Available online at www.sciencedirect.com
SCIENCE DIRECT
Biochemical and Biophysical Research Communications 346 (2006) 819–828
BBRC
www.elsevier.com/locate/ybbrc

Neighbored phosphorylation sites as PHF-tau specific markers in Alzheimer's disease ☆,☆☆

David Singer [a,1], Jörg Lehmann [b,1], Katja Hanisch [a], Wolfgang Härtig [c], Ralf Hoffmann [a,*]

[a] *Bioanalytics, Center for Biotechnology and Biomedicine (BBZ), Faculty of Chemistry and Mineralogy, University of Leipzig, Deutscher Platz 5, 04103 Leipzig, Germany*
[b] *Priontype GmbH and Co. KG, BioCity, Deutscher Platz 5b, 04103 Leipzig, Germany*
[c] *Paul Flechsig Institute for Brain Research, University of Leipzig, Jahnallee 59, 04109 Leipzig, Germany*

Received 22 May 2006
Available online 9 June 2006

Abstract

Neurofibrillary tangles, which represent a major pathological hallmark in Alzheimer's disease (AD), are deposits of the hyperphosphorylated microtubule-associated tau protein (PHF-tau). However, a link between the phosphorylation pattern and the cause or the progress of AD is still missing. The work reported here focused on PHF-tau specific local phosphorylation patterns at Thr212/Ser214 and Thr231/Ser235 using monoclonal antibodies (mAb) generated against correspondingly modified peptides. The binding motifs of the obtained six mAbs were characterized with non-, mono-, and double-phosphorylated peptides as well as terminally shortened sequences. Five mAbs stained neurofibrillary tangles, neuritic plaques, and neuropil threads from autoptic brains of AD cases. Four mAbs recognized PHF-tau without significant cross-reactivity towards normal human tau, bovine tau, and dephosphorylated PHF-tau in ELISA and Western blot analysis. Thus, double phosphorylation is sufficient to distinguish PHF-tau from all other tau versions and there is no need to postulate any PHF-tau specific conformation for this region.

Keywords: AD; Immunohistochemistry; Monoclonal antibody (mAb); Paired helical filaments; Phosphopeptides; Tau; Immunoblot

The family of nervous system-specific microtubule-associated tau proteins promotes microtubule assembly and is a critical regulator of microtubule dynamics [1,2]. The adult human brain contains six isoforms with apparent molecular masses from 55 to 68 kDa in SDS–PAGE formed by alternative splicing of a single gene [1–3]. Human tau is phosphorylated in a remarkably heterogeneous manner at more than 30 serine and threonine residues, which are mostly grouped in three regions of the C-terminal half [4–6]. These heavily phosphorylated regions are present in all splicing forms spanning from positions 198 to 218, 231 to 238, and 396 to 412 (numbering according to the longest human isoform with 441 residues). Clearly, the most heavily phosphorylated tau forms are found in neurofibrillary tangles (NFTs) in the brains of Alzheimer's disease (AD) patients [7,8] and other tauopathies [9]. The NFTs are composed of paired helical filaments (PHFs) containing mostly hyperphosphorylated tau versions, the so-called PHF-tau. The actual sites of abnormal phosphorylation of PHF-tau are still one of the most studied and debated areas of AD research. The lack of microtubule-binding of PHF-tau is attributed

☆ This work was funded by the Deutsche Forschungsgemeinschaft (DFG) and the European Regional Development Fund (EFRE, European Union and Free State Saxonia).
☆☆ *Abbreviations:* AD, Alzheimer's disease; DTT, 1,4-dithio-D,L-threitol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; ELISA, enzyme-linked immunosorbent assay; HPT, hyperphosphorylated tau; mAb, monoclonal antibody; MALDI, matrix-assisted laser desorption/ionization; MS, mass spectrometry; NFT, neurofibrillary tangle; pAb, polyclonal anti-serum/antibody; PHF, paired helical filaments; pS, phosphoserine; pT, phosphothreonine; TBS, Tris-buffered saline.
* Corresponding author. Fax. +49 (0) 341 9731339.
E-mail address: Hoffmann@chemie.uni-leipzig.de (R. Hoffmann).
[1] These authors contributed equally to this work.

