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Assembly of Alzheimer-like filaments from full-length tau protein

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Abstract

The principal fibrous component of neurofibrillary pathology in Alzheimer's disease, the paired helical filament, is formed from hyper-phosphorylated microtubule-associated protein tau. Here we show that recombinant tau protein either in a non-phosphorylated state or following phosphorylation with brain extract can be assembled in vitro into filaments resembling those seen in Alzheimer's disease.

Key words: Alzheimer's disease; Microtubule-associated protein tau; Paired helical filament; Hyper-phosphorylation

1. Introduction

Extensive neurofibrillary pathology, in the form of neurofibrillary tangles, neuritic elements in plaques and a network of neuropil threads in affected areas of the brain, is one of the defining characteristics of Alzheimer's disease. Paired helical filaments (PHFs) form the principal abnormal fibrous component of such fibrillar deposits. Using preparations of PHFs from tangles it was shown that microtubule-associated tau protein forms a component of the PHF [1–3]. Biochemical analysis of preparations of dispersed PHFs produced by sarcosyl extraction [4] indicated that abnormally hyper-phosphorylated tau protein (known as PHF-tau or A68) is likely to be the sole component of the PHF [5].

In normal adult human brain six isoforms of tau protein are produced by alternative mRNA splicing [6,7]. On gels PHF-tau runs as three retarded bands [4,5,8] but after extensive de-phosphorylation generates six bands, which align with the six recombinant isoforms [9], indicating that PHF-tau consists of all six isoforms in an hyper-phosphorylated state. Some of the sites of hyper-phosphorylation have been identified by using antibodies specific for the phosphorylated sites [5,10,11], others have been identified by direct sequencing [12]. Many of these sites can be phosphorylated in vitro by brain extract [10,11] or by purified kinases [13–17]. The isoforms of tau protein contain towards the carboxyl-terminus three or four tandem repeats of 31 or 32 amino acids, which represent the microtubule binding domain of the protein [18,19]. The known phosphorylation sites, with the exception of Ser^{262} [12,20], all lie in regions flanking the repeats. Proteolytic digestion of PHFs monitored by labelling with antibodies specific for various regions of the protein indicates that the amino-terminal half of the protein and the carboxyl terminal form a fuzzy coat around the filament, while the repeat region forms the core of the structure [8,9,21]. Sequence analysis of tau fragments extracted from pronase treated tangle PHFs indicated that both three and four repeat isoforms contribute to the core but that in both cases a length corresponding to only three repeats is protected [22].

These observations suggested that it might be possible to produce PHF-like filaments in vitro using just the repeat region of tau protein, without the flanking regions and their attendant phosphorylation sites. Two different recombinant fragments of tau protein, each spanning approximately three repeats, produced filaments like Alzheimer PHFs [23,24]. To date it has not proved possible to form PHFs in vivo in either transfected cell lines or transgenic animals. However, the ability to phosphorylate recombinant tau protein in vitro in a way similar to its abnormal phosphorylation in Alzheimer's disease allows attempts to assemble filaments from intact protein in different phosphorylation states. We show here that, using assembly conditions different from those successful for the repeat region, it is possible in vitro to produce twisted filaments resembling Alzheimer PHFs from full length recombinant tau protein, either in a non-phosphorylated state or after phosphorylation with brain extract. This observation raises the question of the

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role of the phosphorylation state of tau protein in the assembly of PHFs in vivo.

