Developmental and Regional Expression of Multifunctional Ca^{2+} /Calmodulin-Dependent Protein Kinase Isoforms in Rat Brain

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Multifunctional Ca^{2+} /calmodulin-dependent protein kinase (CaM kinase) participates in diverse calcium signaling pathways in neurons. The α - and β -CaM kinase isoforms are neuron-specific and highly abundant in rat brain. The variable domain of CaM kinase is a potential site for the generation of isoform diversity by alternative splicing of its N- and/or C-terminal segments. We used specific PCR primers which span the variable domain of either α - or β -CaM kinase and isolated three new isoforms from rat brain, namely α_B -, β_e - and β'_e -CaM kinase. α_B -CaM kinase contains 11 amino acids, likely inserted by alternative splicing, at the C-terminal segment of the variable domain. This insertion introduces a nuclear localization signal (NLS) that targets α_B -CaM kinase to the nucleus of transfected cells; α -CaM kinase is excluded from the nucleus. The mRNA and the protein corresponding to this isoform are detected only in the diencephalon/midbrain regions. We have also identified two alternatively spliced isoforms of β -CaM kinase that lack the 24 amino acid sequence at the N-terminal segment of the variable domain. Alternative splicing of these two isoforms occurs with a three base pair shift of the 3'-splice site. Our analysis shows that these new β -CaM kinase isoforms are expressed primarily in early developmental stages, and we therefore term them β_e - (embryonic) and β'_e -CaM kinase. Recombinant α_B -, β_e - and β'_e -CaM kinase expressed in COS-7 cells exhibit characteristic Ca^{2+} /calmodulin-dependent protein kinase activity and autophosphorylation.

[Key words: CaM kinase, alternative splicing, isoforms, development, nucleus, calcium]

Multifunctional Ca^{2+} /calmodulin-dependent protein kinase (CaM kinase or CaM kinase II) is a major mediator of calcium signaling in neurons. Increases in the intracellular concentration of calcium lead to the activation of the kinase which in turn phosphorylates multiple substrates on serine/threonine residues. CaM kinase is involved in diverse functions in neurons, including neurotransmitter synthesis and neurotransmitter release, modulation of ion channels

activity, synaptic plasticity, and gene expression (reviewed in Hanson and Schulman, 1992a; Braun and Schulman, 1995). The kinase has been localized to both the presynaptic and the postsynaptic compartments of neurons. In adult brain, about 75% of CaM kinase activity is associated with cytoskeletal elements, postsynaptic densities (PSD), and synaptic vesicles, and the remaining activity is cytosolic (reviewed in Rostas and Dunkley, 1992).

The diversity in the function and the subcellular localization of CaM kinase suggests a structural heterogeneity of the enzyme. Structural complexity arises in part from the expression of CaM kinase from four distinct genes— α , β , γ , and δ (Bennett and Kennedy, 1987; Hanley et al., 1987; Lin et al., 1987; Tobimatsu et al., 1988; Tobimatsu and Fujisawa, 1989). All the isoforms share a domain structure consisting of a catalytic domain at the N-terminal half followed by a regulatory domain which contains an autoinhibitory segment overlapping a calmodulin binding site. The C-terminal part of the protein is called the association domain and is thought to be involved in the oligomerization of CaM kinase isoforms to a holoenzyme (Lin et al., 1987; Yamauchi et al., 1989).

The least conserved region across CaM kinase sequences is at the border of the regulatory and association domains, termed the variable domain. It consists of a 12–14 amino acid “core” or linker sequence that is present in all the isoforms and may be flanked by N-terminal and/or C-terminal segments. Each of the four CaM kinase genes can give rise to several isoforms by putative alternative splicing of the mRNA at the variable domain. Alternatively spliced isoforms of CaM kinase which contain different insertions in their variable domain include β and β' isoforms from rat brain (Bennett and Kennedy, 1987), CaM kinase isoforms from *Drosophila* (Griffith and Greenspan 1993; Ohsako et al., 1993), γ isoforms from human lymphocytes (Nghiem et al., 1993) and δ isoforms from rat heart (Mayer et al., 1993; Schworer et al., 1993; Edman and Schulman, 1994).

Are the α -, β -, γ -, and δ -CaM kinase isoforms functionally redundant or do they subserve specific physiological roles? Differences in kinetic properties, substrate specificity, subcellular localizations and/or expression patterns between the isoforms would argue for their functional significance. While little is known about unique kinetic properties or substrate specificity, several studies have described discrete expression patterns for each of the CaM kinase isoforms. α - and β -CaM kinase isoforms are neuron-specific and very abundant in the brain while γ - and δ -CaM kinase isoforms are expressed at low levels in all tissues including brain (Tobimatsu and Fujisawa, 1989). Furthermore, the expression of α - and β -CaM kinase isoforms is not uniform across regions of the brain (Miller and Kennedy, 1985). In the adult rat, α -CaM kinase is enriched in the forebrain, leading to

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a α : β subunit ratio of 3:1. In the cerebellum, the level of α -CaM kinase expression is much lower resulting in a α : β subunit ratio of 1:3-4. The γ - and δ -CaM kinase isoforms each exhibit a distinct regional distribution in rat brain (Sakagami and Kondo, 1993). Other studies have shown that CaM kinase expression is developmentally regulated (Sahyoun et al., 1985; Kelly et al., 1987). In the neonatal rat, β -CaM kinase is expressed at a higher level than α -CaM kinase throughout the brain. However, while β -CaM kinase expression stays almost constant thereafter, the α -CaM kinase level in the forebrain increases by approximately 10 fold during the second postnatal week. In addition, α - and β -CaM kinase may also differ in their subcellular localization since mRNA for α - but not β -CaM kinase is found in the dendrites of hippocampal and cortical neurons (Burgin et al., 1990; Benson et al., 1992).

