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τ is phosphorylated by GSK-3 at several sites found in Alzheimer disease and its biological activity markedly inhibited only after it is prephosphorylated by A-kinase

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Abstract Alzheimer disease is characterized by a specific type of neuronal degeneration in which the microtubule associated protein τ is abnormally hyperphosphorylated causing the disruption of the microtubule network. We have found that the phosphorylation of human τ (τ3L) by A-kinase, GSK-3 or CK-1 inhibits its microtubule assembly-promoting and microtubule-binding activities. However, the inhibition of these activities of τ by GSK-3 is significantly increased if τ is prephosphorylated by A-kinase or CK-1. The most potent inhibition is observed by combination phosphorylation of τ with A-kinase and GSK-3. Under these conditions, only very few microtubules are seen by electron microscopy. Sequencing of ^{32}P-labeled trypsin phosphopeptides from τ prephosphorylated by A-kinase (using unlabeled ATP) and further phosphorylated by GSK-3 in the presence of [γ-^{32}P]ATP revealed that Ser-195, Ser-198, Ser-199, Ser-202, Thr-205, Thr-231, Ser-235, Ser-262, Ser-356 and Ser-404 are phosphorylated, whereas if τ is not prephosphorylated by A-kinase, GSK-3 phosphorylates it at Thr-181, Ser-184, Ser-262, Ser-356 and Ser-400. These data suggest that (i) prephosphorylation of τ by A-kinase makes additional and different sites accessible for phosphorylation by GSK-3; (ii) phosphorylation of τ at these additional sites further inhibits the biological activity of τ in its ability to bind to microtubules and promote microtubule assembly. Thus a combined role of A-kinase and GSK-3 should be considered in Alzheimer neurofibrillary degeneration.

Key words: Alzheimer disease; Tau phosphorylation; Microtubule assembly; Glycogen synthase kinase-3; Protein kinase A; Microtubule associated protein tau-1

1. Introduction

Alzheimer disease is characterized histopathologically by the presence of intraneuronal neurofibrillary tangles and the extracellular deposition of β-amyloid in the cerebral cortex, especially the hippocampus. The neurofibrillary tangles are made up of paired helical filaments (PHFs), the major protein subunit of which is the abnormally phosphorylated and glycosylated microtubule associated protein τ [1–3]. Two functions have been described for τ based on its ability to interact with tubulin: the microtubule assembly promoting activity in vitro [4,5] and the microtubule stabilizing activity in vivo [6]. Phosphorylation of τ depresses its ability to promote the in vitro assembly of microtubules [7]. Alzheimer abnormally phosphorylated τ is not only microtubule assembly incompetent [8–10], but also inhibits assembly and disassembles the preassembled microtubules in vitro [11–13]. In the tangle-bearing neurons in Alzheimer disease brain, the normal cytoskeleton is disrupted and replaced with PHFs. Thus it is likely that the abnormal hyperphosphorylation of τ in Alzheimer disease brain leads to the depolymerization of microtubules, impaired axonal transport and neuronal degeneration [13].

The protein kinases involved and the phosphorylation sites responsible for this malfunction of τ are currently not understood. Phosphorylation at Ser-262 is reported to cause the decreased microtubule binding of τ [14,15]. However, phosphorylation of Ser-262 only induces about 40% inhibition in microtubule binding activity [16], suggesting that phosphorylation of other sites is necessary to completely inhibit its biological activity. The effect of these phosphorylations on the biological activity of τ and the sites phosphorylated are, however, not known.

Twenty-one phosphorylation sites in PHF-τ have been identified using reactivity with antibodies to various phosphorylation sites and protein sequencing techniques. Among them, 10 sites are on Ser/Thr-Pro motifs and 11 are on non-Ser/Thr-Pro motifs (see [17,18]). The Ser/Thr-Pro and non-Ser/Thr-Pro sites are probably phosphorylated by proline-dependent protein kinases (PDPKs) and non-PDPKs, respectively. The various PDPKs and non-PDPKs that can phosphorylate τ include MAP kinase [19,20] cdc-2 kinase [21], cdk-2, cdk-5 [22] and GSK-3 [23–25]. The non-PDPKs include A-kinase [26–28], C-kinase [27–30], CaM kinase II [27,28,31], CK-1 [27,28] and CK-2 [27,32]. The interaction of PDPK and non-PDPK in τ hyperphosphorylation has been reported [28,33]. However, the effects of these combination phosphorylations on the biological activity of τ and the sites phosphorylated were not mapped.

In the present study, we have investigated the effect of phosphorylation of τ by individual kinase or combination of kinases on the biological activity of τ and mapped the corresponding sites phosphorylated by these kinases.

2. Materials and methods

2.1. Isolation of τ, CK-1, GSK-3 and protein determination

Human τ3L (cDNA kindly provided by M. Goedert), that has three tandem C-terminal repeats plus two 29-amino acid N-terminal inserts was expressed in *E. coli* and the protein isolated from the bacterial lysate as described previously [16]. CK-1 [34] and GSK-3 [35] were purified from bovine brain as previously described. The GSK-3 prep-

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aration contained both the α and β isoforms in the ratio 1:3. The catalytic subunit of A-kinase was purchased from Sigma Chemical (St. Louis, MO, USA). One unit of either CK-1 or GSK-3 is defined as the amount of kinase required to catalyze the incorporation of 1 nmol ^{32}P per min at 30°C into dephosphocasein (4 mg/ml) or myelin basic protein (1 mg/ml), respectively. Protein was estimated by the modified Lowry method [36].

