Phosphorylation of specific sets of tau isoforms reflects different neurofibrillary degeneration processes

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Abstract Tau proteins are the basic components of filaments that accumulate within neurons during neurofibrillary degeneration, a degenerating process with disease-specific phenotypes. This specificity is likely to be sustained by both phosphorylation state and isoform content of tau aggregates that form neuronal inclusions. In the present study, characterization of tau isoforms involved in neurofibrillary degeneration in Alzheimer's disease, Pick's disease, corticobasal degeneration and progressive supranuclear palsy was performed. Both analyses by immunoblotting using specific tau antibodies and cell transfection by tau isoform cDNAs allowed us to demonstrate the aggregation of (1) the six hyperphosphorylated tau isoforms in Alzheimer's disease, (2) tau isoforms without exon 10-encoding sequence in Pick's disease and (3) hyperphosphorylated exon 10-tau isoforms in corticobasal degeneration and progressive supranuclear palsy. Thus, neurofibrillary degeneration phenotypes are likely to be related to the phosphorylation of different combinations of tau isoforms (with and/or without exon 10-encoding sequence) in subpopulations of neurons.

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Key words: Alternative splicing; Alzheimer's disease; COS cell transfection; Corticobasal degeneration; Phosphorylation; Pick's disease; Progressive supranuclear palsy; Tau protein

1. Introduction

Hyperphosphorylated microtubule-associated tau proteins are the main components of the aggregated filaments found in neurofibrillary tangles (NFT) in Alzheimer's disease (AD) and are referred to as paired helical filaments (PHF) [1–3]. Similar tau immunoreactivity is observed in NFT in other neurodegenerative disorders including corticobasal degeneration (CBD) and progressive supranuclear palsy (PSP) (for review [4]). In CBD and PSP, tau aggregation results in the formation of non-PHF filaments. Other neuronal inclusions such as Pick bodies in Pick's disease (PiD) are also tau-immunoreactive [5].

The human tau gene is located over 100 kb on the long arm of chromosome 17 at band position 17q21 and contains 16 exons [4,6]. In the brain, alternative splicing of three exons (exons 2, 3 and 10) allows six combinations (2−3−10−; 2+3−10−; 2+3+10−; 2−3−10+; 2+3−10+; 2+3+10+) [7,8]. Thus, tau proteins constitute a family of six isoforms which range from 352 to 441 amino acids. They differ from each other by either the presence of three (10−) or four re-

peat-regions (10+) in the carboxy-terminal part of the molecule and one (2+) or two (2+3+) inserts (29 or 58 amino acids) in the amino-terminal part [7,8]. Furthermore, the six tau isoforms may not be equally expressed in neurons. For instance, tau mRNAs containing exon 10 (E10+) are not detected in granular cells of the dentate gyrus suggesting that these particular tau isoforms are weakly expressed or lacking in these neurons [7]. Thus, tau isoforms may be differentially distributed in neuronal subpopulations and/or cell compartments.

In AD, the six tau isoforms are hyperphosphorylated and aggregate into PHF. By immunoblotting, PHF-Tau are characterized by a tau triplet Tau 55, 64, 69 and a minor electrophoretic variant at 74 kDa [9]. In PiD, aggregated tau proteins are made of a major tau doublet Tau 55, 64 and a minor variant at 69 kDa [5,10], whereas in PSP, CBD and in some cases with fronto-temporal dementia with parkinsonism linked to chromosome 17 (FTDP-17), another tau doublet Tau 64, 69 with a minor variant at 74 kDa, characterizes neuronal and glial filaments [5,11,12]. Recent data indicate that the tau electrophoretic profiles observed in neurodegenerative disorders may be related to specific tau isoforms in particular subsets of neurons [13].

We investigated this hypothesis (1) by transfection of COS cells, using different combinations of tau isoform cDNAs, and followed by a treatment with okadaic acid (OA), a phosphatase 1 and 2A inhibitor, and (2) by using monoclonal and polyclonal antibodies to compare tau immunoreactivity in cell extracts and brain homogenates from AD, CBD, PSP and PiD.

2. Materials and methods

2.1. Patients
All of the patients (AD, PSP, CBD, PiD) used in the present study have been described in previous works [5,14–16]. PiD cases showing only Pick bodies were used in the present study.

