanes 1-12). Therefore, in contrast to poly dG/dC or *f*-ECFP DNA, only poly dI/dC and α-satellite DNA competed and displaced tau from tau-satellite complexes. This result was reproducible in different assays, for tau complexes with both γ-satellite DNA α-satellite DNA. The poly dI/dC polymer shares strong structural similarities with DNA rich in A-T bases (Lavery and Pullman, 1981). The specific displacement of tau and satellite DNA complexes with an excess of poly dI/dC sequences suggests a particular affinity of tau protein for sequences with a high adenine (A) and thymidine (T) base content.

Discussion

It is well known that the microtubule-associated tau protein participates in the cytoskeleton integrity, and that abnormally modified forms of tau are involved in the pathogenesis of AD (Maccioni and Cambiazo, 1995; Maccioni et al., 2001b). The common function attributed to tau protein is the participation in neuronal polarity, the stabilization of microtubules and the control of the dynamic instability by affecting the polymerization rates of microtubules (Mitchison, 1992). However, as previously discussed in the Introduction, the function of tau protein at the nuclear level has not yet been established. The significance of the nuclear localization of tau has gained more relevance now based on findings on the capacity of tau to bind to DNA, a capacity that was originally suggested by Corces et al. (Corces et al., 1980) when the effect

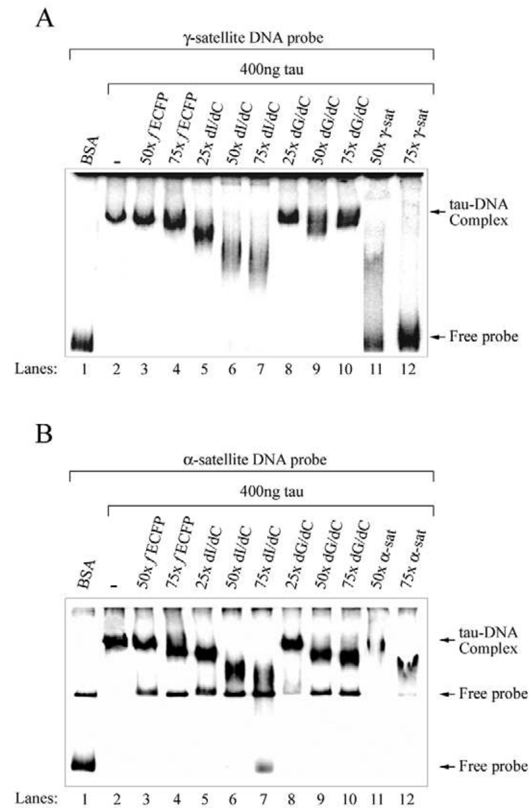

Fig. 7. Tau protein specifically interacts with γ or α-satellite DNA sequences. (A) 400 ng purified tau protein were incubated with ^{32}P-labeled γ-satellite DNA probe in the absence (lane 2) or presence of an excess of unlabeled *f*ECFD DNA fragment (lanes 3,4), unlabeled sonicated poly dI/dC (lanes 5-7), unlabeled sonicated poly dG/dC (lanes 8-10) or unlabeled γ-satellite DNA (lanes 11,12). (B) 400 ng purified tau protein were incubated with ^{32}P-labeled α-satellite DNA probe in the absence (lane 2) or presence of an excess of unlabeled fECFD DNA fragment (lanes 3,4), unlabeled sonicated poly dI/dC (lanes 5-7), unlabeled sonicated poly dG/dC (lanes 8-10) or unlabeled α-satellite DNA (lanes 11,12). The arrows indicate the shift observed for the complexes between the satellite probe and tau and the free-labeled probe at the bottom of the gel. The EMSA gels were dried and radioactivity was visualized using PhosphorImager and the computer program ImageQuant.

of DNA in microtubule assembly was analyzed. In this work we have investigated the possibility of a specific association of nuclear tau with pericentromeric DNA sequences.

