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Evidence for the Existence of Ca^{2+}/Calmodulin-Dependent Protein Kinase IV Kinase Isoforms in Rat Brain[1]

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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 system and immune system, is markedly activated upon phosphorylation by the action of CaM-kinase IV kinase. Northern and Western blot analyses of CaM-kinase IV kinase showed relatively weak reactions in the rat cerebellum, where the activity of CaM-kinase IV kinase has been demonstrated to exist, indicating that CaM-kinase IV kinase isoforms distinct from the enzyme cloned from the cerebral cortex may exist in the cerebellum. When the crude extracts of rat cerebral cortex, brain stem, and cerebellum were immunotitrated with antibody against the cloned enzyme, only approximately 46, 56, and 25% of the enzyme activity of the respective extracts were immunoprecipitated. Thus, at least two distinct isoforms of CaM-kinase IV kinase appear to exist in the brain.

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

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Calmodulin-dependent protein kinase IV (CaM-kinase IV) is a Ca^{2+}-responsive multifunctional protein kinase (*1*) which plays important roles in the functioning of Ca^{2+} in the brain, along with another Ca^{2+}-responsive multifunctional protein kinase, CaM-kinase II (see Refs. *2-4* for reviews). The recent discovery of CaM-kinase IV kinase in the brain (*5*) suggested the existence of a Ca^{2+}/calmodulin-dependent protein kinase kinase cascade which is involved in the activation of CaM-kinase IV in the brain. More recently, CaM-kinase I, a Ca^{2+}-responsive multifunctional protein kinase that is more widely distributed than CaM-kinase II and IV (see Ref. *6* for review), has been reported to be activated upon phosphorylation by CaM-kinase I kinase (*7, 8*). The fact that both CaM-kinase IV kinase and CaM-kinase I kinase can activate both CaM-kinase IV and CaM-kinase I (*9, 10*) suggests that CaM-kinase IV kinase and CaM-kinase I kinase may be the same enzyme. On the other hand, the molecular weight (66,000) of CaM-kinase IV kinase purified from rat brain estimated by SDS-polyacrylamide gel electrophoresis (*11*) is significantly higher than that of CaM-kinase I kinase (52,000) purified from pig brain (*8*), indicating that the two kinases are distinct enzymes. In the present study, the existence of at least two distinct isoforms of CaM-kinase IV kinase in rat brain is demonstrated by immunoblot and immunotitration analyses.

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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) was from Amersham International. Phosphocellulose paper (P81) was from Whatman. Microbial protease inhibitors (pepstatin A, leupeptin, antipain, and chymostatin) were from the Peptide Institute (Osaka). Peptide-γ (KSDGGVKKRKSSSS) (*1*) was synthesized by use of a Shimadzu PSSM-8 automated peptide synthesizer. Syntide-2 (PLARTLSVAGLPGKK) (*12*) was synthesized by the American Peptide Company. TPCK-trypsin was from Worthington Biochemical. 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 (*13*), essentially as described by Gopalakrishna and Anderson (*14*). The cDNA encoding chicken brain calmodulin was kindly donated by A.R. Means (*15*). Recombinant rat brain CaM-kinase IV expressed in *E. coli* was partially purified as described previously (*5*). CaM-kinase IV kinase was purified from rat cerebral cortex as described previously (*11*).

Cloning and Sequencing of CaM-Kinase IV Kinase cDNA—Approximately 100 μg of purified rat brain CaM-kinase IV kinase was digested with about 4 μg of TPCK-trypsin, the digested peptides were separated on a C_{18} reverse-phase HPLC column (TSK gel ODS-80Ts, Tosoh) with a 0-100% acetonitrile gradient in 0.1% trifluoroacetic acid, and the amino acid sequences of several purified peptides were determined with an Applied Biosystems model 477A protein/peptide sequenator. Two degenerate

