Calcium/Calmodulin-dependent Protein Kinase I

cDNA CLONING AND IDENTIFICATION OF AUTOPHOSPHORYLATION SITE*

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Marina R. Picciotto, Andrew J. Czernik, and Angus C. Nairn†

From the Laboratory of Molecular and Cellular Neuroscience, The Rockefeller University, New York, New York 10021

Ca⁺⁺/calmodulin-dependent protein kinase I (CaM kinase I) was previously purified from bovine brain (Nairn, A. C., and Greengard, P. (1987) J. Biol. Chem. 262, 7273–7281) based on its ability to phosphorylate the synaptic vesicle protein, synapsin I at site 1. The cDNA for this protein kinase has now been cloned from both a rat and a bovine brain cDNA library and the complete amino acid sequence of rat CaM kinase I determined. The rat cDNA encoded a protein of 331 amino acids with a calculated M₀ of 37,545, and the encoded kinase was expressed in bacteria as a glutathione S-transferase fusion protein. The resulting fusion protein was purified by Sepharose-CaM affinity chromatography and shown to be totally dependent on Ca⁺⁺ and CaM for activity. Furthermore, the purified kinase phosphorylates synapsin I at the same site (site 1) as the endogenous brain enzyme. CaM kinase I is homologous to other known protein kinases and contains all nine invariant amino acids conserved in the catalytic domain of this class of enzymes. CaM kinase I was most identical to CaM kinase II both in the catalytic domain and in a short region at the COOH-terminal that is predicted to be the calmodulin-binding domain. CaM kinase I appeared to be encoded by a single gene. RNase protection assays detected the mRNA encoding CaM kinase I in all tissues examined. High concentrations of the kinase mRNA were found in all regions of the brain with frontal cortex showing the greatest level. CaM kinase I was autophosphorylated in a Ca⁺⁺/CaM-dependent manner at a threonyl residue (Thr-177) which is located at a position equivalent to that of the threonyl residue (Thr-197) autoposphorylated in cAMP-dependent protein kinase.

Ca⁺⁺ is widely recognized as an essential intracellular messenger in eukaryotic systems regulating many processes such as muscle contraction, neurotransmitter release, cellular metabolism, gene expression, and cell proliferation (for reviews, see Campbell (1983) and Davis (1992)). Many effects of Ca⁺⁺ are mediated via interaction with calmodulin (CaM),¹ and

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† Present address: Institut Pasteur, Paris, France.

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1. To whom correspondence should be addressed: Laboratory of Molecular and Cellular Neuroscience, The Rockefeller University, 1230 York Ave., New York, NY 10021. Tel: 212-327-8871; Fax: 212-327-7888.

The abbreviations used are: CaM, calmodulin; β-ME, β-mercaptoethanol; PAGE, polyacrylamide gel electrophoresis; CaM kinase, strong evidence indicates, in turn, that the effects of Ca⁺⁺/CaM are often achieved through the regulation of protein phosphorylation. A family of CaM-dependent protein kinases has been identified which is known to regulate glycogen metabolism, smooth muscle contraction, and neurotransmitter release. These include myosin light chain kinase, phosphorylase kinase, CaM kinase I, and CaM kinase II (for reviews, see Nairn et al. (1985), Blackshear et al. (1988), Nairn (1990), and Hanson and Schulman (1992)). In addition, calcineurin, a CaM-dependent protein phosphatase, has been identified (for review, see Shenolikar and Nairn (1991)). CaM kinase I was originally identified in rat brain based on its ability to phosphorylate site 1 of the synaptic vesicle-associated protein, synapsin I (Huttner and Greengard, 1979; Huttner et al., 1981). The kinase was purified to homogeneity from bovine brain and found to consist of major polypeptides of ~37 and 39 kDa and a minor polypeptide of ~42 kDa on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Nairn and Greengard, 1987). Based on its presence in brain and its ability to phosphorylate synapsin I, CaM kinase I was suggested to be involved in the regulation of neurotransmitter release. CaM kinase I activity in brain extracts was found to be primarily cytosolic and activity was detected at lower levels in several other tissues (Nairn and Greengard, 1987). When CaM kinase I was first purified, many proteins were tested as substrates in vitro, but only the members of the synapsin family, synapsins I and II, were effectively phosphorylated (Nairn and Greengard, 1987). The amino acid sequences surrounding the phosphorylation site for CaM kinase I in synapsins I and II have been identified and shown to be identical (Czernik et al., 1987; Sudhof et al., 1989). Recently, two other good substrates for CaM kinase I have been identified in vitro: CREB, the CAMP-response element-binding protein (Sheng et al., 1991), and CF-2, a portion of the R-domain of the cystic fibrosis transmembrane conductance regulator (CFTR) (Picciotto et al., 1992). The identification of these potential target substrates and the fact that CaM kinase I activity has been found in tissues other than the brain raise the possibility that CaM kinase I may be a multi-functional kinase with widespread tissue distribution and potential function. In all three substrates, CaM kinase I appears to phosphorylate the same residue that is phosphorylated by cAMP-dependent protein kinase (protein kinase A), suggesting that the consensus phosphorylation site sequences may share common determinants. However, CaM kinase I appears to phosphorylate only a subset of the proteins that are substrates for protein kinase A.

Ca⁺⁺/calmodulin-dependent protein kinase; protein kinase A, cAMP-dependent protein kinase; PCR, polymerase chain reaction; CREB, cAMP-response element binding protein; CFTR, cystic fibrosis transmembrane regulator; TPCK, tosylphenylalanyl chloromethyl ketone; HPLC, high performance liquid chromatography; bp, base pair; kb, kilobase.
In the present study, CaM kinase I has been purified from bovine brain and amino acid sequence has been obtained. A partial-length cDNA encoding CaM kinase I has been cloned from a bovine brain cDNA library, and this cDNA was used to isolate a full-length CaM kinase I cDNA from a rat brain library. The encoded kinase has been expressed in bacteria and shown to have the same properties as CaM kinase I purified from bovine brain. Finally, the level of the mRNA encoding the enzyme has been examined in several brain and body regions and found to have widespread distribution. Elucidation of the primary structure of CaM kinase I will permit further understanding of the structural, catalytic and regulatory properties of the family of CaM kinases.

