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A cdc2-related kinase PSSALRE/cdk5 is homologous with the 30 kDa subunit of tau protein kinase II, a proline-directed protein kinase associated with microtubule

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We previously reported that tau protein kinase II (TPKII) from bovine brain was composed of 30 kDa and 23 kDa subunits. The 30 kDa subunit of TPKII can be regarded as a catalytic subunit because of its ATP-binding activity. Antibodies directed against TPKII-phosphorylated tau also reacted with tau phosphorylated by cdc2 kinase obtained from starfish oocytes, indicating that TPKII and cdc2 kinase phosphorylate the same sites. We determined the amino acid sequence of the 30 kDa subunit and found it to be homologous with a cdc2-related kinase, PSSALRE/cdk5. Moreover, an antibody against PSSALRE/cdk5 reacted with the 30 kDa subunit. These results indicate that the 30 kDa subunit of TPKII is bovine homologue of PSSALRE/cdk5. Expression of the 30 kDa subunit mRNA was enhanced in juvenile rat brain. This result supports our previous hypothesis that the kinase works actively in juvenile brain.

Tau protein; Paired helical filament; Alzheimer's disease; cdc2-relaed protein kinase; Cyclin-dependent kinase 5

1. INTRODUCTION

Paired helical filaments (PHFs) accumulate in the brains of Alzheimer's disease patients. One of their major components is abnormally phosphorylated tau protein. It is important to determine the kinase phosphorylating tau for elucidation of the mechanisms underlying the formation of PHF. From microtubule protein we have purified a protein kinase phosphorylating tau into the PHF-like state [1,2]. We call the enzyme tau protein kinase (TPK) I. TPKI has been found to be identical to glycogen synthase kinase (GSK) 3β [3]. Other groups also reported that GSK3 is involved in PHF formation [4,5]. In addition to GSK3, mitogen-activated protein (MAP) kinase has also been considered as a candidate for an enzyme involved in abnormal phosphorylation of tau [6–9]. During the purification of TPKI/GSK3β, we identified another protein kinase named TPKII [2]. Prior phosphorylation of tau by TPKII enhanced phosphorylation by TPKI/GSK3β, indicating that TPKII may regulate the phosphorylation state of tau not only in normal brain but also in Alzheimer's diseased brain [10], though indirectly. Moreover, we determined phosphorylation sites on tau by TPKII, Ser-202, Thr-205, Ser-235 and Ser-404 [11], and later phosphorylation sites by TPKI/GSK3β, Ser-199, Thr-231, Ser-396 and Ser-413 [12]. The amino acid numbering is in the longest human tau isoform [13]. Phosphorylation of most of these sites in PHF-tau are supported by other groups [14–17]. In order to understand the phosphorylation mechanism observed in PHF-tau, it is important to determine the identity of TPKII.

The kinase is composed of two subunits, whose molecular weights are 30 kDa and 23 kDa determined by their mobilities on SDS-PAGE. The 30 kDa subunit bound to an ATP-analogue, suggesting that the subunit was a catalytic one. One of the best substrates of TPKII was histone H1 [2]. The kinase phosphorylated serine/threonine proline sequence [11,18]. These results suggest that the kinase resembles cdc2 kinase. Here, we show that the sequence of 30 kDa subunit is identical to brain-proline directed protein kinase from bovine brain [19], which is just homologous with PSSALRE from human cell line [20], cyclin-dependent kinase 5 (cdk5) of human fibroblasts [21] and neuronal cdc2-like kinase (nclk) from rat brain cDNA library [22].

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2. MATERIALS AND METHODS

2.1. *Materials*

Bovine tau, TPKI and TPKII were purified by the method described previously [2]. Starfish oocyte cdc2 kinase was a kind gift from Drs.

T. Kishimoto and S. Hisanaga (Tokyo Institute of Technology). The kinase was prepared according to the method described elsewhere [23] with some modification (Okumura and Kishimoto, in preparation). Antibodies specific to TPKII-phosphorylated tau sites, anti-PS202, anti-PT205, anti-PS404, were described in our previous paper as anti-PS144, anti-PT147 and anti-PS315, respectively [10]. The amino acid numbering in this paper is in the longest human tau [13], whereas that in the previous paper was in the shortest human tau [24]. Anti-human cyclin D antibody was purchased from UBI.

