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Molecular cloning of a brain-specific calcium/calmodulin-dependent protein kinase

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ABSTRACT **A calcium/calmodulin-dependent protein kinase type II (CaM-K) α-subunit cDNA has been cloned from rat brain. This enzyme is encoded by a 5.1-kilobase mRNA expressed exclusively in the brain. Hybridization histochemistry reveals that the CaM-K mRNA expression corresponds to the distribution of the immunoreactive α-subunit protein, suggesting that the high enzyme levels in specific brain areas reflect regional differences in gene expression. The sequence of CaM-K α-subunit cDNA indicates a 478-amino acid (54-kDa) protein with three functional domains. The domain organization suggests a structural model for calcium/calmodulin-dependent and independent states that might subserve short- and long-term responses to transient stimuli.**

Neuronal phenotype and function are controlled by extracellular stimuli, which activate diverse intracellular effectors, including protein kinases (1, 2). Because of the important role of calcium in regulating neural function, it is likely that calcium/phospholipid-dependent and calcium/calmodulin-dependent protein kinases have assumed critical regulatory functions (1). One isozyme of the calcium/calmodulin-dependent protein kinase type II (CaM-K) is a serine and threonine kinase that is expressed specifically in the brain (3). The ability of activated CaM-K to sustain calcium/calmodulin-independent autophosphorylation raises questions regarding its potential role in short- and long-term events characteristic of neurons (4). The role of covalent modification of proteins in complex events such as memory has been the subject of speculation (5, 6).

CaM-K is a holoenzyme of over 500 kDa composed of "51"-kDa (CaM-K-α) and "60"-kDa (CaM-K-β) structurally distinct subunits in various ratios (7–11). CaM-K-α appears to be exclusively expressed in neural tissues at high intracellular concentrations, constituting, for example, about 2% of all protein in the hippocampus (3). The enzyme appears to be distributed in several cellular compartments, including the cytosolic surface of synaptic vesicles, but is most concentrated in the postsynaptic density (12). Cam-K substrates include the neuronal cytoskeletal proteins synapsin I and microtubule-associated protein 2 (MAP-2), as well as tyrosine hydroxylase, τ, trytophan hydoxylase, and myelin basic protein (4). CaM-K enzymatic activities in nonneuronal tissues appear to be isozymes with different subunit size and composition (3, 13).

In this manuscript we report the molecular cloning of DNA complementary to mRNA encoding CaM-K-α from rat brain.‖ The predicted primary structure of CaM-K-α suggests a model for regulation of CaM-K function.

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METHODS

Protein Sequencing. CaM-K was purified to apparent homogeneity from rat forebrain as previously described (14). Tryptic fragments were separated by using reverse-phase high-pressure liquid chromatography, consisting of a μBondapak C_{18} column (3.8 × 300 mm) with a gradient of acetonitrile in 0.1% aqueous trifluoroacetic acid. The amino acid sequences of the peptides forming the major 215-nm absorbance peaks were determined by a gas-phase sequencer (Applied Biosystems, Foster City, CA) by automated serial Edman degradation.

Cloning of CaM-K cDNA. cDNA complementary to rat brain poly(A)-containing RNA [poly(A)-selected RNA] was generated by using reverse transcriptase from avian myeloblastosis virus or Moloney murine leukemia virus and cloned in λgt10 or λgt11 arms as previously described (15) or by using a variant procedure (16). On the basis of codon usage the following mixed- and unique-sequence oligonucleotides were constructed: 5′-TGG CA(A or G) AT(A, T, or C) GTG CA(C or T) TT(C or T) CA(C or T) AA-3′, corresponding to P_{14}; and 5′-GA(A or G) TGG GA(C or T) ACG GT(A, G, C, or T) AC(A, G, C, or T) CCG GA(A or G) GC-3′ and (antisense) 5′-CTT GGC CTC AGG GGT CAC TGT GTC CCA CTC AGG GGA GGG GAA GTC ATA GGC GCC AGC-3′, corresponding to P_6. Subsequently, 5′-CTT GGC TTC CGG GGT GAC GGT GAC CCA TTC TGG TGA TGG GAA ATC-3′ was constructed. Screening with kinase-treated oligonucleotides was performed with washing at 40°C to 50°C in 2× SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7.4). Inserts were subcloned in phage M13-based vectors for nick-translation (15) or sequencing by the enzymatic dideoxynucleotide method of Sanger *et al.* (17) with deazaguanosine.

