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Molecular Cloning of Ca^{2+}/Calmodulin-Dependent Protein Kinase Kinase β[1]

Takako Kitani,[2] Sachiko Okuno, and Hitoshi Fujisawa[3]

Department of Biochemistry, Asahikawa Medical College, Asahikawa, Hokkaido 078

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Calmodulin-dependent protein kinase IV (CaM-kinase IV), which plays crucial roles in the functioning of Ca^{2+} in the central nervous and immune systems, is markedly activated upon phosphorylation through the action of CaM-kinase kinase. Our previous immunotitration analysis suggested the existence of an isoform different from CaM-kinase kinase α, the β isoform, in rat brain [Okuno, S., Kitani, T., and Fujisawa, H. (1996) *J. Biochem.* 119, 1176-1181]. In the present study, cDNA for CaM-kinase kinase β was cloned from a rat cerebellar cDNA library. The coded protein consisted of 587 amino acids with a molecular weight of 64,445. Western blot analysis revealed that CaM-kinase kinase β significantly existed only in the brain. The enzyme was not significantly detected in the retina where CaM-kinase kinase α exists.

Key words: brain, Ca^{2+}/calmodulin, CaM-kinase kinase, cDNA sequence, isoform.

Ca^{2+}/calmodulin-dependent protein kinases (CaM-kinases) I, II, and IV play important roles as Ca^{2+}-responsive multifunctional protein kinases (see Refs. *1-4* for reviews), controlling a variety of cellular functions in response to an increase in intracellular Ca^{2+}, and regulation of their activities is therefore very important. Among the three CaM-kinases, CaM-kinases IV (*5-7*) and I (*8, 9*) are activated through phosphorylation by upstream Ca^{2+}/calmodulin-dependent protein kinases, whereas CaM-kinase II is activated through phosphorylation by itself (*10-13*). One of the CaM-kinase kinases, CaM-kinase kinase α (originally called CaM-kinase IV kinase), was cloned from a rat cerebral cDNA library and the nucleotide sequence encoding the entire protein was determined (*14, 15*). On the other hand, most of the CaM-kinase IV kinase activity in the cerebellum was revealed to be due to an enzyme (CaM-kinase kinase β) different from the cloned enzyme (CaM-kinase kinase α) on immunotitration analysis (*15*), and CaM-kinase kinase β was purified from the cerebellum (*16*). In the present study, CaM-kinase kinase β was cloned from a rat cerebellar cDNA library and the nucleotide sequence encoding the entire protein was determined.

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[2] Affiliated with the Laboratory for Radioactive Isotope Research, Asahikawa Medical College.

[3] To whom correspondence should be addressed.

Abbreviations: CaM-kinase I, calmodulin-dependent protein kinase I; CaM-kinase II, calmodulin-dependent protein kinase II; CaM-kinase IV, calmodulin-dependent protein kinase IV; HPLC, high performance liquid chromatography; Mops, 3-(*N*-morpholino)propanesulfonic acid; PCR, polymerase chain reaction; TPCK-trypsin, tosylphenylalanyl chloromethyl ketone-treated trypsin; IPTG, isopropyl β-D-thiogalactoside.

EXPERIMENTAL PROCEDURES

Materials—[γ-^{32}P]ATP (5,000 Ci/mmol), [α-^{32}P]dCTP (3,000 Ci/mmol), and thermo sequenase were from Amersham International. Phosphocellulose paper (P81) was from Whatman. Microbial protease inhibitors (pepstatin A, leupeptin, antipain, and chymostatin) were from the Peptide Institute (Osaka). Restriction enzymes and other DNA modifying enzymes were purchased from Takara Shuzo, Toyobo, or New England Biolabs. Peptide-γ (KSDGGVKKRKSSSS) (*17*) and the PKIV peptide (KKKKEHQVLMKTVCGTPGY) (*18*) were synthesized by means of a Shimadzu PSSM-8 automated peptide synthesizer. TPCK-trypsin was from Cooper Biomedical. All other reagents were of the highest grade commercially available. Wistar rats were purchased from the Shizuoka Laboratory Animal Center.

Protein Preparations—Calmodulin was purified from *Escherichia coli* transformed with expression vector pET11d carrying a cDNA encoding chicken brain calmodulin (*19*), essentially as described by Gopalakrishna and Anderson (*20*). The cDNA encoding chicken brain calmodulin was kindly donated by A.R. Means (*21*). Recombinant rat brain CaM-kinase IV expressed in *E. coli* was partially purified as described previously (*5*). CaM-kinase kinase α was purified from rat cerebral cortex as described previously (*6*). CaM-kinase kinase β was purified from rat cerebellum as described previously (*16*). A crude extract of bacteria transformed with a vector carrying rat CaM-kinase kinase α cDNA and antibodies against CaM-kinase kinase α were prepared as described previously (*15*).

