FEBS Letters 349 (1994) 104–108

FEBS 14264

Alzheimer paired helical filaments

Restoration of the biological activity by dephosphorylation

Khalid Iqbal[a,*], Tanweer Zaidi[a], Christian Bancher[b], Inge Grundke-Iqbal[a]

[a]*New York State Institute for Basic Research in Developmental Disabilities, 1050 Forest Hill Road, Staten Island, New York, NY 10314, USA*
[b]*Ludwig Boltzmann Institute of Clinical Neurobiology, Lainz Hospital, Vienna, Austria*

Received 10 May 1994

Abstract

In a normal mature neuron, microtubule associated protein tau promotes the assembly of tubulin into microtubules and maintains the structure of microtubules. In Alzheimer disease brain, tau is abnormally hyperphosphorylated and is the major protein subunit of paired helical filaments (PHF). In the present study, the biological activity of tau in PHF and the effect of dephosphorylation on this activity were examined. PHF were isolated from Alzheimer disease brains and tau from the untreated or alkaline phosphatase-treated PHF was extracted by ultrasonication in microtubule assembly buffer. Tubulin was isolated by phosphocellulose chromatography of three cycled microtubules from bovine brain. PHF-tau did not promote assembly of bovine tubulin into microtubules whereas tau from the dephosphorylated PHF produced a robust microtubule assembly. These studies suggest (i) that in Alzheimer disease tau in PHF is functionally inactive because of abnormal phosphorylation and (ii) that the abnormally phosphorylated site(s) in PHF that inactivates PHF-tau is accessible to enzymatic dephosphorylation in vitro.

Key words: Alzheimer disease; Paired helical filament; Microtubule associated protein tau; Protein dephosphorylation; Microtubule assembly

1. Introduction

Alzheimer disease (AD) is the single largest cause of dementia in the elderly. The diagnosis of this neurodegenerative disease is confirmed by the presence of numerous neurofibrillary changes of paired helical filaments (PHF) in the brain, especially the neocortex [1]. These accumulations of PHF occur (a) as neurofibrillary tangles in neuronal cell bodies, (b) as neuropil threads in the dystrophic neurite of the affected neurons in the neuropil, and (c) as dystrophic and degenerating neurites of neuritic (senile) plaques, surrounding a dense core or several wisps of extracellular amyloid. Although neurofibrillary tangles of PHF are also seen in a few other human neurodegenerative diseases and in small numbers in normal aged humans, for reasons presently not known, these lesions are not seen in aged animals or in any experimentally induced conditions [2].

Based on solubility, there are two general populations of PHF, PHF-I and PHF-II in AD brain. PHF-I and PHF-II are readily and sparingly soluble in sodium dodecyl sulfate (SDS), respectively; PHF-II are solubilized by ultrasonication and repeated extractions [3]. The major protein of PHF is the microtubule-associated protein tau [4–6]. Tau is a family of closely related polypeptides with a molecular weight range of 50,000–68,000 on SDS-PAGE [7,8]. The cDNA-derived sequences have revealed that there are at least six molecular species of human tau [9]. All of these tau isoforms are products of a single gene and result from differential splicing of the mRNA [10,11]. Tau is a phosphoprotein. Depending on the degree of phosphorylation, tau has different electrophoretic mobilities on SDS-PAGE. Tau in AD brain, especially PHF, is abnormally phosphorylated [5,6, 12,13]. The abnormal phosphorylation of tau in PHF occurs at several sites [5,6,12].

In vitro, tau stimulates microtubule assembly and co- assembles with tubulin into microtubules. Tau microinjected into cells increases assembly and decreases the rate of disassembly of microtubules in vivo [14]. Previously, we did not observe any in vitro assembly of microtubules from brain cytosol of AD cases and predicted abnormal phosphorylation of tau to be a cause of this defect [13]. In the present study, we report in vitro dephosphorylation of PHF from AD brain and a marked increase in the ability of the dephosphorylated PHF-tau to promote in vitro assembly of microtubules.

* Corresponding author. Fax: (1) (718) 494 1080.

