Tau-tubulin kinase phosphorylates tau at Ser-208 and Ser-210, sites found in paired helical filament-tau

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Abstract Hyperphosphorylated tau protein is known to be a major component of the paired helical filaments (PHFs) that accumulate in the brain of Alzheimer's patients. The kinase that phosphorylated Ser-208 and Ser-210 in PHF-tau had remained unknown. We used anti-pS208 and anti-pS210 antibodies and Western blots to confirm that the tau-tubulin kinase (TTK) phosphorylates tau at Ser-208 and at Ser-210. Using partial amino acid sequences of purified bovine brain TTK, a mouse cDNA of TTK was isolated and the sequence was determined. Its 963 bp coding region is composed of 320 amino acids and encodes a 36 kDa protein indistinguishable in size from authentic bovine brain TTK. Our immunoblot analysis demonstrated that TTK is ubiquitously distributed in the rat tissues, and that it is developmentally regulated in the rat brain.

Key words: Tau-tubulin kinase; Tau protein kinase I/glycogen synthase kinase-3β; Phosphorylation of tau; Paired helical filament; Mouse brain cDNA cloning

1. Introduction

Paired helical filament (PHF)-tau accumulates in the brains of Alzheimer's disease (AD) patients where it is highly phosphorylated [1–3]. Recently 25 phosphorylation sites on PHF-tau have been identified: 12 are proline-directed sites, the remainder non-proline sites [4–6]. As candidates acting in the PHF formation, various kinases such as tau protein kinase I/glycogen synthase kinase-3β (TPKI/GSK-3β) [7–11], TPKII/cdk5 [12], mitogen-activated protein (MAP) kinases [11,13], and protein kinase C [14] have been reported to be involved in the phosphorylation of the several sites listed. However, the kinase which phosphorylates Ser-208 and Ser-210 remained unknown. Tau-tubulin kinase (TTK) is a non-proline-directed Ser/Thr kinase which has been purified from bovine brain [15]. TTK phosphorylates a serine that is followed by an arginine residue, thus recognizing an SR-motif. Among the non-proline-directed phosphorylation sites of PHF-tau, Ser-208 and Ser-210 are both within the context of an SR-motif. We previously reported that TTK phosphorylates tau and the K2 fragment/tau 1 portion containing Ser-208 and Ser-210. In order to confirm these phosphorylation sites by Western blot, anti-pS208, anti-pS210 and anti-pS208, pS210 antibodies were raised in rabbits. Furthermore, the interaction between TTK and TPKI/GSK-3β was also examined. Here we report the complete amino acid sequence of TTK deduced from mouse brain cDNA and the developmental distribution of TTK in rat tissues based on Western blot analyses.

2. Materials and methods

2.1. Materials

Wistar rats were obtained from the SCL Corp. TTK, TPKI and TPKII were purified from bovine brain following procedures described previously [8,15]. Recombinant GSK-3β was purchased from Sigma. Tau and tubulin were prepared from bovine brain extracts [8], and human recombinant Tau-441 was purchased from Panvera. The tau peptide K2 (191–224) and the partial phosphopeptides of tau pS202, pT205 [12], pS208 (CPGDPGTPGS^{P}RSRTPSL), pS210 (CPGDPGTPGSRS^{P}RTPSL), and pS208/210 (CPGDPGTPG S^{P}RS^{P}RTPSL) were chemically synthesized employing the solid-phase method and the Biosearch Model 9500 or ABI Model 431A peptide synthesizer as described by Tsukamoto et al. [16] and Sato et al. [17]. The numbering is based on the longest human tau isoform.

2.2. Preparation of antibodies

The synthetic phospho-oligopeptides of tau (pS208, pS210, pS208/210) and the synthetic oligopeptide of mouse TTK (TTK-C; CSDPFDWEKSGTDGSLTTTTTSAT) were covalently cross-linked to the carrier protein keyhole limpet hemocyanin. Rabbits were immunized with these conjugates and the anti-serum purified using affinity columns of Sepharose 4B coupled to each antigenic peptide as described previously [18]. The antibodies pS199 and pT205 have been described before by Ishiguro et al. [12].

2.3. Preparation of rat brain extracts and partial purification of the extracts

Rat brain extracts (TS-Triton extracts) were prepared with TS buffer as described previously [19]. Protein concentrations were determined by the method of Bradford [20]. The extracts were diluted with the same volume of 50 mM Tris–HCl, pH 7.6 containing protease inhibitors and mixed with SP Sepharose (Pharmacia Biotech) equilibrated in the same buffer. The mixture was rotated overnight at 4°C and centrifuged at 15 000 rpm for 5 min. The beads were then washed with 50 mM Tris–HCl, pH 8.5. The proteins bound to the Sepharose were then extracted with the same buffer containing 0.5 M NaCl (SP-extracts).