2.2. Phosphorylation of τ

Human τ3L was separately incubated in the absence or presence of CK-1 (400 munits/ml), A-kinase (6 μg/ml) and GSK-3 (350 munits/ml) in a reaction mixture containing 1 mg/ml of τ, 7 mM $MgCl_2$, 12 mM 2-mercaptoethanol, 0.5 mM ATP, 20 mM HEPES (pH 7.5). Reaction was initiated by the addition of kinase. After incubation at 30°C for 2 h, the reaction was stopped by heating at 95°C for 5 min and denatured kinase removed by centrifugation (10 000×*g* for 10 min). The phosphorylated heat-stable τ was also further phosphorylated with GSK-3 for 4 h at 30°C. In order to determine the stoichiometry of phosphorylation in parallel τ was phosphorylated using identical conditions as above except [γ-^{32}P]ATP.

2.3. Preparation of ^{32}P-labeled tryptic phosphopeptides

τ (300 μg) was phosphorylated as described above by A-kinase and non-radioactive ATP followed by GSK-3 and [γ-^{32}P]ATP (2000 cpm/pmol). In parallel τ incubated without any kinase and heat treated was then phosphorylated by GSK-3 as above. τ was then precipitated with 30% cold trichloroacetic acid and collected by centrifugation at 15 000×*g* at 4°C for 5 min. The pellets were washed twice with 0.5 ml of 20% trichloroacetic acid and twice with 0.5 ml 80% acetone. The washed pellets were resuspended in 0.15 ml of 50 mM ammonium bicarbonate buffer, pH 8.0, containing 250 μg TPCK-treated trypsin and incubated at 37°C for 12 h. Additional aliquots of trypsin (20 μg each) were added to the reaction mixture at 12 h and 24 h. At 36 h a final 10 μg trypsin was added and the incubation continued for another 4 h. The trypsin digests of [^{32}P]-τ were diluted with 3 ml buffer A (0.1 M acetic acid/NaOH, pH 3.1) and then applied to the Fe^{3+} affinity column prepared according to Andersson and Porath [37]. A 1-ml bed column volume of Chelating Sepharose Fast Flow (Pharmacia Biotech) was washed with five column volumes of distilled water and charged with five column volumes of 50 mM $FeCl_3$ solution. The column was then equilibrated with five column volumes of buffer A. After absorption of the sample, the column was washed with buffer A, and consecutively eluted with 0.1 M sodium acetate (pH 5.0), 1% ammonium acetate (pH 6.3), 1% ammonium acetate (pH 7.2), 1% ammonium acetate (pH 8.5) and 0.2 M EDTA (pH 8.0), respectively. The fractions eluted at pH 8.5 were pooled and concentrated by a Speed-Vac concentrator (Savant). The phosphopeptides were resuspended in 0.15 ml of deionized water, followed by centrifugation at 16 000×*g* in ultra free Millipore tubes containing a filter that retained peptides of $M_r > 10\,000$. The peptides in the filtrate were then loaded onto a reverse-phase C_{18} HPLC column (0.46×25 cm) and eluted with a linear gradient of 0–35% acetonitrile (0.5% acetonitrile/min) in 0.1% trifluoroacetic acid (TFA) using Waters HPLC system. The flow rate was 1 ml/min and 0.3-ml fractions were collected. ^{32}P-labeled peaks were detected by counting (Cerenkov cpm) aliquots from fractions in a liquid scintillation spectrophotometer.

2.4. Amino acid sequence analysis and mapping of phosphorylation sites

Radio-sequencing of ^{32}P-labeled phosphopeptides was performed by sequential manual Edman degradation as essentially described by Laursen and Machleidt [38]. The ^{32}P-peptide fractions eluted from RPLC were resuspended in 50% acetonitrile and loaded on Sequelon AA Disk (Sequelon AA Reagent Kit, GEN920033, Millipore). The N-terminal amino acid was derivatized with PITC and cleaved with TFA. The ^{32}P radioactivity released from each cycle of the Edman degradation was determined by Cerenkov counting. Aliquots of the same phosphopeptides were subjected to amino acid sequencing by an automated gas-phase sequencer as described previously [39]. The phosphorylated sites were then identified by matching the ^{32}P peaks from manual radio-sequencing with the amino acid sequences determined by automated gas-phase sequencing.

2.5. Phosphoamino acid analysis

The phosphopeptides isolated from tryptic digests of ^{32}P-τ protein were hydrolyzed in 6 N HCl under N_2 at 110°C for 2 h. The hydrolysate was then subjected to high voltage electrophoresis in the first dimension on TLC plate in formic acid/acetic acid/H_2O (50:160:1794, v/v), pH 1.9, at 1.5 kV and 20°C for 25 min, and the second-dimensional electrophoresis in acetic acid/pyridine/H_2O (10:1:189, v/v), pH 3.5, at 1.3 kV for 20 min followed by autoradiography. The position of the phosphorylated amino acids in the plates were localized by ninhydrin staining of the phosphorylated amino acid standards.