oligonucleotides, AT(T/C/A)GC(T/C/A/G)GA(T/C)TT-(T/C)GG(T/C/A/G)GT as a sense primer and TG(T/C/A/G)GC(A/G)TC(A/G)TT(T/C/A/G)CC(T/C)TC(A/G)AA as an antisense primer, were synthesized on the basis of the amino acid sequence of one of the tryptic peptides, IADFG-VSNQFEGNDAQLSST, and oligonucleotides were synthesized by 40 cycles of PCR using the sense and antisense primers from the first strand cDNA, which had been prepared from poly(A)$^+$ RNA obtained from rat cerebral cortex. The resulting PCR products were subcloned into pGEM-T vector (Promega) and the nucleotide sequences of the inserts of six clones were determined to be AT(C_3/A_3)-GCCGACTTTGGTGTCAGCAACCAGTT(C_4/T_2)GAGG-G(G_3/A_1/T_1/C_1)AA(C_5/T_1)GA(T_3/C_3)GC(C_4/T_2)CA. Accordingly, an oligonucleotide, CCCTCGAACTGGTTGC-TGACACCAAAGTCGGC, was synthesized for use as a probe for screening CaM-kinase IV kinase cDNA. An oligo(dT)-primed cDNA library was constructed in λgt10 using poly(A)$^+$ RNA, which was isolated from rat cerebral cortex by the use of Oligotex™-dT30 (Takara Shuzo) according to the manufacturer's manual, and approximately 6×10^6 plaques were screened with the 32-bp probe 5′ end-labeled with [γ-^{32}P]ATP. Four positive clones, of 3.3, 2.9, 2.5, and 2.0 kbp, were isolated, and their nucleotide sequences were determined by the dideoxynucleotide chain-termination method (*16*). However, even the 3.3-kbp clone contained a 3′ noncoding sequence including a poly(A) tail but did not contain a 5′ noncoding sequence and an initiation ATG. During the course of this study, the nucleotide sequence for the entire rat brain enzyme-coding cDNA was reported by Tokumitsu *et al.* (*9*). To obtain a clone coding the entire CaM-kinase IV kinase protein for expression vector pET11d (*17*), two oligonucleotides were synthesized: TGAAGCCATGGAGCGCAGTCCAGCCGT-CTG, in which an *Nco*I site was introduced into the initiator methionine codon of the sequence corresponding to nucleotides −7-23 of the reported sequence (*9*), as a sense primer, and AGGCAGCTCTGGCCCGGGGT, corresponding to nucleotides 135-154, as an antisense primer; and an oligonucleotide was synthesized by 40 cycles of PCR using the sense and antisense primers from the first strand cDNA prepared from rat cerebral cortex poly(A)$^+$ RNA. The 161-bp PCR product was cloned into pGEM-T vector and the nucleotide sequence was determined.

Expression of CaM-Kinase IV Kinase cDNA in E. coli—To create a *Bam*HI site for introduction into an expression vector, pET11d, the 3.3-kbp clone was digested with *Eco*RI and *Sph*I followed by T4 DNA polymerase, and *Bam*HI linkers were ligated to the resultant blunt-ended fragment. The *Xma*I-*Bam*HI fragment of the processed 3.3-kbp clone, the *Nco*I-*Xma*I fragment of the 161-bp PCR product amplified in pGEM-T vector, and *Nco*I-*Bam*HI-digested pET11d were ligated to generate pETCaMKKα. This construct was transformed into *E. coli* strain BL21 (DE3) (*17*). 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 4 h, the bacteria were harvested by centrifugation, suspended in 6 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, antipain A, and chymostatin), and then disrupted by sonic oscillation. The residue was removed by centrifugation to generate the crude extract.

Production and Purification of Antibody against CaM-Kinase IV Kinase—Approximately 1.15 mg of a peptide, CGEGGKSPELPGVQEDEAAS, corresponding to the carboxyl-terminal 20 amino acids of CaM-kinase IV kinase, was conjugated to 1.6 mg of keyhole limpet hemocyanin (Sigma) using *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (Pierce) as the coupling reagent (*18*) under an argon atmosphere after reduction of the peptide by sodium borohydride (*19*), and the conjugate was used to immunize Japanese white rabbits. Approximately 275 μg of the conjugate in Freund's complete adjuvant was injected intradermally, followed 4 weeks later by 275 μg of the conjugate in Freund's incomplete adjuvant. Beginning 10 days later, three intravenous boosters of 140 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 the peptide-coupled Cellulofine which was prepared by coupling 1.67 mg of the peptide to 5 ml of FMP-activated Cellulofine (Seikagaku). Approximately 7.5 mg of the antibody was purified from 20 ml of the antiserum.

Preparation of Crude Tissue Extracts—The 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.