2.4. Assay of kinase activity

Phosphorylation of tau and tubulin was measured by quantifying radioactive phosphate incorporated from [γ-^{32}P]ATP (Amersham) at 30°C as described previously [9]. The reaction mixture contained 200

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Abbreviations: PHF, paired helical filament; TTK, tau-tubulin kinase; TPK, tau protein kinase; GSK, glycogen synthase kinase; AD, Alzheimer's disease; MTP, microtubule-associated protein; SDS, sodium dodecyl sulfate; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; CBB, Coomassie brilliant blue; GST, glutathione *S*-transferase

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μM [γ-^{32}P]ATP and 0.2–0.3 mg/ml substrate in buffer B (100 mM MES-NaOH, pH 6.5, 5 mM Mg-acetate, 1 mM EGTA, 5 mM 2-mercaptoethanol, protease inhibitors).

2.5. Western blot analysis

Sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) and Western blot were carried out as described previously [19]. The immunodetection was performed with the Vectastain ABC-PO system (Vector Laboratories) or the Prot Blot AP system (Promega). The antibodies against pS208, pS210, and pS208/210 were diluted to 30 μg per ml, and anti-TTK-C antibody was diluted to 15 μg per ml. The anti-pS199 and anti-pT205 antibody preparations were diluted 1:1000 and AT8 (Innogenetics) 1:20. For the absorption test of the antibodies, each antibody was incubated with the peptide (50 μg/ml) for 1 h at 4°C, and the supernatant after centrifugation was analyzed. For Western blot analyses of phosphorylated tau, 1 mM benzaidine (Sigma) was added to the reaction mixtures.

2.6. Sequencing of the TTK peptides from bovine brain

The TTK fractions (0.5 mg/5 ml) were partially purified by AF heparin column chromatography [15] and concentrated by 5% SDS–PAGE for 6.5 h at 18 mA in a Y-shaped gel which has been developed by P. James of the ETH-Zentrum [21] for mass spectrometry and was modified by us. The main bands between 40 and 28 kDa were separated again by 3%/9% SDS–PAGE for 3 h at 10 mA. The gel was stained with Coomassie brilliant blue (CBB, Sigma) and a single band around 32 kDa was cut out, digested with lysylendopeptidase, AP-I (Wako, 0.3 μg/band), in 100 mM Tris–HCl buffer, pH 9.0, overnight at 37°C. Several peptides were obtained from the digest and purified by reverse-phase column (C8, Inertsil, 2.1×150 mm, GL Sciences Inc., Tokyo, Japan) chromatography. The amino acid sequences were determined by a pulse-liquid-phase amino acid sequencer (Applied Biosystems ABI 492 cLC protein sequencer).

2.7. Amplification and isolation of TTK cDNA by PCR

The cDNA clones encoding TTK were isolated as follows. A degenerate oligonucleotide pair corresponding to peptides B-1 and B-4 of TTK was chosen as primers (5′-GA(A/G)TT(C/T)GGIGA(A/G)ATITA(C/T)GA(A/G)GCIATGGA-3′ as the sense primer and 5′-TAITGIC(G/T)IGCIA(A/G)ICC(A/G)AA(A/G)TCIA(A/G)CAT-3′ as the antisense primer) for PCR. PCR was performed on adult mouse brain cDNA, and the amplified product of 419 bp was inserted into the pPCR-Script Amp SK (+) vector (Stratagene) for DNA sequence analysis. To obtain the full length of the cDNA sequence another primer corresponding to the 419 bp PCR fragment was designed for rapid amplification of cDNA ends (RACE). Primers used for the 3′ region were: primer 1, 5′-TGGAGAAATTTACGATGCCTTGGACATGC-3′; primer 2, 5′-GGAGAATGTGGCGCTGAAGGTGGAGTCAGCTCAGC-3′, and primer 3, 5′-GCAGCCAAAGCAGGTTCTGAAGATGGAGG-3′. The PCR primers used for the 5′ end amplification were: primer 4, 5′-GTCTTCCCATTTCCCTGTTCCGATGAGC-3′; primer 5, 5′-CTCCAAGGCAGTTGGCCAACCACAAACTCCACCAAC-3′; primer 6, 5′-GAGCCTGTGGTCATATCTCTCCTTAATGG-3′. The 3′ RACE and the 5′ RACE were performed using the Marathon-Ready mouse brain cDNA amplification kit (Clontech). Each PCR product was ligated into the pGEM-Teasy vector (Promega) and subcloned for DNA sequencing.

2.8. Preparation and purification of a glutathione S-transferase (GST)–TTK fusion protein

The plasmid pGEX-6p-2/TTK (amino acids 11–320) was constructed by digesting pGEX-6p-2 (Pharmacia) with *Sma*I and *Xho*I and ligating the *Eco*RV/*Sal*I fragment from pGEM-Teasy/TTK (fragment D) into it. TTK was expressed in *Escherichia coli* strain BL21 as a fusion protein to GST. The GST–TTK fusion protein was purified and digested with PreScission protease (Amersham Pharmacia Biotech) according to the instructions for the Bulk GST Purification Module. The GST–TTK fusion protein was immobilized on glutathione Sepharose 4B beads (Amersham Pharmacia Biotech). The beads were divided into two tubes (tube 1 and tube 2). The beads in tube 1 were washed with phosphate-buffered saline (PBS), and the GST–TTK fusion protein was eluted by elution buffer. The beads in tube 2 were washed with cleavage buffer. The TTK was cleaved by PreScission protease, and eluted by the same buffer. Aliquots of each elution step were collected and processed by SDS–PAGE for product analysis.

