FEBS Letters 580 (2006) 107–114

P70 S6 kinase mediates tau phosphorylation and synthesis

Jin-Jing Pei[a,*], Wen-Lin An[a], Xin-Wen Zhou[a], Takeshi Nishimura[b], Jan Norberg[c], Eirikur Benedikz[a], Jürgen Götz[d], Bengt Winblad[a]

[a] *Department of Neurotec, Division of Experimental Geriatrics, Karolinska Institutet, KFC Novum, Plan 4, SE-141 86, Huddinge, Sweden*
[b] *Karolinska Institutet and Sumitomo Pharmaceuticals Alzheimer Center (KASPAC), Department of Neurotec, Karolinska Institutet, KFC Novum, Plan 4, SE-141 57, Huddinge, Sweden*
[c] *Department of Biosciences at Novum, Karolinska Institutet, KFC Novum, Plan 4, SE-141 57 Huddinge, Sweden*
[d] *Brain and Mind Research Institute, University of Sydney, Camperdown, NSW 2050, Australia*

Received 13 October 2005; revised 21 November 2005; accepted 21 November 2005

Available online 6 December 2005

Edited by Jesus Avila

Abstract **Currently, we found that the 70-kDa p70 S6 kinase (p70S6K) directly phosphorylates tau at S262, S214, and T212 sites in vitro. By immunoprecipitation, p-p70S6K (T421/S424) showed a close association with p-tau (S262 and S396/404). Zinc-induced p70S6K activation could only upregulate translation of total S6 and tau but not global proteins in SH-SY5Y cells. The requirement of p70S6K activation was confirmed in the SH-SY5Y cells that overexpress wild-type htau40. Level of p-p70S6K (T421/S424) was only significantly correlated with p-tau at S262, S214, and T212, but not T212/S214, in Alzheimer's disease (AD) brains. These suggested that p70S6K might contribute to tau related pathologies in AD brains.**

Keywords: p70 S6 kinase; Tau; Phosphorylation; Translation; Alzheimer's disease

1. Introduction

The cytoplasmic isoform of ribosomal S6 kinase 1 (RSK1), the 70-kDa S6 kinase (p70S6K), is a Ser/Thr (S/T)-directed kinase that regulates the phosphorylation of the 40S ribosomal protein S6 [1]. The nuclear isoform of RSK1, the 85-kDa S6 kinase (p85S6K), is docked to the nucleus by an additional 23-amino-acid sequence at its amino-terminus [1,2]. Both p70S6K and p85S6K are translated from the same transcript by two different start codons. So far, more is known about p70S6K. Through phosphorylation of S6 that directly regulates translation of mRNAs with 5′-terminal oligopyrimidine tracts (5′TOP) that generally encode ribosomal proteins and elongation factors, p70S6K activation plays a crucial role in cell growth, cell differentiation, and cell cycle control [3,4].

p70S6K contains acidic, catalytic, regulatory, and autoinhibitory domains (Fig. 1). Phosphoinositide-dependent protein kinase 1 (PDK1) is constitutively active regardless of extracellular stimulation. It is known that PDK1 activation can phosphorylate T229 of the catalytic domain of p70S6K [5,6], mammalian target of rapamycin (mTOR) [6,7], PDK1 [8], and Never in Mitosis gene A related kinases (NEK6/7) [9] can phosphorylate T389 of the regulatory domain, and tau protein kinases such as extracellular signal-regulated protein kinase 1/2 (ERK1/2) and c-jun amino-terminal kinase (JNK) 1/2 can phosphorylate S411, S418, T421, S424, S429, and T427 of the autoinhibitory domain [10–12]. Our previous studies indicated that both phosphoinositol 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathways are aberrantly regulated in tau-associated pathologies such as Alzheimer's disease (AD) [13–19]. This prompted us to investigate the role of p70S6K in the pathogenesis of AD. Sequential phosphorylation and activation of p70S6K were suggested to be mediated by the PI3K pathway [20]. Most recently, we found that phosphorylation and activation of p70S6K are preferentially regulated by the PI3K pathway, together with the MAPK pathway [21].

The major tau related abnormalities in AD are hyperphosphorylation, accumulation, assembly into paired helical filaments (PHFs) and neurofibrillary tangle (NFT) formation, accompanied by microtubule disruption. In support of the involvement of p70S6K in tau related pathogenesis in AD, we previously reported a concurrent occurrence of phosphorylated (p)/activated p70S6K with the progression of tau associated pathologies in brains staged according to Braak and Braak criteria [22], a colocalization of p-p70S6K with PHF-tau in neurons bearing NFTs and pretangles, and a significant correlation between p-p70S6K (T421/S424) and levels of total and abnormally hyperphosphorylated taus [17]. p70S6K activation and tau hyperphosphorylation were induced by 100 μM zinc in SH-SY5Y cells and primary cultured neurons [17,21], as well as by a selective protein phosphatase (PP)-2A inhibition in rat brain slices [18]. Tau is known to be associated with the phosphatase of the p70S6K upstream kinase mTOR: PP-2A [23–25], which was also found to be associated with p70S6K [26]. Taken together with data obtained in AD brain of the localization of p70S6K and hyperphosphorylated tau,

*Corresponding author. Fax: +46 8 58583880.
E-mail address: Jin-Jing.Pei@neurotec.ki.se (J.-J. Pei).

Abbreviations: AD, Alzheimer's disease; PHFs, paired helical filaments; NFT, neurofibrillary tangle; p70S6K, the 70-kDa p70 S6 kinase; mTOR, mammalian target of rapamycin; PI3K, phosphoinositol 3-kinase; MAPK, mitogen-activated protein kinase; ELISA, enzyme-linked immunosorbent assay; 5′UTR, the 5′ untranslated region; CaMKII, calcium/calmodulin-dependent protein kinase II; MARK, microtubule-affinity regulating kinase; 5′TOP, 5′-terminal oligopyrimidine tract

0014-5793/$32.00
doi:10.1016/j.febslet.2005.11.059

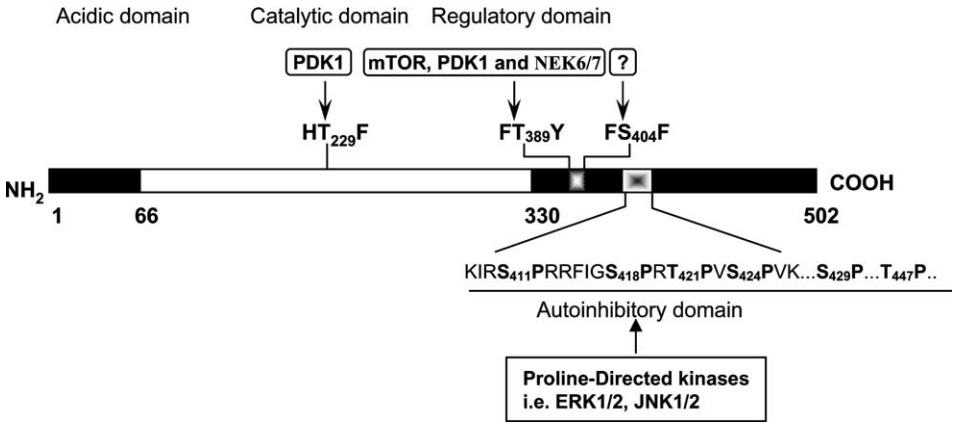

Fig. 1. Diagram of p70 S6 kinase domains. ERK1/2 and JNK1/2 phosphorylate the S411, S418, T421, S424, S429, T447 sites of the autoinhibitory domain of p70 S6 kinase. Phosphoinositide-dependent protein kinase 1 (PDK1) phosphorylates T229 site of the catalytic domain. mTOR, PDK1 and NEK6/7 phosphorylate T389 and additional uncharacterized kinase(s) phosphorylate S404 of the regulatory domain.