2.2. Antibodies
Phosphorylation-dependent monoclonal antibodies (12E8, AD2, and AT100) were used to characterize tau proteins. 12E8 recognizes phosphorylated Ser-262 [17]. AD2 is directed against phosphorylated Ser-396 and 404 [15]. AT100 binds to a conformational epitope including phosphorylated Thr-212 and Ser-214 [18].
Polyclonal antibodies, E2, E3 and E10 respectively, raised against tau sequences encoded by spliced exons (2, 3 and 10) were also used [19].

2.3. Cell culture and transfection
COS-7 cells were grown in 25 cm² flasks in Dulbecco's modified Eagle medium (Life Technologies) with 10% fetal calf serum (Boehringer Mannheim) in a 5% CO_2 incubator at 37°C. cDNA of the six human tau isoforms were cloned in pSG5 vector (Stratagene). They

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are a kind gift from Dr. Michel Goedert (Cambridge, UK). Tau cDNAs were transiently transfected in COS cells using the DEAE-dextran method. Following 42 h transfection, cells were treated or not by OA (Sigma), a phosphatase 1 and 2A inhibitor, for 6 h in serum-free medium [20]. Cells were then harvested in Tris-EDTA solution at 4°C and centrifuged. Cell pellets were homogenized in Laemmli sample buffer and boiled for 10 min.

2.4. Immunoblotting

Electrophoresis and immunoblotting were performed as previously described [15]. Briefly, samples were loaded onto 10% SDS-PAGE (Pharmacia Biotech). After transfer and blocking, membranes were incubated with the primary antibody 90 min at room temperature. Horseradish peroxidase-conjugated antibody (Sigma) was used as secondary antibody and reaction product was detected using the Amersham ECL Western blotting system.

3. Results

Immunochemical characterization of tau variants in neuro-degenerative disorders using AD2 was consistent with that performed in previous studies [1–5,9–11,14,16]. A major tau triplet made of Tau 55, 64 and 69 and a minor variant at 74 kDa were found in AD (Fig. 1A, Fig. 3A). Only the two major variants of higher MW, Tau 64 and 69, were found in CBD and PSP (Fig. 1B, Fig. 3A). The 74 kDa electrophoretic variant was poorly detected. In AD, all electrophoretic variants were immunoreactive with antibodies AD2, AT100 and 12E8 (Fig. 1A, Fig. 3A,B). In CBD and PSP, similar tau immunoreactivity was found indicating that the same tau epitopes as in AD are hyperphosphorylated (Fig. 1B, Fig. 3A,B). In PiD, Tau 55 and 64, and a minor 69 kDa electrophoretic variant were previously described [5,10]. They are labeled by AD2 and AT100 whereas 12E8 did not bind to the tau variants suggesting that Tau 55 and 64 are not phosphorylated at Ser-262 (Fig. 3B). In the present study, all data were similar in CBD and PSP. Thus, we will use in the next paragraphs the abbreviation CBD/PSP for both disorders to facilitate the description.

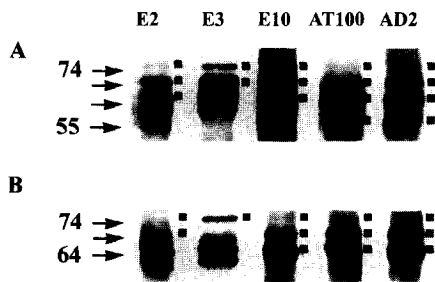By immunoblotting, it was previously shown that in AD, all

