Multiple Ca$^{2+}$-Calmodulin-dependent Protein Kinase
Kinasestrom Rat Brain

PURIFICATION, REGULATION BY Ca$^{2+}$-CALMODULIN, AND PARTIAL AMINO ACID SEQUENCE*

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We have purified to near homogeneity from rat brain
twoca$^{2+}$-calmodulin-dependent protein kinase I (CaM kinase I) activating kinases, termed here CaM kinase I
kinase-$\alpha$ and CaM kinase I kinase-$\beta$ (CaMKIK$\alpha$ and CaMKIK$\beta$, respectively). Both CaMKIK$\alpha$ and CaMKIK$\beta$
are also capable of activating CaM kinase IV. Activation of CaM kinase I and CaM kinase IV occurs via phospho-
rylation of an equivalent Thr residue within the “activation loop” region of both kinases, Thr-177 and Thr-196, respectively. These activities of CaMKIK$\alpha$ and CaMKIK$\beta$
are themselves strongly stimulated by the presence of ca$^{2+}$-CaM, and both appear to be capable of Ca$^{2+}$-CaM-
dependent autophosphorylation. Automated microsequence analysis of the purified enzymes established that
CaMKIK$\alpha$ and -$\beta$ are the products of distinct genes. In addition to rat, homologous nucleic acids corresponding
to these CaM kinase kinases are present in humans and
the nematode, Caenorhabditis elegans. CaMKIK$\alpha$ and CaMKIK$\beta$ are thus representatives of a family of en-
zymes, which may function as key intermediaries in Ca$^{2+}$-CaM-driven signal transduction cascades in a wide
variety of eukaryotic organisms.

Ca$^{2+}$-calmodulin (CaM)$^{2+}$-dependent protein kinases (CaM kinases) I and IV are distinguished among members of the
CaM kinase subfamily by their dependence upon phosphoryl-
ation by distinct protein kinases (CaM kinase kinases) for

maximal activity (1–11). Activating phosphorylation occurs at
an identically positioned Thr residue in both cases: Thr-177 in
CaM kinase I (8, 13) and Thr-196 in CaM kinase IV (11),
although for the latter, Ser phosphorylation in the NH$_2$-ter-
minal region may also contribute to activation (10, 14). The activ-
ating Thr is three amino acids NH$_2$-terminal to a highly
conserved GTPXXXAPE sequence present in protein kinase
-catalytic subdomain VIII (15). Phosphorylation in this region,
termed the "phosphorylation lip" or "activation loop," has been
shown to be essential for the regulation and activity of a num-
ber of protein kinases including protein kinase A (16), protein
kinase C $\beta$ II (17, 18), members of the mitogen-activated protein
kinase family (19–21), and the cyclin-dependent protein
kinases (22–24).

In the context of CaM kinase regulation, an important un-
resolved issue is the number and identity of the CaM kinase
kinases. An activating kinase of approximately 52 kDa was
purified from pig brain using CaM kinase I as substrate (5, 9),
whereas a 66–68 kDa was purified from rat brain using CaM
kinase IV as substrate (6, 7). It was subsequently reported,
however, that CaM kinase I kinase purified from pig brain (11),
or an enzyme partially purified from rat brain (13), was capable
of phosphorylating and activating CaM kinase IV. Conversely,
a recently cloned and expressed, rat brain CaM kinase IV
kinase was found to be capable of activating CaM kinase I (25).
This raised the possibility that a single kinase enzyme may
be responsible for phosphorolyzing both CaM kinases I and IV.

However, multiple chromatographic peaks of CaM kinase
kinase activity were observed during its purification from pig
brain (5), suggesting that multiple CaM kinase kinases with
overlapping substrate specificities (11) could be present.

We have now purified CaM kinase kinases from rat brain
using CaM kinase I activation as an assay and report that two
such kinases are present, both having the ability to activate
CaM kinase I and CaM kinase IV. We also show by direct
amino acid sequencing that they are isoenzymes encoded by
different genes and are likely to be representatives of a family
of enzymes widely distributed among eukaryotic organisms.