**EXPERIMENTAL PROCEDURES**

**Materials**—ATP, Triton X-100, diethiothreitol, 2-mercaptoethanol (β-ME), EDTA, EGTA, Tris, Coomassie Brilliant Blue R-250, SDS, and bovine serum albumin were from Sigma. HEPES, hydrogenated Triton X-100, and phenylmethylsulfonyl fluoride were from Calbiochem. [γ-32P]ATP was from DuPont NEN. Tosylphenylalanyl chloromethyl ketone (TPCK)-trypsin and chymotrypsin were from Worthington. AspGn protease was from Boehringer Mannheim. DEAE-cellulose (DE-52) and phosphocellulose P-81 paper was from Whatman. DEAE-Sepharose and Sepharose 4B-Agarose were from Pharmacia LKB Biotechnology Inc. Bio-Gel HTP hydroxyapatite was from Bio-Rad. Trifluoroacetic acid was from Pierce Chemical Co. Acetonitrile was from Burdick-Jackson. XAR-5 X-ray film and cellulose thin-layer chromatogram sheets were from Eastman Kodak. Leupeptin, chymostatin, pepstatin, and antiapin were from Chemicon. Trasylol was from Miles Inc.

CaM kinase I was purified from bovine brain essentially as previously described (Nairn and Greengard, 1987). CaM kinase was purified from rabbit brain as described (Grand et al., 1979). Synapsin I was purified from bovine brain as described (Bähler and Greengard, 1987).

**Synthesis of Peptide**—Peptide-AMC was synthesized on the Biopolymers Facility or by Operon Technologies. The sequences of the oligonucleotides used were as follows:

- GTC TTC ATC CAG GCT
- GGT ACA AGC

**Peptide Sequencing**—Phosphorylation assays were carried out for 2–10 min at 30 °C. The standard reaction mixture contained, 50 mM HEPES (pH 7.5), 10 mM magnesium acetate, 1 mM EGTA, 5 mM diethiothreitol, 5–50 μM [γ-32P]ATP (specific activity, 2–5 × 10^6 cpm/μl) or [β-32P]ATP (specific activity, 2–5 × 10^5 cpm/μl) and 1 μg synapsin I as indicated. After 2 min of preincubation at 30 °C, reactions were initiated by addition of [γ-32P]ATP, allowed to incubate at 30 °C, and stopped by addition of either acetic acid to a final concentration of 10% or SDS-stop solution (1% SDS, 60 mM Tris-HCl (pH 6.8), 5% glycerol, 0.2 M β-ME, trace of pyromellitic acid, 0.5% SDS) and placed on ice for 2 min with 5 s added per cycle to the elongation at 72 °C. The amplified product (approximately 180 bp) was purified by electrophoresis through low melting temperature agarose (FMC) and then by phenol extraction and ethanol precipitation. The DNA was cleaved using EcoRI and BamHI, and the fragment was subcloned into the EcoRI site of pBlueScript vector and transformed into XLI blue cells. Clones containing an insert were detected by addition of 50 μl of 2% X-gal and 50 μl of 100 mM isopropyl β-D-thiogalactopyranoside to agar plates when plating. White colonies were streaked onto three identical ampicillin-containing agar plates. One plate was used for colony hybridization using agar plates when plating. White colonies were streaked onto three identical ampicillin-containing agar plates. One plate was used for colony hybridization using agar plates when plating. White colonies were streaked onto three identical ampicillin-containing agar plates. One plate was used for colony hybridization using agar plates when plating. White colonies were streaked onto three identical ampicillin-containing agar plates.

**Phosphorylated CaM Kinase I**—Approximately 150 μg of CaM kinase I was autophosphorylated for 20 min and then separated by SDS-PAGE using 11.6% polyacrylamide gels. Gels were dried without fixing, and autoradiography was performed to localize the phosphorylated protein. Excised gel pieces containing 32P-labeled CaM kinase I (37–39 kDa doublet) were rehydrated in 1 ml of water and then frozen in liquid nitrogen. Water was added to a 50 ml mixture: 5 pl of 10× buffer (0.6 M Tris-HCl (pH 7.8), 6.1 M MgCl2, 0.14 M β-ME), 12 μl of [γ-32P]ATP (120 Ci/mmol), 20 units of T4 polynucleotide kinase, and water to 50 μl. Inclusion of 1 mol of ATP/1 mol of oligonucleotide allowed almost stoichiometric incorporation of [γ-32P]ATP.

**Labeling of Oligonucleotides**—40 pmol of the oligonucleotide of interest was labeled by incubation for 30 min at 37 °C in the following mixture: 5 μl of 10× buffer (0.6 M Tris-HCl (pH 7.8), 6.1 M MgCl2, 0.14 M β-ME), 12 μl of [γ-32P]ATP (120 Ci/mmol), 20 units of T4 polynucleotide kinase, and water to 50 μl. Inclusion of 1 mol of ATP/1 mol of oligonucleotide allowed almost stoichiometric incorporation of radioactive ATP.

**Hybridization Using Oligonucleotide Probes**—Hybridization was performed using 1 μl of labeled oligonucleotide/ml of hybridization solution. The temperature of hybridization was 30 °C less than the melting temperature for each oligonucleotide. For degenerate oligo-
nucleotides, the melting temperature of the most A/T rich oligonucleotide in the mixture was calculated and hybridization was carried out at 5 °C below that temperature. Hybridization buffer contained 0.1 M Tris, pH 7.4, 1.0 M NaCl, 6 mM EDTA, 0.5% Nonidet P-40, 2 × Denhardt's solution, 0.2% SDS, 0.05% sodium pyrophosphate, 70 μg/ml tRNA, 100 μg/ml salmon sperm DNA. Washes were performed in 2 × SSC at 65 °C for 20 min and in 0.1 × SSC at 65 °C for 1 h. Dried filters were autoradiographed. DNA sequence was confirmed by sequencing of both strands of the DNA.

