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The microtubule binding repeats of tau protein assemble into filaments like those found in Alzheimer's disease

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The paired helical filament, which comprises the major fibrous element of the neurofibrillary tangle in Alzheimer's disease, contains abnormally phosphorylated microtubule-associated protein tau as its principal constituent. The repeat region of tau protein, which represents the microtubule binding domain, forms the core of the filament. Here we show that an expressed fragment of tau protein spanning the repeat region can assemble in vitro into filaments like those found in Alzheimer's disease

Alzheimer's disease; Microtubule-associated protein tau; Paired helical filament

1. INTRODUCTION

Neurofibrillary tangles constitute one of the defining pathological characteristics of Alzheimer's disease, where their presence in large numbers is indicative of dementia. The principal fibrous component of the tangle is a structure not normally seen in the cytoskeleton known as the paired helical filament (PHF) [1]. PHFs also occur in neuropil threads and in neuritic elements of plaques. PHFs have been prepared from the brains of Alzheimer patients by two quite distinct methods. One approach is to isolate from tangles long PHFs with a core structure that is pronase resistant [2]. From such preparations it was shown directly that the microtubule-associated protein tau forms a component of the PHF [2–4]. The other approach is to use a sarcosyl extraction procedure to produce dispersed PHFs, while discarding the tangle fragments [5]. Such PHFs are shorter and more pronase sensitive than those in tangle fragments and are more tractable, in particular being soluble in denaturing agents such as guanidine or urea. Biochemical analysis of dispersed PHF preparations indicated that abnormally phosphorylated tau protein (known as PHF-tau or A68) was likely to be the sole component of the PHF [6].

In normal adult human brain microtubule-associated protein tau comprises six isoforms, produced from a single gene by alternative mRNA splicing [7,8]. Tau protein from dispersed PHF preparations runs more slowly on gels than normal tau [5,6,9–11] but after extensive de-phosphorylation aligns with the six recombinant isoforms [12], indicating that PHF-tau consists of all six isoforms in an abnormally phosphorylated state. The isoforms 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 [13,14]. 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 region form a fuzzy coat around the filament, while the repeat region forms the core of the structure [10,12,15]. PHF cores from tangle fragments are more resistant to proteolysis than are those from dispersed PHFs, which are therefore likely to represent an earlier and less cross-linked stage of the PHF.

Sequence analysis of tau fragments recovered from pronase treated tangle PHFs indicated that both three and four repeat isoforms contribute to the core of the PHF but that in both cases a length of protein containing only three repeats is protected [16]. Accordingly we thought that if the PHF did indeed contain only tau protein, then it might be possible to assemble filaments in vitro from a fragment of protein corresponding to three tandem repeats. We show here that, by using appropriate buffer conditions, it is possible from an expressed three repeat fragment of tau protein to produce filaments like those observed in Alzheimer's disease.

2. MATERIALS AND METHODS

2.1. *Expression and purification of a fragment of tau protein*

A full-length cDNA clone (htau23 [7]) corresponding to a three-repeat isoform of tau protein was subcloned into the *Eco*RI site of M13mp18. To express a fragment of tau protein corresponding to the three-repeat region, site-directed mutagenesis was used to introduce an NdeI site in the context of the codon for Met-192 (using the

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numbering of the shortest 352 amino acid isoform), two residues before the repeat region. A terminator codon was introduced instead of the codon for Glu-291. Following cleavage with *Nde*I and *Eco*RI the resulting DNA fragments were subcloned downstream of the T7 RNA polymerase promoter in the expression plasmid pRK172 and the recombinant plasmids were transformed into *E. coli* BL21 (DE3) cells. The bacterial cultures were grown, induced and harvested as described [17].

The pellet from a 500 ml culture was resuspended in 20 ml buffer A (50 mM PIPES, pH 6.8, 1 mM DTT, 1 mM EDTA, 0.2 mM PMSF, 0.5 μg/ml leupeptin, 0.5 μg/ml pepstatin), and sonicated for 2 × 3 min using a Kontes micro ultrasonic cell disrupter. Following centrifugation (15,000 rpm for 15 min), the supernatant was passed through a DE-52 cellulose column. The flow-through was loaded onto a P11 phospho-cellulose column, eluted with a 0–500 mM NaCl gradient and 5 ml fractions were collected. Purification was monitored by SDS-polyacrylamide gradient gels (10–20%) and immuno-blotting with polyclonal antibody 135 [8], raised against a synthetic peptide from the repeat region. Fractions containing the tau protein fragment were pooled and dialysed against 50 mM phosphate buffer, pH 6, or 50 mM PIPES buffer, pH 6. Subsequently, the protein was concentrated 5- to 10-fold by freeze-drying.