3. Results

3.1. Specificity of anti-PS208 and anti-PS210 antibodies and Western blot analysis of tau phosphorylated by TTK

The K2 fragment/tau 1 portion of tau was being phosphorylated by TTK [15]. It seemed likely that TTK phosphorylates no other sequence besides Ser-208 and Ser-210 which are within an SR-motif of the K2 peptide. Therefore, antibodies

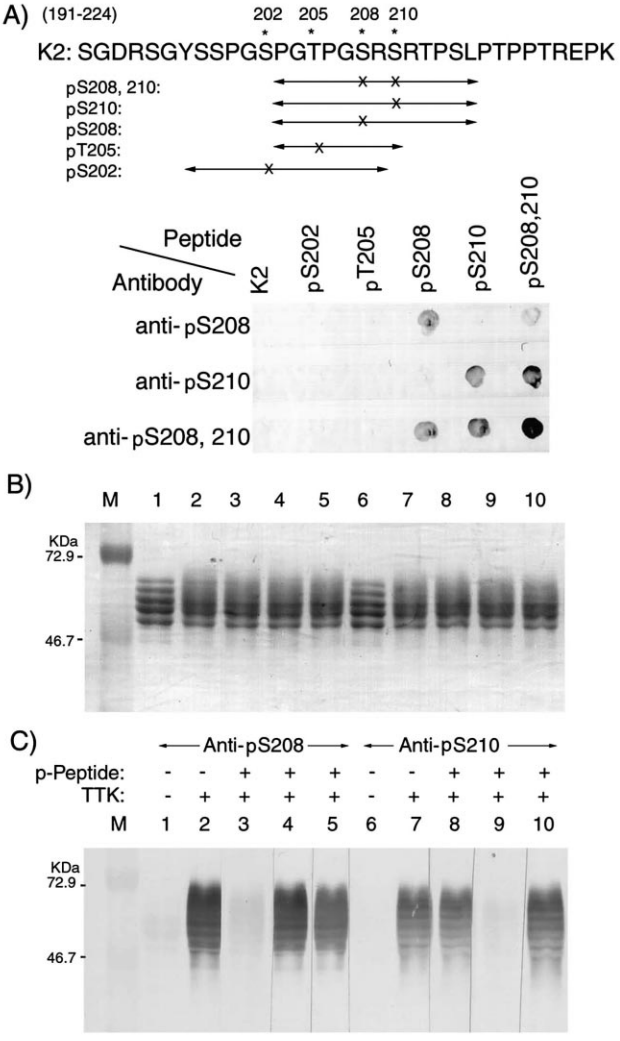

Fig. 1. Phosphorylation of Ser-208 and Ser-210 of tau by TTK. (A) Dot blot analysis of the antibodies against phosphopeptides. Each peptide sequence of the K2 fragment of tau is indicated by arrows. The phosphorylation sites are marked with an X on the arrows. The peptide spots (0.2 μg) on a nitrocellulose sheet were allowed to react with the purified primary antibodies anti-pS208, anti-pS210, and anti-pS208/210. The reactions were detected using the Vectastain ABC-PO kit. (B) Tau (0.8 μg) was incubated at 30°C overnight with (lanes 2–5 and 7–10) or without (lanes 1 and 6) TTK in a reaction mixture (9 μl) containing 200 μM ATP. The reaction mixtures were subjected to SDS–PAGE, transferred onto a nitrocellulose sheet and stained with Ponceau S. (C) Immuno-analyses of a sheet as in B were carried out with antibodies in the absence or presence of excess phosphopeptides (50 μg/ml). Anti-pS208 (lanes 1 and 2), anti-pS208 plus pS208 peptide (lane 3), anti-pS208 plus pS210 peptide (lane 4), anti-pS208 plus pT205 peptide (lane 5), anti-pS210 (lanes 6 and 7), anti-pS210 plus pS208 peptide (lane 8), anti-pS210 plus pS210 peptide (lane 9) and anti-pS210 plus pT205 peptide (lane 10). The reactions were detected using the Prot Blot AP system. M, pre-stained size markers from BRL.

specific to pS208 or/and pS210 in tau were prepared. Fig. 1A shows the sequence of the K2 peptide and the specificities of the anti-pS208, anti-pS210, and anti-pS208/210 antibodies to the various regions of tau determined by dot blot tests. Anti-pS208 bound to the pS208 peptide and less so to the pS208/210 peptide, but not to pS202, pT205, or pS210. Anti-pS210 bound to the pS210 and the pS208/210 peptide, but not to the pS202, pT205, or pS208 peptides. Anti-pS208/210 bound to pS208, pS210, and the pS208/210. The results, therefore, indicate that the antibodies were specific to their corresponding antigens and that they did not cross-react with other phosphorylation sites.

