Gene of rat Ca^{2+}/calmodulin-dependent protein kinase II α isoform — its cloning and whole structure

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Abstract The gene encoding the α isoform of rat Ca^{2+}/calmodulin-dependent protein kinase II was cloned, and its exon-intron organization was analyzed. The coding region of cDNA consists of 18 exons spanning more than 50 kilobase pairs. Each of the discrete functional units, such as the ATP-binding site, the autophosphorylation site responsible for Ca^{2+}-independent activity, the calmodulin-binding site, and link structure is encoded by a single exon. The largest and smallest exons consist of 229 and 41 base pairs, respectively. All splice junction sequences flanking the introns conform to the consensus splice junction sequence and the GT-AG splice rule.

Key words: Ca^{2+}/calmodulin-dependent protein kinase II; α Isoform; Gene structure; Brain specific expression

1. Introduction

Ca^{2+}/calmodulin-dependent protein kinase II (CaM kinase II) is one of the most abundant protein kinases in mammalian brain [1–5]. Neuronal CaM kinase II regulates a broad array of nerve functions, including the synthesis and secretion of neurotransmitters, receptor function, structural modification of the cytoskeleton, axonal transport, long-term potentiation, and gene expression (for reviews, see [6,7]). Recent studies using transgenic mice further indicate that the α isoform of CaM kinase II may play a role in spatial learning [8].

At least four distinct genes that give rise to multiple isoforms (α, β, γ, and δ isoforms) of CaM kinase II have been defined [9–12]. All four isoforms have a high degree of sequence homology and contain three structural domains referred to as the catalytic, regulatory, and association domains [9].

The gene for the α and β isoforms is expressed almost exclusively in the nervous system, and the α isoform is the most predominant isoform in the brain. Marked differences are observed in the expression of two isoforms during brain development and in different adult brain regions [13–16]. The enzyme level increases most rapidly in postnatal brain during the most active phase of synapse formation. It is important to determine the gene structure of CaM kinase II in order to understand the regulation of neuron specific gene expression of the enzyme.

Previously, we characterized the gene of CaM kinase II of *Drosophila* which consists of at least 16 exons spanning about 20 kbp [17]. However, the gene of mammalian CaM kinase II has been only partially defined, and its complete exon-intron organization remains to be determined [18–20]. In the present study, we cloned the gene that encodes the α isoform of rat CaM kinase II cDNA and determined its whole structure.

2. Materials and methods

2.1. Materials

Rat genomic DNA library was purchased from Clontech. Autoread sequencing kit was from Pharmacia LKB Biotechnology. Megaprime DNA labeling kit and Hybond membrane were from Amersham Life Science. λ Phage mapping kit was from Wako Pure Chemicals, Osaka, Japan. Oligonucleotide primers were synthesized by Biologica Co. Nagoya, Japan. Genomic DNA was prepared from rat liver [21].

2.2. Screening of the rat genomic DNA library

1.4×10^6 recombinant phages were screened using fragments of the α isoform of CaM kinase II cDNA, isolated in our laboratory, as a probe [22]. cDNA of the α isoform of CaM kinase II was digested with restriction endonucleases and 7 fragments were isolated by agarose or polyacrylamide gel electrophoresis. The isolated cDNA fragments used as probes were: *Eco*RI/*Bgl*I (the cloning site of the vector – 1), *Bgl*I/*Sma*I (2–310), *Sma*I/*Bgl*I (311–790), *Bgl*I/*Sph*I (791–843), *Sph*I/*Pvu*II (844–1068), *Pvu*II/*Bgl*II (1069–1405), and *Bgl*II/*Eco*RI (1406 – the cloning site of the vector). The numbers correspond to the nucleotides in the α isoform of CaM kinase II [9]. The cDNA fragments were labeled with [α-^{32}P]dCTP using a Megaprime DNA labeling kit to a specific activity of about 5×10^8 cpm/mg. Phages were screened under high stringency hybridization conditions. Positive plaques were purified by further rounds of screening. All recombinant DNA manipulations were performed according to standard procedures [21].

2.3. Southern blot analysis

To identify exons included in each genomic clone, Southern hybridization was carried out using cDNA fragments of the α isoform of CaM kinase II. DNA samples were digested with restriction enzymes, separated on agarose gel by electrophoresis, and transferred to a Hybond membrane. Membranes were hybridized in 50% formamide, 5×SSC, 5×Denhardt's solution, 1% SDS, 0.1 mg/ml denatured salmon sperm DNA, and 2.5×10^5 cpm/ml of ^{32}P-labeled cDNA fragment. After incubation for 20 h at 42°C, membranes were washed, and radioactive bands detected by autoradiography.