Abbreviations: AD, Alzheimer disease; MES, 4-morpholineethane-sulfonic acid; PHF, paired helical filaments; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

2. Materials and methods

2.1. Isolation of bovine tubulin and tau

Bovine brains obtained fresh from a local slaughter house were cleaned free of meninges, rinsed in disassembly buffer and cerebral

SSDI 0014-5793(94)00650-K

hemispheres processed for in vitro assembly-disassembly cycles to three cycled microtubules by the glycerol assembly method of Shelanski et al. [15]. For isolation of tubulin, the final microtubule pellet was homogenized using a Dounce homogenizer (20 strokes) in an equal volume of 200 mM 4-morpholineethane-sulfonic acid (MES), 0.2 mM EDTA, 2 mM EGTA, 2 mM β-mercaptoethanol, 2 mM $MgCl_2$, pH 6.4. The homogenate was then left on ice for 30 minutes, centrifuged at 400,000 × g for 10 min in a Beckman TL-100 Ultracentrifuge to remove any non-disassembled microtubule proteins and passed through phosphocellulose column as described by Weingarten et al. [16]. Tubulin, which does not bind to phosphocellulose, was collected and concentrated by filtration through Centriprep-30 Concentrator (Amicon, Denver, MA).

For isolation of tau, the three-cycled microtubule pellet was diluted with two volumes of the pH 2.7 buffer (100 mM MES, 0.5 mM $MgCl_2$, 1 mM EGTA, 0.1 mM EDTA, 0.75 mM NaCl, 2 mM dithiothreitol, and 0.1 mM phenylmethylsulphonyl fluoride (PMSF), pH 2.7) and heat treated as described by Grundke-Iqbal et al. [4]. Tau, which stays in the heat stable fraction, was then concentrated by ultrafiltration using Centricon-10 Concentrator from Amicon (Denver, MA).

2.2. Isolation of PHF

PHF were isolated from histopathologically confirmed cases of AD obtained within six hours postmortem and stored frozen at −75°C. Cerebral cortex, cleaned free of meninges and underlying white matter, was processed for the isolation of PHF by the long method of Iqbal et al. [3]. PHF isolated by this method, that involves treatment with 2% SDS at room temperature, are PHF-II type. Because of the SDS treatment, the isolated PHF are free from any soluble proteins.

2.3. Dephosphorylation and extraction of PHF polypeptides

PHF were suspended (2 mg protein/ml) in an extraction buffer, pH 6.4, containing 100 mM MES, 0.1 mM EDTA, 1 mM EGTA, 1 mM $MgCl_2$, 2 mM β-mercaptoethanol, 75 mM NaCl, 0.1 mM GTP, 25 μg/ml each of leupeptin, pepstatin and antipain and 1 mM PMSF. One half of the suspension was dephosphorylated by incubating it with alkaline phosphatase (Bovine intestinal mucosa, Type VII-S from Sigma, St. Louis, MO) (0.2 mg/ml) for 3 h at room temperature. At the end of the incubation, the phosphatase was removed from PHF by centrifugation at 9,500 × g for 30 min in a Sorvall RC-5B superspeed centrifuge using rotor HS-4. The pellet was washed 3 times each with 250 mM sodium potassium phosphate buffer, pH 7.0, and the extraction buffer (see above). The final pellet was then suspended in the extraction buffer (1 ml buffer per 1 mg of starting PHF protein) and ultrasonicated for 30 min using 8 s on and 4 s off cycles at 50% pulse and input 2 with a Branson Ultrasonifier equipped with a microprobe. Following ultrasonication, the suspension was centrifuged at 400,000 × g for 10 min in a Beckman TL-100 ultracentrifuge to remove any bundles and intact PHF. The pellet was re-extracted four more times as above at the end of which practically no residue was left. All five supernatants were pooled and concentrated using Centricon-10 Concentrator, (Amicon, Denver, MA) and used as a source of dephosphorylated PHF tau. One half of each PHF preparation was treated identically to the dephosphorylated PHF tau except the alkaline phosphatase treatment which was omitted.

Dephosphorylation of PHF was confirmed by Western blots developed with monoclonal antibody Tau-1 as described previously [12]; Tau-1 labels PHF-tau only when it is dephosphorylated at abnormal phosphorylation sites Ser^{199}/Ser^{202}.

2.4. Dephosphorylation of tau

Aliquots of bovine tau were treated for dephosphorylation with alkaline phosphatase as described above for PHF. Following the phosphatase treatment, the tau-phosphatase mixture was heat treated at 95°C for 5 min to inhibit and remove phosphatase from tau.