0006-291X/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2006.05.201

to hyperphosphorylation, as tubulin binding can be restored by phosphatases [10,11] and Pin1 [12]. Recently, it was suggested that N-glycosylation of tau enhances phosphorylation by kinases, whereas O-glycosylation may protect some sites from phosphorylation [13–15]. Despite these *in vitro* data, there is still no fully compelling evidence that phosphorylation of tau has any casual relation to tau aggregation and formation of PHF. However, the abundance of NFTs corresponds very well to the pathogenesis of AD and describes the severity of the dementia better than any other neuropathological alteration [16–18]. Nevertheless, in the end of the transformation process, abnormally hyperphosphorylated tau variants emerge in PHF. From the hundreds of monoclonal antibodies (mAbs) obtained by immunization with PHF-tau preparations only few mAbs are considered PHF-tau specific, as they do not significantly cross react with normal- or biopsy-derived tau. Whereas mAb Alz50 recognizes a structural epitope consisting of a N-terminal sequence and the microtubule-binding region [19,20], mAbs PHF-27, AT100 (former AT10; both IgG), and TG3 (IgM) recognize distinct phosphorylation sites [21,22]. We could show that mAbs PHF-27 and TG3 bind to the phosphorylation site at Thr231 [21,22], very similar to mAbs AT180 and M4, which recognize both normal tau phosphorylated at Thr231 and PHF-tau. Similarly mAb MC-6 recognizes Ser235 without being PHF-tau specific. The PHF-tau specificity of mAbs PHF-27 and TG3 was explained by a conformational change induced by the neighbored phosphorylation site Ser235 [23]. In this regard, the PHF-tau specificity was attributed to the doubly phosphorylated epitope although only phospho-Thr231 was part of the epitope. This concept was recently extended for TG3 towards recognition of a PHF-tau specific conformation as part of a disease related structural change transforming tau gradually towards PHF-tau in conjunction with phosphorylation of several sites [24]. On the other hand, AT100 binds only to tau versions simultaneously phosphorylated at both Thr212 and Ser214 [21], which was independently confirmed on the protein level [25]. Again the PHF-tau specificity was explained by a PHF-tau specific conformation involving sequential phosphorylation of the tau199-205 region [25]. Based on these interpretations linking the PHF-tau specificity of several mAbs with a structural change of the whole protein and not only a conformational change close to the phosphorylation site, we designed two double-phosphorylated peptides corresponding to either region and generated anti-phosphopeptide mAbs specific for these phosphorylation patterns. These mAbs proved valuable tools to distinguish PHF-tau from both normal tau and dephosphorylated PHF-tau in both ELISA and Western blot analysis. The mAbs stained also specifically tau deposits in AD brain by immunohistochemistry. Moreover, the new mAbs will be valuable to quantify all four phosphorylation sites by distinguishing double- and mono-phosphorylated sequences.

Materials and methods

Reagents. 1,4-Dithio-D,L-threitol (DTT) and 2-mercaptoethanol were from Fluka (Neu-Ulm, Germany). Tween® 20 and all other chemicals used for gel electrophoresis were from Serva (Heidelberg, Germany). The following antibodies were used: mAbs AT100, AT8 (Innogenetics, Hilden, Germany), Tau5, biotinylated goat-anti-mouse IgG (Dianova, Hamburg, Germany), HT7 (Perbio, Bonn, Germany), and goat anti-mouse IgG (Fcγ-specific, Sigma Taufkirchen, Germany). PVDF-membranes were obtained from Millipore GmbH (Schwalbach, Germany).

