Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochimica et Biophysica Acta 1639 (2003) 159–168
BIOCHIMICA ET BIOPHYSICA ACTA
BBA
www.bba-direct.com

Akt/PKB kinase phosphorylates separately Thr212 and Ser214 of tau protein in vitro

Hanna Ksiezak-Reding*, Han Kyoung Pyo, Boris Feinstein, Giulio M. Pasinetti

Neuroinflammation Research Laboratories, Department of Psychiatry, Mount Sinai School of Medicine, Box 1230, One Gustave L. Levy Place, New York, NY 10029, USA

Received 23 June 2003; received in revised form 8 September 2003; accepted 12 September 2003

Abstract

Microtubule-associated protein tau contains a consensus motif for protein kinase B/Akt (Akt), which plays an essential role in anti-apoptotic signaling. The motif encompasses the AT100 double phospho-epitope (Thr212/Ser214), a specific marker for Alzheimer's disease (AD) and other neurodegenerations, raising the possibility that it could be generated by Akt. We studied Akt-dependent phosphorylation of tau protein in vitro. We found that Akt phosphorylated both Thr212 and Ser214 in the longest and shortest tau isoforms as determined using phospho site-specific antibodies against tau. Akt did not phosphorylate other tau epitopes, including Tau-1, AT8, AT180, 12E8 and PHF-1. The Akt-phosphorylated tau retained its initial electrophoretic mobility. Immunoprecipitation studies with phospho-specific Thr212 and Ser214 antibodies revealed that only one of the two sites is phosphorylated per single tau molecule, resulting in tau immunonegative for AT100. Mixed kinase studies showed that prior Ser214 phosphorylation by Akt blocked protein kinase A but not GSK3β activity. On the other hand, GSK3β selectively blocked Ser214 phosphorylation, which was prevented by lithium. The results suggest that Akt may be involved in AD-specific phosphorylation of tau at the AT100 epitope in conjunction with other kinases. Our data suggest that phosphorylation of tau by Akt may play specific role(s) in Akt-mediated anti-apoptotic signaling, particularly relevant to AD and other neurodegenerations.
© 2003 Elsevier B.V. All rights reserved.

Keywords: Tau; Akt/protein kinase B; Protein kinase A; Glycogen synthase kinase 3β; AT100 epitope; Alzheimer's disease

1. Introduction

Tau is a microtubule-associated protein that plays a regulatory role in microtubule assembly and stability in neuronal and glial cells. Normal adult brain contains six tau isoforms, which are derived from alternative splicing. Expression of tau isoforms is developmentally regulated and only a single tau isoform is found in fetal human brain [1]. Interactions of tau with microtubules are regulated by the length of the microtubule-binding domain [2] and by phosphorylation [3]. Specific roles of different phosphorylation sites in tau have been demonstrated in the development of cell processes [4], axonal transport [5] and Fyn-mediated signal transduction [6].

Deposits of tau, designated as paired helical filaments (PHFs), characterize a number of late onset neurodegenerative disorders, including Alzheimer's disease (AD) and other age-related dementias with tauopathy [7,8]. PHFs are composed of highly phosphorylated species of tau but the molecular mechanism leading to hyperphosphorylation and deposition of tau in late onset dementias remains unclear. Hyperphosphorylated tau in PHFs contains a unique double-site phospho-epitope AT100 (Thr212/Ser214). This epitope merits particular attention since it is specific to PHFs and, unlike other phospho-epitopes associated with PHFs, is absent in biopsy-derived normal tau [9]. Studies in vitro have shown that the AT100 epitope could be generated in a sequential phosphorylation first by glycogen synthase kinase-3β (GSK3β) at Thr212, then by protein kinase A (PKA) at Ser214 [10].

Studies have demonstrated that tau phosphorylation sites containing Ser–Pro or Thr–Pro motifs have only a moderate effect on the dynamic instability of microtubules as compared with non-proline sites, e.g., Ser214 and Ser262 [11]. Phosphorylation at Ser214 is of particular importance

* Corresponding author. Tel.: +1-212-241-0240; fax: 1-212-876-9042.
E-mail address: hanna.reding@mssm.edu (H. Ksiezak-Reding).

0925-4439/$ - see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbadis.2003.09.001

because it alone potently disrupts microtubule binding, reducing tau's affinity for microtubules by a factor of 10 [12]. Moreover, unlike Ser–Pro or Thr–Pro motifs phosphorylated by GSK3β and other kinases, phosphorylation at Ser214 has been reported to have a protective effect against tau assembly into PHFs [13]. Effects of Thr212 on microtubule binding and PHF-like aggregation have not been examined mostly due to difficulties in generating tau phosphorylated at a single site.

Akt/protein kinase B (Akt) has not been considered as a potential kinase in generating AT100 epitope or in tau phosphorylation studies. However, the AT100 epitope encompasses a consensus motif for Akt [14] strongly suggesting that phosphorylation of tau in this region could result from activation of a signaling pathway involving Akt [15]. Akt is a 57 kDa Ser/Thr kinase and a downstream effector of the PI3K/Akt anti-apoptotic signaling cascade [16–18]. It is widely expressed, with all tissues containing at least one form of Akt and highest levels in brain, thymus, heart, and lung [16,19]. The activation of Akt is achieved by phosphorylation at Thr308 and Ser473 by a mechanism either dependent or independent of PI3K activation by growth factors. Downstream targets of Akt include transcription factors, anti-apoptotic gene expression and regulation of the cell cycle. Akt effectively inhibits GSK3α/β by phosphorylating Ser21/9 [20]. Furthermore, Akt redistributes hexokinase activity to the mitochondria, couples glycolysis to oxidative phosphorylation and blocks release of cytochrome *c* to inhibit apoptosis [21,22].

In the present studies, using site-specific antibodies, we demonstrate that Akt is able to phosphorylate tau in at least two sites, Thr212 and Ser214, but not simultaneously. Akt-phosphorylated tau can be separated into two pools each containing only a single phospho-site, either Thr212 or Ser214. The results suggest that activation of Akt may partially contribute to specific tau phosphorylation at the AT100 epitope characteristic of PHFs.

2. Materials and methods

2.1. Materials

Alkaline phosphatase (*E. coli*, type III), GSK3β (rabbit recombinant), PKA (bovine heart, P5511), protease inhibitors and other general chemicals were purchased from Sigma, St. Louis, MO. H-7 was purchased from Seikagaku, Tokio, Japan and KT5720 from Calbiochem, La Jolla, CA. Fat-free milk was from Carnation. Nitrocellulose membranes, secondary antibodies conjugated to horseradish peroxidase and protein assay kit were from BioRad, Redding, CA. Enhanced chemiluminescence (ECL) kit was purchased from Amersham, Arlington Heights, IL. Akt kinase was from Panvera Corporation, Madison, WI. Protein G Sepharose was purchased from Pharmacia, Uppsala, Sweden. GSK3α/β fusion protein and Akt kinase assay kit were obtained from Cell Signaling Technology, Inc., Beverly, MA (New England Biolabs).

2.2. Recombinant tau

Recombinant human tau was expressed in *E. coli* BL21 (DE3 cells) using an expression plasmid pRK172 containing a tau cDNA (clone htau40). Clone htau40 encodes the longest tau isoform of 441 amino acid residues and was a generous gift from Michel Goedert [1]. The induction, expression and the isolation of recombinant tau was performed as previously described [23,24]. Protein concentration was determined using a protein assay based on Coomassie brilliant blue G-250 binding.