2. Materials and methods

2.1. Expression, purification and phosphorylation of tau protein

Human tau protein with four repeats corresponding to the 441 amino acid isoform (clone htau40) was expressed in *E. coli* and purified by a modification of the published procedure [25]. Frozen *E. coli* pellets were sonicated in 10 ml/g extraction buffer (50 mM piperazine-*N*,*N*′-bis-2-ethanesulphonic acid (PIPES), pH 6.9, 1 mM dithiothreitol (DTT), 0.1 mM EDTA, 0.1 mM phenylmethylsulphonyl fluoride (PMSF), 1 μM leupeptin, 1 μM pepstatin). After a 15 min spin (10,000 × *g*), the supernatant was brought to 0.5 M NaCl and incubated for 10 min in a boiling water bath. After another centrifugation the supernatant was dialysed twice at 4°C against buffer A (20 mM PIPES, pH 6.9, 50 mM NaCl, 2 mM DTT, 1 mM EGTA, 1 mM $MgSO_4$, 0.1 mM PMSF) and filtered through a 0.2 μm Arcodisc. The filtrate was applied to a Mono-S HR5/5 column equilibrated in buffer A at a flow rate of 1 ml/min and then eluted with a 0–0.5 M gradient of NaCl in buffer A. One ml fractions were collected and aliquots run on SDS-PAGE. The peak tau fractions were dialyzed at 4°C against 40 mM HEPES, pH 7.4, 1 mM DTT, 0.1 mM PMSF and stored frozen until use.

The tau protein was phosphorylated by an extract from adult rat brain as described [11]. Purification and phosphorylation were monitored by SDS-polyacrylamide gels and immuno-blotting with antibodies 134 (a general anti-tau antibody directed against the C-terminus) [7], AT8 [10,11,26] or T3P [5] (against epitopes phosphorylated at serine residues 202 or 396, respectively). Protein concentrations were estimated by Bradford assay (Biorad). In some cases the phosphorylated protein was purified by boiling to remove most of the brain extract proteins prior to concentration. After dialysis in 2 mM Tris-Cl, pH 7.5, tau samples for assembly trials were concentrated to approximately 10 mg/ml by freeze drying and resuspension.

2.2. Assembly of filaments and electron microscopy

Filaments were grown by vapour diffusion in hanging drops in the standard way used for protein crystallization [27]. Trials were carried out with a range of buffers of different pH and ionic strength. Filaments were obtained at room temperature with the well buffer containing 0.5 M Tris-Cl, 1.25 M potassium acetate, pH 6.9 or pH 7.4.

Specimens were deposited on C-coated grids, which were sometimes glow-discharged prior to use, washed with a few drops of distilled water and stained with 1% LiPTA. Micrographs were recorded at a nominal magnification of × 45,000 on a Philips EM301 microscope.

3. Results and discussion

Tau protein corresponding to the 441 amino acid isoform from human brain was expressed and purified. It was phosphorylated using an extract from adult rat brain. The purification and phosphorylation were monitored by gel electrophoresis and immuno-blotting. The characteristic shift to lower mobility after phosphorylation is shown by staining with the general tau antibody 134 (Fig. 1). Similar gels stained with antibodies specific for phosphorylated serine residues 202 (antibody AT8) or 396 (antibody T3P) indicated that the incubation with brain extract produced a high level of phosphorylation at these sites (Fig. 1). Under the conditions used, approximately 8 mol of phosphate per mol of tau were incorporated (data not shown).

Assembly trials over a range of buffer conditions were made both with non-phosphorylated tau and with tau

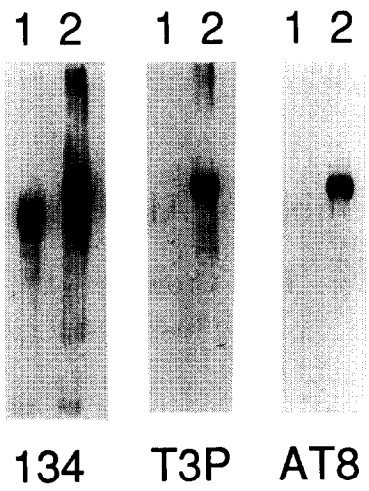

Fig. 1. Immuno-blots of expressed human tau protein before and after phosphorylation. In each panel lane 1 represents the 441 amino acid isoform before phosphorylation, lane 2 after phosphorylation. Staining with the general tau antibody 134 shows a shift to lower mobility after phosphorylation. Phosphorylation specific antibodies T3P and AT8 show no staining before phosphorylation but strong staining afterwards.

phosphorylated by brain extract. Filaments were found for both forms of protein at neutral or slightly alkaline pH 6.9–7.4 in 0.5 M Tris, 1.25 M potassium acetate. These are different from the conditions used previously for producing filaments from the repeat region fragments, which involved acidic conditions in the range pH 4.5–5.

