# A novel pancreatic β-cell isoform of calcium/calmodulin-dependent protein kinase II ($\beta_3$ isoform) contains a proline-rich tandem repeat in the association domain

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**Abstract** **There is evidence for a role for calcium/calmodulin-dependent protein phosphorylation in regulation of insulin secretion but the molecular nature of the kinase(s) responsible is unknown. In this study, the screening of a neonatal rat islet cDNA library resulted in the isolation of a 2 kb clone that was 99% homologous to the β′ isoform of calcium/calmodulin-dependent protein kinase II. The predicted 589 amino acid sequence with a calculated mass of 64,976 Da contained a 24 amino acid deletion in addition to the 15 amino acid deletion that differentiates the β′ from the β isoform, and included an 86 amino acid novel domain consisting of a tandem repeat of proline-rich residues. The expression of this new isoform of calcium/calmodulin-dependent protein kinase II ($\beta_3$) was confirmed in β-cell lines and testis by DNA amplification of the sequence encoding the inserted domain by reverse transcriptase-polymerase chain reaction, followed by Southern analysis.**

*Key words:* CaM kinase II; Insulin secretion; Protein phosphorylation; β-cell

## 1. Introduction

It is well established that insulin secretagogues such as glucose, which lead to an increase in intracellular $Ca^{2+}$ via depolarization of the β-cell, are able to initiate insulin release [1]. There is growing evidence that a calcium/calmodulin-dependent protein kinase (CaM kinase), the activity of which has been detected in association with the cytoskeleton in isolated pancreatic islets [2,3], plays an important role in glucose-regulated insulin secretion. That inhibition of β-cell CaM kinase results in inhibition of insulin release has been demonstrated in pancreatic islets with the use of the diabetogenic agent, alloxan, and its analogue, dehydrouramil [4,5] and at the single cell level with a specific peptide inhibitor of CaM kinase [6]. The activation of islet CaM kinase has been shown to exhibit a close correlation with glucose-induced insulin secretion [7]. It has been suggested that β-cell CaM kinase may be similar or identical to CaM kinase II [8]. This multifunctional protein kinase, although predominantly expressed in neurons, is present in a variety of mammalian tissues and has been implicated in the regulation of multiple cellular processes, including contractility, and neurotransmitter release and synthesis [9,10]. CaM kinase II is a multimer of 4–12 subunits [10,11], each containing a catalytic, a regulatory and an association domain. The association domain is believed to be required for the assembly of subunits and for specific targeting to subcellular locations. Molecular cloning indicates that at least four distinct genes encode CaM kinase II isoforms, designated as α, β, γ and δ. Alternatively spliced variants of each subunit, which differ in insertions or deletions between the calmodulin binding site in the regulatory domain and the association domain, are expressed in different tissues [11–16], suggesting specific functional roles for each isoform. A sequence inserted in the variable domain of $\delta_B$ CaM kinase II appears to be responsible for its nuclear targeting [17]. The γ and δ subunits appear to be the predominant forms in peripheral tissues, and although the α and β/β′ isoforms have only been cloned from neural tissues, β-subunit-specific mRNA species have also been detected in skeletal muscle, diaphragm, small intestine and testis [18].

In this study we present conclusive evidence that β-cells express CaM kinase II. We report here the cloning from rat pancreatic islets of a novel isoform of CaM kinase II that we designate as $\beta_3$ that appears to be the product of alternative splicing of the β-subunit gene transcript. This is the first time that a β-isoform of CaM kinase II has been cloned from a non-neuronal source.

## 2. Materials and methods

### 2.1. Isolation and sequencing of cDNA clones

A neonatal rat islet cDNA library constructed in pCDM8 (InVitrogen) was screened using random-primer $^{32}$P-labelled probes corresponding to two contiguous *Eco*RI–*Bsa*HI restriction fragments (730 and 815 bp) of a cDNA clone of mouse brain CaM kinase II α-subunit. Eleven putative CaM kinase clones were isolated after three rounds of screening, and subcloned into pBluescript (Stratagene). The 5′ and 3′ ends of three clones were sequenced by the dideoxynucleotide chain termination method [19] using Sequenase 2.0 (US Biochemicals). Two clones had identical nucleotide composition and the third consisted of an internal fragment that was 100% homologous to the CaM kinase II β-subunit. The sequence of both strands of one of the clones, encompassing the entire CaM kinase II coding region, was determined and compared to DNA sequences of the cloned CaM kinase II isoforms using the University of Wisconsin Genetic Computers Group software package [20].

