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Identification of the 23 kDa subunit of tau protein kinase II as a putative activator of cdk5 in bovine brain

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Abstract

Tau protein kinase II (TPKII) was reported previously to be composed of a neuron-rich cdc2-related kinase (PSSALRE/cdk5) and 23 kDa subunit. Here we show that the 23 kDa subunit is a putative activator for the kinase activity. Amino acid sequence analysis revealed that the protein was novel and included a partial similarity of amino acids to a cyclin box important for the interaction with cdc2-related kinase. These results suggest that the 23 kDa subunit, but not cyclin, activates cdk5 in neuronal cells, which no longer exhibit cell cycling but are terminally differentiated cells.

Key words: Tau protein; Paired helical filament; Alzheimer's disease; Cyclin-dependent kinase 5; Tag sequence

1. Introduction

Paired helical filaments (PHF), β amyloid and neuronal death are characteristics of the brains of Alzheimer's disease patients. PHF contain abnormally phosphorylated tau protein. The phosphorylation reflects the physiological state in the brain of an Alzheimer's disease patient. An enzyme catalyzing this phosphorylation was found to be tau protein kinase (TPK, EC 2.7.1.135) in microtubule proteins of bovine brain [1]. Later, from the kinase fraction, we purified two protein kinases, TPKI and TPKII [2]. TPKI phosphorylated native tau in a PHF-like manner, indicating that TPKI is a strong candidate for an enzyme catalyzing the abnormal phosphorylation. TPKI is identical to glycogen synthase kinase (GSK) 3β [3]. TPKI/GSK3β was found to be involved in β amyloid-mediated neuronal death [4]. These results suggest that TPKI/GSK3β might be a key enzyme in the disease.

Native tau was already phosphorylated to some extent, and this normal phosphorylation enhanced the abnormal phosphorylation by TPKI/GSK3β, because TPKI/GSK3β recognized the normally phosphorylated sites on tau [5]. As a candidate for a kinase involved in the normal phosphorylation, we found TPKII. TPKII phosphorylates four SerPro or ThrPro sites in tau [6]. Prior phosphorylation of tau by TPKII enhanced phosphorylation by TPKI, indicating that TPKII may regulate the phosphorylation state of tau in Alzheimer's disease brain indirectly. Phosphorylation sites by TPKI [7] and TPKII [6] were close to each other and found to be phosphorylated in human PHF-tau [8] and rat juvenile-tau [9], suggesting that the TPKI/TPKII-phosphorylation system works both in Alzheimer's disease and juvenile brain. Two common features of these brains are sprouting [10] and neuronal death. It is likely that these TPKs are involved in one or both of these phenomena.

TPKII is known to have a cdc2-like kinase activity. This is supported by its high substrate specificity for histone H1 [2], its recognition of the Ser/ThrPro sequence [6,11] and its kinase activity towards the neurofilament-H subunit (NF-H) [12,13] in a manner similar to cdc2 kinase [14].

TPKII is composed of a 30 kDa catalytic subunit and a 23 kDa subunit [2]. The 30 kDa subunit is a cdc2-related kinase known as PSSALRE/cdk5 [15]. The involvement of PSSALRE/cdk5 in the abnormal phosphorylation on PHF-tau in brain has been recently supported also by other groups [16,17]. This kinase was originally found as a cdc2-related kinase having a PSSALRE sequence instead of a PSTAIRE sequence shared by the well-known cdc2-related kinase [18]. Later, the PSSALRE kinase was reported to be associated with D type cyclin in human fibroblasts and has been called cyclin-dependent kinase 5 (cdk5) [19]. Recently, PSSALRE/cdk5 was also cloned from a rat brain cDNA library as a neuronal cdc2-like kinase (nclk) [20]. Lew et al. purified a proline-directed protein kinase homologue from bovine brain, using a good substrate peptide (pro-

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Src) for detecting cdc2-related kinase activity [21]. The kinase (brain proline-directed protein kinase, BPDK) was also composed of cdk5 and another subunit (25 kDa) [22]. Although the estimation of the molecular weight was different, the accessory protein seems to be identical to the 23 kDa subunit. The cDNA cloning and the enzyme purification from brain indicate that cdk5 is the most prominent cdc2-related protein kinase in the brain.