2.3. Tau preparations

Tau fraction was isolated from fetal human brain (19–21 gestational age) as previously described using perchloric acid extraction [25] and used without further treatments (Ftau). Some Ftau preparations were subjected to alkaline phosphatase treatment for 24 h to dephosphorylate endogenous sites as described [25]. After dephosphorylation, samples were adjusted to 0.3 M NaCl and 2% β-mercaptoethanol, boiled for 10 min and centrifuged at $15{,}000 \times g$ for 15 min. The supernatant was dialysed in 50 mM Tris/HCl, pH 7.4 and the obtained fraction designated FtauAP.

2.4. Polyacrylamide gel electrophoresis and Western blotting

Proteins were separated by electrophoresis using SDS-polyacrylamide gels containing 10% polyacrylamide. Separated proteins were electrotransferred onto nitrocellulose membranes. Immunoblotting was performed using 5% fat-free milk in Tris-buffered saline (TBS) to block nonspecific binding sites and to dilute the primary and secondary antibodies. Specific protein signals were detected by ECL.

2.5. Antibodies

Monoclonal antibodies raised against phospho-tau included AT8 (Ser202/Thr205), AT100 (Thr212/Ser214) and AT180 (Thr231) from Innogenetics, Belgium. Other antibodies against phospho-tau included the rabbit anti-pThr212 (Calbiochem), the rabbit anti-pSer214 (Abcam), the monoclonal antibody 12E8 (Ser262/356, Athena Neuroscience), and the monoclonal antibody PHF-1 (Ser396/404, Albert Einstein Coll. Med.). The monoclonal antibody raised against non-phosphorylated tau was Tau 46 (the C-terminal residues 428–441; Zymed). Other antibodies included the rabbit anti-phospho-GSK3α/β (Ser21/9), the monoclonal antibody, 4E2, raised against phospho-Akt (Ser473) and the rabbit antibody raised against phospho-(Ser/Thr) Akt substrate (PAS) from Cell Signaling. The rabbit anti-phospho-Akt1/PKBα (Ser473) was obtained from Upstate and

the rabbit antibody against total PKB (PKBα/Akt1) was purchased from Sigma.

2.6. Kinase assays

Tau and other substrates were incubated with respective kinases in a kinase buffer mixture containing 25 mM Tris/HCl, pH 7.5, 5 mM β-glycerophosphate, 2 mM dithiothreitol, 0.1 mM sodium vanadate, 10 mM $MgCl_2$, 0.1 mg/ml heparin and either 3 mM ATP (Akt and GSK3β) or 3 mM bdcAMP (PKA). Tau protein was added at 2–5 μg and GSK3α/β fusion protein at 5–10 μg per 50 μl final volume. Akt kinase was an active form of human recombinant Akt1 obtained as >90% purified preparations [26] and was used at 0.54 μg/50 μl (equivalent to the activity of 0.25 nmol/min at 30°C with Crosstide peptide as substrate). PKA was used at 10 μg/50 μl, and GSK3β at 1 unit/50 μl. Except for immunoprecipitation studies, the enzymatic reactions were terminated by adding SDS-sample buffer. In some experiments, specific kinase inhibitors were used, lithium chloride (50 mM) for GSK3α/β, KT5720 (3 μM) for PKA, staurosporine (10 nM) for PKC and other kinases (e.g., cdc2), and H-7 (30 μM) for both PKA and PKC [27–29].

2.7. Immunoprecipitation

Five-microliter samples from the kinase reaction containing phospho-tau were diluted 10-fold with the lysis buffer (20 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM Na_3V0_4, 1 μg/ml leupeptin and 0.1 mM PMSF) and incubated with the rabbit anti-tau antibodies (anti-pThr212 and anti-pSer214) for 3–5 h at 4°C. After the incubation, the mixture was combined with 40–20 μl pellet of protein G Sepharose prewashed twice in the lysis buffer. Samples were rocked gently for 2 h at 4°C and then separated into the supernatants and pellets (immune complexes). The supernatants were used either for immunoblotting or to perform the second kinase reaction. Pellets of the immune complexes were washed three times with 250 μl lysis buffer and then suspended in the SDS-sample buffer.

3. Results

3.1. Tau protein contains the Akt kinase consensus motif

The Akt consensus motif has been described in a number of proteins considered as substrates for Akt [14]. The examination of the primary sequence of tau reveals the presence of a single Akt motif, which coincides with the AT100 epitope (Fig. 1) [30]. Among Akt consensus motif, there is a stringent requirement for Arg residues at positions *n*-3 and *n*-5, where *n* is the site of phosphorylation. In tau, only Ser214 conforms to this requirement. It was uncertain whether Thr212, which has Arg residues at positions *n*-1 and *n*-3, and is also a part of the AT100 epitope, could be phosphorylated by Akt. In the present studies, we sought to determine directly whether one or both amino acid residues could indeed be phosphorylated by Akt in vitro.

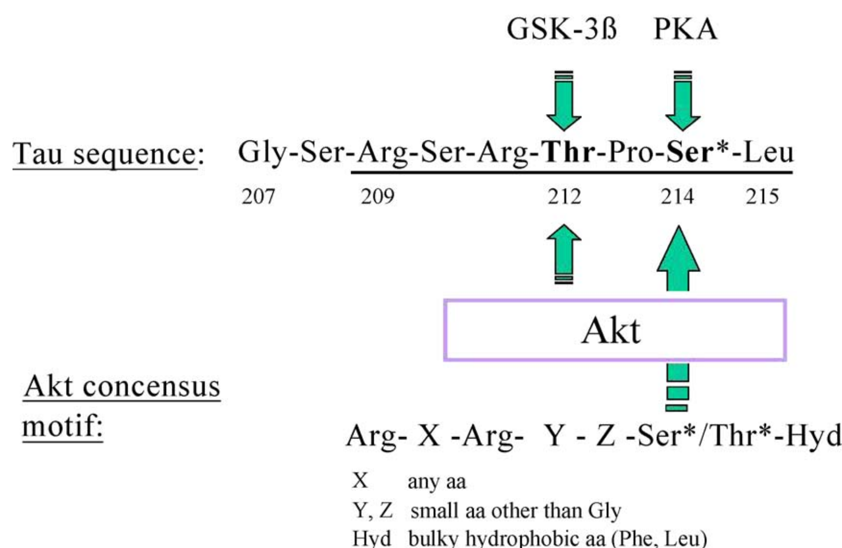

Fig. 1. A diagram illustrating the region of tau molecule encompassing the AT100 epitope (double-phospho site Thr212/Ser214) and the consensus sequence for phosphorylation by Akt based on Alessi et al. [14]. The underlined tau sequence comprises the consensus motif with Ser214 conforming to the stringent requirements for Akt-dependent phosphorylation, with Arg residues at positions *n*-3 and *n*-5. Thr212 only partially conforms to this requirement with Arg residues at positions *n*-1 and *n*-3. GSK3β and PKA are protein kinases previously shown to phosphorylate Thr212 and Ser214, respectively [10,32,33].