RNA and DNA Analysis. After size fractionation, hybridization analysis was performed on poly(A)-selected RNA and restriction digests of rat brain genomic DNA, as previously described (15, 18). ^{32}P-labeled probes used were nick-translated C3 cDNA (2×10^8 cpm/μg) which corresponds to nucleotides −41 to 943, and 3′-C31 complementary RNA (cRNA) "run-off" transcripts (1.4×10^9 cpm/μg), which correspond to nucleotides 1077 to 1471 (19). Washing was performed in 0.2× SSC/0.2% $NaDodSO_4$ at 65°C. For *in situ* hybridization histochemistry a male Sprague–Dawley rat (475 g) was perfused with a formaldehyde buffer, and the brain was postfixed overnight, without glutaraldehyde, as previously

Abbreviations: CaM-K, the multifunctional calcium/calmodulin-dependent protein kinase type II; CaM-K-α, the α subunit of CaM-K; CaM-K-β, the β subunit of CaM-K; MLCK, myosin light chain kinase.

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‖This sequence is being deposited in the EMBL/GenBank data base (Bolt, Beranek, and Newman Laboratories, Cambridge, MA, and Eur. Mol. Biol. Lab., Heidelberg) (accession no. J02942).

described (20). A 1 in 10 series of frozen sections (30 μm) was hybridized to the 3'-C31 [^{32}P]cRNA (10^8 cpm/ml) as previously described (19), washing with 0.1× SSC at 55°C.

RESULTS

Cloning of CaM-K. CaM-K was purified to apparent homogeneity from rat forebrain (14). Tryptic fragments were resolved by using high-performance liquid chromatography and subjected to microsequencing analysis (Fig. 1*A*). cDNA libraries from rat cortex or rat brain, excluding cerebellum, were screened with synthetic oligonucleotides whose sequences were based on the sequences of two peptides. A total of 58 reactive recombinants were identified from about 2 × 10^6 plaques with the oligonucleotides and subsequent nick-translated probes. Six clones were subjected to DNA sequencing; the sequences of four clones permitted the deduction of the entire coding sequence for CaM-K-α (Fig. 1*B*). An open reading frame composed of 1434 nucleotides indicated a 478-amino acid protein (Fig. 2*A*). The initiator methionine was identified by the presence of a preceding stop codon. Distributed throughout the predicted protein of actual M_r 54,111 were sequences corresponding to 15 tryptic peptides (Fig. 1*A* and 2*A*). A cell-free translation product of an *in vitro* RNA transcript encompassing the entire coding region is a 54-kDa protein that is specifically immunoprecipitated by anti-Cam-K-α-specific sera (data not shown).

