Binding of Tau Protein to the Ends of *ex vivo* Paired Helical Filaments

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Abstract. Human recombinant tau can bind to the end of isolated human paired helical filaments (PHF). The sequential binding of tau protein to PHF could result in an elongation of the previously polymerized PHF. However, we have observed that the elongation takes place in a different way on different types of PHF. We have found that there are at least three populations of PHF. For one population, tau protein is able to bind to the ends of the filament and to elongate that filament. In the second PHF population, tau protein binds but does not elongates the filament. In the third, neither tau binding nor elongation was observed.

Keywords: Elongation, paired helical filaments, tau protein

INTRODUCTION

The presence of filamentous particles composed of polymerized tau protein is a hallmark of Alzheimer's disease (AD) and other tauopathies [1]. These AD related filamentous particles, which are known as paired helical filaments (PHF) [2], are typically coiled into superhelical structures observed by electron microscopy as a pattern oscillating between 20 nm and 10 nm widths every 80 nm in length [3]. The overall length of these filaments is, however, highly variable [3].

Tau protein from recombinant means has been shown to polymerize *in vitro* [4] from concentrated solution into filaments that share some of the morphological features of PHF [5–7]. This *in vitro* polymerization reaction seems to require the participation of additional compounds, thereby termed tau polymerization inducers, such as heparin [5,8,9], quinones such as ubiquinone [10] or fatty acids [11]. The *in vitro* nucleation and growth of tau filaments, even in the presence of inducers, like heparin, occurs very slowly [5,8]. As a result, periods of up to two weeks are typically required to reach steady-state in the polymerization of tau under these conditions [12].

It has been recently proposed that the elongation of PHF polymers takes place through the addition of tau protein at the ends of the filament [13]. However, no experimental evidence is available to directly support this idea, neither on *in vitro* grown filaments nor on *ex vivo* PHF. Here we are interested in investigating the mechanism of tau polymerization on pre-nucleated PHF. Particularly, we have studied the incorporation of human tau obtained by recombinant means to human *ex vivo* PHF isolated from AD patients. Using a simple *in vitro* assay, we find a very slight elongation of *ex vivo* PHF upon incubation with recombinant tau for 10 days in the absence of tau polymerization inducers. To directly monitor the binding and/or incorporation of recombinant tau to PHF we use tau protein that has been chemically tagged with a fluorophore. Our results show that fluorescent tau readily binds to the surface of PHF. This binding, however, appears to be weak and non-specific. In addition, we observe preferential (i.e.,

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higher affinity and more specific) binding of fluorescent tau to the ends of pre-existing PHF, again in the absence of tau polymerization inducers. Remarkably, recombinant tau accumulates in one of the two PHF ends, thus suggesting the existence of a growth polarity similarly to the polymerization of other filamentous structures like microtubules.

MATERIALS AND METHODS

Materials

Thioflavin S was obtained from Aldrich (Steinheim, Germany). Gelatin Granular was obtained from Electron Microscopy Science (PA, USA). To detect tau protein we used antibodies ab7.51 (a kind gift of Dr. Wischik, UK; 1:1000) [14] and abT14 (antibody against a peptide containing aminoacids 83 to 120 of human tau; 1:500, Zymed, CA, USA). The antibody used (at a dilution among 1:500 to 1:1000) to detect both Cy3 and Cy5 dyes (Anti-Cy3/Cy5) was purchased from Sigma. The chemical composition of the phosphate buffered saline (PBS) used in the present work was 136 mM NaCl, 2.68 mM KCl, 8 mM Na_2HPO_4 $2H_2O$ and 1.46 mM KH_2PO_4.

Paired helical filament isolation and labeling with Cy5 fluorescent dye

PHF were isolated by preparation of sarkosyl-insoluble extracts, as previously described [15].The isolated PHF were characterized by electron microscopy as previously indicated [16]. Protein content was measured by the BCA (bicinchoninic acid) method at 37°C. PHF samples were labeled with the sulfoindocyanine dye Cy5 (Amersham Biosciences) using the same method described to label purified tau samples [17]. PHF samples were visualized by Transmission Electron and fluorescence microscopy as described below.