the spatial overlap of p70S6K and tau may provide a ground for tau to be phosphorylated by p70S6K, and this process may be involved in pathogenesis of tau related abnormalities in AD. It is also hypothesized that aberrant p70S6K activation might, at least in part, contribute to the continuous production of tau in degenerating neurons in AD brains, so that a large amount of tau including a significant amount of normal tau is accumulated in NFT-bearing neurons [21,27].

To address whether or not p70S6K mediates tau phosphorylation and synthesis, in vitro phosphorylation of tau by p70S6K was carried out in the present study. The possible underlying mechanism of the significant correlation between p70S6K (T421/S424) and PHF-1/tau found in AD brains [17] was clarified by immunoprecipitation of the lysates of SH-SY5Y cells treated with 100 μM zinc, and role of p70S6K in tau synthesis was investigated both in SH-SY5Y cells treated with 100 μM zinc and in SH-SY5Y cells overexpressing wild-type human tau. We found firstly that active p70S6K can directly phosphorylate tau at S262, S214 and T212 sites. Secondly, only p-tau at S396/404 or S262 could be co-immunoprecipitated with p-p70S6K (T421/S424). Thirdly, the synthesis of tau was found to be upregulated by zinc-induced p70S6K activation in SH-SY5Y cells, and in SH-SY5Y cells that overexpress wild-type human tau, levels of both p-p70S6K (T421/S424) and total S6 protein were dramatically increased. Finally, the level of p-p70S6K showed a significant correlation with p-tau at Ser262, S214, and T212 sites in AD brains.

2. Materials and methods

2.1. Antibodies and reagents

Polyclonal rabbit antibodies against tau phosphorylated at S262, T212, and S214, were from Biosource Nordic (Stockholm, Sweden); and polyclonal rabbit antibodies against p70S6K phosphorylated at T389 and at T421/S424, and total p70S6K were purchased from Cell Signalling Technology (Beberly, MA). Mouse monoclonal antibody (mAb) AT100 was from Innogenetics (anti-human PHF-tau, Zwujndrecht, Belgium). Rabbit antiserum R134d specific for the longest isoform of recombinant human tau was a gift from Drs. Khalid and Inge Grundke-Iqbal, New York State for Basic Research in Developmental Disabilities, NY, USA. mAb PHF-1 was a gift from Dr. Peter Davies, Albert Einstein College of Medicine (Bronx, NY) and mAb Tau-1 from Dr. L. Binder, North Western University (Chicago, IL). Zinc sulfate were from Sigma–Aldrich (St. Louis, MO).

2.2. In vitro tau phosphorylation by p70 S6 kinase

0.1 μg/μl recombinant full-length human tau (Invitrogen AB, Stockholm, Sweden) and 1 ng/μl recombinant active p70S6K (T412E) (Upstate, Lake Placid, NY) were incubated in the presence of 2.5 mM ATP in 25 mM Tris–HCl buffer (pH 7.5), containing 5 mM β-glycerophosphate, 2 mM DTT, 0.1 mM sodium vanadate, 10 mM MgCl2, 0.1 mg/ml heparin at 30 °C for 0, 30 min, 2 h or 4 h. The reaction was terminated by adding an equal amount of 2× electrophoresis sample buffer containing 125 mM Tris–HCl (pH 6.8), 4% SDS, 10% glycerol, 0.006% bromophenol blue, 1.8% β-mercaptoethanol, and heated at 95–100 °C for 5 min. Tau phosphorylation was monitored using phospho-site-specific antibodies to tau. Anti-p-tau (S262), 1:2000; Anti-p-tau (S214), 1:10000; Anti-p-tau (T212), 1:2000; AT100 (T212/S214), 1:100; PHF-1 (S396/404), 1:100; R134d, 1:5000.

2.3. Cell culture and preparation of cellular extracts

SH-SY5Y human neuroblastoma cells were cultured and treated with zinc as described [21], and the harvested cell lysates were then kept at −80 °C. Protein concentration was determined by BCA kit from Pierce (Pierce Chemical, Rockford, IL). The mock and wild-type tau overexpressing SH-SY5Y cells [28] were cultivated in DMEM/F12 (1:1) with 2 mM L-glutamine, 10% FBS, 5% horse serum, 1% P/S at 37 °C/5% CO_2/95% humidity.

2.4. Immunoprecipitation

The cell lysates of SH-SY5Y cells treated with 100 μM zinc for 30 min or 4 h were harvested as described [21]. The cell lysates were briefly sonicated, kept on ice for a further 30 min, and then centrifuged at 10000 × *g* at 4 °C. The protein concentrations in the supernatants were determined by the BCA kit (Pierce Chemical). Supernatants with equal amounts of protein (1 μg/ml) were aliquoted into three vials per group. Protein A-sepharose beads (50 μl/ml) were then added, followed by incubation at 4 °C for 3 h with shaking. After spinning down the beads, the supernatants were transferred to a new tube, and incubated with primary polyclonal antibodies directed against p-p70S6K (T389 or T421/S424) at 4 °C overnight with shaking. The complexes were precipitated by incubating with protein A-sepharose beads (50 μl/ml). After washing with cell lysis buffer 3 times, the beads were resuspended in 80 μl cell lysis buffer and 80 μl of 2× electrophoresis sample buffer, and boiled at 95 °C for 5 min. After removing the beads by centrifugation, the supernatants were collected and kept at −20 °C for Western blotting.

2.5. Western blotting

Samples derived from in vitro tau phosphorylation assays with p70S6K, immunoprecipitation, and cell lysates from SH-SY5Y cells treated with zinc, with mocking transfection or htau40 transfection were subjected to 10% or 12% SDS–polyacrylamide gel electrophoresis, as described [21].

2.6. Measurement of protein content per cell

Protein content per cell was determined as described [29]. Briefly, after treatment with 100 μM zinc for 4 h, SH-SY5Y cells were detached with trypsin-EDTA (Invitrogen AB). The rounded cells were prepared into a single cell suspension using a pipette. 50 μl of a single cell suspension were used for cell counting after fixation with 10% formalin. The remaining cells were pelleted by centrifugation at $1000 \times g$, and protein concentrations were measured by BCA kit (Pierce Chemical). Protein content per cell was determined by dividing the total amount of protein by the total number of cells in each sample. The relative levels of total p70S6K, S6, and tau were quantified by Western blotting in the same groups of samples with or without zinc treatment.