Tau was incubated with or without TTK, and the phosphorylated epitopes were analyzed by Western blot using these antibodies. Fig. 1B shows the tau protein bands detected by Ponceau S staining. The bands of tau, which have been incubated with TTK in lanes 2–5 and 7–10, appear smeared and slightly shifted in comparison to the bands obtained without TTK (lanes 1 and 6). Fig. 1C shows the results of the Western blot of the membrane in Fig. 1B. The tau not treated with TTK was not stained by anti-pS208 (lane 1) or anti-pS210 (lane 6). But the tau phosphorylated by TTK was clearly stained by anti-pS208 (lane 2) and anti-pS210 (lane 7). The anti-pS208 antibody was completely absorbed after incubation with the peptide pS208 (lane 3) but not after incubation with peptide pS210 (lane 4) or pT205 (lane 5). Conversely, the anti-pS210 antibody was completely absorbed by incubation with peptide pS210 (lane 9) but not with peptide pS208 (lane 8) or pT205 (lane 10). The results, therefore, prove that TTK phosphorylates tau at Ser-208 and Ser-210.

3.2. Phosphorylation of tau by TTK, TPKII, and TPKI/GSK-3β

Tau was phosphorylated by TTK, TPKII, and TPKI/GSK-3β from bovine brain or recombinant GSK-3β. The products were analyzed by immunoblot using anti-pS199, anti-pT205, and anti-AT8 (Fig. 2). In Fig. 2B, Western blot analysis showed that the anti-pS199 antibody stained tau phosphorylated by TPKI/GSK-3β from bovine brain (lane 4) and recombinant GSK-3β (lane 5), the site of which is known to be specific for TPKI/GSK-3β [12], the antibody did not stain tau phosphorylated by TTK (lane 2). Anti-pT205 stained tau phosphorylated by TPKII (lane 7) and by both of the GSK-3β proteins (lanes 8 and 9), but not tau phosphorylated by TTK (lane 6). Anti-AT8 (specific for pS203 and pT205) stained tau phosphorylated by GSK-3β (lanes 12 and 13), but it did not stain tau phosphorylated by TPKII (lane 11) and not by TTK either (lane 10). These results confirm that TTK does not phosphorylate tau at Ser-199, Ser-203 and Thr-205.

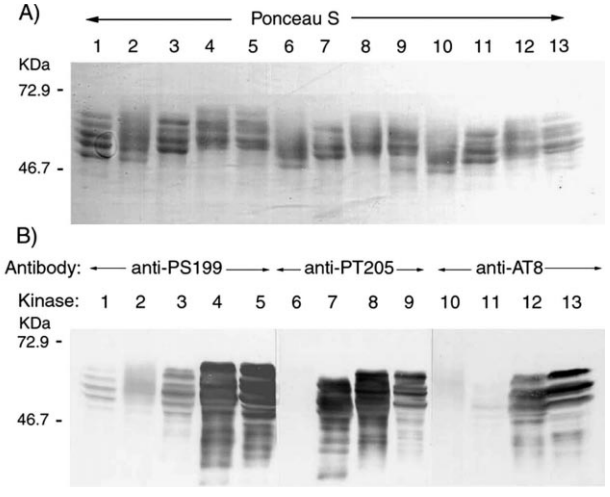

Fig. 2. Immunoblot analyses of tau phosphorylated by TTK, TPKII, and TPKI/GSK-3β. (A, B) Tau from bovine brain (lane 1) was phosphorylated by TTK (lanes 2, 6, and 10), TPKII (lanes 3, 7, and 11), TPKI/GSK-3β from bovine brain (lanes 4, 8, and 12), or recombinant GSK-3β (lanes 5, 9, and 13). The products were first stained with Ponceau S (A) and then analyzed by immunoblot (B) with anti-pS199 (lanes 1–5), anti-pT205 (lanes 6–9), and anti-AT8 (lanes 10–13) antibodies.

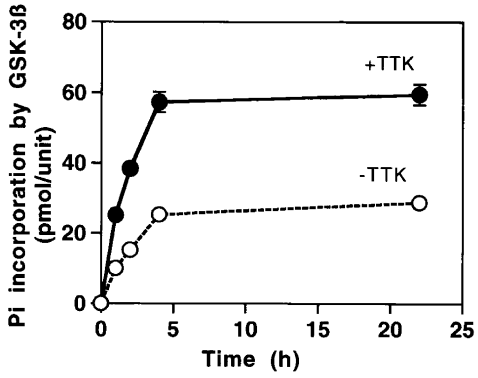

Fig. 3. Effect of prior phosphorylation of tau by TTK on its phosphorylation by GSK-3β. First the reaction mixture containing recombinant tau was incubated at 30°C overnight without (○) or with (●) TTK. After boiling for 10 min, recombinant GSK-3β (2 U/μl) was added to the reaction mixtures and the phosphorylation of tau by GSK-3β was measured as a function of time. Data points are means ± S.D.

3.3. Phosphorylation of tau in combination with TTK and TPKI/GSK-3β

It has been reported that the prior phosphorylation of tau by TPKII enhances the phosphorylation by TPKI/GSK-3β [22]. Recombinant tau was treated with or without TTK, after which the reactivity of the recombinant GSK-3β was compared (Fig. 3). It can be seen that, when tau was phosphorylated by TTK, the rate of P_i incorporation into tau was higher in comparison to tau which had not been prephosphorylated. The data suggest that TTK influences the TPKI/GSK-3β phosphorylation of tau in a similar manner as does TPKII.

