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Regulation of phosphorylation of tau by cyclin-dependent kinase 5 and glycogen synthase kinase-3 at substrate level

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Abstract Microtubule associated protein tau, which is expressed in six alternatively spliced molecular isoforms in human brain, is abnormally hyperphosphorylated in Alzheimer disease and related tauopathies. Here, we show (i) that GSK-3α and neither GSK-3β nor cdk5 can phosphorylate tau at Ser262 and phosphorylation at Ser235 by cdk5 primes phosphorylation at Thr231 by GSK-3α/β; (ii) that tau isoforms with two N-terminal inserts (τ4L, τ3L) are phosphorylated by cdk5 plus GSK-3 at Thr231 markedly more than isoforms lacking these inserts (τ4, τ3); and (iii) that Thr231 is phosphorylated ~50% more in free tau than in microtubule-bound tau, and the phosphorylation at this site results in the dissociation of tau from microtubules. These findings suggest that the phosphorylation of tau at Thr231 and Ser262 by cdk5 plus GSK-3, which inhibits its normal biological activity, is regulated both by its amino terminal inserts and its physical state.

Keywords: Alzheimer disease; Glycogen synthase kinase-3; Cyclin-dependent protein kinase-5; Tau hyperphosphorylation; Neurofibrillary degeneration; Tau kinases

1. Introduction

Alzheimer disease (AD) and related tauopathies are characterized by neurofibrillary degeneration of abnormally hyperphosphorylated tau [1]. The AD abnormally hyperphosphorylated tau does not bind to tubulin but inhibits in vitro microtubule assembly and disrupts preformed microtubules (MT) by sequestering normal tau, and high molecular weight microtubule associated proteins (MAPs), MAP1 and MAP2 [2–4]. These inhibitory effects of the AD hyperphosphorylated are, however, abolished by in vitro dephosphorylation by protein phosphatases (PP), especially PP-2A [5,6]. Despite its importance, the molecular mechanism of the abnormal hyperphosphorylation of tau is not fully understood.

To date, over 25 abnormal sites have been identified at which tau in paired helical filaments (PHF) is phosphorylated [7]. The phosphorylation of tau at only some of these sites might play a significant physiological role. Tau can be phosphorylated at one or more of these abnormal sites by several protein kinases (see [8]). Among these protein kinases, glycogen synthase kinase-3 (GSK-3) and cyclin-dependent protein kinase 5 (cdk5) are believed to be major enzymes involved in Alzheimer abnormal hyperphosphorylation of tau. Cdk5 and GSK-3 co-localize with Alzheimer neurofibrillary degeneration [9–12] and both kinases have been shown to produce neurofibrillary degeneration of hyperphosphorylated tau in transgenic mice [13,14].

Phosphorylation of tau at some of the sites inhibits its binding to MT and its ability to promote microtubule assembly [2,15]. Thr231 and Ser262 are the two major sites phosphorylation of which by cdk5 followed by GSK-3 inhibits binding to MT [16–18]. An important factor that might influence the phosphorylation of tau at specific sites that have received little attention to date is that tau is present in the brain in different substrate forms. In adult human brain, six isoforms of human tau are present. These isoforms, which are generated from a single gene through alternate mRNA splicing [19], vary in length between 352 and 441 amino acids and differ in whether they contain three (τ3, τ3S, τ3L) or four (τ4, τ4S, τ4L) microtubule binding domains of 31 or 32 amino acids each and none (τ3, τ4), one (τ3S, τ4S) or two (τ3L, τ4L) N-terminal inserts of 29 amino acids each. Furthermore, tau in brain is present in at least two major pools: (i) as free tau; and (ii) as tau bound to MT.

In the present study, we compared (i) the phosphorylation of tau isoforms with or without the N-terminal inserts by cdk5 and GSK-3, singly or in combination and their binding to MT, and (ii) the phosphorylation of free and microtubule-bound taus by these two protein kinases. We found (i) that GSK-3α but neither GSK-3β nor cdk5 could phosphorylate tau at Ser262; (ii) that both cdk5 and GSK-3α could phosphorylate Ser-235; (iii) that phosphorylation of tau by cdk5 markedly enhanced the phosphorylation of tau at Ser235 and resulted in a robust phosphorylation of tau at Thr231 by GSK-3, especially the GSK-3β; and (iv) that after phosphorylation by cdk5 and GSK-3, human tau isoforms bound to MT differentially. The phosphorylation at Thr231 and Ser262 by the above kinases and the consequent inhibition of binding to MT were more in tau isoforms with the two amino terminal inserts than the isoforms lacking the inserts. The phosphorylation of Thr231 and Ser262 was decreased in MT-bound tau