2.6. In vitro assembly of microtubule and negative stain electron microscopy

τ (20 μg/ml) that was incubated in the absence or presence of different kinases as described above, was mixed at 37°C in 1-cm quartz microcuvettes with freshly isolated tubulin (see below; 2 mg/ml) in an assembly buffer containing 100 mM MES, 1 mM EGTA and 1 mM GTP. The assembly was followed up to 10 min by recording the turbidity changes at 350 nm in a Cary 1 spectrophotometer. At the end of the assembly reaction, aliquots of the incubation mixture were examined by negative stain microscopy as described previously for microtubules [9,40].

2.7. Preparation of taxol-stabilized microtubules and microtubule binding assay

Rat brain tubulin was isolated through two temperature-dependent cycles of microtubule polymerization-depolymerization [41] followed by removal of the microtubule-associated proteins (MAPs) by phosphocellulose ion-exchange chromatography [42]. Taxol-stabilized microtubules were prepared by incubating MAPs-free tubulin (~4 mg/ml) with taxol (20 μM) in the presence of 1 mM of PMSF at 37°C for 30 min. The microtubules were collected by centrifugation for 30 min at 50 000×*g* at 32°C. For microtubule binding assay, non-phosphorylated or phosphorylated τ (10 μg/ml) was incubated with taxol-stabilized microtubules (0.4 mg/ml) at 37°C for 30 min in a buffer containing 0.1 M HEPES (pH 6.8), 2 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 20 μM taxol and 1 mM GTP. Microtubule-associated τ and unbound τ were separated by centrifugation at 50 000×*g* for 30 min at 32°C over 125 mM sucrose cushion. The pellet (in the sucrose cushion) was washed once with water. The two combined supernatants and the pellet were dried in a Speed-Vac concentrator. The samples were resuspended in Laemmli sample buffer and electrophoresed on a 10% SDS-polyacrylamide gel. The blots were developed with the polyclonal anti-τ primary antibody

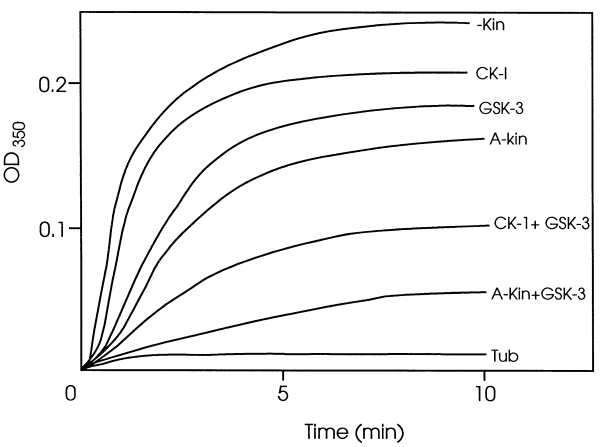

Fig. 1. Inhibition of microtubule assembly promoting activity of τ phosphorylated by various kinases. τ was phosphorylated with the individual kinases A-kinase, GSK-3 and CK-1, or CK-1 plus GSK-3, A-kinase plus GSK-3 combinations as described in Section 2; τ, identically treated but without any kinase served as a control. Microtubule assembly was carried out by incubating at 37°C rat brain tubulin (2 mg/ml) with τ (20 μg/ml). Microtubule assembly promoting activity was inhibited by all the kinase-treated τ samples. The most potent inhibition was seen in the combination phosphorylation of τ by A-kinase plus GSK-3. The curves show microtubule assembly in the presence of τ treated with no kinase, τ phosphorylated with CK-1, GSK-3, A-kinase alone, τ phosphorylated with CK-1 plus GSK-3, A-kinase plus GSK-3 and assembly with tubulin alone.

92e (1:5000 dilution [43]) and ^{125}I-labeled donkey anti-rabbit secondary antibody (0.1 μg/ml). Blots were quantitatively analyzed with the aid of a computerized bioimage analyzer (Fuji BAS-1500).