2.2. *Sequencing*

The TPKII-30 kDa subunit was separated from 23 kDa subunit by reverse-phase column (C4, Aquapore BU-300, 2.1 × 30 mm, Applied Biosystems) chromatography with 0.9 ml linear gradient of 0–70% acetonitrile in 0.1% trifluoroacetic acid, and digested with Acromobacter protease I (API) (Wako Pure Chemical Industries) or protease V8 (Boehringer Mannheim) in the reaction mixture of 4 M urea, 20 mM methylamine, 1 mM ethylenediaminetetraacetic acid and 40 mM sodium phosphate buffer (pH 8.5) at 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 mentioned above. The amino acid sequences were determined by a pulse-liquid-phase amino acid sequencer (Applied Biosystems 477A protein sequencer).

Primers 1 and 2 and probe B were chemically synthesized based on the sequences of human cdc2-related kinase, PSSALRE [20] (see Fig. 1). Probe A was synthesized as a double-stranded form by primer extension of the annealed complex of primers 1 and 2 using the Klenow fragment and 4 dNTPs including labelled dCTP. cDNA of bovine TPKII-30 kDa subunit was cloned from a bovine brain cortex cDNA library (Clontech) using probes A and B. DNA was sequenced by dideoxy-mediated sequencing [25].

2.3. *Other methods*

Peptides were synthesized by the solid phase method, using a Biosearch model 9500 peptide synthesizer. For immunization, the synthetic oligopeptides were covalently crosslinked to the carrier protein, keyhole limpet hemocyanin, and injected into the footpad and fingers of rabbits every three weeks and for a total of three times. Immunoblotting was performed with ABC kit (Vector Laboratories).

Total RNA was extracted from rat brain by the acid guanidinium thiocyanate-phenol-chloroform method [26]. Using magnetic oligo dT beads (Dynabeads, Dynal), poly(A)$^+$ RNA was separated from the total RNA.

3. RESULTS

TPKII resembles cdc2 kinase as judged from two aspects: (i) that it is also a proline-directed Ser/Thr kinase [11] and (ii) that its best substrate is histone H1 [2].

