Identification of alternative splicing variants of the β subunit of human Ca^{2+}/calmodulin-dependent protein kinase II with different activities

Ping Wang[1], Ya-Lan Wu[1], Tian-Hua Zhou, Yue Sun, Gang Pei*

Shanghai Institute of Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, 320 Yue Yang Road, Shanghai 200031, PR China

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Abstract The β subunit of human Ca^{2+}/calmodulin-dependent protein kinase II (β CaMKII) was identified by searching through an expressed sequence tag database and rapid amplification of cDNA 5′-ends and was assigned to chromosome 7. Reverse transcription-polymerase chain reaction and sequencing analysis identified at least five alternative splicing variants of β CaMKII (β, β6, βe, β′e, and β7) in brain and two of them (β6 and β7) were first detected in any species. When expressed in HEK 293 cells, the Ca^{2+}/calmodulin-dependent kinase activity of β7, the shortest variant, was much lower than that of either β (the longest one) or βe (the medium one), suggesting possible regulation of β CaMKII activity by alternative splicing.

Key words: Ca^{2+}/calmodulin-dependent protein kinase II; β Isoform; Alternative splicing; Kinase activity

1. Introduction

Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), as a major intracellular mediator of calcium action, regulates a broad array of functions, especially in long-term potentiation and neurotransmitter release [1–8]. In mammalian cells, CaMKII has four subunits: α, β, γ and δ [2]. A critical neuronal function of the α subunit of CaMKII (α CaMKII) has been clearly demonstrated by studying mice that were either lacking α CaMKII or expressed mutated α CaMKII [3–8]. In contrast to α CaMKII, much less is known about β CaMKII though it is also a prominent kinase in the central nervous system. Even up to now, except its partial cDNA sequence [9], the full-length of human β CaMKII cDNA has not been reported yet.

It is known that CaMKII isozymes are encoded as single polypeptides, each containing a catalytic, a regulatory, and an association domain [10]. These three domains are highly conserved in evolution. A variable region, composed of the different alternative spliced insertion, has been found between the calmodulin binding site in the regulatory domain and the association domain. The functional implications of the variable region in CaMKII remain largely unknown except that these variable inserts could influence the cellular localization of the kinase and its interaction with calmodulin [11,12]. This study thus was undertaken to identify the full coding sequence of human β CaMKII and its alternative splicing variants.

*Corresponding author. Fax: (86)-21-6471 8563.
E-mail: gangpei@sunm.shcnc.ac.cn

[1] These authors should be considered as equal first authors.

2. Materials and methods

2.1. cDNA cloning of human β CaMKII and its splicing variants

The expressed sequence tag (EST) database of GenBank was searched with the mouse CaMKIIβ (GenBank accession number X63615) as probe by Basic local alignment search tool (BLAST) [13] at the website (http://www.ncbi.nlm.nih.gov) of the National Center for Biotechnology Information (NCBI). The 5′ rapid amplification of cDNA ends (5′ RACE) [14] was performed using the human brain Marathon cDNA library (Clontech) with two gene specific primers (5′-CCCCATTTGGTGACAATGGAGA-3′ and 5′-GTTCTCCGGCTTGAGGTCTCT-3′) according to the manufacturer's protocol. PCR bands obtained were gel purified and ligated into pGEM-T vector (Promega) by TA cloning. Reverse transcription-polymerase chain reaction (RT-PCR), using the primer pairs: 5′-GCCATGGCCACCACGGTGA-3′ and 5′-GAGGCAGACACAAACATGCGA-3′, was then used to clone the full-length cDNA of human β CaMKII. With enzymatic digestion, some inserts with different length were found, and at least five variants of human β CaMKII were later identified by DNA sequencing. The presence of these variants in mRNA was confirmed by RT-PCR analysis with primers: the sense (5′-GTCTGCCAACGCTCCACGGTAG-3′) and the antisense (5′-CGTGAGCCGGATGTAAGCGATGC-3′). The chromosomal localization and the intron–exon mapping of β CaMKII were carried out by bioinformatics with search in the NCBI sequence tagged site (STS) database [15].