Nucleolar tau interacts with AT-rich pericentromeric satellite sequences

The capacity of nuclear tau to associate and interact with pericentromeric α-satellite DNA sequences was analyzed by using fluorescence microscopy and gel retardation. In fluorescence confocal microscopy experiments we observed that nucleolar tau localized closely to perinucleolar heterochromatin, partially overlapped with clusters of pericentromeric α-satellite sequences in primary human fibroblasts and lymphoblasts as well as in transformed HeLa cells. In vitro gel-retardation experiments clearly demonstrated that tau had the capacity to bind to α-satellite DNA sequences directly. The interaction of tau with α-satellite DNA occurred

even in the presence of a great excess of double-stranded sonicated salmon sperm DNA suggesting a potential sequence specificity of tau towards pericentromeric α-satellite sequences. Under the same conditions, tau also formed protein-DNA complexes with murine pericentromeric γ-satellite DNA sequences. Even though human α- and murine γ-satellite repeats do not share the same DNA sequence, both display a strong AT-rich base content. The affinity of tau towards AT-rich DNA was confirmed by the capacity of poly dI/dC polymers to fully compete for tau-α-satellite and tau-γ-satellite complexes whereas, under the same conditions, neither salmon sperm DNA nor random plasmid DNA fragment or poly dG/dC DNA were able to fully compete for these complexes. Synthetic poly dI/dC polymers share common structural properties with poly dA/dT molecules (Lavery and Pullman, 1981) so that proteins that bind DNA through the minor groove, displaying a marked preference for (A+T)-rich DNA fragments also display a high affinity for poly dI/dC polymers (Brown and Anderson, 1986; Bailly et al., 1996). Specificity for AT-rich DNA sequences has been previously described for several architectural chromatin-associated proteins such as HMG proteins, especially HMGI/Y (Bustin, 1999; Maher and Nathans, 1996) as well as for linker histone H1 (Churchill and Travers, 1991; Käs et al., 1989). We speculate that this could also be the case for nuclear tau protein when interacting with human and murine satellite DNA sequences.

A possible role for nuclear tau during nucleolar spatial organization

An important role for heterochromatin during nucleolus formation has been suggested (Carmo-Fonseca et al., 2000). Centromeric heterochromatin displays a perinucleolar localization in human cells, especially in neuronal cells (Manuelidis, 1984; O'Keefe et al., 1992; Leger et al., 1994; Payen et al., 1998). A fraction of rRNA genes, which are silenced in nucleoli of all cell types regardless of their metabolic activity, is organized as dense heterochromatin-like structures. They are located at a nucleolar region adjacent to the centromeric perinucleolar heterochromatin (Akhmanova et al., 2000; Carmo-Fonseca et al., 2000). Coalescence of clusters of rRNA genes from different chromosomes would be required for nucleolar formation and integrity in human cells (Mirre et al., 1980). The mechanism governing this coalescence remains undetermined, however a possible link between perinucleolar heterochromatin-silencing proteins and nucleolar integrity has been proposed (Carmo-Fonseca et al., 2000). Proteins such as *Drosophila* Modulo (Perrin et al., 1998; Perrin et al., 1999) and Polycomb (Dietzel et al., 1999) and mammalian proteins pKi-67 (Bridger et al., 1998) and ATRX (McDowell et al., 1999) are heterochromatin-associated proteins that have been reported to be able to display a nucleolar or NOR localization and therefore could potentially participate in rRNA gene silencing. Nonetheless, none of these proteins, with the exception of the murine homolog of pKi-67, which in interphase cells associates with the nucleolus and with perinucleolar heterochromatin (Starborg et al., 1996), have been described as being able to interact with perinucleolar centromeric heterochromatin while localized inside the nucleolus.