2.4. Nucleotide sequencing analysis of genomic DNA

The genomic DNA sequence of the α isoform of CaM kinase II was cut with restriction enzymes, inserted into appropriately digested pUC19 or pBluescript II SK(−) vectors, and sequenced by the dideoxy chain termination method using an Autoread sequencing kit, according to the manufacturer's instructions.

2.5. Polymerase chain reaction (PCR)

We designed PCR primers to amplify the region of the cDNA of the α isoform of CaM kinase II corresponding to nucleotides 412–693. The specific 5′ sense primers were: ag1, 5′-GGAATTCTGTGCTACACTGTCACCAG-3′ (nucleotides 365–384); ag3, 5′-GGAATTC-

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Abbreviations: CaM kinase II, Ca^{2+}/calmodulin-dependent protein kinase II; PCR, polymerase chain reaction

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GAAGCTCAAGGGTGCTGC-3′ (nucleotides 434–452); ag7, 5′-GGAATTCCCCCATTCTGGGATGAG-3′ (nucleotides 630–648). The specific 3′ antisense primers were: ag4, 5′-CCTCGAGCAGCAC-CCTTGAGCTTCG-3′ (nucleotides 452–434); ag8, 5′-CCTCGAG-TACTGGCGGTGCTGGTC-3′ (nucleotides 666–649), ag10, 5′-CCT-CGAGCAGGCAGGGGTTCTCA-3′) (intron 9). 5′ and 3′ primers contained *Eco*RI and *Xho*I restriction sites, respectively.

PCR was performed in a solution of total volume 100 μl, containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 1 unit of Taq DNA polymerase, 0.4 μM 5′ and 3′ oligonucleotide primers, and 1 μg of genomic DNA. The amplification profile involved initial denaturation at 94°C for 2 min, then 98°C for 20 s, annealing at 55°C for 1 min, and extension at 72°C for 5 min. An automated DNA thermal cycler was used. The reaction was repeated for 30 cycles. PCR products were analyzed by electrophoresis on a 1% agarose gel and excised. Purified PCR products were subcloned into pBluescript SK(−) vectors and sequenced.

3. Results and discussion

To determine the structure of the gene encoding the α isoform of rat CaM kinase II, we screened an EMBL3 genomic library. Of 1.4×10^6 recombinant phages screened, 14 clones hybridized to the probe and were isolated. Restriction enzyme mapping analysis indicated that there were 5 independent clones (designated as CL1–CL5). The gene organization of these clones was investigated in the phage DNA partially digested with *Sac*I, using a λ phage mapping kit. *Sac*I fragments of each clone were subcloned into pUC19 or pBluescript II

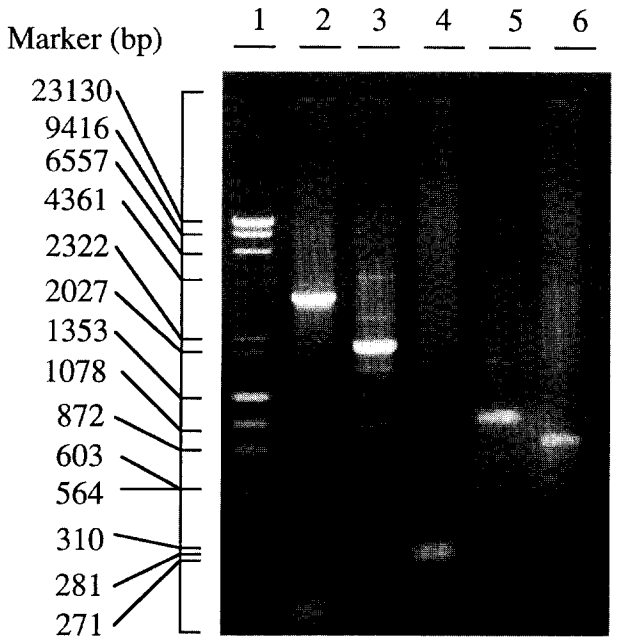

Fig. 1. Analysis of PCR products on agarose gel electrophoresis. PCR was performed using primers ag1/ag4 (lane 2), ag3/ag8 (lane 3), ag5/ag8 (lane 4), ag5/ag10 (lane 5), and ag7/ag10 (lane 6) as described under Section 2. λ *Hin*dIII and φX174 *Hae*III digests were used as size markers (lane 1).