2.2. Expression and cellular localization of human β CaMKII variants

Human β CaMKII variants were subcloned into pcDNA3 containing the coding sequence for hemagglutinin (HA) epitope or green fluorescence protein (GFP) at the amino acid terminus. Indicated plasmids were transiently transfected into HEK293 cells using calcium phosphate–DNA coprecipitation method as described [16]. After transfected with GFP-tagged β CaMKII variant plasmids, the cells were fixed with 4% polyformaldehyde in phosphate-buffered saline (PBS). Images of subcellular localization of the β CaMKII variants were recorded by using a Leica TCSNT laser confocal scanning microscope [17].

2.3. Kinase activity of β CaMKII variants

Kinase activity of CaMKII was determined as previously described [18]. HEK293 cells, 48 h after transfection with HA-tagged β CaMKII variants, were lysed and subjected to Western blot analysis and CaMKII activity assay. Expression of β CaMKII variants was monitored by Western analysis. Ca^{2+}/calmodulin-dependent or independent kinase activity was measured using specific peptide substrate autocamtide-2 (synthesized by Genemed Synthesis) [19]. All reactions were initiated by addition of 20 μg of cell extracts containing a comparable amount of β CaMKII variants and incubated at 30°C for 30 s. Phosphorylation was terminated by spotting an equal amount of sample onto p81 phosphocellulose paper and immediately immersing into 75 mM H_3PO_4. Then the radioactivity of samples was quantified by liquid scintillation counting.

3. Results

3.1. Cloning of human β CaMKII and its splicing variants

By searching against the non-redundant EST database of

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GenBank, we identified a group of human ESTs, whose deduced amino acid sequence was highly homologous to mouse CaMKIIβ (X63615). The contig analysis showed that these ESTs constructed human β CaMKII but its 5′ sequence was unknown (Fig. 1A). 5′ RACE was then performed to obtain the full-length coding sequence of this kinase, and the product of 5′ RACE (Fig. 1B) was cloned and sequenced. The ATG start codon of human β CaMKII was deduced according to the mouse's. RT-PCR analysis further confirmed the presence of full-length β CaMKII mRNA in human brain (Fig. 1C).

During identification of β CaMKII clones, some inserts with different length were found after enzymatic digestion. At least five variants of human β CaMKII were later identified by DNA sequence analysis (β: AF078803, β′e: AF081572, βe: AF083417, β6: AF081924, β7: AF140350). Two of these variants were novel and designed as CaMKIIβ6 and CaMKIIβ7. Further RT-PCR analysis revealed the presence of β, β′e, and β7 mRNA in brain tissue, β′e and β7 mRNA in SH-SY5Y neuroblastoma cells, and β′e mRNA in SK-N-SH neuroblastoma and HEK293 cells (Fig. 2A). In contrast, mRNA of βe or β6 was not detected probably due to their lower expression levels in the samples tested. It is interesting to point out that no expression of CaMKIIβ was detected in two kinds of neuroblastoma cells although it was expressed abundantly in brain.