Do alternatively spliced isoforms of CaM kinase have unique patterns of expression? The only alternatively spliced isoforms described thus far in the brain, β - and β' -CaM kinase, seem to have identical expression patterns. In addition, alternatively spliced γ_β - and γ_c -CaM kinase isoforms have similar expression patterns in non-neuronal tissues (Nghiem et al., 1993). However, the splicing of non-neuronal δ -CaM kinase mRNA is regulated in a tissue-specific manner (Schworer et al., 1993). Furthermore, we have recently demonstrated differential intracellular localization for alternatively spliced δ -CaM kinase isoforms from heart (Srinivasan et al., 1994). Insertion of 11 amino acids in the variable domain of δ_β -CaM kinase generates a nuclear localization signal which targets the protein to the nucleus in transfected cardiac myocytes. In contrast, δ_α - and δ_c -CaM kinase isoforms which lack this particular insert are cytosolic. Benson et al. (1991a) isolated a PCR fragment of monkey α -CaM kinase variable domain, termed α_{33} , which included a similar NLS-generating insert.

We describe here three new alternatively spliced isoforms of CaM kinase that were identified by RT-PCR using specific primers designed to span the variable domain of α - or β -CaM kinase. Each of the isoforms, termed α_β -, β_c -, and β'_c -CaM kinase, contains insertions or deletions in its variable domain. Interestingly, the expression patterns of the new isoforms are unique and differ from those of previously identified isoforms. While α -CaM kinase is expressed in throughout the brain, α_β -CaM kinase can be detected only in the diencephalon/midbrain regions. β_c - and β'_c -CaM kinase are expressed earlier in development than β - and β' -CaM kinase isoforms. In addition, we demonstrate that α_β -CaM kinase isoform is targeted to the nucleus in transfected cells. These observations provide the first example of differential expression of alternatively spliced isoforms of CaM kinase in the brain and support the idea that they are functionally significant.

Materials and Methods

Materials. Restriction enzymes and other DNA modifying enzymes were purchased from GIBCO BRL (Gaithersburg, MD) or New England BioLabs (Beverly, MA). γ - 32 P-ATP (6000 Ci/mmol) was obtained from New England Nuclear (Boston, MA) and α - 35 S-ATP was obtained from Amersham (Arlington Heights, IL). Rat forebrain CaM kinase was purified as described previously (Schulman, 1984). Monoclonal antibodies for CaM kinase are anti α -CaM kinase monoclonal antibody (CB α 2) and anti β -CaM kinase monoclonal antibody (CB β 1) (Baitinger et al., 1990) (also available from Gibco BRL). A second anti α -CaM kinase monoclonal antibody (6G9) was purchased from Boehringer-Mannheim (Indianapolis, IN).

PCR primers and PCR amplification. We designed PCR primers to amplify across the variable domain of α - or β -CaM kinase. The 5'

Figure 1. Approximate position of the PCR primers used in cloning new α - and β -CaM kinases. The coding region (open box) contains a variable domain with a core or linker segment (lightly striped box) and/or additional inserts (dark striped boxes). Lines represent 5' and 3' untranslated sequences. PCR primers are marked as arrows, depicting the direction of elongation.

primer anneals to the calmodulin binding site (CBS) which is identical at the nucleotide level in both α and β -CaM kinase sequences. Therefore, this primer can be used to amplify cDNA of both isoforms. The 3' primers are specific for either α - or β -CaM kinase under our PCR conditions, since they correspond to a region with less sequence identity between α - and β -CaM kinase sequences differing in 7 out of 20 bases. Approximate positions of all PCR primers along CaM kinase sequences are summarized in Figure 1. CBS (5'-GTCCGGAATTCATCCTCACACTATGCTG-3') corresponds to nucleotides 905-924 in α -CaM kinase sequence (Lin et al., 1987; Bulleit et al., 1988) and 908-927 in β -CaM kinase sequence (Bennett and Kennedy, 1987) and contains an EcoRI restriction site. α -CaM kinase specific primers are: α 1 5'-AAGGATCCATCGATGAAAGTCCAGGCC-3' (1170-1189; BamHI restriction site); α 2 5'-CCGAATTCAGGATGGCTACCATCACCTGC-3' (-4-18; EcoRI restriction site); α 3 5'-CAGGATCCACATTCATGGACAAAG-3' (1484-1502; BamHI restriction site); α 4 5'-GAGTGAACGCGTAACCTCTCCGGAGGGAAG-3' (928-951; MluI restriction site is introduced by the replacement of C in position 930 with G, A in position 931 with C, and G in position 933 with T. These changes do not interfere with the amino acid sequence); α 5 5'-GGTGGTGTAGTACTCTCAGAGGATTC-3' (985-1011; ScaI restriction site is introduced by the replacement of C in position 999 with T and C in position 1002 with T. These changes do not interfere with the amino acid sequence). β -CaM kinase specific primers are: β 1 5'-AAGTGAATTCGTGGAAATCCATCCC-3' (1363-1380; EcoRI restriction site); β 2 5'-CCGAATTCATGGCCACCACGGTGACCTG-3' (-1-20; EcoRI restriction site); β 3 5'-CTCAGAAGGGCCCCGAATCTGTCCGGTGAAACAGGC-3' (1637-1656; ApaI restriction site); β 4 5'-GCCAATAAACCTGATCAACCATG-3' (733-756; BclI restriction site is introduced by the replacement of C in position 744 with G. This change does not interfere with the amino acid sequence); β 5 5'-AACATCTGGTTGATCAGGTTTTTGGC-3' (733-758; BclI restriction site as described for β 4); β 6 5'-CCGAACCTGCAGAATTCACGCCGAGCCCAATCGCCAC-3' (-25-5; PstI and EcoRI restriction sites).