Northern Blot Analysis—Northern blot analysis was carried out essentially as described by Sambrook *et al.* (*20*). Poly(A)$^+$ RNA was denatured with 1 M glyoxal and 50% dimethyl sulfoxide for 1 h at 50°C, electrophoresed on a 1% agarose gel, then transferred to a nylon membrane filter (NYTRAN NY13N, Schleicher & Schuell). The *Pvu*II-*Pvu*II fragment of the 3.3-kbp clone, corresponding to nucleotides 919-1329 (Fig. 1), was radiolabeled by primer extension with random primers (hexanucleotide, Boehringer Mannheim), and the labeled *Pvu*II fragment (about 4.6×10^7 cpm/65 ng) was used as a probe for hybridization. Hybridization was performed in a solution consisting of 5×SSC, 50 mM sodium phosphate (pH 7.0), 5× Denhardt's reagent, 0.1% SDS, 250 μg/ml salmon sperm DNA, and 50% formamide for 24 h at 45°C.

Western Blot Analysis—Western blot analysis was performed essentially as described by Winston *et al.* (*21*). Crude tissue extracts were subjected to SDS-PAGE on 7.5% acrylamide gel, then the protein bands were transferred onto 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, then incubated with 0.358 μg/ml antibody to CaM-kinase IV kinase in the blocking buffer for 2 h, followed by incubation with 30 μg/ml goat anti-rabbit immunoglobulins (IgA+IgG+IgM) conjugated with peroxidase (Organon Teknika) at 4°C overnight. The 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

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                                                                  -106 AAGCGGGCAGGGCGGG
 -90 GCGCGCCCAGCGGGAGCGAGCCCGGCGCCCACCGCAGAGACTGTCGCCTGGTGCCTAGGTTCCCAACAGGAATATCCACGGACTGAAGTG
   1 ATGGAGCGCAGTCCAGCCGTCTGCTGCCAAGACCCTCGAGCGGAACTGGTAGAGCGGGTGGCAGCCATCAGTGTGGCCCACCTAGAAGAG
   1 M  E  R  S  P  A  V  C  C  Q  D  P  R  A  E  L  V  E  R  V  A  A  I  S  V  A  H  L  E  E

  91 GCAGAGGAGGGCCCAGAGCCTGCCAGCAATGGTGTGGACCCCCCACCCCGGGCCAGAGCTGCCTCTGTGATCCCTGGCAGTGCTTCAAGA
  31 A  E  E  G  P  E  P  A  S  N  G  V  D  P  P  P  R  A  R  A  A  S  V  I  P  G  S  A  S  R

 181 CCCACACCAGTGCGTCCCAGCCTCTCTGCTAGAAAGTTCTCCCTGCAGGAAAGACCAGCGGGAAGCTGTCTAGAGGCTCAGGTTGGGCCT
  61 P  T  P  V  R  P  S  L  S  A  R  K  F  S  L  Q  E  R  P  A  G  S  C  L  E  A  Q  V  G  P

 271 TACTCTACAGGACCTGCCAGTCACATGTCTCCTCGGGCTTGGCGGAGACCCACCATCGAGTCCCACCATGTGGCCATCTCAGACACAGAG
  91 Y  S  T  G  P  A  S  H  M  S  P  R  A  W  R  R  P  T  I  E  S  H  H  V  A  I  S  D  T  E

 361 GACTGTGTGCAACTGAACCAGTACAAGCTGCAGAGTGAGATTGGCAAGGGTGCCTATGGTGTGGTGAGGCTGGCCTACAATGAAAGGGAA
 121 D  C  V  Q  L  N  Q  Y  K  L  Q  S  E  I  G  K  G  A  Y  G  V  V  R  L  A  Y  N  E  R  E

 451 GACAGACACTATGCAATGAAAGTTCTTTCCAAAAAGAAGTTACTGAAGCAGTATGGCTTTCCTCGCCGTCCTCCCCCGAGAGGGTCCCAA
 151 D  R  H  Y  A  M  K  V  L  S  K  K  K  L  L  K  Q  Y  G  F  P  R  R  P  P  P  R  G  S  Q

 541 GCTCCTCAGGGAGGGCCAGCCAAACAGCTGCTGCCCCTGGAGCGTGTATACCAGGAGATTGCCATTTTAAAGAAACTGGACCACGTGAAT
 181 A  P  Q  G  G  P  A  K  Q  L  L  P  L  E  R  V  Y  Q  E  I  A  I  L  K  K  L  D  H  V  N