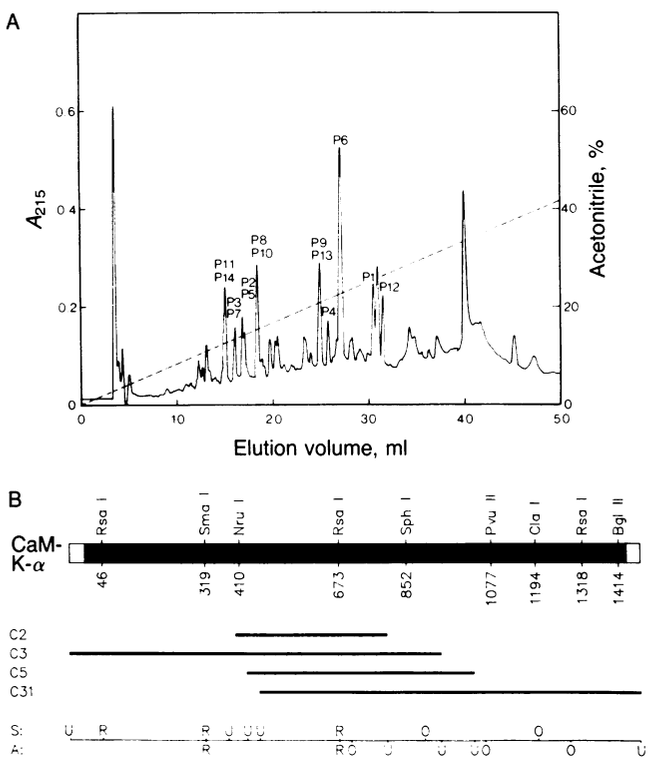

FIG. 1. Cloning of CaM-K-α. (*A*) Fourteen tryptic fragments of purified CaM-K were fractionated by HPLC. Sequences of these peptides, in the standard one-letter code, are as follows: P_1, FTEQYQLFE; P_2, VLAGQEYAAK; P_3, HPNIVR; P_4, DLKPENLLLA; P_5, LYQQIK; P_6, AGAYNFPSPEWDTVTPEAK; P_7, DLINK; P_8, ITAAEALK; P_9, ITAAEALKHPWI; P_{10}, HPWISHR; P_{11}, KQQIIK; P_{12}, FYFENLWS; P_{13}, VWHR; P_{14}, WQIVHFHR. An additional peptide was found on a separate column fractionation: SGAPSV. (*B*) Representation of four of the six clones analyzed to confirm the sequence. DNA sequencing was performed by utilizing the "universal primer" oligonucleotide (U) to sequence the borders of the insert or ends created by restriction enzyme digestion (R) or by utilizing synthetic oligonucleotides (17-mer) corresponding to information from the clones (O). Clones were sequenced on both sense (S) and antisense (A) strands.

Analysis of CaM-K-α mRNA Expression. A probe corresponding to nucleotides −41 to 943 of Cam-K-α cDNA (amino acids 1–314) hybridized to a predominant 5.1-kilobase (kb) brain mRNA, which appears to represent the CaM-K-α mRNA. A 3.9- to 4.2-kb RNA hybridized at <3% of the signal strength of the 5.1-kb RNA (Fig. 3*A*), and other RNAs, 7.1, 6.1, and 3.3 kb in size, were detected in low variable levels. In human fetal brain, the migration of the dominant species is coincident with the rat brain 7.1-kb species. The ratio of signals from the 5.1- and 3.9-kb species in cerebellum was about 1.5:1. When a riboprobe corresponding to nucleotides 1077–1471 (amino acids 360–478) was used, the hybridization to the 3.9-kb RNA relative to the 5.1-kb RNA was greatly enhanced (Fig. 3*B*); it is suggested that the 3.9-kb RNA encodes the CaM-K-β (60-kDa) protein. In most rat tissues no RNA species hybridizing to the CaM-K-α probe was detected, including liver, kidney, spleen, and heart (Fig. 3*A*) and lung and adrenal gland (not shown); even if expressed at levels <1000-fold the level in the brain, CaM-K-α mRNA would have been detectable under these conditions. In pituitary (not shown) and skeletal muscle, a faintly hybridizing 3.9-kb RNA species comigrated with the putative brain CaM-K-β mRNA. In addition, 4.1-, 4.5-, and 4.9-kb homologous RNAs were detected in pituitary, skeletal muscle, and testes, respectively (Fig. 3*A*). A number of cell lines of different origin (Fig. 3*A*), including rat adrenal medulla (PC12), rat thyroid C cells (CA 1037), and mouse lymphocytes (A20) express reactive 3.9- to 4.5-kb RNA species, but no 5.1-kb CaM-K-α mRNA. Restriction analysis of rat genomic DNA fragments by using the C3 probe, which includes the kinase and regulatory domains of CaM-K-α (see below and Fig. 5A), suggests that these domains alone span >15 kb of genomic information (Fig. 3*C*).