2.7. Dot blotting and indirect enzyme-linked immunosorbent assay

Tissue blocks of the medial temporal cortex from 10 controls and 22 AD patients were homogenized as described [17]. Dot blotting was performed as described [30]. The membranes with dotted samples (3 μg/dot in triplicate) were incubated with phospho-site-specific antibodies (1:5000 for S262, 1:1000 for S214, 1:2500 for T212; 1:500 for T212/S214) directed against S262, S214, S212, and S214/212 sites at 4 °C overnight, followed by secondary antibody linked with horseradish peroxidase (Amersham Biosciences AB, Uppsala, Sweden) at room temperature for 1 h. Immunoreactive proteins were detected according to the enhanced chemiluminescence protocol (Amersham Biosciences AB). Intensities of dots were quantified with Quantity One 4.3.0 software (Bio-Rad Laboratories Inc., Hercules, CA). Levels of p70S6K in AD and control cases were previously measured by indirect enzyme-linked immunosorbent assay (ELISA) [17].

2.8. Statistics

Level of global proteins (μg protein/1 000 000 cells) and levels of total p70S6K, S6 and tau between treated and untreated groups, and levels of p70S6K and different antibodies to p-tau in brain homogenates between AD and control groups were compared with Student's independent *t* test. The Pearson correlation analysis was used to estimate the correlation between p70 S6 kinase and tau levels.

3. Results

3.1. Tau phosphorylation is mediated by p70 S6 kinase

It is well established that S6 can be phosphorylated at S^{235} and S^{236} sites by p70S6K. Taking S6 as the substrate, the consensus motif (R/KXR/KXXS*/T*) of p70S6K was determined using the NetPhos2.0program (http://www.cbs.dtu.dk/services/NetPhos/). To investigate whether or not p70S6K can directly phosphorylate tau, the sequences of full-length human tau (Genbank: NP_005901) and S6 (Genbank: CAA47719) were firstly compared with the consensus motif (R/KXR/KXXS*/T*) of p70S6K [31]. We predicted that the T135, T153, S214, T245, S262, and S352 phosphorylation sites of tau might be directly phosphorylated by p70S6K. Of these sites, the S262 site that lies in the KXGS motif (circled by dot-box) is located in the microtubule-binding repeat domain of tau (Fig. 2A).

Following incubation of tau with active p70S6K in the presence of ATP, the predicted phosphorylation sites S262 and S214 of tau were confirmed using phospho-site-specific antibodies directed against tau (Fig. 2B). Furthermore, tau phosphorylation at S262 and S214 sites was time-dependent, especially the S262 site (Fig. 2B and C). However, T212 not predicted to be a substrate of p70S6K was also rapidly phosphorylated by p70S6K, reaching a plateau after 30 min

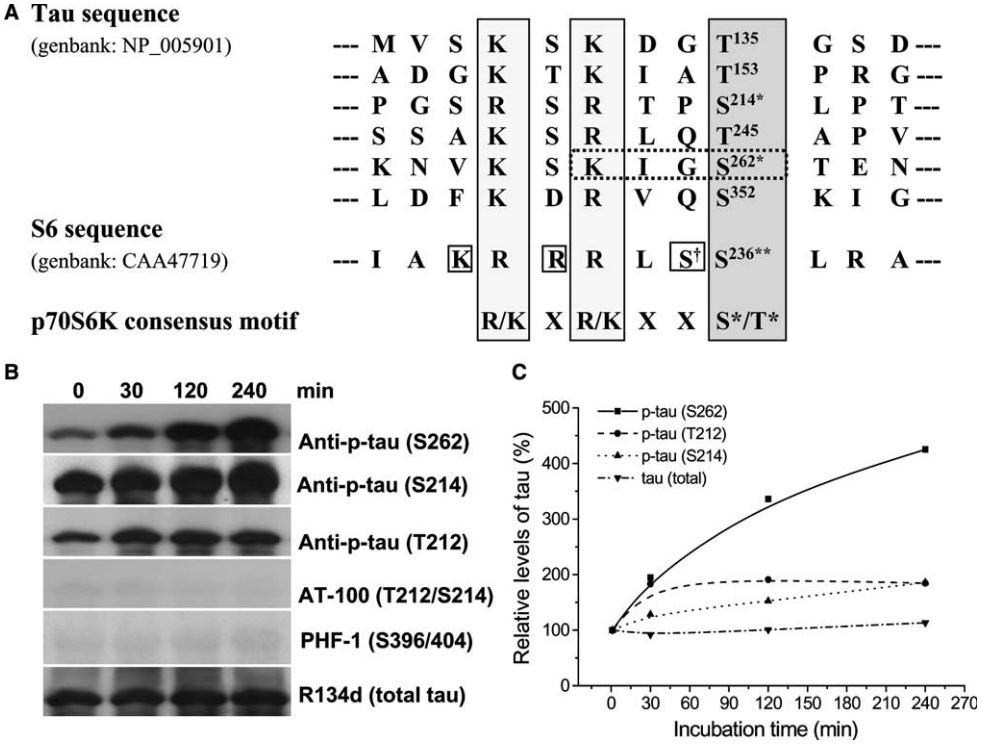

Fig. 2. Phosphorylation of tau by p70 S6 kinase. (A) Alignment of tau sequence (Genbank: NP_005901) with the consensus motif of p70 S6 kinase (p70S6K) (R/KXR/KXXS*/T*). Possible phosphorylation sites such as T135, T153, S214, T245, S262, and S352 on tau by p70S6K are indicated. S235 (†) and S236 (**) on S6 are well-known phosphorylation sites of p70S6K. (B) In vitro phosphorylation of tau by p70S6K. After incubation of p70S6K with tau in the presence of ATP, samples (20 μl/lane) were subjected to Western blotting, and monitored using different antibodies against tau phosphorylated at S262, Anti-p-tau (S262); S214, Anti-p-tau (S214); T212, Anti-p-tau (T212); T212/S214, AT-100; and S396/404 sites, PHF-1. R134d recognizes total tau. (C) Quantification of the relative density of each band shown in B.

(Fig. 2B and C), whereas the unpredicted S396/404 epitope recognized by antibody PHF-1 could not be phosphorylated by p70S6K (Fig. 2B). Although both T212 and S214 sites could be phosphorylated by p70S6K, respectively, no detectable signals were found with phospho-specific antibody against phosphorylated tau at T212/S214 (AT100).