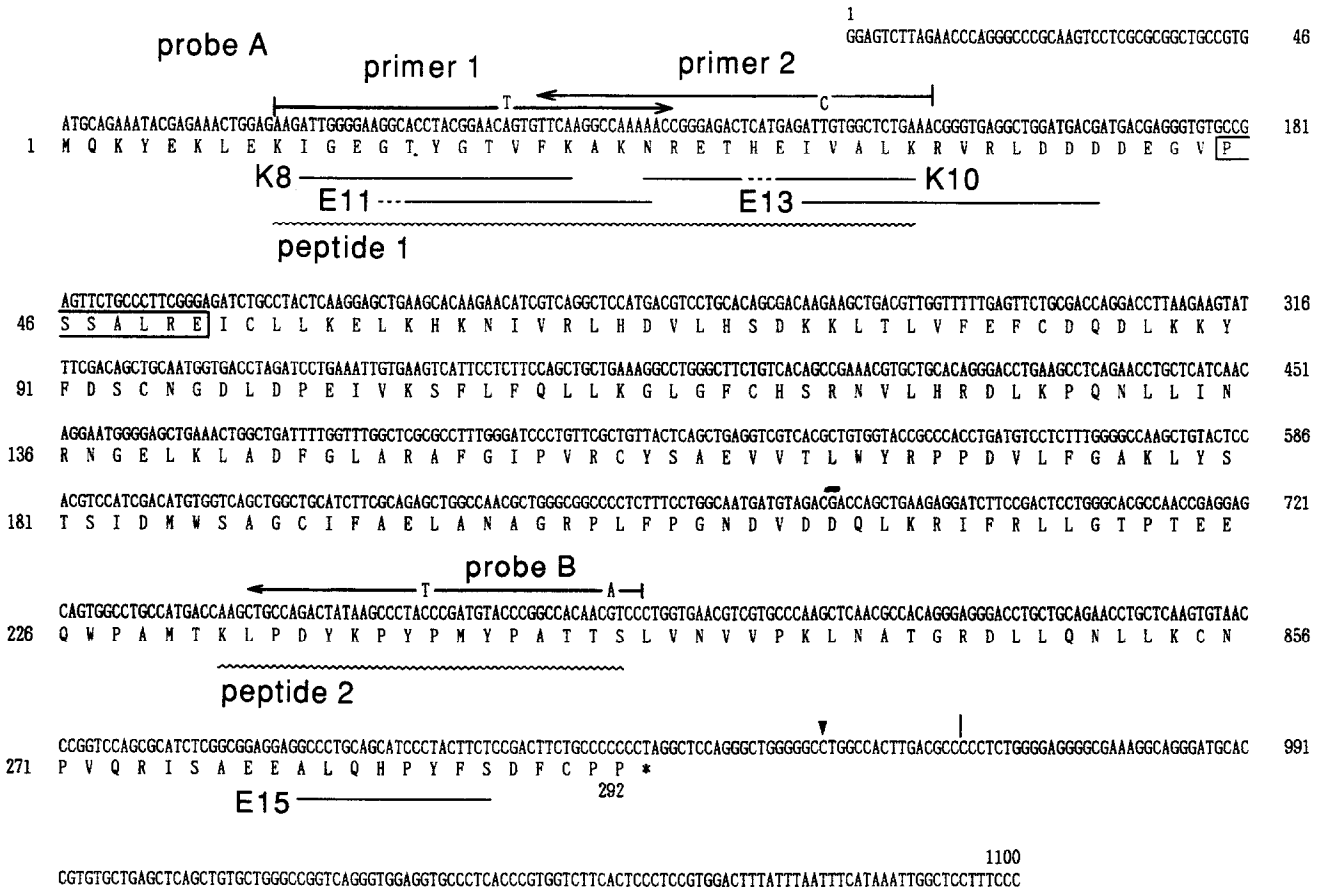

Fig. 1. Sequence of bovine TPKII-30 kDa subunit. K8 and K10 indicate sequences of peptides obtained from API digests. E11, E13 and E15 indicate sequences of peptides obtained from V8 digests. Peptides 1 and 2 are antigens for preparation of anti-30 kDa subunit and anti-PSSALRE/cdk5, respectively. An oligonucleotide probe A was prepared by primer extension of the annealed complex of primer 1 and primer 2. Probes A and B were used for cDNA cloning. Differences in sequence between probes or primers and cDNA are indicated by characters in arrows. The sequence of 5′-non-coding and coding regions is completely identical to that of brain-proline directed protein kinase [19]. In the 3′-non-coding region, a triangle indicates the addition of a single nucleotide, and the downstream sequence beyond the vertical line has not yet been reported.

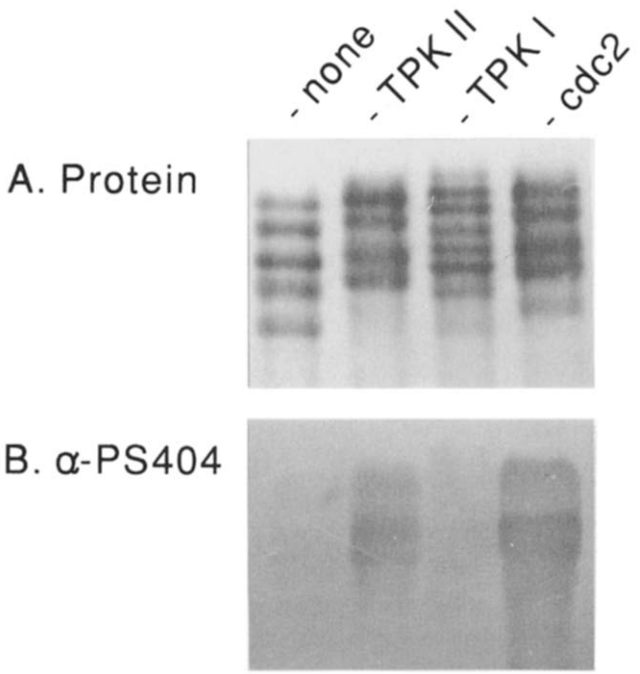

Fig. 2. Comparison between TPKII and cdc2 kinase on phosphorylation of tau. Bovine tau was phosphorylated by TPKII, TPKI or cdc2 kinase. (A) Protein staining of these tau preparations on SDS-PAGE by Ag stain Daiichi (Daiichi Kagaku). (B) Immunostaining of tau with anti-PS404 (α-PS404), an antibody specific to the phosphorylation site, Ser-404.