Fig. 1. Immunoblotting of tau proteins using antibodies raised against exon-encoding sequences (E2, E3 and E10) and phosphoryl-ation-dependent monoclonal antibodies AD2 and AT100. A: In AD, Tau 74 and 69 are labeled by all antibodies E2, E3 and E10. Tau 64 is labeled by both E2 and E10. Tau 55 is never labeled. All phosphorylation-dependent antibodies label the tau triplet Tau 55, 64 and 69 with the minor 74 kDa variant. B: In CBD, E2 labels Tau 69 and 74. E3 only labels Tau 74. The tau doublet Tau 64 and 69 and the minor 74 kDa variant are labeled by E10. All phospho-rylation-dependent antibodies label the tau doublet Tau 64 and 69 with the minor 74 kDa variant in CBD. In all disorders, minor tau species are also labeled by the different antibodies and correspond to non-phosphorylateed tau proteins as described in [9,10,13]. Molecular weights (55–74 kDa) are indicated on the left side of the figure (arrowheads). Black dots at the right part of each immunoblots indicate the position of labeled hyperphosphorylated tau variants.
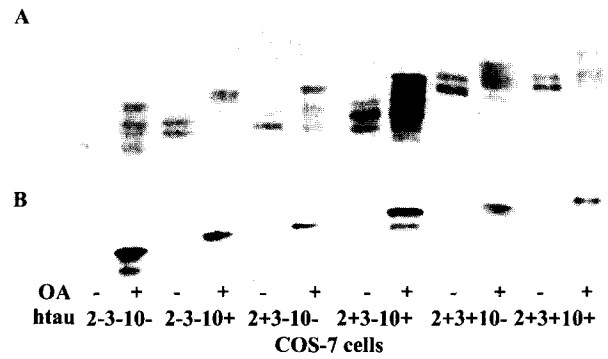

Fig. 2. Immunoblotting of tau proteins in transfected COS cells using the tau polyclonal antibody M19G (A) and the phosphoryla-tion-dependent monoclonal antibody AD2 (B). A: Each tau isoform in OA treated (+) and non OA treated (−) transfected COS cells is characterized by different electrophoretic variants that are likely to reflect different degrees of tau phosphorylation. However, there is a notable shift of MW between tau isoforms from non OA treated (−) and OA treated (+) transfected COS cells. This shift is induced by tau hyperphosphorylation. B: The phosphorylation-dependent antibody AD2 only labels the upper hyperphosphorylated tau var-iants in OA treated transfected COS cells (+). In some cases (2–3–10− and 2+3–10+), lower hyperphosphorylated tau variants are also detected.

six isoforms are phosphorylated to obtain the major tau trip-let and the 74 kDa variant [9] (Fig. 1A) whereas in PiD, Tau 55, 64 and the minor 69 kDa variant are made of tau isoforms without the exon 10 translated sequence since they are not labeled by antibody E10 [13]. Conversely, antibody E10 strongly labeled Tau 64 and 69 in AD and CBD/PSP and to a lesser extent the 74 kDa variant (Fig. 1). Further investiga-tion using E2 and E3 antibodies suggested that tau electro-phoretic variants in CBD/PSP are only made of tau isoforms with exon 10 translated sequence. E2 labeled both Tau 69 and 74 whereas E3 only labeled Tau 74 (Fig. 1B). Other lower MW peptides were also detected. They correspond to non-phosphorylated tau proteins as demonstrated, in AD, by two-dimensional electrophoresis analysis [9].

To confirm at the molecular level these immunochemical data, we performed cell transfections with different tau iso-form cDNAs (Fig. 2). We used OA, a phosphatase 1 and 2A inhibitor, to mimic tau hyperphosphorylation found in neuro-degenerative disorders [20]. In OA treated transfected cells, tau isoforms displayed after SDS-PAGE a shift in MW indi-cating a hyperphosphorylation (Fig. 2A). This was confirmed using the phosphorylation-dependent tau antibody AD2 which only labels tau isoforms of OA treated cells (Fig. 2B).