3.2. Characterization of tau substrates

To determine the dependence of Akt activity on the size of the tau molecule, we selected the longest (rtau) and the shortest (Ftau and FtauAP) human tau isoforms as the potential Akt substrates. Rtau (441 amino acid residues) contains the N-terminal inserts and four repeats in the microtubule binding domain. The shortest tau isoform (352 amino acid residues) contains only three repeats in the microtubule binding domain and lacks the N-terminal inserts. Furthermore, to determine whether Akt activity depends on prior phosphorylation state of tau, fetal tau preparations either contained endogenous phosphorylation sites (Ftau) or were subjected to prior dephosphorylation (FtauAP). Characterization of tau substrates by SDS-PAGE and immunoblotting demonstrated their purity, well-established migration pattern, and immunoreactivity with phospho-dependent and -independent tau antibodies (Fig. 2). Rtau but not Ftau or FtauAP preparations contained degraded tau fragments. These fragments were Tau 46-negative and most likely devoid of the C-terminal end. The preparations differed in the phosphorylation status of tau. Ftau immunoreacted with more phospho-dependent tau antibodies than the other two preparations (Figs. 2C and 3D). This was consistent with Ftau having a high content of up to seven phosphate residues per molecule and other preparations either having no or very low phosphate content [25,31]. A low phosphorylation status of FtauAP was additionally confirmed by its faster electrophoretic mobility than that of Ftau (48 vs. 55 kDa). Neither of the tau preparations were immunoreactive for pThr212 (Fig. 2D) but all were immunoreactive for pSer214 (Fig. 2E). Of the three, Ftau displayed the most intense pSer214 immunoreactivity, whereas rtau and FtauAP were only weakly immunoreactive (Fig. 2D,E). The weak binding could be either due to a low phosphorylation level of pSer214 in these preparations or to cross-reactivity of non-phosphorylated Ser214 and/or surrounding amino acid residues.

3.3. Akt phosphorylates tau at Thr212 and Ser214

We characterized the activity of purified >90% preparations of full-length human Akt (Fig. 3A) with its GSK3α/β substrate. We used the ~30 kDa GSK3α/β fusion protein containing Ser21/9, the site specific for Akt phosphorylation, and found that incubation with Akt for 1 and 5 h increased its phospho-Ser21/9 immunoreactivity in a time-dependent manner (Fig. 3B).

Incubation of tau protein substrates (rtau, Ftau, FtauAP) with the same preparation of Akt elevated the immunoreactivity of tau with anti-pThr212 and anti-pSer214 antibodies, regardless of whether tau contained endogenous phosphorylation sites or varied in its molecular size (Fig. 3C). The increases appeared to reach a plateau by approximately 1 h of incubation under our experimental conditions. Other phospho-epitopes probed with a panel of antibodies against tau (Tau-1, AT8, 12E8, AT180, PHF-1, AT100) were unaltered by incubation with Akt except for Ftau which showed decreases in the PHF-1 immunoreactivity (Fig. 3D). It is still possible that Akt could phosphorylate other sites, which were not tested. The Akt-dependent phosphorylation had no apparent effect on the electrophoretic mobility of tau.

3.4. Comparisons of Akt with GSK3β and PKA

In order to confirm that Akt-dependent phosphorylation of tau was not due to potentially contaminating protein kinases PKA or PKC we used specific and general inhibitors (KT5720, H-7 and staurosporine). As shown in Fig. 4, the inhibitors were without a significant effect on Akt-dependent phosphorylation of tau at the two phospho-epitopes. We concluded that Thr212 and Ser214 were unlikely to be phosphorylated by PKA or PKC potentially contaminating preparations of Akt. We also concluded that the potential role of tau phosphorylation by GSK3β is minimal since Akt is a potent inhibitor of this protein kinase. Moreover, we did

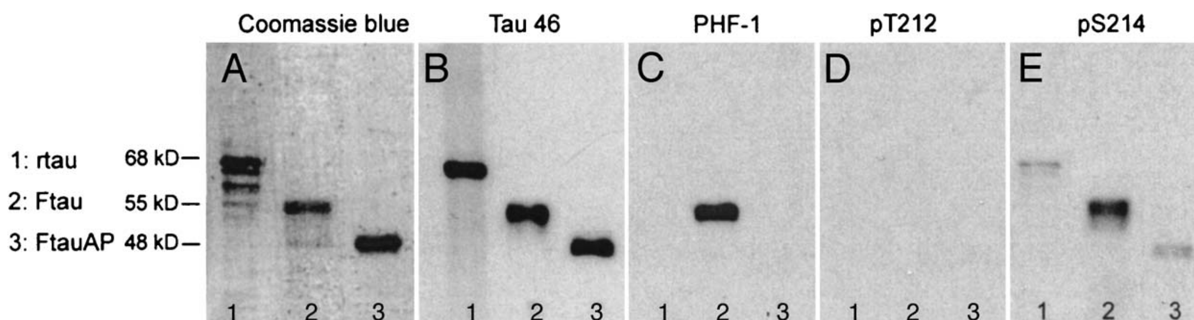

Fig. 2. Characterization of tau preparations. Samples were separated by SDS-gel electrophoresis and either stained with (A) Coomassie blue, or (B–E) immunoblotted with tau antibodies for total tau (Tau 46) and phosphorylated tau epitopes (PHF-1, anti-pThr212, and anti-pSer214). Lane 1, recombinant human tau (rtau); lane 2, fetal tau isolated from human brain (Ftau); and lane 3, fetal tau treated with alkaline phosphatase (FtauAP). Rtau differs from Ftau/FtauAP in molecular size (441 vs. 352 amino acid residues). FtauAP has a faster mobility than Ftau due to dephosphorylation of endogenous sites. Note that anti-pSer214 but not other phospho-tau antibodies cross-react with non-phosphorylated Ser214 in rtau. Weak pSer214 staining of FtauAP may be due to a similar cross-reactivity or a low phosphorylation level at this site. Protein loading was 0.25–0.5 μg for Coomassie blue and 0.1 μg for immunoblots.