Cloning and Sequencing of CaM-Kinase Kinase β cDNA—Approximately 15 μg of purified rat brain CaM-kinase kinase β was digested with about 0.6 μg of TPCK-trypsin, the digested peptides were separated on a C_{18} reverse-phase HPLC column (TSK gel ODS-80Ts, Tosoh)

with a 0-80% acetonitrile gradient in 0.06% trifluoroacetic acid, and the amino acid sequences of several purified peptides were determined with a Hewlett Packard G1005A peptide sequenator. Two degenerate oligonucleotides, ATG(T/C)T(A/G/C/T)GA(T/C)AA(A/G)AA(T/C)CC as a sense primer and CC(T/C)TC(A/G)AA(A/G/C/T)GG-(A/G)TT(A/G/C/T)CC as an antisense primer, were synthesized on the basis of the amino acid sequences of the tryptic peptides, MLDKNPESR and SFGNPFEGSR, respectively, and oligonucleotides were synthesized by 40 cycles of PCR using sense and antisense primers from the first strand cDNA, which had been prepared from poly(A)$^+$ RNA obtained from rat cerebellum. The resulting PCR products were subcloned into the pGEM-T vector (Promega) and the nucleotide sequences of the inserts of several clones were determined by the dideoxynucleotide chain-termination method (*22*), using a LI-COR model 4000L DNA sequencer. A clone possessing the sequence of GAGTCCAGGATTGTGGTGCCTGAAATCAAGCTGCAC-CCTTGGGTCACGAGGCACGGGGCCGAGCCACTGCCG-TCGGAGGACGAGAACTGCACACTGGTCGAGGTGACC-GAAGAGGAGGTCGAGAATTCAGTCAAACACATTCCC-AGCCTGGCAACTGTGATCCTAGTGAAGACCATGATTC-GGAAACGGTCTTTT was used as a probe for screening CaM-kinase kinase β cDNA, because the amino acid sequence deduced from the clone showed high homology with the sequence of CaM-kinase kinase α (amino acid residues 395-459) (*15*). An oligo(dT)-primed cDNA library was constructed in λgt10 using poly(A)$^+$ RNA, which was isolated from rat cerebellum by the use of Oligotex™-dT30 (super) (Takara Shuzo) according to the manufacturer's manual, and approximately 1×10^6 plaques were screened with the probe. Nine positive clones of 1.8 to 4.3 kbp were isolated, and their nucleotide sequences were determined.

Expression of CaM-Kinase Kinase β cDNA in E. coli—An *Nde*I restriction site was created at the start codon of the CaM-kinase kinase β cDNA, according to the method of Kunkel *et al.* (*23*), and the cDNA fragment containing the entire coding sequence was introduced into an expression vector, pET11a (*24*), to generate plasmid pETCaMKKβ. This construct was transformed into *E. coli* strain BL21 (DE3) (*24*). The bacteria were grown to an A_{600} of 0.6 to 1.0 at 30°C in M9ZB medium containing 200 μg/ml ampicillin, and then IPTG was added to a final concentration of 1 mM. After 3 h, the bacteria were harvested by centrifugation, suspended in 7 volumes of 20 mM Tris-HCl buffer (pH 7.5 at 4°C) containing 1 mM EGTA, 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each of microbial protease inhibitors (leupeptin, pepstatin A, antipain, and chymostatin), and then disrupted by sonic oscillation. The residue was removed by centrifugation to generate the crude extract.

Expression of CaM-Kinase Kinase α or β cDNA in Sf9 Cells—A cDNA fragment containing the entire coding sequence of CaM-kinase kinase α (*15*) or β was introduced into a vaculovirus, AcNPV, using a Bac-To-Bac Baculovirus Expression System (Life Technologies). Sf9 cells infected with the recombinant baculovirus were grown at 27°C in Grace's medium comprising 10% fetal bovine serum, 0.35 g/liter $NaHCO_3$, 100 μg/ml kanamycin, and 2.6 g/liter Bacto tryptose phosphate broth (Difco) (pH 6.1). After 3 days, the cells were harvested, washed once with phosphate-buffered saline, suspended in approximately 10 volumes of 20 mM Tris-HCl buffer (pH 7.5 at 4°C) containing 1 mM EGTA, 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each of microbial protease inhibitors (leupeptin, pepstatin A, antipain, and chymostatin), and then disrupted by sonic oscillation. The residue was removed by centrifugation to generate the crude extract.

Production and Purification of Antibodies against CaM-Kinase Kinase β—Approximately 1 mg of a peptide, CGA-PAPGSPPRTPPQQPEEAMEPE, corresponding to the carboxyl-terminal 24 amino acids of CaM-kinase kinase β, was conjugated to 1.6 mg of keyhole limpet hemocyanin (Sigma) using *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (Pierce) as the coupling reagent (*25*) under an argon atmosphere after reduction of the peptide with sodium borohydride (*26*), and the resultant conjugate was used to immunize Japanese white rabbits. Approximately 260 μg of the conjugate in Freund's complete adjuvant was injected intradermally, followed 3 weeks later by 260 μg of the conjugate in Freund's incomplete adjuvant. Beginning 10 days later, two intravenous boosters of 130 μg each of the conjugate in buffered saline were given at 10-day intervals, and the antiserum was harvested 1 week after the final injection. The antibody was purified by affinity chromatography on peptide-coupled Cellulofine, which was prepared by coupling 1.53 mg of the peptide to 5 ml of FMP-activated Cellulofine (Seikagaku). Approximately 9.1 mg of the antibody was purified from 20 ml of the antiserum.

Preparation of Crude Tissue Extracts—Tissues obtained from 10-week-old female Wistar rats, and testis from male rats were homogenized with a Potter-Elvehjem homogenizer in 3 volumes of 20 mM Hepes-NaOH (pH 7.5 at 4°C) containing 1 mM dithiothreitol, 0.1% Triton X-100, and 20 μg/ml each of the microbial protease inhibitors. The residues were removed by centrifugation to generate the crude extracts.