The majority species of filament produced under these conditions from non-phosphorylated tau is thin, with images from negatively stained specimens showing alternating regions of strong and weak stain exclusion, which give the images a dashed appearance (Fig. 2). The apparent axial period of these filaments is approximately 25 nm and their width alternates between about 4.5 nm and 9 nm. Their appearance is rather like very tiny PHFs and we propose to call them 'dashed mini-filaments'. The arrangement of tau molecules in such structures is unknown but we are not suggesting that it is related to the arrangement of tau in PHFs. Amongst the dashed mini-filaments, examples of much more clearly twisted ribbon-like filaments are found (Fig. 2). The images of these filaments alternate in width between about 4.5 nm and 13 nm with an apparent period of 165 nm. Such filaments are reminiscent of Alzheimer PHFs, though the relative proportions of the features are different. However, their existence indicates that non-phosphorylated tau can be assembled into twisted filaments.

The majority species assembled from phosphorylated tau under these conditions is also the dashed mini-filament (Fig. 3a). Again amongst these are found twisted filaments but now with proportions much more like those of Alzheimer PHFs. The example in Fig. 3a alter-

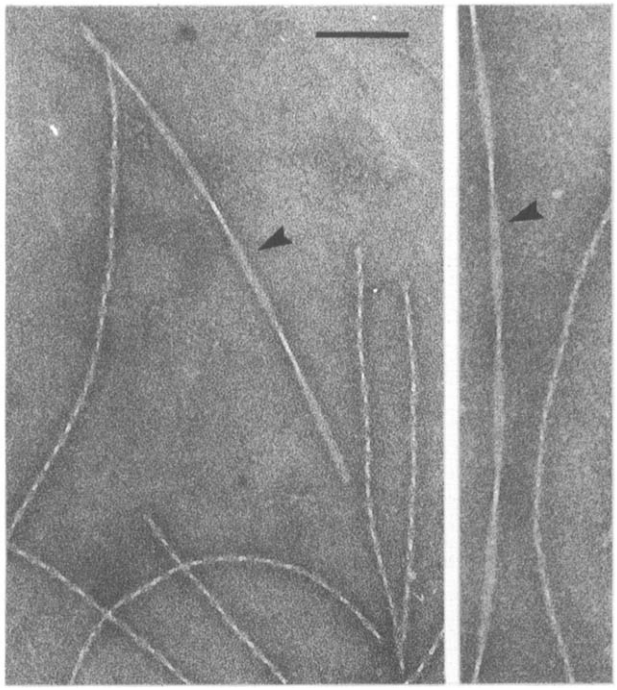

Fig. 2. Filaments assembled in vitro from the non-phosphorylated 441 amino acid isoform of human tau. The majority of filaments are of the dashed mini-filament type (see text) but some larger twisted filaments are found (arrows). Bar = 100 nm.

nates in width between about 7 nm and 18 nm with an apparent period of 83 nm. The example in Fig. 3b has a longer pitch but shows clearly a single stranded region at one end, indicating that these structures are 'paired helical filaments' consisting of two strands of subunits. Fig. 3d shows a filament with much longer pitch which resembles the un-wound ribbons formed from Alzheimer PHFs by treatment with formic acid [28] and in particular shows a region which clearly exhibits two pairs of stain excluding lines of density running parallel to the axis of the filament. Such an appearance is characteristic of Alzheimer PHFs and arises from the intrinsic arrangement of subunits within the PHF [29]. The twisted filaments formed from phosphorylated tau (Fig. 3) appear more similar to Alzheimer PHFs than do those made from non-phosphorylated tau (Fig. 2), in that their proportions are more like those of PHFs and that the narrow cross-over region shows a greater stain excluding density.