Localization of CaM-K mRNA in the Brain. Immunohistochemistry suggests a striking regional distribution of Cam-K, with highest levels in hippocampal pyramidal cells and cortex. Because of the high concentration in postsynaptic densities in the neuropil associated with these regions, the quantitative distribution could reflect either differential stability of the sequestered protein or regional variation in gene expression. To distinguish between these possibilities, hybridization histochemical analysis of a series of coronal rat brain sections was performed; several sections are shown in Fig. 4. In the forebrain, the hybridization was most predominant within the hippocampus and the superficial layers of the cortex. There was also considerable hybridization to the caudate-putamen, amygdaloid complex, thalamus, and hypothalamus. Relatively light hybridization was detected in the colliculi and several brainstem nuclei. Within the cerebellum there was hybridization in the Purkinje cell layer and, to a lesser extent, in the molecular and granular layers. In the spinal cord, light hybridization was detectable only in the superficial layers of the dorsal horns. The quantitative pattern of CaM-K RNA is in agreement with the immunohistochemical analysis of CaM-K protein (3, 31), implying that regulation of CaM-K gene expression in the brain determines the extremely high level of the enzyme in the hippocampus and cortex.

DISCUSSION

The cloning of the CaM-K-α cDNA has allowed us to predict a 54-kDa protein that encodes a protein kinase and contains three apparent domains. The NH_2-terminal domain is highly homologous to other known protein kinases, but it is most closely related to the γ subunit of phosphorylase *b* kinase (PbK-γ) and myosin light chain kinase (MLCK). Conserved regions are diagramed in Fig. 2*B*. Two interesting sequence variations occur in this protein. First, the third Gly in the ATP-binding site sequence (2) is replaced by Ser in CaM-K-α and PbK-γ (Fig. 2*B*, block I). Likewise, the so-called "APE box" (Fig. 2*B*, block XII) is uniquely Ser-Pro-Glu in CaM-

