Isoform-specific Activation and Structural Diversity of Calmodulin Kinase I

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We earlier confirmed that there are isoforms of Ca\(^{2+}\)/calmodulin (CaM)-dependent protein kinase 1 (CaM kinase I) (CaM kinase I\(\beta\)) and I\(\gamma\) beside CaM kinase I\(\alpha\) by cDNA cloning (Yokokura, H., Terada, O., Naito, Y., and Hidaka, H. (1997) Biochim. Biophys. Acta 1338, 8–12). Here, we demonstrate the existence of an isoform-specific activation mechanism of CaM kinase I and alternative splicing specifically regulating CaM kinase I (CaM kinase I\(\beta\)) in the central nervous system. To cast light on isoform structure-enzyme activity relationships, CaM kinase I\(\beta\), I\(\alpha\), and I\(\gamma\) were expressed separately using a baculovirus/Sf9 cell expression system. The novel CaM kinase I\(\beta\) isoform demonstrated similar catalytic activity to those of CaM kinase I\(\beta\) and I\(\alpha\). Interestingly, CaM kinase I\(\beta\) and I\(\beta\) both can activate CaM kinase I\(\alpha\) activity via phosphorylation at Thr\(^{177}\). Reverse transcribed-polymerase chain reaction analysis showed that CaM kinase I\(\beta\) is dominant in the cerebrum and cerebellum, whereas CaM kinase I\(\beta\) is present in peripheral tissues such as liver, heart, lung, kidney, spleen, and testis. CaM kinase I\(\beta\) was also detected with an anti-CaM kinase I\(\beta\) antibody in PC12 cells. The results indicate that alternative splicing is a means for tissue-specific expression of CaM kinase I\(\beta\). Thus the Thr\(^{177}\) residue of CaM kinase I\(\alpha\) is phosphorylated by not only CaM kinase I\(\beta\) but also CaM kinase I\(\beta\) for activation of the enzyme.

The Ca\(^{2+}\)/calmodulin (CaM)-dependent protein kinase (CaM kinase) is known to mediate signals associated with elevation of intracellular Ca\(^{2+}\). CaM kinases constitute a family of structurally related enzymes that include phosphorylase kinase, myosin light chain kinase, and CaM kinases I–IV. CaM kinase I was first identified in rat brain based on its ability to phosphorylate the synaptic vesicle-associated protein, synapsin I (1). It has since been purified and characterized from bovine (2) and rat brain (3, 4). This monomeric enzyme has been revealed to demonstrate multiple 37–43-kDa polypeptides by SDS-PAGE (2–5). We have purified and reported partial amino acid sequences of a novel CaM kinase, closely related to CaM kinase I (5, 6). Following the cloning of one cDNA for CaM kinase I (CaM kinase I\(\alpha\)) (7, 8), we have recently cloned two isoforms of CaM kinase I from a rat fetal brain cDNA library and termed them CaM kinase I\(\beta\) and I\(\gamma\) (9).

It has been recognized that CaM kinase I\(\alpha\) must be phosphorylated at the Thr\(^{177}\) residue for maximal activity (10–14) and that this occurs via autophosphorylation or by the action of CaM kinase I\(\gamma\). Several groups have identified and purified CaM kinase I\(\alpha\) (10, 11, 15–17), and a cDNA for a 68-kDa rat CaM kinase I\(\alpha\) has been cloned (18). Some kinases are known to be able to phosphorylate both CaM kinase I and I\(\gamma\) (14, 18, 19), suggesting the existence of a CaM kinase cascade, similar to the mitogen-activated protein kinase cascade. Moreover, we have reported that CaM kinases I and I\(\gamma\) can phosphorylate each other (14). For further understanding of the relationships between the members of the CaM kinase cascade and identification of the role of CaM kinase I isoforms, more information on their characteristics is necessary.