We first checked that cdc2 kinase phosphorylated the same sites on tau that TPKII phosphorylated. We reported three antibodies specific for the phosphorylation sites; anti-PS202, anti-PT205 and anti-PS404. Previously we called these anti-PS144, anti-PT147 and anti-PS315, respectively [10], according to the amino acid numbering of the shortest human tau [24]. Fig. 2 shows that anti-PS404 also reacted with tau phosphorylated by starfish oocyte cdc2 kinase, as anti-PS202 and anti-PT205 did (data not shown), indicating that TPKII has a similar function to cdc2 kinase.

The 30 kDa subunit was separated from the 23 kDa subunit using reverse-phase column chromatography. The 30 kDa subunit was digested with lysylendopeptidase (API) or protease V8, and several peptides obtained from the digests were sequenced. A search for homologous proteins based on the sequences of these peptides revealed that these sequences are found in PSSALRE, a cdc2-like protein kinase, which was discovered from human cDNA clones with polymerase chain reaction (PCR) amplification using two oligonucleotide primers corresponding to conserved sequences of the cdc2 kinase family [20] (Fig. 1). Later, this kinase was found to associate with cyclin D, so it has been called cyclin-dependent kinase 5, cdk5 [21].

To confirm that the 30 kDa subunit is identical to PSSALRE/cdk5, we next prepared antibodies against the 30 kDa subunit and PSSALRE/cdk5. One antibody was prepared against peptide 1 corresponding to the sequence which we had determined. Another antibody was raised against peptide 2, present in the sequence of PSSALRE/cdk5 but not found in the digests of TPKII. Immunoblot analysis showed that both antibodies reacted with the 30 kDa subunit, confirming the identity between the 30 kDa subunit and PSSALRE/cdk5 (Fig. 3).

Next we cloned TPKII cDNA from a library of bovine brain cortex using two oligonucleotide probes A and B corresponding to peptides 1 and 2, respectively. From 6×10^5 cDNA clones, we obtained two clones which hybridized with both probes. Sequencing of the cDNAs revealed that the nucleotide sequence is almost identical to that of PSSALRE/cdk5 [20], and that amino acid sequence deduced from the nucleotide sequence is identical except for a few residues. At the sequence 86 to 92, our bovine clone has DLKKYFD, while human PSSALRE has DLK-NFD [20]. Amino acid residue 229 is Ala in ours, but Ser in human PSSALRE. The nucleotide sequence of our clone is completely identical to that of bovine brain-proline directed protein kinase [19], except that ours is 14 nucleotides shorter in the 5′-noncoding region and 143 nucleotides longer in the 3′-noncoding region including one nucleotide addition at no. 943. Based on the sequence, the molecular weight of TPKII-30 kDa subunit is ca. 33,300.

These results indicate that the 30 kDa subunit is the bovine homologue of PSSALRE/cdk5. cdk5 associates with cyclin D in fibroblasts [21]. However, the purified TPKII fraction from brain contained a 23 kDa protein