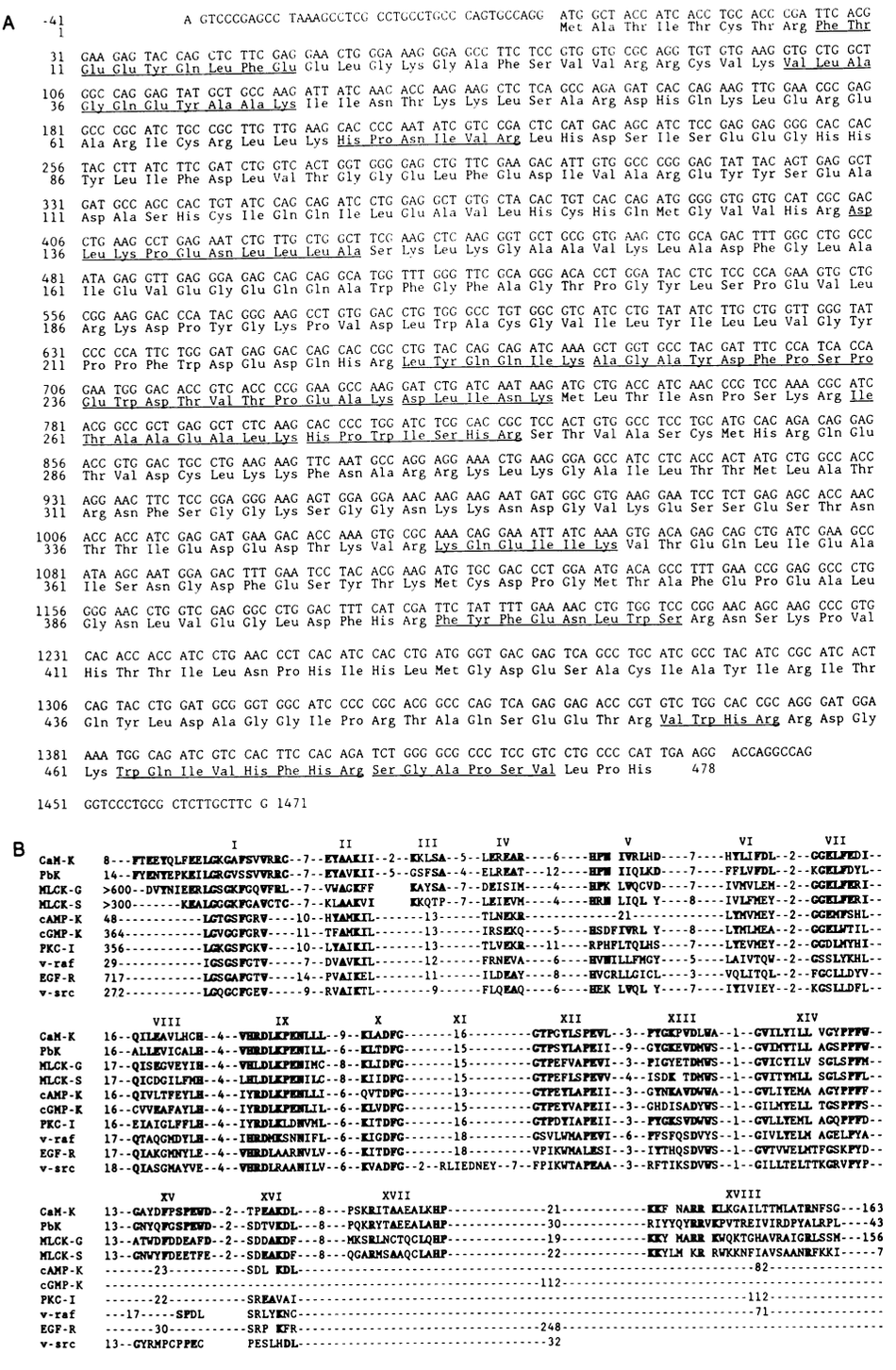

FIG. 2. Primary structure of CaM-K-α. (*A*) The nucleotide sequence encompassing the coding region of CaM-K-α and the predicted encoded protein. The 15 peptides listed in the legend to Fig. 1*A* are present in numerical order in the predicted sequence and are underlined. (*B*) Homology between CaM-K-α and other protein kinases, including the following: PbK, γ subunit of rabbit phosphorylase *b* kinase (21); MLCK-G, chicken gizzard myosin light chain kinase (22); MLCK-S, rabbit skeletal muscle myosin light chain kinase (23); cAMP-K, catalytic subunit of bovine cardiac muscle cAMP-dependent protein kinase (24); cGMP-K, catalytic subunit of bovine lung cGMP-dependent protein kinase (25–27); PKC-I, rat brain protein kinase C type I (28); v-raf, murine sarcoma virus v-raf (29); EGF-R, human epidermal growth factor receptor (15, 30); v-src, Rous sarcoma virus strain Prague C v-src [data of D. Schwartz, R. Tizard, and W. Gilbert in EMBL/GenBank Genetic Sequence Database (1982) GenBank (Bolt, Beranek and Newman Laboratories, Cambridge, MA), Tape Release 47]. Numbers indicate the number of intervening residues not listed due to lack of obvious homology, and blank spaces indicate an artifical gap created in alignment. Identity between CaM-K-α and PbK-γ or between CaM-K-α and any two other listed kinases is highlighted by bold print.

K-α and rabbit skeletal muscle MLCK. A series of homologies are found within the protein kinase domain (amino acid residues 1 to 220–250, Fig. 2*B*, blocks I to XIV–XVI). Amino acid residues 250 to 320–350 appear to reflect a second domain, unique to the calcium/calmodulin-dependent protein kinases. This domain includes a basic amino acid-rich sequence, *Lys-Lys*-Phe-Asn-Ala-*Arg-Arg-Lys*-Leu-*Lys* (Fig. 2*B*, block XVIII), which is postulated to represent the calmodulin-binding site. In support of this assignment, proteolytic fragments of PbK-γ and MLCK containing portions of block XVIII have been reported to bind calmodulin (32–34). Synthetic peptides corresponding to this domain of CaM-K-α bind calmodulin and inhibit kinase activity. Finally, the 163-residue COOH-terminal region, lacking homology to other protein kinases, is highly hydrophilic and contains two areas of high charge density (Fig. 5*A*). The function of this region is unknown, but it could be important in the assembly of subunits of holoenzyme or in the association of the holoenzyme with other proteins or cellular organelles.