3. Results

3.1. Inhibition of microtubule assembly and microtubule binding activities of τ by phosphorylation

In two hours, the ^{32}P incorporation by CK-1, A-kinase and GSK-3 was determined to be 3.4, 1.1 and 1.2 mol/mol of τ, respectively. The subsequent ^{32}P incorporation by GSK-3 in 4 h was 1.5 and 3.2 mol/mol in τ prephosphorylated by CK-1 and A-kinase, respectively. We examined the effect of the phosphorylation of human τ3L by various kinases on its microtubule assembly-promoting and microtubule-binding activities. When acting singly, τ phosphorylated by the different kinases inhibited microtubule assembly in the order A-kinase > GSK-3 > CK-1-phosphorylated τ (Fig. 1). A greater inhibition of microtubule assembly was observed when τ was phosphorylated by a combination of CK-1 and GSK-3 or A-kinase and GSK-3. τ phosphorylated by the latter combination of kinases was most potent in inhibiting microtubule assembly (Fig. 1). When viewed by electron microscopy, only an occasional microtubule was observed when the A-kinase and GSK-3 were acting in combination (Fig. 2). We also examined the binding of τ to taxol-stabilized microtubules (Table 1). When τ was phosphorylated by CK-1, GSK-3 or A-kinase acting alone, the subsequent binding of the phosphorylated τ to microtubules was decreased by ∼10–13%. Like microtubule assembly seen in Fig. 1, the strongest inhibition of binding of τ to microtubules was observed when τ was phosphorylated by a combination of CK-1 and GSK-3 (∼39%) or A-kinase and GSK-3 (∼50%) (Table 1). These data suggest that the inhibition of τ function requires the concerted actions of both PDPKs (such as GSK-3) and non-PDPKs (such as A-kinase and CK-1).

3.2. Purification of ^{32}P-labeled phosphopeptides of τ

As described above, τ phosphorylated by the combination of A-kinase and GSK-3 was most effective in inhibiting microtubule assembly (Fig. 1) and inhibition of binding to microtubules (Table 1). Hence, we chose to identify the sites phosphorylated in τ by this combination of kinases. τ phosphorylated by GSK-3 alone was used as a control. As outlined in Section 2, τ (0.3 mg) was first incubated in the absence or presence of A-kinase and non-radioactive ATP for 2 h. Then reaction mixtures were heated at 95°C for 5 min to inactivate A-kinase and centrifuged. GSK-3 and [γ-^{32}P]ATP were then added to the τ, which is heat stable and is recovered in the supernatant, and the incubation continued for another 4 h. τ was then precipitated with trichloroacetic acid and digested with trypsin. After trypsin digestion, ^{32}P-labeled peptides were isolated by $FeCl_3$ affinity column chromatography. The recovery of ^{32}P peptides was about 97% for τ phosphorylated by a combination of A-kinase and GSK-3 (Fig. 3a), and about 87% for τ phosphorylated by GSK-3 alone (Fig. 3b). The ^{32}P-labeled peptides eluted from $FeCl_3$ affinity column were pooled, lyophilized and resuspended in 150 μl of 0.1% TFA and further purified by reverse phase liquid chromatography (RPLC) using C_{18} column previously equilibrated in 0.1% TFA. Eight major radioactive peaks which contained phosphorylated τ sequences (labeled I–VIII) could be resolved on RPLC for A-kinase plus GSK-3 preparation (Fig. 4a). Under identical conditions, four radioactive τ peptides (labeled IX–XII) were seen in GSK-3 alone (control) (Fig. 4b).

Table 1
Binding to microtubules of τ3L before and after phosphorylation by different kinases

τ phosphorylated by	% τ bound
None	94 ± 5.2
CK-1	87 ± 6.1
GSK-3	86 ± 4.6
A-kinase	81 ± 10.1
CK-1+GSK-3	61 ± 5.2
A-kinase+GSK-3	50 ± 6.6

Taxol-stabilized microtubules (0.4 mg/ml) were incubated with τ (10 μg/ml) for 30 min at 37°C. Microtubule-bound and unbound τ were separated by centrifugation at 50 000 × *g* for 30 min at 32°C over 125 mM sucrose cushion. The pellet and the supernatant dried in a speed-vac concentrator were resuspended in Laemmli sample buffer and electrophoresed on a 10% SDS-polyacrylamide gel. The blots were developed with 92e and quantitated with the aid of a computerized bioimage analyzer (Fuji BAS-1500). The data are expressed as mean ± S.D. of nine values.