2.5. In vitro assembly of microtubules

Microtubule assembly was carried out by incubating at 37°C in 1 cm quartz microcuvettes bovine tubulin (4 mg/ml) with various dephosphorylated or non-dephosphorylated tau and PHF preparations (0.1–0.2 mg/ml) in an assembly buffer consisting of 100 mM MES, 0.1 mM EDTA, 1 mM EGTA, 1 mM $MgCl_2$ and 1 mM β-mercaptoethanol, pH 6.4, and 1 mM GTP. The rate of the assembly reaction was monitored by recording turbidimetric changes at 350 nm in a spectrophotometer. Each assembly reaction was followed for up to 20 min. At the end of the turbidimetric measurements an aliquot of the incubation mixture was examined by negative staining with 2% phosphotungstic acid, pH 7.2, on carbon coated grids used for electron microscopy. Two to three grids were prepared from each assembly reaction and examined with a Philips EM 300 electron microscope as described earlier [17].

2.6. Protein assays and Western blots

Protein concentrations were determined by a modification of the method of Lowry [18]. SDS-PAGE and Western blots were carried out as described previously [19].

3. Results

PHF isolated by the long procedure of Iqbal et al. [3] resulted in a highly purified PHF preparation. Morphological characterization of the isolated PHF was reported previously [3]. The contaminants in this PHF fraction were small amounts of lipofuscin, membranes and a few fragments of capillaries. A yield of 5–10 μg protein/g AD neocortex was obtained.

Treatment of isolated PHF with alkaline phosphatase dephosphorylated PHF-tau at Ser^{199}/Ser^{202} as determined by Western blots of the phosphatase-treated and untreated PHF with antibody Tau-1 (Fig. 1). Dephosphorylation of PHF did not dissociate the tangles apparently, and at the end of the dephosphorylation reaction the tangles of PHF could be sedimented at 9,500 × g, enabling removal of the phosphatase from the PHF.

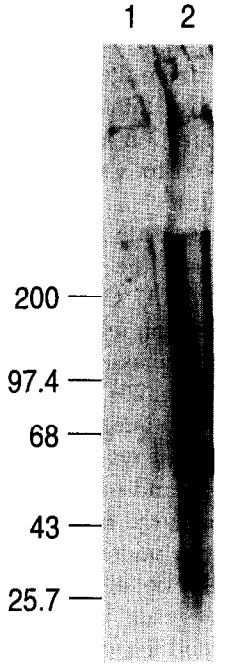

Fig. 1. Western blot of PHF, untreated (lane 1) or dephosphorylated (lane 2) with alkaline phosphatase. Each gel lane contained 10 μg protein. The blot was prepared and developed with the monoclonal antibody Tau-1 as described in section 2. The antibody Tau-1 recognizes PHF-tau only when it is dephosphorylated at Ser^{199}/Ser^{202}. A smear of tau immunoreactivity, typical of PHF, can be seen in lane 2. The positions of the molecular weight markers in kilodalton are indicated on the left margin of lane 1.