Recombinant tau isolation and Cy3 labeling

The expression and purification of recombinant tau protein was performed as previously described [8]. The isolated proteins were characterized by gel electrophoresis and Western blot, using antibodies raised against tau protein (7.51). Purified tau was labeled with sulfoindocyanine Cy3; Amersham Biosciences as described in [17]. The assembly of Tau-Cy3 was done as indicated in reference [20] but in the absence of trifluoroethanol. The binding of tau-Cy3 to PHF was characterized by Immunoelectron Microscopy (IM) and also by immunofluorescence analysis as described below.

Immunofluorescence analysis for isolated PHF, PHF-Cy5 and, Tau-Cy3

A modified version of the protocol previously described was carried out [13]. Briefly, a suspension of isolated PHF previously incubated with tau-Cy3 (or without tau-Cy3 for the negative control) was adsorbed to gelatinized glass slides, and then let to dry at 37 °C. The protein adsorbed to the slide was processed for double immunofluorescence experiments with an antibody raised against tau (T14)(1: 500) [13] and 0.01 % thioflavin S for samples with only PHF, and only with 0.01 % thioflavin S when PHF were mixed with tau-Cy3. Experiments on PHF labeled with Cy5 were done the same way, but eliminating the steps including staining with thioflavin S and the incubation with antibodies. Samples were imaged with a Zeiss Axiovert200 fluorescent microscope. Fluorescence images were captured through a 100X oil-immersion objective on a high- resolution CCD camera (SPOT RT Slider, Diagnostic). The same fluorescent microscope and CCD camera were used to measure fluorescence intensity of filamentous polymers treated with T14 antibody (an anti-human tau antibody obtained from Zymed, CA, USA) and thioflavin-S. The images were saved for later analysis and quantification. Camera exposure and light settings were kept constant during each experiment. Fluorescence intensity was determined with the image analysis software Metamorph 6.1 r6 (Universal Imaging).

Electron and immunoelectron microscopy

PHF samples were placed on a carbon-coated grid for 2 min and then stained with 2% (w/v) uranyl acetate for 1 min. Transmission electron microscopy was performed in a JEOL Model 1200EX electron microscope operated at 100 kV. Immunoelectron microscopy was performed after adsorption of the samples to electron microscopy carbon-coated grids. After blocking (incubation with PBS supplemented with 1% ovoalbumine in order to block unspecific binding sites) for 1 hour, samples were incubated with the first antibody (antibody dissolved in blocking solution (1/40)) for 1 h at room temperature. After extensive washing with phosphate buffered saline, the grids were incubated with the secondary antibody (1/40) conjugated with 10-nm diameter gold particles. Finally, the samples were negatively stained and observed, as described above.

Gel electrophoresis and Western blot analysis

Purified tau and Cy3 labeled tau were resuspended in loading buffer and heated at 100 °C for 5 min before sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins fractionated on gels were transferred to nitrocellulose membranes, and then the membranes were blocked for non-specific binding with 5% dry milk in PBS and 0.05% Tween-20 detergent. Primary antibodies were used in this same blocking buffer and incubated with membranes overnight at 4°C. After incubation with horseradish peroxidase (HRP)-linked secondary antibodies (Dako), proteins were detected using ECL detection reagent (Perkin Elmer Life Sciences). Gel single fluorescence images were captured on an Amershan Typhoon 9410 workstation.

RESULTS AND DISCUSSION

The kinetics of tau polymerization *in vitro* has been investigated in previous work [18,19]. Nucleation was determined from the number of assembled filaments, and elongation from the average length increase of the filaments as a function of time. In this study, we aim at determining the elongation of *ex vivo* human PHF isolated from AD patients by the *in vitro* incorporation of recombinant tau protein. We thus need to isolate *ex vivo* PHF and then develop an assay to determine the potential length increase of the PHF upon incubation with tau.