EXPERIMENTAL PROCEDURES

CaM Kinase Activation Assay—CaM kinase kinases were assayed by their ability to enhance the activity of expressed CaM kinases I and IV
or their respective mutants toward a synthetic peptide substrate base
on site 1 of synapsin I (5, 8, 11). A coupled procedure was used in which
a standard amount (typically 0.2 ng/μl) of CaM kinase I, CaM kinase IV,
or their respective mutants was added to CaM kinase kinase-containing
fractions in the presence or absence of Ca$^{2+}$-CaM as described in the
figure legends and incubated at 30 °C in a mixture containing 50 mM
Tris, pH 7.6, 0.5 mM DTT, 0.5 mM of bovine serum albumin (BSA)/ml, 10
mM MgCl$_2$, 200 μM $[\gamma-^{32}$P]ATP (DuPont NEN, ~100 cpm/μmol), and 50
μM synapsin I site 1 peptide (NYLRRRLSDSNF). Quantification of $^{32}$P

1 The abbreviations used are: CaM, calmodulin; CaM kinase, Ca$^{2+}$-
calmodulin-dependent protein kinase; CaMKIK$\alpha$, CaMKIK$\beta$, Ca$^{2+}$-CaM-
dependent protein kinase I with M, $^2$ values by SDS-PAGE of
~69,600 and ~73,200, respectively; CaMKIKV, CaMKIK$\alpha$, Ca$^{2+}$-CaM-
dependent protein kinase IV; DTT, dithiothreitol; BSA, bovine serum albu-
mín; HPLC, high pressure liquid chromatography; AMPK, S-AMP-
activated protein kinase; hBRAIN, assembled overlapping human
DNA, human cDNA, human cDNA, human cDNA with accession num-
ber R56818; CELC05H8–2 (accession number U11029), part of C. el-
igious cosmid C05H8 containing genomic DNA; BLAST, basic local
alignment search tool (41); PAGE, polyacrylamide gel electrophoresis;
MOPS, 3-(N-morpholinio)propanesulfonic acid.
incorporation into synapsin I site 1 peptide was by a phosphocellulose filter paper method (1). Activating activity is defined as CaM kinase activity in the presence of CaM in kinase minus activity in its absence and is given in units/μg CaM kinase-containing fraction where 1 unit = 1 pmol of 32P incorporated into synapsin I site 1 peptide/minute/μg of CaM kinase I or IV. Synapsin I site 1 peptide was custom synthesized and purified by HPLC at the Biomedical Research Core Facilities of the University of Michigan. CaM kinase I (wild type), its activation site mutant (T177A), and CaM-independent COOH-terminal truncation mutant (1–294) were expressed and purified from Escherichia coli as described in Haribabu et al. (8). CaM kinase IV and its respective activation site mutant (T196A) were expressed and purified from E. coli as described in Selbert et al. (11).