Using conventional and confocal fluorescence microscopy, we demonstrate here that in human cells, clusters of nucleolar tau are systematically surrounded by and partially colocalize with α-satellite pericentromeric heterochromatin. In mitotic chromosomes, tau associates with NOR regions of acrocentric chromosomes that contain rDNA immediately juxtaposed to pericentromeric sequences. We hypothesized that nucleolar tau could create a link between rDNA repeats and pericentromeric heterochromatin, and by doing so participate in rRNA gene silencing and/or nucleolar organization and integrity.

Double fluorescence labeling of human primary fibroblasts and proliferating HeLa cells with anti-nucleolin and Tau-1 antibodies confirmed the nucleolar localization of tau in contact with the internal periphery of the nucleolus partially colocalizing with nucleolar protein nucleolin. This colocalization was almost total in non-dividing fibroblasts whereas it was only partial in dividing HeLa cells. As nucleolar tau, nucleolin localizes at the dense fibrillar component of nucleoli. It interacts with nascent pre-rRNA transcripts as well as with several ribosomal proteins and has been implicated in chromatin structure, rDNA transcription, rRNA maturation, ribosome assembly and nucleo-cytoplasmic transport (Bouvet et al., 1998; Ginisty et al., 1999; Roger et al., 2003; Johansson et al., 2004). Colocalization of tau with nucleolin suggests that these two proteins could interact with one another, reinforcing the hypothesis of a possible role of tau during nucleolar organization.

Using sense and anti-sense strategies Thurston et al., (Thurston et al., 1997) have analyzed the nucleolar localization of tau in neuroblastoma CG cells and established that nucleolar Tau-1 staining was due to the presence of tau in the nucleolus and not to a nonspecific cross-reaction. Even though nucleolar staining of tau completely disappeared after transient transfection of anti-sense tau, nucleolar morphology remained unchanged. Nucleoli are complex structures whose formation is regulated during the cell cycle and require the participation of several factors. Therefore, it would not be expected that transient knockout of only one of the factors participating in nucleolar structure would induce strong nucleolar morphology changes. Similar observations have been made with cytoplasmic tau. Tau-deficient mice did not display any particular phenotype (Dawson et al., 2001). Defects in axonal elongation and neuronal migration were observed only after knocking out tau and MAP1B protein simultaneously (Takei et al., 2000). We believe that, rather than tau knockout experiences, data obtained from pathological situations (particularly AD) as well as during cell cycle stages on tau, tau-nucleolin interactions and their corresponding subcellular distributions should help to gain an insight into the role of nucleolar tau on nucleolar organization and/or function.

Potential links between nucleolar tau, AD and trisomy 21

Interaction of tau with DNA has been reported to be aggregation-dependent so that aggregated tau loses its capacity to interact with DNA (Hua and He, 2002). Since aggregates of tau are formed during AD it would be most interesting to analyze the capacity of nucleolar tau to interact with pericentromeric α-satellite heterochromatin in AD neuronal cells.

It has been suggested that the heterochromatin structure of silenced rRNA genes protects rDNA repeats from illegitimate recombination. In yeast, recombination of rDNA repeats induces cellular aging. As suggested by Akhamanova et al.

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(Akhamanova et al., 2000), such a phenomenon would have serious consequences in cells with long life spans, such as neurons. A role of cellular aging in AD has been reported (Harman, 2002). A potential link could therefore exist between pathogenesis of AD, tau aggregation-dependent disruption of tau-α-satellite interactions and cellular aging.

