Table 2. Reversibility of TGF-β inhibition of mammary ductal growth. Ten 4-week-old C57 female mice were implanted with 200 ng of TGF-β in the number 3 and 4 glands on the right-hand side of the animal and with BSA-containing implants in the contralateral glands. At 4 days, five animals were killed, and glands were scored for end bud number and growth. TGF-β implants were surgically removed from the other five animals and replaced with a marker implant containing India ink particles to facilitate later estimation of growth. Values are shown with 95% confidence intervals.

Treatment	Number of end buds per gland	Distance grown (mm)
4 Days		
TGF-β	2.64 ± 2.3	0
Control	9.90 ± 3.0	0.98 ± 0.73
15 Days		
TGF-β (implant removed at day 4)	14.9 ± 2.6	2.2 ± 1.1
Control	16.1 ± 3.1	5.8 ± 1.5

to suggest that the effects of implanted TGF-β reflect interference with unidentified mammogenic peptides. In addition, TGF-β can stimulate a fibrotic response in vivo (*13*) and collagen (*22*) and fibronectin (*23*) synthesis in vitro. Normal inhibition of ductal growth is accompanied by the progressive envelopment of the end bud by a collagen-rich (*24*) and fibronectin-rich (*25*) tunic, a process that may in itself inhibit growth and that may be accelerated by implanted TGF-β.

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26. To prepare slow-release implants, we mixed human platelet TGF-β, purified by high-performance liquid chromatography (HPLC), with 15 mg of BSA dissolved in 0.25 ml of distilled water and lyophilized the mixture. The lyophilized material was mixed with 0.125 ml of a 20% solution of EVAc in dichloromethane; the mixture was quick-frozen in acetone–dry ice; and the resulting pellet was dried. Pellets typically weighed about 35 mg and implants weighed between 0.5 and 1 mg. Implants prepared with the acetonitrile–trifluoroacetic and HPLC carrier plus BSA had no effect on ductal growth.

27. We thank A. Roberts and M. Sporn for their gift of TGF-β, DuPont for their gift of EVAc (Elvax 40P), and P. Strickland for helpful discussions concerning this work. Supported by PHS grant 01050 from the National Institute on Aging and by NSF grant PCM83-0814.

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Functional Analysis of a Complementary DNA for the 50-Kilodalton Subunit of Calmodulin Kinase II

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The calcium-calmodulin–dependent protein kinase II is a major component of brain synaptic junctions and has been proposed to play a variety of important roles in brain function. A complementary DNA representing a portion of the smaller 50-kilodalton subunit of the rat brain enzyme has been cloned and sequenced. The calmodulin-binding region has been identified and a synthetic analog prepared that binds calmodulin with high affinity in the presence of calcium. Like the 50-kilodalton kinase polypeptide, the concentration of the messenger RNA varies both neuroanatomically and during postnatal development of the brain. The broad tissue and species cross-reactivity of the complementary DNA suggests that the 50-kilodalton subunit found in rat brain is evolutionarily conserved and is the product of a single gene.

CALCIUM-CALMODULIN–DEPENDENT protein kinase II (CaM-KII) belongs to a family of enzymes known as Ca^{2+}- and calmodulin-dependent multifunctional protein kinases because of their broad substrate specificity (*1, 2*). First detected in mammalian brain (*3*), enzymes closely related to CaM-KII are present in many vertebrate tissues and have also been found in a variety of invertebrate organisms (*1, 2, 4–6*). The CaM-KII enzyme has been proposed to play a role in synaptic vesicle translocation and neurotransmitter release in neural tissue (*5*), neuronal modulation in *Aplysia* (*4*), and visual adaptation in *Drosophila* (*6*). In rat forebrain, the enzyme is concentrated at asymmetric synaptic junctions where it comprises a large part of the submembranous structure called the postsynaptic density (*7*). Whereas the native molecular weight of CaM-KII is 650,000 to 750,000, it is an oligomer of two subunits with molecular weights of 50,000 and 60,000, respectively. The stoichiometry of subunits varies between brain regions (*8*). The two subunits are related, since they share antigenic determinants and common tryptic and chymotryptic peptides (*7*). The Ca^{2+}-bound form of calmodulin (Ca^{2+}-calmodulin) interacts with and promotes autophosphorylation of each subunit and is required to activate the enzyme (*1, 2, 9, 10*). Once autophosphorylated, CaM-KII no longer requires Ca^{2+} or calmodulin for activity (*11*). These observations imply a functional interaction between autophosphorylation and calmodulin-binding domains. However, in molecular terms, the precise relations between the subunits and their functional domains are poorly understood.