Purification of CaM Kinase Kinases—The procedure for purifying CaM kinase kinases from rat brain was a modification of the method for purification of pig brain CaM kinase I kinase (5). CaM kinase kinase activity was monitored during purification by CaM kinase I activation as described above. All procedures were at 0–4 °C. Frozen Sprague-Dawley rat brains (202 g) were homogenized in 4 volumes of buffer A (20 mM MOPS, pH 7.2, 1 mM DTT, 1 mM EDTA, 1 mM EGTA, 0.5 mM phenylmethylsulfonyl fluoride, and 5 mg of leupeptin/liter) and centrifuged at 40,000 × g for 30 min. The supernatant was filtered through a 100% cotton handkerchief and chromatographed at ~40 ml/h on a DEAE-Sepharose CL-6B column (2.5 × 36.5 cm) equilibrated in buffer A. After sample application, the column was washed with 1,400 ml of buffer A and eluted with a 1,000-ml linear gradient of 0–0.35 M NaCl in buffer A. Active fractions were pooled, adjusted with glycerol and Triton X-100 to final concentrations of 10 and 0.025%, respectively, and chromatographed at ~25 ml/h on a column of hydroxyapatite (2.5 × 12 cm, BioGel HT, Bio-Rad). The pool was applied to the column equilibrated in buffer B (buffer A, 10% glycerol, 0.025% Triton X-100) containing 0.2 M NaCl and washed with 165 ml of buffer B containing 0.2 M NaCl. Elution was with a 500-ml linear gradient of 20–225 mM sodium phosphate, pH 6.8, in buffer B (MOPS omitted). Pooled fractions were dialyzed against buffer C (10 mM MOPS, pH 7.0, 0.1 mM DTT, 0.05 M NaCl, 10% glycerol, 0.025% Triton X-100, 0.5 mM phenylmethylsulfanyl fluoride, and 2 mg of leupeptin/liter) and adjusted to contain: 2 mM MgCl2, 1.5 mM CaCl2, 10 mg of leupeptin/liter, and 5 mg/liter each of calpain inhibitors I and II. The dialyzed pool was then chromatographed at ~15 ml/h on a CaM-Sepharose (Pharmacia Biotech Inc.) column (1.5 × 8.5 cm) equilibrated in buffer D (buffer C, 2 mM MgCl2, 0.5 mM CaCl2, and 5 mg/liter each of calpain inhibitors I and II). After sample application, the column was washed with 100 ml of buffer C containing 0.2 M NaCl, followed by 15 ml of buffer C containing 50 mM NaCl, and eluted with buffer C containing 2 mM EGTA. Active fractions were chromatographed at ~12 ml/h on a heparin-Sepharose (Pharmacia) column (1.5 × 17 cm). The pool was applied to the column equilibrated in buffer B, and the column was washed with 7,500 ml of this buffer. Elution was with a 300-ml linear gradient of 0–0.6 M NaCl in buffer B.

Peptide Sequencing—Peptides were derived from stained bands cut from SDS-PAGE gels by a modification of the previously described in situ proteolysis method (26). The excised bands were destained in 50% acetonitrile, 200 mM NH4HCO3, and dried in a vacuum centrifuge. The dried gel pieces were rehydrated in a minimum volume of 50 mM NH4HCO3 in 10% acetonitrile, pH 8, containing 1–4 μg (depending on protein load, presence to protein ratio of 1:10, w/w) of modified sequencing grade trypsin (Promega). Digestion was allowed to proceed at 37 °C overnight, and peptides were extracted with sequential washes of 500 μl each of 20, 40, and 60% acetonitrile in water in a sonicator water bath. Extracts were pooled and dried in a vacuum centrifuge. Peptides were dissolved in 6 μl of guanidine HCl and chromatographed using reversed phase HPLC as described previously with manual peak collection. Peptides were sequenced on either an Applied Biosystems 471A protein sequencer or a Hewlett Packard G1000A protein sequencer. Other Methods—SDS-PAGE was performed as described previously (5). M, values were calculated by a plot of log M, versus electrophoretic mobility2 on a 7.5% acrylamide SDS gel using the following values for protein standards: Bio-Rad, 97,000, 66,000, 43,000; Life Technologies, Inc., 90,400, 70,000, 60,000, 50,000, 40,000. The concentrations of CaM kinases I and IV and their respective mutants were determined by the method of Lowry et al. (27) with some modifications (28) using BSA as standard.

2 The plot of log M, versus electrophoretic mobility was best fitted by a linear relationship (r2 = 0.983).
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(Mr = 69,600) than the BSA standard whereas activity in the second peak was associated with a more slowly migrating band (Mr = 73,200). Note that some of the most active fractions (e.g. fractions 18–20, 23–25) contain exclusively the 69- or 73-kDa protein, indicating that the the two-peak pattern is not explainable by carryover of one or the other protein into the adjacent peak. We have designated the faster migrating band as CaM kinase I kinase-α (CaMKIKα) and the slower migrating band CaM kinase I kinase-β (CaMKIKβ). Based on the level of purity obtained, the purification procedure described here is suitable for the preparation of both CaM kinase kinases to near homogeneity.

