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

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Calmodulin-dependent protein kinase (CaM-kinase) phosphatase dephosphorylates and concomitantly deactivates CaM-kinase II activated upon autophosphorylation, and CaM-kinases IV and I activated upon phosphorylation by CaM-kinase kinase [Ishida, I., Okuno, S., Kitani, T., Kameshita, I., and Fujisawa, H. (1998) *Biochem. Biophys. Res. Commun.* 253, 159-163], suggesting that CaM-kinase phosphatase plays important roles in the function of Ca^{2+} in the cell, because the three multifunctional CaM-kinases (CaM-kinases I, II, and IV) are thought to be the key enzymes in the Ca^{2+}-signaling system. In the present study, cDNA for CaM-kinase phosphatase was cloned from a rat brain cDNA library. The coded protein consisted of 450 amino acids with a molecular weight of 49,165. Western blot analysis showed the ubiquitous tissue distribution of CaM-kinase phosphatase. Immunocytochemical analysis revealed that CaM-kinase phosphatase is evenly distributed outside the nucleus in a cell.

Key words: Ca^{2+}/calmodulin-dependent protein kinase, CaM-kinase phosphatase, cDNA sequence, protein kinase phosphatase, protein phosphatase.

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 hence regulation of their activities is very important. Among the three CaM-kinases, CaM-kinase II is activated through phosphorylation by itself (*5-8*), and the other two, CaM-kinases IV (*9-11*) and I (*12, 13*), are activated through phosphorylation by upstream Ca^{2+}/calmodulin-dependent protein kinases. Recently, we found a novel protein phosphatase that deactivates CaM-kinase II activated upon autophosphorylation in rat brain (*14, 15*), and furthermore found that this protein phosphatase can also deactivate CaM-kinases IV and I activated upon phosphorylation by CaM-kinase kinase (*16*), suggesting that the protein phosphatase (CaM-kinase phosphatase) plays important roles in regulating the activities of the three multifunctional CaM-kinases. In the present study, CaM-kinase phosphatase was cloned from a rat brain cDNA library and the nucleotide sequence encoding the entire protein was determined.

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Abbreviations: CaM-kinase, calmodulin-dependent protein kinase; *E. coli*, *Escherichia coli*; HPLC, high performance liquid chromatography; PCR, polymerase chain reaction; TPCK-trypsin, tosylphenylalanyl chloromethyl ketone-treated trypsin; poly(Lys), poly-L-lysine.

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EXPERIMENTAL PROCEDURES

Materials—[α-^{32}P]dCTP (3,000 Ci/mmol), [γ-^{32}P]ATP (5,000 Ci/mmol), and a Thermo Sequenase™ fluorescent labelled primer cycle sequencing kit containing 7-deaza-dGTP were obtained from Amersham Pharmacia Biotech. Microbial protease inhibitors (pepstatin A, leupeptin, antipain, and chymostatin) were from the Peptide Institute (Osaka). TPCK-trypsin was from Cooper Biomedicals. Restriction enzymes and other DNA modifying enzymes were purchased from Takara Shuzo, Toyobo, or New England Biolabs. The λZAPII undigested vector kit was from Stratagene. The pGEM-T vector kit and Gene-Editor™ *in vitro* site-directed mutagenesis system were from Promega. The pET11a and pET11d vectors were from Novagen. ATP and poly(Lys) (average molecular weight, 87,000) were from Sigma. Dulbecco's modified Eagle medium, fetal bovine serum, and horse serum were from Gibco BRL. PC-12 cells (RCB0009) were from the Riken Gene Bank. Cover-slips coated with type I collagen were from Iwaki (Japan). Other reagents were of the highest grade commercially available. Wistar rats were purchased from the Shizuoka Laboratory Animal Center.