In COS cells, co-transfection of the six tau isoform cDNAs and cell treatment by OA led to the formation of large amounts of all electrophoretic variants, Tau 55, 64, 69 and 74, that were immunoreactive for all phosphorylation-depend-ent antibodies (Fig. 3C,D). In AD, Tau 74 is poorly detectable (Fig. 3A,B). Thus, this electrophoretic profile did not reflect quantitatively the one found in AD since tau isoforms that aggregate in AD brain are not expressed in equal quantities. Different amounts of tau isoforms were tested in our trans-fection experiments and the electrophoretic profile the most comparable to the one found in AD was observed with the following combination: 35% (2–3–10− tau isoform), 15% (2+3–10−), 15% (2–3–10+), 15% (2+3+10−), 15% (2+3–10+) and 5% (2+3+10+) (data not shown). In other
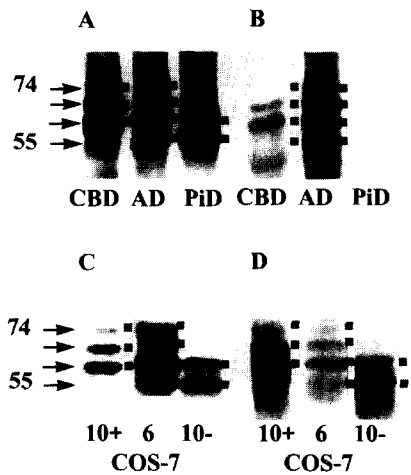
Fig. 3. Comparison of tau immunoreactivity from brain homogenates (A, B) and OA treated transfected COS cells (C, D) by immunoblotting using phosphorylation-dependent monoclonal antibodies AD2 (A, C) and 12E8 (B, D). A: AD2 labels the tau triplet Tau 55, 64 and 69 with the minor 74 kDa electrophoretic variant in AD, the tau doublet Tau 55 and 64 with the minor 69 kDa variant in PiD and the tau doublet Tau 64 and 69 with the minor 74 kDa variant in CBD. B: Similar immunoreactivity is found with 12E8 with the exception of PiD where Tau 55 and 64 are not labeled. For both antibodies, it should be noted that Tau 64 in PiD corresponds to the upper part (65 kDa) of the AD Tau 64 variant, whereas Tau 64 in CBD corresponds to its lower part (63 kDa). Similar observations are available for Tau 69. These data are consistent with the electrophoretic profile of tau isoforms observed in OA treated transfected COS cells. Using OA treated transfected COS cells, similar data are obtained for both antibodies AD2 (C) and 12E8 (D). The six hyperphosphorylated tau isoforms (6) run on SDS-PAGE as four variants at 55, 64, 69 and 74 kDa. Hyperphosphorylated E10– tau isoforms (10–) run at 55, 65 and 70 kDa whereas hyperphosphorylated E10+ tau isoforms (10+) run at 63, 68 and 74 kDa. These data are consistent with the results obtained in PiD and CBD/PSP. Molecular weights (55 and 74 kDa) are indicated on the left part of each immunoblots (arrowheads). Black dots indicate the position of labeled hyperphosphorylated tau variants either Tau 55, 64 and 69 in PiD or Tau 64, 69 and 74 in CBD.

transfection studies using specifically E10– or E10+ tau isoform cDNAs to mimic PiD and CBD/PSP pathology respectively, the following combination was used (45% (2–3–), 45% (2+3–) and 10% (2+3+)). Triple transfection of E10– tau isoform cDNAs in COS cells led to the formation, after OA treatment, of the characteristic electrophoretic tau profile found in PiD showing AD2 immunoreactivity (Fig. 3C). However, hyperphosphorylated E10– tau isoforms (Tau 55 and 64 and the minor variant at 69 kDa) obtained in transfected COS cells, were also 12E8-immunoreactive (Fig. 3D). This is a striking difference with PiD where the hyperphosphorylated tau variants are not 12E8-immunoreactive (Fig. 3B).

Triple transfection of E10+ tau isoform cDNAs (45% (2–3–), 45% (2+3–) and 10% (2+3+)) in COS followed by OA cell treatment led to the formation of tau variants exhibiting the same electrophoretic tau profile as in CBD/PSP, labeled by all phosphorylation-dependent monoclonal antibodies: Tau 64 and 69 and a minor tau variant at 74 kDa (Fig. 3C,D). Thus, the tau electrophoretic profile observed in CBD resulted from the hyperphosphorylation of E10+ tau isoforms (Fig. 1B, Fig. 3C,D).

For all antibodies, it should be noted that Tau 64

(2+3–10–) in PiD corresponds to the upper part of the Tau 64 variant (mixture of 2+3–10– and 2–3–10+) in AD whereas Tau 64 (2–3–10+) in CBD/PSP corresponds to its lower part (Fig. 3C,D). Similar observations are available for Tau 69. These data are consistent with the electrophoretic profile of tau isoforms observed in OA treated transfected COS cells (Fig. 2) and further support our findings.