3.4. Amino acid sequencing of peptide fragments of TTK

The TTK protein was digested with lysylendopeptidase. Four peptides obtained from the digest were sequenced as B-1 [IMPSEFGEIYEAMDLLT(R)E(N)], B-2 [MEVAVLK], B-3 [(R/S)ILESIE(A/L)(I/S)XX(V/G)(G)(F)] and B-4 [HYMLDFGLARQYT(N/G)TXXXV]. A search for homologous proteins based on the sequences of these four peptides yielded no exact matches with other protein kinases. However, the peptides B-1, B-2, B-3, and B-4 have homologous sequences, in this order which is based on the numbering of the amino acid sequence deduced from cDNA of *Caenorhabditis elegans*, as revealed by a homology search with the Genetyx computer program (S.D.C.). Furthermore, the B-4 peptide contains a sequence similar to one in the central core of the catalytic domain, subdomain VII, of the protein kinase family [23].

By PCR using degenerated primers corresponding to the

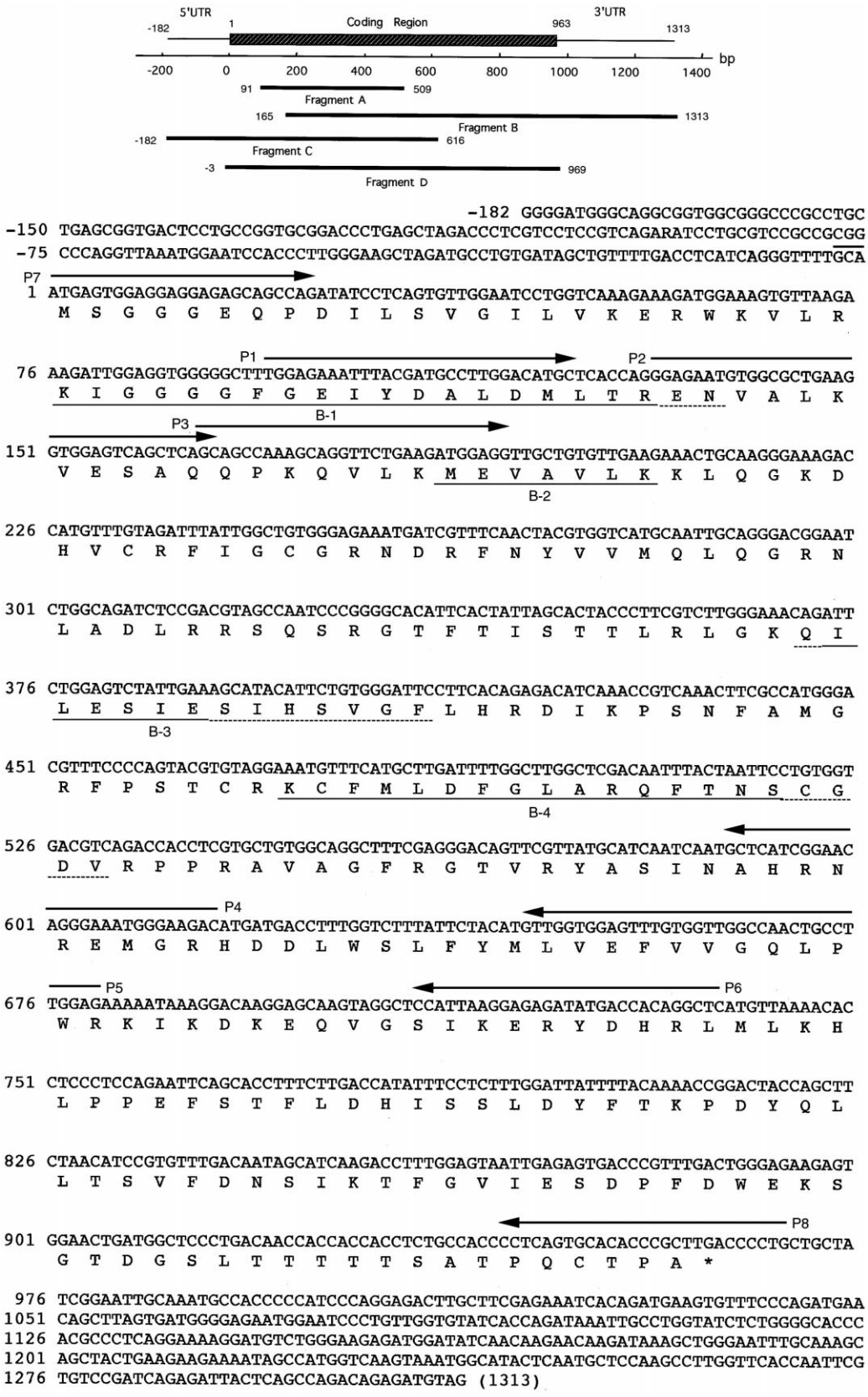

Fig. 4. Structure and sequence of the mouse brain TTK cDNA. The cDNA sequence of TTK was determined as 182 bp of 5′ UTR, 963 bp of coding region (shaded box), and 350 bp of 3′ UTR. The corresponding positions of peptides B-1, B-2, B-3, and B-4 of the bovine brain TTK are indicated by underlining below the mouse amino acid sequence. The positions of primers used are indicated by arrows above the nucleotide sequence. Fragment A is a PCR product utilizing primers derived from peptides B-1 (sense) and B-4 (antisense). Fragments B and C are the products of 3′ RACE (primer 3 as sense and AP2 as antisense) and 5′ RACE (AP2 as sense and primer 4 as antisense), respectively. The primer 7 as sense and the primer 8 as antisense were used in PCR to generate the PCR product represented by fragment D.