PHF isolation and characterization

PHF were isolated as indicated in Materials and Methods and characterized by electron microscopy. Samples of PHF isolated from brain of AD patients show three types of filaments (Fig. 1). One filament type (Fig. 1A) corresponds to PHF with flaky ends. The second filament type corresponds to PHF that maintain the ordered structure throughout the extension of the filament (Fig. 1B). As third type we observed typical straight filaments (Fig. 1C). Figure 2 shows a type of PHF with a structured central region and amorphous or flaky ends that has been stained with thioflavin S (Th-S). A strong staining with Th-S is apparent in the central region whereas the level of staining decreases towards the ends of the filament, in agreement with having structurally more disorganized ends.

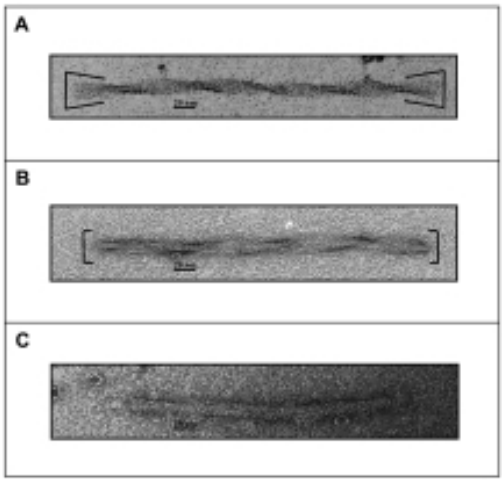

Fig. 1. Presence of different tau filamentous polymers isolated from the brain of AD patients. PHF show in their central part a constant width of about 20 nm. However, it can be found PHF with disorganized ends (A) while also can be found PHF without flaky ends (B). Straight filaments can be also found in these brain samples (C). Bar indicates 20 nm.

In vitro elongation of ex vivo PHF

In a first attempt we incubated samples of *ex vivo* PHF (with average length of 0.30 μm and deviation of 0.2 μm) with human recombinant tau protein for 10 days. The average length (see Fig. 3) of the PHF after incubation was 0.35 μm with a deviation of 0.28 μm, thus indicating slight elongation and increase in heterogeneity upon incubation with tau protein (see also length distribution). We performed the same experiment using a sample of *ex vivo* PHF that had been previously sonicated to produce PHF with an average length of 0.20 μm and standard deviation of 0.2 μm. The distribution of filaments after incubation exhibited an average length of 0.29 μm and standard deviation of 0.28 μm, again, showing signs of an elongation reaction resulting in slightly longer filaments and a more heterogeneous distribution. Although the net filament elongation that we observed was small, the consistency between the results in both experiments strongly supports the observation of *in vitro* PHF elongation.

Characterization of fluorescence labeled tau

Purified recombinant human tau protein was covalently attached to the fluorescent red dye Cy3 (see Materials and Methods). The fluorescence labeled protein