 631 GTAGTCAAGTTGATCGAGGTCCTGGATGATCCTGCTGAAGACAATCTCTATTTGGTGTTTGACCTCCTGAGAAAGGGACCAGTCATGGAA
 211 V  V  K  L  I  E  V  L  D  D  P  A  E  D  N  L  Y  L  V  F  D  L  L  R  K  G  P  V  M  E

 711 GTGCCCTGCGACAAGCCCTTCCCAGAGGAGCAAGCTCGCCTCTACCTCCGGGACATCATCCTGGGCCTCGAGTACTTGCACTGCCAGAAG
 241 V  P  C  D  K  P  F  P  E  E  Q  A  R  L  Y  L  R  D  I  I  L  G  L  E  Y  L  H  C  Q  K

 811 ATTGTGCACAGGGACATCAAGCCGTCCAATCTGCTCCTTGGGGACGATGGGCACGTGAAGATCGCCGACTTTGGTGTCAGCAACCAGTTT
 271 I  V  H  R  D  I  K  P  S  N  L  L  L  G  D  D  G  H  V  K  I  A  D  F  G  V  S  N  Q  F

 901 GAGGGGAATGATGCTCAGCTGTCCAGTACGGCAGGGACCCCAGCATTCATGGCCCCGGAGGCCATTTCTGACACCGGCCAGAGCTTCAGT
 301 E  G  N  D  A  Q  L  S  S  T  A  G  T  P  A  F  M  A  P  E  A  I  S  D  T  G  Q  S  F  S

 991 GGGAAGGCCTTGGATGTATGGGCCACTGGGGTTACACTGTATTGTTTTGTCTACGGGAAGTGCCCATTCATTGATGAGTACATCCTGGCC
 331 G  K  A  L  D  V  W  A  T  G  V  T  L  Y  C  F  V  Y  G  K  C  P  F  I  D  E  Y  I  L  A

1081 CTGCACAGAAAGATCAAGAATGAGGCCGTGGTGTTCCCTGAGGAGCCGGAGGTCAGCGAGGAACTCAAAGACCTGATCCTGAAGATGCTA
 361 L  H  R  K  I  K  N  E  A  V  V  F  P  E  E  P  E  V  S  E  E  L  K  D  L  I  L  K  M  L

1171 GACAAGAATCCTGAAACAAGAATTGGGGTGTCTGATATCAAGTTACACCCTTGGGTGACCAAGCATGGAGAGGAGCCCCTCCCTTCAGAG
 391 D  K  N  P  E  T  R  I  G  V  S  D  I  K  L  H  P  W  V  T  K  H  G  E  E  P  L  P  S  E

1261 GAGGAACACTGCAGTGTGGTAGAGGTGACTGAGGAGGAGGTGAAGAACTCAGTCAAGCTCATCCCCAGCTGGACCACTGTGATCCTGGTC
 421 E  E  H  C  S  V  V  E  V  T  E  E  E  V  K  N  S  V  K  L  I  P  S  W  T  T  V  I  L  V

1351 AAGTCTATGCTGAGAAAGCGTTCCTTTGGAAACCCATTTGAGCCCCAAGCACGCAGGGAAGAAAGATCTATGTCTGCGCCAGGAAACTTA
 451 K  S  M  L  R  K  R  S  F  G  N  P  F  E  P  Q  A  R  R  E  E  R  S  M  S  A  P  G  N  L

1441 CTGTTGAAAGAAGGATGTGGAGAAGGGGGCAAAAGCCCAGAGCTTCCCGGAGTCCAGGAAGATGAGGCTGCATCCTGAGTCCCTGCATGT
 481 L  L  K  E  G  C  G  E  G  G  K  S  P  E  L  P  G  V  Q  E  D  E  A  A  S  *