3.2. Interaction between p70 S6 kinase and tau

Previously, we found a significant correlation between levels of p-p70S6K (T421/S424) and tau phosphorylated at S396/404 sites (PHF-1/tau) in AD brain homogenates [17]. Here, we wanted to investigate whether or not the phosphorylation sites of p-p70S6K and that of tau interact. Based on previous findings that p70S6K can induce phosphorylation at T389 and T421/S424 sites in SH-SY5Y cells treated with zinc [21], we treated the cells with 100 μM zinc for 30 min and 4 h, respectively, and obtained cell lysates, which were then immunoprecipitated with antibodies against p-p70S6K (T389) or p-p70S6K (T421/S424), and then reacted with antibodies specific for tau. We found that PHF-1/tau could be co-immunoprecipitated with p-p70S6K (T421/S424), but not with p-p70S6K (T389) (Fig. 3A). In contrast, unphosphorylated tau (Tau-1/tau) could not be pulled down with p70S6K phosphorylated at either T421/S424 or T389. Tau phosphorylated at S262 could be pulled down with p-p70S6K (T421/S424), but not p-p70S6K (T389) (not shown). A tendency of step-wise increase of both PHF-1/tau and Anti-p-Tau (S262) immunosignals was seen in cells treated with 100 μM zinc. Furthermore, p-tau at T212/S214 labelled by AT100 and at T231 labelled by AT180 (data not shown) could not be co-immunoprecipitated with p-p70S6K (T421/S424). Taken together, p-p70S6K at T421/S424, not at T389, is associated with PHF-1/tau or p-tau at S262.

3.3. Tau synthesis requires p70 S6 kinase activation

Surprisingly, when the 5′ terminus was compared among several human mRNAs including p70S6K, S6, tau, nucleolin, and amyloid precursor protein (APP), all of which are aberrantly changed in AD brains [17], a 5′TOP-like sequence similar to S6 mRNA was found in the 5′ untranslated region (5′UTR) of human tau mRNA (Fig. 4A). However, p70S6K mRNA or APP mRNA does not contain this kind of sequence. To estimate whether or not there is a relative selectivity of total tau increase after p70S6K activation, production of overall proteins per one million cells was measured, and relative levels of total p70S6K, S6, and tau were examined in SH-SY5Y cells treated with 100 M zinc for 4 h. Production of overall proteins per one million cells showed no change as compared with untreated control (Con) (Fig. 4B). However, the relative levels of total S6 and tau, not p70S6K were significantly increased in the cells treated with 100 μM zinc for 4 h (Fig. 4C). These results indicated that zinc-induced p70S6K activation could selectively increase translation of some proteins including tau, which contain a 5′TOP motif in the 5′UTR of their mRNAs. In SH-SY5Y cells that overexpress wild-type htau40, a dramatic increase of p-p70S6K (T421/S424) and total S6 was seen, indicating that the overexpression of stably transfected htau40 requires continuous p70S6K upregulation (Fig. 4D).

3.4. Correlation of p-p70 S6 kinase (T421/S424) with tau phosphorylated at S262, S214, and T212 sites

Since p70S6K could directly phosphorylate tau at S262, S214 and T212 sites, levels of tau phosphorylated at these sites were investigated in homogenates obtained from the medial temporal cortex of AD and control brains, and compared with p-p70S6K (T421/S424). It was found that levels of p-tau at all of these sites (S262, S214, and T212) were significantly increased in AD compared to controls (Fig. 5A). Levels of p-p70S6K (T421/S424) showed a strong correlation with tau phosphorylated at the S262 ($P < 0.05$, Fig. 5B), S214 ($P < 0.001$, Fig. 5C), and T212 ($P < 0.01$, Fig. 5D) in AD cases. Although levels of tau phosphorylated at both T212/S214 (AT100) showed a significant increase in AD as compared with controls (Fig. 5A), its increase did not show a significant correlation with p-p70S6K (T421/S424) (Fig. 5E). An elevated level of p-p70S6K (T421/S424) might contribute to the phosphorylation of tau at S262, S214 and T212 sites in AD brain. In controls, no significant correlation was found between p-p70S6K (T421/S424) and tau phosphorylated at these sites ($P > 0.05$).

4. Discussion

Our in vitro experiments indicated that phosphorylation of tau at S262 (microtubule-binding repeat domain), S214 and T212 (flanking domain) sites, but not at S396/404 and T212/S214, is favored by p70S6K. While phosphorylation of tau at the S214 site compromised its binding ability to microtu-

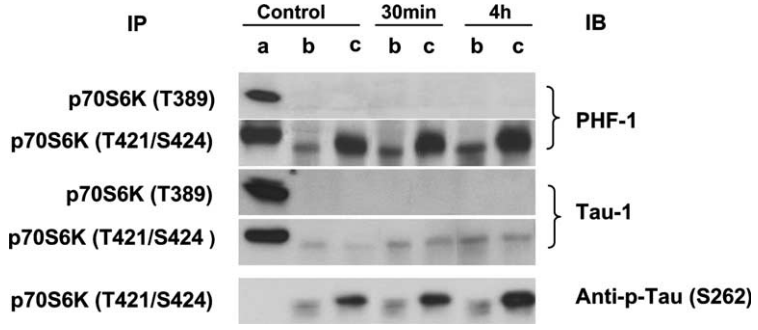

Fig. 3. Interaction between p70 S6 kinase and tau. Association between phosphorylated (p) p70S6K and p-tau. SH-SY5Y cells were treated with 100 μM zinc for 30 min or 4 h. The cell extracts were immunoprecipitated with antibodies against p-p70S6K (T389) or p-p70S6K (T421/S424), subjected to 10% SDS gel electrophoresis (20 μl/lane) and membranes reacted with antibodies to tau phosphorylated at S396/404 (PHF-1/tau) and S262 sites, and tau unphosphorylated at S198/199/202/T205 sites (Tau-1/tau). Cell extract from untreated cells (lane a), negative control (without primary antibody during immunoprecipitation, lane b), immunoprecipitated samples (lane c).

A

The 5' terminus of human mRNAs

Protein	*Sequence (1-20)*	*5'TOP*	*C/U*	*5' UTR*	*Accession number*
P70S6K	GCACGAGGCUGCGGCGGGUC	-	-	27	NM_003161
S6	CCUCUUUUCCGUGGCGCCUC	10	5/5	42	X67309.1
Tau	CCUCCCCUGGGGAGGCUCGC	8	6/2	236	NM_005910.2
Nucleolin	CUUCGGGUGUACGUGCUCCG	4	2/2	111	XM_010858.4
APP	AGUUUCCUCGGCAGCGGUAG	NA	NA	147	Y00264.1

