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Identification of novel human tumor cell-specific CaMK-II variants

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Abstract

CaMK-II (the (type II) multifunctional Ca^{2+}/CaM-dependent protein kinase) has been implicated in diverse neuronal and non-neuronal functions, including cell growth control. CaMKII expression was evaluated in a variety of human tumor cell lines using RT-PCR (reverse transcriptase coupled polymerase chain reaction). PCR primers which flanked the CaMK-II variable domain were used so that all possible variants of the four mammalian CaMK-II genes (α, β, γ and δ) could be identified. 8 distinct CaMK-II isozymes were identified from human mammary tumor and neuroblastoma cell cDNA, each of which represented a variant of β, γ or δ CaMK-II. They included 2 β isozymes (β_e, β'_e), 4 γ isozymes (γ_B, γ_C, γ_G, γ_H) and 2 δ isozymes (δ_C, δ_E) This is the first report of human β and δ CaMK-II sequences.

A panel of human cell types was then screened for these CaMK-II isozymes. As expected, cerebral cortex predominately expressed α, β and δ_A CaMK-II. In contrast, tumor cells, including those of neuronal origin, expressed an entirely different spectrum of CaMK-II isozymes than adult neuronal tissue. Tumor cells of diverse tissue origin uniformly lacked α CaMK-II and expressed 1–2 β isozymes, at least 3 γ isozymes and 1–2 δ isozymes. When compared to undifferentiated fibroblasts, β_e, β'_e, γ_G and γ_H were preferentially expressed in tumor cells. CaMK-II immunoblots also indicated that neuroblastoma and mammary tumor cells express isozymes of CaMK-II not present in their non-transformed cell or tissue counterpart. The identification of these new, potential tumor-specific CaMK-II variants supports previous indications that CaMK-II plays a role in growth control. In addition, these results provide insight into both splice variant switching and variable domain structural similarities among all CaMK-II isozymes.

Keywords: Calmodulin; CaMK-II; Protein kinase; Breast tumor; Neuroblastoma; Alternative splicing

1. Introduction

Mammalian cell growth is dependent upon Ca^{2+} operating through calmodulin (CaM) during the G1 phase of the cell cycle [1–3]. Transient elevations of Ca^{2+} occur in response to growth factors and CaM peaks in mass during G1 [1–3]. The ablation or inhibition of CaM arrests cells during G1 [4–6] and cellular transformation can lead to elevated levels of CaM [7]. CaM has a number of potential targets through which it could act to channel Ca^{2+} signals into the growth regulatory machinery, including the family of CaM-dependent protein kinases [8]. Of the 6 different CaM kinases, which include CaMK I through IV, myosin light chain kinase and phosphory-

Abbreviations: CaM, calmodulin; CaMK-II, (type II) multifunctional Ca^{2+}/CaM-dependent protein kinase; RT-PCR, reverse transcriptase coupled polymerase chain reaction

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lase kinase, only CaMK-I and CaMK-II are uniformly expressed in proliferative cells [8,9]. CaMK-IV is regulated by other kinases and has been implicated in transcriptional regulation, but is specific to neuronal and reproductive cells [10,11]. In mammalian cells, CaMK-II is encoded by four known genes: α, β, γ and δ, to give rise to over a dozen CaMK-II subtypes, presumably through alternative splicing [12–14].

CaMK-II (the (type II) multifunctional Ca^{2+}/CaM kinase) was originally discovered as a major protein in differentiated neuronal tissue where it was implicated in long-term potentiation and neurotransmitter release [15,16]. CaMK-II has since been discovered in yeast, mold, fruit flies and humans [17–19]. It has been linked to the regulation of carbohydrate metabolism, membrane fusion and the cell cycle in non-neuronal cells [20–24]. CaMK-II activity and polypeptides have been found in proliferative cells in culture [23,25] and CaMK-II has been reported to be activated within 20 sec of growth induction by serum, PDGF, EGF or bradykinin [26,27]. CaMK-II inhibitory drugs arrest mammalian cells in G1 and S phase [22,23,28] and block the expression of early response genes, such as fos, which are necessary for cell growth [11,29].

CaMK-II isozymes are encoded as single polypeptides, each containing a catalytic, a regulatory, a variable and an association domain with predicted masses between 54 and 60 kDa. The catalytic domain has features common to most serine/threonine protein kinases. The regulatory domain is the site of CaM binding and autophosphorylation, through which CaMK-II can sustain its own activity after a Ca^{2+} surge [9,30]. The generation of Ca^{2+}-independent or autonomous activity in this manner is believed to comprise the molecular basis for long-term functions of CaMK-II. The regulatory domain is adjacent to a region of high variability where most differences between the four CaMK-II genes and among the splice variants are located [9]. It is believed that this region serves in part as an elongated tether between the catalytic and association domains and is therefore exposed to other proteins enabling it to associate with sub-cellular organelles or substrates [12,14]. The association domain is responsible for the oligomerization of CaMK-II, a trait unique to this member of the CaM kinase family.

Neuronal-specific functions of multifunctional CaMK-II are believed to be carried out by α and β CaMK-II, while the widespread distribution of γ and δ suggests that they may be involved in functions common to all cells [31]. Non-neuronal functions of β CaMK-II, however, are suggested by the embryonic lethality of a transgenic mouse strain in which β CaMK-II is disrupted [32] and the identification of a non-neuronal isozyme of β CaMK-II in rat pancreatic cells [33]. Tissue-specific expression of CaMK-II genes and of their splice variants has been reported for γ CaMKII's [34,35]. δ CaMK-II isozymes are expressed in both neuronal and non-neuronal cells, with two domains of variability leading to over 6 different splice variants [13,36,37].

Although CaMK-II has been implicated in mammalian growth control during G1, it is not known which CaMK-II isozymes are expressed in proliferative cells and therefore which ones might be involved in regulating growth. A comprehensive analysis of CaMK-II isozyme expression in non-neuronal, undifferentiated cells has been lacking. In this study, the entire spectrum of CaMK-II isozymes expressed in a panel of human tumor cell lines was characterized by using PCR primers which were conserved amongst all CaMK-II's. The partial cDNA sequence of 6 human β, γ and δ CaMK-II splice variants is reported, some of which are novel and may be preferentially expressed in tumor cells.