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 [18]. Trials were carried out over a range of pH, ionic strength, with and without $MgCl_2$, at room temperature and at 4°C. Filaments were obtained at room temperature with the well buffer containing 0.5–0.6 M Tris-HCl, pH 4.5–5.

Specimens were deposited on C-coated grids, washed with 5 drops of distilled water and stained negatively with 1% LiPTA. Micrographs were recorded at a nominal magnification of X45000 on a Philips EM301 microscope.

3. RESULTS AND DISCUSSION

The predicted protein sequence (Fig. 1) coded by the recombinant clone has a length of 99 amino acids and spans the region of tandem repeats found in the three-repeat tau isoforms. The protein was expressed at a high level and was purified as described. Fig. 2 shows a gel of the purified fragment and a corresponding immunoblot confirming the identity of the band. The fragment self-associates to form dimers, as was the case for the tau fragment recovered from PHF cores [16]. The apparent molecular weights based on gel mobility were approximately 13 and 27 kDa for the monomer and dimer, respectively. N-terminal sequencing of the protein indicates that the initiating methionine is removed in the bacteria.

The original reason for producing this protein fragment was to see whether any indication of folded structure could be detected by NMR. Although there was little sign of folded structure in the NMR spectra (D. Neuhaus, personal communication), we noticed that when the solution was buffered to pH 5 the protein showed a strong tendency to precipitate. This suggested that low pH might be an appropriate condition for assembly of filaments. Previously, paracrystals of tau protein had been grown by dialysis against sodium acetate or Tris-HCl buffer [19]. Trials were made over a range of ionic strength and pH both at room temperature and at 4°C. Filaments were found in drops equilibrated at room temperature against well buffers in the range 0.5–0.6 M Tris-HCl, pH 4.5–5. Various filamentous structures were observed including coiled ribbons, short thin unbranched filaments and, most interestingly, filaments resembling the PHFs of Alzheimer's disease.

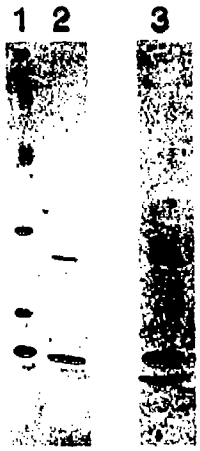

Fig. 2. Coomassie-stained gel and corresponding immunoblot of the purified protein fragment. Lane 1, molecular weight markers of 14, 20, 30, 43, 67 and 94 kDa; lane 2, purified protein; lane 3, immunoblot labelled with antiserum 135 specific for the repeat region of tau protein [8]. Note that the protein fragment tends to form dimers.

A series of such in vitro assembled filaments is shown in Fig. 3b–i, compared with a pronase treated PHF from an Alzheimer tangle preparation (Fig. 3a). The images of assembled filaments show an alternating pattern of wide and narrow regions varying in width between about 16 nm and 5 nm with an apparent period of 70–90 nm (Fig. 3b–g). In some favourable places at the widest part of the image a pattern of four longitudinal white stain excluding lines can be seen, characteristic of the Alzheimer PHF structure [20,21]. This pattern is clearer in the ribbon-like structures in Fig. 3g–h, which resemble the images of untwisted Alzheimer PHFs produced by low pH treatment [22]. The final panel (Fig. 3i) shows what appears to be a 'half-PHF' type of structure of the sort described in [20], in that its width appears to be about half that of the filaments in (b–g) and the character of the stain exclusion at the edges of the filament alternates between sharp and fuzzy. These PHF-like filaments were not frequent and had to be searched for on the microscope grid. They were mostly

HPDLKNVKSKIGSTENLKHQPGGGKVQIVYKPV
DLSKVTSKCGSLGNIHHKPGGGQVEVKSEKLDF
KDRVQSKIGSLDNITHVPGGGNKKIETHKLTFR

Fig. 1. Predicted amino acid sequence (in the single letter code) of the tau protein fragment coded by the recombinant clone. The sequence contains 99 amino acids (residues 192–290 of the shortest isoform) and spans slightly more than three tandem repeats. The characteristic PGGG motif is underlined in each repeat.