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Abbreviations: AD, Alzheimer disease; PHF, paired helical filaments; cdk5, cyclin-dependent protein kinase 5; GSK-3, glycogen synthase kinase-3; PDPK, proline-dependent protein kinase; MT, microtubule; PMSF, phenyl methyl sulfonyl fluoride

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compared to free tau, whereas the phosphorylation of Ser46, Ser396, Ser404 and Ser422 was unchanged. These findings suggest that the phosphorylation of tau at sites which affect its biological activity is regulated significantly at the substrate level.

2. Materials and methods

2.1. Materials

Tau monoclonal antibodies (mAb) employed were as follows: M4 to tau phosphorylated at Thr231 and Ser235 [20] was a gift from Dr. Y. Ihara (University of Tokyo, Japan), 12E8 to tau phosphorylated at Ser262 and/or Ser356 [21] was a gift from Dr. Dale Schenk of Elan Pharmaceuticals (San Francisco, CA), and PHF-1 to tau phosphorylated at Ser396 and/or Ser404 [22] was a gift from Dr. P. Davies, Albert Einstein College of Medicine (Bronx, NY). Polyclonal tau antibodies 92e to total tau and 102c to tau dephosphorylated at Ser46 were raised in rabbits, as previously reported [23,24]. Polyclonal rabbit R145 to tau phosphorylated at Ser422 was described previously [25]. Rabbit antibodies pT^{231}, pS^{236}, and pS^{262} to tau phosphorylated at Thr231, Ser235 and Ser262, respectively, were obtained from Biosource Intl. (Camarillo, CA). Rabbit antibodies 127d to GSK-3β and 133d to GSK-3α and GSK-3β were raised in our lab [10]. Polyclonal antibody to GSK-3α was from Upstate Biotechnology, Inc. (Lake Placid, NY), and monoclonal antibody to GSK-3β was from Transduction Labs (San Diego, CA). Recombinant GSK-3β was purchased from Calbiochem (San Diego, CA). ^{125}I-labeled anti-mouse and anti-rabbit IgG antibodies were purchased from Amersham (Arlington Heights, IL). [γ-^{32}P] ATP was purchased from ICN Biomedicals (Costa Mesa, CA).

2.2. Methods

The human tau clones 23, 24, 39 and 40 (kindly provided by Dr. M. Goedert) that encode for the isoforms τ3, τ4, τ3L and τ4L, respectively, [19] were subcloned in *E. coli* and purified from cell extracts as described previously [26]. pGEX-2T plasmids containing cdk5 and p25, kind gifts from Dr. Jerry H. Wang (The Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong) were expressed and purified from E.coli as GST fusion proteins [27]. The purification of GSK-3 and cdk5 was as described [28]. The GSK-3 preparation contained both α and β isoforms in 3:2 ratio [26]. Cdk5 and GSK-3 were also purified by immunoprecipitation with appropriate antibodies from rat brain extract. The $16000 \times g$ brain extract, 50 μg protein was incubated with 2 μg of specific antibodies in 50 mM Tris, pH 7.4, 150 mM NaCl, 10 mM NaF, 1 mM Va_3VO_4, 2 mM EGTA, 1 mM phenylmethyl sulfonyl fluoride (PMSF) and 5 μg/ml each of leupeptin, pepstatin and aprotinin. After incubation overnight at 4 °C, 30 μl of protein G immobilized on agarose beads (Pierce, Rockford, IL) was added and the reaction mixture was further incubated for 3 h with constant mixing. The incubated mixture was then centrifuged and the beads were washed three times with immunoprecipitation buffer, resuspended in 10 μl of the phosphorylation reaction buffer, and used as a kinase.

MAPs-free tubulin was isolated from rat brain extract through two temperature-dependent cycles of microtubule polymerization–depolymerization, followed by chromatography on cellulose [2]. Taxol-stabilized MT were prepared by incubating MAPs-free tubulin (4 mg/ml) with taxol (20 μM) in the presence of MES (0.1 M, pH 6.8), EGTA (1 mM) and PMSF (1 mM) for 30 min at 37 °C. The MT were collected by centrifugation over a cushion of 0.125 M sucrose at $50000 \times g$ for 30 min at 32 °C.