Nucleolar tau has been reported to localize to the short arms at the NOR regions of acrocentric chromosomes number 13, 14, 15, 21 and 22 (Thurston et al., 1996). Nondisjunction of these chromosomes leads to trisomy, of which only trisomy 21 is viable. Most patients with trisomy 21 or Down syndrome (DS) that live beyond their fourth decade develop AD (Wisniewski et al., 1985). Although the association between trisomy 21 and AD pathogenesis is not clear, the presumable reason is the lifelong overexpression of the amyloid precursor protein (APP) gene localized on chromosome 21, and the consequent overproduction of β-amyloid in the brains of these patients. However another link between both pathologies could be related to nuclear tau because the incidence of trisomy 21 is enhanced in the offspring of families displaying familial AD (Wang et al., 1993 and references therein). Also preferential occurrence of chromosome 21 missegregation has been described in the lymphocytes of AD patients (Migliore et al., 1999). The cause of nondisjunction of acrocentric chromosomes remains undetermined, however, in human meiotic oocytes, NOR regions and satellite sequences from several acrocentric chromosomes (homologous and non-homologous) associate in a common nucleolus, a conformation that could favor acrocentric chromosomal anomalies such as translocation and nondisjunction (Mirre et al., 1980). A role for tau during coalescence of NOR regions through the interaction with α-satellite DNA of acrocentric chromosomes, would imply a participation of abnormally modified nuclear tau during nondisjunction of acrocentric chromosome 21, establishing nucleolar tau as another potential molecular link between AD and trisomy 21.

In summary, we found a specific association of tau protein and pericentromeric satellite DNA. Our results suggest a putative role for nuclear tau protein in the conformation of the nucleolar structure and/or heterochromatinization of a fraction of ribosomal genes. This hypothesis was reinforced by the observation that tau and nucleolin, a major nucleolar organizer protein, colocalized inside the nucleolus. Interestingly phosphorylated nucleolin alongside with abnormally phosphorylated tau, are both early markers for neurofibrillary tangles (NFT) during AD development, both proteins being epitopes of the monoclonal antibody TG-3 generated against the NFT found in AD (Dranovsky et al., 2001; Hamdame et al., 2003). These findings considered together with previous data shed new light on mechanisms leading to tau-aggregation-dependent pathologies such as AD. Our results suggest a nuclear role for a variant of the microtubule-associated tau protein localized in the nucleus.

Materials and Methods

Cell culture

HeLa cells were grown in monolayer, in Dulbecco's modified Eagle's culture medium (Gibco) supplemented with 5% fetal bovine serum (FBS) (Gibco), 2 mM L-glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin. Human skin fibroblasts were grown in Minimum Essential Medium (Gibco), supplemented with 15% fetal bovine serum, 2 mM L-glutamine, penicillin/streptomycin and 0.1 mM of non-essential amino-acids. Human lymphoblasts were grown in RPMI medium (Gibco) supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, 20 mM HEPES and 15% FBS (Sigma). Cultures were maintained at 37°C in a humidified incubator with 5% CO_2 atmosphere. For immunofluorescence analysis, cells were removed from culture dishes by trypsinization and plated on glass coverslips 48 hours before use.

Fluorescent in situ hybridization coupled to immunofluorescence (immunoFISH)