Protein Preparations—CaM-kinase phosphatase was purified from rat brain as described previously (*15*). Recombinant rat CaM-kinase IV (K71R), in which Lys^{71} (ATP-binding site) was replaced with Arg, expressed in Sf9 cells was purified as described previously (*17*). Recombinant CaM-kinase kinase α expressed in *Escherichia coli* (*18*) was purified as described previously (*17*). Calmodulin

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was purified from *E. coli* transformed with expression vector pET11d carrying cDNA encoding chicken brain calmodulin (*19*). Crude tissue extracts were prepared by homogenization of tissues obtained from 10-week-old female Wistar rats and testes from male rats 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 (pepstatin A, leupeptin, antipain, and chymostatin), followed by centrifugation. A crude extract of PC-12 cells was prepared by homogenization of cells cultured as described previously (*20*) with a Potter-Elvehjem homogenizer in 3 volumes of 5 mM sodium phosphate buffer (pH 7.5) containing 20 μg/ml each of the microbial protease inhibitors, followed by centrifugation at 100,000 $\times g$ for 1 h and removal of low-molecular-weight materials by gel filtration on Bio-gel P-10.

Cloning and Sequencing of CaM-Kinase Phosphatase—Approximately 10 μg of purified rat brain CaM-kinase phosphatase was subjected to SDS-PAGE on a 10% acrylamide gel, and then the stained band corresponding to the position of CaM-kinase phosphatase was excised from the gel and digested in the gel matrix with TPCK-trypsin, essentially as described by Rosenfeld *et al.* (*21*). The resulting peptides were extracted and separated on a C_{18} reversed-phase HPLC column (TSK gel ODS-80TM, Tosoh) with a 0-50% acetonitrile gradient in 0.1% trifluoroacetic acid, and the amino acid sequences of several purified peptides were determined with a Hewlett Packard G1005A peptide sequenator. Two degenerate oligonucleotides, GG(A/C/T/G)GA(C/T)GT(A/C/T/G)TT(C/T)CA(A/G)AA(A/G)CC as a sense primer and ACCAT(A/C/T/G)AC(A/C/T/G)GT(A/T/G)AT(A/G)TT(A/G)TC as an antisense primer, were synthesized on the basis of the amino acid sequences of the tryptic peptides, AIGDVFQKPYVVXGEADAAXR and DRGSHDNITVMVVFLR, 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 brain stem. 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 TTATGTGTCTGGCGAGGCAGATGCAGCATCCAGAGAGCTGACAGGCTTGGAGGACTACCTGCTGCTTGCCTGTGACGGTTTCTTCGATGTCGTCCCCCACCATGAAATCCCGGGTCTTGTCCATGGCCACTTGCTCAGGCAGAAGGGCAGTGGGATGCACGTTGCTGAGGAGCTGGTGGCTGTAGCCCGTGACCGGGGTTCCCAT was used as a probe for the screening of CaM-kinase phosphatase cDNA. An oligo(dT)-primed cDNA library was constructed in λ ZAPII (Stratagene) using poly(A)$^+$ RNA, which was isolated from rat brain stem by use of Oligotex™-dT30 (super) (Takara Shuzo) according to the manufacturer's manual, approximately 7×10^5 plaques being screened with the probe. Eight positive clones of 3.5 to 4.5 kbp were isolated, and their nucleotide sequences were determined. When an MTN membrane (Clontech), on which 2 μg each of poly(A)$^+$ RNA isolated from rat heart, brain, spleen, lung, liver, skeletal muscle, kidney, and testis had been blotted, was hybridized with the ^{32}P-labeled probe used for the screening of CaM-kinase phosphatase cDNA in a solution consisting of 5 $\times$ SSC, 50 mM sodium phosphate (pH 7.0), 5 $\times$ Denhardt's reagent, 0.1% SDS, 250 μg/ml heat-denatured salmon sperm DNA, and 50% formamide for 24 h at 45°C, a mRNA species of about 4.5 kb was found in all the tissues examined, as shown in Fig. 1.

Expression of CaM-Kinase Phosphatase cDNA in E. coli—An *Nde*I restriction site was created in the start codon of the CaM-kinase phosphatase cDNA, using the GeneEditor™ *in vitro* site-directed mutagenesis system (Promega), and the cDNA fragment containing the entire coding sequence was introduced into an expression vector, pET11a (*23*), to generate plasmid pETCaMKPase. This construct was transformed into *E. coli* strain BL21 (DE3) (*23*). 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 isopropyl β-D-thiogalactoside (IPTG) was added to a final concentration of 1 mM. After 2.5 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 the microbial protease inhibitors, and then disrupted by sonic oscillation. The residue was removed by centrifugation to generate a crude extract.