2. Materials and methods

2.1. Cell lines and culture

The following cell lines were used to prepare RNA and are indicated with tumor type of origin and American Type Culture Collection (ATCC) number. Human breast cancer cell lines included: MCF7: breast adenocarcinoma (HTB 22); ZR 75-1: ductal carcinoma (CRL 1500); DU4475: metastatic cutaneous nodule (HTB 123); MDA-MB361: adenocarcinoma (HTB 27) and SK-BR-3: adenocarcinoma (HTB 30). Other cell lines included CCRF-CEM: lymphoblastic leukemia (CCL 119); HT-29: colon adenocarcinoma (HTB 38); MCF10A: mammary epithelial cells (CRL 10317) and WI-38 embryonic lung

fibroblasts (CCL75). NIH 3T3 mouse embryo fibroblasts (CRL 1658) and the human neuroblastoma LAN5 cell line [38] were also used. Cells were cultured on polystyrene dishes or in suspension in polystyrene flasks in DMEM or RPMI-1640 (BioWhitaker, Walkersville, MD) and 10% fetal bovine serum (GIBCO-BRL, Bethesda, MD), supplemented with penicillin/streptomycin in a 5% CO_2 humidified chamber @37°C.

2.2. RT-PCR

Total RNA was isolated from exponentially growing cells as described [39]. Poly A^+ RNA was isolated directly from cell lysates using oligo-dT cellulose (Invitrogen, San Diego, CA). First strand cDNA was prepared from 5 μg total RNA or approx. 50 ng poly A^+ RNA using oligo dT_{16}-primer and AMV reverse transcriptase (Life Technologies, Gaithersburg, MD). Oligo-dT-primed adult human cerebral cortex and human mammary tissue cDNA were obtained from commercial sources (Clontech, Palo Alto, CA).

For the primary PCR reactions used to identify CaMK-II classes (Fig. 2), 1 μl of cDNA (1–2 ng) was subjected to nested PCR [40,41]. PCR reactions were conducted in 50 μl containing 1.5 mM $MgCl_2$, 0.2 mM dNTP, 4 μg/ml sense and anti-sense primers and 0.2 Units Taq polymerase (Perkin Elmer Inc., Foster City CA). Primary PCR reactions were conducted for 20 cycles with 1 min at 92°C, 1.5 min at 42°C and 2 min at 72°C. Nested secondary PCR reactions were conducted with 2 μl of the primary reaction diluted in 50 μl total volume and cycled 35 times with 1 min at 92°C, 1.5 min at 50°C and 2 min at 72°C. PCR amplification conditions were optimized for $MgCl_2$ concentration and hybridization temperature using CaMK-II clones and for the minimum cycle number and cDNA concentration necessary to achieve strong bands from brain cDNA, which was known to express all CaMK-II mRNAs. PCR primers were designed from the variable domain of CaMK-II, which is at the C-terminal end of the CaM-binding domain and where almost all of the variability exists among isozymes. The sense primer for the primary PCR reactions was 5′-GGGACA-CAGTGACACCTGAAGC-3′, corresponding to amino acids WDTVTPEA (residues 238–245 in human γ_B CaMK-II) and the anti-sense primer was 5′-GGTT[C/T]AGGATG[A/G][A/G]T [A/G]G-T[A/G]TG-3′ corresponding to HTTILNP (residues 451–457 in γ_B CaMK-II). The secondary (nested) PCR reaction used the same anti-sense primer with the following sense primer: 5′-GGTGCCA-TC[C/T]T[C/G]AC[C/G/A]AC[C/T]ATGCT-3′ corresponding to the amino acid sequence GAILTT-ML (γ_B CaMK-II$^{302-309}$). Products were separated on 3% low melting point agarose for 2 h at 80 V and visualized with ethidium bromide.

To further define isozyme expression in various cell lines (Fig. 4), nested PCR was again used under the same conditions described above. After the same

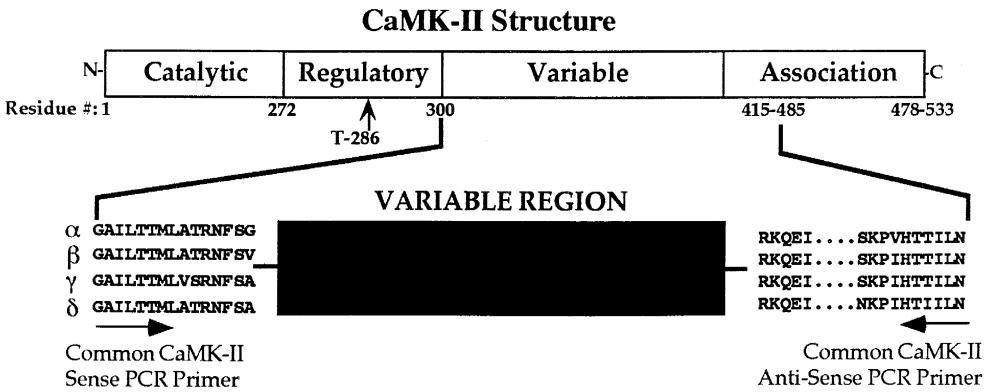

Fig. 1. CaMK-II primary structure and PCR primer design. All 4 mammalian CaMK-II genes are composed of catalytic, regulatory, variable and association regions. PCR primers (arrows) which were common to all CaMK-II's were used to amplify sequences contained within the variable and a portion of the association region.