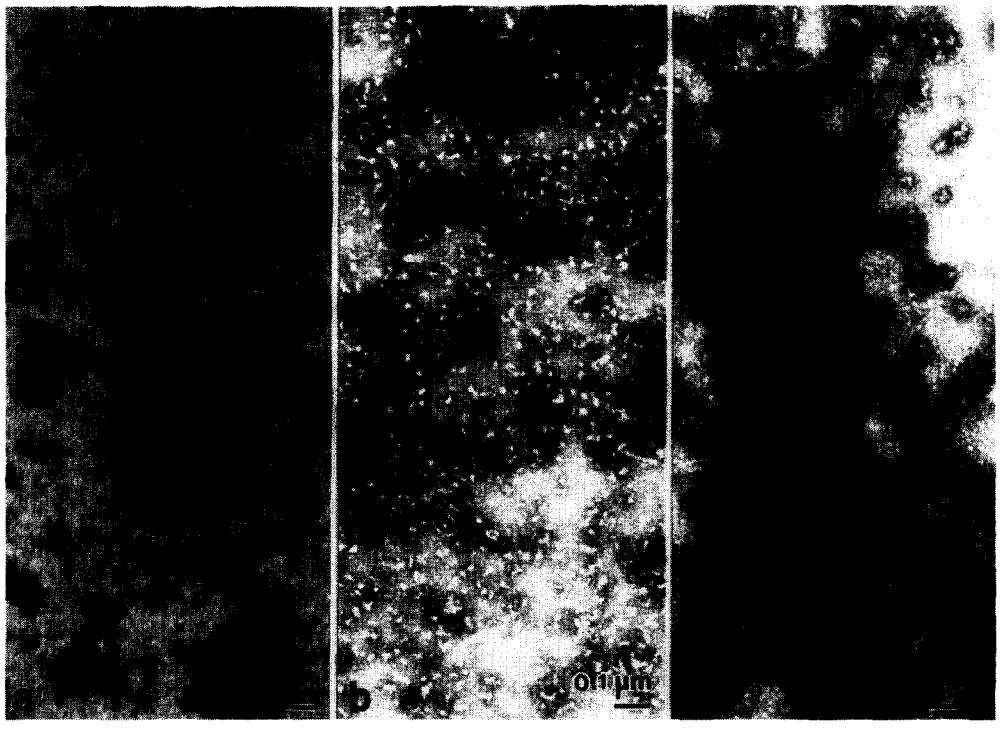

Fig. 2. Electron micrographs of ultrasonicated PHF preparation alone, untreated with alkaline phosphatase (panel a); the PHF preparation plus bovine tubulin, showing no assembly of microtubules (panel b); and dephosphorylated PHF preparation plus bovine tubulin showing assembly of microtubules. Not shown in this figure, no microtubules were detected in the tubulin alone assembly control. The negative staining was carried out using phosphotungstic acid as described in section 2.

Ultrasonication of the PHF preparation resulted in the dissociation of PHF from the tangles and fragmentation of the fibrils. Negative stain electron microscopy of the ultrasonicated preparation revealed that most of the PHF were fragmented to a single twist length, i.e. into 80 nm pieces (Fig. 2a).

Incubation of the ultrasonicated PHF preparation with bovine tubulin in the assembly buffer generated increase in turbidity at 37°C over tubulin control (Fig. 3a). However, on electron microscopy of the incubation mixture at the end of the assembly reaction, no microtubules were detected (Fig. 2b). In contrast, the incubation mixture in which alkaline phosphatase dephosphorylated PHF preparation was employed, both an additional increase in rate and net turbidity at 350 nm (Fig. 3a) and as well as a large number of microtubules by negative stain electron microscopy were observed (Fig. 2c). Unlike PHF, the bovine tau treated with alkaline phosphatase did not result in any increase but a small decrease in the rate and the amount of assembly as determined by turbidity assay (Fig. 3b).

4. Discussion

The exact relationship between neurofibrillary degeneration and β amyloidosis, the two histopathological hallmarks of AD is presently not understood. However, increasing evidence suggests that the neurofibrillary degeneration is required for the clinical expression of the disease, and that the β amyloidosis alone does not produce the disease clinically [20,21]. Understanding of the mechanism of neurofibrillary degeneration is therefore critical to developing a rational treatment for AD.

Tau is a phosphoprotein, the biological activity of which is regulated by its degree of phosphorylation [22]. Soluble non-PHF abnormally phosphorylated tau isolated from AD brain has been previously shown to be dephosphorylated by alkaline phosphatase [23] and phosphoseryl/phosphothreonyl protein phosphatases -1, -2A and -2B [24–26]. Dephosphorylation of PHF-tau by alkaline phosphatase on Western blots has also been observed previously [12]. The present study demonstrates that the microtubule associated protein tau in neurofibrillary tangles/PHF is functionally inactive, however, its biological activity can be restored by enzymatic dephosphorylation.

The isolation of PHF from AD brain involved treatment with 2% SDS. The PHF preparation is, therefore, unlikely to be contaminated with any soluble tau. Ultrasonication was necessary for the extraction of tau from PHF. The stimulation of the microtubule assembly by the dephosphorylated PHF was most likely due to the extracted tau and not PHF fragments because no micro-