Production and Purification of Antibodies against CaM-Kinase Phosphatase—Approximately 1 mg of a peptide, CSQDLSTGLSELEINTSQRS, consisting of the carboxyl-terminal 19 amino acids of CaM-kinase phosphatase with a cysteinyl residue added to the amino terminus for coupling to a carrier protein, was conjugated to 1.6 mg of keyhole limpet hemocyanin (Sigma) using *m*-maleimido-benzoyl-*N*-hydroxysuccinimide ester (Pierce) as the coupling reagent (*24*) under an argon atmosphere after reduction of the peptide with sodium borohydride (*25*), 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 incom-

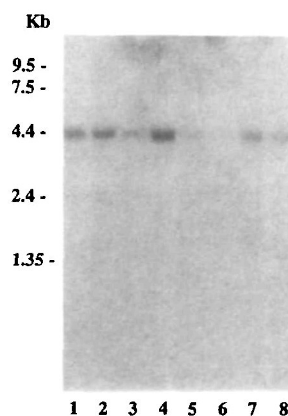

Fig. 1. **Northern blot analysis.** An MTN membrane (Clontech), on which 2 μg each of poly(A)$^+$ RNA isolated from rat heart (lane 1), brain (lane 2), spleen (lane 3), lung (lane 4), liver (lane 5), skeletal muscle (lane 6), kidney (lane 7), and testis (lane 8) had been blotted, was hybridized with a ^{32}P-labeled probe as described under "EXPERIMENTAL PROCEDURES." The result was visualized by autoradiography. RNA sizes in kilobases are given on the left.

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plete adjuvant. Beginning 10 days later, two intravenous boosters of 130 μg each of the conjugate in buffered saline were given with a 10-day interval, 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 mg of the peptide to 5 ml of FMP-activated Cellulofine (Seikagaku). Approximately 4 mg of the antibody was purified from 20 ml of the antiserum.

Western Blot Analysis—Western blot analysis was performed essentially as described by Winston *et al.* (*26*). 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 phosphatase 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 $CoCl_2$.

Assaying of CaM-Kinase Phosphatase—The activity of CaM-kinase phosphatase was determined by measuring the dephosphorylation of CaM-kinase IV (K71R), which had been phosphorylated with [γ-^{32}P]ATP and CaM-kinase kinase α essentially as described previously (*16*), by the trichloroacetic acid precipitation assay method (*15*).

Immunocytochemical Analysis of CaM-Kinase Phosphatase—PC-12 cells grown on coverslips coated with type I collagen were rinsed with phosphate-buffered saline (PBS), fixed with 4% formaldehyde for 30 min, washed three times in PBS, and then permeabilized with methanol for 5 min. After washing in PBS, the cells were incubated with 0.5% nonfat milk in PBS for 30 min, incubated overnight at 4°C with 1.7 μg/ml of antibodies against CaM-kinase phosphatase prepared as described above, washed three times in PBS, and then incubated with 6 μg/ml of fluorescein isothiocyanate (FITC)-conjugated swine anti-rabbit IgG (DAKO) in PBS containing 0.5% nonfat milk for 3 h. After washing three times in PBS, cell nuclei were visualized by staining with 0.5 μg/ml of Hoechst 33258 (Polysciences) for 30 min, followed by washing in three changes of PBS. The cells were mounted in Vectashield (Vector Laboratories), and then examined by fluorescence microscopy under a Zeiss Axioskop microscope (Carl Zeiss) equipped with a standard FITC filter set, #487909 (Carl Zeiss), and a Hoechst 33258 filter set, #487902 (Carl Zeiss), using a Zeiss 100×Plan-Neofluar lens (Carl Zeiss).

Other Analytical Procedures—SDS-PAGE was carried out according to the method of Laemmli (*27*). The concentration of calmodulin was determined spectrophotometrically using an absorption coefficient, A_{280} (1 mg/ml), of 0.21 (*28*), and a molecular weight of 16,700 (*29, 30*). Other proteins were determined by the method of Lowry *et al.* (*31*) as modified by Peterson (*32*) with bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Nucleotide Sequence of cDNA Encoding CaM-Kinase Phosphatase—A λZAPII cDNA library constructed from rat brain was screened by plaque hybridization with an oligonucleotide, which was prepared on the basis of the