The PCR mix contained 1/20 vol. from the reverse transcription reaction (see below) in 50 μ l of 50 mM potassium chloride, 10 mM Tris-HCl, pH 8.4, 1.5 mM magnesium chloride, 0.001% (w/v) gelatin, 0.125 μ M each dATP, dCTP, dGTP, and dTTP (Pharmacia, Piscataway, NJ), 0.5 μ M each 5' and 3' primers, and 1 U of Taq DNA polymerase (Perkin-Elmer, Norwalk, CT). All amplifications were performed in a TwinBlock System thermal cycler (Ericomp Inc., San Diego, CA) involving an initial step of 5 min at 94°C followed by 25-35 cycles of 30 sec at 94°C, 30 sec at 55°C, and 1 min at 72°C, and ending with a 15 min incubation at 72°C. Samples of 8-12 μ l from each PCR mixture were loaded on a 5% polyacrylamide gel and the products were visualized by ethidium bromide staining. In some experiments, the 5' primer was prelabeled with γ - 32 P-ATP using T4 polynucleotide kinase. Labeled primers were separated from unincorporated ATP on a G-25 Sephadex Quick Spin column (Boehringer-Mannheim, Indianapolis, IN) before use in PCR. When the reaction contained a radioactive primer, the gel was dried and autoradiographed.

RNA purification and cDNA synthesis. Brains of Sprague-Dawley rats at the indicated age were dissected to separate the cerebellum, diencephalon/midbrain, hippocampus, and frontal and occipital cortices. For isolation of caudate putamen, hypothalamus and thalamus, the brain of a 6 week old rat was sectioned using a sliding vibratome and 500 μ m thick slices were carefully microdissected to obtain the desired region. Total RNA was isolated from the each brain sample using RNazol reagent (Cinna/Biotech Laboratories International, Friendswood, TX) according to the manufacturer's instructions. An additional step of digestion with RNase-free DNase (Boehringer-Mannheim, Indianapolis, IN) was performed to remove any residual DNA. The RNA samples were then phenol extracted and ethanol precipitated. For the preparation of first strand cDNA, 0.1–0.25 μ g of total RNA was subjected to reverse transcription using random primers and SuperScript enzyme (GIBCO BRL, Gaithersburg, MD). Primary cultures of hippocampal neurons were prepared and maintained essentially as described (Margaroli and Tsien, 1992). mRNA was extracted from the cells after 8–10 d in culture using RNazol reagent.

Subcloning. α -CaM kinase clones: PCR products amplified with CBS and α 1 primers from diencephalon/midbrain regions were cut with EcoRI and BamHI, inserted into appropriately digested pBluescript (Stratagene, La Jolla, CA) and sequenced. Longer fragments of α -CaM kinase obtained by PCR amplification using CBS and α 3 primers for the C-terminal half of the cDNA or α 1 and α 2 primers for the N-terminal half (see Fig. 1), were digested and ligated into pBluescript. We verified by restriction digest which clones contained the 33 bp insertion and sequenced the variable domain of several clones.

β -CaM kinase clones: PCR products amplified with CBS and β 1 primers from E18 cDNA were cut with EcoRI, subcloned into pBluescript, and sequenced. The N-terminal half of the mRNA was amplified using β 2 and β 1 primers and the C-terminal half using CBS and β 3 primers (see Fig. 1). After subcloning, we sequenced the variable domain of several clone as well as 50–60% of the rest of the coding region.

Sequencing of all the double-stranded DNA constructs were carried out using T₃ and T₇ oligonucleotides and Sequenase reagents (USB, Cleveland, OH) according to manufacturer's instructions.

Analysis of the β -CaM kinase gene. Rat genomic DNA was used as a template for PCR amplification of the intron between exons X and IX and the intron between exons IX and VIII. CBS primer and a specific primer for exon IX (β ex9.1 5'-CGGAATTCATTGTGGCCGGAGCGGTGGTC-3'; 957–978, EcoRI restriction site) were used to amplify a band of approximately 1 Kb that was subcloned into pBluescript. The genomic clones were sequence from the exons up to 200 nucleotides into the intron from each side. We used a different strategy to amplify the intron between exons IX and VIII, since this intron was very long. Therefore, we amplified only the exon/intron boundaries using a PCR primer that annealed to the exon (β ex9.3 5'-ACAGAATTCGCTCCGGCCACAATGTC-3'; 962–980, EcoRI restriction site; β ex8.1 5'-GCAAGCTTGACTCCGTCTGCTTCTTG-3'; 1038–1059, HindIII restriction site) and a PCR primer that annealed approximately 200 bp inside the intron (β int9.4 or β int8.3). The intron-specific primers were designed based on the partial sequence of this intron from mouse β -CaM kinase gene (K. Harbers, personal communication). A 210 bp fragment that spanned the exon/intron boundary at the end of exon IX was amplified by β ex9.3 and β int9.4 PCR primers, and a 220 bp fragment that spanned the exon/intron boundary at the beginning of exon VIII was obtained using β int8.3 and β ex8.1 PCR primers. Both fragments were subcloned into pBluescript and sequenced.

Expression in COS-7 cells. The variable domain of α -CaM kinase was amplified using α 4 and α 5 primers and inserted into α -CaM kinase-MS PCR 1000.12 vector via unique MluI and ScaI restriction sites (Srinivasan et al., 1994). These sites were introduced into α -CaM kinase sequence by site directed mutagenesis. The α -CaM kinase cDNA was subcloned into the EcoRI site of pSR α .BKS expression vector (Nghiem et al., 1993). Full length cDNA for β -CaM kinase in pUC-18 was a gift from Dr. M. N. Waxham (University of Texas, Houston). We subcloned the EcoRI fragment from this construct into pSR α .BKS expression vector. In order to obtain full length cDNAs for β - and β' -CaM kinase, the N-terminal half of β -CaM kinase was amplified from rat cerebellum using β 6 and β 5 primers and digested with PstI and BclI. The C-terminal half (including the variable domain) was amplified from E18 brain using β 3 and β 4 primers and digested with BclI and ApaI. The two halves were ligated into PstI-ApaI sites of pSR α .BKS expression

Figure 2. Structure and regional distribution of α -CaM kinase isoforms in rat brain. *a*, RT-PCR products that encompass the variable domain of α -CaM kinase isoforms were amplified from the indicated brain regions and visualized by ethidium bromide staining of a 5% polyacrylamide gel. CBS and α 1 primers amplify a band of 301 bp for α -CaM kinase (position marked by " α "). The intensity of the band in cerebellum is lower, reflecting lower level of α -CaM kinase mRNA expression in this region. An additional band of 334 bp (position marked by " α_B ") corresponding to α_B -CaM kinase is detected in diencephalon/midbrain but not in other brain regions; *b*, Schematic of the variable domain of α -CaM kinase isoforms. PCR products corresponding to the variable domain of α -CaM kinase obtained from diencephalon/midbrain were subcloned and sequenced. In comparison to α -CaM kinase, α_B -CaM kinase contains an 11 amino acid insertion at the C-terminal side of the linker sequence (lightly striped box).

vector. Full length clones corresponding to β - and β' -CaM kinase were isolated and verified by sequencing of the variable domain.