two peptides B-1 and B-4 a DNA fragment of 419 bp was amplified from mouse brain cDNA. The predicted amino acid sequence encoded in the 419 bp DNA fragment (fragment A in Fig. 4) contains regions with high homology to two of the TTK peptides from the bovine brain (B-2 and B-3). These results confirmed, therefore, that the PCR product was indeed derived from TTK cDNA.

The 419 bp cDNA encoding a sequence of TTK was used to probe Northern blot of total RNA from adult mouse brain. A single, approximately 1.7 kb mRNA transcript hybridized to the TTK probe (data not shown).

3.5. cDNA sequence of the mouse brain TTK

Further attempts were undertaken to extend the sequence of the TTK cDNA. Primary and nested PCRs were carried out using the adapter primers (AP1 and AP2, Clontech) coupled with several gene specific primers (primers 1–6). The RACE-PCR amplification products were subcloned, and all clones containing TTK fragments were sequenced. As shown in Fig. 4, the product of the 5′ RACE represented a cDNA of 798 bp (fragment C) which contained the initiation codon ATG. The product of the 3′ RACE was 1149 bp long (fragment B) and contained the termination codon TGA. Both cDNA fragments contained the DNA sequence of fragment A, and the overlapping sequences of fragments B and C (165–616) were identical. We, therefore, prepared by PCR fragment D using primer 7, 5′-GCAATGAGTGGAGGAGGAGAG-CAGCCAG-3′, as sense and the primer 8, 5′-CAGGGGT-CAAGCGGGTGTGCACTGAGG-3′, as antisense to confirm the sequence of the coding region. Finally, the cDNA sequence of the TTK was determined as containing 182 bp of 5′ untranslated region (UTR), 963 bp of coding region, and 350 bp of 3′ UTR. The entire open reading frame encodes a 320 amino acid polypeptide with a molecular mass of 36 251 Da and an isoelectric point (p*I*) of 9.56. The results of a homology search indicated that sequences homologous to TTK are present in various kinase-like sequences as deduced from DNA sequences obtained from mouse testis, humans, zebrafish, *C. elegans*, and *Drosophila melanogaster* (Fig. 5). A partial amino acid sequence (141–298) showed 99% identity between brain and testis from the mouse. Another partial amino acid sequence of the mouse brain TTK is also highly homologous to a sequence of human-1 (86% identity, 3–108), human-2 (100% identity, 140–196), and zebrafish (79%, 1–155). The results thus suggest that TTK may be distributed ubiquitously among various species. The nucleotide sequence of the mouse brain TTK cDNA has been deposited in the GenBank/EMBL/DDBJ databases (accession no. AB046593).

3.6. Preparation of a GST–TTK fusion protein and assay of TTK activity

A GST–TTK fusion protein was prepared as described under Section 2. The fusion protein was contained in inclusion bodies. Nevertheless, a small amount of GST–TTK fusion protein was purified using glutathione Sepharose 4B. The products were then analyzed by SDS–PAGE (Fig. 6A). The GST–TTK fusion protein as detected in lane 4 is about 62 kDa in size, while the digests of the fusion protein (TTK at around 36 kDa and GST at 26 kDa) and the PreScission protease (46 kDa) are seen in lane 7. As shown in Fig. 6B, in the Western blot analysis the anti-TTK-C antibody stained the GST–TTK fusion protein at 62 kDa in lane 3 and the cleaved TTK at around 36 kDa in lane 4. The partially purified SP Sepharose 4B fractions of rat brain extracts (lanes 1 and 2), of bovine brain extract (lane 5), and of the microtubule-associated protein (MTP) (lane 6) also show the stained band corresponding to TTK around 36 kDa.

The activities of TTK on tau and tubulin were then examined for the GST–TTK fusion protein and the cleaved TTK. Fig. 6C-1 shows that the GST–TTK fusion protein phosphorylated both tau and tubulin, but the phosphorylating activity on tau was much higher than that on tubulin. While the cleaved TTK exhibited similar activities on tau and tubulin (Fig. 6C-2), the ratio for both substrates was mostly the same as was exhibited by the purified TTK from bovine brain reported in a previous paper [15]. The fragment not containing inserted TTK did not show any phosphorylating activity. The data in Fig. 6, therefore, prove that the recombinant TTK protein based on the mouse brain cDNA sequence has the same TTK epitopes as the TTK in bovine brain extracts and that it is able to phosphorylate tau and tubulin.