### 2.2. Reverse transcription and polymerase chain reaction

Total RNA was purified from rat tissues and β-cell lines [21] and used for reverse transcription-polymerase chain reaction (RT-PCR) [22] with Moloney murine leukemia virus reverse transcriptase (Life Technologies) and the antisense primer complementary to nucleotides 1413–1434 of $\beta_3$ CaM kinase II. One twentieth of the volume of the reverse transcription reactions was subjected to amplification with the sense primer, corresponding to nucleotides 1131–1154 of $\beta_3$ CaM kinase II and the antisense primer (primer pair I, each primer included *Bam*HI sites and 4 extra nucleotides at their 5′ ends). Coding regions of rat brain CaM kinase cloned subunits α and β, and γ, were amplified with the sense

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primer 5′-ATGGCTACCATCACCTGCACC-3′ and the antisense primer 5′-TCAATGCGGCAGGACGGAGG-3′ (primer pair II). The $\beta_3$ CaM kinase II clone was amplified using the sense primer 5′-AGCCCAATCGCCACCGCCATG-3′ (nucleotides 27–47) and the antisense primer 5′-ACTCTGTCCGGTGAAACCAGGCG-3′ (complementary to nucleotides 1821–1843) (primer pair III). Thirty cycles of amplification were conducted with one unit of Vent DNA polymerase per reaction (New England Biolabs) in the reaction buffer supplied by the manufacturers. Each cycle consisted of a 1-min denaturation at 94°C, 45-s annealing at 58°C, and 90-s extension at 73°C. A negative control that lacked template was included for every set of PCR reactions.

### 2.3. Southern analysis

PCR products electrophoresed in an agarose gel were transferred and UV fixed onto a nylon membrane (Hybond N; Amersham). Hybridization was carried out at 42°C with a *Bam*HI–*Hin*cII restriction fragment (nucleotides 1081–1460) of $\beta_3$ CaM kinase II) that had been Klenow filled-in with [$^{32}$P]dCTP. After hybridization, the membrane was washed twice with 0.1× SSC, 0.1% SDS at 65°C for 1 h and subjected to autoradiography.

### 2.4. Sequence homology searches

Database searches were conducted with the MPsrch program against the Swiss-Prot protein sequence database using the best local similarity algorithm [23]; with BLAST [24] against Brookhaven Protein Data Bank, PIR, Swiss-Prot and the SBASE protein domain database; and with Motifs [20] against the PROSITE dictionary of protein sites.

## 3. Results

### 3.1. Nucleotide and deduced amino acid sequence of rat islet CaM kinase II ($\beta_3$ isoform)

Sequencing of the 5′ and 3′ ends of two of the 11 putative CaM kinase cDNA clones obtained by screening a neonate rat islet library showed that one clone (1979 base pairs) encompassed the entire coding region. Analysis of the nucleotide sequence (Figs. 1 and 2) revealed that it was identical to that of rat brain $\beta'$ CaM kinase II [12] with the exceptions of a 72 nucleotide deletion (24 amino acids) corresponding to residues 947–1019, which is also found in the $\alpha$, $\delta$ and some of the $\gamma$ isoforms, and a 258 nucleotide insertion (86 amino acids) at position 1227 of the brain enzyme, suggesting that both messages originate from alternative splicing of the $\beta$-isoform gene transcript. The clone encodes a predicted protein of 589 amino acids with a calculated molecular mass of 64,975 Da. The only substitution between the two sequences consists of a change from valine at position 317 of the $\beta'$ CaM kinase II to alanine, as a result of the splicing event occurring within the GTG codon at position 946. The 86 amino acid insertion in the association domain (corresponding to nucleotides 1155–1412 in the $\beta_3$ CaM kinase II) comprises two repeated motifs of 43 amino acids each, in a tandem arrangement which are 84% identical (Fig. 2). Proline accounts for 27% of the residues in this domain, and 48% of the sequence is hydrophobic. The second repeat motif contains the peptide PPVGPPPCP (amino acids 436–444) that conforms to the consensus sequences PXXXPPXXP/PXXPPXXP derived from the alignment of putative SH3 (src homology 3) domain-binding sites that are present in dynamin, 3BP1, mSOS1 [25], and in SPRK [26]. A potential CaM kinase II phosphorylation site (consensus RXXS/T) is present in the sequence RRGS (residues 424–427). The search for motifs in the Prosite dictionary of protein sites revealed that the novel domain provides 4 additional potential protein kinase C phosphorylation sites in serines 379, 413, 422 and 456.