Table 1
Exon/intron organization of the gene encoding the α-isoform of CaM kinase II

Exon	Size (bp)	Donor sites	Intron	Size (bp)	Acceptor sites	
ex. 1	209	A CTG GGA AA gtgagtcaca	int. 1	>5000	tgtcctccag G GGA GCC TTC	ex. 2
ex. 2	95	TCA GCC AGA G gtaggttctg	int. 2	~5700	tgcatttcag AT CAC CAG AA	ex. 3
ex. 3	60	CCC AAT ATC G gtgagcagag	int. 3	~4000	cttcccacag TC CGA CTC CA	ex. 4
ex. 4	55	TC TTC GAT CT gtgagtcttt	int. 4	~700	gtctccccag G GTC ACT GGT	ex. 5
ex. 5	66	CT GAT GCC AG gtgagtaccg	int. 5	86	ttctctccag C CAC TGT TAC	ex. 6
ex. 6	73	C GAC CTG AAG gtgagtaacc	int. 6	~3600	tcctgggaag CCT GAG AAT C	ex. 7
ex. 7	103	GCA TGG TTT G gtgagtgggg	int. 7	~1900	tggggagcag GG TTC GCA GG	ex. 8
ex. 8	84	TGG GCC TGT G gtaagtccat	int. 8	111	ctctccccag GC GTC ATC CT	ex. 9
ex. 9	95	T GCC TAC GAT gtgagtgttg	int. 9	836	cccttccag TTC CCA TCA C	ex. 10
ex. 10	123	C TGG ATC TCG gtgagctttc	int. 10	319	ctctctccag CAC CGC TCC A	ex. 11
ex. 11	84	G AAA CTG AAG gtaacccgtc	int. 11	~1200	tctgtttcag GGA GCC ATC C	ex. 12
ex. 12	43	AAC TTC TCC G gtaggtggtg	int. 12	~4000	tcctccacag GA GGG AAG AG	ex. 13
ex. 13	41	T GGC GTG AAG gtaagtcccc	int. 13	~6500	tctttggcag GAA TCC TCT G	ex. 14
ex. 14	49	GAC ACC AAA G gtagggggac	int. 14	~7000	taccttacag TG CGC AAA CA	ex. 15
ex. 15	76	AA TCC TAC AC gtgagttagc	int. 15	~2000	cttccccag G AAG ATG TGC	ex. 16
ex. 16	95	TTT GAA AAC C gtgagcaatc	int. 16	~4500	ctgcctctag TG TGG TCC CG	ex. 17
ex. 17	229	TC CTG CCC CA gtaagaactc	int. 17	246	tgtcttacag T TGA AGGACC	ex. 18
ex. 18	39+					

Exon and intron sequences are shown in uppercase and lowercase letters, respectively. Conserved gt and ag nucleotides are double underlined.