Here we report cDNA cloning of a novel isoform of CaM kinase I, CaM kinase I\(\beta\), which is expressed in the central nervous system, along with an analysis of the kinetic character of the expressed enzyme. The relationship between CaM kinases I\(\alpha\) and I\(\beta\) is described, and evidence is presented that CaM kinase I\(\beta\) may have a significant role in the CaM kinase cascade.

EXPERIMENTAL PROCEDURES

Materials—The syntide-2 (PLARTLSVAGLPGKK), CREB peptide (L\(\delta\)RRP\(\delta\)SYKRLNDL), synapsin site 1 peptide (NY\(\delta\)LLRR\(\delta\)LDSNFP), and myosin light chain peptide (KKA\(\delta\)ARATSSNVFA) were synthesized with a model 431A peptide synthesizer (Applied Biosystems Inc.). [\(\alpha\]\(^{-32}\)P]dCTP and [\(\gamma\]\(^{-32}\)P]ATP were purchased from Amersham Corp., and all other chemicals were purchased from standard suppliers.

cDNA Cloning of CaM Kinase I Isoform—Oligo(dT)-primed cDNA library of embryonic rat brain in \(\lambda\)ZAP II were kindly provided by Dr. S. Nakashima (Institute of Immunology, Kyoto University Faculty of Medicine, Kyoto, Japan). About 5.4 × 10\(^5\) plaques were screened using the EcoRI-SacI fragment (430 bp) of clone N2 (9). CaM kinase I\(\beta\), labeled with [\(\alpha\]\(^{-32}\)P]dCTP as a probe. A single cDNA clone of insert size 1.6 kb was isolated and sequenced.

RT-PCR and Northern Blot Analysis—Total cellular RNA from rat tissues was isolated by the guanidine thiocyanate method. Total cellular RNA from PC12 cells was isolated using the MagExtractor System (Toyobo, Osaka, Japan). Total RNAs (30 \(\mu\)g) were electrophoresed on formaldehyde-containing 1% agarose gels and blotted onto Hybond-N (Amersham Corp.). The filters were hybridized with a [\(\alpha\]\(^{-32}\)P]dCTP random primed fragment as a probe, washed in 0.1 × SSC, 0.1% SDS at 50 °C, and autoradiographed. RT-PCR was performed using 2.3 \(\mu\)g of total RNA and RT-PCR High-Plus (Toyobo, Osaka, Japan) under the following conditions: 1 cycle of 60 °C for 30 min, 94 °C for 2 min; 40 cycles of 94 °C for 1 min, 60 °C 1.5 min. The sequences of the PCR primers used were: P2, 5′-G\(\delta\)AC\(\delta\)AC\(\gamma\)CT\(\delta\)G\(\alpha\)G\(\gamma\)G\(\gamma\)AG\(\gamma\)G\(\gamma\)C\(\gamma\)AG\(\gamma\)A\(\gamma\)G-3′ (sense); P1, 5′-C\(\gamma\)G\(\delta\)AC\(\delta\)G\(\delta\)F\(\gamma\)AC\(\gamma\)G\(\gamma\)C\(\gamma\)G-3′ (antisense).

Construction of Plasmids—CaM kinase I\(\beta\) and I\(\beta\) cDNA were inserted into the PsI-NotI cut PVL1392 baculovirus transfer vector.
CaM kinase Iα cDNA was inserted into BamHI-EcoRI cut PVL1393. The expression plasmid for CaM kinase Iα was made as follows. Two primers were synthesized: sense 5'-ATGATTCATGAGGCGAGTTCCACGGGCTCCAGGACCTCTCAT-3' and antisense 5'-ATGCAGGCGCTCAGGATGCAGCCTCATCTTT-3', based on the published cDNA sequence of CaM kinase Iα (18). A PCR reaction was performed using LA PCR kit ver. 2 (Takara Shuzo, Shiga, Japan) with a cDNA library of embryonic rat brain as the template under the following conditions: 30 cycles of 72 °C for 3 min, 94 °C for 1 min, and 55 °C for 2 min. After digestion with EcoRI and NotI, the PCR fragment was subcloned into the bacterial expression vector, pGEX-5T-1 (Pharmacia Biotech Inc.). A Thr^{T177}→Ala mutant of CaM kinase Iα was made using the Sculptor in vitro mutagenesis system (Amersham Corp.), a cDNA of CaM kinase Iα subcloned into the BamHI-EcoRI site of pGEX5T (Pharmacia Biotech Inc.) and a mutagenic oligonucleotide (5'-AGTGTCCTCTCCAGGACCTCTTGGGATT-3').