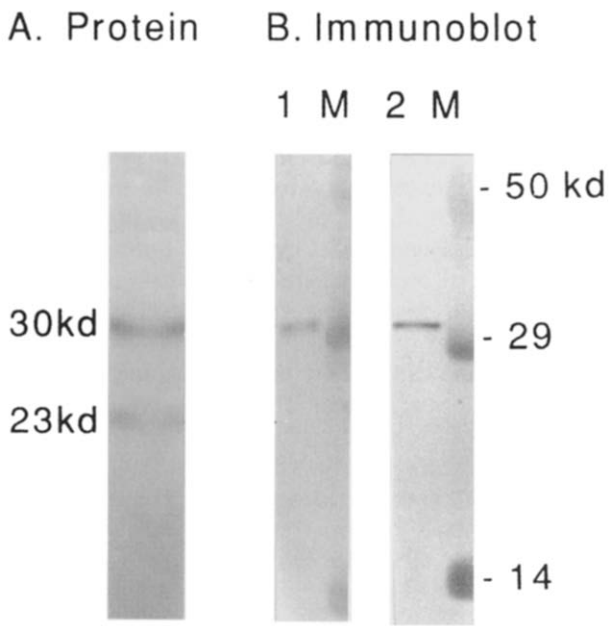

Fig. 3. Immunoblot analyses of TPKII. (A) Protein staining of TPKII on SDS-PAGE. Two polypeptides (30 kDa and 23 kDa) of TPKII are indicated. (B) Immunoblotting of 30 kDa polypeptide. TPKII were immunostained with antibodies raised against peptide 1 (lane 1, 1:30) and against peptide 2 (lane 2, 1:30). Lanes M indicate positions of prestained molecular weight markers (BRL, 50, 29 and 14 kDa).

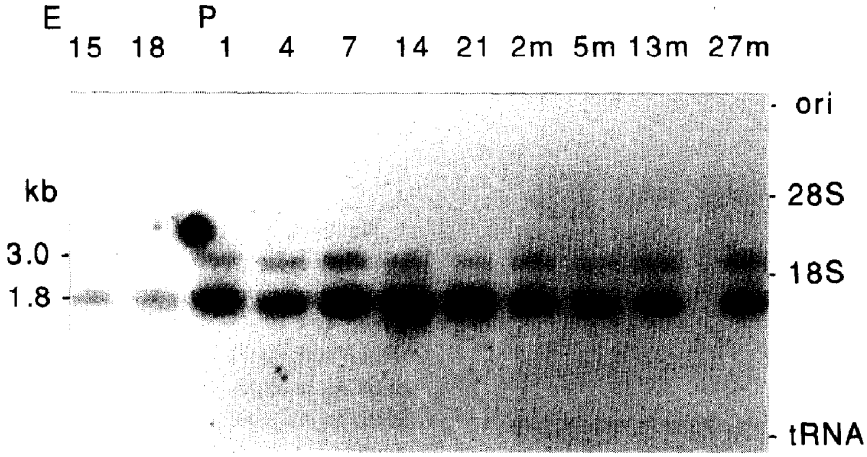

Fig. 4. Developmental change of expression of mRNA of TPKII-30 kDa subunit in rat brain. Poly(A)$^+$ RNA was prepared from rat brains at embryonic day 15 and 18, and postnatal day 1, 4, 7, 14, 21, and month 2, 5, 13, 27. 1.5 μg of poly(A)$^+$ RNA were subjected to Northern analysis using an *Eco*RI fragment (1.1 kbp) of bovine TPKII cDNA as a probe.

[2] instead of cyclin D (M.W. 35 kDa). The 23 kDa protein was not immunostained with anti-human cyclin D antibody.

Northern analysis using the cDNA probe revealed that the major form of mRNA of PSSALRE/cdk5 is 1.8 kb with the minor form of 3.0 kb in rat brain. These sizes are identical to those of rat PSSALRE/cdk5 (nclk) mRNAs [22]. The Northern analysis also revealed that the mRNAs were the most abundant in the juvenile period (Fig. 4), confirming our previous observation that the kinase works actively at this period [10].

4. DISCUSSION

We present here evidence that the 30 kDa subunit of TPKII is the bovine homologue of PSSALRE/cdk5. PSSALRE/cdk5 was originally found as a cdc2-related kinase having PSSALRE sequence instead of the PSTAIRE sequence shared by the wellknown cdc2-related kinases [20]. Later, the PSSALRE kinase was reported to be associated with cyclin D in human cells and has been called cyclin-dependent kinase 5 (cdk5) [21]. Considering this history, we will call the 30 kDa subunit PSSALRE/cdk 5.