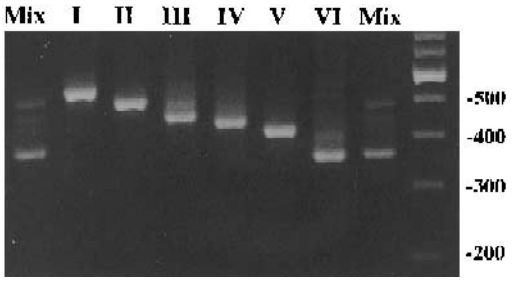

Fig. 2. CaMK-II variable region amplification and cloning by RT-PCR from mammary tumor cells. Oligo dT-primed first strand cDNA from ZR-75-1 mammary tumor cells was subjected to PCR using the primers designated in Fig. 1. The resulting PCR products (lanes 1 and 8: MIX) were directly cloned. Clones were then re-amplified with the same primer set and separated on a 3% agarose gel to reveal 6 size classes (I–VI).

PCR primers used in Fig. 2 were used in primary reactions on first strand cDNA, the following antisense primers for α, β, γ and δ CaMK-II were used in the secondary PCR reaction with the 'GAILTTML' sense primer. For α CaMK-II: 5′-TTGTTTCCTCC[A/G]CTCTTCCC-3′, corresponding to rat α CAMK-II$^{315-322}$: GGKSGGNK; for β CaMK-II: 5′-TTTGGTGCTGTTTGTCTGGG-3′, corresponding to human β_e CaMK-II$^{329-335}$: PQTNSTK; for γ CaMK-II: 5′-TACCACGTGGTTTGTGGCTC-3′, corresponding to human γ_B CaMK-II$^{354-360}$: EPQTTVV and for δ CaMK-II: 5′-TTTATTGATTTTGGACAAAGC-3′, corresponding to human δ_C CaMK-II$^{404-409}$: KSNKPI.

2.3. Cloning and sequencing

PCR products were cloned directly into the 'T/A' cloning vector, pCR-II (Invitrogen, San Diego CA). Plasmid DNA was then purified from isolated clones using selective isopropanol precipitation [39]. Sequencing was conducted in duplicate by SP6 or T7 primed fluorescently tagged dideoxynucleotide terminator sequencing reactions [42] for automated sequencing (Applied Biosystems, Foster City, CA). Consensus sequences from at least 3 separate clones were used to compile the final sequences reported. GenBank Accession Numbers are U50358 for β_e, U50359 for γ_G, U50360 for γ_H, U50361 for δ_C and U73738 for δ_E.

2.4. Cytosol preparation

In order to examine both CaMK-II activity and immunoreactive polypeptides, cytosolic fractions were prepared from cells harvested by either scraping or detaching with trypsin-EDTA and rinsing centrifugally ($2000 \times g$ for 5 min) with PBS (phosphate buffered saline) containing 5 mM EDTA. Cellular pellets were dissolved in 3 volumes of homogenization buffer, which consisted of 20 mM Hepes, pH 7.4, 2.6 mM EDTA, 20 mM $MgCl_2$, 80 mM β-glycerol phosphate, 50 mM NaF, 0.1 mM dithiothreitol, 0.01 mg/ml each chymostatin, leupeptin, aprotinin, pepstatin and soybean trypsin inhibitor (Sigma, St. Louis MO). Dissolved samples were then sonicated (two 5-s bursts on ice), centrifuged at $10\,000 \times g$ for 15 min at 4°C and either assayed immediately or stored at −70°C. Lysates prepared by sonication solubilized over 90% of the total CaMK-II as measured by solution assays [23].

2.5. CaMK-II immunoblots

1–10 μg cytosol was diluted with an equal volume of preheated 2× sample buffer [43], followed by boiling for 3 min. Protein concentrations were determined by BCA assay (Pierce, Rockford IL) in triplicate. Discontinuous SDS gels were prepared with 7% polyacrylamide. Proteins were transferred to nitrocellulose and nitrocellulose was blocked by a 1 h incubation with 5% bovine serum albumin (BSA), 2% normal goat serum in Tris-buffered saline containing 0.05% Tween-20 (TBST). The primary antibody incubation was overnight at room temperature with a rabbit polyclonal anti-CaMK-II antibody (UBI, Lake Placid, NY) diluted to 1 μg/ml in TBST/1% BSA. This antibody was prepared against an N-terminal and C-terminal peptide immunogen common to all known CaMK-II isozymes. Blots were rinsed 3 times with TBST and then incubated for 1 h at room temperature with 0.25 μg/ml biotinylated goat anti-mouse IgG (KPL, Gaithersburg, MD). Blots were rinsed 3 times with TBST and then incubated for 1 h at room temperature with 2.5 μg/ml streptavidin-alkaline phosphatase (Molecular Probes, Eugene, OR). After 3 rinses in TBST, blots were rinsed once in 0.1 M NaCl, 50 mM $MgCl_2$, 30 mM Tris, pH 9.2

and then developed with 50 μg/ml nitro blue tetrazolium and 50 μg/ml 5-Br, 4-Cl indolyl phosphate in this buffer.

3. Results

3.1. Human tumor cells express as many as 8 different isozymes of CaMK-II

CaMK-II activity has previously been detected in non-neuronal proliferative cells [23,25] and has been implicated in the regulation of growth control [22,23]. In order to further investigate the role of CaMK-II in growth control, this study used a comprehensive strategy to identify all expressed CaMK-II isozymes in human tumor and in other non-transformed cell lines. Since isozyme complexity is believed to be accomplished by alternative splicing in each of the four mammalian (α, β, γ and δ) CaMK-II genes [9] in a domain which spans the variable domain and a portion of the association domain, PCR primers were chosen which bracketed this entire region and were common to all CaMK-II's (Section 2, Fig. 1). Nested sets of PCR primers were used to amplify sequences from oligo dT-primed cDNA. PCR amplification conditions were optimized using brain cDNA (see Section 2). When RNA from the human mammary tumor cell line, ZR-75-1, was used as the template for cDNA synthesis and PCR, a mixture of bands between 350 and 550 bp (Fig. 2, lanes 1 and 8: Mix) was observed. This entire mixture was directly cloned, without fractionation and 85 positive clones were then evaluated. Upon amplification with the same primer set, each clone exhibited one of the bands from the PCR mixture in the size range of 350 to 550 bp. A representative clone from each one of the 6 size classes (I–VI) is shown (lanes 2–7). Approx. 70% of these clones were in class VI, reflecting the predominance of this size band in the initial PCR product mixture. All other classes were approximately equally represented.