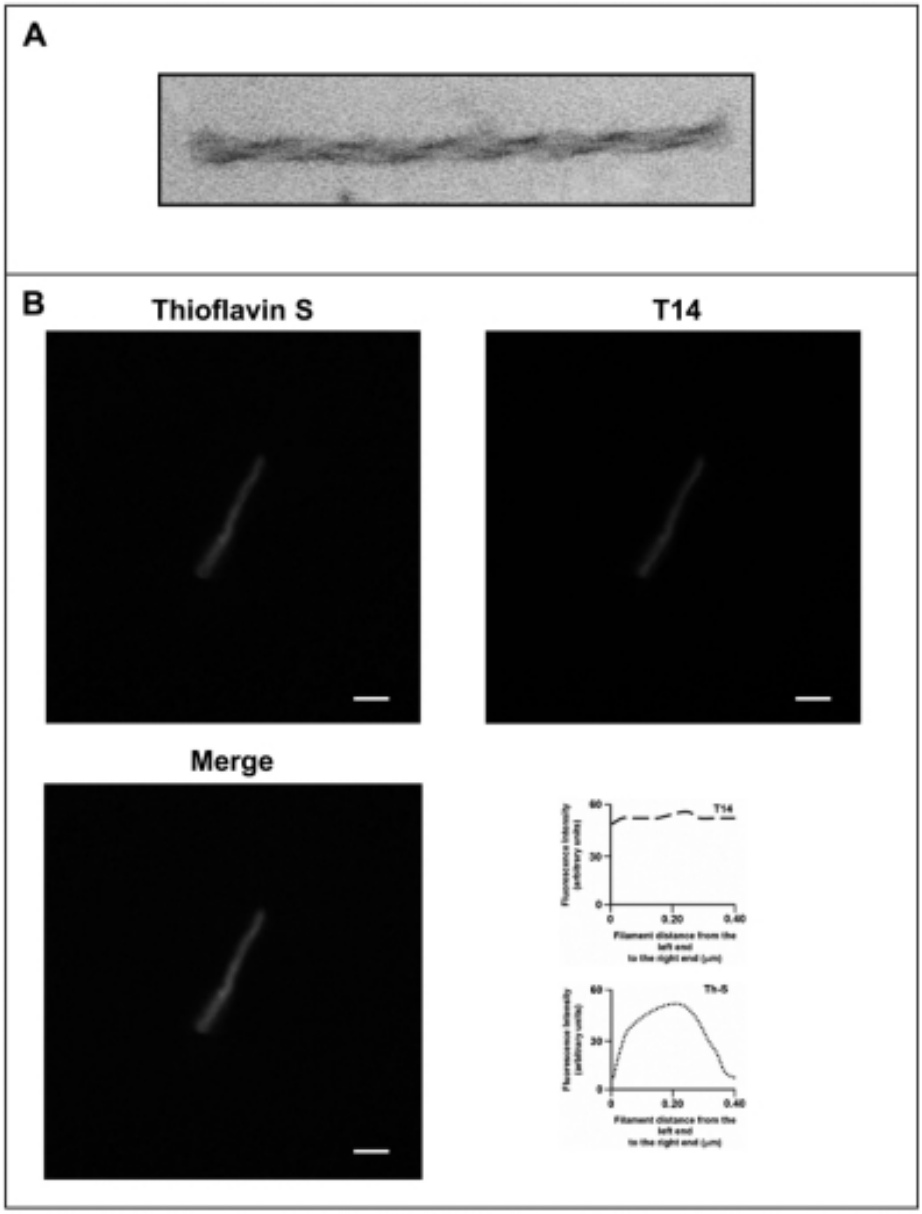

Fig. 2. Characterization of PHF by electron and immunofluorescence microscopy. A) A negatively stained PHF after its isolation and purification. B) PHF were visualized by inmunofluorescence (see Materials and Methods) using tau antibody T14 and thioflavin-S. The merge of T14 plus thioflavin-S images is shown. It is observed in the plot, a decrease in the fluorescence intensity of Th-S staining at the ends of the PHF. Bars indicate 1 μm.

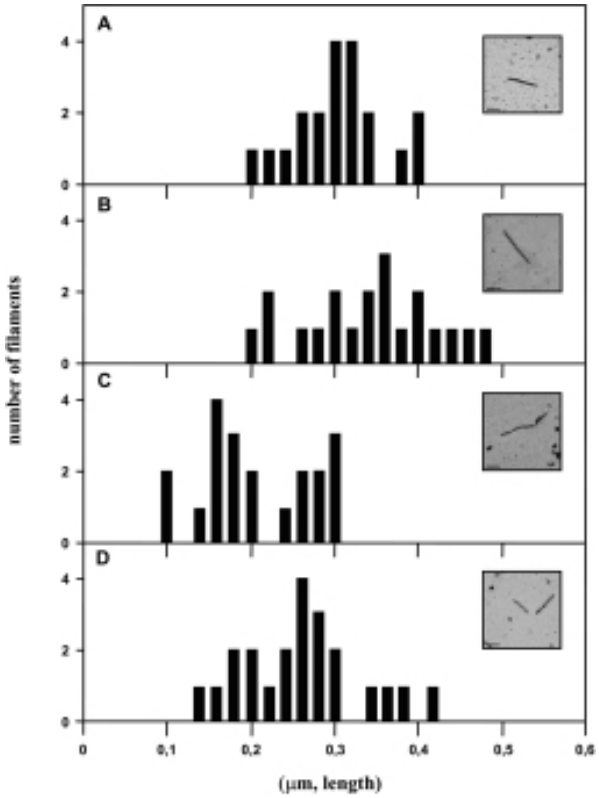

Fig. 3. Length increase for PHF incubated with tau. Isolated PHF were incubated in the absence (A), or the presence (B) of recombinant tau, for 10 days. After that time the average length of tau filaments was measured (see text). In addition, PHF were sonicated (C) to obtain a shorter average length and afterwards in the absence (C) or the presence (D) of tau. The average length of each sample is indicated in the text. Insets show examples of PHF incubated with or without tau (Bars, in electron micrographs, indicate 0.2 μm).