We have used antibodies to obtain and identify complementary DNA (cDNA) clones encoding a large part of the 50-kD subunit of rat brain CaM-KII. Partial re-

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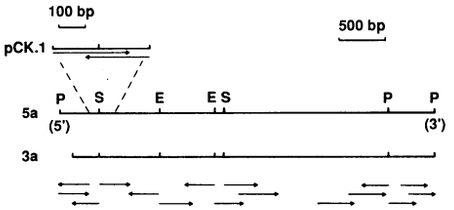

Fig. 1. Restriction mapping of cDNAs encoding the 50-kD subunit of CaM-KII (pCK.1, 5a, and 3a). Horizontal arrows indicate inserts and direction of sequencing; the endonuclease sites indicated are: E, Eco RI; S, Sph I; P, Pst I. A rat brain cDNA library expressed in λgt11 (ClonTech) was screened with affinity-purified antibodies to CaM-KII (*1*) by the procedure described by Guerriero *et al.* (*12*). A second rat brain cDNA library (from R. Akeson and G. Shull) was prepared as described (*23*); cDNAs were size-selected to include the 2- to 5-kb fraction before subcloning into pBR322. This cDNA library was screened with ^{32}P-labeled pCK.1. Restriction fragments were labeled by random oligo-priming.

striction maps of three positive cDNA clones are shown in Fig. 1. The smallest cDNA, pCK.1, was initially isolated from a rat brain λgt11 cDNA library with an affinity-purified polyclonal antibody (*1*). The 354-bp insert was subsequently used to obtain two additional clones from a rat brain cDNA library in pBR322. The single Sph I site in pCK.1 was used to align the clones. Additional mapping of the two pBR322 cDNAs suggested that they were very similar, if not identical, in those regions of overlap.

At the time these clones were isolated, no sequence information was available regarding either subunit of CaM-KII. Because the polyclonal antibody used to obtain pCK.1 recognized both subunits of the enzyme (*1*), we utilized a series of monoclonal antibodies to determine which subunit might be encoded by the cDNA. The protein encoded by pCK.1, which is composed of a portion of β-galactosidase fused to the CaM-KII peptide (fusion protein), was induced with isopropyl-β-D-thiogalactopyranoside (IPTG) and analyzed on immunoblots (Fig. 2). This protein was recognized by the polyclonal antibody used to screen the library (lane 3) as well as by four monoclonal antibodies that either react with both 50- and 60-kD kinase subunits (lane 7) or are 50-kD subunit specific (lanes 4 to 6). No reactivity was found to a monoclonal antibody that recognized only the 60-kD subunit (lane 8). Controls demonstrated that λgt11 lacking cDNA inserts produced β-galactosidase (upon induction with IPTG) that did not react with either polyclonal (lane 9) or monoclonal antibodies (lane 10). Because both subunits of CaM-KII bind calmodulin in a Ca^{2+}-dependent manner, the fusion protein from pCK.1 was examined for this property. With the gel overlay method adapted for nitrocellulose transfers (*9*), the fusion protein bound ^{125}I-labeled calmodulin in the presence (lane 14) but not in the absence (lane 13) of Ca^{2+}. An equivalent amount of the 50-kD subunit of the rat brain enzyme bound similar levels of ^{125}I-labeled calmodulin in a Ca^{2+}-dependent fashion (lane 15). On the basis of these criteria, we tentatively identified pCK.1 as encoding the portion of the 50-kD subunit cDNA that contained the calmodulin-binding region.

We next sequenced the pCK.1 insert and regions of pBR322 clone 5a (Fig. 1, arrows). The sequence of the 588-nucleotide open reading frame, as well as the deduced amino acids encoded, are shown in Fig. 3. The entire pCK.1 cDNA was present within clone 5a. In addition, beyond the 3′ boundary of pCK.1, 3.6 kb of DNA was contained in clone 5a. The Pst I–Pst I fragment at the 3′ end of clone 5a ended with 29 A residues. Eighteen nucleotides upstream from these A residues was the sequence AATAAA, believed to be the recognition site for the polyadenylation enzyme. Primer extension studies with a synthetic oligonucleotide derived from the 5′ end of clone 5a indicated that the insert was approximately 450 nucleotides short of a full-length cDNA. These data predicted that the messenger RNA (mRNA) for the subunit would be approximately 5 kb.