The three-domain structure of the protein suggests a model for the mechanism of the known regulation of CaM-K enzyme (Fig. 5). In the inactive state the regulatory region of each subunit may be folded into a closed configuration, with the calmodulin-binding site sterically blocking the entry of substrates into the active site (Fig. 5*B*), analogous to similar events in MLCK (34). Binding of calcium/calmodulin displaces the inhibitory domain and activates the enzyme. Kinetic studies have shown that autophosphorylation at one or two threonine residues (L. L. Lou and H.S., unpublished data) converts the holoenzyme to an autonomous state (4, 36). Because limited proteolysis of CaM-K releases a monomeric 30-kDa autonomous enzyme devoid of autophos-

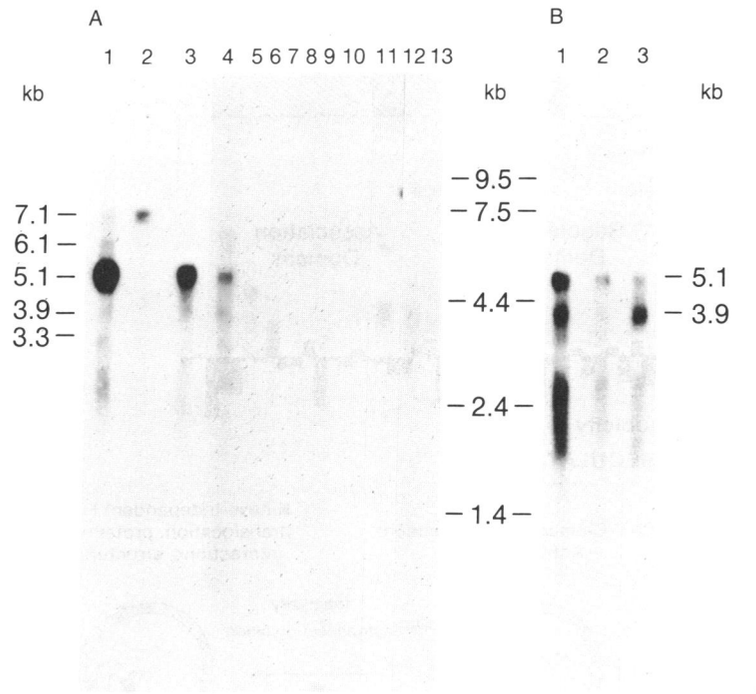

FIG. 3. RNA and genomic DNA hybridization to CaM-K-α cDNA. (*A* and *B*) Poly(A)-selected RNA prepared from various tissues (5 μg from rat, unless otherwise specified, size-fractionated, transferred to nitrocellulose, and hybridized to ^{32}P-labeled probes. In *A*, the probe was C3 cDNA. Autoradiographs were exposed for 10 hr (lanes 1–3) or 24 hr (lanes 4–13). Lanes: 1, temporal lobe; 2, human fetal brain; 3, brain without cerebellum; 4, cerebellum; 5, skeletal muscle; 6, testes; 7, kidney; 8, liver; 9, spleen; 10, heart; 11, adrenal medullary cell line PC12; 12, thyroid C cell line CA 1037; 13, mouse lymphoma cell line A20. In *B*, the probe was a 3′-C31 cRNA. Lanes: 1, brain without cerebellum (5-hr exposure); 2, brain without cerebellum (1 μg, 5 hr); 3, cerebellum (20 hr). Migration of RNA standards (Bethesda Research Laboratories RNA Ladder) is indicated. (*C*) Rat brain genomic DNA was digested to completion with *Bam*HI (lane 1), *Eco*RI (lane 2), or *Hin*dIII (lane 3), size fractionated and hybridized with C3 nick-translated probe. Migration of DNA standards (Bethesda Research Laboratories 1-kb DNA Ladder) is indicated.

phorylation sites (37), it is likely that these sites are in the regulatory or association domains. The four sequences most homologous to the consensus sequences for cAMP-dependent protein kinase and CaM-K substrates (4) are marked with asterisks in Fig. 5*A* and are found clustered throughout the "hinge" region and calmodulin-binding site. Phosphorylation of one or more of these residues may be the modification necessary to sustain the open configuration required for autonomy of CaM-K, potentially permitting short-term enhancement of responses to transient calcium signals (4, 6).