Two to five clones representing each cDNA class were sequenced (Fig. 3). Sequencing confirmed that each of the 6 separate size classes shown in Fig. 2 represented distinct CaMKII cDNA types. Of the 6 cDNA classes, I,II,III and V were most similar to known γ CaMK-II isozymes, IV was most like β CaMK-II and VI like δ CaMK-II. Classes II and V were identical to human CaMK-II γ_B and γ_C, respectively, at the nucleotide level [34]. Two additional CaMK-II isozymes were cloned in a similar way from cDNA from the LAN5 neuroblastoma cell line. These two additional clones were most like a β CaMK-II and a δ CaMK-II, respectively. Fig. 3

Sequence of Novel Human Tumor Cell CaMK-II Variable Region

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                  301
Human β'e: GAILTTMLATRNFSAAKSLLNKKADGVKPQTNSTKNSAAATSPKGTLPPAAL----------------ESSDSANTTIEDEDAKA
Human βe: GAILTTMLATRNFSAAKSLLNKKADGVKPQTNSTKNSAAATSPKGTLPPAALEPQATVIHNPVDGIKESSDSANTTIEDEDAKA
Human γG: GAILTTMLVSRNFSAAKSLLNKKSDGGVKPQSNNKNSL---------------EPQTTVVHNATDGIKGSTESCNTTTEDEDLKARSPEGRSSRDRTAPSAG
Human γH: GAILTTMLVSRNFSAAKSLLNKKSDGGVKPQSNNKNSL---------------EPQTTVVHNATDGIKGSTESCNTTTEDEDLKV
Human δC: GAILTTMLATRNFSAAKSLLKKPDGVK-----------------------------------------ESTESSNTTIEDEDVKA
Human δE: GAILTTMLATRNFSAAKSLLKKPDGVK-------------------------EPQTTVIHNPDGNKESTESSNTTIEDEDVKA

                                                                                  441
Human β'e: ------------RNQEIIKTTEQLIEAVNNGDFEAYAKICDPGLTSFEPEALGNLVEGMDFHRFYFENLLAKNSKPIHTTILNP..
                                                                                456
Human βe: ------------RNQEIIKTTEQLIEAVNNGDFEAYAKICDPGLTSFEPEALGNLVEGMDFHRFYFENLLAKNSKPIHTTILNP..
                                                                                471
Human γG: MQPQPSLCSSAMRKQEIIKITEQLIEAINNGDFEAYTKICDPGLTSFEPEALGNLVEGMDFHKFYFENLLSKNSKPIHTTILNP..
                                                                                443
Human γH: ------------RKQEIIKITEQLIEAINNGDFEAYTKICDPGLTSFEPEALGNLVEGMDFHKFYFENLLSKNSKPIHTTILNP..
                                                                                416
Human δC: ------------RKQEIIKVTEQLIEAINNGDFEAYTKICDPGLTAFEPEALGNLVEGMDFHRFYFENALSKSNKPIHTIILNP..
                                                                                430
Human δE: ------------RKQEIIKVTEQLIEAINNGDFEAYTKICDPGLTAFEPEALGNLVEGMDFHRFYFENALSKSNKPIHTIILNP..
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Fig. 3. Predicted amino acid sequence of human CaMK-II β_E, γ_G, γ_H and δ_C. Clones representing all 6 size classes from ZR-75-1 cDNA (Fig. 2) were sequenced. Classes II and V represented known human CaMK-II sequences (γ_B and γ_C CaMK-II). The other 4 classes represent novel human sequences. Two additional novel human cDNA's were cloned from LAN5 neuroblastoma cells. The predicted amino acid sequences of the 6 unpublished human CaMK-II's (β'_e, β_e, γ_G, γ_H δ_C, and δ_E are shown aligned to each other.

shows the predicted amino acid sequences across the variable domain of the 6 novel human CaMK-II isozymes reported here. There are two variants of γ CaMK-II, (γ_G and γ_H), two variants of β CaMK-II, (β_e and β'_e), and two variants of δ CaMK-II, (δ_C and δ_E) identified in this study [13,35,37].

CaMK-II β_e has a structure which would be predicted for a splice variant lacking two exons, when compared to the rodent β CaMK-II gene [32]. This isozyme has been called β_e since it was originally discovered in embryonic rat brain, along with CaMK-II β'_e [44]. Where the sequences align, human β CaMK-II was identical to the corresponding sequence in rodent β CaMK-II in 91% of 423 nucleotides and in 96% of 141 amino acids [32] excluding the two gaps. The LAN5 β'_e clone lacks an additional exon when compared to β_e. None of the β clones detected here contained the proline-rich domain found in CaMK-II β_3 [33]. There is no evidence for β_3 CaMK-II in ZR-75-1 or LAN5 cells as it would have a variable domain PCR product size (675 bp) outside of the range observed (Fig. 2). This is the first report of human β CaMK-II sequences and the second example of non-neuronal β CaMK-II [33].

Both γ_G and γ_H CaMK-II contain inserts which are missing from previously described human non-neuronal γ CaMK-II's (γ_B and γ_C), but are otherwise identical in nucleotide sequence [34]. These isozymes are distinct from γ_D and γ_E CaMK-II, isozymes recently identified in human biliary epithelial cells [35].

δ_C CaMK-II was the most predominant clone found in this screen. Also known as δ_2, this cDNA was identical to rodent CaMK-II in 93% of the nucleotides and in 98% of the amino acids [13,37]. An additional CaMK-II δ isozyme, δ_E is also reported here. The partial cDNA encoding this isozyme was cloned from the neuroblastoma cell line, LAN5. δ_E represents a novel variant of δ CaMK-II and contains an additional domain when compared to δ_C CaMK-II. These are also the first reports of any human δ sequences.