COS-7 cells were transiently transfected with 15 μ g of DNA per 10 cm plates by calcium phosphate precipitation essentially as described (Hanson et al., 1989); 70–80 hr after transfection cells were collected and disrupted by sonication with a water cup sonicator (Heat Systems-Ultrasonics) in a lysis buffer containing 50 mM PIPES pH 7.0, 1 mM EGTA, 10% glycerol, 1 mM PMSE, 10 μ g/ml leupeptin, 10 μ g/ml pepstatin, and 2 mM DTT. Cell extracts were centrifuged at 12,000 $\times$ g for 20 min and the supernatant was frozen at -80°C . Protein concentrations were routinely determined using Bradford assay (Bradford, 1976) with IgG as a standard.

Immunoprecipitation and protein gels. Whole brain from E18 Sprague-Dawley rat was homogenize in 10 volumes of buffer A (40 mM Tris/HCl pH 7.6, 1 mM DTT, 1 mM EGTA, 1 mM EDTA, 1 mM PMSE, 10 μ g/ml leupeptin, and 10 μ g/ml pepstatin). After centrifugation at 12,000 $\times$ g for 20 min at 4°C , the supernatant was frozen at -80°C (cytosolic fraction). The pellet was resuspended in 10 volumes of buffer A containing 0.1% Triton X-100 (particulate fraction). Immunoprecipitation of α - and β -CaM kinase from E18 protein extracts

Figure 3. Structure and expression pattern of β -CaM kinase isoforms in rat brain. *a*, Autoradiograph of RT-PCR products that correspond to the variable domain of β -CaM kinase isoforms from embryonic day 18 (*E18*) and adult (*A*) rat brain. 32 P-Labeled CBS and β 1 primers amplify bands of 489 bp for β -CaM kinase and 444 bp for β' -CaM kinase, as well as two lower bands (417 and 372 bp). In *E18*, only the two lower bands are detected; *b*, structure of the variable domain of β -CaM kinase isoforms. N-Terminal and C-terminal segments (dark striped boxes) flank the linker sequence (light striped box). The deletions in β' -, β_e -, and β'_e -CaM kinase isoforms are shown as dashes. Residue 316 is changed from valine in the adult isoforms to alanine in the embryonic isoforms (underlined); *c*, immunoprecipitation of β -CaM kinase isoforms from *E18*. β -CaM kinase was immunoprecipitated from cytosolic (*cyt*) or particulate (*par*) protein fractions using CB α 2 and CB β 1 monoclonal antibodies, subjected to SDS-

were carried out in RIPA buffer using CB α 2 and CB β 1 monoclonal antibodies. Protein samples, either immunoprecipitated from E18 brain or COS-7 cell extracts, were subjected to electrophoresis on 8% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. A calmodulin overlay assay using biotinylated calmodulin and enhanced chemiluminescence was performed as described previously (Nghiem et al., 1993).

CaM kinase activity assay. CaM kinase activity was assayed using a synthetic peptide substrate, autocalmitide-2, with sequence based on an autophosphorylation site of CaM kinase (Hanson et al., 1989). Standard assay mixes contained 50 mM PIPES pH 7.5, 10 mM magnesium acetate, 10 μ g/ml calmodulin, 10 μ M autocalmitide-2, 20 μ M γ -³²P-ATP (1 Ci/mmol) and either 0.5 mM CaCl₂ for kinase activity or 0.5 mM EGTA as control. The reaction was started by the addition of protein extract to a final volume of 50 μ l and incubated for 1 min at 30°C. Reactions were stopped with trichloroacetic acid, applied to phosphocellulose paper, washed, and measured for Cerenkov radiation. For detection of autophosphorylation, the reactions were incubated for 15 sec at 30°C and stopped by the addition of SDS-protein loading buffer.

Transfection of neuroblastoma cells and immunostaining. Human neuroblastoma cell line (SH-SY5Y) was kindly provided by Dr. S. K. Fisher (University of Michigan, Ann Arbor) and cultured as described (Martin et al., 1993). Calcium phosphate transfections and immunostaining of the transfected cells were carried out as described previously (Srinivasan et al., 1994). Briefly, transfected neuroblastoma cells expressing α - or α _{β} -CaM kinase were transferred onto glass coverslips and were fixed 48 hr posttransfection with 2% formaldehyde followed by methanol. The fixed cells were stained with CB α 2 antibody and goat anti-mouse antibody coupled to rhodamine. The cells were viewed and photographed using Zeiss Axioplan Universal microscope equipped for fluorescence detection with 63 $\times$ and 100 $\times$ objectives.

Immunohistochemistry. Rats (Sprague-Dawley, 6 week old) were perfused with 4% paraformaldehyde + 0.1% glutaraldehyde in phosphate buffer. Brains were fixed overnight and incubated for an hour in 30% sucrose in 0.1 M phosphate buffer; 30 μ m sections were permeabilized with 0.2% Triton-X100 in Tris-saline buffer for 15 min and then preincubated with 1% horse serum in Tris-saline for 1 hr. Incubations with CB α 2 mAb (1:1000) or 6G9 mAb (1:200) were carried out for 18 hr at 4°C followed by washing and incubation with biotinylated anti mouse IgG (Vector Laboratories, Burlingame, CA) for 2 hr. After washing and incubation with avidin-HRP for 1 hr, the slices were incubated in 0.02% 3,3'-diaminobenzidine tetrahydrochloride in 50 mM Tris buffer and 0.003% hydrogen peroxide for 6–8 min. Finally, the sections were mounted onto slides, dehydrated, and photographed using a Leitz DIALUX 20 microscope.