Autonomous autophosphorylation at a site(s) distinct from the aforementioned threonine residues results in the inhibition of enzyme activity for exogenous substrates (4, 5). This inhibitory autophosphorylation may occur within the calmodulin-binding site at the serine residue marked by an asterisk (Fig. 2*B*, block XVIII, and Fig. 5*A*), potentially inducing a conformational alteration of the COOH-terminal domain. Phosphorylation of gizzard MLCK by cAMP-dependent protein kinase at a comparable serine residue within the calmodulin-binding site leads to enzyme inhibition (32).

Autophosphorylation of Cam-K may also result in translocation of the holoenzyme to other areas of the neuron (38), altered protein–protein interactions, or activation of structural function(s) of the protein. The high concentration of such an autophosphorylated or translocated protein in neurons provides a potential facilitory mechanism for short-term and long-term neural response to stimuli. The cloning of CaM-K-α cDNA permits a structure–function analysis of its potential biological functions.

Note Added in Proof. Comparison of the sequence of CaM-K-α with the recently published CaM-K-β sequence (39) suggests that they are the products of two highly homologous transcription units, with amino acid insertions carboxyl-terminal to the calmodulin-binding domain accounting for the larger size of the β subunit.

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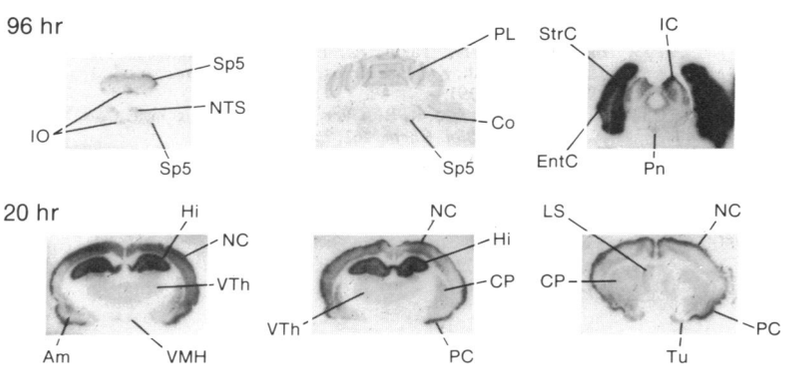

FIG. 4. *In situ* hybridization histochemistry of CaM-K RNA. Rat brain sections were hybridized to ^{32}P-labeled 3′-C31 riboprobe and exposed for 96 hr (*Upper*) or 20 hr (*Lower*) to Cronex 4 film without an intensifying screen. Brainstem sections (medulla, cerebellum, and midbrain, left to right) are in *Upper*, and forebrain sections (cortex, hippocampus, and neostriatum) are in *Lower*. Am, amygdaloid complex; Co, cochlear nucleus; CP, caudate-putamen; EntC, entorhinal cortex; Hi, hippocampus (including CA pyramidal cells and the dentate gyrus); IC, inferior colliculus; IO, inferior olive; LS, lateral septum; NC, neocortex (both frontoparietal-motor and frontoparietal-somato-sensory areas); NTS, nucleus tractus solitarius; PC, piriform cortex; PL, Purkinje layer of the cerebellum; Pn, pontine nuclei; Sp5, spinal tract of trigeminal nerve; StrC, striate cortex; Tu, olfactory tubercle; VMH, ventromedial hypothalamic nucleus; VTh, ventral thalamic nuclei (not exclusive).