1531 GCCCAGGGCCATCGGCAGCATGCTCATTCCGCTCCTCCAGAGGCCCACCGCCCTCATGCAATCATCGCCCCTGCAGGCAGGGGGCTGGGG
1621 ACTGCAGCCCCTCTCCTGGCCCCCCTCACCCATTGTGCTGCATGACCTGCGCACAGGCATATCCAGGGACAGGATTGGAATGTGTATCAT
1711 TTGGGGCTCGGGGGTCCCGGTTGGTTCTCTCTGTTCTGATCTATCTTGTGGGGAATATTAGAAACCCTACCTGACTGGCTGGTGAGGCCC
1801 AGGGCAGAAGGCAAGAGACCAAAATAGCAGGTGGAGACCTGGTGGGCTGCTTCTCAGAGAACTCCCATAGTGATCGGTGGCCTGCCCAGC
1891 TGCTGCACACGAGGAGTGATTGTGTCACTTCTGTGTGGAAGGTGCTGAGTGCTGGAGTCCTCAGATCTCTAGTGTGGATGCTACAAGTAA
1981 TGAGGCGTGCCTGGGAGAAGACTGATCTTGGAGAATCCAGATTCCTCTCATTGTCTTTTACCTGAGGGCTTGGGTGGGAGCGGCTGTTAT
2071 GGAGAGCATCTTCTGGCTTTGGGTAGTCCGTGGTACACTGTACGGCAGTCACGAGGATCTGACATTCATAGACTGTGTTTTACACTTGGC
2161 ACTCGCTGGAGAAAGGACCAAAGTGAATCCAGCTTAGAGCATAGACCTGAGCCTGGAGCAGTGTACTTCTCCTGCCAACCTCCCTTCCTC
2251 TCTCACGGATCTCCAGAATGAAGGTTACAGAACACCAGGAGTGACAAGGAGGGCCACGGACTCTCAGCACTGTGGCCCTGCAGATGTGGA
2341 AGTAACTGGGATCTAGGCGCTGAGCCGACACTGGCTTACTCACTACATGCTCTGATGGAGGAGGCTCAGCCAGATGAGGGACCGTACAAG
2431 ACGCACTCCTGTCAGGCTGGTGGCCCTTCTGTCAGTTGCTTCAGTACAGGACATCAGACAGTCAGCCCACACAGGTCAGAGCTGCACAGT
2521 TGGGACACACGTGGACACTGCCCTTTCAGAACAGATGTCAGCTCTCTGGATTCTGTGGTCGTCTGGCTATTCTAATGTTTACTGATAATG
2611 CTCATAGGGATTGGTCCCCAAAGGGAGATGAGCAGGACTCAGGTAGCCCTCTCTGAAAGCCTCCCATAAGAGATCCTGGCTGCCCACATG
2701 GGTTTCAAGAATGCAGGAGGGCTAGGCTAGAAAAGAGAGAGGCTGTGCGGGAGCGCTGGTTCCCACTCTTATCTCTGTGCCCATGGGTGT
2791 ACATTGCTCCTTCATGTGCACATGCGTGTGTGTGCCCATCTGCTGTATACAGCACACTTGCATGTCTCATACTGGCACATGCATCTGTAA
2881 TATAGTTTCTATGTCAACTTATGCCTAGGAGCAGAAGGTAGCCCATTGTCAAAGGGCTCCCATTTCAACCCAACTCCTCTGCACTAAGGC
2971 AGCCAGTGTAACCCAGGGCTTGAAAACAAATAATCTAATCCTATCTCTAAGAATACTTTTATAGAGTGAATAGGAGGCAAGTTATGCCAA
3061 CCAATAAATAGCAAAGAGTCAGGGAGCTTCACCGATGGGACCCACACAGGAAAAGCTCGGAGTTGGGTGGCTGGCTCATTGTCCCCAGCA
3151 GAGCCAGGAAGGCATGCTCACTGTCACCTGTTGTTGAGAGCTTTGGTTGGCTTTGGTTTTGAATGTAGCACAAGTGAACAAACTCCATAA
3241 GAGTGTTTATAAAAATTAACTTCTTAGGAAGTGAATTAAAAACAATAAAAGCCCTTTGTTGAGTTAAAAAAAAAAAAAAAAA
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Fig. 1. **Nucleotide sequence and deduced amino acid sequence of rat CaM-kinase kinase α.** 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 sequence determined by PCR as described under "EXPERIMENTAL PROCEDURES." The sequence upstream of the underlined region is quoted from the report by Tokumitsu *et al.* (9). The arrows indicate nucleotides and amino acids different from the reported sequence (9).

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antiserum to CaM-kinase IV kinase in a mixture (final volume of 70 μl) containing 10 mM Mops-NaOH (pH 7.0), 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, 70 μl of a 10% suspension of *Staphylococcus aureus* was added, and the mixture was incubated for 30 min with shaking. An aliquot (4 μl) of the supernatant obtained on centrifugation for 10 min at 15,000$\times g$ was assayed for CaM-kinase IV kinase.