Tau isoforms were phosphorylated at 30 °C in a reaction mixture normally containing 4.0 μM tau, 6 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 0.25 mM [γ-^{32}P] ATP, and 40 mm HEPES (pH 7.5). Reactions were initiated at 30 °C by the addition of kinases. For determination of ^{32}P incorporation into protein, aliquots of the reaction mixtures were removed at different times, spotted on strips of Whatman filter paper (31ET) and free [γ-^{32}P] ATP separated from ^{32}P-labeled protein by ascending chromatography [26]. The ^{32}P-labeled tau on the filters was quantitated by Cerenkov counting in a scintillation counter. When prephosphorylation of tau was required, [γ-^{32}P] ATP was replaced with unlabeled ATP. After incubation for 2 h at 30 °C, the reaction was stopped by heating at 95 °C for 5 min and denatured kinases removed by centrifugation ($10000 \times g$ for 10 min). The extent of tau phosphorylation was determined in parallel experiments using [γ-^{32}P] ATP. The heat-stable phosphorylated tau was then used as a substrate for the second kinase. In controls, the incubation was carried out without the first kinase but otherwise treated identically as the samples for combination phosphorylation.

To analyze the binding of tau to MT, 0.47 μM tau (non-phosphorylated or phosphorylated) was incubated with 7.4 μM taxol-stabilized MT for 30 min at 37 °C in buffer A (0.1 M HEPES, pH 6.8, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM PMSF, 20 μM taxol and 1 mM GTP). Tau bound to MT was separated from unbound tau by centrifugation at $50000 \times g$ for 30 min at 32 °C on a cushion of 10% glycerol. The pellet was washed twice with buffer A and dried in a Speed Vac. The combined supernatants were precipitated by acetone, centrifuged and the precipitate was again dried by a vacuum concentrator. All samples were electrophoresed in 10% SDS-polyacrylamide gels, transferred to nitrocellulose and immunoblots probed with the rabbit polyclonal phosphorylation-independent tau antibody, 92e (1/5000). The immunoreactive bands were quantitated. In each set, the supernatant (free tau) and the pellet (bound tau) were calculated as percent of total tau. To assay the release of MT-bound tau, the phosphorylation reaction was stopped by cooling to 4 °C and immediately centrifuged ($50000 \times g$) to separate tau which remained bound to microtubules (pellets) from free released tau (supernatant).

Immunoblotting of tau by the different antibodies was carried out as described previously [1]. The following dilutions of the primary antibodies were used: MT (1/1000), 12E8 (1/500), PHF-1 (1/500), 92e (1/5000), 102c (1/1000) and anti-P-Ser422 (1/3000). Blots were then further probed with anti-mouse or anti-rabbit IgG secondary antibodies which were labeled with ^{125}I. The immunoreactive bands were visualized and quantitated by Fuji 1500 Phosphorimager Imaging System.

3. Results

3.1. Phosphorylation of free and MT-bound tau by a combination of cdk5 and GSK-3

To examine the regulation of the phosphorylation of tau by its state of conformation as a substrate, we investigated the phosphorylation of free and MT-bound taus by cdk5 and GSK-3. For this purpose, we chose two tau isoforms (see Fig. 1), τ3 and τ3L as free proteins and as bound to MT stabilized with taxol. Prephosphorylation of MT-bound tau by cdk5 increased the rate of subsequent phosphorylation by GSK-3 by ~25% in the case of τ3L but not τ3 (Fig. 2). On the other hand, prephosphorylation of free τ3 and τ3L by cdk5 increased subsequent phosphorylation by GSK-3 by ~30% and ~50%, respectively (Fig. 2C). These results suggest that prephosphorylation by cdk5 stimulates the subsequent phosphorylation by GSK-3 (i) more in tau with the N-terminal inserts than the same protein without the inserts, and (ii) more in free tau than the same as MT-bound protein.

3.2. Binding of different phosphorylated species of tau isoforms to microtubules

Since the phosphorylation of tau at only some and not all sites might be of physiological significance, we investigated whether the upregulation of the phosphorylation of tau due to N-terminal inserts inhibited its binding to MT. We examined the effect of phosphorylation by cdk5, GSK-3 or by combination of cdk5 and GSK-3 on the binding of tau to MT using τ3, τ4, τ3L, and τ4L. For all the four tau isoforms, the kinase combination cdk5 followed by GSK-3 was most effective for causing inhibition of tau binding to MT (Fig. 3A). In contrast, no significant increase in inhibition was observed