### 3.2. Evidence for expression of the inserted domain in the β-cell by RT-PCR

Total RNA from various tissues and cultured $\beta$-cell lines was reverse transcribed and then amplified by PCR with primers flanking the 258 nucleotide insertion. Amplified products of

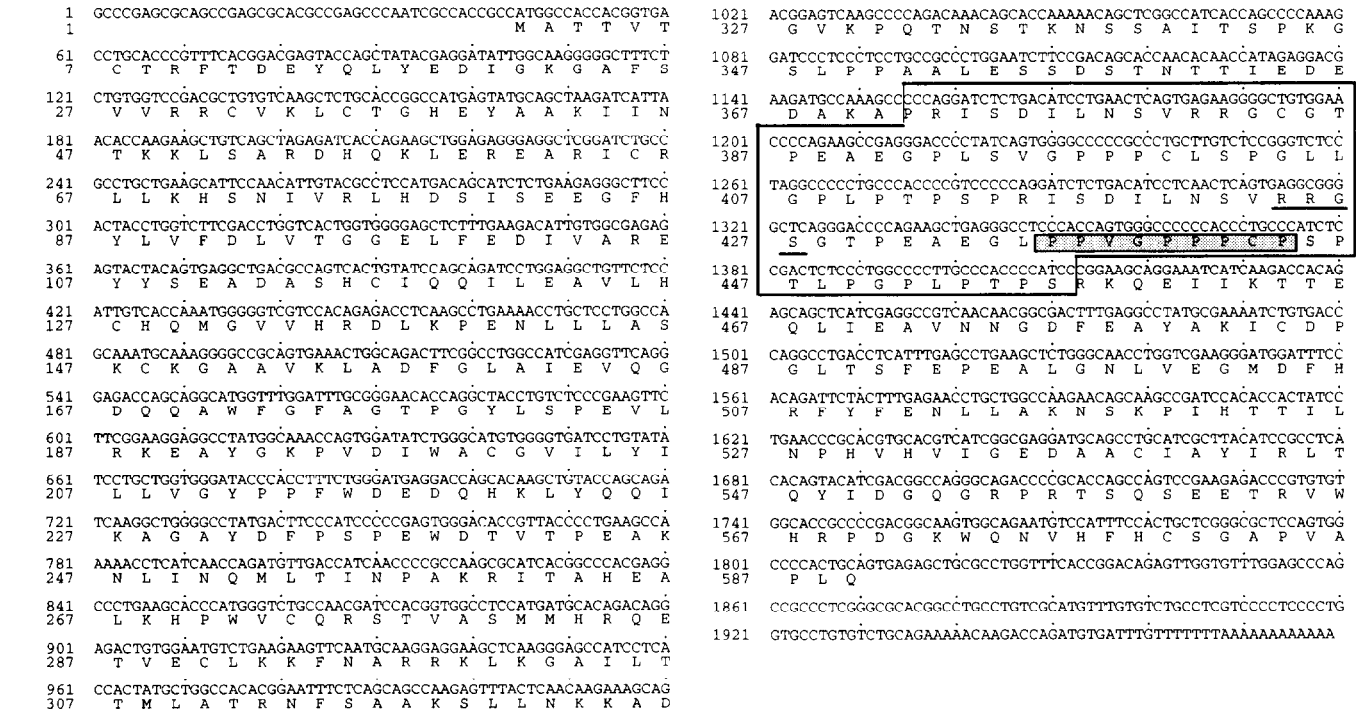

Fig. 1. Nucleotide and deduced amino acid sequence of the $\beta_3$ isoform of CaM kinase II. The proline-rich insertion is boxed. The putative CaM kinase II phosphorylation site is underlined and the putative SH3-binding site is shaded.