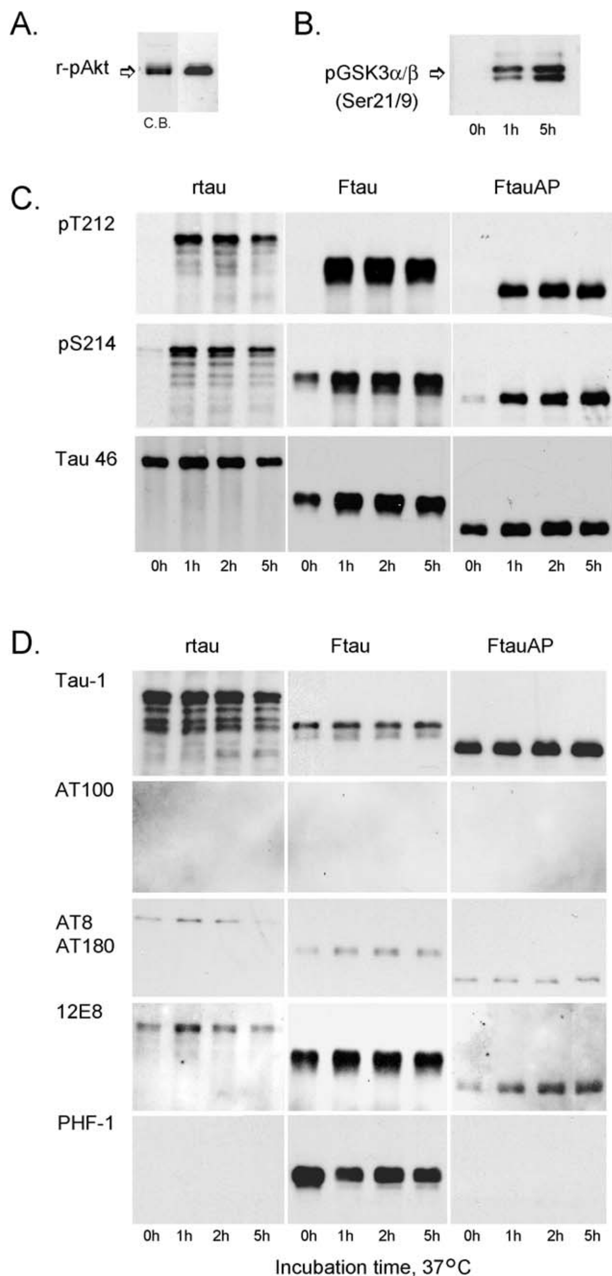

Fig. 3. Akt kinase activity with various substrates. (A) Preparation of active Akt1 (human recombinant, r-pAkt) stained with Coomassie blue (CB) or immunoblotted with the antibody against pSer473-Akt. Loading was 0.5 and 0.05 μg, respectively. (B) GSK3α/β fusion protein incubated with Akt for 0–5 h and immunoblotted for phospho-Ser21/9. (C) Three tau preparations incubated with Akt for 0–5 h and immunoblotted with anti-pThr212, anti-pSer214 and Tau 46 (total tau) antibodies as indicated. Note the time-dependent elevation of phospho-tau immunoreactivity in all preparations. In Ftau, a range of Tau 46-reactive tau species was significantly wider at 1–5 h than 0 h and the immunoreactivity appeared stronger. This could be due to endogenous protease or phosphatase activities generating faster migrating tau species. (D) Samples in C immunoblotted with a panel of phospho-dependent tau antibodies and Tau-1, whose binding is blocked by phosphorylation. No significant increases were noted over the period of 0–5 h incubation, suggesting that Akt was inactive at these sites. In Ftau, decreases in the PHF-1 immunoreactivity during the incubation suggest the presence of phosphatase activities in this preparation.

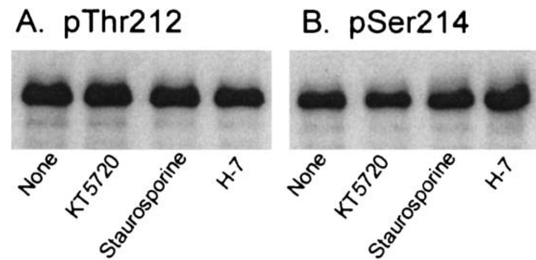

Fig. 4. Akt and protein kinase inhibitors. FtauAP was incubated with Akt for 1 h in the absence (None) or presence of inhibitors KT5720 (3 μM), staurosporine (10 nM), and H-7 (30 μM) as indicated. The immunoreactivity with (A) anti-pThr212 and (B) anti-pSer214 showed no significant effect of these inhibitors on tau phosphorylation.

not detect any pGSK3α/β in Akt preparations by immunoblotting (Fig. 3B, 0 h).

GSK3β and PKA have previously been shown to phosphorylate Thr212 and Ser214 of tau, respectively [10,32,33]. We selected these two enzymes to compare their site specificity with that of Akt. The results obtained using FtauAP showed that both Akt and GSK3β phosphorylated Thr212 in a similar time-dependent manner between 0 and 60 min incubation (Fig. 5A,B). In contrast to Akt, however, the incubation with GSK3β resulted in an upward mobility shift of FtauAP from 48 (arrows) to 55 kDa, reaching that of Ftau. The results indicate that besides Thr212, other epitopes of tau were also phosphorylated by GSK3β as shown earlier [34,35]. This conclusion was confirmed by finding elevated immunoreactivity with PHF-1 in parallel samples (Fig. 5B). Unlike Akt, GSK3β showed negligible phosphorylation of Ser214. Similar studies with PKA revealed phosphorylation of tau at Ser214 but not Thr212 (Fig. 5C), consistent with the previous evidence [33]. PKA caused the upward mobility shift of a fraction of tau protein indicating the involvement of other tau epitopes besides Ser214, as reported earlier [12,32]. The shift in mobility with PKA was less prominent than with GSK3β. These comparisons underline a unique specificity of Akt to phosphorylate both Thr212 and Ser214 of tau. Our results also indicate that anti-pThr212 and anti-pSer214 antibodies bind their specific tau epitopes in a selective manner, without apparent overlap.

3.5. Akt phosphorylates Thr212 and Ser214 separately

It was interesting to note that Akt-dependent phosphorylation of tau at both Thr212 and Ser214 was ineffective in inducing immunoreactivity with AT100 (see Fig. 3D, second row). This was not due to a low concentration of the antibody used since parallel samples of positive control (PHF-tau fraction isolated from AD brain) were found highly immunoreactive (not shown). The negative results with AT100 could be explained if Thr212 and Ser214 were phosphorylated in two separate rather than in a single tau molecules. In order to test this idea, we immunoprecipitated

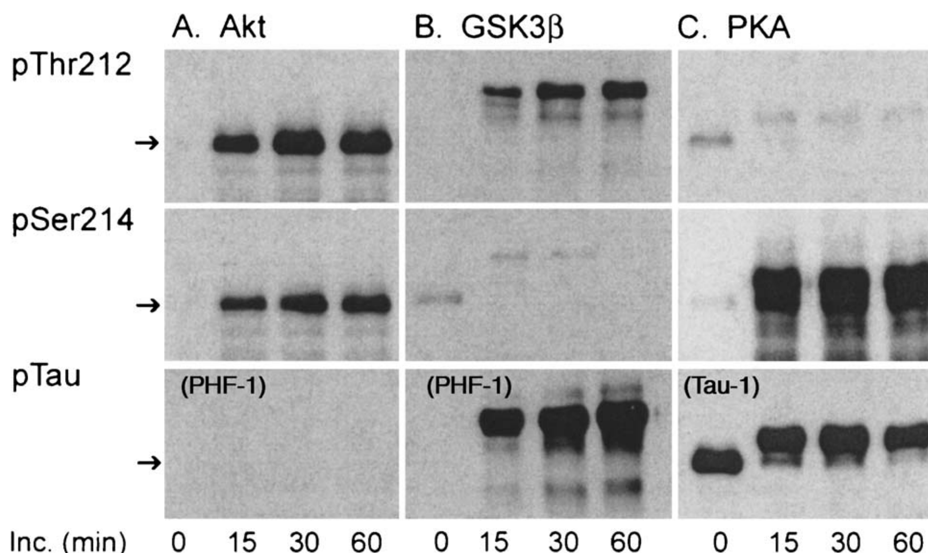

Fig. 5. Comparisons of site-specificity of Akt, GSK3β and PKA. FtauAP was incubated with respective kinases for 0–60 min and immunoblotted with tau antibodies as indicated. (A) Akt, (B) GSK3β, and (C) PKA. Note a differential phosphorylation of Thr212 and Ser214 by GSK3β and PKA but not Akt. Except for Akt, other kinases resulted in upward mobility shift of tau from 43 kDa to 55 kDa (GSK3β) or 52 kDa (PKA). In C, in order to demonstrate PKA-induced shift in the mobility of tau, blots were developed with Tau 1 (FtauAP-positive) instead of PHF-1 (FtauAP-negative). Arrows indicate the initial FtauAP mobility at 48 kDa.