ImmunoFISH was carried out essentially as described (Robert-Fortel et al., 1993) with minor modifications. For the immunofluorescence procedure, cells were fixed in 1% paraformaldehyde in phosphate-buffered saline (PBS) for 20 minutes, then in methanol at –20°C for 10 minutes, permeabilized with 0.1% Triton in PBS for 5 minutes, blocked in PBS with 5% bovine serum albumin (BSA) for 45 minutes and labeled with the primary monoclonal antibody against tau, Tau-1, that recognizes unphosphorylated tau epitopes at amino acids Pro189-Gly207 (generous gift from Lester Binder of Northwestern University, Chicago) and rabbit anti-dimethylK9H3 polyclonal antibody (Upstate 07-212) or rabbit anti-nucleolin C23 polyclonal antibody (Santa Cruz Biotechnology, sc-13057) for 1 hour. Conditions of nucleolin immunodetection in Fig. 2H-J were as described (Barboule et al., 2005). Cells were fixed with 3.7% formaldehyde for 15 minutes. After washing with PBS, cells were permeabilized with 0.2% Triton X-100 in PBS for 5 minutes, washed and incubated with 100% methanol for 10 minutes at –20°C. After extensive washing, coverslips were blocked in PBS with 5% BSA for 45 minutes and labeled with the Tau-1 and C23 antibodies. As secondary antibodies, we used Alexa Fluor 488 goat anti-mouse IgG (Molecular Probes) and Cy3 goat anti-rabbit IgG (Chemicon International) for 1 hour, washing with PBS after each step. Then, cells were post-fixed with 4% paraformaldehyde in PBS for 3 minutes, permeabilized in 0.1% Triton X-100 in PBS for another 3 minutes, treated with 0.1 M Tris-HCl, pH 7.0 for 2 minutes and washed with 2× SSC twice for 2 minutes. Cells were dehydrated in 70, 80, 90 and 100% ethanol at 4°C for 2 minutes each and dried. Then cells were treated with 100 mg/ml RNase A for 45 minutes at 37°C, washed, dehydrated, dried and subjected to in situ hybridization. Cells were hybridized with 50 ng α-satellite p82H plasmid, a generous gift from Mariano Rocchi (University of Bari, Italy), directly labeled by nick translation with fluoroRED (Amersham-Pharmacia). The probe in 75% formamide, 10% dextran sulfate, 2× SSC, 2.5 μg single-stranded DNA from salmon sperm (ssDNA) (Boehringer) (final volume 50 μl) was denatured at 95°C for 5 minutes. Hybridization was performed on slides for 5 minutes at 80°C (to denature DNA in cells) and then overnight at 37°C. After hybridization, coverslips were washed once in 2× SSC for 30 minutes at 37°C, then in 1× SSC for 30 minutes at room temperature, and in 0.5× SSC for another 30 minutes at room temperature, and the preparation was mounted.

Confocal microscopy analysis

The cells were observed with a Leica-DMRBE microscope with TCS 4D confocal head. The merged images were analyzed by the Scanware (LeicaLasertechnik GmbH) or Image J programs. Double-labeled pixels were identified from pixel fluorograms as described (Demandolx and Davoust, 1997). Double-labeled pixels appeared white on the colocalization overlay images.

Purification of the microtubule-associated tau protein

Microtubule-associated tau protein was purified from fresh bovine brain, following the procedure of Grundke-Iqbal et al. (Grundke-Iqbal et al., 1986) with some modifications (Farias et al., 1992). Brain tissue was homogenized at 4°C in 0.1 M Mes buffer, pH 6.8, 1 M glycerol, 1 mM $MgCl_2$ in a volume equivalent to tissue weight, in the presence of protease inhibitors (10 μg/ml pepstatin, 10 μg/ml leupeptin, 100 μg/ml PMSF and 1 μg/ml aprotinin). The homogenates were centrifuged at 42,000 ***g*** for 30 minutes at 4°C to sediment cell debris. The supernatant was adjusted to 1 mM GTP, 2.5 M glycerol, 0.5 mM EGTA, 1 mM $MgCl_2$ plus protease inhibitors, and incubated at 37°C in a thermoregulated bath for 1 hour. Microtubules were pelleted at 42,000 ***g*** for 30 minutes at 30°C, and the supernatant was discarded. Microtubular pellets were resuspended at 4°C using a glass homogenizer, in 0.1 M Mes buffer, pH 2.7, 1 mM EGTA, 0.5 mM $MgCl_2$, 1 mM EDTA, 0.75 mM NaCl and 2 mM DTT. The solution was boiled for 5 minutes, and then centrifuged at 42,000 ***g*** for 30 minutes at 4°C. The supernatant was adjusted to 2.5% perchloric acid and then centrifuged at 42,000 ***g*** for 30 minutes at 4°C. The supernatant was finally dialyzed against 2.5 mM Tris-HCl, pH 7.6, in dialysis membranes at 4°C for 24 hours. The protein was concentrated using an ultrafiltration cell (Amicon® models 8050 and 8003), with the appropriate membranes. The protein concentration was evaluated by Bio-Rad Protein Assay.