4. Discussion

The present study demonstrates at the molecular level that hyperphosphorylated tau isoforms with exon 10-encoding sequence exhibit the same electrophoretic profiles as those found in CBD/PSP. It also confirms that all six hyperphosphorylated tau proteins aggregate in AD whereas only tau isoforms lacking exon 10 translated sequence are hyperphosphorylated in PiD. Thus, the tau electrophoretic profiles encountered in neurodegenerative disorders may be obtained in cell models using specific tau isoforms (with or without exon 10 translated sequence) and OA cell treatment. However, differential tau phosphorylation (i.e. Ser-262 phosphorylation in PiD) was not reproduced suggesting that not only tau isoforms but also particular kinases are involved in the pathological process.

In AD, all hyperphosphorylated tau isoforms (E10– and E10+) aggregate into PHF and are biochemically characterized by a tau triplet that is labeled by all phosphorylation-dependent tau monoclonal antibodies (for review [4]). All neurodegenerative disorders with PHF-like filaments including Guamanian amyotrophic lateral sclerosis/parkinsonism dementia complex and postencephalitic parkinsonism are also characterized by this tau triplet [14,16]. Tau cDNA from each of the six tau isoforms was previously transfected into COS cells leading to the formation of different peptides that are likely to be related to different degrees of phosphorylation [22]. After transfection of tau isoform cDNAs, a cell treatment by OA was performed and allowed us to induce the highest degrees of phosphorylation [20,23]. Using the method with all six tau isoforms, an electrophoretic profile similar to that found in AD was observed. These data are comparable to those describing tau phosphorylation by GSK3β [24]. Following this approach, the different tau electrophoretic profiles found in CBD and PiD may be explained. In PiD, Pick bodies consist of random coiled filaments made of aggregated E10– tau isoforms that are found in these characteristic neuronal populations [13,19]. For instance, granular cells of the dentate gyrus are one of the cell types that exhibit Pick bodies in PiD: they do not express E10+ isoforms [7]. Our transfection studies indicate that hyperphosphorylated E10– isoforms lead to the same electrophoretic profile as that found in PiD (Tau 55 and 64). Thus, this is the molecular demonstration that E10– tau isoforms are found in Pick bodies. Conversely, in CBD and likely in PSP, only E10+ tau isoforms aggregate into straight filaments. The characteristic tau doublet Tau 64 and 69 are obtained after transfection of E10+ tau isoforms and their hyperphosphorylation. It was reported that exon 3 translated sequence was not found in CBD [25]. This controversial finding may be explained by the very low amount of the 2+3+10+ isoform found phosphorylated in CBD at 74 kDa. Thus, E10+ tau isoforms are likely to aggregate in subsets of neurons that are degenerating in PSP/CBD. It should also be noted that particular tau gene polymorphisms in PSP may

modify alternative splicing and lead to an increase in E10+ tau isoforms formation [26]. Similarly, in FTDP-17, point mutations in introns close to exon 10 may also increase levels of E10+ tau isoforms further leading to their aggregation [27,28]. A point mutation in exon 10 was also reported in one family with FTDP-17. It is located in the microtubule binding domain and is likely to induce important conformational changes leading to a lack of binding to microtubules. In this family, E10+ tau proteins aggregate into filaments and are mainly characterized by the tau doublet Tau 64 and 69 [27]. These data suggest that tau isoforms that do not bind to microtubules may aggregate into filaments. Thus, our data are consistent with recent genetic analyses suggesting that particular tau isoforms may aggregate in subsets of neurons.

However, it should be noted that differences in tau phosphorylation were observed between pathology and transfection studies. The 12E8 immunoreactivity was not found in PiD in two studies [13,21] whereas it was present in a neuropathological analysis in one case [29] and also in the present study, after transfection of E10− tau isoforms and cell treatment by OA. Conversely, AD2 and AT100 immunoreactivities were obtained in our cell model suggesting that the sequential phosphorylation GSK3β and pKA responsible for AT100 epitope formation [18] occurs in COS cells. Such cascades of kinases were already suggested in COS cells [23]. Thus, transduction signals involved in kinases activation/phosphatases inhibition are much more complex than a single hyperphosphorylation process.

In conclusion, degeneration of neurons leads to the aggregation and hyperphosphorylation of their constitutive tau isoforms. Thus, the biochemical signatures (Tau 55 and 64, Tau 64 and 69, Tau 55, 64 and 69) found in neurodegenerative disorders are related to the presence of both different combinations of tau isoforms (E10− and/or E10+) and particular kinases in subpopulations of neurons.

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