Cdc2-related kinases (cyclin-dependent kinases) are active only in complexes with their regulatory subunits known as cyclins [23]. It seems reasonable that the 23 kDa subunit may act as a regulatory factor for TPKII phosphorylation. In order to understand the role of the 23 kDa subunit, it is important to determine the amino acid sequence and function of the 23 kDa subunit.

Here, we show its sequence and suggest a role for this subunit.

2. Materials and methods

2.1. Materials

Bovine TPKII was purified by the method described previously [2]. The anti-30 kDa subunit antibody was also described previously as an antibody against peptide 2 [15]. Anti-cyclin D1 antibody was purchased from UBI.

2.2. Sequencing

The TPKII-23 kDa subunit was separated from the 30 kDa subunit by reverse-phase column (C4, Aquapore BU-300, 2.1 × 30 mm, Applied Biosystems) chromatography with a linear gradient (0.9 ml) of 0–70% acetonitrile in 0.1% trifluoroacetic acid, and digested by *Acromobacter lyticus* protease I (API) (Wako Pure Chemical Industries) and protease V8 (Boehringer Mannheim) in a reaction mixture of 4 M urea, 20 mM methylamine, 1 mM ethylenediaminetetraacetic acid and 40 mM sodium phosphate buffer (pH 8.5) at a protein/weight ratio of 10:1 at 37°C for 18 h. Several peptides were purified from the digest by reverse-phase column (C8, Aquapore RP-300, 2.1 × 30 mm, Applied Biosystems) chromatography under the same condition as mentioned above. The amino acid sequences were determined by a pulse-liquid-phase amino acid sequencer (Applied Biosystems 477A protein sequencer).

Probes 1 and 2 were guessmers chemically synthesized based on the amino acid sequences of the corresponding peptide fragments 1 and 3 of the 23 kDa subunit, respectively. Sequences of probe 1 and 2 were 5′-ATGCT[C/G]CAGAT[C/T]AA[C/T]GA[C/T]CC[C/T/A]CACTAC-TTCAC-3′ and 5′-GC[C/T]CAGCC[C/T]CC[C/T]CC[C/T/A]GC[C/T]CAGCC-3′, respectively. cDNA of the bovine TPKII-23 kDa subunit was cloned from a bovine brain cortex cDNA library (Clontech) using probes 1 and 2.

The cDNA was ligated to the universal linker that was cut at the *Eco*RI sites and inserted into the vector, pUC19. DNA was sequenced by dideoxy-mediated sequencing [24]. PCR was done by a GeneAmp DNA amplification reagent kit (Perkin-Elmer Cetus) using a cDNA clone as a template and two primers. Sequences of the sense primer (primer 1) and antisence (primer 2) were 5′-GCAGCTATCAGAAC-AACATCACGCACC-3′ and 5′-TTCCTGCGGAGCTGACGGCAG-GGTGC-3′, respectively.

2.3. Other methods

Peptides were synthesized by the solid phase method, using a Biosearch model 9500 peptide synthesizer. The sequence of the synthetic peptide substrate (pro-Src) was RRPDAHRTPNRAF. An antibody against the peptide (KAQPPPAQPPAPPASQLSGSQTGV) was prepared by the method described previously [15]. Immunoblotting was performed with an ABC kit (Vector Laboratories).

3. Results

The 23 kDa subunit was separated from cdk5 using reverse-phase column chromatography. A digest of the 23 kDa subunit was made using lysylendopeptidase (API) and V8 protease. Three peptides obtained from the digest were sequenced (Fig. 1). The N-terminal amino acid sequence of the 23 kDa protein is identical to that of fragment 1. A search for homologous proteins based on the sequences of these peptides revealed that these sequences are novel. We prepared an antibody against the peptide corresponding to amino acid residue number 1 to 23 of fragment 1. This antibody was confirmed to react with the 23 kDa subunit.