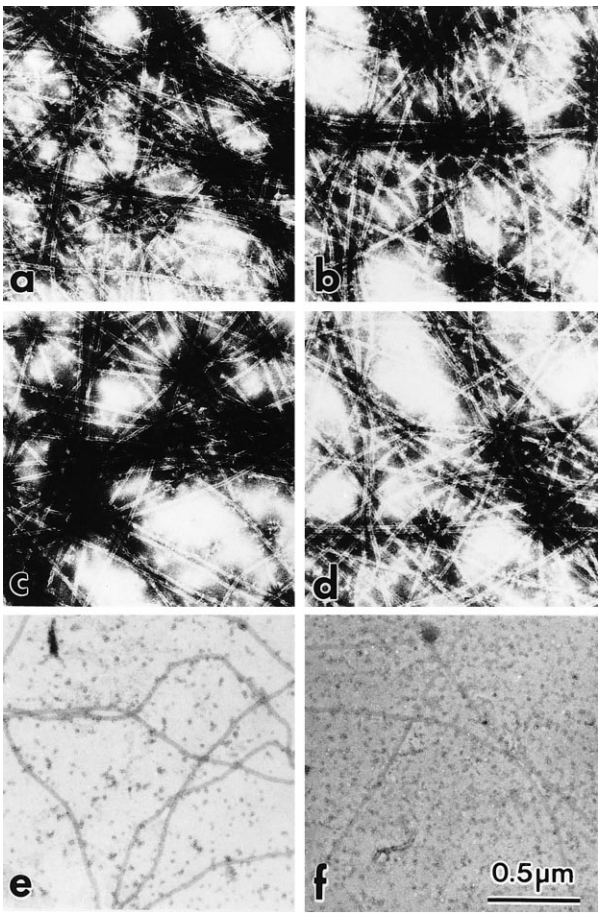

Fig. 2. Electron micrographs showing the products of the microtubule assembly reaction negatively stained with phosphotungstic acid. Aliquots of each sample (from Fig. 1) were taken at the steady state of polymerization (∼10 min) and stained negatively with 2% phosphotungstic acid. A large mass of microtubules was seen in assembly promoted by non-kinase treated τ (panel a), CK-1 phosphorylated τ (panel b), GSK-3 phosphorylated τ (panel c) and A-kinase phosphorylated τ (panel d). Whereas only an occasional microtubule was seen in the assembly promoted by CK-1 plus GSK-3 (panel e) and A-kinase plus GSK-3 (panel f) treated τ. Bar, 0.5 μm.

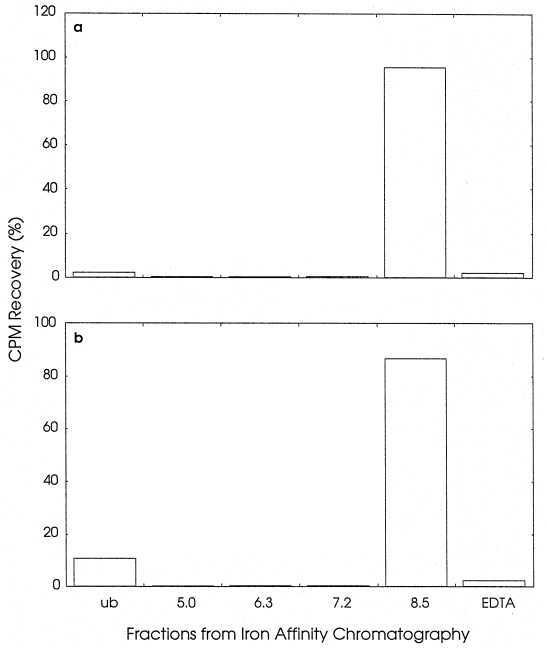

Fig. 3. $FeCl_3$ affinity column chromatography to separate non-phosphorylated peptides from phosphorylated ones. $FeCl_3$ affinity column was prepared as described in Section 2. The tryptic digests of ^{32}P peptides in 0.1 M of acetic acid were applied to the column. ub is the unbound and 0.1 M acetic acid wash fractions; 5.0, 6.3, 7.2, 8.5 and EDTA represent the fractions eluted from the column with 0.1 M sodium acetate, pH 5.0, 1% ammonium acetate, pH 6.3, 1% ammonium acetate, pH 7.2, 1% ammonium acetate, pH 8.3 and 0.2 M EDTA, pH 8.0, respectively. The recovery of ^{32}P in the pH 8.5 elutes was 97 ± 1% (panel a, A-kinase plus GSK-3) and 87% (panel b, GSK-3 alone).

3.3. *Phosphoamino acid analysis and sequential manual Edman degradation of ^{32}P peptides isolated by RPLC*

Analysis of the phosphopeptide peaks by high-voltage electrophoresis on thin-layer silicone plates revealed that peaks I–V and IX contained both p-Ser and p-Thr, whereas peaks VI–VIII and X–XII contained only p-Ser (Table 2). Amino acid sequence analysis and sequential manual Edman degradation of ^{32}P fractions isolated by RPLC demonstrated that S(p)GYS(p)S(p)PGS(p)PGT(p)PGSR, T(p)PPKS(p)PSSAK, IGS(p)TENLK, IGS(p)LDNITHVPGGGNK, SPVVSGDT-S(p)PR were the parent peptides containing the sites phosphorylated by A-kinase plus GSK-3, whereas T(p)PP-S(p)SGEPPK, SPVVS(p)GDTSPR, IGS(p)TENLK and IGS(p)LDNITHVPGGGNK were the peptides that contained sites phosphorylated by GSK-3 alone. Peaks I and II had the same sequence of Ser-198–Arg-209 (Fig. 5a); only peptide I contained one more phosphorylation site (Ser-202) than peptide II. Peaks III and IV shared the same sequence of Ser-195–Arg-209. Peak IV might have been contaminated by a small amount of peak III which smeared a little on the RPLC separation; 'tailing' of this type is not uncommon. Peaks V, VI, VII and VIII represented τ fragments of Thr-231–Lys-240, Ile-260–Lys-267, Ser-396–Arg-406 and Ile-354–Lys-369, respectively (Fig. 5a). Peaks IX–XII corresponded to the sequences of Thr-181–Lys-190, Ser-396–Arg-406, Ile-260–Lys-267 and Ile-354–Lys-369, respectively (Fig. 5b). Alignment of the phosphorylated peptides of ^{32}P-τ by A-kinase plus GSK-3 demonstrated that the corresponding phosphorylated sites were Ser-195, Ser-198, Ser-199, Ser-202, Thr-205, Thr-231, Ser-235, Ser-262, Ser-356 and Ser-404. By the same procedure, it was demonstrated that τ phosphorylated by GSK-3 alone generated sites at Thr-181, Ser-184, Ser-262, Ser-356 and Ser-400 (Fig. 6).