Tau preparation. Tau proteins were isolated from AD brain (total AD tau), human non-AD brain (normal tau), and calf brain (bovine tau) using the published protocol for total tau [3]. Briefly, brain samples were mixed 1:1 (w/v) with cold buffer (100 mmol/L PIPES, pH 6.9; 1 mmol/L EGTA, 0.5 mmol/L EDTA, 0.5 mmol/L $MgSO_4$, 2 mmol/L DTT, 0.1 mmol/L PMSF, 10 mmol/L benzamidine, and 750 mmol/L NaCl), homogenized in a Waring blender (VWR, Darmstadt, Germany), and centrifuged at 100,000*g* (4 °C) for 60 min (Optima, Beckman Coulter GmbH, Unterschleißheim, Germany). The supernatant was heated in a boiling water bath for 10 min. The precipitated proteins were removed by centrifugation (35,000*g*, 4 °C, 30 min; Avanti, Beckman–Coulter). By addition of ammonium sulfate to a final concentration of 50% (w/v), the tau-protein was precipitated overnight at 4 °C. After centrifugation (30,000*g*, 4 °C, 20 min), the pellet was dissolved in 50 mmol/L ammonium hydrogen carbonate buffer (pH 7.0) and concentrated in a Centriprep™ concentrator (Amicon®, Millipore) using the same buffer. The tau samples referred to as total AD tau, normal tau, and bovine tau were stored in aliquots at −20 °C.

PHF-tau was prepared from three AD brains, which were homogenized with a Potter-homogenisator on ice using 1.5 mL cold buffer (750 mmol/L NaCl, 100 mmol/L 2-(*N*-morpholino)ethanesulfonic acid (MES), pH 6.8; 1 mmol/L EGTA, 50 mmol/L NaF, 1 mmol/L sodium orthovanadate, 0.5 mmol/L $MgSO_4$, 2 mmol/L 1,4-dithio-D,L-threitol (DTT), 0.5 mmol/L phenylmethylsulfonylfluoride (PMSF), and 250 μL Protease Inhibitor Mix M) per gram brain sample. After 20 min stirring on ice, the homogenate was centrifuged (11,000*g*, 4 °C for 20 min) and the supernatant centrifuged again at 100,000*g* (4 °C, 60 min). The combined pellets were extracted with the tenfold volume of extraction buffer (10 mmol/L Tris–HCl, pH 7.4, 10% (w/v) sucrose, 850 mmol/L NaCl, 1 mmol/L EGTA, 50 mmol/L NaF, and 1 mmol/L sodium orthovanadate), centrifuged (15,000*g*, 4 °C, 20 min) and PHF-tau precipitated with 1% sarkosyl at room temperature for 1 h, and centrifuged again (100,000*g*, 4 °C, 30 min). The pellet containing PHF-tau was dissolved in 10 mmol/L Tris–HCl (pH 7) and stored in aliquots at −80 °C.

Dephosphorylation of PHF-tau. One hundred and twenty nanograms PHF-tau were incubated with 6 mU alkaline phosphatase (Fluka) in Tris-buffer (10 mmol/L Tris, pH 8.0, 1 mmol/L $MgCL_2$, and 0.1 mmol/L $ZnCl_2$) at 67 °C. The reaction was stopped after 4 h by acetone precipitation and the pellet dissolved in Tris-buffer (50 mmol/L Tris, pH 7.5, 0.1 mmol/L Na_2EDTA, 5 mmol/L DTT, and 0.01% BRIJ 35, and 2 mmol/L $MnCl_2$). This solution was incubated with 12 U recombinant lambda protein phosphatase (expressed in *Escherichia coli*, SIGMA, Deisenhofen) at 40 °C. After 30 min the reaction was stopped by heat denaturation at 95 °C for 5 min. The dephosphorylated PHF-tau was either directly analyzed by SDS–PAGE or stored at −20 °C.

Peptide synthesis. Peptides were synthesized on solid-phase with fluorenylmethoxycarbonyl (Fmoc) as temporary protecting group and *tert.*-butyl (^{t}Bu) based permanent protecting groups by di-isopropyl carbodiimide/1-hydroxy-benzotriazole (DIC/HOBt) activation and global post-synthetic phosphorylation using the phosphoramidite approach [26] (Table 1). Briefly, all side chains of trifunctional amino acids were protected using 2,2,5,7,8-pentamethylchroman-6-sulfonyl (Pbf) for Arg, ^{t}Bu for Asp, Glu, Ser, and Thr, trityl (Trt) for Cys and His, and *tert.*-butyloxycarbonyl (Boc) for Lys. Positions to be phosphorylated were incorporated with an unprotected hydroxyl-group, i.e., Fmoc-Ser-OH or Fmoc-Thr-OH. After cleavage of the N-terminal Fmoc-group of the full-length peptide, the free hydroxyl group was phosphitylated with 20 equivalents