Protein TAU variants present in paired helical filaments (PHFs) of Alzheimer brains

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Polyclonal anti-TAU antisera directed against native Tau protein and the NH_2-terminal side of the mouse TAU sequence were used to determine the nature of the TAU variants present in Alzheimer brains and in PHFs. These antibodies labelled specifically neurofibrillary tangles and plaque neurites in Alzheimer brains. On immunoblots of PHF extracts, two entities of 69 and 130 kDa were identified. These TAU-related species were absent from control brains. Protein immunoblot of total Alzheimer and control supernatants were shown to contain the same 4–5 TAU variants but none of the 69 and 130 TAU-related entities found in PHFs. These data suggest that specific TAU species are present in PHFs.

Alzheimer disease; Paired helical filament; Protein TAU

1. INTRODUCTION

The Alzheimer disease is characterized by two types of lesions: the neurofibrillary tangles (NFTs) and the senile plaques. Senile plaques are formed with distrophic neurites surrounding an 'amyloid core'. NFTs are made of 10 nm paired helical filaments (PHFs) which are present both in the perikarya of affected neurons and in the distrophic neurites of the senile plaques (see [1] for a review). The finding that PHFs contain TAU epitopes [2] has received strong support during the past few years [3,4].

In the mature brain, TAU proteins consist of 4–5 variants (50–70 kDa) [5] whereas at immature stages only 2–3 variants of 45–48 kDa are expressed [6]. The C-terminal end of all these variants is highly conserved and contains repeats which have been shown to be the tubulin binding sites [7–9]; three and four repeats are present, respectively, in the immature and mature TAU variants. Two other microtubule-associated proteins known as MAP2 [10] and MAP4 (C. Bulinski, personal communication) also contain repeated sequences in their C-terminal domain. These repeats are highly homologous to those found in TAU proteins. Several studies suggest that more than one TAU variant is present in PHFs [11,12] and that the entire molecule is incorporated into NFTs [12,13]. However, the exact nature of TAU isoforms found in PHFs remains unclear. To answer this question the first antibody (1E9) used in this work was raised against the whole TAU sequence. This antibody was therefore potentially able to detect not only TAU proteins but also MAP2, MAP4 and eventually other microtubule-associated proteins containing repeated sequences homologous to those present in TAU proteins. Another antibody (B19-1) was raised against a short sequence present in the N-terminal end of TAU protein which is absent in both MAP2 and MAP4. This N-terminal sequence is present in all mouse [7], rat [13], bovine [8,14] and human [12] TAU sequences established so far and is specific of TAU proteins. Using these two antibodies we show in this work that a major TAU variant of 69 kDa is present in the PHFs together with an immunologically larger TAU-related protein of 130 kDa.

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Abbreviations: NFTs, neurofibrillary tangles; PHFs, paired helical filaments; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

2. MATERIALS AND METHODS

Brains from 5 Alzheimer (80–98 years old) and 5 control patients (61–88 years old) who had died of non-neurological diseases were obtained 7–24 h post-mortem, and were stored at −70°C. The clinical diagnosis was confirmed histologically by the presence of plaques and tangles in temporal cortex and hippocampus.

Total and heat-treated supernatants were prepared as previously described [6].

A polyclonal antiserum (B19-1) was raised in rabbits against an oligopeptide corresponding to amino acids 29–48 of the mouse TAU sequence [7]. The respective peptide was generated by solid phase synthesis (Multiple Peptide Systems, San Diego, CA) and covalently linked to keyhole limpet hemocyanine (KLH) (Pierce, Rockford, IL) [12]. Male rabbits were immunized with 250 μg antigen in the presence of a synthetic glycolipid, BAY R-1005 (Bayer AG, Leverkusen) as adjuvant (1 mg/kg). Booster injections were given every three weeks. After 18 weeks the rabbits were sacrificed and the

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serum collected. A polyclonal antibody (1E9) directed against native rat brain TAU was raised as previously described [6] and purified by affinity chromatography.

Antisera (1/5000 for B19-1 and 1/200 for 1E9) were applied on sections of paraffin-embedded, formalin-fixed Alzheimer hippocampus. Sections were successively incubated with a biotinylated anti-rabbit antibody and with horseradish peroxidase-streptavidin (Amersham) and the peroxidase was localized with diaminobenzidine [15].