**Protein Preparation**—CaM kinase Iα was purified from bovine brain as described previously (20). CaM kinase Iβ, Iγ, and Iα were expressed in the baculovirus expression system (Invitrogen). Sf9 cells expressing recombinant enzymes were harvested by centrifugation. Soluble protein extracts were prepared by sonication of the cells in buffer A (10 mM HEPES, pH 8.0, 1 mM EGTA, 5 mM 2-mercaptoethanol, 10 μg/ml leupeptin, 1 μg/ml pepstatin, and 0.4 mM phenylmethylsulfonyl fluoride (PMSF)). After centrifugation at 8,000 × g for 15 min, the supernatant was incubated with 2 μM CaCl2 and a GST-Sephrose 4B (Sigma) and washed with 40 mM Tris-HCl, pH 8.0, 1 mM CaCl2, 1 mM NaCl. Elution was carried out with buffer B (40 mM Tris-HCl, pH 8.0, 2 mM EGTA, 50 mM NaCl). To evaluate CaM dependence, CaM kinase Iβ was purified with phenyl Sepharose (Pharmacia Biotech Inc.) and glutathione-S-transferase fusion proteins were prepared as follows. JM109 cells containing the expression plasmids were grown overnight at 37 °C. The fully grown culture was diluted 100-fold in fresh LB medium and cells containing the expression plasmids were harvested by centrifugation. Soluble protein extracts were prepared by sonication of the cells in buffer A (10 mM HEPES, pH 8.0, 1 mM EGTA, 5 mM 2-mercaptoethanol, 10 μg/ml leupeptin, 1 μg/ml pepstatin, and 0.4 mM phenylmethylsulfonyl fluoride (PMSF)). After centrifugation at 8,000 × g for 15 min, the supernatant was loaded onto glutathione-Sepharose 4B (Pharmacia Biotech Inc.), washed with 40 mM Tris-HCl, pH 8.0, 2 mM EGTA, 50 mM NaCl. The kinase activity was determined at 30 °C for 10 min using standard assay conditions as described above with 50 μM syntide-2 and 100 μM [γ-32P]ATP as the substrate. For stoichiometrical analysis, 200 nM CaM kinase Iα was incubated in 300 μM [γ-32P]ATP with 20 μM CaM kinase Iβ or CaM kinase Iγ for 60 min. Reactions were stopped by the addition of loading buffer followed by electrophoresis on SDS-PAGE. Gels were stained with Coomassie Blue and dried. Quantitation of 32P incorporation into CaM kinase Iα was achieved by cutting out the appropriate gel pieces and determining their radioactivity by scintillation counting.

**Other Methods**—DNA sequences were determined for both strands by automatic sequencing using a model 373A (Applied Biosystems Inc.). SDS-PAGE was carried out by the method of Laemmli (23). Protein concentrations were measured by the Bradford (24) method.

**RESULTS**

**Isolation and Analysis of cDNA Clones of CaM Kinase Iβ**—We earlier reported the existence of isoforms of CaM kinase I (CaM kinase Iγ and I) other than CaM kinase Iα by cDNA cloning. Further screening of a rat brain oligo(dT)-primed cDNA library yielded a 1.6-kb clone. Sequencing of this clone indicated that it contained the appropriate nucleotide sequence of CaM kinase Iβ with an additional 137-bp sequence after nucleotide 954. This sequence contains a stop codon and codes for 25 amino acids that do not share significant sequence homology with CaM kinase Iβ (Fig. 1). The
From the image, there is a textual content that describes the isoform-specific regulation of CaM kinase I. The text is excerpted from a scientific article, likely discussing the tissue distribution, kinetic properties, and substrate specificities of CaM kinase I. Here is a transcription and translation of the text:

**Tissue Distribution of CaM Kinase I**

![Image](66x597 to 290x729)

**FIG. 3.** SDS-PAGE of purified recombinant CaM kinase Is and Western blot analysis. A, purified recombinant CaM kinase Ia, Ib1, and Ib2 were separated by SDS-PAGE. B, crude extracts of PC12 cells, as well as aliquots (10 ng) of CaM kinase Ib2, Ib1, and Ia were subjected to Western blot analysis as described under “Experimental Procedures.”

**TABLE I**

| CaM kinase | CREB | Syntide-2 |
|------------|------|-----------|
|            | $K_m$ (CREB) | $V_{max}$ (CREB) | $K_m$ (Syntide-2) | $V_{max}$ (Syntide-2) | $K_m$ (ATP) | $V_{max}$ (ATP) |
| Ia         | 482  | 5.29      | 235         | 573         | 2.53       | 2.55       |
| Ib1        | 265  | 1.11      | 341         | 297         | 2.00       | 2.60       |
| Ib2        | 273  | 1.24      | 347         | 276         | 2.56       | 2.56       |

**FIG. 4.** Ca$^{2+}$/CaM activation of CaM kinase Ib1 and Ib2. CaM kinase Ib1 (●) and Ib2 (○) were assayed for their activities with 1 mM Ca$^{2+}$ and the indicated concentrations of CaM (other conditions as given under “Experimental Procedures”). Mean ± S.E. values from two to three independent experiments performed in duplicate are shown.

**FIG. 5.** Comparison of substrate specificities of CaM kinase Ia, Ib1, and Ib2. Kinase activities for CaM kinase Ia (■), Ib1 (■), and Ib2 (□) were measured using 200 μM of syntide-2, site 1 peptide, CREB peptide, or MLC peptide as substrates. Mean ± S.E. values from two to six independent experiments performed in duplicate are shown.

**FIG. 6.** Activation of CaM kinase Ia, Ib1, and Ib2 by CaM kinase Ia. 1.6 μM aliquots of CaM kinase Ia, Ib1, and Ib2 were preincubated with or without CaM kinase Ia (21 nM) for 0 or 10 min (preincubation) in a kinase activation reaction (see “Experimental Procedures”). After terminating the activation, the activities of 21 nM CaM kinase Ia, Ib1, or Ib2 were assayed for their activities with 50 μM syntide-2 and 100 μM [γ-32P]ATP in the presence of 1 mM EGTA (open bars) or 1 mM CaCl2 (closed bars). Mean ± S.E. values from two to three independent experiments performed in duplicate are shown.

CaM kinase Ib reported previously and this novel isoform are tentatively termed CaM kinase Ib1 and CaM kinase Ib2, respectively.

**Tissue Distribution of CaM Kinase Ib1 and Ib2**—Northern blot analysis of rat tissues was performed using a cDNA fragment of CaM kinase Ib2 sharing 561 bp with CaM kinase Ib1 as a probe. An approximately 1.8-kb mRNA was detected in the cerebrum and cerebellum, and a 4-kb faint band in heart, lung, kidney, spleen, and testis (Fig. 2).

To evaluate the tissue-specific expression of CaM kinase Ib1 and Ib2, we conducted RT-PCR of rat tissues with overlapping pairs of primers spanning the alternatively spliced segment. In all samples we examined, cDNAs were amplified. The larger fragment amplified from cerebrum and PC12 cells exhibited the same apparent size as that from the CaM kinase Ib2 cDNA (Fig. 2). The fragment was extracted and the sequence was analyzed and found to be identical to CaM kinase Ib2. The smaller fragment, exhibiting the same apparent size as that from CaM kinase Ib1, was dominantly amplified in the liver, heart, kidney, lung, spleen, and testis (Fig. 2).