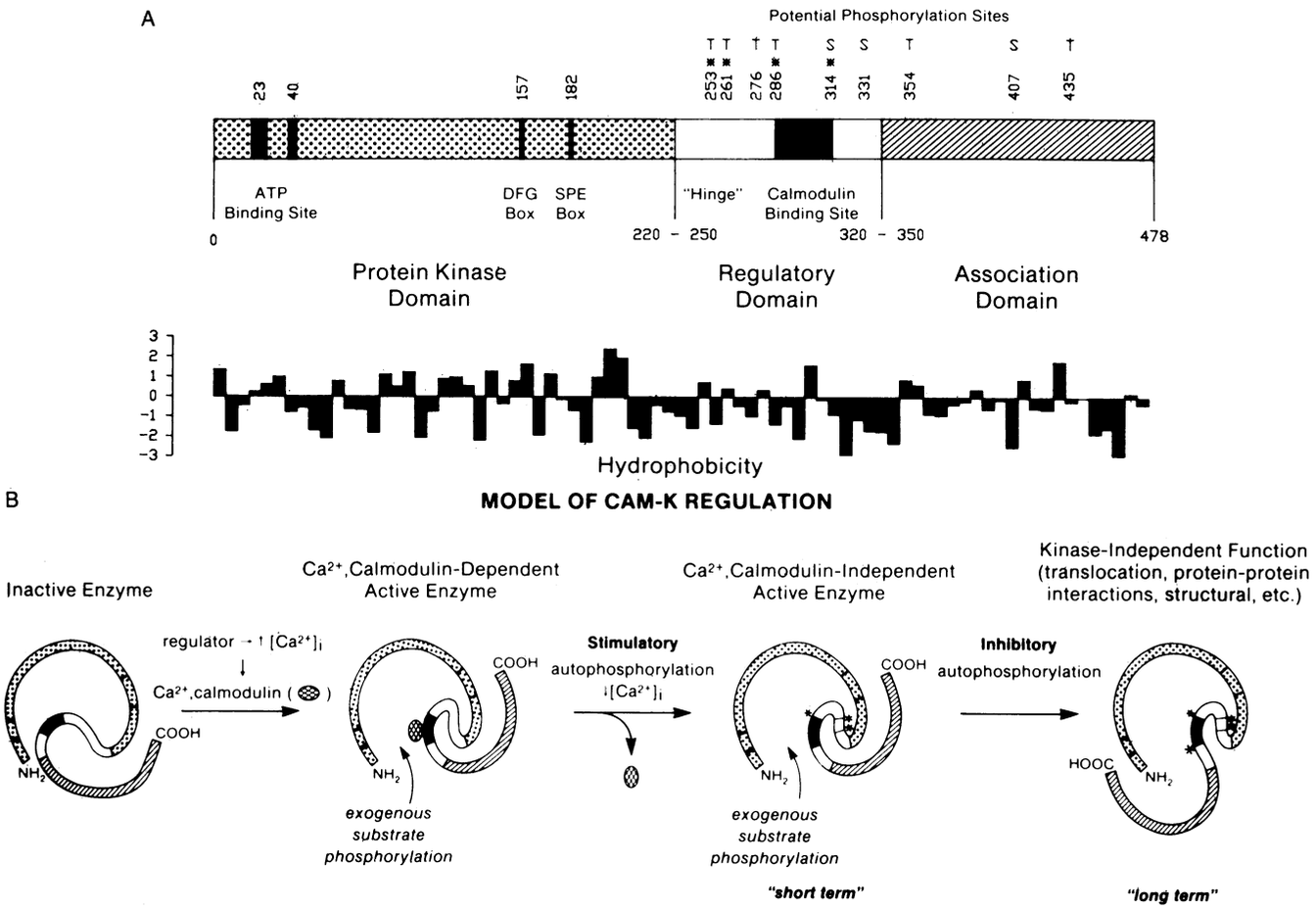

FIG. 5. Model of domain structure of CaM-K-α. (*A*) Potential serine (S) and threonine (T) sites for phosphorylation are noted; those with the greatest site homology are marked with an asterisk. The putative "hinge" region represents a region where the regulatory domain may bend so that further COOH-terminal regions may block the kinase active site (see *Discussion*). Below is a hydrophobicity plot drawn to scale and based on a window of six residues generated by the algorithm of Kyte and Doolittle (35). (*B*) A model for alternative conformations of CaM-K-α (see *Discussion*). Asterisks correspond to the threonine and serine sites shown in *A*.

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