Table 2
Phosphoamino acid analysis of phosphopeptides separated by RPLC

Phosphopeptides	P-Ser	P-Thr
I	+	+
II	+	+
III	+	+
IV	+	+
V	+	+
VI	+	−
VII	+	−
VIII	+	−
IX	+	+
X	+	−
XI	+	−
XII	+	−

Phosphoamino acid analysis of the phosphopeptide peaks resolved from C_{18} (peaks I–VIII) or GSK-3 alone (peaks IX–XII). RPLC of the tryptic digests of ^{32}P-τ phosphorylated by A-kinase plus GSK-3. The phosphopeptide peak fractions shown in Fig. 4 were hydrolyzed in 6 N HCl under N_2 at 110°C for 2 h. The hydrolysate was spotted on a TLC plate and subjected to high voltage two-dimensional electrophoresis followed by autoradiography to localize the ^{32}P spots. The positions of the phosphorylated amino acid standards were visualized by ninhydrin staining. P-Ser: phosphoserine; P-Thr: phosphothreonine.

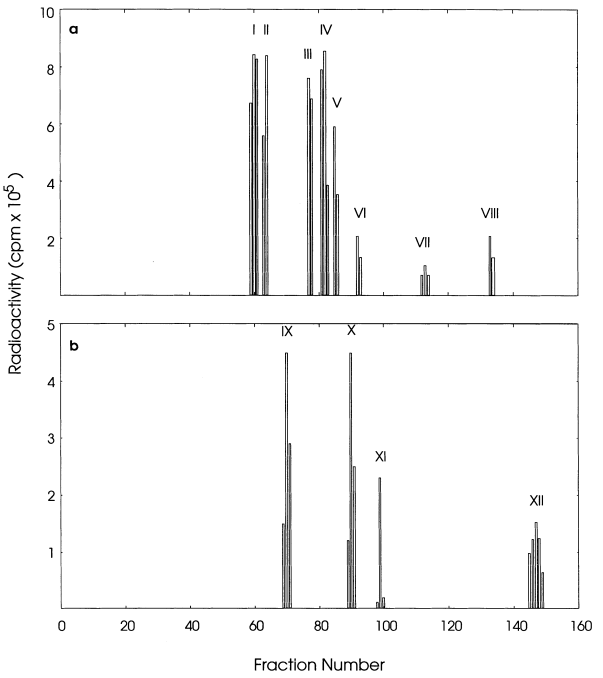

Fig. 4. ^{32}P τ fragments phosphorylated by A-kinase and GSK-3 (panel a) or GSK-3 alone (panel b) resolved on RPLC. The tryptic digests of ^{32}P-τ (0.3–0.5 mg) eluted at pH 8.5 from $FeCl_3$ column were lyophilized, resuspended in 0.1% TFA and applied to C_{18} reverse phase column. Eight (panel a, A-kinase plus GSK-3) and four (panel b, GSK-3 alone) major radioactive peaks were seen after the separation, respectively. Peaks either not sequenceable or which did not yield any identifiable τ sequence are not shown.