Screening of Brain cDNA Libraries—Rat and bovine brain λ-GT10 cDNA libraries (Stratagene) were screened. For the bovine brain library, 1.1 × 10^6 recombinants were screened on 100-mm plates. For the rat library, 3.5 × 10^6 recombinants were screened on 25 cm 2 × 3-cm L-broth/magnesium agar plates (0.5% NaCl, 0.5% yeast extract, 1% Tryptone, 10 mM magnesium sulfate) as described elsewhere (Sambo, 1989). Briefly, about 40 ml of a phage lawn was plated in 40 ml of L-broth/magnesium agar plates. Plates were incubated for 20 min at 37 °C with 2.5 ml of an overnight culture of C600 Hfl that had been concentrated 2-fold and resuspended in 10 mM magnesium sulfate. Twenty-five ml of 0.7% molten agarose in L-broth/magnesium were added, and the mixture was plated on agar plates. Plates were incubated for 8 h at 37 °C and then cooled for 1 h at 4 °C. Nitrocellulose sheets were cut to fit the plates, and duplicate lifts were taken from each plate. The nitrocellulose sheets were air dried, fixed for 2 min in the autoclave, and baked for 2 h at 80 °C in a vacuum oven. Hybridization was performed at 65 °C in 6 × SSC, 0.025% powdered milk containing 250,000 cpm of probe/ml (Sambo, 1989). Washing was performed with several changes of 1 × SSC, 0.5% SDS at 65 °C. The radioactive probe was synthesized using a random priming kit (Boehringer Mannheim). In screening the bovine library the template for random priming was the EcoRI/BamHI insert from the subcloned bovine CaM kinase I PCR product described above. The 1.4-kb clone obtained from the bovine library was sequenced and determined to be a partial length clone. It was then used to screen the rat brain cDNA library. One positive clone was obtained from the rat library and was plaque-purified. Phage DNA was isolated using the mini-prep method as previously described (Maniatis et al., 1982). Briefly, a positive plaque was eluted into 5 ml of TE buffer (10 mM Tris-HCl (pH 7.6), 1 mM EDTA), sonicated, and centrifuged at 10,000 g. The resulting supernatant was recovered and mixed with 250 pl of glutathione-agarose beads for 2 h. The supernatant was recovered and mixed with 200 μl of glutathione-agarose beads for 10 min at room temperature. Then three washes with 2 × 5 ml of phosphate-buffered saline were done. The protein was eluted from the beads with 250 μl of 5 mM glutathione. Activity and autophosphorylation of the fusion proteins were assayed as described above. Alternatively, pellets cells from larger scale preparations (1–2 liters of LB with appropriately larger amounts of overnight culture) were harvested, washed as described above (Smith and Johnson, 1988). Briefly, 25 ml of LB were incubated with 250 μl of an overnight culture of the clone of interest and allowed to grow for a further 3 h at 37 °C. Cells were then pelleted, resuspended in 0.5 ml of phosphate-buffered saline, and lysed by mild sonication and addition of Triton X-100 to a final concentration of 1%. Debris was pelleted by centrifugation for 5 min in a microcentrifuge. The supernatant was recovered and mixed with 250 μl of glutathione-agarose beads for 10 min at room temperature. Then three washes with 2 × 5 ml of phosphate-buffered saline were done. The protein was eluted from the beads with 250 μl of 5 mM glutathione. Activity and autophosphorylation of the fusion proteins were assayed as described above.
The DNA solution was dialyzed against several changes of 50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 10 mM NaCl. The sample was then treated with 100 μg/ml of RNase A for 3 h at 37 °C, extracted with phenol/chloroform, and precipitated with ethanol. Thirty pg of genomic DNA were digested with either BamHI, EcoRI, or HindIII and run on a 0.7% agarose gel. Southern blotting was performed using GeneScreen Plus by following the manufacturer’s recommendations. Hybridization was performed using standard conditions for GeneScreen Plus that are described above for Northern blotting. The probe used was the entire 1.4-kb insert of the bovine CaM kinase I cDNA. Hybridization was performed at 60 °C, and washing was performed as described for Northern blotting.

**RNA Probes for Protection Assays**—The plasmid containing the rat CaM kinase I PCR product in the pBluescript vector was linearized using EcoRI. An in vitro transcription kit (Ambion), containing T7 polymerase and using [32P]UTP, was used to synthesize radioactive antisense RNA from the plasmid. A linearized mouse actin plasmid provided in the kit was also used to transcribe radioactive actin RNA. Probes were purified away from unincorporated nucleotides using a NucTraps™ probe purification column (Stratagene).

**RNase Protection Assays**—Several rat brain regions and body tissues were used to prepare total RNA, and RNA was quantitated spectrophotometrically. RNase protection assays were performed using a kit from Ambion and following the manufacturer’s recommendations. RNA samples were analyzed using the rat CaM kinase I probe. To test for RNA integrity and to quantify the RNA precisely, parallel samples were also analyzed using the β-actin probe included with the kit. Briefly, 100,000 cpm of RNA probe were vacuum dried with 20 μg of RNA from the brain regions or 40 μg of RNA from the body regions and resuspended in 20 μl of buffer supplied by the manufacturer. Probe controls included 20 μg of yeast tRNA. Hybridization was performed overnight at 42 °C. A mixture of RNase T1 and RNase A was used at a dilution of 1:100 to digest away unhybridized probe. A control sample was left undigested to ensure probe integrity. Reactions were stopped, and protected fragments were precipitated using a solution provided by the manufacturer. Protected fragments were separated using a 6% sequencing gel (Sequagel), electrophoresed for 2 h at 2000 V, dried, and autoradiographed.

Quantitation was performed using a PhosphorImager (Molecular Dynamics).