These results indicate that twisted filaments resembling Alzheimer PHFs can be assembled at physiological pH but high ionic strength from both phosphorylated and non-phosphorylated full-length tau. The Alzheimer-like filaments assembled from fragments corresponding to the repeat region were produced at much lower pH [23,24]. The majority species, the dashed mini-filament, produced from both states of the full-length protein appears very different from PHFs. Its existence emphasizes the range of filamentous structures formed by tau and mirrors the polymorphism found in the disease, where both PHFs and straight filaments are found [30]. The observation that both non-phosphorylated and phosphorylated tau can be assembled into twisted filaments raises the question of the role of the hyper-phosphorylation of tau in the pathology of Alzheimer's disease. While the markedly reduced affinity of PHF-tau for microtubules that results from its hyper-phosphorylation [31,32] probably affects the integrity of neuronal microtubules, it is not clear whether the phosphorylation of tau per se also promotes PHF assembly. The cluster of actual and potential phosphorylation sites just N-terminal of the repeat region overlaps with a strongly basic region of the protein. We have suggested that one result of phosphorylation might be to neutralize this region partially, thus overcoming a charge repulsion that might normally inhibit assembly of the non-phosphorylated protein [33]. The present results do not necessarily cast doubt on such a model, because the in vitro assembly conditions used

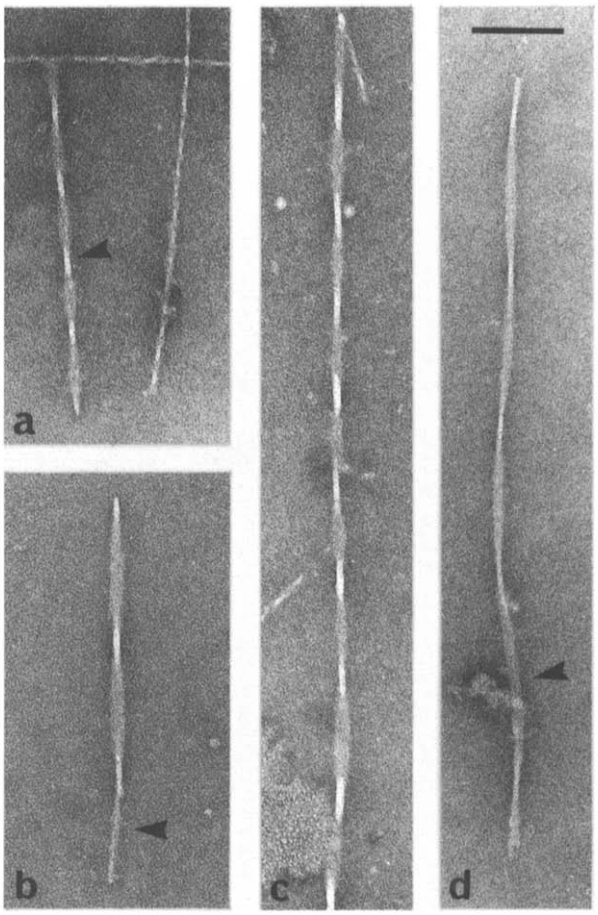

Fig. 3. Filaments assembled in vitro from the 441 amino acid isoform of human tau phosphorylated by brain extract. (a) shows two dashed mini-filaments and a PHF-like twisted filament (arrow). (b–d) Show other examples of twisted filaments with a range of pitches. In (b) the arrow indicates a region of 'half-filament'. In (d) the arrow indicates a region of ribbon-like filament, in which two pairs of white stain-excluding tracks run parallel to the axis of the filament. Bar = 100 nm.

here, though at physiological pH, are otherwise far from physiological, particularly with respect to high protein concentration and high ionic strength. The abnormal hyper-phosphorylation of tau in the brain in Alzheimer's disease may in some way lower the concentration necessary for assembly in vivo, such that, in the long time scale of the disease, hyper- phosphorylated tau assembles into PHFs whereas normal tau does not. Further experiments are in progress in an attempt to improve the efficiency of assembly and to investigate more closely the differences that phosphorylation makes to assembly.

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