At about the same time we completed the nucleotide sequence shown in Fig. 3, K. Tatemoto and H. Schulman (Stanford) purified and sequenced a 19–amino acid tryptic peptide from the 50-kD subunit of rat forebrain CaM-KII. This sequence is identical to the underlined residues in Fig. 3 (amino acids 96 to 114). This confirmed that the cDNAs we had cloned represent the 50-kD subunit of the enzyme. The deduced amino acid sequence was analyzed for homologous sequences present in a molecular biology information resource database (Fig. 3). The highest homologies were found with other members of the protein serine–kinase family of enzymes (Fig. 3). Although stretches of homology were found throughout the CaM-KII open reading frame, the greatest homologies were restricted to two regions. The first began with a six–amino acid sequence, Asp-Leu-Lys-Pro-Glu-Asn (underlined in Fig. 3), that is present in the catalytic domain of many protein serine–kinases (*12, 13*). These six amino acids are present near the 5′ end of the CaM-KII sequence, and the primary homology extends for about 80 amino acids (Fig. 3). Within this region direct amino acid homologies varied from 40 to 50% or, when allowing for conservative substitutions, were between 62 and 75%. The highest homologies exist with chicken smooth muscle myosin light chain kinase (SM MLCK) and rabbit skeletal muscle phosphorylase b kinase (Phos K), whereas the lowest homology is with CDC28, a yeast protein kinase involved in cell cycle progression (*14*). Intermediate homologies were observed with rabbit skeletal muscle myosin light chain kinase (SK MLCK), protein kinase C (PKC), adenosine 3′,5′-monophosphate–dependent protein kinase (cAMP K), and guanosine 3′,5′-monophosphate–dependent protein kinase (cGMP K).

The second homologous region of CaM-KII begins approximately 36 amino acids from the end of the sequence in Fig. 3. This region extends for 25 residues and only occurs in SM MLCK, SK MLCK, and Phos K. Thirty percent of the residues are identical; with conservative substitutions, 60 to 72% are homologous. This 25-residue region constitutes most of the calmodulin-

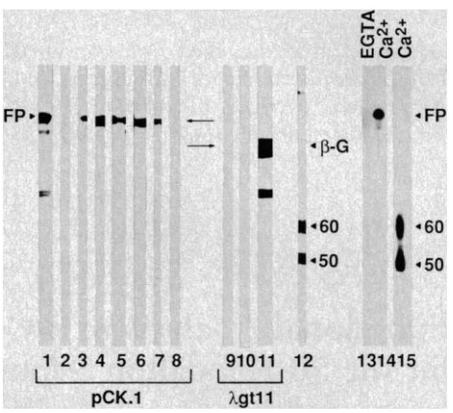

Fig. 2. Immunoblot analysis of pCK.1-generated fusion protein (FP). Fusion proteins were produced from pCK.1 as described (*12*), separated on 8 to 16% gradient polyacrylamide slab gels, and transferred to nitrocellulose. Immunoblots were prepared and analyzed as described (*1*). Each lane contained 150 ng of FP and was immunostained with antibodies to the following proteins: lane 1, β-galactosidase (β-G); lane 2, control without primary antibody; lane 3, CaM-KII (affinity-purified antibody); lane 4, 50-kD subunit of CaM-KII [monoclonal antibody (Mab) CB-12]; lane 5, 50-kD subunit (Mab CB-10); lane 6, 50-kD subunit (Mab CB-12); lane 7, 50-kD and 60-kD subunits (Mab C42.1); lane 8, 60-kD subunit (Mab CB-7). Lanes 9 through 11 contain protein lysates from IPTG-induced λgt11 without cDNA inserts and stained with: lane 9, affinity-purified antibody to CaM-KII; lane 10, Mab CB-12; lane 11, antibody to β-galactosidase (Mab). Lane 12 contains synaptic junction CaM-KII (1 μg of protein) stained with affinity-purified antibody to CaM-KII. Immunoreactive bands were visualized with antibodies to immunoglobulin G conjugated to alkaline phosphatase (Promega Inc.). ^{125}I-labeled calmodulin overlays of pCK.1-derived fusion protein (FP; lanes 13 and 14) and control overlay showing the Ca^{2+}-dependent binding of ^{125}I-labeled calmodulin to purified CaM-KII (300 of ng protein; lane 15). CaM overlays (*1*) were carried out in the presence of Ca^{2+} (0.5 m*M*) or EGTA (5 m*M*).