**RESULTS**

**Amino Acid Sequencing of CaM Kinase I and Identification of the Autophosphorylation Site**—A peptide sequence was obtained using approximately 150 μg of CaM kinase I, purified from bovine brain as previously described (Nairn and Greenberg, 1987). The kinase was autophosphorylated using [γ-32P]ATP and further purified by SDS-PAGE. The purified protein was transferred to nitrocellulose and digested sequentially with AspN, trypsin, and chymotrypsin. The resulting peptides from each digest were purified by reversed-phase HPLC, and an amino acid sequence was obtained for several peaks. CaM kinase I was previously shown to be autophosphorylated only on threonine (Nairn and Greenberg, 1987). A single peptide from each proteolytic digest was found to be 32P-labeled, consistent with the presence of one autophosphorylation site in CaM kinase I. Twenty-six of 29 residues of the 32P-labeled peptide from the AspN digest (peptide D) were unequivocally identified as follows: DPSSVLXAXGTYPVAPLQAK-PYXRA. The sequences obtained for the 32P-labeled peptides from the tryptic and chymotryptic digests were included within that of peptide D, although fewer amino acid acids were identified (data not shown). In each 32P-labeled peptide one common amino acid residue position was not identified (X in the sequence LSXA). Based on the high yield of the phenylthiohydantoin-derivative from the other threonyl residue in peptide D, we conclude that the unidentified amino acid in the sequence LSXA is the autophosphorylated threonyl residue (see below).

Cloning of CaM Kinase I—Peptide D was highly homologous to a conserved protein kinase domain that contains the APE motif (Hanks et al., 1988; Hanks and Quinn, 1991; Hanks and Lindberg, 1991), confirming that the purified protein was a protein kinase. However, this peptide contained 9 amino acids using several combinations of oligonucleotides based on the amino acid sequences of peptide D and other sequenced non-radioactive peptides. Furthermore, direct screening of a bovine brain library using these oligonucleotides did not result in identification of any CaM kinase I cDNA clones. In order to obtain partial sequence of the gene encoding CaM kinase I, degenerate oligonucleotides, based on the amino acid sequence obtained from a conserved part of peptide D (oligo 394) and a second conserved motif of other CaM-dependent protein kinases (the HRD motif) (oligo Kin-I) (Fig. 1), were used to amplify a 180-bp product from bovine forebrain cDNA. The PCR product was subcloned and individual subclones isolated by hybridization to a third degenerate oligonucleotide (oligo 202) based on the partially conserved middle region of peptide D (Fig. 1). Random sequencing of 10 subclones revealed that all encoded CaM kinase II. All subclones were then hybridized to a fourth degenerate oligonucleotide (oligo 395) based on the unique NH₂-terminal region of peptide D. One subclone was obtained which encoded all of the amino acid sequence of peptide D. DNA sequences corresponding to the oligonucleotides used to amplify the PCR product (394 and Kin-I) and to the two oligonucleotides that hybridized to this subclone (395 and 202) were present in this subclone. This...
subclone also included the DNA sequence encoding the HRD motif (as expected since one of the oligonucleotides used to amplify the PCR product was based on this sequence) and the DFG motif which lies between the HRD and APE motifs in protein kinase catalytic domains (see Fig. 1). Approximately one copy of the CaM kinase I PCR product was obtained for every 50 subclones of the 180-bp amplification product.

The same procedure described above was used to amplify a PCR product from rat forebrain cDNA. A rat CaM kinase I subclone was isolated by hybridization to an oligonucleotide (oligo CK-1A) corresponding to part of the bovine CaM kinase I PCR product sequence. The rat sequence was isolated in order to facilitate localization studies which are most easily done on rat tissues. In addition, this sequence enabled us to examine the degree of homology between the rat and bovine cDNA. The bovine PCR product sequence. The rat sequence was isolated in a serine to glycine substitution in the unique region of peptide D. The bovine PCR product was used to screen a rat brain cDNA library, and one positive clone was obtained from 1 million plaques. The bovine clone was subsequently used to screen a rat brain cDNA library, and one positive clone was obtained from 3.5 million clones. This phage was found to contain a 1.65-kb EcoRI insert that encoded the full coding region of CaM kinase I (Fig. 2). The initiation AUG was part of an initiation consensus sequence (Kozak, 1986). No polyadenylation site was found, consistent with the fact that the library had been generated by random priming. The predicted molecular weight of the protein was 37,545, consistent with the mobility of purified CaM kinase I on SDS-PAGE. In addition to the sequence of peptide D, peptide sequence derived from other proteolytic fragments could be identified within the coding region of this cDNA. A threonyl residue was predicted at amino acid 177, and this corresponds to the position of the unidentified amino acid in the \(^{32}P\)-labeled peptide (peptide D). Therefore, it is likely that this is a major site of autophosphorylation in CaM kinase I.

**Bacterial Expression of CaM Kinase I**—To verify that the cloned cDNA did indeed encode CaM kinase I, the coding region of the rat cDNA was subcloned into the pGEX 2T expression vector, and the resulting fusion protein was purified by affinity chromatography using glutathione-agarose (Smith and Johnson, 1988). The predicted open reading frame was verified by subcloning fragments of the cDNA that corresponded to two different reading frames. The fragment that started with the ATG at nucleotide 92 of the cDNA produced active fusion protein that phosphorylated synapsin I (residues 3–13) efficiently in the presence of Ca\(^{2+}\) and CaM, but was essentially inactive in their absence. As expected, the fusion protein produced from the cDNA fragment that started with the ATG at position 150 was inactive (Fig. 3). The fragment that started with the ATG at position 92 produced a protein of the expected size (~67 kDa, corresponding to ~30 kDa of glutathione S-transferase plus ~37 kDa of CaM kinase I) (Fig. 4). The fragment that started with the ATG at position 150 produced a protein of ~30 kDa, corresponding to the expected size of glutathione S-transferase alone (data not shown). The 67-kDa polypeptide was purified essentially to homogeneity by affinity chromatography using Sepharose 4B-CaM (Fig. 5).