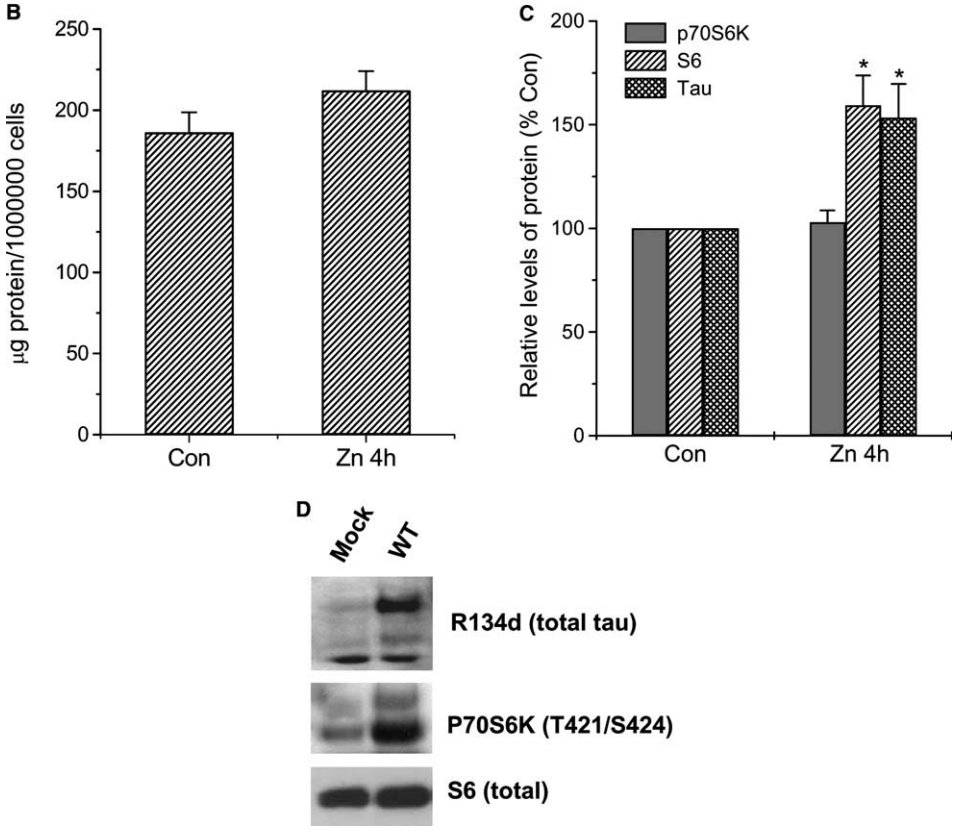

Fig. 4. Tau protein translation mediated by p70 S6 kinase. (A) Comparison of the 5′ terminus sequence of p70S6K, S6, tau, nucleolin, and amyloid precursor protein (APP) mRNAs. 5′UTR of human tau mRNA contains a sequence similar to the 5′TOP of S6. (B) No changes of global proteins in SH-SY5Y cells treated with 100 μM zinc for 4 h. Protein concentrations were measured with the BCA kit. Protein content per 1 million cells was determined by dividing the total amount of protein by total cell numbers. (C) Changes of total levels of p70S6K, S6, and tau determined by Western blotting for the same group as in B. (D) Increased expression of p-p70S6K (T421/S424) and total S6 in SH-SY5Y cells overexpressing wild-type htau40 (10 μg protein/lane). The results suggested that overexpression of stably transfected htau40 requires upregulation of p70S6K activation. $^{*}P < 0.05$.

bules [32], tau phosphorylated at the S262 site could abolish the ability to promote microtubule assembly and stability [33]. Although the T212 site appeared to have a relatively minor effect on microtubule assembly, prephosphorylation at this site has been shown to enhance S214 phosphorylation [34]. It is possible that microtubule disruption as seen in AD brains is synergistically caused by phosphorylase kinase [35], calcium/calmodulin-dependent protein kinase II (CaMKII) [36–38], microtubule-affinity regulating kinase (MARK) [33,39], protein kinase A [36], and finally p70S6K, all of which are capable of phosphorylating tau at the S262 site.

In SH-SY5Y cells and rat brain primary cultured neurons, 100 μM zinc was able to induce increased tau phosphorylation at S396/404 (PHF-1) sites, and this increase could be dramatically inhibited by the mTOR inhibitor rapamycin [17,21]. In metabolically active rat brain slices, selective PP-2A inhibition by okadaic acid could induce increased tau phosphorylation at S396/404 (PHF-1), S422 (R145d), and S262/356 (12E8) sites. Among these sites, inhibition of ERK1/2 with U0126 did not affect tau phosphorylation at PHF-1 sites, but increased tau unphosphorylation level at Tau-1 sites (S198/199/202/T205), and decreased tau