binding site for both skeletal muscle (*15*) and smooth muscle (*16, 17*) MLCKs. Both phosphorylase b kinase and CaM-KII are also calmodulin-binding proteins. It seemed likely that this 25-residue domain would also encode the calmodulin-binding region of these enzymes. Because the pCK.1 fusion protein that binds calmodulin includes these amino acids, this was directly tested for CaM-KII by preparing a synthetic peptide corresponding to the 25 residues of CaM-KII bracketed in Fig. 3 (amino acids 159 to 183).

The synthetic peptide from CaM-KII inhibits the calmodulin-dependent activation of bovine brain phosphodiesterase (Fig. 4A). The kinetics of this inhibition are indistinguishable from those seen with synthetic peptides prepared to the homologous 25–amino acid regions present in the MLCKs. In each case the calculated dissociation constant (K_d) is less than 1 n*M*. Thus, the overall charge distribution of synthetic peptides may be more important than the precise amino acid sequence for binding calmodulin in a competition assay. If this is true, it is not surprising that the peptide analogs have higher affinity for calmodulin than do the enzymes from which they are derived (*15–17*). To confirm the calmodulin-binding properties of the CaM-KII peptide analog, the synthetic peptide was electrophoresed in the presence or absence of calmodulin, and the Ca^{2+}-dependence of this interaction was assessed in the presence of this cation or EGTA (Fig. 4B). The nondenaturing conditions described by Davis and Ornstein [see (*18*)] were chosen because the basically charged peptide would migrate in the opposite direction from the acidic calmodulin. Stained protein bands were not observed in lanes containing the peptide alone (lanes 1 and 4), because it migrated toward the cathode and off the gel. Lanes 2 and 5 show the electrophoretic properties of calmodulin. The presence of