Cdk5 was also found to be a catalytic subunit of BPDK [22], cdc2-related kinase purified from bovine brain by Lew et al.[21]. BPDK was reported to contain a 25 kDa subunit. In order to compare the 25 kDa subunit with the TPKII-23 kDa subunit, the anti-23 kDa subunit antibody was used as a probe monitoring the presence of the 23 kDa subunit during the purification of cdk5 from new-born bovine brain by their method [21]. We found that the kinase activity phosphorylating cdc2 kinase-specific substrate (pro-Src peptide) was eluted at an apparent molecular weight of 50,000, together with the 23 kDa subunit immunoreactivity at gel filtration step (Fig. 2), indicating that the active kinase contains the 23 kDa subunit. On the other hand, most of the cdk5 immunoreactivity was eluted at a molecular weight of 30 kDa, indicating that most of cdk5 exists as an inactive monomer. The result suggest that TPKII is

cycle	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
fr. 1	A	Q	P	P	(P)	A	Q	P	P	A	P	P	A	S	Q	L	S	G	S	Q	(T)	G	V	(S)	(S)	(S)
fr. 2	K	A	P	(H) (P)	P	A	V	S	(S)	A	G	T														
fr. 3	M	L	Q	I	N	A	D	P	(H)	(Y)	(F)	(T)														

Fig. 1. Amino acid sequence of three peptide fragments (fragment 1, 2 and 3) obtained from a digest of the TPKII-23 kDa subunit. Residues of which the identities are uncertain from protein sequence analysis are in parentheses.

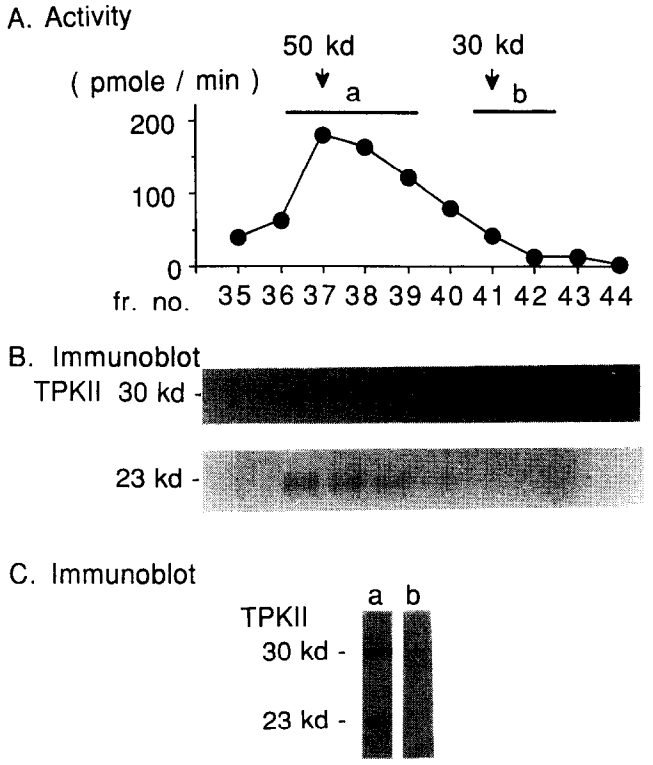

Fig. 2. Gel filtration of cdc2-related kinase activity. (A) The kinase activity. The activity phosphorylating pro-Src peptide was eluted at apparent molecular weight of 50 kDa. (B) 5 μl and 20 μl of the fractions were immunoblotted with anti-30 kDa subunit (cdk5) and anti-23 kDa subunit antibodies, respectively. (C) Fractions a and b in panel A were collected and double-immunoblotted with anti-30 kDa subunit and anti-23 kDa subunit.

identical to BPDK. In addition, the active fraction did not react with anti-cyclin D1 antibody by immunoblot analysis (data not shown), though cyclin D1 was reported to associate with cdk5 in fibroblast [19]. In neural cells, the 23 kDa subunit may be an activator for cdk5 in a manner similar to cyclin for cdc2-related kinase.