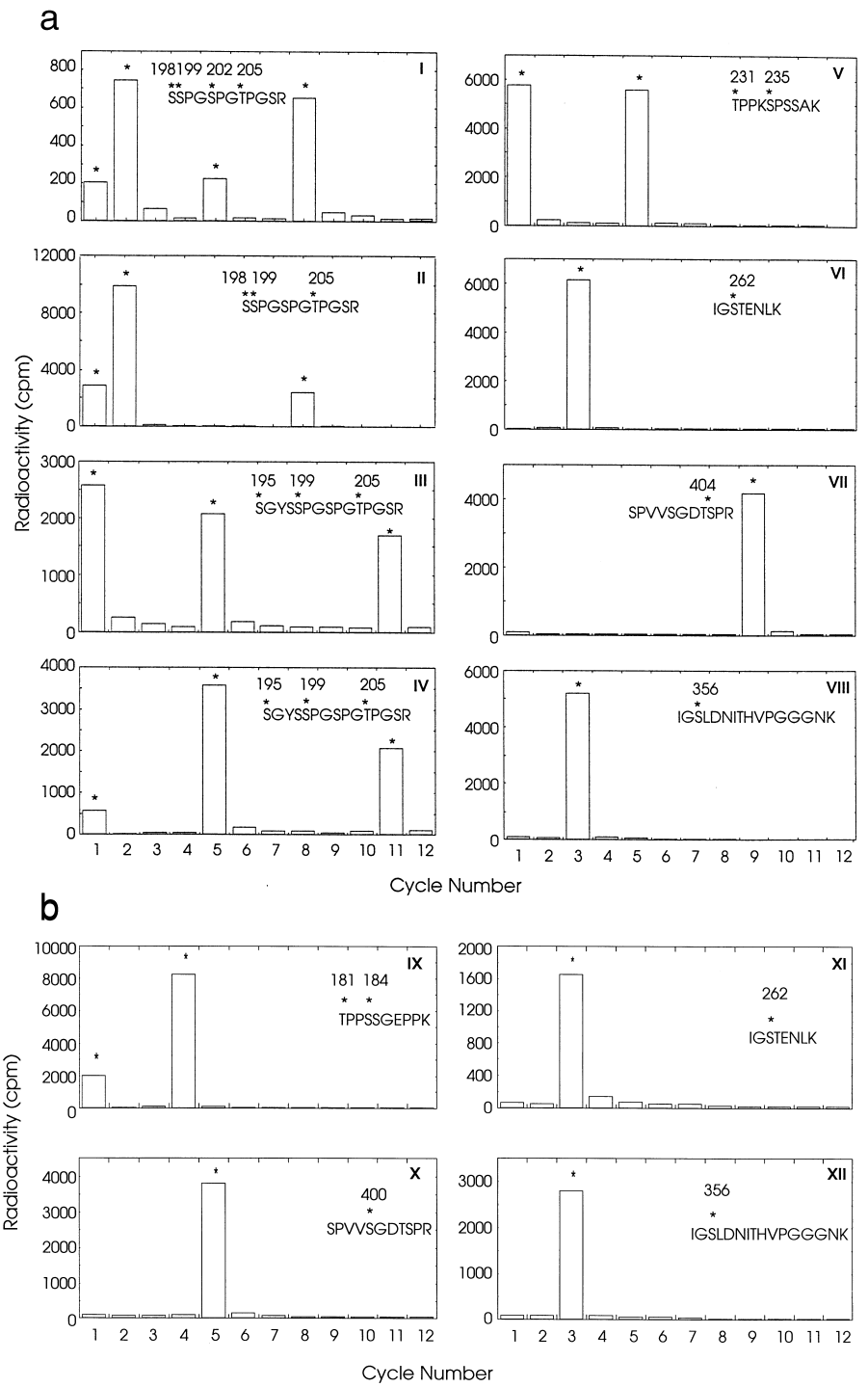

Fig. 5. Identification of phosphorylation sites in the phosphopeptides separated by C_{18} RPLC of the tryptic digests of τ. The ^{32}P peptides eluted from the RPLC column were subjected to sequential manual Edman degradation and amino acid sequence analysis to identify phosphorylation sites as described in Section 2. Peaks I–VIII (a) are from A-kinase plus GSK-3 whereas peaks IX–XII (b) are from GSK-3 alone phosphorylation. The phosphorylated amino acids are indicated by asterisks in each peptide sequence.

4. Discussion

Previously, we have shown that both non-PDPKs and PDPKs phosphorylate τ at some of the same sites as in PHF-τ as determined by immunoreactivity with phosphorylation-dependent τ antibodies [28,44]. Furthermore, a prephosphorylation of τ by various non-PDPKs (A-kinase, C-kinase, CK-1 and CaM KII) served to stimulate a subsequent phosphorylation catalyzed by GSK-3. The highest level of such stimulation was obtained with τ3L that was prephosphorylated by A-kinase [28]. In the present study, we demonstrate that the interaction of A-kinase with GSK-3 phosphorylates τ at 7 of 21 sites seen in PHF-τ and markedly inhibits its ability to promote in vitro assembly and binding to microtubules.

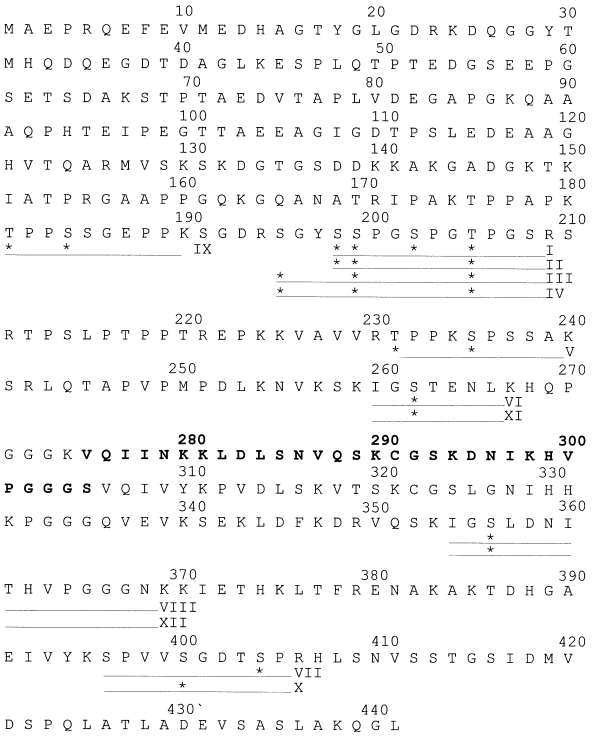

Fig. 6. Alignment in τ sequence of the phosphopeptides identified from τ phosphorylated by A-kinase plus GSK-3 (I–VIII) or GSK-3 alone (IX–XII). The amino acid numbering is according to τ4L. Amino acid residues Val-275–Ser-305 which comprise the second microtubule binding repeat and is missing in τ3L are highlighted.