When a crude extract of PC12 cells was subjected to Western blot analysis with antibody against CaM kinase I, which can recognize CaM kinase Ib1, Ib2, a clear band at the position corresponding to CaM kinase Ib2 (M, 38,000) and a weaker band at the position corresponding to CaM kinase Ia (M, 41,000) were detected (Fig. 3).

**Characterization of Recombinant CaM Kinase Ib2**—To examine their enzymatic characteristics, CaM kinase Ia, Ib1, and Ib2 were expressed in Sf9 cells and purified with CaM-Sepha-
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FIG. 7. Phosphorylation and activation of CaM kinase Iα by CaM kinase Iβ2. A, 1.6 μM aliquots of CaM kinase Iα and Iβ2 were incubated with CaM kinase kinase for 10 min in a kinase activation reaction (see “Experimental Procedures”). 21 nM activated CaM kinase Iα (act. CaMKIα) and activated CaM kinase Iβ2 (act. CaMKIβ2) and CaM kinase kinase (CaMKK) were assayed for their ability to phosphorylate CaM kinase Iα or CaM kinase Iβ2 with 100 μM [γ-32P]ATP. Reaction products were analyzed by SDS-PAGE and subjected to autoradiography. B, 1 μM aliquots of CaM kinase Iα and Iβ2 were incubated with 21 nM activated CaM kinase Iα, activated CaM kinase Iβ2, or CaM kinase kinase in a kinase activation reaction. Subsequently, the activities of 20 nM of CaM kinase Iα or Iβ2 were measured using 50 μM substrate-2, 100 μM [γ-32P]ATP. Mean ± S.E. values from two or three independent experiments performed in duplicate are shown.

rose. These enzymes had similar catalytic activity (Table I) and were activated by CaM in a dose-dependent manner (Fig. 4). CaM kinase Iβ2, Iβ1, and Iα demonstrated CaM requirements with EC50 values of approximately 35, 80, and 150 nM, respectively.

For analysis of substrate specificity, we tested four peptides. The phosphorylation capacities of CaM kinase Iα, CaM kinase Iβ1, and CaM kinase Iβ2 were similar except with CREB (Fig. 5), which was found to be a highly preferred substrate for CaM kinase Iα, but not Iβ1 or Iβ2. Kinetic analysis data showed the Vmax of the CaM kinase Iα for the CREB peptide to be 5 times larger than those for the CaM kinase Iβ1 and Iβ2 (Table I).

Activation of CaM Kinase Iβ2 by CaM Kinase Kinase—It is well established that CaM kinase Iα and Iβ are phosphorylated and activated by CaM kinase kinase. To examine the effects of CaM kinase kinase on the activity of CaM kinase Iβ2, we expressed a glutathione S-transferase-CaM kinase kinase fusion protein in Escherichia coli and purified it on glutathione-Sepharose. CaM kinase Iβ2 was incubated with or without CaM kinase kinase for 10 min, and its activity was determined. CaM kinase Iβ2 was activated 6.7-fold by autophosphorylation and 30-fold by CaM kinase kinase (Fig. 6). Under the same conditions, CaM kinase Iα and Iβ1 were activated 2.9- and 2.9-fold by autophosphorylation and 40- and 16-fold by CaM kinase kinase, respectively (Fig. 6). Unlike CaM kinase II or CaM kinase IV, CaM kinase Iβ1 or Iβ2 activated by CaM kinase kinase did not generate Ca2+/CaM-independent activity.

Phosphorylation of CaM Kinase Iα by CaM Kinase Iβ1 and Iβ2—We have suggested the existence of a new CaM kinase isoform that is phosphorylated by CaM kinase Iβ2 (Fig. 8) and was not activated (data not shown). mutant CaM kinase Iα was not phosphorylated by CaM kinase Iβ2 (Fig. 8) and was not activated (data not shown).