Protein electrophoresis and western blots

The identification and purity of bovine brain tau protein preparations were analyzed by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Gels were stained with Coomassie blue or electrophoretically transferred to nitrocellulose membranes for the western blots. After blocking the non-specific binding sites with 5% non-fat dry milk in PBS, the membranes were incubated overnight with the primary monoclonal antibody Tau-5 (1:1000) (generous gift from Lester Binder, Northwestern University, Chicago, IL) that reacts with residues 210-

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230 in the proline-rich domain of tau protein (Thurston et al., 1996), in PBS with 1% BSA at 4°C in a wet chamber. Then the membranes were washed and incubated with horseradish peroxidase secondary antibody, rabbit anti-mouse, diluted in PBS with 1% BSA and analyzed with the ECL western blotting analysis system (Bio-Rad) using enhanced chemiluminescence.

Gel retardation assays

Purified tau protein (100-400 ng) were incubated with 0.5 μg ssDNA, as a competitor, in 20 μl (final volume) of 50 mM Tris-HCl (pH 7.5), 50 mM NaCl, 5 mM EDTA, 10% glycerol, and 5 mM dithiothreitol for 10 minutes at room temperature, before adding the corresponding 5′-^{32}P-labeled probe (0.05 pmol). Two satellite DNA fragments were used as labeled probes. These DNA fragments were: (i) a murine γ-satellite DNA of 936 bp (four copies of 234 bp γ-satellite repeat) obtained by digestion of the plasmid PBS (a generous gift from Niall Dillon, Imperial College, London, UK), with the restriction enzyme *Eco*RI, and (ii) a fragment of human α-satellite DNA of 700 bp generated by PCR amplification with the primers αSATsense (5′-GGAAACGGGAATTCCTTCACATAAAGAT-3′) and αSATantisense (5′-TCTCTCTAGGGATCCTGGAAGATACTCC-3′), using the p82H plasmid as template. The PCR conditions were as follows: 1 cycle of 94°C for 5 minutes, 30 cycles of 94°C for 1 minute, 50°C for 1 minute, and 72°C for 1 minute and 1 cycle of 72°C for 10 minutes. After adding the labeled probe, the mixture was incubated at room temperature for 15 minutes. Electrophoresis was carried out in an 8% polyacrylamide gel in 0.25× Tris-borate-EDTA. For the competition experiments 400 ng purified tau protein were incubated with a 25-, 50- or 75-fold excess of the corresponding unlabeled DNA fragments for 10 minutes. As a competitor DNA we used: (i) a restriction fragment of 600 bp (*f*-ECFP), of random sequence, obtained by digestion of the plasmid pECFP with the enzyme *Pvu*II; (ii) sonicated poly dI/dC sequences; (iii) sonicated poly dG/dC sequences; or (iv) the α- and γ-satellite unlabeled DNA fragments. Afterwards, the 5′ ^{32}P-labeled γ- and α-satellite DNA probe were added to the tau/unlabeled competitor DNA mix, and incubated for 10 minutes at room temperature before electrophoresis was carried out. Each fragment of DNA was purified from agarose gel and ethanol precipitated. The labeling of the DNA fragments (5 pM) previously dephosphorylated with alkaline phosphatase (Promega), was performed at the 5′ end with [γ-^{32}P] dATP (3000 Ci/mmol, Perkin Elmer) with the enzyme T4 polynucleotide kinase (Promega) (5-10 U/μl) at 37°C for 30 minutes. After labeling, the probes were phenol extracted, ethanol precipitated and resuspended in 10 mM Tris-HCl pH 8.0, 1 mM EDTA before use.

Gel retardation assays before western blotting were carried out as described above, except that 100 and 200 ng of purified tau were incubated with 0.5 μg of ssDNA and 5 pmol of unlabeled γ-satellite DNA probe. After the electrophoresis, the gels were soaked in transfer buffer (50 mM Tris-HCl, 40 mM Glycine, 1 mM SDS and 20% methanol) for 40 minutes at room temperature, before being subjected to western blotting with the primary antibody Tau-1 (1:1000).

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