Hippocampi (7 g) were homogenized in 5 vols of buffer A (0.1 M Mes, 0.5 mM $MgCl_2$, 1 mM EGTA, 0.1 mM EDTA, 1 mM GTP, 0.4 mM PMSF, 5 μM leupeptine, 10 mM benzamidine, 1 mM mercaptoethanol, 0.25 mg/ml trypsin inhibitor, pH 6.4) plus 0.34 M sucrose and filtered through a 100 μm muslin screen. The sucrose concentration of the filtrate was brought to 1 M and 7 ml of the mixture were layered over a discontinuous sucrose gradient of 1.2 M and 1.8 M (9 ml each). After centrifugation at 5000 × g for 1 h at 4°C, the 1/1.2 M interface was diluted to 0.5 M with buffer A and material was pelleted at 110000 × g for 45 min at 4°C. The pellet was suspended in 0.34 M sucrose, layered over an equal volume of 1 M sucrose and centrifuged at 190000 × g for 90 min at 4°C. The pellet was resuspended in one volume of 0.34 M sucrose, layered over a gradient of 1.2 M and 1.8 M sucrose (9 ml each) and centrifuged at 190000 × g for 2 h. The 1.2/1.8 M interface was pelleted as above. The pellet was diluted in 1 ml of buffer A, incubated with 100 μl of 2% SDS for 5 min at 20°C and centrifuged at 11000 × g for 10 min to collect the PHFs. Such mild SDS treatment allows one to obtain apparently non-contaminated PHF preparations and, subsequently, much clearer protein immunoblots.

PHF fractions were absorbed onto carbon-coated grids and stained with 1% phosphotungstic acid (pH 7.4).

Heat-treated supernatants and sonicated PHFs were boiled for 5 min in 2.3% SDS, 10% glycerol and 5% mercaptoethanol and subjected to SDS-PAGE (10% and 12%, respectively). Transfer to the nitrocellulose and treatment with antibodies (dilutions: 1/5000 for B19-1, 1/200 for 1E9) were performed as previously described [6].

3. RESULTS

The polyclonal antiserum B19-1 used in this study is directed against the NH_2-terminal side of the mouse TAU sequence described by Lee et al. [7] whereas polyclonal 1E9 was raised against the entire TAU molecule. On Western blot, B19-1 (Fig. 1A and 3A) and 1E9 (not shown) reacted with the juvenile (48 kDa) and adult (50–70 kDa) TAU variants present in total and heat-stable supernatants from 6 days and adult rat brains as well as with the adult human brain variants. Staining of tangles by the B19-1 (Fig. 1B) and 1E9 (not shown) was tested on hippocampus tissue sections of patients with Alzheimer disease. Both antisera strongly labelled NFTs and numerous neurites surrounding senile plaque cores.

PHFs were purified from the hippocampus region which is rich in NFTs from 5 Alzheimer brains. The same procedure of purification was applied to 4 non-Alzheimer brains as control. The three successive sucrose gradients greatly enriched the pool of PHFs. The interface 1.2/1.8 of the last gradient contained cellular components contaminating PHFs. Mild treatment of this fraction with SDS for 5 min at room temperature solubilized most cellular fragments giving a fraction highly enriched in NFTs of different size. These NFTs retained their characteristic Congo red

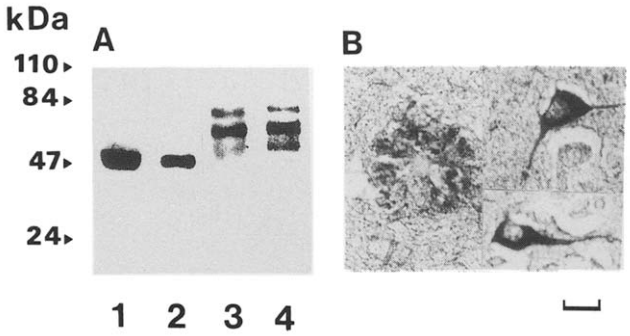

Fig. 1. (A) Immunoblot with anti TAU B19-1 of 6 days (1, 2) and adult (3, 4) rat brain extracts. (1, 3) Total 10^5 g supernatants; (2, 4) heat-treated supernatants. (B) Immunocytochemical staining of NFTs and senile plaques in hippocampal sections of Alzheimer disease patient. Bar = 20 μm.

birefringence (Fig. 2A). At a higher magnification, the tangles have elongated forms with filaments visible inside (Fig. 2B). At the electron microscopic level, NFTs were constituted of bundles of 23-nm paired helical filaments with a half-periodicity of 85–100 nm (Fig. 2C, D).