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                                                                                    -93 GTG
 -90 GGGAGCATTGCGGCAGGCTGGCAGAGTAAAGACTTGACTGAAGACCATCTCACCCCAGCCTAGCTGCAAGCCCCCCGGAATGCACTGGGC
   1 ATGGCCTCTGGAGCCCCACAGAACAGCAGCCAGATGGCCTGTGACGGCGAGATCCCAGGTTTCCTGGACACACTCCTCCAAGACTTCCCA
   1  M  A  S  G  A  P  Q  N  S  S  Q  M  A  C  D  G  E  I  P  G  F  L  D  T  L  L  Q  D  F  P

  91 GCCCCACTGAGCCTGGAGAGCCCTTTGCCATGGAAGGTCCCAGGGACAGTCCTGGGCCAGGAGGAGGTGGAGGCTGAGCTGACGGAGCTG
  31  A  P  L  S  L  E  S  P  L  P  W  K  V  P  G  T  V  L  G  Q  E  E  V  E  A  E  L  T  E  L

 181 GCAATGGGTTTCCTGGGCAGCAGGAATGCACCCCCAGCAGTTGCTGCGGCTGTGACCCACGAGGCAATTTCCCAGCTGCTTCAGACAGAC
  61  A  M  G  F  L  G  S  R  N  A  P  P  A  V  A  A  A  V  T  H  E  A  I  S  Q  L  L  Q  T  D

 271 CTCTCTGAATTTAAGAGGTTGCCTGAACAAGAGGAAGAGGAGGAGGAGGAGGAAGAGAGAGTCCTCACGACCCTACTGGATGCCAAAGGC
  91  L  S  E  F  K  R  L  P  E  Q  E  E  E  E  E  E  E  E  E  R  V  L  T  T  L  L  D  A  K  G

 361 CTGTCCCGGAGCTTCTTTAACTGTCTTTGGGAAGTATGCAGCCAGTGGCAGAAACGGGTGCCACTGACTGCCCAGGCCCCTCAGCGCAAG
 121  L  S  R  S  F  F  N  C  L  W  E  V  C  S  Q  W  Q  K  R  V  P  L  T  A  Q  A  P  Q  R  K

 451 TGGCTGGTCTCTATCCATGCCATCCGGAACACACGACGAAAGATGGAGGATCGACACGTGTCCCTTCCTGCTTTCAACCACCTCTTCGGC
 151  W  L  V  S  I  H  A  I  R  N  T  R  R  K  M  E  D  R  H  V  S  L  P  A  F  N  H  L  F  G

 541 TTGTCTGACTCCGTGCACCGCGCCTACTTCGCTGTGTTTGATGGTCATGGAGGTGTGGATGCAGCACGGTATGCCTCTGTCCACGTGCAC
 181  L  S  D  S  V  H  R  A  Y  F  A  V  F  D  G  H  G  G  V  D  A  A  R  Y  A  S  V  H  V  H

 631 ACCAACGCCTCTCACCAGCCAGAGCTGCTCACAGACCCTGCAGCAGCTCTCAAAGAAGCCTTCCGGCATACTGACCAGATGTTTCTCCAG
 211  T  N  A  S  H  Q  P  E  L  L  T  D  P  A  A  A  L  K  E  A  F  R  H  T  D  Q  M  F  L  Q

 721 AAAGCCAAGCGAGAGCGACTGCAGAGCGGTACCACAGGTGTGTGTGCGCTCATCACAGGAGCAGCCCTGCATGTTGCCTGGCTTGGAGAC
 241  K  A  K  R  E  R  L  Q  S  G  T  T  G  V  C  A  L  I  T  G  A  A  L  H  V  A  W  L  G  D

 811 TCCCAGGTCATCCTGGTACAGCAAGGACAGGTAGTGAAGCTGATGGAGCCACACAAACCGGAACGACAGGATGAGAAATCACGCATTGAA
 271  S  Q  V  I  L  V  Q  Q  G  Q  V  V  K  L  M  E  P  H  K  P  E  R  Q  D  E  K  S  R  I  E

 901 GCGCTGGGCGGCTTTGTTTCTCTCATGGACTGCTGGAGAGTCAACGGGACCCTGGCAGTGTCCAGAGCCATTGGGGATGTCTTCCAGAAG
 301  A  L  G  G  F  V  S  L  M  D  C  W  R  V  N  G  T  L  A  V  S  R  A  I  G  D  V  F  Q  K