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                          20                  40
Bovine                          --MPSE-----E-M-L--- --
Mouse    MSGGGEQPDILSVGILVKERWKVLRKIGGGGFGEIYDALDMLTR ENVALK
Human-1   -----A---PANYV--D-----K-----------E-M-L--- ------
C.ele.   MTSSSEGE--Q--QVIR----IKA----------E-T-VQNHH-R--I-
Z.fish   ---TV--T---A--SM-R---R--K----------EV--HVNQ VS----
D.mel.     MTSE-L-QP-HV-------V-----------EGQ-LI-- -Q----

             60                  80                  100
Bovine          --------
Mouse    VESAQQPKQVLKMEVAVLKKLQGKDHVCRFIGCGRNDRFNYVVMQLQGRN
Human-1  ------------------------------S--EKL-----------
C.ele.   ---SKAT----------RR----K-A-K-Y------K---L--S---K-
Z.fish   ----------R----------V----------E-----
D.mel.   ----R-----------------E-----------------K-

                        120                 140
Bovine                       --------A-
Mouse    LADLRRSQSRGTFTISTTLRLGKQILESIESIHSVGFLHRDIKPSNFAMG
 testis                                          ----------
Human-1  --------
Human-2                                          ----------
C.ele.   ------EAPKQC-NL--AV-V-I---NG-RE---I------V--------
Z.fish   ------NM-H---SV------XRH---A--------------X-------
D.mel.   --E---A-P--A-SL-------L---KA-----------------SV-

             160                 180                 200
Bovine         -HY---------Y--TXXX-
Mouse    RFPSTCRKCFMLDFGLARQFTNSCGDVRPPRAVAGFRGTVRYASINAHRN
 testis ------------------------------------------------
Human-2  ----------------------------------------------CTSE
C.ele.   -TSQ-M-NVY---------YL-AK-EI-S--SA----------AVT--K-
Z.fish   PTYR-
D.mel.   -L-YN--RVY---------Y-TGT-E--C---A----------

                        220                 240
Mouse    REMGRHDDLWSLFYMLVEFVVGQLPWRKIKDKEQVGSIKERYDHRLMLKH
 testis ------------------------------------------------
Human-2  QGN-K
C.ele.   K----Q----------T--LQ----------DE--KM--EA-LVVL-DD
D.mel.   ----------N---------------LT--K-----L---

             260                 280                 300
Mouse    LPPEFSTFLDHISSLDYFTKPDYQLLTSVFDNSIKTFGVIESDPFDWEKS
 testis --------------X-----------------------------
C.ele.   C-H-MHL-VA-LKV-G-ADT---VY-E-LLNKIVAENEISWDE-Y---LG
D.mel.   --SDLKQ--E--Q--T-GDR---LM-IGL-ERCM-RR--K----Y----

                        320
Mouse    GTDGSLTTTTTSATPQCTPA
C.ele.   YDNMATRQKQQANGNLSARL
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Fig. 5. Alignment of amino acid sequences with the mouse brain TTK sequence. Amino acid residues 1–320 of the mouse brain TTK in Fig. 4 were aligned with the amino acid sequences of bovine brain TTK and various kinase-like amino acid sequences deduced from DNA sequences. Each GenBank accession number is indicated in parentheses: mouse testis (AI385971), human-1 (AI197837), human-2 (AA234451), zebrafish (z. fish, AW305688), *C. elegans* (C. ele., CER90), and *D. melanogaster* (D. mel., AC013215). Amino acid numbers are given at the top. Amino acids identical to those in the mouse brain TTK are indicated by dashes. X, not determined.