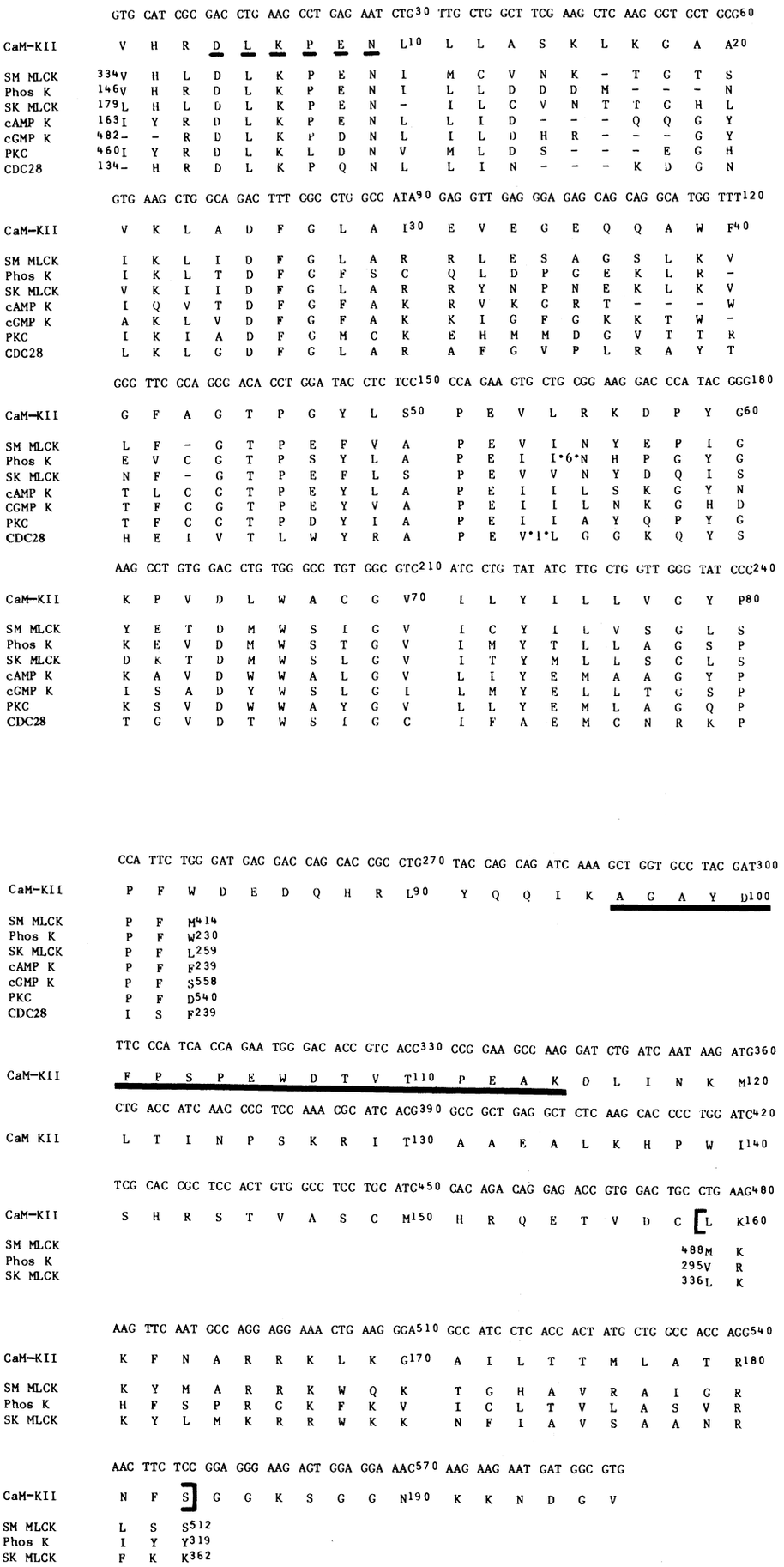

Fig. 3. Nucleotide sequence of CaM-KII cDNA and comparison of the deduced amino acid sequence with other selected members of the protein serine–kinase family. The sequence strategy is shown in Fig. 1, and the dideoxy method was utilized (*24*). The nucleotide sequence was entered into a molecular biology information resource database, which was searched for homology at the amino acid level (*25*). The underlined sequence in CaM-KII is the sequence of the tryptic peptide provided by K. Tatemoto and H. Schulman. The peptide analog representing the calmodulin-binding region and used in the experiments in Fig. 4 is bracketed. The numbers represent the amino acid residue of the sequences in the database. The single letter code for amino acids is: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and T, Tyr.

Ca^{2+} (lane 5) retarded the migration of calmodulin relative to that achieved in the presence of EGTA (lane 2). Mixtures of peptide and calmodulin were added to lanes 3 and 6. In the absence of Ca^{2+} (lane 3) only calmodulin was observed, whereas, in the presence of Ca^{2+}, the migration of calmodulin was further retarded due to formation of the ternary Ca^{2+}-calmodulin-peptide complex. We conclude from these results that the 25–amino acid synthetic peptide constitutes most, if not all, of the calmodulin-binding domain of the 50-kD CaM-KII subunit. Because of the high degree of similarity, we suggest that the homologous region of phosphorylase b kinase is probably a calmodulin-binding site in the catalytic subunit of this enzyme.