Akt-phosphorylated tau protein using anti-pThr212 or anti-pSer214 antibodies. The resulting immuno-pellets and -supernatants were tested for the presence of tau protein with specific phospho-epitopes. The results showed that tau fraction could indeed be separated into two distinct pools, each containing tau with a single phospho-epitope, either pThr212-tau or pSer214-tau. For example, immunoprecipitation with anti-pThr212 resulted in the immunoprecipitate positive for pThr212 but not pSer214 (Fig. 6A, pellet). Moreover, tau remaining in the supernatant was immunore-

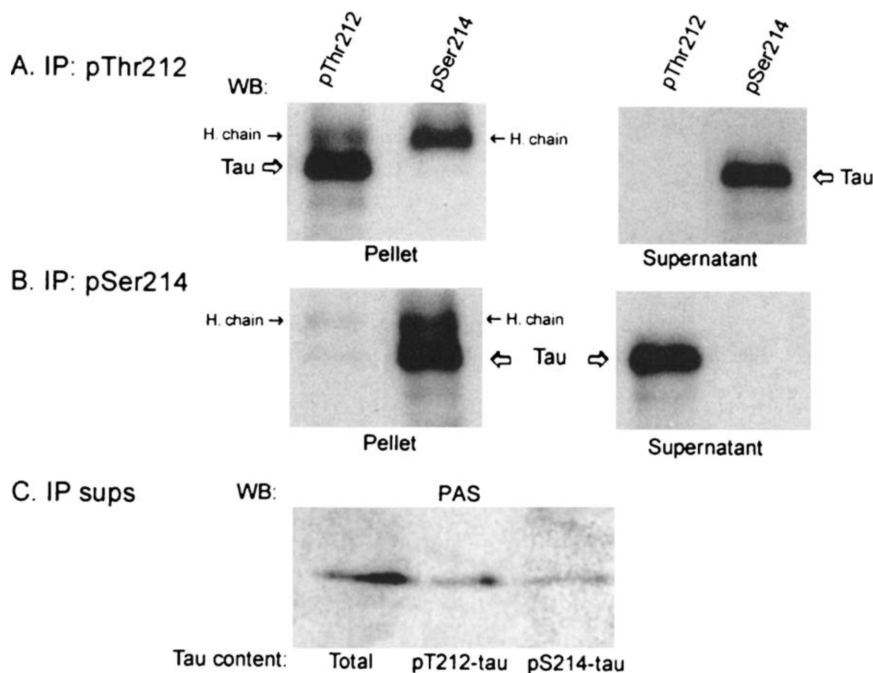

Fig. 6. Immunoprecipitation of distinct species of phospho-tau. FtauAP incubated with Akt for 1 h and immunoprecipitated with (A) anti-pThr212 or (B) anti-pSer214 antibodies. Immunopellets and supernatants demonstrate distinct pools of tau enriched either for pThr212- or pSer214-tau. Note the complementary pattern of immunoreactivity between pellet and supernatant fractions in A and B. Black arrows mark the position of immunoglobulin heavy chain (H. chain) detected only in pellets. (C) The total and supernatant fractions from A and B immunoblotted with an antibody against a generic phospho-Akt substrate (PAS). Note that both pools of tau bind the PAS antibody.

active with anti-pSer214 but not anti-pThr212 (Fig. 6A, supernatant). A complementary pattern of immunoreactivity was obtained using an immunoprecipitation assay with anti-pSer214 (Fig. 6B). These results indicate that Akt phosphorylates two sites of tau Thr212 and Ser214, but each site is on a different tau molecule. Therefore, only one site, either Thr212 or Ser214 can be phosphorylated in a single tau molecule.

It was unclear which of the two sites was the preferred phosphorylation site for Akt. To determine the ratio between two pools of phospho-tau, we quantified the total tau immunoreactivity (Tau 46) in the corresponding immunoprecipitates and supernatants. The obtained ratio between the pThr212- and pSer214-enriched pools of tau was 4.3:1 ($n=8$), indicating that Thr212 was the preferred phosphorylation site for Akt.

3.6. Akt-phosphorylated tau binds PAS antibody

To further confirm that both sites of tau Thr212 and Ser212 can be recognized as potential substrates for Akt, we used the antibody PAS. This antibody has been raised against a generic phospho-Akt substrate peptide and shown to preferentially bind proteins containing phospho-Thr/Ser preceded by Lys/Arg at positions -5 and -3. The pThr212- and pSer214-enriched pools of tau were obtained by immunoprecipitation and collection of supernatants (see also Fig. 6A,B, IP supernatants) and analyzed by Western blotting with PAS. The results showed that both pools of tau bound PAS and therefore could be recognized as generic Akt substrates (Fig. 6C). The PAS binding occurred despite the fact that only Ser214 was preceded by Arg residues required at positions -5 and -3, whereas Thr212 was preceded by Arg at positions -1 and -3. This finding additionally supports our conclusion based on direct Akt phosphorylation studies that both sites in the AT100 epitope are recognized by Akt and may serve as phosphate acceptors.

3.7. Akt blocks PKA- but not GSK3β-dependent phosphorylation of tau

Our present studies (Fig. 5) and previous reports indicate that both GSK3β and PKA phosphorylate multiple sites in tau and cause a significant upward shift in the electrophoretic mobility of this protein. Since Akt was able to phosphorylate only selected sites recognized by GSK3β and PKA, we asked whether prior Akt activity could block tau from further interactions with these kinases and prevent changes in tau mobility. To address this question, we first incubated Akt with FtauAP for 1 h to obtain a mixture of pThr212-tau and pSer214-tau and then separated individual species of phospho-tau by immunoprecipitation as described in Fig. 6. The supernatant enriched in pThr212-tau was incubated with GSK3β and this treatment caused the upward mobility shift of tau (Fig. 7A, lane 2). The supernatant