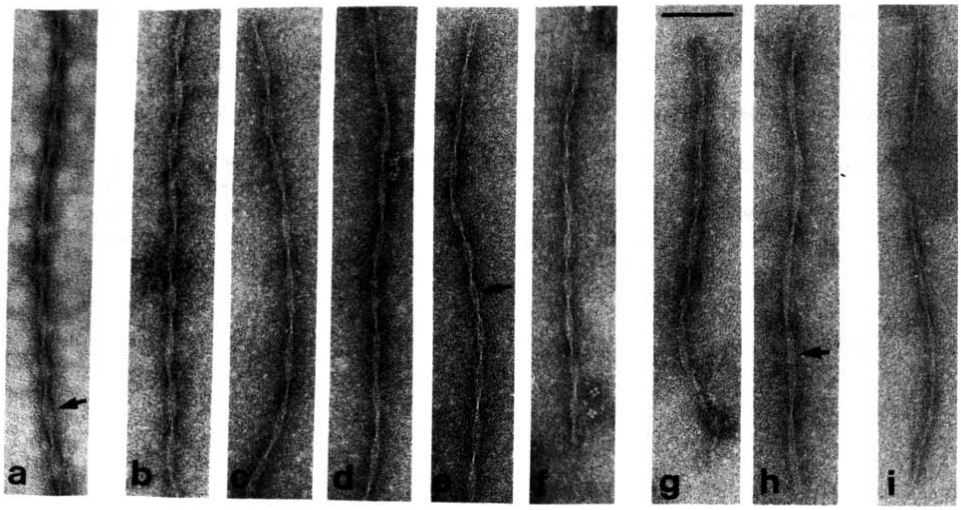

Fig. 3. Alzheimer PHF and filaments assembled in vitro from the expressed fragment of tau protein. (a) Pronase treated Alzheimer PHF from the preparation described in [15]. (b–f) Filaments resembling PHFs assembled from expressed protein. (g–h) In vitro assembled filaments, one partially and the other more fully untwisted, resembling the untwisted ribbon-like form of PHFs [22]. (i) In vitro assembled filament resembling the 'half-PHFs' described in [20]. The arrows indicate regions of filament where the characteristic pattern of four white stain excluding lines parallel to the axis can be seen. Scale bar 100 nm.

about 1 μm long and no short fragments were observed, suggesting that, not unexpectedly, it is the nucleation step which is limiting the assembly of filaments.

Although, as outlined above, the in vitro assembled filaments resemble Alzheimer PHFs in many characteristic structural respects, the assembled filaments appear to have less stain excluding mass per unit length than pronase treated Alzheimer PHFs (Fig. 3). Pronase treated PHFs have the amino- and carboxyl-terminal parts of tau protein removed, but the fragment of protein spanning the repeat region that is left behind is larger than that used here for the in vitro assembly. This additional mass will contribute directly to the increased mass per unit length and may affect detailed staining of the filaments; more importantly, by changing the interactions between the polypeptide chains, it may affect the density of packing of the protein in the filament. Further experiments with different expressed proteins from the repeat region of tau are necessary to determine exactly which parts affect the detials of packing.

The fragment of tau used here does not contain any of the sites believed to be phosphorylated in PHF-tau. To date, all the sites that are known to be abnormally phosphorylated in PHF-tau are serine residues followed by a proline (SP sites) [6,23]. The longest human brain tau isoform [8] contains 17 SP and TP sites that are distributed through the amino-terminal half and the carboxy-terminal region but are absent from the repeat microtubule binding region. This striking distribution of potential abnormal phosphorylation sites may indicate that the abnormal phosphorylation of tau in Alzheimer's disease leads to a conformational change in the molecule that favours self-aggregation over microtubule binding and leads to filament assembly by association of the non-phosphorylated microtubule binding region. This interpretation is consistent with the results reported here, which show that the repeat region of tau protein, without the phosphorylated flanking regions, can assemble into filaments like Alzheimer PHFs. The present findings strongly support the idea that tau protein in an abnormally phosphorylated state is the only component necessary to form PHFs.

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