3.2. Human tumor cells express a unique pallern of CaMK-II isozymes

Based in part on the human sequences reported here, four additional PCR primer sets were prepared in order to screen cDNA's from a wide variety of different human cell types for the expression of CaMK-II variants from the four mammalian CaMK-II genes. These additional primers were also nested within the first primer set used in Fig. 1 (see Section 2) and were specific for each of the 4 (α, β, γ and δ) mammalian CaMK-II genes. Approximately equivalent amounts of cDNA from neuronal tissue, tumor cells, embryonic fibroblasts and non-malignant mammary epithelial cells and tissue were examined with each of these 4 primer sets under identical conditions of amplification (Fig. 4). Although this study was not strictly quantitative PCR, these results indicate that a completely different spectrum of CaMK-II cDNA's was found in adult neuronal tissue (lane 2) than in tumor or 'normal' cells, including those of neuronal origin (lanes 3–11).

Human cerebral cortex (lane 2) and rat cerebral cortex cDNA (not shown) expressed identical spectra of CaMK-II isozymes. α CaMK-II expression is indicated by co-migration with a rodent α CaMK-II cDNA standard (lane 12). β CaMK-II was identified by a principal band at 195 bp from cerebral cortex, as expected for neuronal β CaMK-II. A weak band at 110 bp was also observed which co-migrated with that amplified from the β_e CaMK-II cDNA standard (lane 12). Multiple γ CaMK-II variants were expressed in neuronal cells. γ_A CaMK-II (205 bp) was weakly amplified from rat and human neuronal cDNA. The 185 bp band co-migrated with the standard for γ_B (lane 12) and the 145 bp band co-migrated with the γ_G/γ_H CaMK-II standard (lane 13). γ_C CaMK-II yields a band at 115 bp (lane 14), but this was absent from cerebral cortex. The major bands at 250 and 170 bp are most likely neuronal β and δ_A CaMK-II, respectively, which are the principal representatives of their respective genes and which share sequences with the γ primer pair. Due to the complexity in γ CaMK-II variants, as revealed by this study, not all γ CaMK-II variants can be distinguished by the PCR primers used here. Rather, gel analysis of PCR products defines γ CaMK-II subclasses as indicated in Fig. 4. δ_A CaMK-II (420 bp) is the principal δ CaMK-II in neuronal tissue and as with β CaMK-II, there are trace amounts of the truncated variants δ_E and δ_C CaMK-II at 380 and 320 bp, respectively.

α CaMK-II was never detected in any other cell

type examined (lanes 3–11), including those derived from a neuronal tumor (lane 3). Likewise, the neuronal β CaMK-II was never observed in cells other than adult brain. β_e (or β'_e) CaMK-II (110 bp) was the only β CaMK-II seen in these cells and its expression was variable. β_e/β'_e was observed in neuroblastoma (lane 3) and mammary tumor cells (lane 7), but was absent from all other tumor cell lines, including small cell lung carcinoma (lane 4), colon carcinoma (lane 5) and leukemic (lane 6) cells. cDNA from non-malignant mammary tissue (lane 8) or a non-transformed breast epithelial cell line, MCF10 A (lane 9), also contained this band, but it was consistently less intense when compared under identical amplification conditions to the ZR-75-1 cell line. Other mammary tumor cells were screened under identical conditions and expressed similar levels of β_e CaMK-II to ZR-75-1 cells (data not shown). Embryonic human (lane 10) or rodent (lane 11) fibroblasts also contained β_e CaMK-II. These PCR primers cannot distinguish β_3 CaMK-II [33] from β_e or β'_e CaMK-II, since they share sequences in this region. However, β_3 CaMK-II was not detected in ZR-75-1 or LAN5 tumor cells, since a band with the predicted size (675 bp) for the β_3 variable domain was not observed with the common variable domain primer set (Fig. 2).

γ_B and γ_C CaMK-II (185 and 115 bp, respectively) were consistently seen in every nonneuronal cell line examined, in contrast to the absence of α CaMK-II and the variable expression of β CaMK-II. Since these comparative studies were done simultane-