**Fig. 2. Nucleotide and deduced amino acid sequence of rat CaM kinase I cDNA.** A CaM kinase I cDNA was isolated from a rat forebrain A-GT10 cDNA library as described under "Experimental Procedures." The complete DNA sequence of the clone is shown here with the deduced amino acid sequence below. The initiation methionine is encoded by ATG at nucleotide 92. The termination codon is at nucleotide 1066 (*). The encoded protein has a predicted molecular weight of 37,545. The amino acid sequences obtained from proteolytic fragments of purified bovine CaM kinase I are underlined.
one S-transferase/CaM kinase I fusion protein by Sepharose-CaM affinity chromatography. A glutathione S-transferase/CaM kinase I fusion protein was expressed in Escherichia coli as described under "Experimental Procedures." The bacteria were lysed by sonication and cell debris removed by centrifugation. Ca²⁺ was added and the sample applied to Sepharose 4B-CaM. The pure fusion protein was eluted from the affinity column in the absence of Ca²⁺. Aliquots of the relevant fractions were separated by SDS-PAGE using 10% acrylamide and the gel stained with Coomassie Brilliant Blue and destained. A major polypeptide of ~67 kDa is present in the supernatant prepared from the bacterial lysate (E. coli Supn.). This polypeptide was specifically absorbed to the affinity chromatography resin (compare the applied sample with the flow-through (Seph-CaM FT) and the fraction eluted by buffer plus EGTA (Seph-CaM EGTA)).

**Fig. 4. Purification of glutathione S-transferase/CaM kinase I fusion protein by Sepharose-CaM affinity chromatography.** A glutathione S-transferase/CaM kinase I fusion protein was expressed in Escherichia coli as described under “Experimental Procedures.” The bacteria were lysed by sonication and cell debris removed by centrifugation. Ca²⁺ was added and the sample applied to Sepharose 4B-CaM. The pure fusion protein was eluted from the affinity column in the absence of Ca²⁺. Aliquots of the relevant fractions were separated by SDS-PAGE using 10% acrylamide and the gel stained with Coomassie Brilliant Blue and destained. A major polypeptide of ~67 kDa is present in the supernatant prepared from the bacterial lysate (E. coli Supn.). This polypeptide was specifically absorbed to the affinity chromatography resin (compare the applied sample with the flow-through (Seph-CaM FT) and the fraction eluted by buffer plus EGTA (Seph-CaM EGTA)).

**Fig. 5. Substrate specificity of bacterially expressed glutathione S-transferase/CaM kinase I fusion protein.** The glutathione S-transferase/CaM kinase I fusion protein was purified by Sepharose 4B-CaM affinity chromatography as described under “Experimental Procedures” (see Fig. 3). The purified fusion protein as well as CaM kinase I purified from bovine brain were used to phosphorylate bovine brain synapsin I in the presence of Ca²⁺ and CaM. The samples were separated by SDS-PAGE, and the phosphorylated synapsin I was localized by autoradiography, excised, and digested with S. aureus V8 protease. The autoradiogram shows that both CaM kinase I preparations phosphorylated synapsin I only at site 1 which is present in the 10-kDa V8 fragment (in each case, 96% of the radioactivity was present in the 10-kDa fragment; 4% of the radioactivity is present in the 30-kDa fragment that contains sites 2 and 3).

indicating that the expressed protein contained a functional CaM-binding domain. In addition, the purified fusion protein phosphorylated synapsin I only at site 1, consistent with the substrate specificity of CaM kinase I purified from bovine brain (Fig. 5). These results, together with the observations that amino acid sequences corresponding to peptides isolated from purified CaM kinase I were encoded by the isolated cDNA and that the deduced protein sequence exhibited a high degree of homology with the catalytic domains of other known protein kinases, lead us to conclude that we have isolated the full-length rat CaM kinase I cDNA.

**Northern and Southern Blotting Using the CaM Kinase I cDNA**—The bovine cDNA hybridized to a single DNA fragment in digests of rat genomic DNA with BamHI or HindIII (Fig. 6). One large DNA fragment and a second smaller fragment were detected in the digest with EcoRI.

Two mRNA species, of ~1.8 and ~5.5 kb, were detected using the rat PCR product on a blot rat forebrain RNA (Fig. 6). In addition, all probes tested, including a unique 35-bp oligonucleotide made from the sequence of the rat PCR product, were able to detect a ~1.8-kb RNA species in forebrain poly(A⁺) RNA (data not shown). This RNA species probably corresponds to the cDNA isolated from the rat forebrain library. Both bands appear in Northern blots of RNA isolated from all tissues tested (data not shown). The identity of the larger band remains to be determined.

**Tissue Distribution of CaM Kinase I mRNA**—The distribution of CaM kinase I mRNA in several brain regions as well as in non-neuronal tissues was analyzed by RNase protection assay (Fig. 7). RNA samples were hybridized to mouse β-actin and rat CaM kinase I probes. Radioactivity detected with the CaM kinase I probe was normalized to that detected with the mouse β-actin probe (Ponte et al., 1989). Frontal cortex contained the highest level of CaM kinase I mRNA, and all values were normalized to the levels found in frontal cortex. Most brain regions contained similar levels of CaM kinase I mRNA; however, the levels varied in the body tissues examined. Significant mRNA levels were detected in every tissue tested. The highest levels of CaM kinase I mRNA in non-neuronal tissues were found in adrenal gland, liver, and lung.