S. aureus (Cowan I strain) was cultured as described by Goding (*22*). The cells were collected and fixed with formaldehyde essentially as described by Kessler (*23*), and stored as a 10% (wet weight/volume) suspension at -80°C. Before use, the cells were washed once and suspended in 10 mM Hepes-NaOH (pH 7.2) containing 10% glycerol, 0.05% Tween 80, and 0.1 mM EDTA, 0.1 mM dithiothreitol, 0.1 M NaCl, and 5 μg/ml each of the microbial protease inhibitors.

Assay of CaM-Kinase IV Kinase—The activity of CaM-kinase IV kinase was assayed by measuring the CaM-kinase IV activity generated by incubation with recombinant CaM-kinase IV under Ca^{2+}/calmodulin-dependent protein phosphorylation conditions, as described previously (*11*). The assay mixture contained, in a final volume of 50 μl, 50 mM Mops-NaOH (pH 7.0 at 30°C), 5 mM magnesium acetate, 0.2 mM [γ-^{32}P]ATP (10^5 cpm/nmol), 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, and a suitable amount of CaM-kinase IV kinase. After incubation for 5 min at 30°C, a sample of 30 μl was withdrawn and the incorporation of [^{32}P]phosphate into peptide-γ was determined by the phosphocellulose paper method of Roskoski (*24*).

Other Analytical Procedures—SDS-PAGE was carried out according to the method of Laemmli (*25*). The concentration of calmodulin was determined spectrophotometrically using an absorption coefficient, A_{280} (1 mg/ml), of 0.21 (*26*) and the molecular weight of 16,700 (*27, 28*). Other proteins were determined by the method of Lowry *et al.* (*29*) as modified by Peterson (*30*) with bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Nucleotide Sequence of cDNA Encoding CaM-Kinase IV Kinase—A λgt10 cDNA library constructed from rat cerebral cortex was screened by plaque hybridization with a 32-bp oligonucleotide, which was prepared on the basis of the sequence of a PCR product obtained using two degenerate oligonucleotides corresponding to the amino acid sequence of a trypsin-digested peptide of CaM-kinase IV kinase purified from rat cerebral cortex as primers as described under "EXPERIMENTAL PROCEDURES," and four positive clones of 3.3, 2.9, 2.5, and 2.0 kbp were isolated from about 6×10^6 plaques. Even the 3.3-kbp clone contained nucleotides 108–3323 (Fig. 1) but did not contain the region coding the amino-terminal part of the enzyme. During the course of this study, Tokumitsu *et al.* (*9*) reported the nucleotide sequence coding the entire protein. The homology of the coding sequence between our and the reported clones is higher than 99% and there are only two differences between the two, nucleotide 133 and 223, indicating the identity of the two clones. In order to obtain bacterial expression plasmid containing the entire protein-coding cDNA possessing an *Nco*I site at the initiator methionine codon, the 161-bp oligonucleotide was synthesized by PCR using a sense primer designed on the basis of the sequence of nucleotide -7–23 of the reported sequence (*9*) and an antisense primer corresponding to the sequence of nucleotide 135–154 (Fig. 1). The 161-bp oligonucleotide, the 3.3-kbp clone, and pET11d were ligated to generate the expression plasmid, pETCaMKKα, as described under "EXPERIMENTAL PROCEDURES." When *E. coli* BL21(DE3) was transformed with pETCaMKKα and grown under the inducing conditions, the crude extract from the *E. coli* gave an intense protein band corresponding to the position of CaM-kinase IV kinase purified from rat cerebral cortex upon SDS-PAGE, as shown in Fig. 2A, and showed the activity of CaM-kinase IV kinase, as shown in Fig. 2B. These results indicate that the cDNA does indeed encode CaM-kinase IV kinase.

Tissue Distribution of CaM-Kinase Kinase α—Tissue distribution of CaM-kinase IV kinase was examined by Western blot analysis with antibody raised against a synthetic peptide corresponding to the carboxyl-terminal 20 amino acids of CaM-kinase IV kinase, as shown in Fig. 3. Among a number of rat tissues tested, significant immunoreactivity was detected at a position corresponding to CaM-kinase IV kinase in cerebral cortex, brain stem, cerebellum, and retina. The intensity of the immunoreactivity was highest in cerebral cortex and brain stem, and those in cerebellum and retina were approximately one-fifth of those in cerebral cortex and brain stem. These results, together with our previous observation that the ratio of the activity of CaM-kinase IV kinase in rat cerebral cortex, brain stem, and cerebellum is approximately 1.0 : 0.69 : 0.55 (*5*), raised the possibility that most of the CaM-kinase IV kinase activity in cerebellum and some of