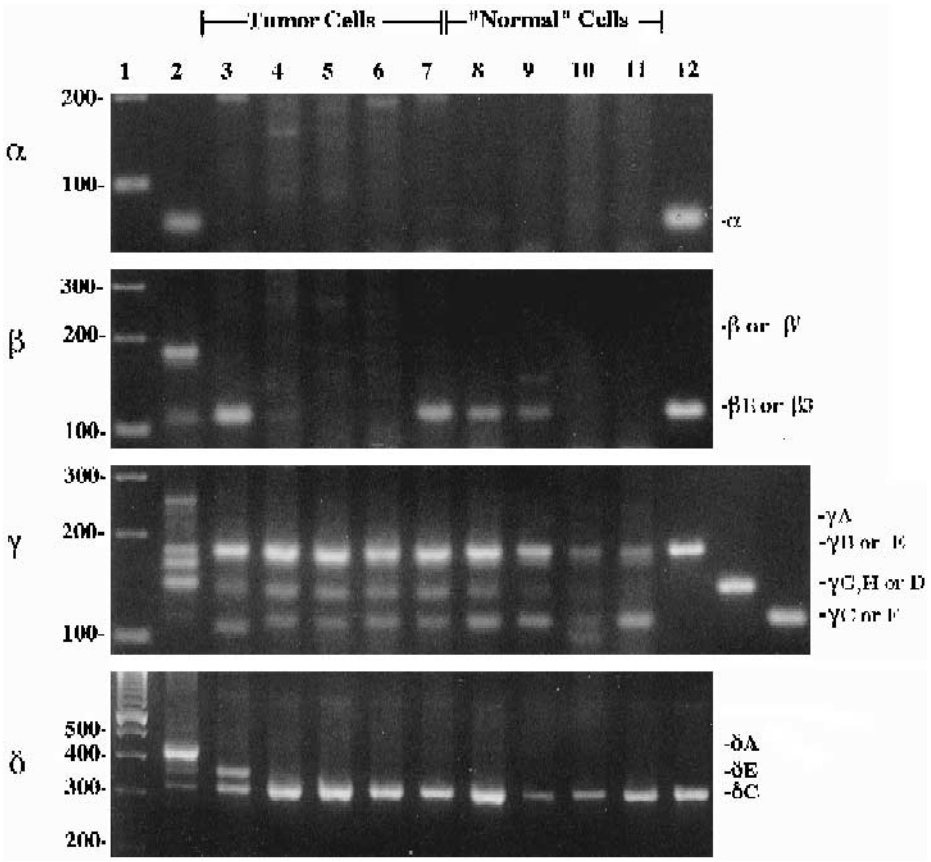

Fig. 4. RT-PCR analysis of α, β, γ and δ CaMK-II in human cells and tissues. Oligo dT-primed cDNA from adult human cerebral cortex (2), LAN5 neuroblastoma (3), H146 small cell lung carcinoma (4), HT29 colon carcinoma (5), CEM leukemic (6); ZR751 mammary tumor cells (7), normal mammary tissue (8), non-malignant mammary epithelial cells (9), WI-38 human fibroblasts (10) and rodent NIH 3T3 cells (11) was subjected to nested PCR as described in Section 2. α, β_E, γ_B, and δ_C were amplified with their respective primer sets and are shown in lane 12. Lanes 13 and 14 in the γ CaMK-II panel are γ_G and γ_C, respectively. Expected PCR products from all known CaMK-II variants are indicated.

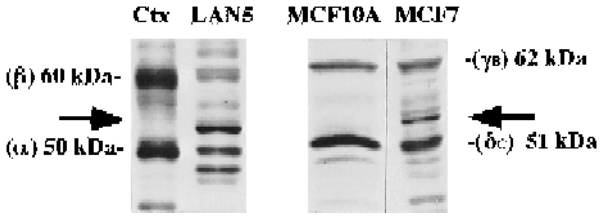

Fig. 5. Identification of CaMK-II polypeptides in human tumor cells. 4 μg of cytosol from rat brain (Ctx) and 8 μg from each of the indicated cells was separated by SDS-PAGE, transferred to nitrocellulose and probed with a pan-specific polyclonal antibody against CaMK-II. Samples included rat brain cortex: (Ctx); neuroblastoma (LAN5); immortalized mammary epithelial cell line (MCF10 A) and mammary adenocarcinoma (MCF7). Relative masses are indicated for α, β, γ_B and δ_C. Large arrows indicate bands which are expressed in tumor cells, but absent from comparable non-transformed tissue or cells.

ously from the same source of cDNA, γ_B and γ_C CaMK-II serve as excellent internal controls for the variability in the expression of other genes. As previously shown by RNase protection [34] and by the relative level of amplification of γ variants shown here, γ_B is the predominant γ splice variant in nonneuronal cells. Tumor cells preferentially expressed bands which co-migrated with the γ_G CaMK-II standard (lane 13), when compared to non-malignant fibroblasts.

δ_C CaMK-II was also consistently seen in every non-neuronal cell line examined, regardless of malignant status. Although neuroblastoma cells expressed both δ_C and δ_E CaMK-II, not a single malignant or non-neuronal proliferative cell line expressed δ_A CaMK-II, the primary δ CaMK-II in rodent and human cerebral cortex.

CaMK-II immunoblots were also conducted on selected tissues and cell lines (Fig. 5) in order to qualitatively corroborate the RT-PCR findings at the protein level. Soluble lysates were prepared from rat cerebral cortex and from the human cell lines LAN5 (neuroblastoma), MCF10A (mammary epithelial cell line) and MCF7 (mammary adenocarcinoma). Fresh rat cerebral cortex was used since it yields an identical spectrum of CaMK-II isozymes to human cerebral cortex when compared by RT-PCR. Results of this experiment indicated that transformed cells expressed CaMK-II proteins not present in their non-transformed counterparts. For example, cerebral cortex (Ctx) contains major CaMK-II polypeptides of M_r 50 and 60 kDa and less intense bands at 51 and 58 kDa, as expected. The 50 kDa band is believed to represent α CaMK-II, the 60 kDa band represents β CaMK-II while δ_A CaMK-II has a M_r of 59 kDa [13,45]. In contrast, LAN5 cells lack the 50 and 60 kDa α and β CaMK-II bands; they are replaced by reactive proteins at 61/62 kDa (doublet), 57 kDa, 54 kDa (arrow; strongest reactivity) and 51 kDa. The band at approx. 48 kDa is unidentified. From RT-PCR, LAN5 cells express β_e or β'_e at least three γ isozymes and the two δ isozymes, δ_C and δ_E (Fig. 4, lane 3). The 62 kDa band co-migrates with that expected for CaMK-II γ_B and the 61 kDa band with that predicted for CaMK-II γ_G. Rat embryonic brain β_e and β'_e CaMK-II have M_r of 58 and 56 kDa, respectively [44]. δ_C CaMK-II has a predicted M_r of 51 kDa [13] and δ_E CaMK-II an M_r of 53 kDa. Although it is unclear which isozyme the major immunoreative 54 kDa CaMK-II (arrow) represents, it is clear that this and other bands are absent from adult brain. Likewise, although both MCF7 and MCF10 A cells express the 51 and 62 kDa CaMK-II proteins, there are CaMK-II proteins in MCF7 cells which are not present in their non-transformed counterpart, MCF10 A (lanes 3 and 4). The spectrum of CaMK-II proteins, like mRNA's, is similar amongst the human tumor cell lines, LAN5 and MCF7 and indicate that while δ_C and γ_B are uniformly expressed in all proliferative cells, β_e/β'_e, γ_G and γ_H are preferentially expressed in tumor cells.