FIG. 8. Substitution of Thr177 by Ala abolishes phosphorylation of CaM kinase Iα by CaM kinase Iβ2. Wild type and Thr177 → Ala mutant forms of CaM kinase Iα, fused with glutathione S-transferase, were expressed in E. coli and partially purified by glutathione-Sepharose (see “Experimental Procedures”). 1 μM aliquots of wild-type and Thr177 → Ala mutant (T177A) were incubated with 21 nM of activated CaM kinase Iβ2 (act. CaMKIβ2) or CaM kinase kinase (CaMKK) with 100 μM [γ-32P]ATP in a kinase activation reaction. Reaction products were analyzed by SDS-PAGE and subjected to autoradiography.

DISCUSSION

The data presented in this study show the existence of a novel isoform of CaM kinase I, generated by alternative splicing between nucleotide 955 and 956 of the CaM kinase Iβ1 cDNA. Northern blot analysis revealed 1.8- and 4-kb mRNAs for the CaM kinase Iβ2 (Fig. 2). Considering the RT-PCR data, the 1.8-kb mRNA that is highly expressed in the central nervous system but lacking in the liver, heart, lung, kidney, spleen, and testis might be CaM kinase Iβ2. The 4-kb mRNA could encode CaM kinase Iβ1, but the possibility that it represents an unspliced intermediate or a spurious hybridization could not be excluded. Western blot analysis revealed that CaM kinase Iβ2 was expressed in PC12 cells and RT-PCR data showed the form to be Iβ2. The results indicate that CaM kinase Iβ2 might have a significant role as an effector enzyme for brain specific functions.

CaM kinase Iβ1 and Iβ2 contain the same catalytic and putative CaM binding domains and show similar kinetic activities. CaM kinase Iβ2, however, has a lower CaM requirement (Fig. 4). Since the amino acid sequence of the putative CaM binding domain in CaM kinase Iβ2 is similar to that in CaM kinase Iα (91% similarity), the difference in the CaM requirement is interesting. Thus, CaM may interact with as yet un-
known domains of CaM kinase Iβ2 or Iβ1, in addition to the canonical CaM kinase binding domain. In this context, the C-terminal sequence in CaM kinase Iβ2 might be a good candidate since it is absent in CaM kinase Iβ1. The C-terminal sequences of CaM kinase Iα and Iβ1 contain proline-rich sections, whereas CaM kinase Iβ2 does not. It is therefore also possible that this prevents CaM kinase Iα or Iβ1 from forming a secondary structure necessary for additional CaM binding.

Activation of CaM kinase I and IV is dependent upon phosphorylation by CaM kinase kinase for maximal activity (10–18, 25–27). Our data demonstrated that CaM kinase I also possesses CaM kinase kinase activity. Recently, another two CaM kinase kinases were purified from rat brain (28) and from rat cerebellum (29). The molecular weight of the former is about 73,000 and that of the latter is about 66,000 as determined by SDS-PAGE. The predicted molecular weight of CaM kinase I is 73,000 and that of the latter is about 66,000 as determined by cerebellum (29). The molecular weight of the former is about 73,000 and that of the latter is about 66,000 as determined by SDS-PAGE. The predicted molecular weight of CaM kinase Iβ2 is 38,423 and the lack of any sequence identity indicates that it is a totally different protein. The level of phosphorylation of CaM kinase Iα catalyzed by CaM kinase kinase appears greater than that catalyzed by CaM kinase Iβ2. Stoichiometric analysis showed that 1 molecule of $^{32}$P was incorporated per CaM kinase Iα molecule by CaM kinase Iβ2, whereas CaM kinase kinase provided additional phosphorylation. The T177A mutant was also phosphorylated by CaM kinase kinase, although the phosphorylation level of the mutant was lower than that catalyzed by CaM kinase Iα.

Recently, Aletta et al. (30) reported the existence of an intracellular CaM kinase I phosphorylation cascade in PC12 cells, and we showed, here, that they express CaM kinase Iβ2 (Fig. 3). These data suggest that CaM kinase Iβ2 might be involved in a CaM kinase I cascade in PC12 cells. However, CaM kinase Iβ2 phosphorylates peptides other than those targeted by CaM kinase kinase, so it is possible that there might be specific substrates. Further work is required to clarify these points.

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