Western Blot Analysis—Western blot analysis was performed essentially as described by Winston *et al.* (*27*). Samples were subjected to SDS-PAGE on a 7.5% acrylamide gel, and then the protein bands were transferred to a polyvinylidene difluoride membrane (Fluorotrans, Pall Bio Support). The membrane was blocked with 5% nonfat milk in phosphate-buffered saline for 30 min at 24°C, and then incubated with antibodies to CaM-kinase kinase α or β in the blocking buffer at 4°C overnight, followed by incubation with 30 μg/ml goat anti-rabbit immunoglobulins (IgA+IgG+IgM) conjugated with peroxidase (Organon Teknika) at 24°C for 1 h. Positive bands were detected with diaminobenzidine tetrahydrochloride and H_2O_2 in the presence of $CoCl_2$.

Immunotitration—Immunotitration was carried out by incubation of crude extracts with various amounts of antibodies to CaM-kinase kinase α or β in a final volume of 60 μl of 10 mM Mops-NaOH (pH 7.0) containing 0.15 M NaCl, 0.05% Tween 80, 0.1 mM EDTA, 0.1 mM dithiothreitol, and 5 μg/ml each of the microbial protease inhibitors at 24°C. After incubation for 20 min, 20 μl of a 10% suspension of *Staphylococcus aureus* was added, and then the mixture was incubated for 30 min with shaking. An aliquot (5 μl) of the supernatant obtained on centrifugation for 10 min at 15,000$\times g$ was assayed for CaM-kinase kinase.

S. aureus (Cowan I strain) was cultured as described by Goding (*28*). The cells were collected and fixed with formaldehyde essentially as described by Kessler (*29*), and stored as a 10% (wet weight/volume) suspension at −80°C. Before use, the cells were washed once and suspended in 10 mM Mops-NaOH (pH 7.0) containing 0.15 M NaCl, 0.05% Tween 80, 0.1 mM EDTA, 0.1 mM dithiothreitol, and 5 μg/ml each of the microbial protease inhibitors.

Assaying of CaM-Kinase Kinase—The activity of CaM-kinase kinase was determined by measuring the phosphorylation of the PKIV peptide (*18*) in an assay mixture comprising, in a final volume of 50 μl, 50 mM Mops-NaOH (pH 7.0 at 30°C), 5 mM $Mg(CH_3COO)_2$, 0.2 mM [γ-^{32}P]-ATP (100 cpm/pmol), 0.1 mM EGTA, 1 μM calmodulin, 2 mM dithiothreitol, 0.2 mM PKIV peptide, and a suitable amount of CaM-kinase kinase, in the presence or absence of 0.2 mM $CaCl_2$. After incubation for 5 min at 30°C, a sample of 30 μl was withdrawn and the incorporation of [^{32}P]-phosphate into the PKIV peptide was determined by the phosphocellulose paper method of Roskoski (*30*). For immunotitration analysis, the activity was determined by measuring the CaM-kinase IV activity generated on incubation with recombinant CaM-kinase IV under Ca^{2+}/calmodulin-dependent protein phosphorylation conditions, as described previously (*6*). The assay mixture comprised, in a final volume of 50 μl, 50 mM Mops-NaOH (pH 7.0), 5 mM $Mg(CH_3COO)_2$, 0.2 mM [γ-^{32}P]ATP (100 cpm/pmol), 0.1 mM EGTA, 0.2 mM $CaCl_2$, 2 μM calmodulin, 2 mM dithiothreitol, 40 μM peptide-γ, 2 μg of recombinant CaM-kinase IV (expressed in *E. coli*), and a suitable amount of CaM-kinase kinase. After incubation for 5 min at 30°C, the incorporation of [^{32}P]phosphate into peptide-γ was determined as described above.

Other Analytical Procedures—SDS-PAGE was carried out according to the method of Laemmli (*31*). The concentration of calmodulin was determined spectrophotometrically using an absorption coefficient, A_{280} (1 mg/ml), of 0.21 (*32*), and a molecular weight of 16,700 (*33, 34*). Other proteins were determined by the method of Lowry *et al.* (*35*) as modified by Peterson (*36*) with bovine serum albumin as a standard.

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                                                                -129 CCGGACCGAGTCGAGCCGAGCCAAGCCGAGCCGGGGGCG
 -90 CAGAGCGCGGGAGGCGGCGGCGCGGAGCCCAGGCAGCTCTACTGCTGATGGAAGTGCTCCGGCCTGGCCGATGAAGTTGGTACACACACC
   1 ATGTCATCATGTGTCTCTAGCCAGCCCACCAGCGACCGGGCGGCCCCCCAGGATGAGCTGGGAAGCGGGGGTGTCAGCCGGGAAAGCCAG
   1 M  S  S  C  V  S  S  Q  P  T  S  D  R  A  A  P  Q  D  E  L  G  S  G  G  V  S  R  E  S  Q

  91 AAGCCCTGCGAGGCACTGCGGGGACTCTCATCCTTAAGCATCCACTTGGGCATGGAATCCTTCATCGTGGTCACCGAGTGTGAACCAGGC
  31 K  P  C  E  A  L  R  G  L  S  S  L  S  I  H  L  G  M  E  S  F  I  V  V  T  E  C  E  P  G

 181 CGGGGCGTGGACCTCAGCCTGGCCAGAGACCAACCTCTGGAGGCCGATGGCCAGGAACTCCCTCTGGATGCCTCGGAACCTGAGTCACGG
  61 R  G  V  D  L  S  L  A  R  D  Q  P  L  E  A  D  G  Q  E  L  P  L  D  A  S  E  P  E  S  R

 271 TCCCTGCTTTCTGGTGGCAAGATGTCCCTCCAGGAGCGGTCCCAGGGCGGGCCGGCGTCCAGCAGCAGCCTGGACATGAATGGACGCTGC
  91 S  L  L  S  G  G  K  M  S  L  Q  E  R  S  Q  G  G  P  A  S  S  S  S  L  D  M  N  G  R  C