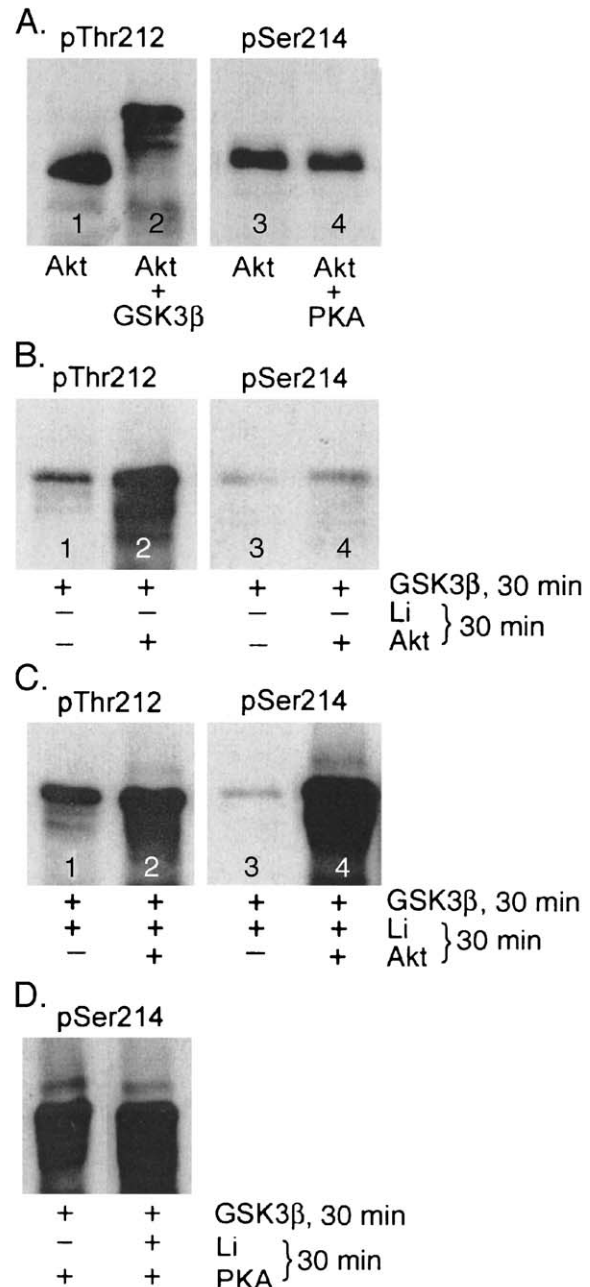

Fig. 7. Mixed kinases. (A) FtauAP incubated with Akt for 1 h and immunoprecipitated to obtain supernatants enriched in pThr212-tau (left panel) or pSer214-tau (right panel). The supernatants incubated for an additional 30 min with none (lanes 1 and 3), GSK3β (lane 2) or PKA (lane 4). Note that GSK3β but not PKA elevated tau immunoreactivity and caused a shift in the mobility of tau. (B) FtauAP incubated with GSK3β for 30 min and then supplemented with either no (lanes 1 and 3) or Akt alone (no lithium) for another 30-min incubation (lanes 2 and 4). Note that Akt was active with Thr212 but inactive with Ser214. (C) FtauAP incubated with GSK3β for 30 min and then supplemented with 50 mM lithium (lanes 1 and 3) or Akt plus 50 mM lithium for another 30-min incubation (lanes 2 and 4). Note that Akt was active with both Thr212 and Ser214 in the presence of lithium. (D) FtauAP incubated with GSK3β for 30 min and then supplemented with PKA alone (lane 1) or PKA plus 50 mM lithium (lane 2) for another 30-min incubation. Note the robust PKA activity under both conditions.

enriched in pSer214-tau was incubated with PKA but the mobility shift of tau was not detected (Fig. 7A, lane 4). The results indicated that prior phosphorylation of tau by Akt had no apparent inhibitory effect on further interactions of tau with GSK3β. Prior phosphorylation by Akt, however, could block further interactions of tau with PKA, as judged by tau mobility.

3.8. GSK3β blocks Akt- but not PKA-dependent phosphorylation of tau

Next, we asked whether prior phosphorylation by GSK3β precluded interactions of tau with Akt. In these experiments, GSK3β was incubated with FtauAP for 30 min, then the reaction mixture was supplemented either with buffer or Akt and incubated for another 30 min. Immunoblotting analysis of the final reaction products showed that GSK3β alone phosphorylated Thr212 but very little of Ser214 (Fig. 7B, lanes 1, 3), as expected. Incubation with both GSK3β and Akt improved phosphorylation of Thr212 but largely inhibited phosphorylation of Ser214 by Akt (Fig. 7B, lanes 2, 4). These results suggest that GSK3β selectively prevented Akt from phosphorylating Ser214 but not Thr212.

We asked whether inhibition of GSK3β by lithium could enable Akt to phosphorylate Ser214. These experiments were performed as above, except that the second 30-min incubation was carried out either in the presence of 50 mM lithium (Fig. 7C, lanes 1, 3) or Akt and 50 mM lithium (Fig. 7C, lanes 2, 4). The immunoblotting analysis showed that the presence of lithium had little stimulatory effect on phosphorylation of Thr212 by Akt (Fig. 7C, lane 2) but dramatically improved phosphorylation of Ser214 (Fig. 7C, lane 4).

Parallel studies with GSK3β and PKA determined that prior incubation of tau with GSK3β had no inhibitory effect on PKA activity with tau and phosphorylation of Ser214, regardless of whether lithium was present or not (Fig. 7D).

4. Discussion

The results of the present studies demonstrate that Akt incorporates phosphate groups in two sites of tau protein in vitro. The phosphorylation of tau is independent of the size of the tau molecule as determined using the longest and the shortest tau isoforms. It is also independent of prior (endogenous) phosphorylation of tau. One site phosphorylated by Akt is Ser214, which fulfills the stringent requirement for Arg residues at positions n-3 and n-5. In addition, Akt also phosphorylates Thr212, which conforms less rigorously to this requirement, with Arg residues at positions n-1 and n-3. Moreover, Thr212 is phosphorylated 4.3-times more efficiently than Ser214 and binds equally well to antibody raised against a generic phospho-substrate of Akt. Further, Akt-dependent phosphorylation of tau has an unusual feature in that only a single site, either Thr212 or Ser214, can be phosphorylated per tau molecule. This is not due to potential contaminating kinases since PKA, PKC and GSK3β inhibitors were ineffective in blocking Akt kinase activity. The results of the present studies strongly suggest that phosphorylation of tau by Akt has a specific physiological role(s).

The putative role of Akt-dependent tau phosphorylation could be related to tau function as a microtubule-associated protein. The phosphorylation of Ser214 or pseudophosphorylation by conversion of Ser214 to Asp have been demonstrated to potently inhibit tau binding to microtubules and suppress tau ability to promote microtubule assembly [12] or microtubule nucleation [36]. Moreover, the phosphorylation of Ser214 has been detected in metaphase but not in interphase of tau-transfected CHO cells, suggesting its potential role in cell cycle activities such as detachment of tau from mitotic microtubules.

The important role of Ser214 is underlined by the fact that at least two kinases, including Akt and PKA, are responsible for the site-specific phosphorylation. For PKA activity, Ser214 appears to be a primary target. Our observations extend this idea by showing that prior phosphorylation of Ser214 by Akt disrupts or inhibits PKA activity towards tau (Fig. 7A). This is particularly evident in comparisons to GSK3β, which has no apparent inhibitory effect on PKA activity (Fig. 7D). Our results suggest that there may be a relationship (competition) between Akt and PKA in regulating tau interactions with microtubules and that Ser214 controls these interactions. Alternatively, phosphorylation of Ser214 may be a part of the redundant mechanism controlling microtubule binding. This mechanism appears to be activated in dividing cells since a significant upregulation of pSer214-tau has been demonstrated in mitotic cells but not in interphase [12]. Similar mechanism controlling microtubule binding via pSer214 and Akt may play a role in regulation of synaptic strength [37]. It is unclear whether Ser214 plays other roles, e.g., in cell apoptosis.

Phosphorylation of Ser214 has been implied to play a protective role in the formation of abnormal tau aggregates in neurodegenerative disorders [13]. The fact that Akt may be involved in this protection suggests that tau is a candidate factor in PI3K/Akt anti-apoptotic signaling. It also suggests that phosphorylation of tau may play a protective role in cell survival. Such a role has been postulated with regard to cultured neurons [38] and neurofibrillary tangle/PHF formation in AD brain [39,40]. It is conceivable that phosphorylation of Ser214 may facilitate microtubule-based trafficking of organelles by causing detachment of tau from microtubules [5]. This could be necessary for neuronal survival. On the other hand, other studies applying a pseudohyperphosphorylation model of AD in cultured cells indicate that phosphorylation of tau may impart toxic gain of function and contribute to neuronal death [41]. Further studies need to resolve the important issue of the role of tau phosphorylation in neurodegeneration.

Recently, the expression of active Akt has been demonstrated in AD brain [42]. The expression followed the pattern of localization in neurons consistent with the early events in the pathogenesis of neurofibrillary changes. Such studies provide independent evidence supporting the view that Akt could be involved in tau metabolism in vivo and play a protective role in cellular responses in neurodegeneration [43].