Using guessmers corresponding to fragment 1 and 3 as hybridization probes (probe 2 and 1, respectively), 3 cDNA clones were obtained from 4×10^5 clones of a bovine brain cortex cDNA library. Sequencing of two of these cDNAs revealed that the cDNA had sequences corresponding to all three peptide fragments that we obtained (Fig. 3). A termination codon was found at position 628 (Fig. 3B). From the Ala at position 1 to the TGA termination codon at 628, there are 209 amino acid residues coding for a protein of 23,000 Da. We have determined the sequence of 183 nucleotides upstream of the Ala codon at position 1, and found neither an initiation codon nor a termination codon in the open reading frame indicated in Fig. 3A. Cleavage of the precursor of the 23 kDa protein is between a phenylalanine and the alanine at position 1.

Cyclins share a common sequence which is thought to be involved in interaction with cdc2-related kinase [25,26]. The region is called a cyclin box. Cdk5 was reported to bind to cyclin D1 in fibroblasts [19]. It is interesting that the 23 kDa subunit contains a region of weak homology with the cyclin box of cyclin D1 (Fig. 4). This region may associate with cdk5.

The 23 kDa subunit has no homologous sequence to human $p13^{suc1}$, another protein binding to cdc2 kinase [27], indicating that the interaction of the 23 kDa subunit with cdk5 is not similar to that of $p13^{suc1}$ with cdc2 kinase.

The C-terminal half of the 23 kDa subunit is homologous to the human expression sequence tag (EST02338) (Fig. 3), recently reported from work involved with the human genome project [28–30]. No protein encoded by the gene containing EST02338 has previously been reported. However, we now have identified EST02338 as a partial sequence encoding the human 23 kDa subunit of TPKII. We observed only 17 differences in a stretch of 281 nucleotide residues (94% identity). The differences do not change amino acid residues, indicating that the protein is highly conserved between bovine and human.

4. Discussion

It is concluded from the experiments reported here that TPKII is comprised of two subunits; cdk5, a catalytic subunit, and a 23 kDa regulatory subunit. Determination of the location of cdk5 upon gel filtration showed that cdk5 can be found in two different size fractions; a 30 kDa monomer which had no kinase activity and a 50 kDa complex (cdk5 and the 23 kDa subunit) which was able to phosphorylate the pro-Src substrate, indicating that the 23 kDa subunit is an activator of PSSALRE/cdk5 in brain cells. This observation is supported by the work of others [17]. It has been well established that cdc2-related kinases use members of the cyclin family as activators. Cdc2-related kinases bind to different members of the cyclin family in different parts of the cell cycle [23] and this interchange of cdc2-related kinases with the different cyclins is thought to regulate the progression of the cell through the cell cycle. The lack of similarity between the 23 kDa subunit of TPKII and cyclins, taken together with the fact that neurons are terminally differentiated cells not progressing through a cell cycle, suggests that the 23 kDa protein is not a cyclin. Association of cdk5 with cyclin D1 in fibroblasts [19] and with the 23 kDa protein in neurons may reflect the need to recognize different substrates in these two distinctly different cell types.

There is weak homology between the 23 kDa protein and cyclins within the cyclin box. The cyclin box [25] has been shown to be sufficient to account for the binding of cdc2-related kinases and cyclins [26]. Interestingly, the cyclin box with most similarity to the 23 kDa protein is that of cyclin D1 [31] which also binds to cdk5 [19]. It