 361 ATCTGCCCATCCCTGTCCTACTCACCAGCCAGCTCCCCACAGTCCTCTCCCCGGATGCCCCGGCGGCCCACAGTGGAGTCGCACCACGTC
 121 I  C  P  S  L  S  Y  S  P  A  S  S  P  Q  S  S  P  R  M  P  R  R  P  T  V  E  S  H  H  V

 451 TCCATCACGGGTTTGCAGGACTGTGTGCAGCTGAATCAGTACACGCTGAAGGATGAAATTGGAAAGGGCTCCTATGGCGTTGTCAAGCTG
 151 S  I  T  G  L  Q  D  C  V  Q  L  N  Q  Y  T  L  K  D  E  I  G  K  G  S  Y  G  V  V  K  L

 541 GCCTACAATGAAAATGACAATACTTATTATGCAATGAAAGTGCTGTCCAAAAAGAAGCTGATCCGACAGGCCGGCTTTCCACGTCGCCCC
 181 A  Y  N  E  N  D  N  T  Y  Y  A  M  K  V  L  S  K  K  K  L  I  R  Q  A  G  F  P  R  R  P

 631 CCACCTCGAGGTACTCGCCCAGCTCCAGGGGGCTGCATCCAGCCCAGGGGCCCCATCGAGCAGGTGTATCAGGAAATTGCCATCCTCAAG
 211 P  P  R  G  T  R  P  A  P  G  G  C  I  Q  P  R  G  P  I  E  Q  V  Y  Q  E  I  A  I  L  K

 721 AAGCTGGATCACCCCAACGTGGTGAAGCTGGTGGAGGTCCTGGATGACCCTAACGAGGACCATCTGTACATGGTGTTTGAACTGGTCAAC
 241 K  L  D  H  P  N  V  V  K  L  V  E  V  L  D  D  P  N  E  D  H  L  Y  M  V  F  E  L  V  N

 811 CAAGGGCCTGTGATGGAAGTTCCCACCCTCAAGCCACTGTCTGAAGACCAGGCCCGGTTCTACTTCCAAGATCTGATCAAAGGCATTGAG
 271 Q  G  P  V  M  E  V  P  T  L  K  P  L  S  E  D  Q  A  R  F  Y  F  Q  D  L  I  K  G  I  E

 901 TACTTACACTACCAGAAGATCATTCACCGGGACATCAAACCCTCCAACCTCCTAGTGGGGGAGGACGGGCACATCAAGATAGCCGACTTC
 301 Y  L  H  Y  Q  K  I  I  H  R  D  I  K  P  S  N  L  L  V  G  E  D  G  H  I  K  I  A  D  F

 991 GGCGTGAGCAATGAGTTCAAGGGCAGCGACGCCTTGCTGTCTAACACCGTGGGCACGCCTGCCTTCATGGCGCCCGAGTCGCTCTCAGAG
 331 G  V  S  N  E  F  K  G  S  D  A  L  L  S  N  T  V  G  T  P  A  F  M  A  P  E  S  L  S  E

1081 ACCCGGAAGATCTTCTCCGGAAAGGCCTTGGATGTTTGGGCCATGGGTGTGACACTATACTGCTTTGTTTTTGGCCAGTGCCCTTTCATG
 361 T  R  K  I  F  S  G  K  A  L  D  V  W  A  M  G  V  T  L  Y  C  F  V  F  G  Q  C  P  F  M

1171 GATGAACGAATCATGTGTTTGCACAGTAAGATCAAGAGTCAGGCCCTGGAGTTTCCAGACCAGCCTGACATAGCCGAAGACTTGAAAGAT
 391 D  E  R  I  M  C  L  H  S  K  I  K  S  Q  A  L  E  F  P  D  Q  P  D  I  A  E  D  L  K  D

1261 CTGATCACCCGGATGTTGGACAAGAACCCAGAGTCCAGGATTGTGGTGCCTGAAATCAAGCTGCCACCCTTGGGTCACGAGGCACGGGGCC
 421 L  I  T  R  M  L  D  K  N  P  E  S  R  I  V  V  P  E  I  K  L  P  P  W  V  T  R  H  G  A

1351 GAGCCACTGCCGTCGGAGGACGAGAACTGCACACTGGTCGAGGTGACCGAAGAGGAGGTCGAGAATTCAGTCAAACACATTCCCAGCCTG
 451 E  P  L  P  S  E  D  E  N  C  T  L  V  E  V  T  E  E  E  V  E  N  S  V  K  H  I  P  S  L

1441 GCAACTGTGATCCTAGTGAAGACCATGATTCGGAAACGGTCTTTTGGGAACCCATTTGAAGGTAGCCGGCGGGAGGAGCGTTCCTTGTCA
 481 A  T  V  I  L  V  K  T  M  I  R  K  R  S  F  G  N  P  F  E  G  S  R  R  E  E  R  S  L  S

1531 GCACCCGGAAACCTGCTCACCAAAAAACCAACCAGGGAGTGGGAGCCCTTGTCTGAGCCCAAGGAAGCAAGGCAGCGAAGACAGCCTCCG
 511 A  P  G  N  L  L  T  K  K  P  T  R  E  W  E  P  L  S  E  P  K  E  A  R  Q  R  R  Q  P  P

1621 GGGCCCAGAGCCAGCCCCTGTGGGGGAGGAGGAAGTGCTCTTGTGAAAGGTGGTCCCTGCGTGGAAAGTTGCGGGGCTCCGGCCCCTGGC
 541 G  P  R  A  S  P  C  G  G  G  G  S  A  L  V  K  G  G  P  C  V  E  S  C  G  A  P  A  P  G