 991 CCTTATGTGTCTGGCGAGGCAGATGCAGCATCCAGAGAGCTGACAGGCTTGGAGGACTACCTGCTGCTTGCCTGTGACGGTTTCTTCGAT
 331  P  Y  V  S  G  E  A  D  A  A  S  R  E  L  T  G  L  E  D  Y  L  L  L  A  C  D  G  F  F  D

1081 GTCGTCCCCCACCATGAAATCCCGGGTCTTGTCCATGGCCACTTGCTCAGGCAGAAGGGCAGTGGGATGCACGTTGCTGAGGAGCTGGTG
 361  V  V  P  H  H  E  I  P  G  L  V  H  G  H  L  L  R  Q  K  G  S  G  M  H  V  A  E  E  L  V

1171 GCTGTAGCCCGTGACCGGGGTTCCCATGACAACATTACAGTCATGGTGGTTTTCCTTAGGGACCCCCTGGAGCTGCTGGAGGGTGGGGG
 391  A  V  A  R  D  R  G  S  H  D  N  I  T  V  M  V  V  F  L  R  D  P  L  E  L  L  E  G  G  G

1261 CAGGGGGCAGGGGGTGCCCAGGCAGATGTAGGAAGTCAGGACTTGTCCACTGGCCTCTCAGAACTTGAGATCAATACCTCACAGAGAAGC
 421  Q  G  A  G  G  A  Q  A  D  V  G  S  Q  D  L  S  T  G  L  S  E  L  E  I  N  T  S  Q  R  S

1351 TAAGTGGTCCAGGCCCCCAGGCCCCATCCCTCTGCCCTTGTGACCCTCCCCTTCAAAGACCTTAGATCCAACAGGTATAGTGGCAGGGG
 451  *
1441 TGCACACCCTCACAGCATTCCCTTAGCACCCCAGCCCTTCATGTTGCCTGCCACAGCCTGTTCTCATGGCTGCAGAACTGTAGGCACCAA
1531 TGGATCTCAAGGAAGCATAGGAAAATGACCTCACAAAAGAATAGATGGCTGAGGTGATCAGGGCAGGACAGGTCTGGGGACAGAAACCAC
1621 AGGCACCTGCTGGCAGCCTAACCAAAAGCATAGGTCAAGTCTTAGGAACCCTCACACAGACCCTGGGGTAGGATCCAGCCCAGACTGTGG
1711 CTTCTCACTGTCCCCAAGTGAGGAGGGTTTGGTCTGTACTAACACACAAGCTCACAGTCCTGCTTGACTGCTGGCTTCCCAAAGAAGTTG
1801 ACATTGGTCTTGCTGGGAGGAGCACCAGGAGAGTGGCCACTG
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Fig. 2. **Nucleotide sequence and deduced amino acid sequence of rat CaM-kinase phosphatase.** 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 the screening of the cDNA library as described under "EXPERIMENTAL PROCEDURES." The double underline indicates a protein phosphatase 2C motif (PROSITE entry no. PS01032).

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sequence of a PCR product obtained using two degenerate oligonucleotides corresponding to the amino acid sequences of trypsin-digested peptides of CaM-kinase phosphatase purified from rat brain as primers, as described under "EXPERIMENTAL PROCEDURES," eight positive clones being isolated from about 7×10^5 plaques. The isolated clones had lengths of 3.5 to 4.5 kbp and proved to be derived from the same gene, as judged on nucleotide sequence analysis. Figure 2 shows the nucleotide sequence of the predicted coding region and the deduced amino acid sequence. The coded protein consisted of 450 amino acids with a molecular weight of 49,165, and contained a protein phosphatase 2C motif (PROSITE entry no. PS01032). When a computer search for sequences homologous with that of the cloned rat CaM-kinase phosphatase was made, a protein (GENBANK accession no. D13640) predicted on the analysis of randomly sampled cDNA clones derived from human immature myeloid cell line KG-1 (*33*) was found to show high sequence homology with CaM-kinase phosphatase. It was recently reported that a 1,025,415-bp segment including the entire human immunoglobulin λ gene locus (GENBANK accession no. D86995) contains the gene for the predicted human protein (*34*). Figure 3 shows the deduced amino acid sequence homology between rat CaM-kinase phosphatase and the predicted human enzyme. When properly aligned, the homologies of the nucleotide sequence for the coding region and the deduced amino acid sequence of rat CaM-kinase phosphatase with those of the predicted human enzyme were estimated to be approximately 82 and 79%, respectively, suggesting that the predicted protein may be the human homologue of CaM-kinase phosphatase.