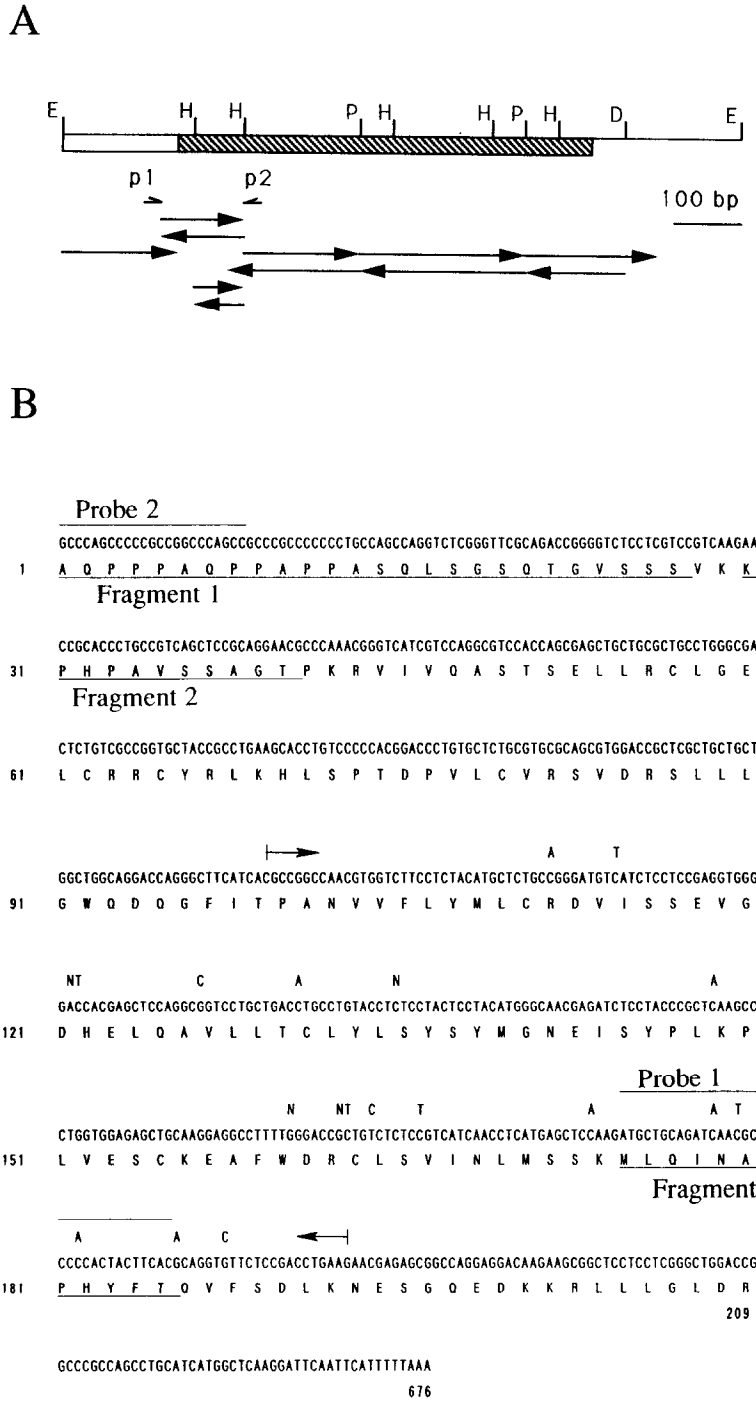

Fig. 3. Structure of cDNA No. 5 of bovine TPKII-23 kDa subunit. (A) Restriction map of the cDNA No.5 of bovine TPKII-23kDa. Its length is about 1020 bp. Arrows indicate the region and direction of DNA strand that the sequencing analysis has been done. Two small arrows, p1 and p2, indicate the hybridizing positions of primers for PCR. The PCR product has also been sequenced to confirm the sequence. Abbreviations of restriction points are as follows: E, *Eco*RI; P, *Pst*I; D, *Dra*I; H, *Hae*III. The box indicates an extensive open reading frame; the hatched portion of the box corresponds to the 23 kDa subunit. (B) Sequence of the bovine TPKII-23 kDa subunit. The nucleotide sequence starts at the site corresponding to the N-terminal of the 23 kDa subunit and ends at the *Dra*I site. The full length of the amino acid sequence of the subunit is shown. Fragment 1, 2 and 3 indicate sequences of peptides obtained from API and V8 digests. Oligonucleotide probes 1 and 2 were used for cDNA cloning. A nucleotide sequence of human tag EST02338 is indicated by the sequence between two arrows below that of the 23 kDa subunit. Only differences in the sequences are indicated by characters in the lines marked EST.

may be that binding of the 23 kDa protein to cdk5 is through these residues that exhibit homology to cyclin D1.