In Fig. 1 we have expressed CaM kinase kinase activities as units/μl column fraction, where 1 unit is defined as 1 pmol of synapsin site 1 peptide phosphorylated/min/ng of CaM kinase I or IV. The differences in scale between CaM kinase I and CaM kinase IV activating activities suggest that both CaMKIKα and CaMKIKβ prefer CaM kinase I as substrate relative to CaM kinase IV. It should be noted, however, that in this coupled assay absolute differences in units can reflect not only the extent of activation of the CaM kinases but also their maximal peptide kinase specific activities achievable under these assay conditions and that the latter may differ between CaM kinases I and IV. Nonetheless there is precedence for the notion that kinase kinases of this class while not absolutely substrate-specific are relatively stringent in their substrate preferences. Hawley et al. (29) demonstrated that the kinase kinase responsible for the phosphorylation and activation of 5'-AMP-activated protein kinase (AMPK) is capable of phosphorylating and activating CaM kinase I and that conversely pig brain CaM kinase I kinase can phosphorylate and activate AMPK but that in both of these heterologous reactions the rate of phosphorylation is 2–3 orders of magnitude slower than that of the corresponding homologous reactions. Whether a similar selectivity of phosphorylation of CaM kinase I or IV by CaMKIKα and CaMKIKβ exists remains to be established through future detailed kinetic comparisons.

Three lines of evidence indicated that the previously characterized pig brain CaM kinase I kinase is itself a Ca2+-CaM-regulated protein kinase. 1) It demonstrates Ca2+-dependent binding to CaM-Sepharose (5). 2) It phosphorylates and activates, in a Ca2+-CaM-stimulated fashion, CaM kinase I (1-294), a form of CaM kinase I that has lost its CaM-binding domain through truncation mutagenesis (8). 3) It phosphorylates and activates, in a Ca2+-CaM-stimulated fashion, a non-CaM-binding enzyme, AMPK (29). We therefore examined whether either or both CaMKIKα and CaMKIKβ are themselves Ca2+-CaM-regulated. As shown in Fig. 2 by two criteria, the activities of both enzymes are strongly stimulated by Ca2+-CaM. First, Ca2+-CaM enhances the abilities of both CaM kinase kinases to activate the CaM-independent fragment, CaM kinase I (1-294) (Fig. 2, top panel), and second, both CaM kinase kinases appear to autoprophosphorylate in a Ca2+-CaM-stimulated fashion (Fig. 2, bottom panel). Consistent with previous reports using pig brain CaM kinase I kinase (8, 29), there is detectable, albeit slight, activity of both CaMKIKα and -β in the absence of Ca2+-CaM. Previous studies have also established that Ca2+-CaM binding to CaM kinase I promotes its phosphorylation and activation by CaM kinase I kinase by inducing exposure of Thr-177 (or a conformation favorable for phosphorylation) (8, 29). Further studies will thus be required to assess the relative importance of these dual effects of Ca2+-CaM in promoting phosphorylation and activation of CaM kinase I by CaMKIKα and -β.

In order to prove that CaMKIKα and CaMKIKβ are distinct proteins, samples of each were subjected to automated microsequencing as described under “Experimental Procedures.” We obtained a sequence of 165 residues of CaMKIKα and 166 residues of CaMKIKβ, which, based on the sequence of the cDNA of CaM kinase IV kinase (CaMKIKV) reported by Tokumitsu et al. (25), could represent 33% of the amino acid sequences of both α and β. The alignment of these amino acid sequences with that of CaMKIKV is presented in the bottom two lines of Fig. 3. Based on a 99% identity over 165 residues, CaMKIKα appears to represent a protein product either identical or highly related to CaMKIKV (25). On the other hand, CaMKIKβ clearly represents a separate gene product relative to CaMKIKα or CaMKIKV showing only 76% identity with CaMKIKα over 67 overlapping residues and 73% identity with CaMKIKV over 166 overlapping residues. Moreover, these differences are distributed throughout the homologous regions. Taken together, Figs. 1–3 demonstrate the existence of two functionally similar but structurally distinct CaM kinase kinases, CaMKIKα and CaMKIKβ.