Results

α -CaM kinase isoforms in rat brain

Previous studies have suggested that isoforms of CaM kinase can be generated by putative alternative splicing at the variable domain of the protein. Therefore, we amplified fragments of α - or β -CaM kinase that include the variable domain using reverse transcription-polymerase chain reaction (RT-PCR). Specific PCR primers for α -CaM kinase, CBS and α 1 (Fig. 1), amplified a single product of the predicted size, 301 bp, from all brain regions (Fig. 2a). An additional, larger product was amplified from diencephalon/midbrain. Sequence analysis of the two PCR products from diencephalon/midbrain reveals that the 301 bp band is identical to nucleotides 905–1189 of α -CaM kinase sequence (Lin et al., 1987). The larger product contains additional 33 nucleotides which are inserted after position 984 (Fig. 2b).

In order to determine whether this larger PCR fragment is part of a distinct CaM kinase isoform, we have amplified by PCR the entire coding region of α -CaM kinase from diencephalon/midbrain (see Materials and Methods). We confirmed by partial sequencing that the 33 bp insertion is present in the vari-

able domain of a full length message for CaM kinase. Since the new isoform, termed α _{β} -CaM kinase, is identical to α -CaM kinase outside the variable domain, it is likely to be generated by alternative splicing of α -CaM kinase mRNA.

The intensity of the PCR signal for α _{β} -CaM kinase isoform in the diencephalon/midbrain suggests that this isoform accounts for a substantial amount of the total α -CaM kinase mRNA (Fig. 2a). Furthermore, about half (7/12) of the subcloned PCR products from diencephalon/midbrain corresponded to α _{β} -CaM kinase (data not shown), suggesting that there are roughly equal amounts of α - and α _{β} -CaM kinase mRNA in the diencephalon/midbrain. In contrast, the PCR signal of α _{β} -CaM kinase in other regions of the brain was undetectable or very weak (Fig. 2a). The expression of α _{β} -CaM kinase appears to be regulated in a region-specific manner.

β -CaM kinase isoforms in rat brain

Based on the β -CaM kinase cDNA sequence, primers CBS and β 1 (Fig. 1) should amplify PCR products of 489 bp and 444 bp representing the variable domains of β - and β' -CaM kinase, respectively. The results of PCR amplification of β -CaM kinase from adult rat brain cDNA are shown in Figure 3a. The two largest bands correspond to β -CaM kinase, which is the major product, and β' -CaM kinase which is smaller and less intense. In addition to the β - and β' -CaM kinase bands there are two other minor, but distinct, smaller-sized products. When cDNA from embryonic day 18 (E18) is used as a template only the two smaller products are apparent (Fig. 3a). These PCR products from E18, termed β_e - and β'_e -CaM kinase because they are more predominantly expressed in embryonic brain, were subcloned and sequenced. The variable domain of β -CaM kinase consists of N-terminal and C-terminal sequences flanking a conserved linker. Unlike β - and β' -CaM kinase isoforms, both β_e - and β'_e -CaM kinase isoforms lack 24 amino acids at the N-terminal segment of the variable domain (Fig. 3b). In addition, the 15 amino acids that are absent in the C-terminal segment of the variable domain β' -CaM kinase are also absent in β'_e -CaM kinase isoform. Thus, β_e - differs from β -CaM kinase and β'_e - differs from β' -CaM kinase by the lack of the same 24 amino acid segment. The deletion of this N-terminal segment changes amino acid 316 from valine to alanine without altering the reading frame of the message.

We next isolated full length cDNAs for β_e - and β'_e -CaM kinase from E18 brain using RT-PCR. Analysis of the cDNA clones by restriction digestion and partial sequencing indicates that there is no difference between our clones and the β -CaM kinase sequence outside the variable domain (data not shown). However, all of our clones have an amino acid at position 522 that differs from the published sequence of β -CaM kinase (Bennett et al., 1987). We find at this position a codon for arginine (CGC) whereas the published sequence of β -CaM kinase encodes for a proline (CCC). Furthermore, Mouse β -CaM kinase sequence encodes for Arg⁵²² as well (Karls et al., 1992). Alignment of the published α -, γ -, and δ -CaM kinase amino acid sequences also reveals an arginine at an equivalent position (Tobimatsu and Fujisawa, 1989).

The abundance of mRNA for these new β -CaM kinase iso-

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polyacrylamide gel electrophoresis and visualized using calmodulin overlay. Purified CaM kinase from adult forebrain (A) is used to mark the migration of β/β' - and α -CaM kinase (60/59 and 54 kDa, respectively).

However, our sequencing analysis found three additional nucleotides (CAG) that are inserted between exons X and VIII (Fig. 4a). This observation was confirmed by analysis of at least three individual clones of β_c - and β'_c -CaM kinase. In order to determine the source of these three nucleotides, we sequenced the relevant intron/exon boundaries of rat β -CaM kinase gene based on the corresponding sequences from the mouse (K. Harbers, personal communication). We observed that the structure of the rat gene around exons X-VIII is identical to that of the mouse (Fig. 4b). Moreover, a repeat of the sequence CAG is found at the end of the intron between exons IX and VIII. These data suggest that the addition of the three nucleotides in β_c - and β'_c -CaM kinase sequences can be due to a slightly shifted 3'-splice site in front of exon VIII. During the processing of the mRNA for the β - and β' -CaM kinase isoforms the entire intron is left out. However, while generating the embryonic isoforms in which exon IX is spliced out, the splicing machinery seems to choose another 3'-splice site that results in the inclusion of the last three nucleotides of the intron in the message (Fig. 4c). Thus, the additional three nucleotides in β_c - and β'_c -CaM kinase appear to be derived from the intron and encode for an additional ala-

Figure 5. Developmental regulation of β -CaM kinase isoform expression. Autoradiograph of RT-PCR products that were obtained using ^{32}P -labeled CBS and $\beta 1$ primers and equal amount of template derived from P1, P4, P13, P22, or adult (A) mRNA; 5 μl from each reaction was separated on a 5% polyacrylamide gel. The migration of the four β -CaM kinase isoforms is marked on the right. A similar pattern of expression is seen in the hippocampus and in the cerebral cortex, with a shift from embryonic to adult isoforms during the second postnatal week. The major β -CaM kinase isoform in primary hippocampal culture (cul) is β'_e .

nine at the N-terminus of the linker sequence (underlined in Fig. 3b).