4. Discussion

This study has identified at least 8 separate CaMK-II isozymes which are expressed in human cell lines derived from a wide variety of tumors, but which are absent from adult neuronal tissue. The 8 tumor cell CaMK-II isozymes described here are products of the β CaMK-II gene (β_e, β'_e), the γ CaMK-II gene (γ_B, γ_C, γ_G and γ_H) and the δ CaMK-II gene (δ_C, δ_E). Three of these isozymes (γ_G, γ_H and δ_E) have not previously been reported. Although only β CaMK-II isozymes have been confirmed as splice variants by comparison to the rodent β CaMK-II gene, it is widely believed that variants of all four CaMK-II genes [13,14,18,19,33,36] are achieved through alternative splicing in the variable domain. The total

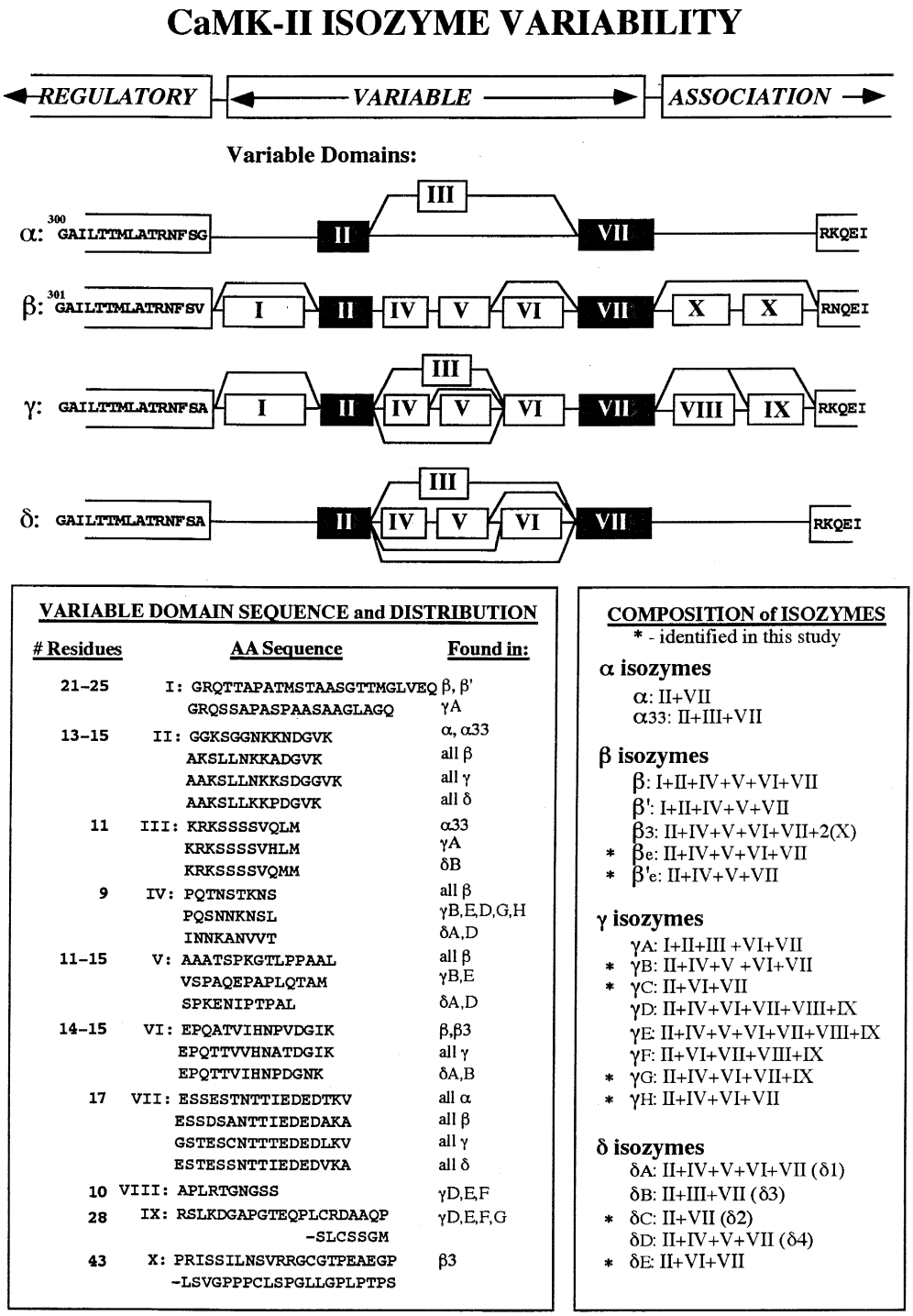

Fig. 6. CaMK-II variable region domain structure. The variable domain structure of all known CaMK-II cDNA's is represented schematically based on the concept that the 4 genes are related. Sequences were obtained from both rodent and human CaMK-II's. Variable domains I through X are based on the β CaMK-II exon structure as well as on cDNA's which contain or omit one of these domains (upper panel). Domains II and VII are darkened to indicate their proposed role as a conserved tether in all isozymes. All known CaMK-II isozymes are indicated schematically by lines which connect domains and are summarized by domain composition (lower right panel). Domain sequence is also indicated in single letter amino acid code (lower left panel).

number of mammalian CaMK-II isozymes achieved through variations in this domain is now at least 20, including 2 α, 5 β, 8 γ and 5 δ. Additional δ CaMK-II isozymes are achieved through variations at the C-terminus [36]. This is also the first report of human β and δ CaMK-II sequences. It also confirms recent and new indications that β CaMK-II is not exclusively expressed in the brain [33].

Although the role of each of these isozymes in cell function is not yet known, the expression of over 20 CaMK-II isozymes is consistent with the multiple functions implicated for CaMK-II, including memory, secretion, metabolism and cell growth control. The results presented here confirm that CaMK-II expression is modulated at the level of mRNA splicing. Tumor cell lines, including the neuroblastoma cell line, LAN5 and the H146 small cell lung carcinoma cell line, which is of neuroendocrine origin, lose almost every neuronal-specific CaMKII isozyme, even though 3 of the 4 CaMK-II genes continue to express product.

Careful examination of the sequence of all known CaMK-II isozymes has revealed ten separate domains which are subject to splicing (Fig. 6). Each of these domains may have a particular function which may be associated with the cell type in which the isozyme(s) which contain that domain are expressed. The boundaries of these domains (I–X) were derived from the known intron-exon structure of β CaMK-II [32] and from cDNA's whose exon boundaries are not known, but were predicted from differences between isozymes. As shown, each of these domains has similar length and sequence between genes, although some domains are gene-specific. Each known CaMK-II variant is composed of between 3 and 7 of these separate domains, presumably achieved through alternative splicing. The lines connecting these domains (Fig. 6) represent all known possibilities.