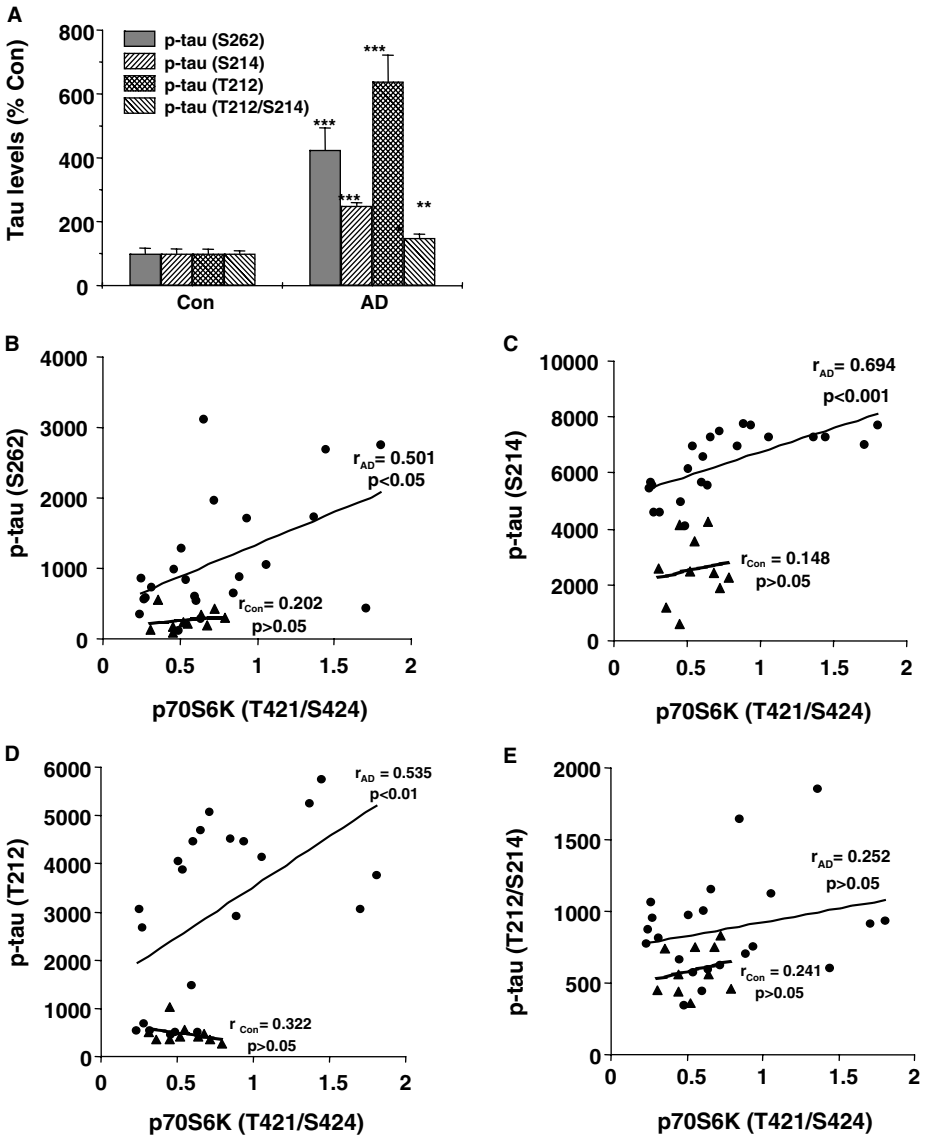

Fig. 5. Correlations between p70 S6 kinase (T421/S424) with tau phosphorylated at S262, S214, T212, and T212/S214. (A) Significant increase of tau phosphorylation at Ser262, S214, T212, and T212/S214 sites in AD brain extracts as compared with control. (B–E) Correlation of phosphorylated (p) p70S6K (T421/S424) with tau phosphorylated at S262, S214, T212 and T212/S214 sites, respectively. Levels of p-p70S6K (T421/S424) in AD brains showed a significant correlation with p-tau at S262 ($P < 0.05$), S214 ($P < 0.001$), and T212 ($P < 0.01$), but not at T212/S214. For comparison, there was no significant correlation between p-p70S6K (T421/S424) and p-tau at these sites in controls ($P > 0.05$). Circle, AD cases; triangle, control case. $^{*}P < 0.05$; $^{**}P < 0.01$; and $^{***}P < 0.001$.

phosphorylation at S422 and 12E8 sites [18]. In transgenic mice with chronically reduced PP2A activity by expressing a dominant-negative mutant form of the PP2A catalytic subunit, endogenous tau was phosphorylated at the epitopes S202/T205 (AT8) and S422, accompanied by ERK1/2 activation [40]. This suggested that tau phosphorylation state at Tau-1, 12E8, AT8, and S422 sites might be favored by ERK1/2. In contrast, tau phosphorylation at PHF-1 sites might be more favored by kinases other than ERK1/2, and that the activity of the kinase(s) regulating tau phosphorylation at PHF-1 sites could be blocked by rapamycin.

Tau protein at a high concentration is capable of self-aggregating in vitro even when present in an unmodified form [41]. However, when tau is phosphorylated at the S262 site in the microtubule-binding repeat domain it can be detached from the microtubules, and may thus be protective in preventing tau aggregation into AD-like PHFs [39]. In contrast, phosphorylation of tau at S214 and T212 sites in the flanking domain may neutralize the basic charge, and thus neutralize the inhibitory effect of S262 phosphorylation and cause tau to self-assemble into filaments [42]. Levels of tau phosphorylated at these sites were dramatically elevated in AD as compared to control (current study). The significant correlation as seen between p-p70S6K (T421/S424), and p-tau at S262, S214, and T212, but not T212/S214 suggested that p70S6K activation might act on the S262 site, and in a similar manner to PKB [34] on the S214 or T212 site, but not T212/S214 sites in AD. Thus, p70S6K activation might play a significant role in

tau hyperphosphorylation at these sites, which might cause microtubule disruption and inhibition of PHF formation in degenerating neurons in AD.

Here, we have found that tau phosphorylated at S396/404 (PHF-1) sites or the S262 site (not Tau-1/tau dephosphorylated at S198/199/202/T205) could only be co-precipitated with p-p70S6K (T421/S424) and not p-p70S6K (T389). Thus, it is reasonable to speculate that after binding of p-p70S6K (T421/S424) with S396/404 or S262, by facilitating site-specific phosphorylation on regulatory (T389) and catalytic (T229) domains, p70S6K activity may be enhanced, which in turn may phosphorylate tau at S262, T212, and S214 sites. As a sticky protein, tau was found to bind to a number of proteins [43] such as actin [44], PP-1 and PP-2A [23,45], α-synuclein [46], and phospholipase C [47,48], and glycogen synthase kinase-3β [49]. The significance of the interactions among these proteins in pathogenesis of tau related abnormalities in AD is not understood.

Comparative analysis of the first 20 nucleotides of mRNAs encoding proteins such as p70S6K, S6 and tau revealed that the 5′UTR of tau mRNA has a 5′TOP-like structure similar to that of S6 mRNA. In the present study, global protein translation was not altered in zinc-treated SH-SY5Y cells, consistent with the concept that p70S6K regulates the translation of a set of 5′TOP-containing mRNAs rather than overall protein synthesis. We found a dramatic increase of levels of total tau and S6, but not total p70S6K, both in zinc-treated SH-SY5Y cells (current study) and in AD brains [17]. These data together suggested that p70S6K activation upregulates translation of a group of proteins including tau. The requirement of p70S6K activation in tau translation was confirmed in SH-SY5Y cells, as when tau was overexpressed, level of total S6 was also increased, together with a dramatic increase of p-p70S6K (T421/S424) levels. Thus, the 5′TOP-like structure might enable tau mRNA to be preferentially translated into protein in response to p70S6K activation similar to S6 5′TOP mRNA. Although tau mRNA level was elevated in the brains of Down syndrome patients [50], it was unchanged in AD brains [51–53]. This indicates that in AD brains a sufficient amount of tau mRNA is available for synthesizing new tau protein.

Aberrant activation of p70S6K in AD brains might be due to dys-regulation of PP-2A activity and the PI3K and MAPK signalling pathways [17,18,21,40]. Our present study suggests that p70S6K mediates both tau phosphorylation and synthesis. To fully understand the role of p70S6K in formation of tau related abnormalities, further studies are needed to identify whether or not the type of self-assembled non-PHF filaments exists in AD brain and other tauopathies.

Acknowledgements: The authors acknowledge Dr. Irina Alafuzoff for providing human brain tissues. This study was supported by Alzheimerfonden, Gamla Tjänarinnor Foundation, Gun och Bertil Stohnes Stiftelse, Loo and Hans Ostermans Foundation, SADF (Insamlingsstiftelsen för Alzheimer- och Demensforskning), SIDA, Socialstyrelsens Stiftelser, Stiftelsen för Ålderssjukdomar, Svenska läkaresällskapet, and Tryggers Stiftelse.