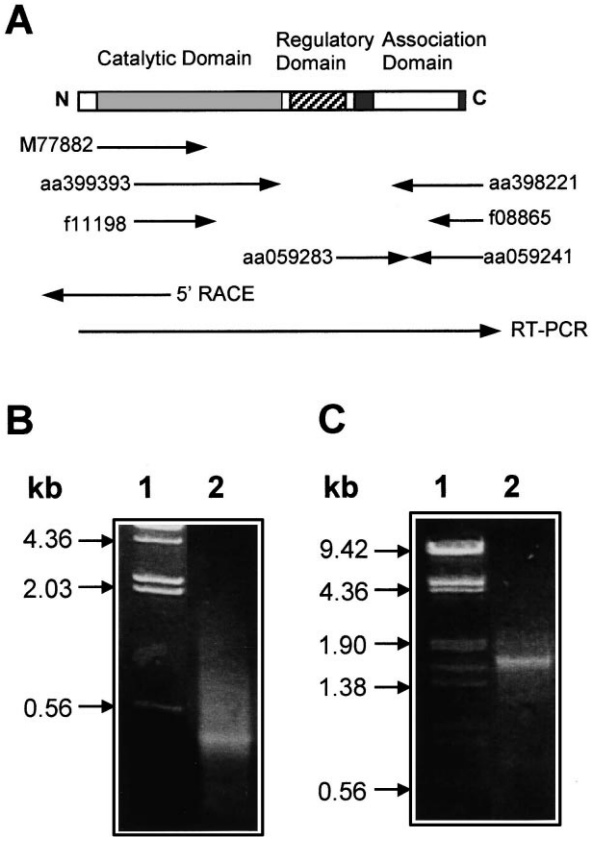

Fig. 1. Cloning of the β subunit of human CaMKII. A: Clones obtained from the EST Database, 5′ RACE and RT-PCR. Products from 5′ RACE (B) and RT-PCR (C) were subjected to agarose gel electrophoresis. The molecular size marker and sample were loaded in lane 1 and 2 respectively.

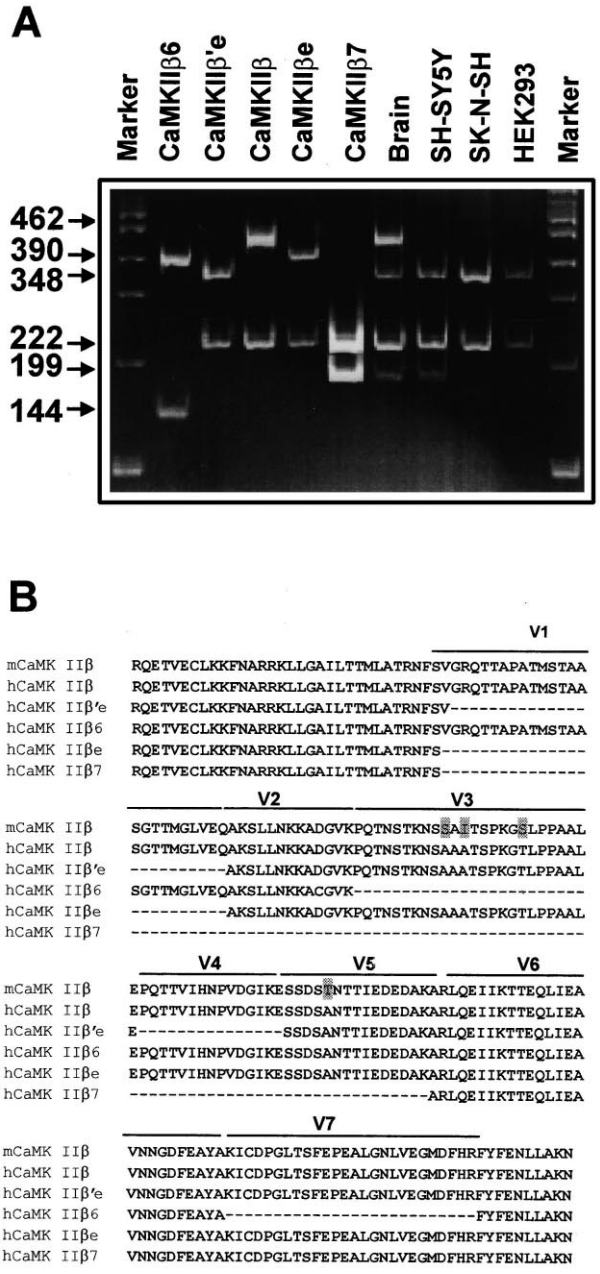

Fig. 2. Expression and alignment of the variable region of the human β CaMKII variants. A: RT-PCR products of samples were analyzed on 9% non-denatured PAGE after enzymatic digestion as compared to PCR products from positive clones. The expected sizes of the digested fragment are indicated on the left side in base pairs. B: Amino acid sequence alignment of the variable region of human β CaMKII variants with the mouse CaMKIIβ. The dashed lines indicate the absence of the alternative spliced insertions. The different amino acids between mouse CaMKIIβ and human β CaMKII are marked in gray.