Previous studies have evaluated the regional distribution of the 50-kD subunit of CaM-KII in rat brain and have demonstrated that the accumulation of this protein is developmentally regulated (*19*). We have, therefore, utilized the CaM-KII cDNA to examine developmental changes in mRNA. Total RNA and polyadenylated [poly(A)$^+$] mRNA fractions were prepared from 7- and 20-day-old rat forebrain and 30-day-old cerebellum (Fig. 5A). Northern hybridization analysis demonstrated that ^{32}P-labeled cDNA probes recognized a 5-kb brain RNA, thus confirming the size of the mRNA deduced from the length of the cDNA clone 5a and its primer extended product. In forebrain from 20-day-old animals the 5-kb mRNA was enriched about 12-fold in poly(A)$^+$ RNA as compared to total RNA (lanes 2 and 5, respectively). During postnatal development, hybridization to the 5-kb mRNA was approximately five times greater in 20-day-old than 7-day-old forebrain preparations of poly(A)$^+$ RNA (lanes 2 and 1, respectively). Even larger differences were observed in comparisons between forebrain and cerebellum; hybridization to 20-day-old forebrain poly(A)$^+$ RNA was about 14-fold greater than analogous RNA fractions from 30-day-old cerebellum (lanes 2 and 3, respectively). Total RNA fractions were also prepared from 1-, 7-, 14-, 21- and 90-day-old rat forebrains and analyzed by Northern hybridization (Fig. 5B). These comparisons demonstrated an approximate tenfold increase in the 5-kb mRNA between days 1 and 21, and an additional 2.5-fold increase between postnatal days 21 and 90. In addition, the level of 5-kb mRNA in adult cerebellum was slightly lower than that observed in 7-day-old forebrain (lanes 6 and 2, respectively). These results on the 5-kb mRNA in rat brain are consistent with information on developmental increases and regional differences in the amount of 50-kD polypeptide in rat brain tissues (*8, 19*). Moreover, the greatest accumulation of kinase mRNA coincides with the most active period of synapse formation (*20*). The additional two- to threefold increase in mRNA observed between days 21 and 90 temporally corresponds to the functional maturation and stabilization of synaptic contacts (*19*). The fact that mRNA levels remain highest in adult forebrain may underlie the large demands placed on CaM-KII for Ca^{2+}-calmodulin–dependent phosphorylation associated with cytoskeletal elements, synaptic transmission, and neuronal plasticity (*4, 5, 21*).

The relatively large family of related enzymes referred to as Ca^{2+}- and calmodulin-dependent multifunctional protein kinases is broadly distributed across tissues and species (*1, 2*). To evaluate the possible relatedness of this family of kinase genes, selected cDNA probes were used to examine cross-hybridizing RNAs from a variety of tissue sources (Fig. 5C). RNAs of approximately 5 kb were identified in a number of tissues and cell lines including: (i) monkey brain (lane 2); (ii) primary cultures of rat hippocampal pyramidal neurons (lane 4); (iii) baboon testis (lane 5); (iv) human medulloblastoma cells (lane 6); and (v) adult mouse brain (Fig. 5A, lane 7). Primary cultures of astrocytes prepared from newborn rat forebrain displayed no detectable cross-reacting RNA (Fig. 5C, lane 3), which is consistent with the notion that the mRNA encoding the 50-kD subunit is highly enriched in neuronal elements compared to nonneuronal elements. Somewhat larger (7 kb) and smaller RNAs (3.8 kb) were detected in 44-day-old chicken brain (Fig. 5C, lane 7). Separate experiments with a ^{32}P-labeled riboprobe constructed with the 3.6-kb Pst I–Pst I fragment of clone 5a revealed 4.5- to 5.7-kb mRNAs in rat heart, liver, and skeletal muscle. These data indicate that the various Ca^{2+}-calmodulin–dependent multifunctional protein kinases are homologous at the mRNA as well as protein level.

Although CaM-KII is composed of two subunits, we have only identified a single mRNA species in mammalian tissue by hybridization of the cDNA representing the smaller (50-kD) subunit. This was true with a variety of stringency conditions. Because the two subunits differ by only 10 kD in size, each could be encoded by the same size mRNA. We cannot rule out the possibility that the hybridization signals seen in Fig. 5 may represent two similar sized mRNAs. However the intensity of this signal varies depending on the anatomical region of the