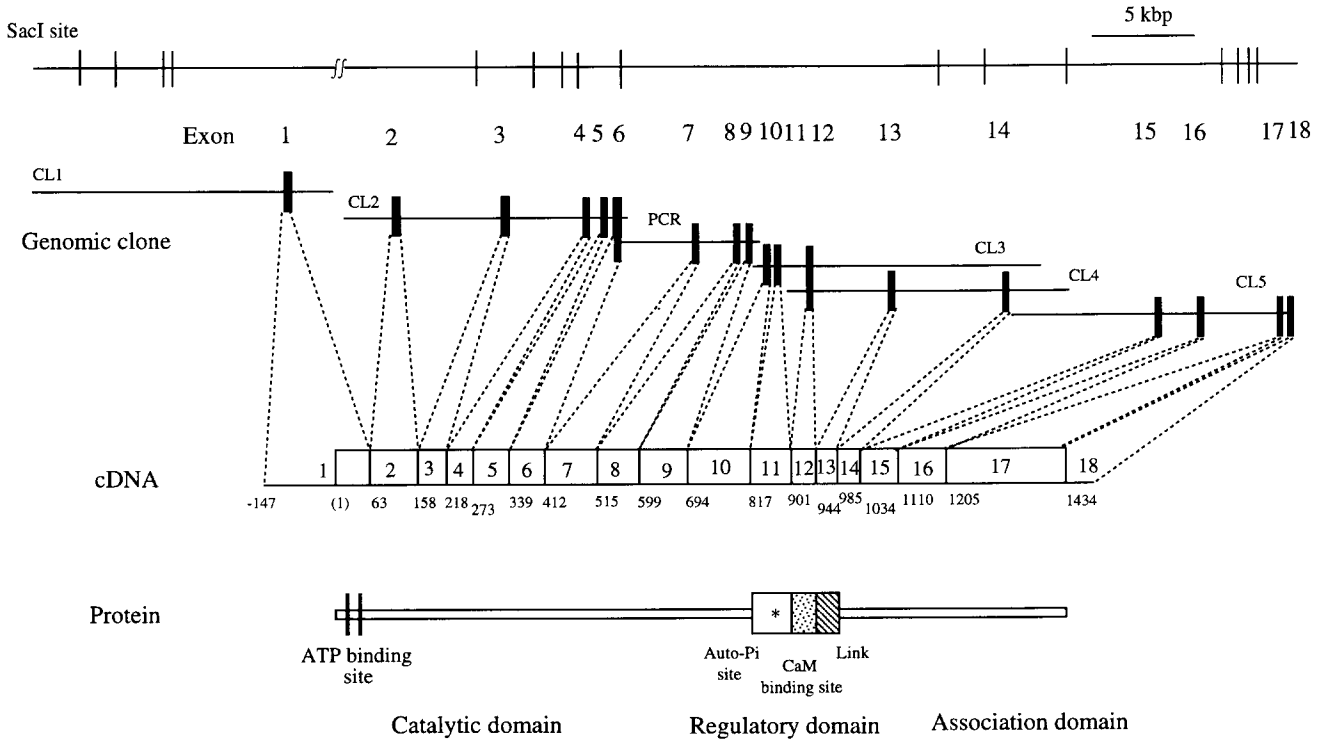

Fig. 2. Organization of the gene encoding the α isoform of the CaM kinase II. *Sac*I sites are shown in the upper panel. The genomic clones and exons identified (black bar), as well as their numbers are shown in the middle panel. The relationship between the exons, the functional domains of the α isoform of CaM kinase II cDNA (lower panel), and protein (lower panel) are also shown. The numbers in the boxes of cDNA indicate the corresponding exon. The numbers below the boxes of cDNA show the position of the first nucleotide of each exon, with the first ATG of cDNA assigned as +1. PCR, the results of PCR analysis; *, autophosphorylation site (Auto-Pi site) responsible for Ca^{2+}-independent activity; dotted box, calmodulin-binding site; cross-hatched box, link structure.

SK(−) vectors. Regions that hybridized with the cDNA fragments were detected by Southern blot analysis. The fragments hybridized with cDNA were isolated, subcloned further, and sequenced. Comparison of genomic DNA and cDNA sequences indicated that the structure of 15 exons of the gene was matched to the cDNA. However, the region corresponding to nucleotides 412–693 of the cDNA was not contained in these genomic clones.

To determine the structure of this region, PCR analyses were performed using 5′ and 3′ primers whose sequences were designed to amplify 3 exons (exons 7–9). Fig. 1 shows the analysis of PCR products by agarose gel electrophoresis. 3.6, 2.0, 0.3, 1.1 and 0.9 kbp products were specifically amplified using primers ag1/ag4, ag3/ag8, ag5/ag8, ag5/ag10, and ag7/ag10, respectively. These bands were confirmed to be hybridized with the cDNA *SmaI*/*BglI* fragment (nucleotides 311–790) by Southern blot analysis. The bands were excised, subcloned into pBluescript II SK(−) and sequenced. Exons 7–9 were identified by sequencing analysis.

We sequenced all the exon-intron junctions and determined the length of introns using restriction mapping, PCR and/or sequencing. We also confirmed overlapping regions of each clone by sequencing. The determined exon sequence contains all of the cDNA sequence determined by Lin et al. [9]. Fig. 2 shows the structure of the gene encoding the α isoform of CaM kinase II. The coding region of cDNA consists of 18 exons spanning more than 50 kbp. In intron 1, the 1.9 kbp sequence downstream from the 3′ end of exon 1 does not overlap the 2.4 kbp sequence upstream from the 5′ end of exon 2. No genomic clones were found to contain the complete intron 1 sequence, and so we were unsuccessful in obtaining the genomic DNA of this region by PCR. Exon 18 encodes the last base (T) of the C-terminal amino acid (His), a stop codon (TGA) and a 3′ non-coding region of cDNA (34 bp). The largest and smallest exons consist of 229 and 41 bp, respectively. The largest, other than intron 1, and the smallest introns are about 7 kbp and 86 bp, respectively.