was characterized by gel electrophoresis and Western blot using ab 7.51 raised against tau protein and an antibody raised against Cy3. Figure 4A shows that tau-Cy3 protein does indeed bind to both antibodies.

The ability of tau-Cy3 to polymerize *in vitro* was tested by incubation of the protein for 10 days (see Methods), followed by characterization of the aggregates by immunoelectromicroscopy with the antibody raised against Cy3 (Fig. 4B). The staining with anti-Cy3 coupled to gold particles of samples from the polymerization reaction shows amorphous aggregates (see reference [20]). A negative control consisting in the incubation of Cy3 alone under the same conditions resulted in no visible aggregates (data not shown). These results demonstrate that the fluorescence labeled version of tau is also capable of self-assembly into amorphous aggregates particles.

Co-incubating PHF and fluorescence labeled tau

PHF and Tau-Cy3 were mixed and incubated for 4 days. After that incubation, the protein sample was stained with Th-S to identify PHF by immunofluorescence analysis. Figure 5A (left) shows that Th-S bind to PHF, thus resulting in green color. PHF co-incubated with tau-Cy3 showed yellow color indicating the binding to both Tau-Cy3 (red) and Th-S (green) (Fig. 5A). It is interesting to note that the ends of the filamentous particles have increased red color, suggesting increased binding to tau-Cy3.

As a control we co-incubated PHF that had been previously fluorescence labeled with Cy5 (green color), with tau-Cy3 (red). The co-incubated samples showed a yellow color (Fig. 5B), indicating that the binding of tau-Cy3 was not mediated by binding of Th-S to

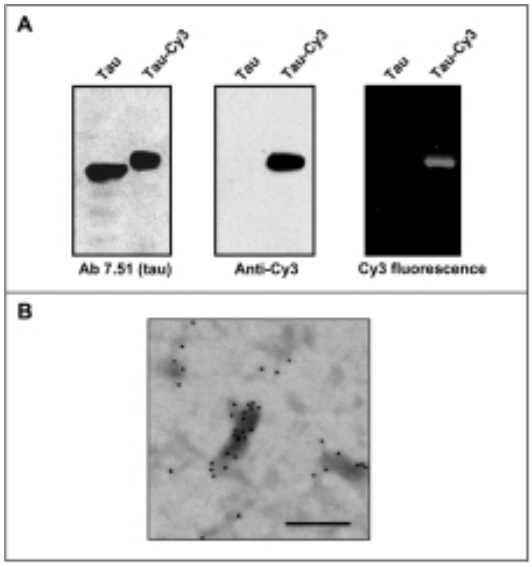

Fig. 4. Characterization of tau-Cy3. A) Tau covalently bound to the dye Cy3 was characterized by western blot using antibodies reacting against tau (ab 7.51) or against Cy3 (anti-Cy3) and also by using a Typhoon 9410 workstation. B) The capacity for self assembly of tau-Cy3 was determined by immunoelectron microscopy using anti-Cy3 and a secondary antibody conjugated to gold beads. Bar indicates 0.1 μm.

PHF. Importantly, the binding of tau-Cy3 to PHF could be eliminated by adding a large excess of unlabeled tau prior to incubation with the labeled tau-Cy3. This is manifested with the just detection of green PHF by immunofluorescence (Fig. 5C), and indicates that the binding to PHF is mediated by interactions with the tau moiety of tau-Cy3.

The overall binding that we observe seems to occur on the surface of the PHF and more or less homogeneously along the filament. However, these experiments had to be undertaken without significant washing after co-incubation to avoid the detachment of the PHF. Repetition of the experiment using gelatinized microscope slides to allow more intensive washing without PHF detachment showed, on the other hand, the preferential binding of tau-Cy3 to the ends of the PHF particles (Fig. 5D), similar to that found in Fig. 5A.