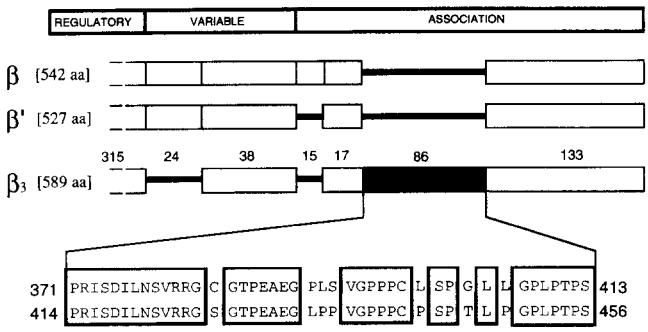


Fig. 2. Comparison of the variable and association domains of three isoforms of the $\beta$ subunit of CaM kinase II. Solid narrow boxes represent deletions. The number of amino acids in each conserved, deleted or inserted region is indicated above the $\beta_3$ isoform. The amino acid composition of the proline-rich insertion in the $\beta_3$ isoform (shaded box) is depicted in the lower panel as an internal alignment of the two 43-residue halves of the fragment, where identical repeats are boxed. The numbers on each side of the alignment correspond to positions in the $\beta_3$ deduced amino acid sequence (Fig. 1).

compatible size with that of the sequence encoding the extra domain plus the primer sequences (324 bp) were obtained with cDNA from HIT T15 and MIN6 $\beta$-cell lines, and from neonatal rat islets and adult rat testis (Fig. 3A). However, no amplification products of this size were detected with reverse transcription products from brain, heart, small intestine, kidney or liver RNA. To confirm the identity of the PCR products, an end-labelled $\beta_3$ CaM kinase II *Bam*HI–*Hin*cII restriction fragment (nucleotides 1081–1460) was used in Southern analysis. In order to assess whether primer sequences in the amplified fragments could account for a positive signal, sequences corresponding to the coding regions of brain CaM kinase II isoforms $\alpha$, $\beta$ and $\gamma$, and of $\beta_3$ CaM kinase II were amplified from cDNA clones and included in the Southern blot as hybridization controls. The results shown in Fig. 3B indicate that a variant of CaM kinase II containing the novel domain described in this study is expressed in both the $\beta$-cell lines and also in testis.

## 4. Discussion

There is growing evidence for the importance for insulin secretion of calcium/calmodulin-dependent protein phosphorylation in the $\beta$-cell [4–7]. Studies on the substrate specificity [7,8] and sensitivity to inhibitors [4,5,27] of CaM kinase activity in islets of Langerhans led to the suggestion that the enzyme may be similar or identical to CaM kinase II [8]. Immunological evidence has supported this possibility [7,28] but the $\beta$-cell CaM kinase II has not hitherto been characterised at the molecular level. In the present study we provide conclusive evidence for the expression of CaM kinase II in the pancreatic $\beta$-cell by molecular cloning of a novel isoform ($\beta_3$) of the enzyme. In contrast to previously cloned CaM kinase II isoforms, in which the differences lie mainly in the variable domain, the major distinguishing feature of the cloned islet CaM kinase II is an 86 amino acid insertion containing two tandem repeats in the association domain.

Database searches conducted in order to predict a structure–function relationship of the novel repeated motif resulted in significant homologies with proline-rich regions (PRRs). Proline-rich sequences containing multiple tandem repeats with minor variations have been implicated in the reversible association of proteins into functional complexes regulated by phosphorylation within the PRR [29]. An example is provided by the synaptic vesicle-associated protein synapsin I, which contains three 17-residue proline-rich repeats. Phosphorylation of two serines in the PRR near the C-terminal region of synapsin I by CaM kinase II causes synapsin I to dissociate from synaptic vesicles [30] and increases neurotransmitter release [31,32]; and the regulatory domain of a synaptic vesicle-associated form of CaM kinase II binds the C-terminal region of synapsin I [33]. Apart from the synapsins, several other substrates for CaM kinase II, such as calcineurin, cardiac ryanodine receptor and microtubule-associated proteins [10], exhibit homologies with the $\beta_3$ proline-rich domain. It is tempting to speculate that the PRR of the $\beta_3$ isoform, and the putative SH3-binding sequence contained within, play a role in the interaction of $\beta_3$ CaM kinase II with signalling pathway or cytoskeleton components, according to the targeting subunit hypothesis [34]. This hypothesis postulates that a part of a protein kinase directs the catalytic domain to a target locus, and is exemplified by the presence of a nuclear localization signal in an 11-amino acid insert at the beginning of the association domain of the $\delta_B$ isoform of CaM kinase II [17].