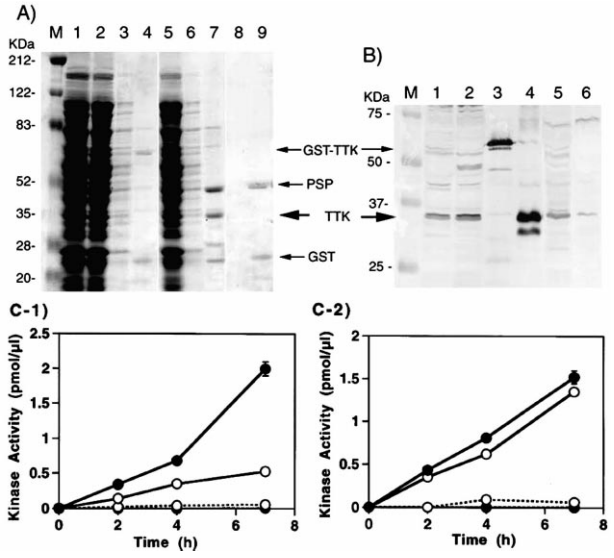

Fig. 6. Preparation of a GST–TTK fusion protein. (A) SDS–PAGE of the GST–TTK. GST–TTK fusion proteins were purified in tube 1 (lanes 2–4) with glutathione Sepharose 4B and then digested with PSP in tube 2 (lanes 5–9). Lane 1, supernatant of the *E. coli* BL21/pGEX-6p-2/TTK; lane 2, unbound fraction; lane 3, PBS wash fraction; lane 4, eluate with elution buffer; lane 5, unbound fraction; lane 6, PBS wash fraction; lanes 7 and 8 (eluates using the cleavage buffer) and 9 (eluate using the elution buffer) for the cleaved TTK. The gel was stained with CBB. M, molecular weight markers, broad range (Bio-Rad). GST–TTK, GST/TTK fusion protein; PSP, PreScission protease (46 kDa). (B) Western blot analysis with anti-TTK-C antibody. Lanes 1 and 2, SP-extracts from rat brain at 20 days and at 1 year of age, respectively; lanes 3 and 4, the same eluate as in lanes 4 and 7 in A, respectively; lane 5, SP-extract from bovine brain extract; lane 6, SP-extract from the MTP. M, molecular weight markers (Bio-Rad). (C) GST–TTK (the same fraction as in lane 4 in A) was used (2 μl/reaction mixture) to measure kinase activities on tau (solid line with ●) and tubulin (solid line with ○) (C-1). The cleaved TTK (the same fraction as in lane 7 in A) was used (2 μl/reaction mixture) to measure kinase activities on tau (solid line with ●) and tubulin (solid line with ○) (C-2). Samples for the control blank for tau (dashed line with ●) and tubulin (dashed line with ○) were obtained from pGEX-6p-2 with GST but no inserted TTK.

3.7. Distribution of TTK in various tissues of the rat

The distribution of TTK in various tissue extracts from the rat was studied by Western blot analysis. TTK is strongly expressed in the brain, heart and muscle in 8 day and 10 week old rats, while its distribution is lower in the liver, thymus, spleen, lung, kidney, testis, and ovary throughout both stages of the development (data not shown). Developmental changes in TTK expression were also examined for the rat brain. The expression increased gradually from the embryonic stage on and remained nearly constant after 15 days to up to 1 year. Phosphorylated tau was observed mostly between postnatal days 1–11 using anti-pS208, but not when using anti-pS210. The brain extracts from rats between 15 days and 1 year of age did not show immunoreactivity with either of the two antibodies (data not shown). The results seem to deviate from the distribution pattern of TTK in the rat brain. However, the distribution of phosphorylated tau in vivo must be considered in balance with kinases and phosphatases.

4. Discussion

TPKI/GSK-3β is a major candidate among the various kinases participating in the PHF formation. TPKI/GSK-3β tends to phosphorylate serine/threonine residues in a proline-rich environment, which is why the enzyme is called a proline-directed protein kinase. Godemann et al. have confirmed that recombinant GSK-3β shows a strong attraction for proline-directed motifs in tau in the order of Ser-396/Ser-404, Ser-46/Thr-50, and Ser-202/Thr-205 followed by others, while GSK-3β does not phosphorylate Ser-262 [10]. Reynolds et al. have reported that nanoelectrospray mass spectrometry was used to identify phosphorylation sites on recombinant tau for three MAP kinases and GSK-3β [11]. They also identified a new phosphorylation site, Thr-175, and one non-proline-directed phosphorylation site, Ser-400, in addition to the known GSK-3β phosphorylation sites on tau. TTK is a non-proline-directed Ser/Thr kinase that has been purified from bovine brain [15] and is, as we show here, the first kinase known to phosphorylate Ser-208 and Ser-210 of tau, both of which sites are PHF phosphorylation sites. Thr-212, which is a neighboring residue close to Ser-208 in tau, has been known to be a phosphorylation site for GSK-3β [24]. The specificity of anti-pS208 has been proven by the absorption test done with peptides pS208 and pS210. Therefore, we can be sure that the phosphorylation site Ser-208 is a site separate from Thr-212.

For accelerated phosphorylation of tau, TPKI/GSK-3β is known to require prior phosphorylation by another proline-directed protein kinase, TPKII [22], and a non-proline-directed protein kinase such as AMP-dependent protein kinase, protein kinase C, and casein kinase-II [25,26]. In our present study we found that TTK is also a kinase that increases the subsequent phosphorylation of tau by TPKI/GSK-3β. It seems that the precisely timed appearance of TTK may be a key event in the regulation of the hyperphosphorylation of tau by TPKI/GSK-3β in vivo. Rat brain extracts during postnatal days 1–11 were immunoreactive to anti-pS208 antibody, but extracts after 15 days and up to 1 year were not. The immunoreactive period corresponds to the peak of the expression of TPKI/GSK-3β and to the appearance of tau phosphorylated at Ser-199 and Ser-396 by TPKI/GSK-3β in the brain extracts [9]. Meanwhile, TTK exists throughout all developmental stages in the rat brain, increasing for up to 15 days after birth and then being maintained at the same level during the adult stage. We found that phosphorylated tau as well as TPKI/GSK-3β are distributed in the cell soma and the apical dendrites in the neocortex and the hippocampus of adults rats, in spite of the lower levels of phosphorylated tau, as determined by Western blot analysis, at the adult stage [19]. The results of the developmental distribution of TTK suggest, therefore, that TTK might contribute to the hyperphosphorylation of tau by TPKI/GSK-3β through the prior phosphorylation of tau in the adult brain and also the AD brain.

We were able to obtain the complete cDNA of mouse brain TTK and to determine the whole amino acid sequence. TTK can be expected to be distributed widely among various species based on our homology search results. This suggests that TTK has an important physiological role. The preparation of a recombinant TTK will help us to come to a clearer understanding of the properties of this kinase, which will contribute to the resolution of the significance of the hyperphosphorylation of tau in PHF.

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