Electron microscopy analysis

The preferential binding of tau-Cy3 to the ends of PHF was further confirmed by electron microscopy. In this case, the binding of tau-Cy3 to PHF after various incubation times was monitored by immunoelectromicroscopy using an antibody against Cy3 followed by incubation with a secondary antibody coupled to gold beads. Figure 6 summarizes the results from these experiments. Some PHF particles exhibited no binding to tau-Cy3 even after 10 days of co-incubation (Fig. 6A). For a large fraction of PHF particles we observed staining with gold particles, which was mostly localized on the ends of the PHF (Fig. 6B). Moreover, the number of gold particles per PHF increased proportionally with the incubation time (Table 1). The distribution of gold beads on this population of PHF after 10 days of incubation clearly demonstrates the preferential binding to the ends of the PHF. In addition, the distribution is not even on both ends of the PHF, but it is strongly polarized to only one end per particle (see Fig. 6B). This is an important result that suggests that PHF elongation upon tau binding occurs with a predetermined growth polarity. A small number of PHF particles showed gold

Table 1
Twenty PHF were analyzed per sample, after incubation of PHF filaments with tau-Cy3 for 1, 4 or 10 days and further incubation of the mixture with anti-Cy3 antibody coupled to gold beads. The percentage of PHF bearing gold beads at their ends, after 1, 4 or 10 days of incubation, is shown. Measurements in 3 different fields of 20 filaments were done

PHF bearing gold beads at their ends			
Number of beads	1	2	3 or more
1 day of incubation	5 ± 5 %	0 %	0 %
4 days of incubation	30 ± 15 %	5 ± 5 %	0 %
10 days of incubation	55 ± 25 %	10 ± 5 %	10 ± 5 %

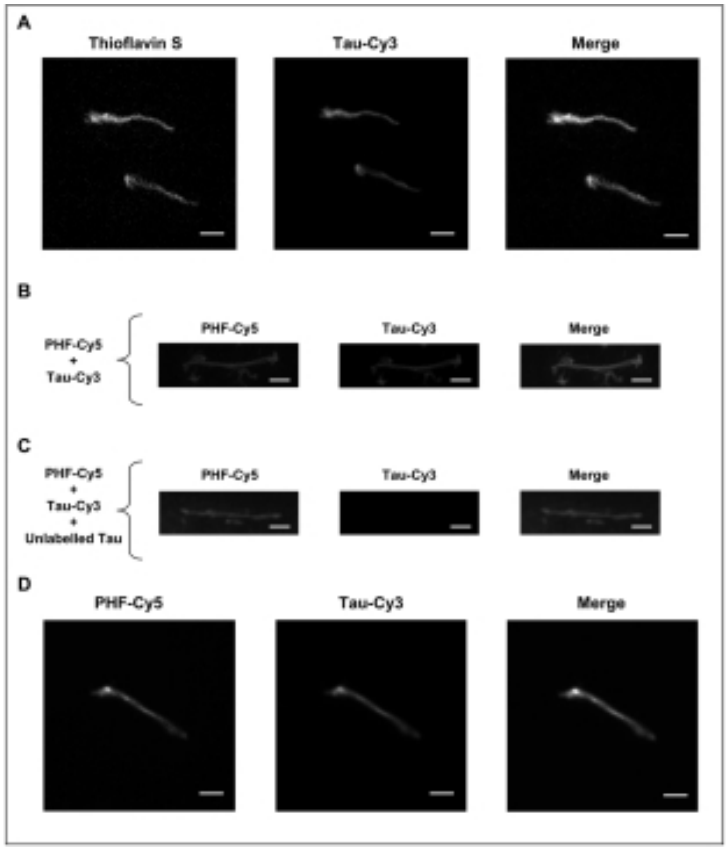

Fig. 5. Characterization by immunofluorescence analysis of tau-Cy3 bound to PHF. A) Tau-Cy3 was mixed with PHF filaments, after washing Th-S was included. The merge of both types of staining is shown to demonstrate perfect overlap. B) In this case, PHF labeled with Cy5 (PHF-Cy5) were mixed with Tau-Cy3. C) as in B, but the staining was determined in the presence of an excess of unlabeled tau protein. D) The study was carried out as in B, but with an extensive washing, after incubation with Tau-Cy3. Bars indicate 1 μm.

particles also bound closer to the center of the PHF. This binding pattern could be interpreted as indicative of a population of PHF with a degree of elongation that is significantly above the mean.