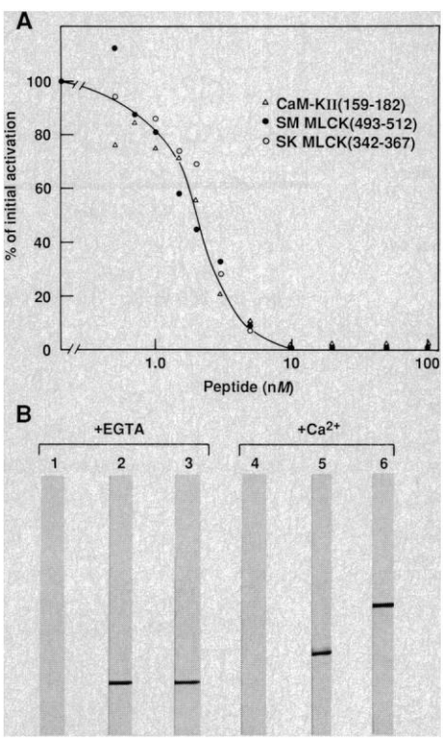

Fig. 4. (**A**) Effect of calmodulin-binding peptide analogs on calmodulin-dependent cyclic nucleotide phosphodiesterase activation. Calmodulin-deficient phosphodiesterase was isolated from bovine brain through the affinity chromatography step as described by Sharma *et al.* (*26*). The enzyme was assayed by a modification of the batch method of Thompson *et al.* (*27*). Phosphodiesterase activity is defined as counts per minute of substrate hydrolyzed in 60 minutes. The reaction mixture contained 40 m*M* tris-HCl, *p*H 8.0, 5 m*M* $MgOAc_2$, 1 m*M* $CaCl_2$, 30 μ*M* cGMP, 0.15 μCi of [^{3}H]cGMP, 1 m*M* dithiothreitol, 20% glycerol, bovine serum albumin (0.64 mg/ml), 2.38 n*M* calmodulin, 50 p*M* calmodulin-deficient phosphodiesterase, and various concentrations of the synthetic peptides in a total volume of 250 μl. The assay was initiated by the addition of substrate and carried out at 30°C for 60 minutes. Percent of initial activation of phosphodiesterase was calculated from the total counts per minute [^{3}H]cGMP hydrolyzed per assay tube, by the following equation: percent of initial activation = $(e - b)/(a - b) \times 100$, where e is phosphodiesterase activity assayed in the presence of 2.38 n*M* calmodulin plus various amounts of synthetic peptides; b is activity of phosphodiesterase assayed in the absence of calmodulin and peptides; and a is phosphodiesterase activity in the absence of peptides but presence of 2.38 n*M* calmodulin. The phosphodiesterase was stimulated over tenfold by a saturating concentration of calmodulin and to about 60% of maximal activity by 2.38 n*M* calmodulin. (**B**) Ca^{2+}-dependent interaction of calmodulin and the CaM-KII peptide analog. Calmodulin and the CaM-KII peptide analog were subjected either alone or in a mixture to electrophoresis under nondenaturing conditions (*18*). Electrophoresis was performed in a 12.5% polyacrylamide slab gel in the presence of 5 m*M* EGTA (lanes 1 to 3) or 5 m*M* $CaCl_2$ (lanes 4 to 6). The anode is at the bottom of the gel. Lanes 1 and 4, 5 μg of the peptide; lanes 2 and 5, 6 μg of calmodulin; lanes 3 and 6, 5 μg of peptide plus 6 μg of calmodulin. Proteins were visualized by staining with Coomassie brilliant blue.