B19-1 was used to compare the soluble TAU variants present in Alzheimer and control brain extracts (Fig. 3A). Western blot analysis of heat-stable supernatants from hippocampus regions revealed that the distribution of the TAU-related proteins was similar in

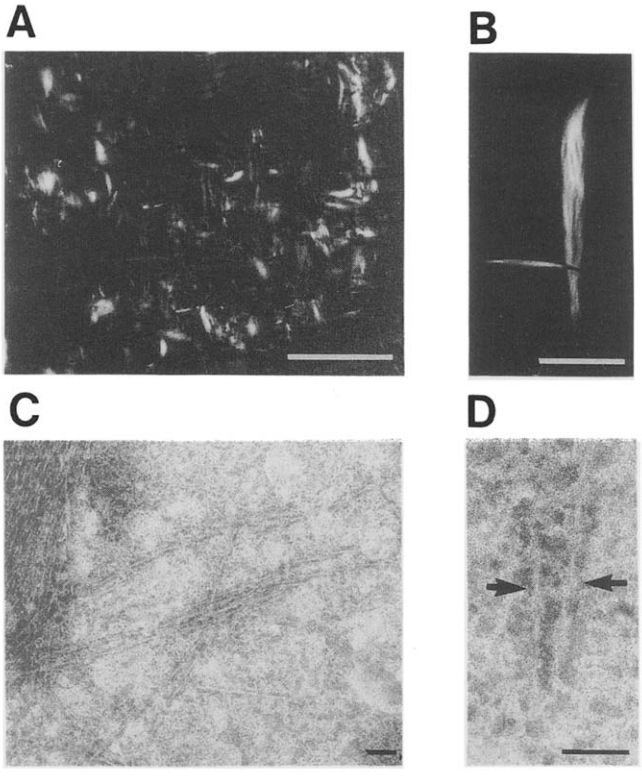

Fig. 2. Congo red staining (A, B) and electron microscopy (C, D) of PHFs. Bars = A: 100 μm; B: 20 μm; C and D: 100 nm.

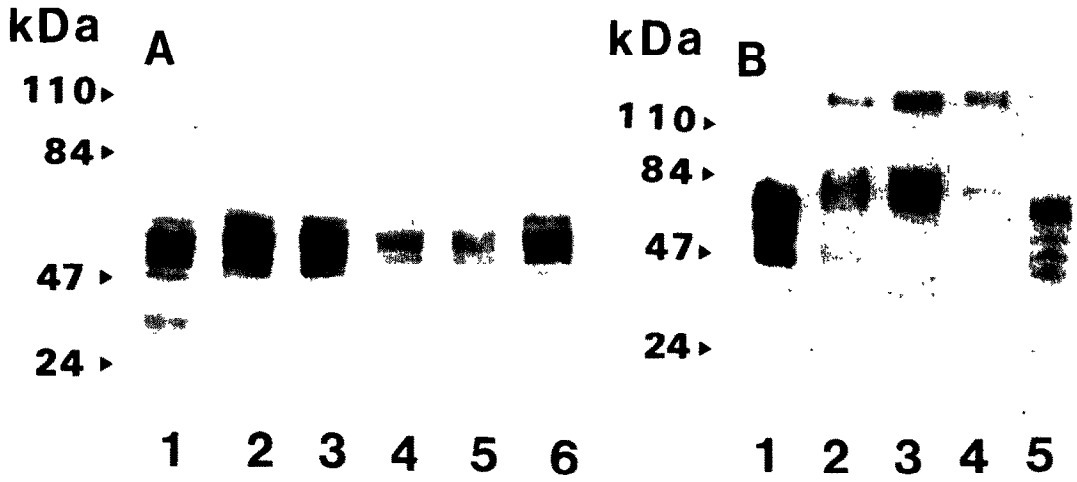

Fig. 3. Western blot analysis with the B19-1 antibody of the TAU variants present in Alzheimer and control brains (A) and in PHFs (B). (A) Heat-treated supernatants (20 μg) of Alzheimer (lanes 1–3) and control (lanes 4–6) brains. (B) Alzheimer brain supernatant (1). Alzheimer (2) and control (5) homogenates. Pellet (3) and supernatant (4) of PHF extracts.

the Alzheimer and the control brain supernatants with several TAU variants (50–65 kDa) present in both cases. The same pattern was obtained with the polyclonal 1E9 (not shown). None of these antibodies detected a TAU variant specific or present in higher quantity in Alzheimer brain supernatants.