In comparison to Ser214, Thr212 appears to have a less significant role in regulating binding to microtubules. In this respect, Thr212 is similar to other Thr–Pro and Ser–Pro sites, targets of proline-directed kinases such as GSK3β [4]. It is conceivable that phosphorylation of Thr212 plays other roles, e.g., modulating phosphorylation of Ser214. Further studies need to determine the role of the Akt-dependent phosphorylation of Thr212.

It was interesting to note that GSK3β effectively blocked interactions of Akt with Ser214, the site not phosphorylated by GSK3β. One explanation is that GSK3β activity could change the conformation of tau and block access to Ser214. This suggestion is less likely, however, since Akt is active towards GSK3β-phosphorylated tau in the presence of lithium (Fig. 7C) and towards endogenously phosphorylated tau as well (Fig. 5A). Alternatively, this inhibition could be due to the presence of GSK3β itself since 50 mM lithium alone was able to reverse the inhibition. For example, GSK3β could sequester tau as demonstrated for non-phosphorylated tau [44] or it could sequester Akt. It is conceivable that lithium binding by GSK3β may release the sequestered proteins and reverse the inhibition. Which of these interpretations is correct will require further studies.

Our results indicate that Akt introduces only one phospho-residue per tau molecule in vitro and thus may generate one part of the double phospho-epitope AT100, either pThr212 or pSer214. Is unclear which other kinase(s) could contribute to the complementary phosphorylation. Our results suggest that the GSK3β-Akt combination may be effective (in the presence of lithium) as well as the Akt–PKA combination under specific conditions. Further examinations need to consider also other kinases not tested in the present studies, e.g., cdk5 or cdc2.

In summary, the ability of Akt to phosphorylate tau not only suggests that tau is a candidate factor in the PI3K/Akt signaling pathway but also supports the recent view that changes in phosphorylation of tau represent an integral part of the normal signal-processing machinery [45,46].

Acknowledgements

The authors thank Drs. Gloria Lee and Michel Goedert for their generous gift of tau clones, Peter Seubert from Athena Neurosciences (San Francisco, CA) for providing 12E8 antibody, Peter Davies for providing antibody PHF-1, A. Van de Voorde and E. Vanmechelen from Innogenetics (Ghent, Belgium) for providing a panel of antibodies from the AT series, AT8, AT100, and AT180, and Michael J. Reding for critically reading the manuscript. This work was supported by the Alzheimer's Association (Zenith Award) and the Society for Progressive Supranuclear Palsy (Grant No. 411) to HKR.

References

[1] M. Goedert, M.G. Spillantini, N.J. Cairns, R.A. Crowther, Tau proteins of Alzheimer paired helical filaments: abnormal phosphorylation of all six brain isoforms, Neuron 8 (1992) 159–168.

[2] B.L. Goode, S.C. Feinstein, Identification of a novel microtubule binding and assembly domain in the developmentally regulated inter-repeat region of tau, J. Cell Biol. 124 (1994) 769–782.

[3] Y. Morita-Fujimura, M. Kurachi, H. Tashiro, Y. Komiya, T. Tashiro, Reduced microtubule-nucleation activity of tau after dephosphorylation, Biochem. Biophys. Res. Commun. 225 (1996) 462–468.

[4] J. Biernat, E.M. Mandelkow, The development of cell processes induced by tau protein requires phosphorylation of serine 262 and 356 in the repeat domain and is inhibited by phosphorylation in the proline-rich domains, Mol. Biol. Cell 10 (1999) 727–740.

[5] K. Stamer, R. Vogel, E. Thies, E. Mandelkow, E.M. Mandelkow, Tau blocks traffic of organelles, neurofilaments, and APP vesicles in neurons and enhances oxidative stress, J. Cell Biol. 156 (2002) 1051–1063.

[6] G. Lee, S.T. Newman, D.L. Gard, H. Band, G. Panchamoorthy, Tau interacts with src-family non-receptor tyrosine kinases, J. Cell. Sci. 111 (1998) 3167–3177.

[7] D.W. Dickson, W.-K. Liu, H. Ksiezak-Reding, S.H. Yen, Corticobasal degeneration: neuropathologic and molecular considerations, in: I. Litvan, C. Goetz, A. Lang (Eds.), Corticobasal Degeneration, Adv. Neurol., vol. 82, Lippincott-Raven Publishers, Philadelphia, 2000, pp. 9–27.

[8] M. Goedert, Neurofibrillary pathology of Alzheimer's disease and other tauopathies [Review], Prog. Brain Res. 117 (1998) 287–306.

[9] E.S. Matsuo, R.-W. Shin, M.L. Billingsley, A. Van deVoorde, M. O'Connor, J.Q. Trojanowski, V.M.-Y. Lee, Biopsy-derived adult human brain tau is phosphorylated at many of the same sites as Alzheimer's disease paired helical filament tau, Neuron 13 (1994) 989–1002.

[10] Q. Zheng-Fischhofer, J. Biernat, E.M. Mandelkow, S. Illenberger, R. Godemann, E. Mandelkow, Sequential phosphorylation of Tau by glycogen synthase kinase-3beta and protein kinase A at Thr212 and Ser214 generates the Alzheimer-specific epitope of antibody AT100 and requires a paired-helical-filament-like conformation, Eur. J. Biochem. 252 (1998) 542–552.

[11] G. Drewes, A. Ebneth, E.M. Mandelkow, MAPs, MARKs and microtubule dynamics, Trends Biochem. Sci. 23 (1998) 307–311(Review).

[12] S. Illenberger, Q. Zheng-Fischhofer, U. Preuss, K. Stamer, K. Baumann, B. Trinczek, J. Biernat, R. Godemann, E.M. Mandelkow, E. Mandelkow, The endogenous and cell cycle-dependent phosphorylation of tau protein in living cells: implications for Alzheimer's disease, Mol. Biol. Cell 9 (1998) 1495–1512.

[13] A. Schneider, J. Biernat, M. von Bergen, E. Mandelkow, E.M. Mandelkow, Phosphorylation that detaches tau protein from microtubules (Ser262, Ser214) also protects it against aggregation into Alzheimer paired helical filaments, Biochemistry 38 (1999) 3549–3558.

[14] D.R. Alessi, F.B. Caudwell, M. Andjelkovic, B.A. Hemmings, P. Cohen, Molecular basis for the substrate specificity of protein kinase B; comparison with MAPKAP kinase-1 and p70 S6 kinase, FEBS Lett. 399 (1996) 333–338.

[15] M. Andjelkovic, T. Jakubowicz, P. Cron, X.F. Ming, J.W. Han, B.A. Hemmings, Activation and phosphorylation of a pleckstrin homology domain containing protein kinase (RAC-PK/Akt) promoted by serum