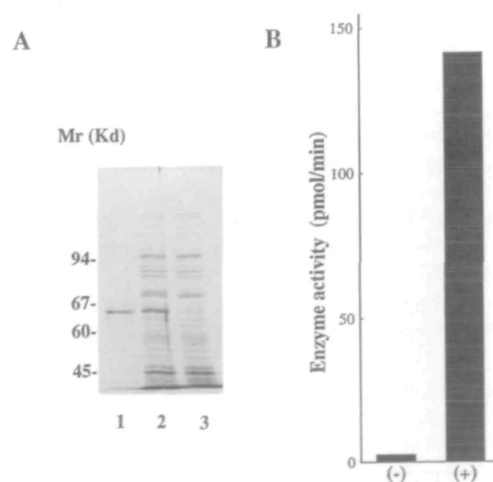

Fig. 2. **Expression of rat CaM-kinase kinase α in *E. coli*.** (A) Approximately 0.5 μg of CaM-kinase IV kinase purified from rat cerebral cortex (lane 1), and 0.3-μl aliquots of the crude extracts of bacteria transformed with pETCaMKKα (lane 2) and pET11d (lane 3) were subjected to SDS-PAGE on 7.5% gels and stained with Coomassie Brilliant Blue. (B) The CaM-kinase IV kinase activity of the crude extracts (0.001 μl) of the bacteria transformed with pETCaMKKα was measured in the standard assay (+). A control was run with the crude extracts of the transformed bacteria omitted (−).

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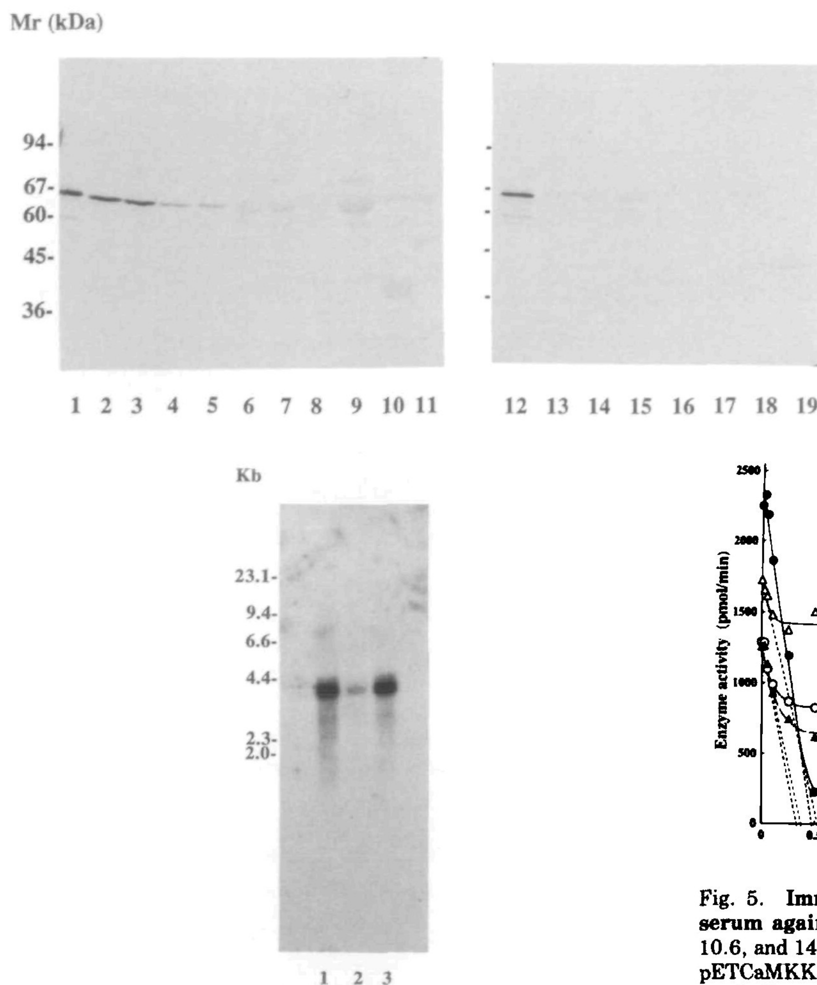

Fig. 3. **Western blot analysis.** Approximately 20 ng of CaM-kinase IV kinase purified from rat cerebral cortex (lanes 1 and 12) and 20 μg protein of the crude extracts of rat cerebral cortex (lane 2), brain stem (lane 3), cerebellum (lane 4), retina (lane 5), thymus (lane 6), testis (lane 7), spleen (lane 8), uterus (lane 9), skeletal muscle (lane 10), pancreas (lane 11), intestine (lane 13), stomach (lane 14), adrenal (lane 15), liver (lane 16), kidney (lane 17), lung (lane 18), and heart (lane 19) were subjected to Western blot analysis as described under "EXPERIMENTAL PROCEDURES." Molecular masses in kilodaltons are given on the left.