References

[1] Ferrari, S. and Thomas, G. (1994) S6 phosphorylation and the p70s6k/p85s6k. Crit. Rev. Biochem. Mol. Biol. 29, 385–413.
[2] Reinhard, C., Fernandez, A., Lamb, N.J. and Thomas, G. (1994) Nuclear localization of p85s6k: functional requirement for entry into S phase. EMBO J. 13, 1557–1565.
[3] Petritsch, C., Beug, H., Balmain, A. and Oft, M. (2000) TGF-beta inhibits p70 S6 kinase via protein phosphatase 2A to induce G(1) arrest. Genes Dev. 14, 3093–3101.
[4] Saucedo, L.J. and Edgar, B.A. (2002) Why size matters: altering cell size. Curr. Opin. Genet. Dev. 12, 565–571.
[5] Pullen, N., Dennis, P.B., Andjelkovic, M., Dufner, A., Kozma, S.C., Hemmings, B.A. and Thomas, G. (1998) Phosphorylation and activation of p70s6k by PDK1. Science 279, 707–710.
[6] Saitoh, M., Pullen, N., Brennan, P., Cantrell, D., Dennis, P.B. and Thomas, G. (2002) Regulation of an activated S6 kinase 1 variant reveals a novel mammalian target of rapamycin phosphorylation site. J. Biol. Chem. 277, 20104–20112.
[7] Isotani, S., Hara, K., Tokunaga, C., Inoue, H., Avruch, J. and Yonezawa, K. (1999) Immunopurified mammalian target of rapamycin phosphorylates and activates p70 S6 kinase alpha in vitro. J. Biol. Chem. 274, 34493–34498.
[8] Romanelli, A., Dreisbach, V.C. and Blenis, J. (2002) Characterization of phosphatidylinositol 3-kinase-dependent phosphorylation of the hydrophobic motif site Thr(389) in p70 S6 kinase 1. J. Biol. Chem. 277, 40281–40289.
[9] Belham, C., Comb, M.J. and Avruch, J. (2001) Identification of the NIMA family kinases NEK6/7 as regulators of the p70 ribosomal S6 kinase. Curr. Biol. 11, 1155–1167.
[10] Mukhopadhyay, N.K., Price, D.J., Kyriakis, J.M., Pelech, S., Sanghera, J. and Avruch, J. (1992) An array of insulin-activated, proline-directed serine/threonine protein kinases phosphorylate the p70 S6 kinase. J. Biol. Chem. 267, 3325–3335.
[11] Dennis, P.B., Pullen, N., Pearson, R.B., Kozma, S.C. and Thomas, G. (1998) Phosphorylation sites in the autoinhibitory domain participate in p70(s6k) activation loop phosphorylation. J. Biol. Chem. 273, 14845–14852.
[12] Eguchi, S., Iwasaki, H., Ueno, H., Frank, G.D., Motley, E.D., Eguchi, K., Marumo, F., Hirata, Y. and Inagami, T. (1999) Intracellular signaling of angiotensin II-induced p70 S6 kinase phosphorylation at Ser(411) in vascular smooth muscle cells. Possible requirement of epidermal growth factor receptor, Ras, extracellular signal-regulated kinase, and Akt. J. Biol. Chem. 274, 36843–36851.
[13] Pei, J.J., Tanaka, T., Tung, Y.C., Braak, E., Iqbal, K. and Grundke-Iqbal, I. (1997) Distribution, levels, and activity of glycogen synthase kinase-3 in the Alzheimer disease brain. J. Neuropathol. Exp. Neurol. 56, 70–78.
[14] Pei, J.J., Braak, E., Braak, H., Grundke-Iqbal, I., Iqbal, K., Winblad, B. and Cowburn, R.F. (1999) Distribution of active glycogen synthase kinase 3beta (GSK-3beta) in brains staged for Alzheimer disease neurofibrillary changes. J. Neuropathol. Exp. Neurol. 58, 1010–1019.
[15] Pei, J.J., Braak, E., Braak, H., Grundke-Iqbal, I., Iqbal, K., Winblad, B. and Cowburn, R.F. (2001) Localization of active forms of C-jun kinase (JNK) and p38 kinase in Alzheimer's disease brains at different stages of neurofibrillary degeneration. J. Alzheimers Dis. 3, 41–48.
[16] Pei, J.J., Braak, H., An, W.L., Winblad, B., Cowburn, R.F., Iqbal, K. and Grundke-Iqbal, I. (2002) Up-regulation of mitogen-activated protein kinases ERK1/2 and MEK1/2 is associated with the progression of neurofibrillary degeneration in Alzheimer's disease. Brain Res. Mol. Brain Res. 109, 45–55.
[17] An, W.L., Cowburn, R.F., Li, L., Braak, H., Alafuzoff, I., Iqbal, K., Grunke-Iqbal, I., Winblad, B. and Pei, J.J. (2003) Up-regulation of phosphorylated/activated p70 S6 kinase and its relationship to neurofibrillary pathology in Alzheimer's disease. Am. J. Pathol. 163, 591–607.
[18] Pei, J.J., Gong, C.X., An, W.L., Winblad, B., Cowburn, R.F., Grundke-Iqbal, I. and Iqbal, K. (2003) Okadaic-acid-induced inhibition of protein phosphatase 2A produces activation of mitogen-activated protein kinases ERK1/2, MEK1/2, and p70 S6, similar to that in Alzheimer's disease. Am. J. Pathol. 163, 845–858.
[19] Pei, J.J., Khatoon, S., An, W.L., Nordlinder, M., Tanaka, T., Braak, H., Tsujio, I., Takeda, M., Alafuzoff, I., Winblad, B., Cowburn, R.F., Grundke-Iqbal, I. and Iqbal, K. (2003) Role of