Variations in the domain use among the four CaMK-II genes occur in a progressively complex manner suggestive of an evolutionary hierarchy. For example, in bctween the two 'tether' domains, II and VII, the only optional domain in α CaMK-II is domain III and in β CaMK-II, domain VI. In contrast, γ and δ CaMK-II optional domains include all of those between III and VI. In other words, γ and δ CaMK-II use a combination of domain options from α and β CaMK-II. Further options, particular to β, γ and δ CaMK-II on both the N-terminal side of domain II and the C-terminal side of domain VII, increase the number of existing and potential gene products. These observations suggest that a shift in splicing signals could act similarly upon all CaMK-II genes to restrict or expand the spectrum of isozymes expressed in different tissues.

All CaMK-II's can be described based on the presence or absence of domains. All 8 of the tumor cell CaMK-II isozymes identified here and implicated from isozyme-specific PCR (Fig. 4), lack domain I. In fact, domain I has only been found in neuronal-specific β and γ isozymes; it is missing from every non-neuronal CaMK-II isozyme identified. It has been suggested that domain I may influence the binding affinity for CaM [13] since it resides on the catalytic side of the invariant tether domain (II). In β CaMK-II, the exon just prior to domain I comprises the C-terminal CaM binding domain [32,46]. From structural analysis, Leu^{300} and Leu^{309} are responsible for interacting with the N- and C-terminal hydrophobic binding pockets of CaM [47]. Leu^{309} is only 7 amino acids from this missing domain in β_e CaMK-II. Thr^{305} and Thr^{306} and Ser^{314} are sites of autophosphorylation in neuronal CaMK-II known to influence catalytic activity [30]. It is possible that phosphorylation or elimination of domain I may influence CaM binding and therefore catalytic activity.

Domain II represents the invariable 'tether' domain found in every CaMK-II isozyme. It is thought to connect the regulatory and variable regions. Domain III is found in only 3 cDNA's and is responsible for nuclear targeting [14]. Of the tumor cells evaluated here, none expressed a putative nuclear targeted CaMK-II isozyme. CaMK-II has been reported in the nucleus of proliferative cells [48] and may contribute to the regulation of gene expression [11], however, none of the 8 CaMK-II isozymes found in human tumor cells contain this nuclear localization sequence and would therefore not be expected to be targeted to the nucleus. CaMK-II need not enter the nucleus to function in regulating cell growth control; it could act through the modification of a cytoplasmic member of the growth signaling cascade, as suggested by its kinetics of activation [26].

Domain IV, rich in basic amino acids is found in γ_B, γ_G, γ_H, β'_e and β_e. Neither this domain nor

domain V have strong tissue-specificity, being found in both neuronal and non-neuronal isozymes. Domain V is rich in proline (20–27%) and is found in half of all CaMK-II's. Of the tumor cell CaMK-II's, β'_e, β_e and γ_B CaMK-II contain insert V. This domain was implied by comparing the sequence of the γ_D, γ_G and γ_H CaMK-II isozymes. This suggests gene or species-specific exon differences since domains IV and V are encoded as a single exon in rodent β CaMK-II [32].

Domain VI is found in all γ isozymes including γ_B, γ_C, γ_G and γ_H Within domain VI, Thr^{382} is rapidly autophosphorylated in β CaMK-II [9,30], but the result of its phosphorylation is not known. Of domains IV, V and VI, each tumor cell CaMK-II contains between 1 and 3 of these domains, except for δ_C, which contains none.

Domain VII is found in every CaMK-II; its length and three quarters of its amino acids are absolutely invariant. This domain has a net charge of -4 to -5 and contains between 29 and 41% serine or threonine residues. It is believed to be at the beginning of the 'association domain' and is therefore analogous to domain II, serving as a conserved tether between the variable and association domains.

Domains VIII and IX are specific to the γ CaMK-II gene. Domain VIII is a 10 amino acid domain specific to γ_D, γ_E and γ_F while domain IX is a 28 amino acid insert specific to γ_D, γ_B, γ_F and γ_G. Among the tumor cell CaMK-II's described in this study, domain IX was found in γ_G CaMKII, but not in γ_H CaMK-II. Unlike other domains, VIII and IX have only been found in nonneuronal γ isozymes and therefore appear to be γ gene-specific. The absence of domain VIII from both γ_G and γ_H CaMK-II defines another potential exon boundary not previously realized.

Domain X is a β CaMK-II-specific proline-rich domain described only in CaMK-II β_3 from the rat pancreas [33]. This domain was not identified, however, in the genomic mouse CaMK-II sequence [32]. Interestingly, this domain conforms to SH3-binding sequences, and may therefore be targeted directly to the growth factor machinery [33]. These domains could also target isozymes to subcellular sites or substrates or influence the oligomeric nature of CaMK-II.

There are several additional mammalian δ CaMK-II isozymes whose variability is additionally conferred at the distal C-terminus, where the last 21 amino acids are either inserted or deleted [36]. These additions are specific to δ CaMK-II's. Fig. 6 reveals that CaMK-II δ_C, like CaMK-II α, contains only 2 of the 7 possible variable domains in the variable region.

In summary, the results presented here suggest that at some point in neuronal tumorigenesis, the α CaMK-II gene is turned off and the splicing signals amongst the other three CaMK-II genes are similarly changed; i.e., all new variants are truncated with similar domain omissions. These results also suggest that the opposite change in CaMK-II splicing accompanies differentiation since undifferentiated embryonic fibroblasts have a pattern distinct from adult differentiated neuronal tissue and much more like that of tumor cells.

Transformed cells have previously been shown to undergo alterations in their dependence on Ca^{2+}, in their level of CaM and in CaM binding proteins [3,49–54]. This report now shows that they also undergo changes in the expression pattern of CaMK-II. Although it is not known which isozyme(s) may regulate cell growth in human tumor cells, the inappropriate expression of CaMKII variants in tumor cells may contribute to the malignant phenotype.

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