Fig. 4. **Northern blot analysis.** Approximately 5 μg of poly(A)$^+$ RNAs from rat cerebral cortex (lane 1), cerebellum (lane 2), and brainstem (lane 3) were subjected to Northern blot analysis using the *Pvu*II-*Pvu*II fragment (nucleotides 919-1329) as a probe as described under "EXPERIMENTAL PROCEDURES." The result was visualized by autoradiography. RNA sizes in kilobases are given on the left.

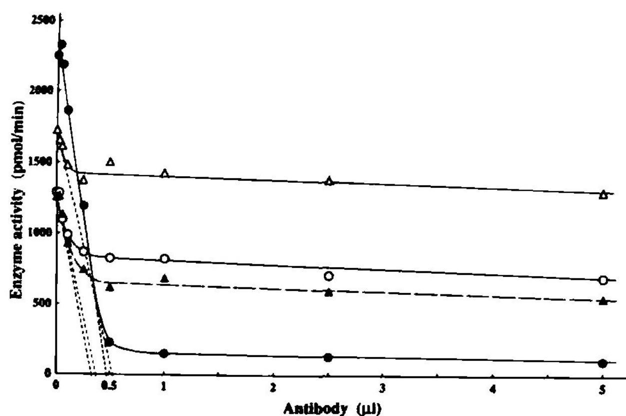

Fig. 5. **Immunotitrations of crude brain extracts with antiserum against CaM-kinase kinase α.** Approximately 0.45, 7.1, 10.6, and 14.0 μg of the crude extracts of bacteria transformed with pETCaMKKα (●), rat cerebral cortex ( ), brain stem (▲), and cerebellum (△), respectively, were immunotitrated with varying amounts of the antiserum against CaM-kinase kinase α as described under "EXPERIMENTAL PROCEDURES."

the activity in cerebral cortex do not arise from the protein cloned in this study. When poly(A)$^+$ RNAs isolated from rat cerebral cortex, brain stem, and cerebellum were subjected to Northern blot analysis, as shown in Fig. 4, a mRNA species of about 3.6 kb was clearly detected in cerebral cortex and brain stem, being more abundant in brain stem than in cerebral cortex, but it was only slightly detected in cerebellum, also indicating the existence of CaM-kinase IV kinase isozyme(s), which is distinct from the enzyme cloned in this study, in cerebellum and cerebral cortex. Further strong support for this contention was provided by immunotitration of the crude brain extracts with antiserum to CaM-kinase IV kinase, as shown in Fig. 5. When the crude extracts of rat cerebral cortex, brain stem, cerebellum, and the bacteria transformed with pETCaMKKα were immunotitrated with antiserum to the cloned enzyme, almost all the activity of the recombinant enzyme was immunoprecipitated but only approximately 46, 56, and 25% of the activity of cerebral cortex, brain stem, and cerebellum, respectively, were immunoprecipitated by the addition of an excess of the antiserum. Thus, the cloned CaM-kinase IV kinase accounted for only one-fourth of total CaM-kinase IV kinase activity in cerebellum, where CaM-kinase IV exists most abundantly, suggesting that most of the activity is attributable to other CaM-kinase kinase. Accordingly, we propose to designate the cloned enzyme as "calmodulin-dependent protein kinase kinase α (CaM-kinase kinase α)" to distinguish it from the other isoforms. The enzyme activity/antibody ratios at the equivalence point were estimated to be 3.5, 3.8, 3.3, and 4.8 nmol/min/μl for the crude extracts of cerebral cortex, brain stem, cerebellum, and the transformed bacteria, respectively (Fig. 5), indicating that the activity of the recombinant enzyme expressed in bacteria was as high as that of the brain enzyme. These results suggest that CaM-kinase kinase α does not require the upstream protein kinase for activity.

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