CaM kinase II consists of three functional domains [9]. The catalytic domain stretches from exon 1 to 10, the regulatory domain from exon 11 to 13, and the association domain from exon 14 to 18. Exon 1 contains the 5′ non-coding region of the cDNA and the 62 bp coding sequence from the first ATG. The structure of the mRNA transcription start site was the same as that reported by Olson et al. [19], confirming that exon 1 includes a 147-bp 5′ non-coding sequence. The ATP binding site is located in exon 1. The autophosphorylation site responsible for Ca^{2+}-independent activity is encoded in exon 11. Exons 12 and 13 encode the calmodulin binding site and the link structure, respectively.

Table 1 shows the exon-intron organization of the α isoform of the CaM kinase II gene. All of the exon-intron boundary sequences of the gene matched the consensus sequence of the GT-AG splice rule.

In the present study, the gene structure of the α isoform of rat CaM kinase II was determined. The coding region of the cDNA consists of 18 exons spanning more than 50 kbp. We have previously defined the CaM kinase II gene of *Drosophila*, which consists of at least 16 exons spanning about 20 kbp

[17]. The *Drosophila* CaM kinase II is expressed in great quantities in the nervous system of the adult fly, and shows considerable homology to rat brain CaM kinase II with more than 70% of the amino acids being identical. The rat CaM kinase II gene is more than twice as large as the *Drosophila* gene, and contains more exons. The splice site in the catalytic domain is conserved between the rat and *Drosophila* genes except that exons 5–8 of the rat gene are composed of a single exon in the *Drosophila* gene. In the regulatory and association domains, however, the exon-intron organization significantly differed between the two species.

On the other hand, the rodent CaM kinase II gene has been only partially defined [18–20]. Sunyer and Sahyoun [18] have determined the structure of 5′ flanking region of rat α CaM kinase II gene. Olson et al. [19] have also determined 5′ flanking region and the first exon, and 5 additional exons that encode the association domain and 3′ non-coding region of the cDNA sequence of the rat α CaM kinase II gene. Karls et al. [20] have determined the structure of 16 exons of the mouse β CaM kinase II gene, which encode about 80% of the cDNA sequence from the 3′ end. However, the genomic clone from the 5′ end has not been obtained [20].

This study has demonstrated for the first time the complete exon-intron organization of the mammalian gene encoding CaM kinase II. The structure of the 5′ flanking region of the α isoform of CaM kinase II gene is the same as that previously reported [18,19]. The structure of exon 1 is almost the same as that reported by Olson et al. [19], except for one base. Olson et al. reported that the exon encodes 63 bp from the first ATG. They also identified 5 exons that encode the association domain and 3′ non-coding region of the cDNA sequence. Our results, however, differ from theirs with regard to the splice site. Although the size of exons 15, 16 and 17 defined in this study did not differ from that of exons 1141–1216, 1217–1311, and 1312–1540 determined by Olson et al., respectively, the splice site of these exons varied by about 40 bp. We could not compare with their sequence, since they did not show the structure of the exon-intron boundaries.

An isoform, CaM kinase II α-33, derived by different splicing has been reported in the monkey forebrain and rat brain by PCR [23,24]. CaM kinase II α-33 was found to contain a 33-bp insert between exons 13 and 14 of the α CaM kinase II gene, the 3′ site of the link structure.

Karls et al. [20] have determined the structure of 16 exons of mouse β CaM kinase II gene. Three out of the 16 exons are specific for the β isoform and one is 3′ non-coding. The remaining 12 exons of mouse β CaM kinase II gene have almost equal size to the corresponding exons (exons 6–17) of the rat α CaM kinase II gene, respectively. Thus, the exon structure is highly conserved between the α and β isoforms of rodent CaM kinase II gene. However, the 5′ untranslated region and 5′ end of the coding region of the β CaM kinase II gene (corresponding to exons 1–5 of the α isoform) have not been sequenced [20]. The α and β isoforms are expressed at different steps during brain development and in different brain regions. Such differential expression of the two isoforms remains to be explained in terms of certain different structure and functions of the two genes.

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