In contrast, analysis of SDS lysates of total homogenates prepared from Alzheimer hippocampi showed the presence of a TAU-related entity of 69 kDa which was the dominant TAU species (Fig. 3B). The same 69 kDa species was detected in the SDS extracts of the purified PHFs fraction. Two other entities of about 68 and 70 kDa were also feintly detected. These bands were absent in total soluble extracts (lane 1) and in fractions obtained from control brains (not shown). Another component of 130 kDa, present in variable quantity in homogenates (lane 2) of the different Alzheimer brains, was also detected in the PHF extracts (lane 3). This component was absent from control brain homogenates (lane 5).

4. DISCUSSION

Our PHF preparations contain one major TAU variant of 69 kDa as well as a protein of 130 kDa immunologically related to TAU. Both entities were detected by a polyclonal antiserum directed against an amino acid stretch present in the N-terminal domain of all the TAU variants sequenced so far [7,8,13,14].

The 69 kDa entity does not seem to be overexpressed in the brain of the demented patients since the immunoblots obtained with supernatants from control and Alzheimer brains were identical. Flament et al. have recently reported that two TAU entities of 64 and 69 kDa are present in Alzheimer brain homogenates [16]. The same authors have observed a decrease in the apparent molecular weight of these entities after phosphatase treatment. It is not clear whether the 69 kDa species described by these authors is identical to the TAU variant extracted from our PHF preparations since phosphatase treatment of our extracts was not conclusive.

Previous studies have reported that PHFs contain several adult TAU forms and at least one juvenile TAU variant [11,12,17]. Such entities were not seen in our preparations and this might depend either on the antibodies or on the purification procedure of the PHFs we used.

Two arguments are against the first assumption: (i) the B19 anti-TAU was raised against a TAU domain which is present in all adult and juvenile TAU forms sequenced so far; (ii) this antibody detected all the adult and juvenile TAU entities when tested with rat and human brain supernatants (Fig. 1A and 3A). We may therefore conclude that only the 69 and 130 kDa TAU entities are present in our PHF preparations. It has been shown recently [14] that the different TAU variants are produced by alternative splicing of a primary transcript generated from a single TAU gene which contains 13 exons. The variant transcripts corresponded to mRNA species missing one or more exons [14]. Four major TAU protein isoforms were identified by comparison with translation products of cDNA constructs [14]. This suggests that the TAU variants extracted from PHFs contain the sequence present in N-terminal region of the TAU gene since they were recognized by B19 anti-TAU. The presence of only two TAU variants in the PHF extracts might be explained by assuming that the other TAU species are released by the mild SDS treatment performed during the last step of our purification procedure. However, analysis of the supernatant obtained after this SDS mild exposure showed that, if TAU proteins were slightly released by this treatment, the 69 kDa component was always the predominant TAU entity (Fig. 3, lane 4). Finally, we come to the conclusion that the 69 kDa variant is an ab-

normal TAU entity or a TAU variant preferentially able to self-assemble in PHFs.

The second entity clearly identified in PHF extracts is a protein of 130 kDa. This protein seems to be specific to the Alzheimer brain: (i) it was constantly found in all Alzheimer samples (homogenates and PHF extracts) tested and never in the controls; (ii) it was absent or present in very low quantitities in total brain supernatants; (iii) it increased in concentration during the successive steps of PHF purification. Nukina et al. have also found high molecular weight entities (160–180 kDa) in Alzheimer brain [18]. They suggested that these entities are TAU aggregates. A TAU-related entity with unusual HMW seems to be present in some regions of normal brains; for instance Kosik et al. have found a 110 kDa entity immunologically related to TAU in the optic nerve [17]. A TAU variant of 120 kDa which is under NGF control is also present in the PC12 pheochromocytoma cells [19]. It is not clear whether our 130 kDa TAU-related protein is identical to one of these HMW entities. However, one might consider the possibility that PHFs are enriched in such a TAU variant because they are preferentially formed in a subset of neurons undergoing degeneration. This assumption is consistent with the observation that prominent abnormalities are spread along the olfactory bulb, hippocampal formation, entorhinal area and the associated cortices [20,21]. Another possibility, which does not contradict the first, would be that both the 130 kDa and the 69 kDa entities are abnormal TAU variants which are able to self assemble to produce PHFs.

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