The 23 kDa subunit was derived from a large precursor by cleavage between a phenylalanine and alanine residue, suggesting that a chymotrypsin-like protease is

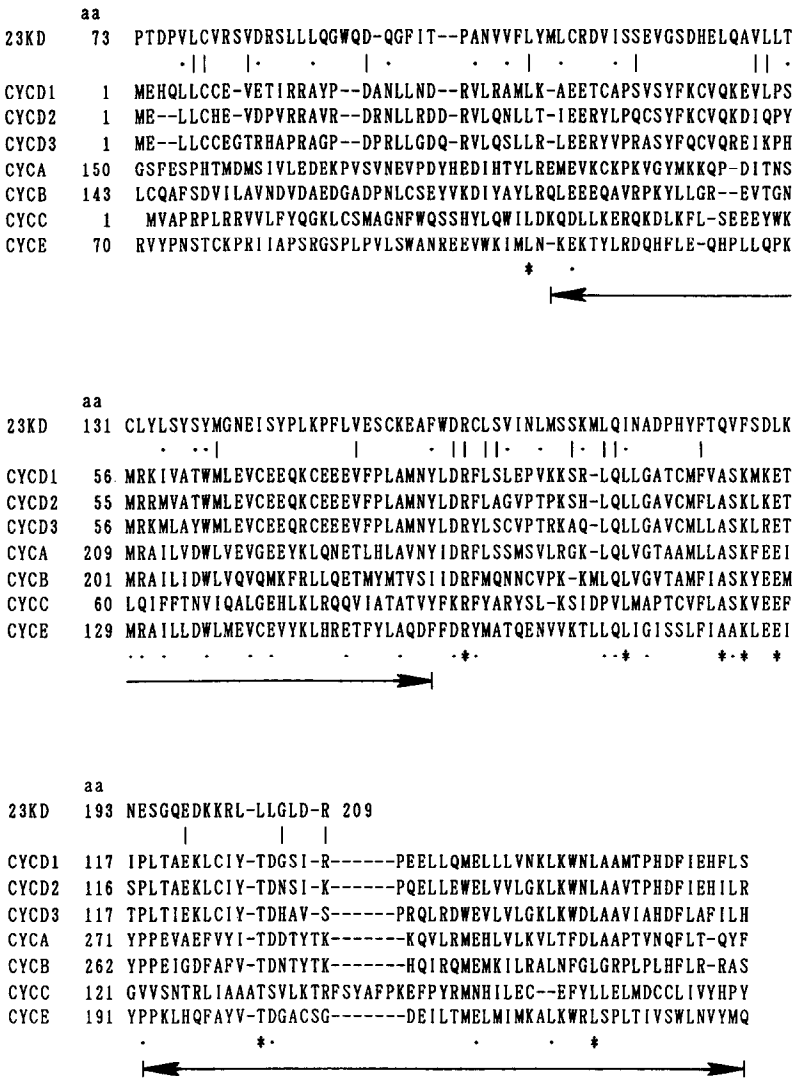

Fig. 4. Homology between the 23 kDa subunit and cyclins. A partial amino acid sequence of 23 kDa subunit (23KD) is compared with those of the cyclin box region of human cyclin A (CYCA) [32], B (CYCB) [33], C (CYCC) [34], D1 (CYCD1) [34], D2 (CYCD2) [35], D3 (CYCD3) [35] and E (CYCE) [34]. Asterisks and dots under the sequences are completely and almost conserved amino acid residues among these cyclins, respectively. Arrows indicate binding regions of cyclin A to cdc2 kinase [26]. Vertical lines and dots between the 23 kDa subunit and cyclin D1 indicate the identical and homologous amino acid residues between the two proteins, respectively, both of which can bind to cdk5.

responsible for the generation of the 23 kDa protein. We know from sequence analysis of our current clones that the precursor must be at least 30 kDa. We are presently in the process of determining the exact size of the precursor.

Comparison of the amino acid sequence with proteins in the data base did not show any protein closely related to the 23 kDa subunit. However, when compared to the expression sequence tag (EST) sequences, we found that EST02338 from a human cDNA library contains a partial coding region for a protein that is 94% identical in sequence to the 23 kDa subunit. ESTs are regarded as a powerful physical mapping strategy in the human genome project [28]. Automated partial DNA sequencing was conducted on many randomly selected human brain cDNA clones to generate ESTs [29]. A proportion of ESTs encode unknown proteins [30]. Now, one of the ESTs has been identified as the partial sequence encoding a homolog of an important component of TPKII, a kinase that is associated with the developmental stage of the neuron and with Alzheimer's disease.

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