Our data showed (i) that A-kinase and GSK-3 combination induced a 2- to 3-fold higher incorporation of ^{32}P to τ than by GSK-3 alone; (ii) that phosphorylation of τ with the combined kinases led to a most potent inhibition of biological activity of τ; and (iii) that the A-kinase and GSK-3 combination was capable of phosphorylating at least 10 sites (Ser-195, Ser-198, Ser-199, Ser-202, Thr-205, Thr-231, Ser-235, Ser-262, Ser-356 and Ser-404), whereas GSK-3 alone only phosphorylated 5 sites (Thr-181, Ser-184, Ser-262, Ser-356 and Ser-400) under identical conditions. Among the 21 phosphorylation sites (Ser-46, Ser-123, Ser-198, Ser-199, Ser-202, Ser-208, Ser-210, Ser-212, Ser-214, Ser-217, Ser-231, Ser-235, Ser-262, Ser-396, Ser-400, Ser-403, Ser-404, Ser-409, Ser-412, Ser-413 and Ser-422) found in Alzheimer PHF-τ [14,18,39,45–49], GSK-3 alone only phosphorylated Ser-262 and Ser-400, whereas A-kinase and GSK-3 combination phosphorylated Ser-198, Ser-199, Ser-202, Thr-231, Ser-235, Ser-262 and Ser-404. These data suggest strongly that the interaction of A-kinase with GSK-3 generates a more Alzheimer-like state of τ, indicating that it might be a potential system involved in Alzheimer neuronal degeneration.

Phosphorylation of τ at specific sites inhibits the microtubule assembly and reduces the binding of τ to taxol-stabilized microtubules (see Fig. 1 and Table 1). Dephosphorylation of PHF-τ restores the biological activity of τ in promoting the assembly of microtubules [8–10]. Recent studies report that Ser-262 is a most potent site in regulating the binding of τ to microtubules [50]. Using recombinant τ3L as a substrate and reactivity with an antibody (12E8) to P-Ser-262 and P-Ser-356, we recently found that phosphorylation at Ser-262 reduces only about 40% of microtubule binding as well as causing a slight inhibition of microtubule assembly-promoting activity of τ [16]. In the present study, we further demonstrated that a more effective inhibition was seen when in addition to Ser-262, Alzheimer sites Ser-198, Ser-199, Ser-202, Thr-231, Ser-235 and Ser-404 were also phosphorylated. It implied that phosphorylation of τ at the above mentioned sites is involved in the regulation of biological activity of τ.

Phosphorylation of τ by individual kinases has been extensively studied recently. It has been found that a kinase phosphorylates different τs at different rates and sites. Such a differential phosphorylation exists between bovine τ and human τ, even among human τ isoforms [18,44,51]. For instance, A-kinase phosphorylated τ3 at Ser-214, Ser-324, Ser-356, Ser-409 and Ser-416 [52] whereas it phosphorylated τ4L at Ser-262, Ser-293, Ser-305, Ser-324 and Ser-356 [15]. Using bovine τ (a mixture of six isoforms) as substrate, it was found that GSK-3α phosphorylated τ at Ser-235 and Ser-404 [53] while GSK-3β phosphorylated τ at Ser-199, Thr-231, Ser-396 and Ser-413 [54]. In the present study, we have mapped biochemically the sites phosphorylated in human τ3L by GSK-3 and A-kinase plus GSK-3 which have not been reported previously (see Table 3).

In conclusion, the present study revealed that phosphorylation of τ3L by various kinases differentially inhibits the biological activity of τ in microtubule assembly promoting and microtubule binding. The most potent inhibition was seen in the combined phosphorylation of τ by A-kinase and GSK-3. Under these conditions, at least seven Alzheimer-like phosphorylation sites (Ser-198, Ser-199, Ser-202, Thr-231, Ser-235, Ser-262 and Ser-404) were obtained, while only two of them (Ser-262 and Ser-400) were seen by GSK-3 alone. The interaction of A-kinase with GSK-3 might be one of the most potent systems responsible for the degeneration of neurons with neurofibrillary/τ pathology in Alzheimer disease.

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Table 3
Comparison of τ sites phosphorylated by A-kinase, GSK-3 or by a combination of the two kinases

Kinases	Phosphorylation sites	τ source	Reference
A-kinase	Ser-214, Ser-324, Ser-356, Ser-409, Ser-416	τ3	Scott et al. [52]
A-kinase	Ser-262, Ser-293, Ser-305, Ser-324, Ser-356	τ4L	Drewes et al. [15]
GSK-3(α)	Ser-235, Ser-404	Bovine	Yang et al. [53]
GSK-3(β)	Ser-199, Thr-231, Ser-396, Ser-413	Bovine	Ishiguro et al. [54]
GSK-3(α+β)	Ser-181, Ser-184, Ser-262, Ser-400	τ3L	Present work
A-kinase+GSK-3	Ser-195, Ser-198, Ser-199, Ser-202, Thr-205, Thr-231, Ser-235, Ser-262	τ3L	Present work

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