Expression of Recombinant CaM-Kinase Phosphatase in E. coli—When *E. coli* BL21(DE3) was transformed with pETCaMKPase and grown under inducing conditions, the crude extract gave an intense protein band at the position of CaM-kinase phosphatase purified from rat brain upon SDS-PAGE, as shown in Fig. 4A, and the crude extract showed high ability to dephosphorylate CaM-kinase IV (K71R), which had been phosphorylated by CaM-kinase kinase α on Thr[196] (*17*), in the presence of 10 μg/ml poly(Lys), as shown in Fig. 4B. The recombinant enzyme showed low dephosphorylating activity toward the phosphorylated CaM-kinase IV (K71R) even in the absence of poly(Lys) (Fig. 4B), and the activation by 10 μg/ml poly(Lys) was about 85-fold, consistent with our earlier observation that the activity of the enzyme purified from rat brain toward autophosphorylated CaM-kinase II is activated about 90-fold in the presence of 10 μg/ml poly(Lys) (*15*). These results, taken together, indicate that the cDNA

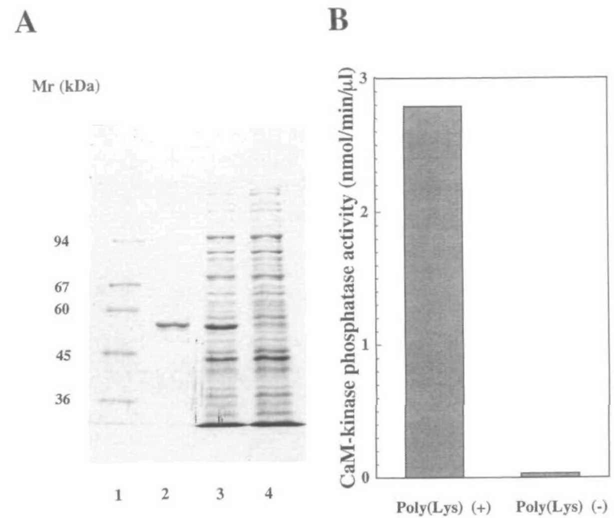

Fig. 4. **Expression of rat CaM-kinase phosphatase in *E. coli* cells.** (A) Approximately 0.5 μg of CaM-kinase phosphatase purified from rat brain as described previously (*15*) (lane 2), 0.5 μl (8.5 μg protein), and 1 μl (8 μg protein) of the crude extracts of *E. coli* cells transformed with pETCaMKPase (lane 3) and pET11a (lane 4), respectively, and marker proteins (lane 1) were subjected to SDS-PAGE on 7.5% gels and stained with Coomassie Brilliant Blue R-250. The marker proteins 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). (B) The CaM-kinase phosphatase activity of the crude extract of *E. coli* cells transformed with pETCaMKPase was measured with CaM-kinase IV (K71R) as a substrate in the presence and absence of 10 μg/ml poly(Lys) as described under "EXPERIMENTAL PROCEDURES."