1711 TCCCCGCCACGCACGCCTCCACAGCAGCCCGAGGAGGCCATGGAGCCGGAGTAGCTGCGCGGACCACCTGACCTCGCATGCCTGGCTGTG
 571 S  P  P  R  T  P  P  Q  Q  P  E  E  A  M  E  P  E  *

1801 TGGCCCTGCACGGGGCTGCTGCACCCTGCGTTTCCATAGCAGCATGTCCTACGGAAACCAAGCACGTGTGTAGAGCCTTGACTGTCATCT
1891 CTGGTTATCAGTTTTTGTTTTTGTTTTTGTTTCTTTTTGTTGTTGCTGTTGTTTTTCTTGTGGTTTTTCTTTTCTTTTTTTTTCTCTCC
1981 CTTTGTTGTCTAAGGGACAAAGAAAAAAAAAAAAAA
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Fig. 1. **Nucleotide sequence and deduced amino acid sequence of rat CaM-kinase kinase β.** Nucleotides are numbered beginning with the first nucleotide of the translational initiation codon. Amino acids are numbered beginning with the predicted amino-terminal residue. The solid underline indicates the region corresponding to the probe used for screening of the cDNA library as described under "EXPERIMENTAL PROCEDURES."

RESULTS AND DISCUSSION

Nucleotide Sequence of cDNA Encoding CaM-Kinase Kinase β—A λgt10 cDNA library constructed from rat cerebellum was screened by plaque hybridization with an oligonucleotide, which was prepared on the basis of the sequence of a PCR product obtained using two degenerate oligonucleotides corresponding to the amino acid sequences of trypsin-digested peptides of CaM-kinase kinase β purified from rat cerebellum as primers, as described under "EXPERIMENTAL PROCEDURES," and nine positive clones were isolated from about 1×10^6 plaques. Among them, two clones of 2.1 kbp contained all the nucleotide sequence of the probe, and showed the same restriction enzyme map. Figure 1 shows the nucleotide sequence and the deduced amino acid sequence. The coded protein consisted of 587 amino acids with a molecular weight of 64,445, this size being larger than that of CaM-kinase kinase α consisting of 505 amino acids with a molecular weight of 55,907 (*15*).

Figure 2 shows the deduced amino acid sequence homology between CaM-kinase kinases α and β. When properly aligned, the homologies of the nucleotide sequence for the coding region and the deduced amino acid sequence of

TABLE I. **The activities of recombinant CaM-kinase kinases β and α expressed in *E. coli* and Sf9 cells.** The CaM-kinase kinase activities of crude extracts of *E. coli* and Sf9 cells transformed with expression vectors carrying CaM-kinase kinase β or α cDNA was measured with the PKIV peptide as a substrate in the presence or absence of Ca^{2+}, as described under "EXPERIMENTAL PROCEDURES."

	Ca^{2+} (+)	Ca^{2+} (−)
	(nmol/min/mg protein)	
CaM-kinase kinase β		
Crude *E. coli* extract	0.87	0.57
Crude Sf9 extract	0.58	0.07
CaM-kinase kinase α		
Crude *E. coli* extract	8.31	1.30
Crude Sf9 extract	1.61	0.08