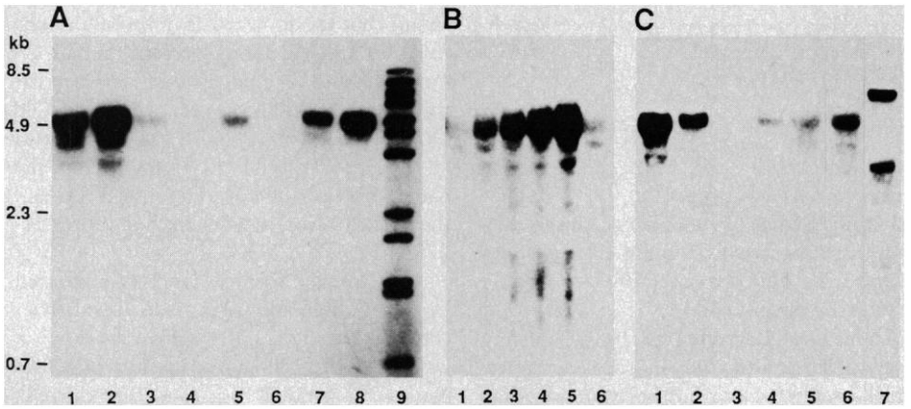

Fig. 5. Northern hybridization analysis of RNA fractions from rat brain and other tissues. Poly(A)$^+$ RNA was isolated, analyzed in 1.25% agarose gels after denaturation with glyoxal and dimethylsulfoxide, and transferred to Biotrans nylon membranes (*28*). (**A**) Lane 1, 10 μg of poly(A)$^+$ RNA from 7-day-old forebrain; lane 2, 10 μg of poly(A)$^+$ RNA from 20-day-old forebrain; lane 3, 10 μg of poly(A)$^+$ RNA from 30-day-old cerebellum; lane 4, 10 μg of total RNA from 7-day-old forebrain; lane 5, 10 μg of total RNA from 20-day-old forebrain; lane 6, 10 μg total RNA from 30-day-old cerebellum; lane 7, 5 μg of poly(A)$^+$ RNA from adult mouse brain; lane 8, 5 μg of poly(A)$^+$ RNA from 20-day-old forebrain; lane 9, ^{32}P-labeled λ DNA standards prepared by digestion with Bst EII endonuclease. (**B**) Lanes 1 to 5 contain 15 μg each of total RNA from 1-, 7-, 14-, 21-, and 90-day-old rat forebrain; lane 6, 15 μg of RNA from 90-day-old cerebellum. (**C**) Lane 1, 10 μg of RNA from 21-day-old rat forebrain; lane 2, 10 μg of RNA from monkey (Macaque) frontal cortex; lane 3, 10 μg of RNA from primary astrocyte cultures from newborn rat brain; lane 4, 3 μg of RNA from 30-day cultures of pyramidal neurons cultured from embryonic day-17 rat hippocampus; lane 5, 10 μg of RNA from baboon testis; lane 6, 10 μg of RNA from human TE-671 medulloblastoma cells; lane 7, 5 μg of poly(A)$^+$ RNA from 44-day-old chicken brain. ^{32}P-labeled pCK.1 was used for (A), and the 600-bp 5′ Pst I–Sph I fragment of 5a was used for (B) and (C). Primary cultures of pyramidal neurons of astrocytes were prepared as described (*29*). Autoradiographic patterns were quantitated by densitometry (*19*).

rat brain in a manner similar to differences observed in the content of 50- versus 60-kD polypeptides (*8*). In addition, only one primer extension product was observed, indicating that the extended sequence for the 50-kD subunit is present in only one mRNA transcript. Taken together it seems likely that the two subunits are the products of separate genes.

Kemp *et al.* (*17*) examined the properties of a synthetic peptide encompassing the calmodulin-binding domain of SM MLCK and showed that the former possesses pseudosubstrate characteristics. In the absence of Ca^{2+} and calmodulin, SM MLCK exists in a conformation in which its substrate-binding and calmodulin-binding domains interact and thus prohibit substrate binding to the enzyme. We speculate that a functional interaction between catalytic and regulatory domains may also impart stringent Ca^{2+}-calmodulin dependency to CaM-KII. Such a relationship may be more complicated than in the case of the MLCKs (*15, 17*). Indeed, the autophosphorylation of CaM-KII converts it to an enzyme that no longer requires Ca^{2+}-calmodulin for activity (*11*). Thus, autophosphorylation of sites near the calmodulin-binding domain may produce the nonregulated kinase. Consistent with the concept of coupled functional domains, peptide mapping has shown that the same peptide generated from the 50-kD subunit by V8 protease digestion contains both autophosphorylation and calmodulin-binding sites (*9*). As predicted from Fig. 2, two potential autophosphorylation sites (Arg-X-X-Thr and Arg-X-X-Ser) flank the calmodulin-binding sequence of the synthetic peptide. Moreover, autophosphorylation of the 50-kD subunit occurs on both Ser and Thr residues (*9*). Therefore, the possibility of functional coupling between autophosphorylation and calmodulin-binding domains provides a framework in which to test the regulatory properties of this prominent neuronal kinase.

Note added in proof: After this manuscript was submitted, Bennett and Kennedy (*22*) published the sequence of a cDNA clone encoding the 60-kD subunit of rat brain CaM-KII. Over the first 183 amino acids shown in Fig. 3, the deduced amino acid sequences of the two clones are 93% identical. Both the 19–amino acid tryptic peptide and the 25–amino acid residues used to construct the synthetic analog of the calmodulin-binding site are identical in the two clones. Comparison of the Northern blots suggests that the two mRNAs are similar in size. The fact that the nucleotide sequences corresponding to the two identical peptide regions are different confirms our hypothesis that the mRNAs must be the products of separate genes. The size difference in the 50- and 60-kD polypeptides appears to be due to variation in a region that begins immediately to the carboxyl terminal side of the calmodulin-binding site in both cDNAs and extends for only about 60 amino acids.

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