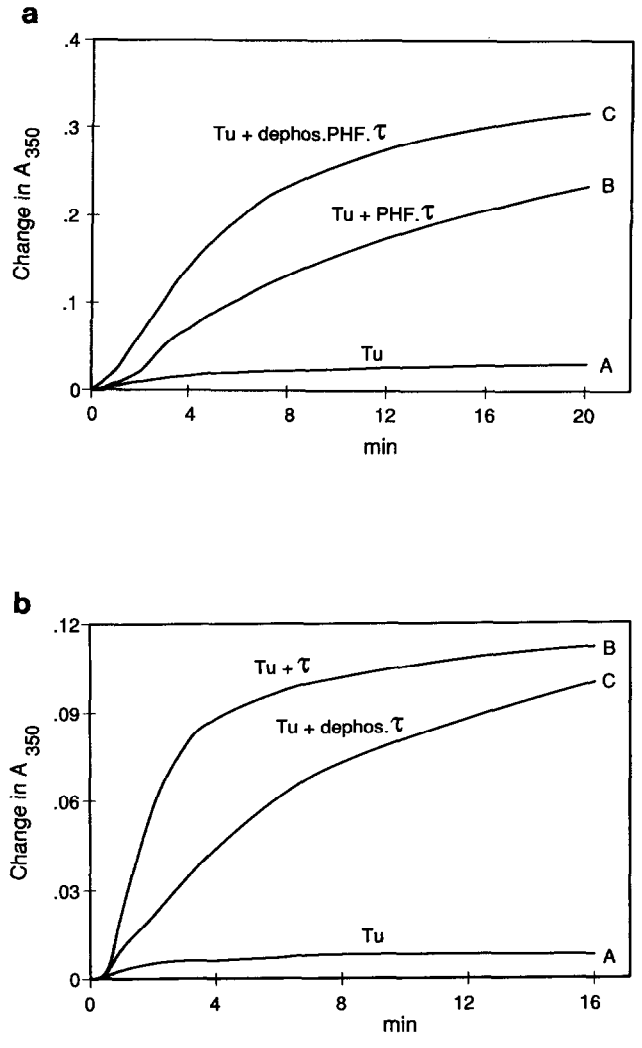

Fig. 3. Turbidity changes at 350 nm showing the rate of the assembly of microtubules. The in vitro assembly of microtubules was carried out by incubating tubulin (tu), 4 mg/ml with or without tau (τ), 0.1 mg/ml at 37°C in 100 mM MES, 0.1 mM EDTA, 1 mM EGTA, 1 mM $MgCl_2$ and 1 mM β-mercaptoethanol, pH 6.4 and 1 mM GTP as described in section 2. Turbidity changes using PHF-tau and bovine tau are shown in panel a and panel b, respectively. Although PHF-tau (untreated with alkaline phosphatase) plus tubulin produced turbidity (panel a, curve B) greater than tubulin alone (panel a, curve A), negative stain electron microscopy failed to reveal any assembly of microtubules (see Fig. 2, panel b).

tubules were seen growing directly from the latter. The cause of the increase in turbidity but no assembly of microtubules obtained using non-dephosphorylated PHF, at present, is not clear. The turbidity increase might be due to self-aggregation of PHF-tau.

Unlike PHF, treatment of bovine tau with alkaline phosphatase did not increase its microtubule assembly-promoting activity. A small loss in the activity of the phosphatase-treated tau might be due to an over dephosphorylation because a certain degree of phosphorylation might be required for the maximal activity of tau [27]. Alternatively, some of the tau might be degraded by proteases in the alkaline phosphatase preparation not completely inhibited by the protease inhibitors used in the dephosphorylation incubation mixture.

Tau is known to be required for the maintenance of the microtubule network and thereby the axoplasmic transport in neurons. Restoration of the biological activity of the inactive tau would be expected to prevent neurodegeneration. Recent studies have shown the presence of a significant pool of soluble abnormally phosphorylated tau in non-PHF form in AD brain, suggesting that tau is most likely phosphorylated prior to its polymerization into PHF [23]. It would thus appear that dephosphorylation of the abnormal tau might prevent both the neurofibrillary degeneration and the formation of the PHF and the neurofibrillary tangles. In situ the beneficiary effect of the dephosphorylation will be restricted mostly to the unpolymerized (non-PHF) pool of the abnormal tau. Tau in PHF is in a beta pleated sheet conformation, and is thus likely to be depolymerized at a very slow rate, if any, and made available for reutilization in the affected neurons. Future studies will reveal the effect of dephosphorylation on the dissociation of PHF.

Acknowledgements: We thank Joanne Lopez and Padmini Reginald for secretarial assistance and the Biomedical Photography Unit of this Institute for the preparations of figures. Autopsied brain specimens from Alzheimer disease and control patients were provided by the Netherlands Brain Bank in the Netherlands Institute for Brain Research, Amsterdam, the Brain Tissue Resource Center (Public Health Science Grant MH/NS 31862), McLean Hospital, Belmont, MA., and the National Neurological Research Bank, VA Medical Center, Wadsworth, Los Angeles. These studies were supported in part by New York State Office of Mental Retardation and Developmental Disabilities. National Institutes of Health Grants AG 05892, AG 08076, and NS 18105; and Zenith Award (to K.I.) from Alzheimer's Association, Chicago.

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