Recently, it has been shown that granular tau oligomers, which have a conformational state different to that of tau filaments grown *in vitro* in the presence of heparin, may function as a polymerization-competent state in the process of tau formation of tau filaments [18, 20]. Such granular tau oligomers are thought to bind to the flaky ends of PHF. Since PHF samples display a wide distribution of lengths for their flaky ends (see Fig. 1 for example) the kinetics for binding of tau protein could be expected to also be different, and proportional to the flakiness of the PHF ends. Therefore, we cannot discard the binding of granular tau oligomers to the ends of some PHF in our experiments, or even that process as the main factor leading to PHF elongation *in vitro*. This interpretation would also be consistent with the different types of PHF shown in Fig. 6.

Concluding remarks

It has been indicated that *in vitro* tau polymerization in the presence of inducers takes place through the third microtubule binding repeat [5]. Besides, peptides comprising only the first part of the third repeat (P1 peptide), residues 306–311 (VQIVYK) [21], or residues 317–335 (KVTSKCGSLGNIHHKPGGG; P2 peptide) from the second part of the third repeat [9] appear to retain such polymerization ability. However, it is not

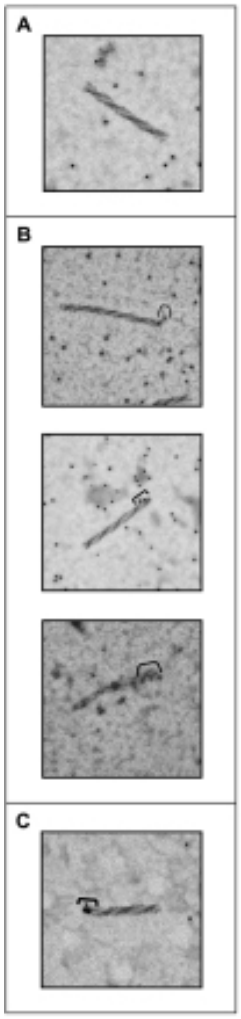

Fig. 6. Binding of tau-Cy3 to PHF analyzed by electron microscopy. Tau-Cy3 was mixed with PHF and incubated for 10 days. Binding was monitored using an antibody against Cy3 followed by incubation with a secondary antibody coupled to gold beads. Three different PHF populations were observed. A) PHF without tau-Cy3 bound to them; B) PHF with increased tau protein bound to the ends; and C) PHF with bound tau proteins that appear unable to elongate the polymer.

clear whether the *in vitro* formation of tau polymers under the presence of inducers reflects the *in vivo* formation of PHF, which seem to include a significant α-helical content [20,22,23], or a less specific aggregation process leading to amyloid-like fibrils [20].

Moreover, whereas polymers grown from peptide P1 stain well with Th-S, polymers grown from peptide P2 do not (R. Cuadros, unpublished observations) suggesting the acquisition of a non β-sheet backbone conformation. Thus, tau polymerization occurring through the P2 sequence should be expected to yield tau polymers that do not bind to Th-S, at least in a first step. Since the flaky ends of PHF show greatly decreased binding to Th-S and are involved in the binding of tau-Cy3 conducive to their elongation, as is shown here, we propose that such slow elongation (occurring in the absence of inducers) proceeds via a route similar to that of peptide P2 resulting in a non β-sheet backbone conformation. This polymerization could be mediated by the aggregation of granular tau oligomers, as it has been shown by other authors [18]. Furthermore, the *in vitro* elongation in the absence of inducers seems to exhibit growth polarity, an observation that deserves further investigation. Another issue that remains to be elucidated is whether the conformation present in the flaky ends of PHF changes into a structure competent for Th-S binding upon further maturation, or rather the Th-S binding ability is gained by formation of a secondary coat around a non β-sheet PHF core.

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