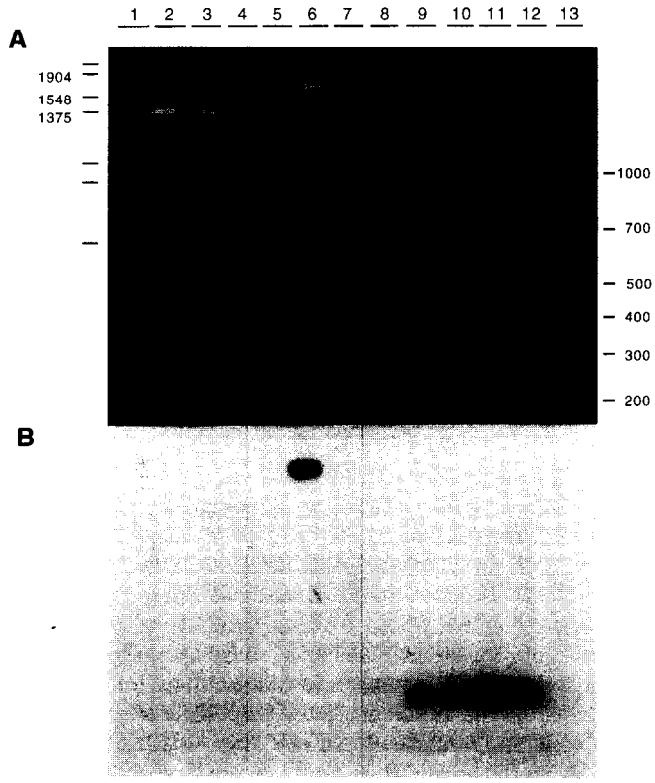


Fig. 3. Expression of the sequence coding for the 86 amino acid insertion of CaM kinase II $\beta_3$ isoform. (A) PCR products obtained with primer pair II (cDNA clones of brain CaM kinase isoforms) (lanes 2–5); with primer pair III (lane 6); and with primer pair I, flanking the 258 bp insertion (RT-PCR) (lanes 9–12) as described in section 2. Lane 2, mouse $\alpha$-subunit; lane 3, rat $\alpha$-subunit; lane 4, rat $\beta$-subunit; lane 5, rat $\gamma$-subunit. Lane 6, $\beta_3$ CaM kinase II; lane 9, HIT T15 $\beta$-cells; lane 10, MIN 6 $\beta$-cells; lane 11, rat testis; lane 12, rat islets. Lanes 1 and 7, lambda *Hin*dIII–*Eco*RI markers; lanes 8 and 13 DNA ladder. The numbers on each side indicate the size of DNA markers in base pairs. (B) Southern blot of the products shown in A with an end-labelled probe consisting of nucleotides 1081–1460 of the $\beta_3$ CaM kinase II.

Expression of the $\beta$-isoform of CaM kinase II has previously been regarded as confined to the nervous system [17]. The $\beta_3$ isoform appears to be derived by alternative splicing of the same primary transcript as the $\beta$ form. Our results with RT-PCR on various tissues suggest that the splicing event giving rise to the $\beta_3$ form has a restricted tissue distribution. It should also be noted that although two independent clones isolated from the rat islet cDNA library corresponded to the $\beta$-subunit, there is no evidence for that to be the only or even the predominant isoform of the enzyme in the $\beta$-cell. It has been shown that the level of $\beta$ message decreases during neuronal development and that the $\alpha$:$\beta$ ratio in forebrain increases more than twofold, the $\beta$-subunit being the dominant isozyme at birth [10]. These facts may therefore account for a higher probability of isolating clones of the $\beta$ isoform from the neonatal rat islet library.

Expression of the cloned $\beta_3$ isoform and studies on subcellular localization of the enzyme will contribute to the understanding of structure–function relationships.

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