From bovine brain, Lew et al. also purified a cdc2 kinase homologue, its components were a 33 kDa subunit and a 25 kDa subunit [27]. Later, the 33 kDa subunit was found to be PSSALRE/cdk5 [19]. Although PSSALRE/cdk5 associated with cyclin D in fibroblasts, TPKII and Lew's enzyme fraction did not contain cyclin D. Instead, PSSALRE/cdk5 was associated with another subunit, the molecular weight of which is estimated with SDS-PAGE to be 23 kDa by us [2] and 25 kDa by Lew et al [27]. The 23 kDa subunit seems to be important for kinase activity. Using antibodies against PSSALRE/cdk5, it was found that most of the 30 kDa protein existed alone as an inactive monomer. On the other hand, the kinase was activated when it coexisted with the 23 kDa protein, suggesting that the 23 kDa component is necessary for the kinase activity (in preparation). Moreover, the molecular weight of TPKII was 50,000 on gel filtration [2]. These results suggest that TPKII is a complex of PSSALRE/cdk5 and the 23 kDa subunit. PSSALRE/cdk5 was also reported to be associated with proliferating cell nuclear antigen (PCNA) in fibroblasts [21]. PCNA (M.W. 36 kDa) is, however, different from the 23 kDa subunit in the molecular weight. The identity of 23 kDa subunit remains unclear. Further study is in progress.

PSSALRE/cdk5 cDNA was also cloned from a rat brain cDNA library as a neuronal cdc2-like kinase (nclk), which was expressed at high levels in neurons [22]. Since PSSALRE/cdk5 was purified because it was enzymatically the most active cdc2-like kinase from brain [19,27], it is quite reasonable to think that it is the most prominent cdc2-related protein kinase in the brain. In neurons, the accessory protein of PSSALRE/cdk5 is not cyclin but a 23 kDa [2] or a 25 kDa subunit [27], which does not function as a cyclin because neurons are terminally differentiated cells and no longer in the cell cycle.

Another substrate for TPKII is neurofilament subunit H (NF-H) [28]. NF-H is highly phosphorylated in axons but not in dendrites and cell bodies [29]. Phosphorylation decreased its electrophoretic mobility significantly and weakened the interaction between neurofilament and microtubule. Hisanaga et al. showed that starfish oocyte cdc2 kinase was responsible for the phosphorylation [30]. Later, TPKII was found to phosphorylate NF-H in a manner similar to cdc2 kinase [28,31]. Further support comes from a report that PSSALRE/cdk5 from bovine brain phosphorylated NF-H [19]. It is likely that TPKII rather than another cdc2-like kinase is responsible for the phosphorylation of tau and NF-H in vivo. Both tau and NF-H are specifically localized to neurons [32,33] with tau being most

abundant in axons. PSSALRE/cdk5 is localized predominantly in neural tissue [22] and TPKII is associated with tau on microtubule surfaces [1] and also seems to be close to NF-H in axons.

A proline-directed protein kinase ($p34^{cdc2}/p58^{cyclin\ A}$) was reported to phosphorylate Ser-202, Thr-205, Thr-231 and Ser-235 on tau [34]. Phosphorylation of these sites is thought to be important for conversion of normal tau into PHF-tau. Others have also pointed out the importance of cdc2 kinase for the conversion [7]. Phosphorylation of tau by TPKII enhanced phosphorylation by TPKI/GSK3β which converts normal tau into PHF-tau [10]. Although TPKI/GSK3β is critical for conversion of tau into PHF-tau [2,4,5], TPKII is also important for the first step of phosphorylation of tau. From this point, it is cooperation of TPKII and TPKI/GSK3β that induces tau into the PHF-like state.

The conversion is also induced by mitogen-activated protein kinase (MAP kinase) [6–9]; sometimes called extracellular signal-regulated protein kinase (ERK). It remains unresolved which kinase(s) works in Alzheimer's disease brain, MAP kinase or TPKI/GSK3β and TPKII. Takashima et al. reported that TPKI/GSK3β increased in β amyloid-mediated neuronal death [35]. If the phosphorylation of tau participates in the neuronal death, not only TPKI/GSK3β but also TPKII should be important for the process.

The phosphorylation state of tau in Alzheimer's disease brain is similar to that in juvenile brain [36]. We found that phosphorylation by TPKII was enhanced in juvenile brain [10]. This is supported by our observation that mRNA of PSSALRE/cdk5 was abundant in young brain. Common features between juvenile brain and Alzheimer's disease brain are sprouting [37] and neuronal death. It is likely that TPKII and TPKI/GSK3β is involved in somatodendritic sprouting and/or neuronal death.

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