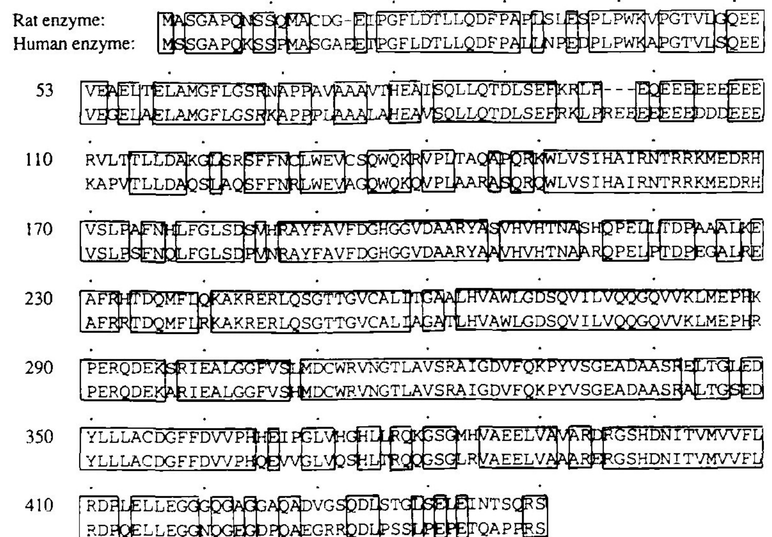

Fig. 3. **Comparison of the deduced amino acid sequence of rat CaM-kinase phosphatase with that of the human homolog predicted on analysis of randomly sampled cDNA clones.** A computer search for sequence homology with the cloned rat CaM-kinase phosphatase revealed that a protein (GENBANK accession no., D13640) predicted on analysis of randomly sampled cDNA clones derived from human immature myeloid cell line KG-1 (*33*) shows high sequence homology. The deduced amino acid sequences of the two enzymes are aligned for maximal homology, matching amino acids being boxed.

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does indeed encode CaM-kinase phosphatase.

Tissue and Subcellular Distributions of CaM-Kinase Phosphatase—The tissue distribution of CaM-kinase phosphatase was examined by Western blot analysis with antibodies raised against the carboxyl-terminal 19 amino acids of CaM-kinase phosphatase, as shown in Fig. 5. Significant immunoreactivity was detected at the position corresponding to CaM-kinase phosphatase in all the tissues examined, and the intensity was highest in the adrenal gland, and relatively high in the lung, thymus, brain, spleen, uterus, and pancreas. Among the three multifunctional CaM-kinases whose activities are regulated by CaM-kinase phosphatase (*16*), only CaM-kinase I is ubiquitous (*35*), the other two not being ubiquitous in tissue distribution. CaM-kinase IV occurs abundantly in the brain (*9, 36, 37*) and thymus (*38*), and CaM-kinase II occurs abundantly in the brain (*39*), although minor isoforms, such as the γ and δ isoforms, show much more ubiquitous tissue distributions (*40*). The targets of CaM-kinase phosphatase (*16*) are thought to be autophosphorylated Thr^{286} of CaM-kinase II, and Thr^{196} of CaM-kinase IV (*17, 41*) and Thr^{177} of CaM-kinase I (*42, 43*), which have been phosphorylated by CaM-kinase kinase. Among the two CaM-kinase kinases so far known, CaM-kinase kinase α occurs in the brain and retina (*18*), and CaM-kinase kinase β only occurs in the brain (*44*), but both were not detected in the other tissues examined, such as the thymus, testis, spleen, uterus, skeletal muscle, pancreas, intestine, stomach, adrenal gland, liver, kidney, lung, and heart, on immunoblot analysis (*18, 44*). Thus, the tissue distribution of CaM-kinase phosphatase did not agree with those of the three multifunctional CaM-kinases (I, II, and IV), which are its targets, or those of CaM-kinase kinases α and β, which should act in cooperation with CaM-kinase phosphatase in regulation of the activities of CaM-kinases I and IV. Since Western blot analysis revealed that CaM-kinase phos-

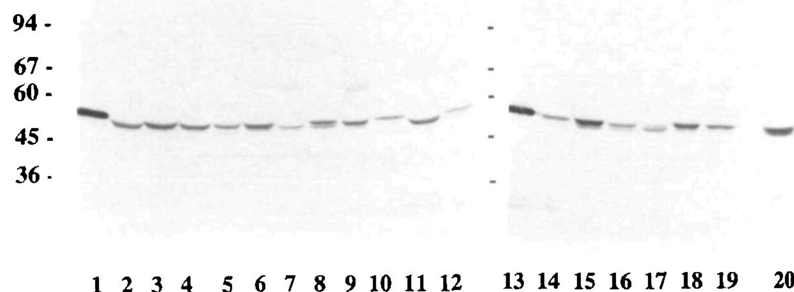

Fig. 5. **Tissue distribution of CaM-kinase phosphatase on Western blot analysis.** Approximately 0.34 μg protein of the crude extract of *E. coli* cells transformed with pETCaMKPase (lanes 1 and 13), 25 μg protein of the crude extracts of rat cerebral cortex (lane 2), brain stem (lane 3), cerebellum (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 12), stomach (lane 14), adrenal gland (lane 15), liver (lane 16), kidney (lane 17), lung (lane 18), and heart (lane 19), and 29 μg of the crude extract of PC12 cells (lane 20), prepared as described under "EXPERIMENTAL PROCEDURES," were subjected to Western blot analysis with approximately 1.7 μg/ml antibodies against CaM-kinase phosphatase.