**DISCUSSION**

Several members of the CaM kinase family have been cloned and all members of the family have a characteristic
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FIG. 6. Southern blot of rat genomic DNA and Northern blot of rat forebrain RNA. The left panel shows a Southern blot. 30 µg of rat genomic DNA were cleaved using BamHI (lane B), EcoRI (lane E) or HindIII (lane H). DNA was separated on a 0.7% agarose gel and blotted to nylon membrane. The blot was hybridized to the 1.4-kb bovine CaM kinase I cDNA at 60 °C, washed in 0.1 X SSC at 60 °C, and visualized by autoradiography. The right panel shows a Northern blot. Two µg of rat forebrain poly(A+) RNA were separated on a 1% formaldehyde-containing agarose gel, and RNA was transferred to nylon membrane. Hybridization was performed at 60 °C using the 180-bp CaM kinase I PCR product amplified from rat forebrain cDNA, and the result was visualized by autoradiography. 18S, the rat 18 S ribosomal RNA; 35S, the rat 35 S ribosomal RNA.

organization (Guerriero et al., 1986; da Cruz e Silva and Cohen, 1987; Lin et al., 1987; Bulleit et al., 1988; Ohmstede et al., 1991; Jones et al., 1991). They each have a highly conserved catalytic domain and a regulatory domain of varying length that is not highly conserved among family members. The regulatory domain is thought to associate with the catalytic domain in a manner that results in inhibition of catalytic activity in the basal state (Colbran et al., 1988, 1989; Kwiatkowski et al., 1990; Means et al, 1991; Bagchi et al., 1992b; Knighton et al., 1992). The CaM-binding domain is found within the regulatory domain and binding of Ca<sup>2+</sup>/CaM causes the dissociation of the regulatory domain and activation of the kinase. In general, CaM-binding domains are not highly conserved at the level of primary structure; however, there is conservation at the level of secondary structure (O'Neil and DeGrado, 1990; Kretsinger, 1992).

The predicted amino acid sequence of CaM kinase I places it within the family of serine/threonine protein kinases and more specifically, in the family of CaM-dependent protein kinases. All the residues that are identical in other protein kinase catalytic domains (Hanks et al., 1988; Hanks and Quinn, 1991) are conserved in CaM kinase I (Fig. 8). Comparison of the predicted amino acid sequence of CaM kinase I with that of the α subunit of rat brain CaM kinase II (Lin et al., 1987) revealed a high percentage of identity (approximately 42%) throughout the catalytic domain (Fig. 8). A region with a relatively high percentage of identity was found overlapping the region known to be the CaM-binding domain of CaM kinase II (approximately 32%) and other CaM-binding proteins (Figs. 8 and 9). In contrast to CaM kinase II, and consistent with the fact that CaM kinase I is active as a monomer, CaM kinase I does not contain an association domain. By analogy with other protein kinases, CaM kinase I might be expected to contain an autoinhibitory domain. The amino acid sequence surrounding the phosphorylated seryl residue of site 1 of synapsin I is RRRRLS(P)DS and recent studies using synthetic peptide analogues suggest that all three arginyl residues are necessary for phosphorylation of...
primary sequence of CaM kinase I did not reveal an obvious this site by CaM kinase I. However, examination of the primary sequence of CaM kinase I did not reveal an obvious pseudosubstrate sequence in the regulatory domain which is highly related to the synapsin I phosphorylation site sequence. Recent analysis of the crystal structure of the catalytic subunit of protein kinase A (Knighton et al., 1991a, 1991b) and modeling studies of myosin light chain kinase (Knighton and Kretsinger, 1992). CaM kinase I (Bulleit et al., 1988, Lin et al., 1989) are also involved in binding to ATP. Lys-72 (Lys-48 in CaM kinase I); Ghu-91 (Glu-65) and Asp-184 (Asp-162) are also involved in binding to ATP. Lys-72 (Lys-48) and Glu-91 (Glu-65) lie in the smaller lobe and Asp-184 (Asp-162) is part of the conserved DFG motif which lies in the large lobe of the kinase and chelates the magnesium in the Mg-ATP complex. The sequence YRDKPEN, corresponding to residues 164-171 in protein kinase A (HRDLKPEN, residues 139-146 in CaM kinase I) directs the catalytic event and guides the peptide substrate into the proper orientation so that catalysis can occur. In this respect, the HRD motif, which is highly conserved among members of the CaM kinase family, is likely to be involved in conferring serine/threonine specificity to the protein kinase (Hanks et al., 1988; Hanks and Quinn, 1991). Finally, CaM kinase I is autophosphorylated at a threonyl residue (Thr-177) which is located at a position equivalent to that of the threonyl residue (Thr-197) autophosphorylated in protein kinase A (Fig. 10). In protein kinase A, the autophosphorylated Thr-197 is highly resistant to dephosphorylation and it has been suggested that the phosphate moiety contributes to the overall stability of the enzyme (Knighton et al., 1991a, 1991b). In CaM kinase I, autophosphorylation of Thr-177 occurs in a Ca2+/CaM-dependent manner suggesting that this residue is relatively more accessible to dephosphorylation by endogenous protein phosphatases. Autophosphorylation of CaM kinase I does not appear to significantly affect its Ca2+/CaM-dependent activity although by analogy with other protein kinases such as mitogen-activated protein kinase or insulin-regulated kinase I which are activated by phosphorylation of threonyl residues in equivalent positions in those enzymes (Fig. 10), it is possible that CaM kinase I may be regulated by autophosphorylation of Thr-177 in an unknown fashion.

CaM kinase I is related to other members of the protein kinase family based on comparison of the sequence of the

\[ \text{CaM Kinase I} \]

\[ \begin{align*}
\text{CaM PHOS} & : \\
\text{CaM Kinase I} & : \\
\text{CaM Kinase II} & : \\
\text{CaM Kinase IV} & : \\
\text{PKA} & : \\
\text{PKC} & : \\
\text{PKC} & : \\
\text{MSK} & : \\
\text{PHOS KINASE} & : \\
\text{PKC} & : \\
\text{PKA} & : \\
\end{align*} \]

Fig. 10. Comparison of autophosphorylation sites. The amino acid sequence surrounding the autophosphorylated threonyl residue in CaM kinase I (Thr-177) is compared with that of one of the autophosphorylation sites of protein kinase A (Thr-197) (Knighton et al., 1991b). Mitogen-activated protein kinase is activated by phosphorylation by mitogen-activated protein kinase at Thr-190 and Thr-192 (Payne et al., 1991). Insulin-dependent protein kinase I (INS. P.K. I) is activated by phosphorylation of Thr-156 by MAP kinase (Sutherland et al., 1993). Conserved residues in domain VIII of each of these protein kinases are boxed.