β -CaM kinase isoform expression is developmentally regulated

Since we had found striking differences in the expression of β -CaM kinase isoforms between embryonic and adult rat brain, we decided to look at their expression during development. Figure 5 shows the expression of β -CaM kinase isoforms in cerebral cortex and hippocampus at various postnatal developmental stages. β_e - and β'_e -CaM kinase isoforms are expressed at high levels from birth until postnatal day 4 (P4). At P13 there is a large increase in the expression of the adult isoforms, β - and β' -CaM kinase, and a corresponding decrease in the expression of the embryonic isoforms. In the adult, only β - and β' -CaM kinase isoforms are detected. We later isolated RNA from P9 in order to narrow down the time window of the change in β -CaM kinase isoform expression. The pattern of PCR bands in P9 is similar to the pattern seen on P4 (data not shown), suggesting that the change to the adult isoforms occurs between P9 and P13. We also looked at β -CaM kinase isoforms in primary hippocampal cultures. Interestingly, after 10 d in culture β'_e is the predominant CaM kinase isoform (Fig. 5).

The developmental upregulation of α -CaM kinase expression occurs primarily in the forebrain and hippocampus during the second postnatal week (Hanley et al., 1987). Using α -CaM ki-

nase specific PCR primers, we observed a large increase in mRNA levels for both α - and α_B -CaM kinase between P9–13 in the diencephalon/midbrain (data not shown).

Functional expression of new α - and β -CaM kinase isoforms

We expressed the three new CaM kinase isoforms in order to determine whether they encode functional CaM kinases. α_B -, β_e -, and β'_e -CaM kinase cDNAs were cloned into the pSR α expression vector and transiently transfected into COS-7 cells. Extracts of transfected cells contained calmodulin-binding proteins of about 55.5 kDa, 58 kDa, and 56 kDa, compatible with the calculated sizes of α_B -, β_e -, and β'_e -CaM kinase isoforms, respectively (Fig. 6a).

We next tested the ability of α_B -, β_e -, and β'_e -CaM kinase to undergo autophosphorylation, a characteristic property of all known CaM kinase isoforms. Indeed, all three isoforms incorporated phosphate in a Ca^{2+} /calmodulin-dependent manner (Fig. 6b). The cytosolic fractions of transfected COS-7 cells were assayed for CaM kinase activity. All three isoforms show normal Ca^{2+} /calmodulin-dependent phosphorylation of the specific CaM kinase substrate autocamtide-2 (Fig. 6c). Thus, each of the new isoforms encodes the protein kinase activity characteristic of CaM kinase.

α_B -CaM kinase is targeted to the nucleus

We have recently demonstrated a role for the variable domain in the subcellular targeting of CaM kinase isoforms (Srinivasan et al., 1994). The variable domain of δ_B -CaM kinase contains a nuclear localization signal (NLS) which targets it to the nucleus in transfected cardiac myocytes. Sequence comparison reveals that α_B - and δ_B -CaM kinase isoforms share a similar NLS-generating segment in their variable domain. This C-terminal segment is KRKSSSSVQMM in δ_B -CaM kinase and KRKSSSSVQLM in α_B -CaM kinase (Fig. 2b). We therefore decided to study the subcellular targeting of transiently transfected α_B -CaM kinase.

Human neuroblastoma cells transfected with either α - or α_B -CaM kinase cDNA were analyzed by immunostaining with an α -CaM kinase specific monoclonal antibody (CB α 2). This antibody recognizes both α - and α_B -CaM kinase on a Western blot (data not shown). α_B -CaM kinase transfected cells exhibit bright nuclear staining with no detectable cytoplasmic signal, indicating that α_B -CaM kinase is specifically targeted to the nucleus (Fig. 7). In contrast, α -CaM kinase is excluded from the nucleus and exhibits an exclusively cytoplasmic pattern of staining. Since α_B -CaM kinase differs from α -CaM kinase by the 11 amino acids insert (Fig. 2b), these results suggest that the variable domain of α_B -CaM kinase is responsible for targeting this isoform to the nucleus. It is important to note that the transfected cells were not stimulated in any way prior to fixation and immunostaining. Therefore, our results also imply that the localization of α_B -CaM kinase to nucleus may not require activation by Ca^{2+} /calmodulin.

Is α_B -CaM kinase localized to neuronal nuclei in the brain? We used α -CaM kinase antibodies to examine the intracellular distribution of α -CaM kinase isoforms in the diencephalon/midbrain structures, where both α - and α_B -CaM kinase isoforms are expressed, and a forebrain structure, where only α -CaM kinase is expressed (Fig. 2a). In hypothalamic and thalamic neurons the immunostaining is both cytoplasmic and nuclear but in the caudate putamen the immunostaining is only cytoplasmic (Fig. 8a–c). RT-PCR analysis demonstrates that α_B -CaM kinase

Figure 6. Functional expression of the new CaM kinase isoforms in COS-7 cells. *a*, Cytosolic extracts of COS-7 cells transfected by the indicated CaM kinase isoform cDNA were subjected to SDS-polyacrylamide gel electrophoresis and visualized using calmodulin overlay. The

mRNA is almost equally abundant as α -CaM kinase mRNA in the thalamus and hypothalamus whereas only α -CaM kinase mRNA is present in the caudate putamen (Fig. 8*d*). Taken together, the immunostaining and the RT-PCR analysis show that only thalamic and hypothalamic neurons which express α_B -CaM kinase isoform exhibit nuclear staining. Therefore, α_B -CaM kinase isoform seems to be targeted to the nucleus in neurons.