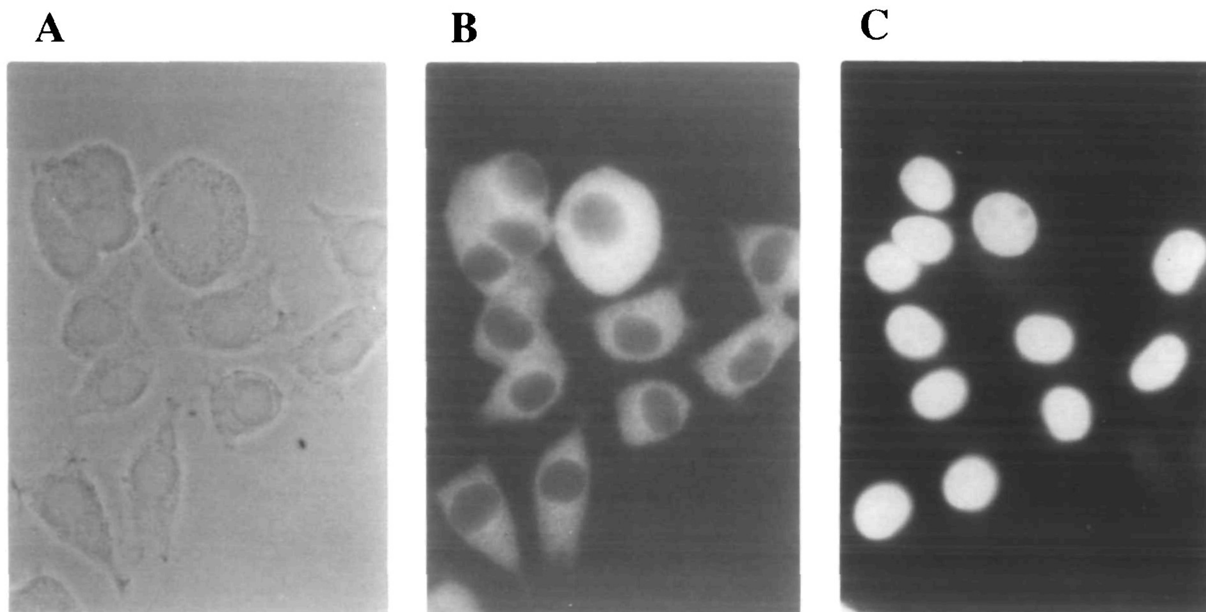

Fig. 6. **Subcellular distribution of CaM-kinase phosphatase on immunocytochemical analysis.** PC12 cells were stained by means of indirect immunofluorescence with an antibody against CaM-kinase phosphatase (B), and the cell nuclei were stained with Hoechst 33258 (C), as described under "EXPERIMENTAL PROCEDURES." A phase-contrast image of the cells is shown in panel A.

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phatase exists abundantly in PC12 cells (Fig. 5, lane 20), the subcellular distribution of the enzyme was examined by immunocytochemical analysis, as shown in Fig. 6. As shown in Fig. 6B, CaM-kinase phosphatase was evenly distributed outside the cell nuclei within PC12 cells. CaM-kinase I exists in the cytosol (*35*), while CaM-kinase IV exists in the nuclei (*45, 46*). In contrast, CaM-kinase II exists in cell membranes, such as postsynaptic density, and the cytosol (*47, 48*), the extent of the association with the postsynaptic density increasing on autophosphorylation (*49-51*). On the other hand, CaM-kinase kinase α is localized in the nuclei of neurons (*52*), while the subcellular localization of CaM-kinase kinase β is currently under investigation. Thus, the tissue and subcellular distributions of CaM-kinase phosphatase coincided with those of only CaM-kinase I, one of the targets of CaM-kinase phosphatase. However, our earlier studies suggested the existence of isoforms of CaM-kinase phosphatase (*14, 53*), and the possible subcellular translocation of CaM-kinases and CaM-kinase kinases on phosphorylation and dephosphorylation have not yet been studied. Thus, much remains to be studied for an understanding of the physiological significance of the cloned CaM-kinase phosphatase.

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