3.2. Structure of the variable region of the β subunit of human CaMKII

The analysis of the sequence revealed that these variants encode proteins with molecular masses from 49 to 60 kDa. Multiple amino acid sequence alignment of these human variants and mouse CaMKIIβ clearly indicated that there are seven potential variable domains (V_1–V_7) in the variable region (Fig. 2B). CaMKIIβ7, a novel splicing variant found in

this study, lacks V_2 and V_5 domains which has been previously reported as invariable 'tether' domains [9]. CaMKIIβ6, another novel variant, is the first reported isoform lacking the variable domain V7.

To further test if these variants are really derived from alternative splicing, the genomic DNA sequence of variable region of β CaMKII was analyzed by bioinformatics. One human DNA contig NT-002349 assigned to human chromosome 7 was identified, which contains all of the β CaMKII gene except its 5′ end. Examination of the variable region between the calmodulin binding site and the association domain showed that seven potential exons (V_1–V_7) identified by the above protein sequence analysis map well with the rule of the intron–exon junction (data not shown). These seven exons were all flanked by the canonical consensus splice donor and acceptor sites: AG at the 3′ splice site and GT at the 5′ splice site [20]. This indicated that five β CaMKII variants identified in this study are indeed derived from the alternative.

3.3. Cellular localization of CaMKII variants

To investigate the relationship between structure and cellular localization of these β CaMKII variants, three of them (the longest one: CaMKIIβ, the medium one: CaMKIIβe and the shortest and novel one: CaMKIIβ7) were fused with GFP. Previous study has shown that the GFP tag does not affect β CaMKII's activity to phosphorylate the peptide substrate and its cellular localization [21]. When expressed in HEK293 cells, all these three variants of β CaMKII distributed primarily in the cytosol and no significant difference in cellular distribution was observed (Fig. 3). In contrast, cells expressing GFP alone showed homogeneous green fluorescence both in the nucleus and in the cytosol.

3.4. The kinase activity of β CaMKII variants

HA-tagged CaMKIIβ, CaMKIIβe, and CaMKIIβ7 were transiently expressed in HEK293 cells, and the level of their expression was detected by Western blot analysis using anti-HA monoclonal antibody (Fig. 4A). The results showed that expressed variants possess different apparent molecular masses from 47 to 60 kDa, which is similar with those predicted based on their amino acid sequences. The peptide autocamtide-2, a CaMKII specific substrate [19], was used as peptide substrate to investigate the kinase activity of three variants. As shown in Fig. 4B, in the absence of Ca^{2+}/calmodulin all three variants did not display significant basal kinase activity towards the peptide autocamtide-2. In the presence of Ca^{2+}/calmodulin, however, CaMKIIβ and CaMKIIβe substantially phosphorylated the peptide substrate whereas CaMKIIβ7 did not exhibit significant kinase activity under the same conditions (Fig. 4B).

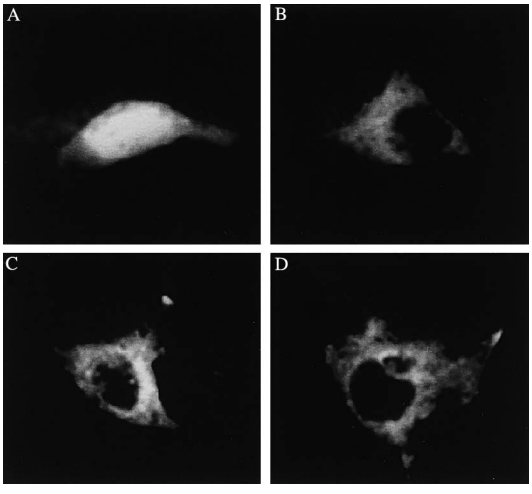

Fig. 3. Cellular localization of the human β CaMKII variants. GFP (A), GFP-tagged CaMKIIβ (B), GFP-tagged CaMKIIβe (C), and GFP-tagged CaMKIIβ7 (D) were transiently transfected into HEK293. Images were recorded by using a laser confocal scanning microscope after fixing with 4% polyformaldehyde in PBS.