Discussion

In this report we describe the isolation and functional expression of three new CaM kinase isoforms from rat brain, termed α_B -, β_e -, and β'_e -CaM kinase. These isoforms are probably generated by alternative splicing of α - and β -CaM kinase mRNAs. α_B -CaM kinase contains an 11 amino acid insertion at the C-terminal segment of the variable domain. The β -CaM kinase isoforms, β_e - and β'_e -CaM kinase, both lack 24 amino acids which form the N-terminal segment in β - and β' -CaM kinase variable domains. The β'_e -CaM kinase, like β' -CaM kinase, lacks 15 amino acids at the C-terminal side of the linker sequence. Thus, the β -CaM kinase isoform has a large variable domain containing exons IX-VI (Karls et al., 1992), β_e - and β'_e -CaM kinase isoforms lack exon IX (24 amino acids), and β' - and β'_e -CaM kinase isoforms lack exon VI (15 amino acids) due to alternative splicing. The existence of additional β -CaM kinase isoforms was suggested by Bulleit et al. (1988). In performing an S1 protection assay using a probe to the variable domain they detected protected fragments that did not correspond to β - and β' -CaM kinase and commented that this finding could be accounted for by an additional deletion.

Generation of the β_e - and β'_e -CaM kinase isoforms involves a small shift in the 3'-splice site, resulting in two alanine residues that are encoded in part by nucleotides derived from the intron. Several other known genes contain two adjacent potential 3'-splice site at the intron/exon boundary that are utilized to include or exclude three base pairs (CAG) in the mRNA (Maurer et al., 1981; Shelleness and Williams, 1984; Cook et al., 1985; Sun and Baltimore, 1991; Manrow and Berger, 1993). The effect of the additional alanine on β_e - and β'_e -CaM kinase proteins remains unclear, although in all the other cases reported no particular function is associated with the choice of the 3'-splice site.

The newly isolated CaM kinase isoforms have different expression patterns from the previously characterized isoforms. α -CaM kinase is the predominant isoform in the brain and has a broad regional distribution with particularly high levels of expression in the hippocampus and cortex. By contrast, the expression of α_B -CaM kinase mRNA in rat brain is restricted to the diencephalon/midbrain with little or no mRNA in the hippocampus and the cortex (Fig. 2*b*). Previous studies surveying α -CaM kinase immunoreactive or autophosphorylated protein noted that in the diencephalon/midbrain, but not in other brain regions, α -CaM kinase migrated as a doublet on SDS-polyacrylamide gels with a new band of slightly lower mobility (Walaas et al., 1983; Erondy and Kennedy, 1985). This band is probably α_B -CaM kinase and appears to be a significant fraction of the

first lane shows the migration of α - and β -CaM kinase purified from adult rat forebrain (*A*); *b*, autoradiograph of autophosphorylated CaM kinase isoforms. Cytosolic extracts from transfected COS-7 cells were incubated with γ - 32 P-ATP in the absence (–) or presence (+) of Ca^{2+} /calmodulin; *c*, phosphorylation of CaM kinase specific peptide substrate by recombinant CaM kinase isoforms.

Figure 7. Localization of α -CaM kinase isoforms in transfected human neuroblastoma cells. α -CaM kinase specific monoclonal antibody (CB α 2) stains the nucleus in cells transfected with α_B -CaM kinase cDNA (*a*, *b*) and the cytoplasm of cells transfected with α -CaM kinase cDNA (*c*). Phase contrast view of the field in *a* is shown in *b*. Scale bar, 40 μ m.

CaM kinase protein in the diencephalon/midbrain. A fragment of α_B -CaM kinase was originally amplified as a PCR product from monkey frontal cortex mRNA (Benson et al., 1991a). In that study, 2 of 8 cDNA clones obtained by PCR amplification across the variable domain of α -CaM kinase were of α_{33} , the fragment with a 33 bp insert relative to α -CaM kinase, suggesting that α_B -CaM kinase is likely to be a significant form of cortical kinase in primates. By contrast, we could not detect this isoform in rat frontal cortex (Fig. 2*a*). This apparent species-specific difference in α_B -CaM kinase distribution may be useful in identifying the possible unique function of α_B -CaM kinase.

We have demonstrated that the alternative splicing of β -CaM kinase mRNA is developmentally regulated. From E18 through the first postnatal week, β_e - and β'_e -CaM kinase are the most highly expressed isoforms. The expression of the adult isoforms, β - and β' -CaM kinase, is upregulated during the second postnatal week while the mRNAs for β_e - and β'_e -CaM kinase almost disappear by P13 and beyond. There appears to be a develop-

mental control of the splicing of exon IX, which is missing in both embryonic isoforms and present in both adult isoforms. Several studies have addressed the expression of the CaM kinase isoforms during development using isoform-specific antibodies (Sahyoun et al., 1985; Weinberger and Rostas, 1986). In neonate brain, β -CaM kinase protein is expressed at a higher level than α -CaM kinase. This level of β -CaM kinase expression does not change through subsequent development and aging. On the other hand, α -CaM kinase protein is expressed at low levels at birth and increases sharply between P5 and P10. However, the antibodies used could not distinguish between β - and β' -CaM kinase isoforms and therefore probably cannot discriminate between these and the embryonic isoforms. *In situ* hybridization and Northern analysis studies were consistent with the immunodetection data (Hanley et al., 1987; Burgin et al., 1990). However, the specific probes used in these studies did not discriminate between the four β -CaM kinase isoforms that we now know exist. For example, Burgin et al. (1990) used a probe which

Figure 8. Intracellular localization of α -CaM kinase immunostaining in several regions of rat brain is correlated with the expression of α -CaM kinase isoforms. Immunostaining with α -CaM kinase specific monoclonal antibodies (CB α 2 or 6G9) of the caudate putamen (*a*), thalamus (*b*), and hypothalamus (*c*) detects nuclear staining only in thalamic and hypothalamic neurons. Scale bar, 55 μ m. *d*, Ethidium bromide staining of RT-PCR products obtained using templates derived from the caudate putamen, thalamus or hypothalamus and α -CaM kinase specific primers (CBS and α 1). A larger band which corresponds to α_B -CaM kinase is detected in the hypothalamus and thalamus but not in the caudate putamen. The control reaction did not contain template.

corresponds to a segment of the variable domain (base pairs 1090–1128) that is common to all β -CaM kinase isoforms. Similarly, Karls et al (1992) amplified a PCR fragment in the association domain (base pairs 1259–1663) present in all β -CaM kinase isoforms.