protein kinase B in Alzheimer's neurofibrillary pathology. Acta Neuropathol. (Berl.) 105, 381–392.
[20] Kim, S., Jung, Y., Kim, D., Koh, H. and Chung, J. (2000) Extracellular zinc activates p70 S6 kinase through the phosphatidylinositol 3-kinase signaling pathway. J. Biol. Chem. 275, 25979–25984.
[21] An, W.L., Bjorkdahl, C., Liu, R., Cowburn, R.F., Winblad, B. and Pei, J.J. (2005) Mechanism of zinc-induced phosphorylation of p70 S6 kinase and glycogen synthase kinase 3β in SH-SY5Y neuroblastoma cells. J. Neurochem. 92, 1104.
[22] Braak, H. and Braak, E. (1991) Neuropathological stageing of Alzheimer-related changes. Acta Neuropathol. (Berl.) 82, 239–259.
[23] Sontag, E., Nunbhakdi-Craig, V., Lee, G., Brandt, R., Kamibayashi, C., Kuret, J., White 3rd, C.L., Mumby, M.C. and Bloom, G.S. (1999) Molecular interactions among protein phosphatase 2A, tau, and microtubules. Implications for the regulation of tau phosphorylation and the development of tauopathies. J. Biol. Chem. 274, 25490–25498.
[24] Hartley, D. and Cooper, G.M. (2002) Role of mTOR in the degradation of IRS-1: regulation of PP2A activity. J. Cell Biochem. 85, 304–314.
[25] Nojima, H., Tokunaga, C., Eguchi, S., Oshiro, N., Hidayat, S., Yoshino, K., Hara, K., Tanaka, N., Avruch, J. and Yonezawa, K. (2003) The mammalian target of rapamycin (mTOR) partner, raptor, binds the mTOR substrates p70 S6 kinase and 4E-BP1 through their TOR signaling (TOS) motif. J. Biol. Chem. 278, 15461–15464.
[26] Peterson, R.T., Desai, B.N., Hardwick, J.S. and Schreiber, S.L. (1999) Protein phosphatase 2A interacts with the 70-kDa S6 kinase and is activated by inhibition of FKBP12-rapamycin-associated protein. Proc. Natl. Acad. Sci. USA 96, 4438–4442.
[27] Khatoon, S., Grundke-Iqbal, I. and Iqbal, K. (1992) Brain levels of microtubule-associated protein tau are elevated in Alzheimer's disease: a radioimmuno-slot-blot assay for nanograms of the protein. J. Neurochem. 59, 750–753.
[28] Ferrari, A., Hoerndli, F., Baechi, T., Nitsch, R.M. and Gotz, J. (2003) Beta-amyloid induces paired helical filament-like tau filaments in tissue culture. J. Biol. Chem. 278, 40162–40168.
[29] Chen, Q.M., Tu, V.C., Wu, Y. and Bahl, J.J. (2000) Hydrogen peroxide dose dependent induction of cell death or hypertrophy in cardiomyocytes. Arch. Biochem. Biophys. 373, 242–248.
[30] Li, X., An, W.L., Alafuzoff, I., Soininen, H., Winblad, B. and Pei, J.J. (2004) Phosphorylated eukaryotic translation factor 4E is elevated in Alzheimer brain. Neuroreport 15, 2237–2240.
[31] Zhang, H., Zha, X., Tan, Y., Hornbeck, P.V., Mastrangelo, A.J., Alessi, D.R., Polakiewicz, R.D. and Comb, M.J. (2002) Phosphoprotein analysis using antibodies broadly reactive against phosphorylated motifs. J. Biol. Chem. 277, 39379–39387.
[32] Illenberger, S., Zheng-Fischhofer, Q., Preuss, U., Stamer, K., Baumann, K., Trinczek, B., Biernat, J., Godemann, R., Mandelkow, E.M. and Mandelkow, E. (1998) The endogenous and cell cycle-dependent phosphorylation of tau protein in living cells: implications for Alzheimer's disease. Mol. Biol. Cell 9, 1495–1512.
[33] Drewes, G., Ebneth, A., Preuss, U., Mandelkow, E.M. and Mandelkow, E. (1997) MARK, a novel family of protein kinases that phosphorylate microtubule-associated proteins and trigger microtubule disruption. Cell 89, 297–308.
[34] Ksiezak-Reding, H., Pyo, H.K., Feinstein, B. and Pasinetti, G.M. (2003) Akt/PKB kinase phosphorylates separately Thr212 and Ser214 of tau protein in vitro. Biochim. Biophys. Acta 1639, 159–168.
[35] Paudel, H.K. (1997) The regulatory Ser262 of microtubule-associated protein tau is phosphorylated by phosphorylase kinase. J. Biol. Chem. 272, 1777–1785.
[36] Sironi, J.J., Yen, S.H., Gondal, J.A., Wu, Q., Grundke-Iqbal, I. and Iqbal, K. (1998) Ser-262 in human recombinant tau protein is a markedly more favorable site for phosphorylation by CaMKII than PKA or PhK. FEBS Lett. 436, 471–475.
[37] Bennecib, M., Gong, C.X., Grundke-Iqbal, I. and Iqbal, K. (2001) Inhibition of PP-2A upregulates CaMKII in rat forebrain and induces hyperphosphorylation of tau at Ser 262/356. FEBS Lett. 490, 15–22.
[38] Yamamoto, H., Yamauchi, E., Taniguchi, H., Ono, T. and Miyamoto, E. (2002) Phosphorylation of microtubule-associated protein tau by Ca2+/calmodulin-dependent protein kinase II in its tubulin binding sites. Arch. Biochem. Biophys. 408, 255–262.
[39] Schneider, A., Biernat, J., von Bergen, M., Mandelkow, E. and Mandelkow, E.M. (1999) Phosphorylation that detaches tau protein from microtubules (Ser262, Ser214) also protects it against aggregation into Alzheimer paired helical filaments. Biochemistry 38, 3549–3558.
[40] Kins, S., Kurosinski, P., Nitsch, R.M. and Gotz, J. (2003) Activation of the ERK and JNK signaling pathways caused by neuron-specific inhibition of PP2A in transgenic mice. Am. J. Pathol. 163, 833–843.
[41] Avila, J., Lim, F., Moreno, F., Belmonte, C. and Cuello, A.C. (2002) Tau function and dysfunction in neurons: its role in neurodegenerative disorders. Mol. Neurobiol. 25, 213–231.
[42] Alonso, A., Zaidi, T., Novak, M., Grundke-Iqbal, I. and Iqbal, K. (2001) Hyperphosphorylation induces self-assembly of tau into tangles of paired helical filaments/straight filaments. Proc. Natl. Acad. Sci. USA 98, 6923–6928.
[43] Avila, J., Lucas, J.J., Perez, M. and Hernandez, F. (2004) Role of tau protein in both physiological and pathological conditions. Physiol. Rev. 84, 361–384.
[44] Correas, I., Padilla, R. and Avila, J. (1990) The tubulin-binding sequence of brain microtubule-associated proteins, tau and MAP-2, is also involved in actin binding. Biochem. J. 269, 61–64.
[45] Liao, H., Li, Y., Brautigan, D.L. and Gundersen, G.G. (1998) Protein phosphatase 1 is targeted to microtubules by the microtubule-associated protein Tau. J. Biol. Chem. 273, 21901–21908.
[46] Jensen, P.H., Hager, H., Nielsen, M.S., Hojrup, P., Gliemann, J. and Jakes, R. (1999) Alpha-synuclein binds to Tau and stimulates the protein kinase A-catalyzed tau phosphorylation of serine residues 262 and 356. J. Biol. Chem. 274, 25481–25489.
[47] Hwang, S.C., Jhon, D.Y., Bae, Y.S., Kim, J.H. and Rhee, S.G. (1996) Activation of phospholipase C-gamma by the concerted action of tau proteins and arachidonic acid. J. Biol. Chem. 271, 18342–18349.
[48] Jenkins, S.M. and Johnson, G.V. (1998) Tau complexes with phospholipase C-gamma in situ. Neuroreport 9, 67–71.
[49] Sun, W., Qureshi, H.Y., Cafferty, P.W., Sobue, K., Agarwal-Mawal, A., Neufield, K.D. and Paudel, H.K. (2002) Glycogen synthase kinase-3beta is complexed with tau protein in brain microtubules. J. Biol. Chem. 277, 11933–11940.
[50] Oyama, F., Cairns, N.J., Shimada, H., Oyama, R., Titani, K. and Ihara, Y. (1994) Down's syndrome: up-regulation of beta-amyloid protein precursor and tau mRNAs and their defective coordination. J. Neurochem. 62, 1062–1066.
[51] Mah, V.H., Eskin, T.A., Kazee, A.M., Lapham, L. and Higgins, G.A. (1992) In situ hybridization of calcium/calmodulin dependent protein kinase II and tau mRNAs; species differences and relative preservation in Alzheimer's disease. Brain Res. Mol. Brain Res. 12, 85–94.
[52] Chambers, C.B., Lee, J.M., Troncoso, J.C., Reich, S. and Muma, N.A. (1999) Overexpression of four-repeat tau mRNA isoforms in progressive supranuclear palsy but not in Alzheimer's disease. Ann. Neurol. 46, 325–332.
[53] Boutajangout, A., Boom, A., Leroy, K. and Brion, J.P. (2004) Expression of tau mRNA and soluble tau isoforms in affected and non-affected brain areas in Alzheimer's disease. FEBS Lett. 576, 183–189.