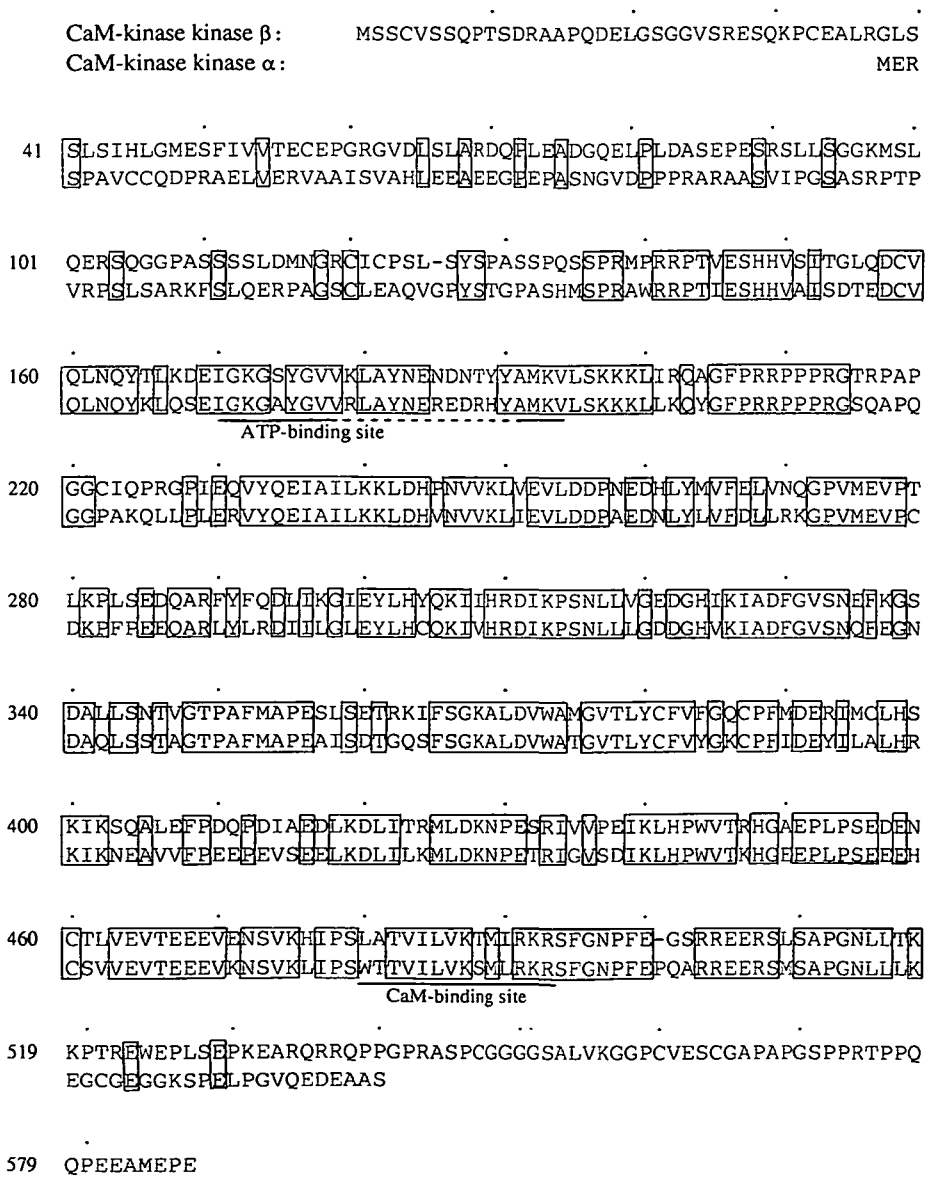

Fig. 2. **Alignment of the deduced amino acid sequences of CaM-kinase kinases α and β.** The deduced amino acid sequences of rat CaM-kinase kinases α (*15*) and β (determined in this study) are aligned for maximal homology, matching amino acids being boxed. The putative ATP-binding and calmodulin-binding sites (*14*) are indicated below the sequences.

CaM-kinase kinase β with those of the α isoform were estimated to be approximately 65 and 69%, respectively. On the basis of the amino acid alignment, the enzyme appears to consist of a highly conserved functional part (the region between amino acid residues 136 and 518 of CaM-kinase kinase β), and divergent amino-terminal and carboxyl-terminal parts.

Expression of Recombinant CaM-Kinase Kinase β in E. coli and Sf9 Cells—When cDNA for CaM-kinase kinase β was expressed in *E. coli* or Sf9 cells, as described under "EXPERIMENTAL PROCEDURES," the crude extracts showed the ability to phosphorylate the PKIV peptide, as shown in Table I, but the bacterial crude extract showed relatively high Ca^{2+}/calmodulin-independent activity, compared with the Sf9 cell extract. When cDNA for CaM-kinase kinase α was expressed, the bacterial extract also showed relatively high Ca^{2+}/calmodulin-independent activity, compared with the Sf9 cell extract, but the ratio of the Ca^{2+}/calmodulin-independent activity (the activity in the absence of Ca^{2+}) to the total activity (the activity in the presence of Ca^{2+}) was much higher when the β isoform was expressed than when the α one was expressed, suggesting that the β isoform was more susceptible to proteolytic attack than the α one, because it has been demonstrated that truncation of the carboxyl-terminal regions of CaM-kinases II (*37, 38*) and IV (*39*) by proteolysis or mutagenesis produces the Ca^{2+}/calmodulin-independent forms of the enzymes. Figure 3A shows Western blot analysis of the bacterial and Sf9 cell extracts with antibodies raised against a synthetic peptide corresponding to the carboxyl-terminal 24 amino acids of CaM-kinase kinase β. Both crude extracts gave a major immunoreactive band corresponding to an apparent molecular weight of approximately 70,000, but the intensity of the Sf9 extract band (Fig. 3A, lane 3) was significantly higher than that of the bacterial extract one (lane 4), although the quantity of enzyme activity of the bacterial extract applied to the gel (approximately 0.087 pmol/min toward the PKIV peptide) was greater than that of the Sf9 extract (0.058 pmol/min), presumably reflecting much more proteolytic degradation of the enzyme protein in the bacteria. The Sf9 extract not only gave the major band corresponding to a molecular weight of about 70,000 but also several bands with lower molecular weights, indicating that proteolytic degradation of the enzyme also occurred in Sf9 cells. The enzyme purified from rat cerebellum gave a doublet at a region corresponding to a molecular weight of about 67,000, and two weak bands, one located at a region of a molecular weight of about 70,000 and the other at one of a lower molecular weight. In contrast, the crude cerebellar extract gave two major bands with molecular weights of 70,000 and 67,000. These results suggest the possibility that most of the enzyme protein with an apparent molecular weight of 70,000 was degraded into a species with an apparent molecular weight of about 67,000 during the purification. The immunoreactive band with a molecular weight of about 94,000 observed for the cerebellar extract