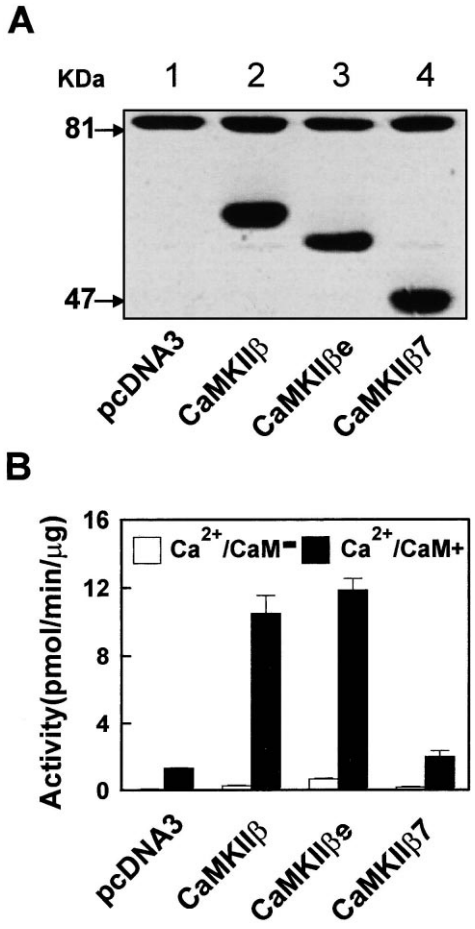

Fig. 4. Kinase activity of human β CaMKII splicing variants. A: Western blot analysis of expression of HA-tagged β CaMKII in HEK293 by anti-HA antibody. B: Ca^{2+}/calmodulin dependent or independent kinase activity of the variants was measured using specific peptide substrate autocamide-2. Results are expressed as mean ± S.D. of three independent experiments.

4. Discussion

Though it is a prominent kinase in the central nervous system, much less is known about β CaMKII as compared with α CaMKII. In this study, at least five variants of β CaMKII, likely derived from alternative splicing, are obtained from brain tissue. It has been shown that the variable region of murine β CaMKII is encoded by four exons [22]. But our results indicate that at least seven exons encode the human β CaMKII variable region, which may thus produce more splicing alternatives. This suggests that variable region

of β CaMKII evolves quickly and also implies that the region plays an important role in the function of β CaMKII.

It is very likely that the pattern of the alternative splicing may be related with the type of tissues and cells, developmental status, and physiological and pathological conditions. For instance, CaMKIIβ′e is shown to be expressed not only in brain tissue and two types of neuroblastoma cells but also in non-neural cells such as HEK293 cells, THP-1 cells (data not shown) and likely in mammary tumor cells [9]. In contrast, CaMKIIβ is abundantly expressed in the brain tissue but is not detected in the two types of neural cells tested in this study. Furthermore, CaMKIIβ7 is detected only in SH-SY5Y cells but not in SK-N-SH cells, indicating the different alternative splicing pattern even between different neuroblastoma cell lines. Interestingly, our data suggest that expression of CaMKIIβ′e may be functionally related to cell proliferation since it is detected in both neuroblastoma cells and embryonic cells.

It has been shown that the 'tether' domains in the variable region, composed of V_2 and V_5 domains, are present in every splicing variant of all four isoforms of CaMKII [9]. It is also proposed that the N-terminus of the 'tether' domain may modify Ca^{2+}/calmodulin binding affinity or substrate specificity while its C-terminus may regulate the multimeric size of the kinase [12,23]. But in one of the two novel splicing variants (CaMKIIβ7) identified in this study, the 'tether' domains appear to be deleted which is further confirmed by finding a corresponding EST (AA399393) in GenBank. Consequently, the deletion of 'tether' domains may thus lead to the loss of kinase activity in vitro as tested in this study. However, it remains unclear why this inactive variant is expressed in brain and how its expression is regulated? It could also not be excluded that this splicing variant may possess other than kinase functions such as a targeting module to localize other members of CaMKII to synaptic and cytoskeletal sites of action [24].

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