and protein phosphatase inhibitors, Proc. Natl. Acad. Sci. U. S. A. 93 (1996) 5699–5704.
[16] P.J. Coffer, J. Jin, J.R. Woodgett, Protein kinase B (c-Akt): a multifunctional mediator of phosphatidylinositol 3-kinase activation [Review], Biochem. J. 335 (1998) 1–13.
[17] T.F. Franke, S.I. Yang, T.O. Chan, K. Datta, A. Kazlauskas, D.K. Morrison, D.R. Kaplan, P.N. Tsichlis, The protein kinase encoded by the Akt proto-oncogene is a target of the PDGF-activated phosphatidylinositol 3-kinase, Cell 81 (1995) 727–736.
[18] B.A. Hemmings, Akt signaling: linking membrane events to life and death decisions, Science 275 (1997) 628–630.
[19] K. Fukunaga, T. Kawano, Akt is a molecular target for signal transduction therapy in brain ischemic insult [Review], J. Pharmacol. Sci. 92 (2003) 317–327.
[20] R. Dajani, E. Fraser, S.M. Roe, N. Young, V. Good, T.C. Dale, L.H. Pearl, Crystal structure of glycogen synthase kinase 3 beta: structural basis for phosphate-primed substrate specificity and autoinhibition, Cell 105 (2001) 721–732.
[21] S.G. Kennedy, E.S. Kandel, T.K. Cross, N. Hay, Akt/protein kinase B inhibits cell death by preventing the release of cytochrome *c* from mitochondria, Mol. Cell. Biol. 19 (1999) 5800–5810.
[22] K. Gottlob, N. Majewski, S. Kennedy, E. Kandel, R.B. Robey, N. Hay, Inhibition of early apoptotic events by Akt/PKB is dependent on the first committed step of glycolysis and mitochondrial hexokinase, Genes Dev. 15 (2001) 1406–1418.
[23] R.A. Crowther, O.F. Olesen, M.J. Smith, R. Jakes, M. Goedert, Assembly of Alzheimer-like filaments from full-length tau protein, FEBS Lett. 337 (1994) 135–138.
[24] H. Ksiezak-Reding, G. Yang, M. Simon, J.S. Wall, Assembled tau filaments differ from native paired helical filaments as determined by scanning transmission electron microscopy (STEM), Brain Res. 814 (1998) 86–98.
[25] W. Gordon-Krajcer, L.S. Yang, H. Ksiezak-Reding, Conformation of paired helical filaments blocks dephosphorylation of epitopes shared with fetal tau except Ser199/202 and Ser202/Thr205, Brain Res. 856 (2000) 163–175.
[26] D. Fabbro, D. Batt, P. Rose, B. Schacher, T.M. Roberts, S. Ferrari, Homogeneous purification of human recombinant GST-Akt/PKB from Sf9 cells, Protein Expr. Purif. 17 (1999) 83–88.
[27] C.C. Kumar, R. Diao, Z. Yin, Y. Liu, A.A. Samatar, V. Madison, L. Xiao, Expression, purification, characterization and homology modeling of active Akt/PKB, a key enzyme involved in cell survival signaling, Biochim. Biophys. Acta 1526 (2001) 257–268.
[28] D.M. Gadbois, H.A. Crissman, R.A. Tobey, E.M. Bradbury, Multiple kinase arrest points in the G1 phase of nontransformed mammalian cells are absent in transformed cells, Proc. Natl. Acad. Sci. U. S. A. 89 (1992) 8626–8630.
[29] V. Stambolic, L. Ruel, J.R. Woodgett, Lithium inhibits glycogen synthase kinase-3 activity and mimics wingless signalling in intact cells, Curr. Biol. 6 (1996) 1664–1668.
[30] H. Ksiezak-Reding, D. He, W. Gordon-Krajcer, Y. Kress, S. Lee, D.W. Dickson, Induction of Alzheimer-specific tau epitope AT100 in apoptotic human fetal astrocytes, Cell Motil. Cytoskel. 47 (2000) 236–252.
[31] A. Kenessey, S.H. Yen, The extent of phosphorylation of fetal tau is comparable to that of PHF-tau from Alzheimer paired helical filaments, Brain Res. 629 (1993) 40–46.
[32] C.W. Scott, R.C. Spreen, J.L. Herman, F.P. Chow, M.D. Davison, J. Young, C.B. Caputo, Phosphorylation of recombinant tau by cAMP-dependent protein kinase. Identification of phosphorylation sites and effect on microtubule assembly, J. Biol. Chem. 268 (1993) 1166–1173.
[33] G.A. Jicha, C. Weaver, E. Lane, C. Vianna, Y. Kress, J. Rockwood, P. Davies, cAMP-dependent protein kinase phosphorylation on tau in Alzheimer's disease, J. Neurosci. 19 (1999) 7486–7494.
[34] D.P. Hanger, K. Hughes, J.R. Woodgett, J.P. Brion, B.H. Anderton, Glycogen synthase kinase-3 induces Alzheimer's disease-like phosphorylation of tau: generation of paired helical filament epitopes and neuronal localisation of the kinase, Neurosci. Lett. 147 (1992) 58–62.
[35] J.S. Song, S.D. Yang, Tau protein kinase I/GSK-3 beta/kinase FA in heparin phosphorylates tau on Ser199, Thr231, Ser235, Ser262, Ser369, and Ser400 sites phosphorylated in Alzheimer disease brain, J. Protein Chem. 14 (1995) 95–105.
[36] J. Leger, M. Kempf, G. Lee, R. Brandt, Conversion of serine to aspartate imitates phosphorylation-induced changes in the structure and function of microtubule-associated protein tau, J. Biol. Chem. 272 (1997) 8441–8446.
[37] Q. Wang, L. Liu, L. Pei, W. Ju, G. Ahmadian, J. Lu, Y. Wang, F. Liu, Y.T. Wang, Control of synaptic strength, a novel function of Akt, Neuron 38 (2003) 915–928.
[38] M. Lesort, C. Blanchard, C. Yardin, F. Esclaire, J. Hugon, Cultured neurons expressing phosphorylated tau are more resistant to apoptosis induced by NMDA or serum deprivation, Brain Res. Mol. Brain Res. 45 (1997) 127–132.
[39] T. Gomez-Isla, R. Hollister, H. West, S. Mui, J.H. Growdon, R.C. Petersen, J.E. Parisi, B.T. Hyman, Neuronal loss correlates with but exceeds neurofibrillary tangles in Alzheimer's disease, Ann. Neurol. 41 (1997) 17–24.
[40] R. Morsch, W. Simon, P.D. Coleman, Neurons may live for decades with neurofibrillary tangles, J. Neuropathol. Exp. Neurol. 58 (1999) 188–197.
[41] T. Fath, J. Eidenmuller, R. Brandt, Tau-mediated cytotoxicity in a pseudohyperphosphorylation model of Alzheimer's disease, J. Neurosci. 22 (2002) 9733–9741.
[42] J.J. Pei, S. Khatoon, W.L. An, M. Norlinder, T. Tanaka, H. Braak, I. Tsujio, M. Takeda, I. Alafuzoff, B. Winblad, R.F. Cowburn, I. Grundke-Iqbal, K. Iqbal, Role of protein kinase B in Alzheimer's neurofibrillary pathology, Acta Neuropathol. 105 (2003) 381–392.
[43] T.D. Stein, J.A. Johnson, Lack of neurodegeneration in transgenic mice overexpressing mutant amyloid precursor protein is associated with increased levels of transthyretin and the activation of cell survival pathways, J. Neurosci. 22 (2002) 7380–7388.
[44] W. Sun, H.Y. Qureshi, P.W. Cafferty, K. Sobue, A. Agarwal-Mawal, K.D. Neufield, H.K. Paudel, Glycogen synthase kinase-3beta is complexed with tau protein in brain microtubules, J. Biol. Chem. 277 (2002) 11933–11940.
[45] E. Planel, K. Yasutake, S.C. Fujita, K. Ishiguro, Inhibition of protein phosphatase 2A overrides tau protein kinase I/glycogen synthase kinase 3 beta and cyclin-dependent kinase 5 inhibition and results in tau hyperphosphorylation in the hippocampus of starved mouse, J. Biol. Chem. 276 (2001) 34298–34306.
[46] Y. Okawa, K. Ishiguro, S.C. Fujita, Stress-induced hyperphosphorylation of tau in the mouse brain, FEBS Lett. 535 (2003) 183–189.