Fig. 9. Comparison of amino acid sequences of CaM-binding domains. The amino acid sequence of the putative CaM-binding domain of CaM kinase I is compared with CaM-binding domains of other CaM kinases as well as a protein phosphate II (Thr-197) (Knights et al., 1993). Conserved residues in domain VIII of protein kinase A (HRDLKPEN, residues 139-146 in CaM kinase I) which is autophosphorylated at a threonyl residue (Thr-177) which is located at a position equivalent to that of the threonyl residue (Thr-197) autophosphorylated in protein kinase A (Fig. 10). In protein kinase A, the autophosphorylated Thr-197 is highly resistant to dephosphorylation and it has been suggested that the phosphate moiety contributes to the overall stability of the enzyme (Knighton et al., 1991a, 1991b). In CaM kinase I, autophosphorylation of Thr-177 occurs in a Ca2+/CaM-dependent manner suggesting that this residue is relatively more accessible to dephosphorylation by endogenous protein phosphatases. Autophosphorylation of CaM kinase I does not appear to significantly affect its Ca2+/CaM-dependent activity although by analogy with other protein kinases such as mitogen-activated protein kinase or insulin-regulated kinase I which are activated by phosphorylation of threonyl residues in equivalent positions in those enzymes (Fig. 10), it is possible that CaM kinase I may be regulated by autophosphorylation of Thr-177 in an unknown fashion.

CaM kinase I is related to other members of the protein kinase family based on comparison of the sequence of the

Fig. 8. Comparison of the deduced amino acid sequences of rat CaM kinase I and the α subunit of rat CaM kinase II. The percentage identity of the two deduced amino acid sequences was 42% when the entire CaM kinase I (1) amino acid sequence was compared with residues 1-328 of CaM kinase II (II) (Bulleit et al., 1988; Lin et al., 1987). The CaM-binding domain is located at the COOH-terminal of this part of CaM kinase II and the corresponding region is predicted to contain the CaM-binding domain of CaM kinase I. Invariant amino acid residues present in the catalytic domain of all known protein kinases and the CaM-binding domain are boxed and shaded. The putative autophosphorylation site in CaM kinase I is Thr-177, and one of the autophosphorylation sites for CaM kinase II, Thr-286 (Thiel et al., 1988) are also boxed and shaded. The association domain of the α subunit of CaM kinase II follows the sequence shown. 3 identical amino acid and 1 conservative amino acid change.

\[ \text{CaM Kinase I} : \\
\text{CaM Kinase II} : \\
\text{CaM Kinase IV} : \\
\text{PKA} : \\
\text{PKC} : \\
\text{PKC} : \\
\text{MSK} : \\
\text{PHOS KINASE} : \\
\text{PKC} : \\
\text{PKA} : \\
\]

this site by CaM kinase I. However, examination of the primary sequence of CaM kinase I did not reveal an obvious pseudosubstrate sequence in the regulatory domain which is highly related to the synapsin I phosphorylation site sequence.

Recent analysis of the crystal structure of the catalytic subunit of protein kinase A (Knighton et al., 1991a, 1991b) and modeling studies of myosin light chain kinase (Knighton et al., 1991a, 1991b) allow several of the invariant amino acids in the catalytic domain of CaM kinase I to be assigned a tentative function. In the smaller lobe of protein kinase A several residues are involved in ATP binding. Gly-50 (residue 27 in CaM kinase I) and Gly-52 (Gly 29 of the GXGXXG motif (GTGAFS in CaM kinase I) lie on a sharp turn between antiparallel β-strands. The nucleotide portion of ATP is buried below the β-sheet and the glycines interact with the non-transferable phosphates in ATP. In addition, the glycine-containing loop anchors the phosphate moiety and positions the γ-phosphate for transfer. Several other residues including Lys-72 in protein kinase A (Lys-48 in CaM kinase I); Ghu-91 (Glu-65) and Asp-184 (Asp-162) are also involved in binding to ATP. Lys-72 (Lys-48) and Glu-91 (Glu-65) lie in the smaller lobe and Asp-184 (Asp-162) is part of the conserved DFG motif which lies in the large lobe of the kinase and chelates the magnesium in the Mg-ATP complex. The sequence YRDLKPEN, corresponding to residues 164-171 in protein kinase A (HRDLKPEN, residues 139-146 in CaM kinase I) directs the catalytic event and guides the peptide substrate into the proper orientation so that catalysis can occur. In this respect, the HRD motif, which is highly conserved among members of the CaM kinase family, is likely to be involved in conferring serine/threonine specificity to the protein kinase (Hanks et al., 1988; Hanks and Quinn, 1991). Finally, CaM kinase I is autophosphorylated at a threonyl residue (Thr-177) which is located at a position equivalent to that of the threonyl residue (Thr-197) autophosphorylated in protein kinase A (Fig. 10). In protein kinase A, the autophosphorylated Thr-197 is highly resistant to dephosphorylation and it has been suggested that the phosphate moiety contributes to the overall stability of the enzyme (Knighton et al., 1991a, 1991b). In CaM kinase I, autophosphorylation of Thr-177 occurs in a Ca2+/CaM-dependent manner suggesting that this residue is relatively more accessible to dephosphorylation by endogenous protein phosphatases. Autophosphorylation of CaM kinase I does not appear to significantly affect its Ca2+/CaM-dependent activity although by analogy with other protein kinases such as mitogen-activated protein kinase or insulin-regulated kinase I which are activated by phosphorylation of threonyl residues in equivalent positions in those enzymes (Fig. 10), it is possible that CaM kinase I may be regulated by autophosphorylation of Thr-177 in an unknown fashion.