The peptide sequences were used to search the available protein data bases for previously doned homologous nucleic acids using BLAST. This search was conducted before the paper by Tokumitsu et al. (25) was published, so only three highly similar sequences were found. These sequences, named CELC05H8–2, hBRAIN, and R56818, are also presented in Fig. 3. R56818 represents a partial cDNA sequenced as part of the Human Genome Project by the Washington University Expressed Sequence Tag Project and doned by the IMAGE.
Consortium at the Lawrence Livermore National Laboratory. hBRAIN is a designation we have given to a sequence assembled from four overlapping partial human cDNAs (R50465, H12132, H19237, and H19394), cloned and sequenced as was R56818, and one additional overlapping clone (F06422). As shown in Fig. 3, hBRAIN aligns with the NH₂-terminal portion of CaMKIVK, and R56818 aligns with the COOH-terminal part without overlap of the two sequences. We have obtained a human cDNA by screening a brain cDNA library with sequences derived from those present in R56818. Whereas the cDNA is not complete, we have obtained enough sequence to ascertain that hBRAIN and R56818 represent parts of the same mRNA (data not shown). Based on the data shown in Fig. 3, the human sequence is much more similar to CaMKIKβ (95% identity over 132 overlapping residues) than to either CaMKIKα (76% identity over 76 overlapping residues) or CaMKIVK (70% identity over 268 overlapping residues). Thus, the putative protein encoded by the human nucleic acid appears to be a homologue of rat CaMKIKβ.

The other sequence shown in Fig. 3, CELC05H8–2, is part of a cosmid containing genomic DNA from the nematode, Caenorhabditis elegans. The cosmid was done and sequenced by the Genome Sequencing Center at Washington University in collaboration with the Sanger Centre in Cambridge, United Kingdom. The putative C. elegans kinase is predicted to be comprised of 357 amino acids and would be 56% identical to CaMKIVK over its entire length. The coding sequences were predicted from computer analysis using the Genefinder program. We have used a fragment of the cosmid containing the nematode gene to screen a C. elegans cDNA library. Sequence analysis of the positive clones reveals a region identical to the predicted sequence from Ile-366 to Arg-465. The likely existence of a protein in the nematode that corresponds to a CaM kinase kinase underscores the idea that this newly discovered family of regulatory protein kinases may be widely distributed among eukaryotic organisms.

It may be inferred from our data that mRNAs for two distinct CaM kinase kinases exist in rat brain. It seems likely that multiple activating kinases are required to regulate the activities of CaM kinases I and IV in different cells and tissues. This idea is consistent with the differential cellular and tissue distributions of CaM kinases I and IV. Whereas CaM kinase IV expression is restricted to brain, thymic lymphocytes, and meiotic male germ cells (30–35), CaM kinase I is present in most,
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if not all, cell types (8, 12). Even within the brain, the regions in which these two CaM kinases are enriched differ markedly. For example, CaM kinase IV is abundant in the cerebellar granule cells but is largely absent from most brain stem nuclei (36). By contrast, CaM kinase I was not detected in cerebellar granule cells (37) where intense immunoreactivity was observed in brain stem nuclei (38). At the subcellular level CaM kinase I is cytosolic (38) whereas CaM kinase IV has a predominantly nuclear localization (39, 40). Our results now make it possible to test, in future studies, whether CaMKIKα and β demonstrate differential tissue and/or subcellular localizations that correlate with the distribution of either of the CaM kinase targets. Differences in distribution, combined with the possibility of tissue-specific mechanisms for initiation of the CaM kinase signal transduction cascades, suggest that future efforts to understand the regulation and function of the CaM kinase family of enzymes will prove to be very rewarding.

Note Added in Proof—While this paper was under review a paper appeared (Tokumitsu, H., and Soderling, T. R. (1996) J. Biol. Chem. 271, 5617–5622) reporting activation of CaM kinase IV by phosphorylation of Thr-196 by the rat brain CaM kinase kinase (CaMKIVK), which appears to correspond to CaMKIKα.

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