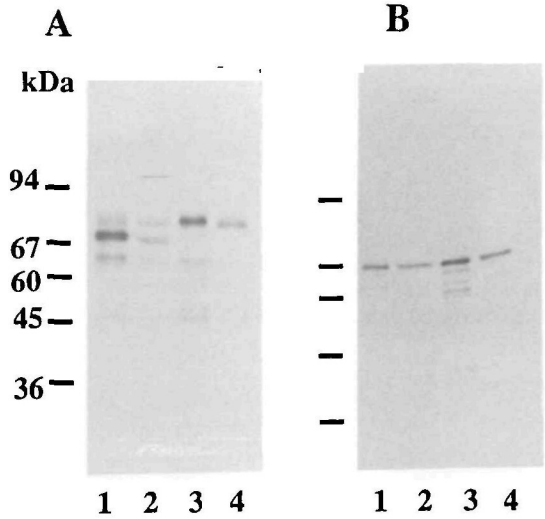

Fig. 3. **Expression of rat CaM-kinase kinases α and β in *E. coli* and Sf9 cells.** (A) Approximately 0.02 μg of CaM-kinase kinase β purified from rat cerebellum (lane 1), 20 μg protein of a rat crude cerebellar extract (lane 2), and 0.1 μg protein each of crude extracts of Sf9 cells (lane 3) and *E. coli* cells (lane 4) transformed with expression vectors carrying CaM-kinase kinase β cDNA were subjected to Western blot analysis with approximately 0.3 μg/ml antibodies against CaM-kinase kinase β. (B) Approximately 0.02 μg of CaM-kinase kinase α purified from rat cerebral cortex (lane 1), 20 μg protein of a rat crude cerebral extract (lane 2), and 0.1 μg protein each of crude extracts of Sf9 cells (lane 3) and *E. coli* cells (lane 4) transformed with expression vectors carrying CaM-kinase kinase α cDNA were subjected to Western blot analysis with approximately 0.4 μg/ml antibodies against CaM-kinase kinase α. The molecular masses in kilodaltons are given on the left. The molecular weight standards used were phosphorylase *b* (M_r 94,000), bovine serum albumin (M_r 67,000), catalase (M_r 60,000), ovalbumin (M_r 45,000), and lactate dehydrogenase (M_r 36,000).

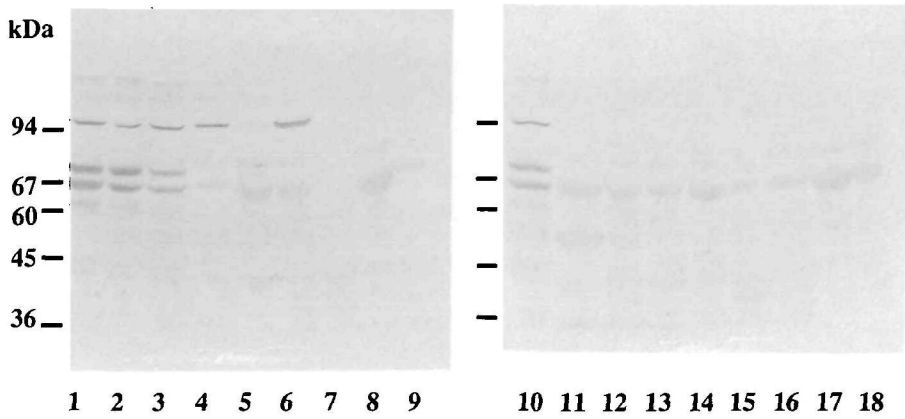

Fig. 4. **Tissue distribution of CaM-kinase kinase β on Western blot analysis.** Approximately 40 μg protein of crude extracts of rat cerebellum (lanes 1 and 10), cerebral cortex (lane 2), brain stem (lane 3), retina (lane 4), thymus (lane 5), testis (lane 6), spleen (lane 7), uterus (lane 8), skeletal muscle (lane 9), pancreas (lane 11), intestine (lane 12), stomach (lane 13), adrenal (lane 14), liver (lane 15), kidney (lane 16), lung (lane 17), and heart (lane 18) were subjected to Western blot analysis with approximately 0.9 μg/ml antibodies against CaM-kinase kinase β.