Our data clearly indicate that although the expression of the sum total of β -CaM kinase isoforms may not change during development, there appears to be a switch from the embryonic isoforms to the adult isoforms (Figs. 3*a*, 5). The developmental switch in the expression of β -CaM kinase isoforms and the increase in α -CaM kinase expression occur at approximately the same period, between P9 and P13. The second postnatal week is considered to be a phase of ongoing synaptogenesis in rat brain development. It is possible that changes in the expression

of CaM kinase isoforms play a role in the process of neuronal maturation.

What is the physiological significance of the multiple isoforms of α - and β -CaM kinase? The variable domain resides between the regulatory and association domains of the enzyme. An electron microscopy study of CaM kinase holoenzyme structure has suggested a planar, flower with petal-like arrangement of the subunits (Kanaseki et al., 1991). According to this model, residues 317–328 in the α -CaM kinase variable domain form a tether that links the globular catalytic/regulatory particle at the periphery to a central, large globular particle composed of all the association domains. In fact, the residues in β -CaM kinase corresponding to this putative linker are encoded by a separate exon (exon VIII). In such a structure, the N-terminal segment of the

variable domain (exon IX) will reside in the outer particle and the C-terminal segment (exons VI and VII) in the inner particle. Sequences derived from exon IX may therefore affect the catalytic functions such as calmodulin affinity whereas exon VI and VIII derived sequences may affect the assembly of the kinase into a multimer or targeting to intracellular sites. Sequences spliced in at either end may introduce phosphorylation sites for modulators of these functions.

Autophosphorylation is an important mechanism for the modulation of CaM kinase activity. The autonomy site (Thr²⁸⁷ in β -CaM kinase sequence) is crucial for Ca²⁺-independent activity of CaM kinase (Fong et al., 1989; Hanson et al., 1989). Other sites of autophosphorylation inhibit CaM kinase activity after dissociation of calmodulin (Patton et al., 1990; Hanson and Schulman, 1992b). It is interesting to note that exon IX contains a potential autophosphorylation sites (R-X-X-S/T). However, phosphopeptide mapping of β -CaM kinase subsequent to Ca²⁺/calmodulin-stimulated autophosphorylation identified a non-consensus site on exon VI (Miller et al., 1988). Although the potential site on exon IX was not found to be phosphorylated, this exon is particularly rich in serine and threonine residues (8 out of 24 amino acids) and some of these may be phosphorylated by other protein kinases or autophosphorylated under other conditions. The β -CaM kinase has a higher affinity for calmodulin than the α -CaM kinase (Miller and Kennedy, 1985; Yamauchi et al., 1989). The calmodulin affinity of the embryonic β -CaM kinase isoforms may be effected by the deletions in the variable domain.

We have demonstrated that α_B -CaM kinase isoform is targeted to the nucleus in transfected cells. α_B -CaM kinase contains a consensus NLS, KKRK (Fig. 2b) which has been reported to target proteins to the nucleus (reviewed in Garcia-Bustos et al., 1991). Two other CaM kinase isoforms, γ_A and δ_B , have NLS-generating inserts in the C-terminal segment of their variable domains. We have recently shown that the KKRK sequence is critical for targeting δ_B -CaM kinase isoform to the nucleus (Srinivasan et al., 1994). These results indicate that a sequence in the C-terminal segment of the variable domain targets CaM kinase to the nucleus. It is possible that different insertions in the C-terminal segment of the variable domains could target other CaM kinase isoforms to distinct non-nuclear sites.

Previous surveys of α -CaM kinase immunohistochemistry did not detect nuclear staining (Erondu and Kennedy, 1985; Benson et al., 1991b). Nuclear CaM kinase activity has been reported by others, although the corresponding isoform was not identified and the forebrain activity was probably cytoskeletal rather than nuclear since there is little or no nuclear CaM kinase in this region (Sahyoun et al., 1984; Ohta et al., 1990). We show here that there is a strong correlation between nuclear staining with α -CaM kinase antibody the regional expression of α_B -CaM kinase isoform in rat brain. In caudate putamen neurons that express only α -CaM kinase isoforms the immunostaining is cytoplasmic. In thalamic and hypothalamic neurons that express both α - and α_B -CaM kinase isoforms the immunostaining is cytoplasmic and nuclear. We have previously shown that cells cotransfected with a nuclear isoform and a cytosolic isoform in a 1:1 ratio exhibit both nuclear and cytosolic targeting of CaM kinase (Srinivasan et al., 1994). Thus, the observed intracellular distribution of α - and α_B -CaM kinase isoforms in the thalamus and the hypothalamus (Fig. 8b-d) is consistent with the localization pattern in transfected cells.

CaM kinase has been implicated in several nuclear functions

including nuclear envelope breakdown, regulation of cell cycle, and gene expression (Baitinger et al., 1990; Sheng et al., 1991; Planas-Silva and Means, 1992). A recent study has found that α -CaM kinase in the nucleus phosphorylates CREB at two sites, one of which blocks transcriptional activation of CREB by cAMP or calcium (Sun et al., 1994). This suggests that the amount of α_B -CaM kinase in the nucleus could determine the effectiveness of other signals in CREB-mediated transcription. CREB phosphorylation in response to elevated cAMP is known to be important for the induction of various neuropeptides in the hypothalamus. Nuclear α_B -CaM kinase in hypothalamic neurons may provide an antagonistic pathway. It will be interesting to investigate the precise distribution of α_B -CaM kinase in the hypothalamic and thalamic nuclei by *in situ* hybridization and assess the specific nuclear functions that may be modulated by the kinase in these cells. Previous studies addressing the role of CaM kinase in gene expression utilized a truncated form of α -CaM kinase lacking the association domain. This form is monomeric and not excluded from the nucleus. Our study demonstrates that there is an isoform of CaM kinase specifically designed to enter the nucleus and mediate neuronal functions.

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