CaM kinase I is related to other members of the protein kinase family based on comparison of the sequence of the
cDNA. However, CaM kinase I appears to be the product of a single gene based on the results obtained from Southern blotting of rat genomic DNA. The enzyme purified from bovine brain was detected using SDS-PAGE as a doublet of two major polypeptides of approximately 37 and 39 kDa with a minor polypeptide at 42 kDa. It was suggested that these different polypeptides were likely to be derived by proteolysis from one species, since the autophosphorylated polypeptides showed identical two-dimensional peptide map patterns (Nairn and Greengard, 1987). In support of this possibility, initial immunoblotting of rat tissues with an antisera made against the bacterially expressed fusion protein revealed one band of approximately 40 kDa (data not shown). It is possible, however, that more than one isoform of CaM kinase I protein exist in brain or in other tissues which result from alternative mRNA splicing. Two RNA species hybridize to the CaM kinase I PCR product in rat tissues. The 1.8-kb message is close in size to the rat brain cDNA which encodes a protein with a predicted molecular mass of ~37 kDa. The 5.5-kb RNA species could encode another CaM kinase I isoform or it is possible that the 5.5-kb band represents an un-spliced intermediate, although it is rare to see such an intermediate present in such high abundance. It is also possible that the upper band may represent spurious hybridization to the 28 S ribosomal subunit which migrates at this position.

A Ca<sup>2+</sup>-calmodulin-dependent protein kinase, termed CaM kinase V, has recently been purified from rat cerebrum using the peptide, syntide-2, as substrate (Mochizuki et al., 1993). The enzyme consisted of a single polypeptide of 41 kDa and had several properties similar to CaM kinase I. Examination of the partial amino acid sequence of four peptides derived from CaM kinase V (Mochizuki et al., 1993) revealed that it is most likely identical to CaM kinase I. Two other CaM-dependent protein kinases have also been purified from rat brain using synapsin I site 1 peptide as a substrate (DeRemeber et al., 1992a, 1992b). These were termed CaM kinase Ia and Ib and had several properties in common with CaM kinase I purified from bovine brain. They both consisted of polypeptides of ~40 kDa and had other chromatographic properties similar to bovine CaM kinase I. Based on the fact that CaM kinase Ia was autophosphorylated on threonine in a CaM-dependent manner it is possible that this is the equivalent of rat CaM kinase I; however, rat CaM kinase Ia was activated by autophosphorylation, whereas bovine brain CaM kinase I is not significantly affected by autophosphorylation. The PCR fragments of CaM kinase I that were amplified from bovine and rat brain cDNA were very similar. In addition, the partial cDNA clone isolated from the bovine brain library which encoded much of the catalytic domain of the kinase was 85% identical at the DNA level and 94% identical at the amino acid level to the equivalent part of the full-length rat cDNA that was obtained. These results suggest that we have identified the primary structure of CaM kinase I and that this is highly conserved in rat and bovine brain. The existence of other CaM kinase I isoforms, as well as the relationship to rat CaM kinase Ia and Ib will await more extensive immunological analysis.

CaM kinase I is present in significantly lower levels than CaM kinase II based on measurement of their relative activities in rat forebrain (Nairn and Greengard, 1987). The low frequency of the CaM kinase I cDNA in rat and bovine libraries also suggests that the mRNA for CaM kinase I is relatively rare. RNase protection assays indicate that CaM kinase I mRNA is ubiquitous, with the highest level being found in frontal cortex. The levels of mRNA in non-neuronal tissues are somewhat higher compared to brain than might have been predicted from the measurements of CaM kinase I activity (Nairn and Greengard, 1987). For example, CaM kinase I activity in lung was approximately 10% of that found in brain; however, the level of mRNA in lung is approximately 70% of that present in frontal cortex. It is possible that measurement of incorporation of 32P into site 1 of holosynapsin I overestimated the activity of CaM kinase I in the brain since CaM kinase IV can also phosphorylate site 1 of synapsin I (Miyano et al., 1992). Future studies using antisera specific for CaM kinase I will allow direct quantitation of the level of protein in tissues and will resolve the potential discrepancy between activity and mRNA levels.

The ubiquitous localization of both mRNA and activity for CaM kinase I argues strongly that other substrates for the kinase must exist besides the neuron-specific synapsins. Recently, it has been reported that CaM kinase I phosphorylates CREB, the cyclic AMP-response element-binding protein (Sheng et al., 1991). CREB binds to a DNA motif called CRE, the CAMP-response element, and increases transcription from several genes in response to elevation of cAMP. Phosphorylation of serine 133 by protein kinase A in CREB greatly increases its ability to stimulate transcription but does not change its ability to bind to CRE. CaM kinase I, CaM kinase II (Sheng et al., 1993) and CaM kinase IV<sup>2</sup> are able to phosphorylate the same residue in CREB<sub>vitr</sub>. Increased transcription of CRE-containing genes occurs in response to elevation of intracellular Ca<sup>2+</sup> as well as cAMP, therefore phosphorylation of CREB by CaM kinase(s) may be important in<sub>vitr</sub>. Of the CaM kinases that can phosphorylate CREB<sub>vitr</sub>, CaM kinase IV<sup>2</sup> is the only one known to have a nuclear localization (Jensen et al., 1991), although the subcellular localization of CaM kinase I remains to be studied. CaM kinase I can also phosphorylate CF-2, a portion of the R-domain of CPTF (Picciotto et al., 1992). The efficiency of phosphorylation of CF-2 is even greater than that of intact synapsin I, previously the best known substrate for CaM kinase I. Ca<sup>2+</sup> is known to regulate chloride flux in epithelial cells (Boucher et al., 1988; Al-Bazzaz and Jayaram, 1981; Willumsen and Boucher, 1989) and CaM kinase I is found in several tissues that exhibit defective electrolyte regulation in cystic fibrosis. The observation that CaM kinase I is widely distributed in mammalian tissues raises the distinct possibility that CREB or CPTF, as well as additional substrates, exist for this enzyme. The elucidation of the structure of CaM kinase I will be useful in further characterizing the physiological function of this novel serine/threonine protein kinase.

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REFERENCES


* M. Greenberg, personal communication.