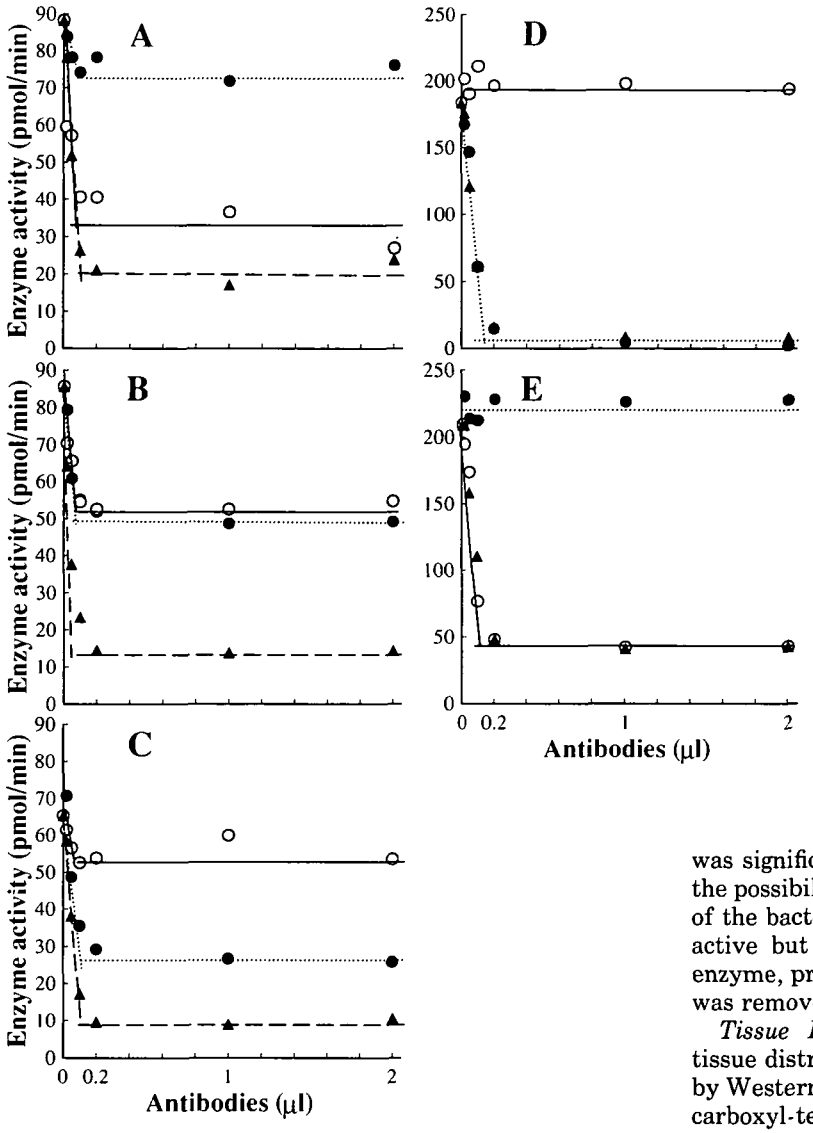

Fig. 5. **Immunotitration of crude brain extracts with antibodies against CaM-kinase kinases.** Approximately 6.8, 3.6, and 5.0 μg protein of crude extracts of rat cerebellum (A), cerebral cortex (B), and brain stem (C), and 54 and 88 ng protein of crude extracts of Sf9 cells transformed with expression vectors carrying CaM-kinase kinase α (D) and β (E) cDNAs were immunotitrated with varying amounts of 1.2 μg/ml antibodies against CaM-kinase kinase α (●), 0.9 μg/ml antibodies against CaM-kinase kinase β (○), and both antibodies (▲), as described under "EXPERIMENTAL PROCEDURES."

may be an immunologically cross-reacting material different from the enzyme. When CaM-kinase kinase α purified from rat cerebral cortex and the crude cerebral extract were examined by Western blot analysis with antibodies raised against a synthetic peptide corresponding to the carboxyl-terminal 20 amino acids of CaM-kinase kinase α, as shown in Fig. 3B, both gave a single immunoreactive band with an apparent molecular weight of 66,000, in agreement with our earlier observation (*6*). Thus, CaM-kinase kinase α, in contrast to the β isoform, does not appear to undergo significant proteolysis during its purification. On the other hand, the crude extract of Sf9 cells transformed with vectors carrying CaM-kinase kinase α gave several minor bands with lower molecular weights in addition to a major band with a molecular weight of 66,000, suggesting the occurrence of proteolytic degradation of the enzyme in Sf9 cells. The activity of CaM-kinase kinase α of the bacterial extract applied to the gel (approximately 0.83 pmol/min toward the PKIV peptide) (Fig. 3B, lane 4) was much higher than that of the Sf9 extract (0.16 pmol/min) (lane 3), but the intensity of the stained band of the former was significantly lower than that of the latter, suggesting the possibility that most of the CaM-kinase kinase activity of the bacterial extract might be due to an enzymatically active but immunologically undetectable species of the enzyme, presumably the enzyme whose carboxyl-terminal was removed through proteolysis.

Tissue Distribution of CaM-Kinase Kinase β—The tissue distribution of CaM-kinase kinase β was examined by Western blot analysis with antibodies raised against the carboxyl-terminal 24 amino acids of CaM-kinase kinase β, as shown in Fig. 4. Among the number of rat tissues examined, significant immunoreactivity was detected at positions corresponding to apparent molecular weights of about 70,000 and 67,000 in the cerebellum, cerebral cortex, and brain stem. The intensity of the immunoreactivity was almost the same in cerebellum and cerebral cortex, but relatively weaker in brain stem. The crude extract of retina, in which significant immunoreactivity with antibodies against CaM-kinase kinase α has been demonstrated (*15*), gave no significant band (Fig. 4, lane 4). An immunoreactive band corresponding to an apparent molecular weight of about 94,000 was found for retina and testis in addition to cerebellum, cerebral cortex, and brain stem. Thus, the cross-reacting material also showed an interesting tissue-specific distribution.

The previous immunotitration of a rat crude cerebellar extract with antibodies against CaM-kinase kinase α revealed the existence of the isoform, CaM-kinase kinase β (*15*), and the cDNA sequence and the deduced amino acid sequence of the β were determined in the present study. The next question of whether a third isoform, CaM-kinase kinase γ, exists in brain was now examined by immunotitration with antibodies against CaM-kinase kinases α and β, as shown in Fig. 5. When the crude extract of rat

cerebellum was immunotitrated with antibodies against CaM-kinase kinase α, only 15% of the activity was immunoprecipitated on the addition of the antibodies, in agreement with our earlier observation (*15*), but approximately 65% of the activity was precipitated with excess antibodies against CaM-kinase kinase β (Fig. 5A). Approximately 20% of the activity remained in the supernatant after precipitation with excess antibodies against the α and β isoforms, but a similar level of activity remained in the supernatant of the crude extract of Sf9 cells, in which recombinant CaM-kinase kinase β was expressed, after reaction with excess antibodies (Fig. 5E), thus indicating that a third isoform of the enzyme does not necessarily exist in rat brain. In contrast, when the extract of Sf9 cells, in which the α isoform was expressed, was immunotitrated with the anti-α antibodies, almost all the activity was abolished with an excess of the antibodies (Fig. 5D). It is thus conceivable that the activity remaining in the supernatant after reaction with excess antibodies is due to CaM-kinase kinase β, whose carboxyl-terminal antigenic determinant for the antibodies used in the present study was removed through proteolysis. Approximately 40 and 60% of the activity of the crude cerebral and brain stem extracts, respectively, was immunoprecipitated with the anti-α antibodies, in agreement with our earlier observation (*15*), and 40 and 20% of the activity of the cerebral and brain stem extracts was immunoprecipitated with the anti-β antibodies (Fig. 5, B and C). The activity remaining in the supernatant of the cerebral or brain stem extract after reaction with excess antibodies was lower than that of the cerebellar extract, presumably reflecting the lower content of the β isoform.

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